CLC **Main** Workbench

USER MANUAL

Manual for

CLC Main Workbench 25.0.2

Windows, macOS and Linux

June 11, 2025

This software is for research purposes only.

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Introduction

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Introduction to *CLC Main Workbench*

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Welcome to *CLC Main Workbench 25.0.2* — a software package supporting your daily bioinformatics work.

CLC Main Workbench 25.0.2 **is for research purposes only.**

The *CLC Main Workbench* provides an easy-to-use graphical interface for running bioinformatics analyses. Tools can be run individually, or chained together in a workflow, making running complex analyses simple and efficient. The functionality of the *CLC Main Workbench* can also be extended using plugins. The built-in Plugin Manager provides an up-to-date listing. A list is also available on our plugin webpage: https://digitalinsights.qiagen.com/products-overview/plugins/.

Supporting documentation and links for the *CLC Main Workbench* can be found under the **Help** menu in the top toolbar. Of particular note when getting started:

- The built-in Workbench user manual can be opened by choosing the **Help** option or by clicking on the **F1** key.
- Manuals for installed plugins can be accessed under the **Plugin Help** option.
- The **Online Tutorials** option opens our tutorials webpage in a browser. Tutorials offer hands-on examples of how to use features of the *CLC Main Workbench*. Alternatively, click on the following link to visit that webpage: https://digitalinsights.qiagen.com/support/tutorials/.

Watch product specialists demonstrates our software in the videos offered via our **Online presentations** area: https://tv.qiagenbioinformatics.com/.

The latest version of this user manual can be found in pdf and html formats at https://digitalinsights.qiagen.com/technical-support/manuals/

The *CLC Main Workbench* is being constantly developed and improved. A detailed list of new features, improvements, bug fixes, and changes for the current version of *CLC Main Workbench* can be found at https://digitalinsights.qiagen.com/technical-support/latest-improvements/.

1.1 Contact information and citation

CLC Main Workbench is developed by:

QIAGEN Aarhus A/S
Kalkværksvej 5, 11.
DK - 8000 Aarhus C
Denmark

https://digitalinsights.qiagen.com/

Email: ts-bioinformatics@qiagen.com

The QIAGEN Aarhus team is continuously improving *CLC Main Workbench* with your interests in mind. We welcome all requests and feedback from users, as well as suggestions for new features or more general improvements to the program.

Getting help via the Workbench If you encounter a problem or need help understanding how *CLC Main Workbench* works, and the license you are using is covered by our Maintenance, Upgrades and Support (MUS) program (https://digitalinsights.qiagen.com/

technical-support/maintenance-and-support/), please contact us by clicking on the Support button at the right hand side of the top toolbar (figure 1.1) or by choosing **Contact Support** under the **Help** menu.

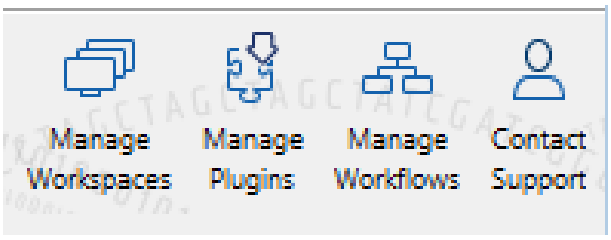


Figure 1.1: *Contact our Support team by clicking on the button at the right hand side of the top Toolbar*

This will open a dialog where you can enter your contact information, and a text field for writing the question or problem you have. On a second dialog you will be given the chance to attach screenshots or even small datasets that can help explain or troubleshoot the problem. When you send a support request this way, it will automatically include helpful technical information about your installation and your license information so that you do not have to look this up yourself. Our support staff will reply to you by email.

Other ways to contact the support team You can also contact the support team by email: ts-bioinformatics@qiagen.com

Please provide your contact information, your license information, some technical information about your installation , and describe the question or problem you have. You can also attach screenshots or even small data sets that can help explain or troubleshoot the problem.

Information about the license(s) being used by a *CLC Workbench* and any installed modules can be found by opening the License Manager:

Help | **License Manager...**

Information about MUS cover on particular licenses is provided in your myCLC account: https://secure.clcbio.com/myclc/login.

How to cite us To cite a CLC Workbench or Server product, use the name of the product, the version number. For example QIAGEN CLC Main Workbench 24.0 or QIAGEN CLC Genomics Workbench 24.0. If a location is required by the publisher of the publication, use (QIAGEN, Aarhus, Denmark). Our website is https://digitalinsights.qiagen.com/.

Further details about citing QIAGEN Digital Insights software can be found in our FAQ at

https://qiagen.secure.force.com/KnowledgeBase/KnowledgeNavigatorPage?id=kA41i000000L63hCAC

1.2 Download and installation

Software installers for Windows, macOS and Linux systems for the current *CLC Main Workbench* release can be downloaded from https://digitalinsights.qiagen.com/downloads/

product-downloads/.

More ways to get installers are described in the Frequently Asked Question entry "Where can I get installer files for the software?": https://qiagen.secure.force.com/KnowledgeBase/KnowledgeNavigatorPage?id=kA41i000000L5uQCAS

To check for available updates from within the software, go to the menu option: **Help** | **Check for Updates...** (⟳).

General information about running software installers, including differences between upgrading to a new minor version compared to upgrading to a new major version, are covered in section 1.2.1. Detailed instructions for running the software installer in interactive mode on each supported operating system then follows.

Information about running the software installers in console mode and silent mode are provided in the Workbench Deployment manual at https://resources.qiagenbioinformatics.com/manuals/workbenchdeployment//current/index.php?manual=Installation_modes_console_silent.html.

1.2.1 General information about installing and upgrading Workbenches

When a Workbench installer is run, it performs the following tasks:

1. **Extracts and copies files to the installation directory** The Workbench software is installed into a directory. It is self contained. The suggested folder name to install into reflects the software name and the major version line. For example, for a *CLC Genomics Workbench* with major version 25, the default installation location offered on each platform would be:

   Windows C:\Program files\CLC Genomics Workbench 25

   macOS /Applications/CLC Genomics Workbench 25

   Linux /opt/CLCGenomicsWorkbench 25

   To install the software into central locations, like those listed above, generally requires administrator rights. Administrator rights will also be needed to install licenses and plugins for installations in central locations. The software can be installed to another location, if desired. When only a single person will use the software, this can be useful. Installing to an area they have permission to write to means that licenses and plugins can then be installed without needing administrator rights.

   General recommendations for installation locations

   - **For minor updates**, you will be asked whether you wish to:
     - **Update the existing installation** *Generally recommended for minor updates.* New files will be installed into the same directory as the existing installation. Licensing information and installed plugins remain in place from the installation already present.
       OR
     - **Install to a different directory**. Configuration will be needed after installation. E.g. licensing needs to be configured, any desired plugins will need to be installed, etc.

   - **For major updates**. The suggested installation directory will reflect the new major version number of the software. *Please do not install a new major version into the same folder as an existing, older version of the Workbench.* Configuration will be needed after installation. E.g. licensing needs to be configured, any desired plugins will need to be installed, etc.

2. **Sets the amount of memory** The installer investigates the amount of RAM on the machine during installation and sets the amount of memory that the Workbench can use.

3. **Establishes shortcuts (optional)** On Windows and Mac systems, an option is provided during installation to create a shortcut for starting the Workbench. On Linux systems, this option is also presented, but it has no effect.

On Macs without Rosetta present on the system, the option of installing it is offered during the installation process. Rosetta enables Intel-based features to run on Apple Silicon Macs. While not needed for the majority of tools, some require it, for example De Novo Assembly, BLAST, Sample Reads and tools for analyzing small RNA.

Updating workflows after upgrading a *CLC Workbench* is described in section 13.6.1.

1.2.2 Installation on Microsoft Windows

To install to a central location, the installer must be run in *administrator mode*. On Windows, right-click on the of the downloaded installer file and choose "Run as Administrator".

To install to a location you have write permission to, double click the icon of the downloaded installer file.

Then walk through the following steps. (The exact order options are presented may differ to that described.)

- Read and accept the License agreement and click **Next**.
- Unless you are installing a minor update to the same folder as an existing installation, you will be prompted to choose where you would like to install the Workbench. If you are upgrading from an earlier version, please refer to section 1.2.1 for information about installing to an existing or different directory. Click on **Next**.
- Choose where you want the program's shortcuts to be placed. Click on **Next**.
- Choose if you would like to associate .clc files to the *CLC Main Workbench*. If you check this option, double-clicking a file with a "clc" extension will open the *CLC Main Workbench*.
- Choose if a desktop icon should be created, and choose whether clc://URLs should be opened by this program by default. Click on **Next**.
- Wait for the installation process to complete, and then choose whether you would like to launch *CLC Main Workbench* right away. Click on **Finish**.

When the installation is complete the program can be launched from the Start Menu or from one of the shortcuts you chose to create.

1.2.3 Installation on macOS

To install to a location you have write permission to, double click the icon of the downloaded installer file.

Then walk through the following steps. (The exact order options are presented may differ to that described.)

- Read and accept the License agreement and click **Next**.
- Choose where you would like to install the application. If you are upgrading from an earlier version, please refer to section 1.2.1 for information about installing to an existing or different directory. Click on **Next**.
- Specify other options associated with the installation such as whether a desktop icon should be created, whether the software should open clc:// URLs. whether .clc files should be associated with the software and whether it should be added to the dock. Click on **Next**.
- Wait for the installation process to complete, choose whether you would like to launch *CLC Main Workbench* right away, and click on **Finish**.

On Apple Silicon Macs without Rosetta present on the system, the option of installing it is offered during the installation process. Rosetta enables Intel-based features to run on Apple Silicon Macs. While not needed for the majority of tools, some require it, for example De Novo Assembly, BLAST, Sample Reads and tools for analyzing small RNA.

When the installation is complete, the program can be launched from the dock, if present there, or by clicking on the desktop shortcut if you chose to create one. The software can also be launched from within the installation folder.

1.2.4 Installation on Linux with an installer

Navigate to the directory containing the installer and launch it. This can be done by running a command similar to:

```
# sh CLCMainWorkbench_25_0_2_64.sh
```

To install to a central location such as /opt or /usr/local, you will normally need to run the above command using sudo. If you do not have sudo privileges you can choose to install in your home directory, or any other location you have write permission for.

Then walk through the following steps. (The exact order options are presented may differ to that described.)

- Read and accept the License agreement and click **Next**.
- Choose where you would like to install the application. If you are upgrading from an earlier version, please refer to section 1.2.1 for information about installing to an existing or different directory. Click on **Next**.

- Choose where you would like to create symbolic links to the program. Click on **Next**.
  DO NOT create symbolic links in the same location as the application.
  Symbolic links should be installed in a location which is included in your environment PATH. For a system-wide installation you can choose for example /usr/local/bin. If you do not have root privileges you can create a 'bin' directory in your home directory and install symbolic links there. You can also choose not to create symbolic links.
- Wait for the installation process to complete and click on **Finish**.

If you choose to create symbolic links in a location which is included in your PATH, the program can be executed by running the command:

```
# clcmainwb25
```

Otherwise you start the application by navigating to the location where you choose to install it and running the command:

```
# ./clcmainwb25
```

1.3 System requirements

The system requirements of *CLC Main Workbench* are:

- Windows: Supported versions of Windows 10, Windows 11, Windows Server 2016, Windows Server 2019, Windows Server 2022 and Windows Server 2025
- Mac: macOS 13, 14 and 15
- Linux: RHEL 8 and later and supported versions of SUSE Linux Enterprise Server 12.5 and later. The software is expected to run without problem on other recent Linux systems, but we do not guarantee this. To use BLAST related functionality, libnsl.so.1 is required.
- 64 bit operating system
- 1 GB RAM required
- 2 GB RAM recommended
- 1024 x 768 display required
- 1600 x 1200 display recommended

System requirements for 3D viewers

A graphics card that supports OpenGL 2.0.

Note: 3D rendering is only supported when the *CLC Main Workbench* is installed on the same machine the viewer is opened on. Indirect rendering (such as X11 forwarding through ssh), remote desktop connection/VNC, and running in virtual machines is not supported.

CLC data location requirements

Requirements for CLC data locations are provided in section 3.1.2.

1.3.1 Limitations on maximum number of cores

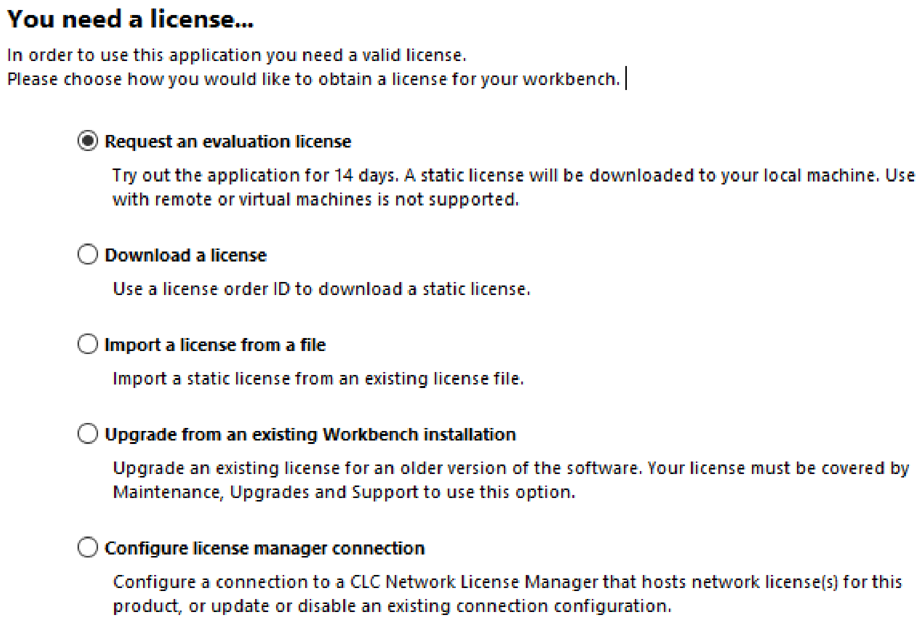
Most modern CPUs implement hyper threading or a similar technology that makes each physical CPU core appear as two logical cores on a system. In this manual the term "core" always refers to a logical core unless otherwise stated.

Licensing: Static licenses can be used on systems with 64 or fewer logical cores. On systems with more than 64 logical cores a network license is needed. See `https://digitalinsights.qiagen.com/licensing/`.

1.4 Workbench Licenses

When you start up the *CLC Main Workbench* for the first time on your system, or after installing a new major release, the **License Assistant**, shown in figure 1.2, will be presented to you. The **License Assistant** can be also be launched during an active Workbench session by clicking on the "Upgrade Workbench License" button at the bottom of the **License Manager**. The **License Manager** can be started up using the Workbench menu item:

Help | **License Manager** (📜)



You need a license...

In order to use this application you need a valid license.
Please choose how you would like to obtain a license for your workbench.

● **Request an evaluation license**

Try out the application for 14 days. A static license will be downloaded to your local machine. Use with remote or virtual machines is not supported.

○ **Download a license**

Use a license order ID to download a static license.

○ **Import a license from a file**

Import a static license from an existing license file.

○ **Upgrade from an existing Workbench installation**

Upgrade an existing license for an older version of the software. Your license must be covered by Maintenance, Upgrades and Support to use this option.

○ **Configure license manager connection**

Configure a connection to a CLC Network License Manager that hosts network license(s) for this product, or update or disable an existing connection configuration.

Figure 1.2: *The License Assistant provides access to licensing options.*

The options available in the **License Assistant** window are described in brief below, and then in detail in the sections that follow.

- **Request an evaluation license** Request a fully functional, time-limited static license.

- **Download a license** Use the license order ID provided when you purchase the software to download and install a static license file.
- **Import a license from a file** Import an existing static license file, for example a file downloaded from the license download webpage.
- **Upgrade from an existing Workbench installation** If you have used a previous version of the *CLC Main Workbench*, and you are entitled to upgrade to a new major version, select this option to upgrade your static license file.
- **Configure license manager connection** If your organization has a *CLC Network License Manager*, select this option to configure the connection to it.

Select the appropriate option and then click on the **Next** button.

To use the **Request an evaluation license**, **Download a license** or the **Upgrade from an existing Workbench installation** options, your machine must be able to access the external network. If this is not the case, please see section 1.4.7.

When using a *CLC Main Workbench* installed in a central location on your system, you must be running the program in administrative mode to license the software. On Linux and Mac, this means you must be logged in as an administrator. On Windows, you can right-click the program shortcut and choose "Run as Administrator".

If you do not have a license order ID or access to a license, you can still use the Workbench in **Viewing Mode**. See section 1.4.8 for further information about this.

Note: Static licenses are tied to the host ID of the machine they were downloaded to. If your license is covered by Maintenance, Upgrades and Support (MUS), please contact our Support team (ts-bioinformatics@qiagen.com) if you need to start using a different machine for working with the *CLC Main Workbench*.

1.4.1 Request an evaluation license

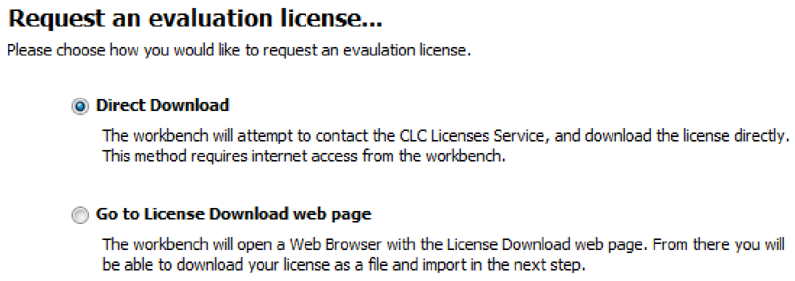
We offer a fully functional version of the *CLC Main Workbench* free of charge for a 14 day period for evaluation purposes. The 14 day period commences when the evaluation license is downloaded. If you have questions about *CLC Main Workbench* features or product licensing options, please send an email to bioinformaticssales@qiagen.com.

When you choose the option **Request an evaluation license**, you will see the dialog shown in figure 1.3.

In this dialog, there are two options:

- **Direct Download**. Download the license directly. This method requires that the Workbench has access to the external network.
- **Go to CLC License Download web page**. The online license download form will be opened in a web browser. This option is suitable for when downloading a license for use on another machine that does not have access to the external network, and thus cannot access the QIAGEN Aarhus servers.

After selecting your method of choice, click on the button labeled **Next**.



Request an evaulation license...

Please choose how you would like to request an evaulation license.

● Direct Download

The workbench will attempt to contact the CLC Licenses Service, and download the license directly. This method requires internet access from the workbench.

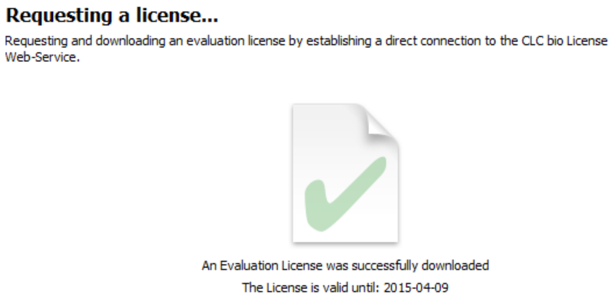
○ Go to License Download web page

The workbench will open a Web Browser with the License Download web page. From there you will be able to download your license as a file and import in the next step.

Figure 1.3: *Choose between downloading a license directly, or opening the license download form in a web browser.*

Direct download

After choosing the **Direct Download** option and clicking on the button labeled **Next**, a dialog similar to that shown in figure 1.4 will appear if the license is successfully downloaded and installed.



Requesting a license...

Requesting and downloading an evaluation license by establishing a direct connection to the CLC bio License Web-Service.

An Evaluation License was successfully downloaded

The License is valid until: 2015-04-09

Figure 1.4: *A license has been successfully downloaded and installed for use.*

When the license has been downloaded and installed, the **Next** button will be enabled.

If there is a problem, a dialog will appear indicating this.

Go to license download web page

After choosing the **Go to CLC License Download web page** option and clicking on the button labeled **Next**, the license download form will be opened in a web browser, as shown in figure 1.5.

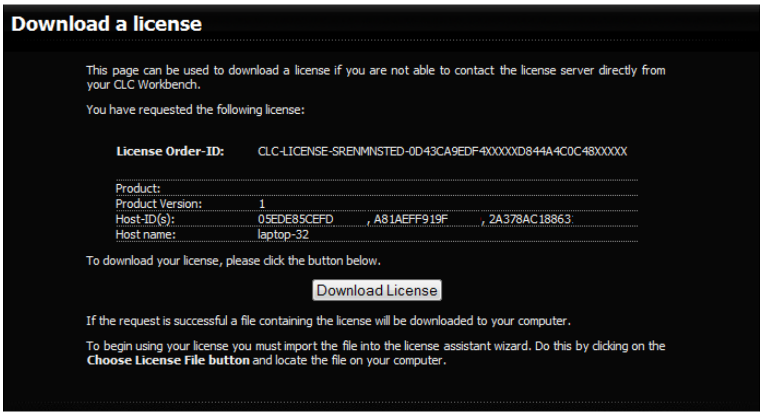
Click on the **Download License** button and then save the license file.

Back in the Workbench window, you will now see the dialog shown in 1.6.

Click on the **Choose License File** button, find the saved license file and select it. Then click on the **Next** button.

Accepting the license agreement

The End User License Agreement (EULA) must be read and accepted as part of the installation process. Please read the EULA text carefully, and if you agree to it, check the box next to the text **I accept these terms**. If further information is requested from you, please fill this in before



Download a license

This page can be used to download a license if you are not able to contact the license server directly from your CLC Workbench.

You have requested the following license:

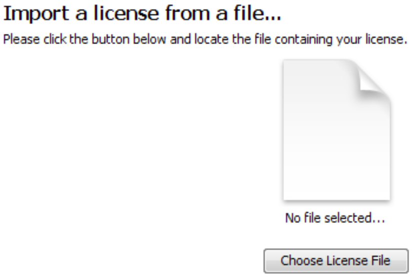
License Order-ID:	CLC-LICENSE-SRENMNSTED-0D43CA9EDF4XXXXXD844A4C0C48XXXXX
Product:	
Product Version:	1
Host-ID(s):	05EDE85CEFD , A81AEFF919F , 2A378AC18863
Host name:	laptop-32

To download your license, please click the button below.

If the request is successful a file containing the license will be downloaded to your computer.

To begin using your license you must import the file into the license assistant wizard. Do this by clicking on the **Choose License File button** and locate the file on your computer.

Figure 1.5: *The license download form opened in a web browser.*



Import a license from a file...

Please click the button below and locate the file containing your license.

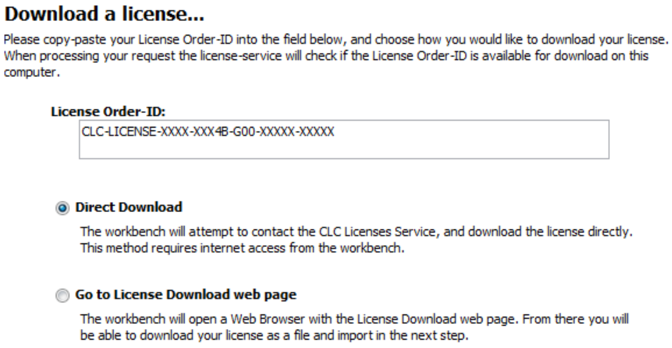
No file selected...

Figure 1.6: *Importing the license file downloaded from the web page.*

clicking on the **Finish** button.

1.4.2 Download a license using a license order ID

Using a license order ID, you can download a license file via the Workbench or using an online form. When you have chosen this option and clicked on the **Next** button, you will see the dialog shown in 1.7. Enter your license order ID into the License Order ID text field. (The ID can be pasted into the box after copying it and then right clicking in the text field and choosing Paste from the context menu, or using a key combination like Ctrl+V, or on a Mac, ⌘ + V).



Download a license...

Please copy-paste your License Order-ID into the field below, and choose how you would like to download your license. When processing your request the license-service will check if the License Order-ID is available for download on this computer.

License Order-ID: CLC-LICENSE-XXXX-XXX4B-G00-XXXXX-XXXXX

● **Direct Download**

The workbench will attempt to contact the CLC Licenses Service, and download the license directly. This method requires internet access from the workbench.

○ **Go to License Download web page**

The workbench will open a Web Browser with the License Download web page. From there you will be able to download your license as a file and import in the next step.

Figure 1.7: *Enter a license order ID into the text field and then click on the Next button.*

In this dialog, there are two options:

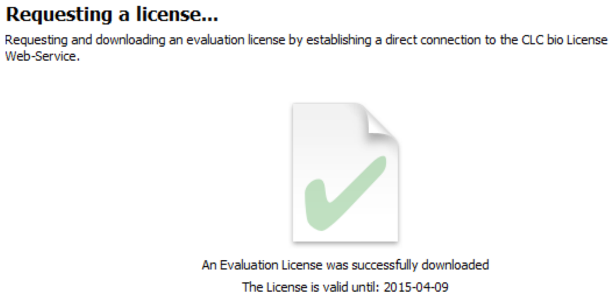
- **Direct Download**. Download the license directly. This method requires that the Workbench has access to the external network.

- **Go to CLC License Download web page**. The online license download form will be opened in a web browser. This option is suitable for when downloading a license for use on another machine that does not have access to the external network, and thus cannot access the QIAGEN Aarhus servers.

After selecting your method of choice, click on the button labeled **Next**.

Direct download

After choosing the **Direct Download** option and clicking on the button labeled **Next**, a dialog similar to that shown in figure 1.8 will appear if the license is successfully downloaded and installed.



Requesting a license...

Requesting and downloading an evaluation license by establishing a direct connection to the CLC bio License Web-Service.

An Evaluation License was successfully downloaded

The License is valid until: 2015-04-09

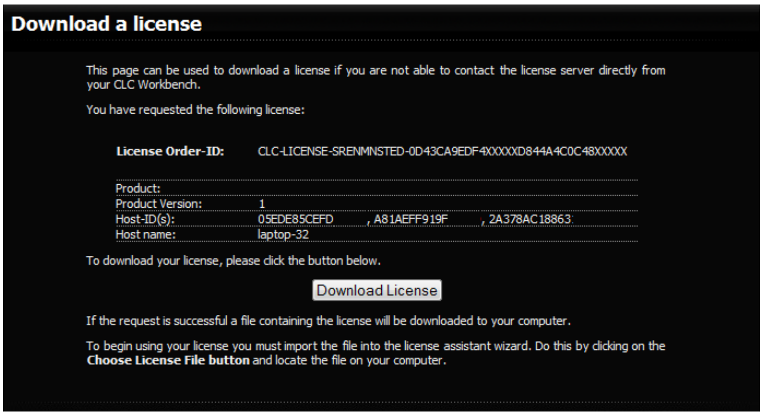
Figure 1.8: *A license has been successfully downloaded and installed for use.*

When the license has been downloaded and installed, the **Next** button will be enabled.

If there is a problem, a dialog will appear indicating this.

Go to license download web page

After choosing the **Go to CLC License Download web page** option and clicking on the button labeled **Next**, the license download form will be opened in a web browser, as shown in figure 1.9.



Download a license

This page can be used to download a license if you are not able to contact the license server directly from your CLC Workbench.

You have requested the following license:

License Order-ID:	CLC-LICENSE-SRENMNSTED-0D43CA9EDF4XXXXXD844A4C0C48XXXXX
Product:	
Product Version:	1
Host-ID(s):	05EDE85CEFD , A81AEFF919F , 2A378AC18863
Host name:	laptop-32

To download your license, please click the button below.

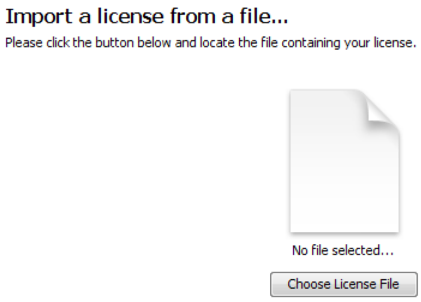
If the request is successful a file containing the license will be downloaded to your computer.

To begin using your license you must import the file into the license assistant wizard. Do this by clicking on the **Choose License File button** and locate the file on your computer.

Figure 1.9: *The license download form opened in a web browser.*

Click on the **Download License** button and then save the license file.

Back in the Workbench window, you will now see the dialog shown in 1.10.



Import a license from a file...

Please click the button below and locate the file containing your license.

No file selected...

Figure 1.10: *Importing the license file downloaded from the web page.*

Click on the **Choose License File** button, find the saved license file and select it. Then click on the **Next** button.

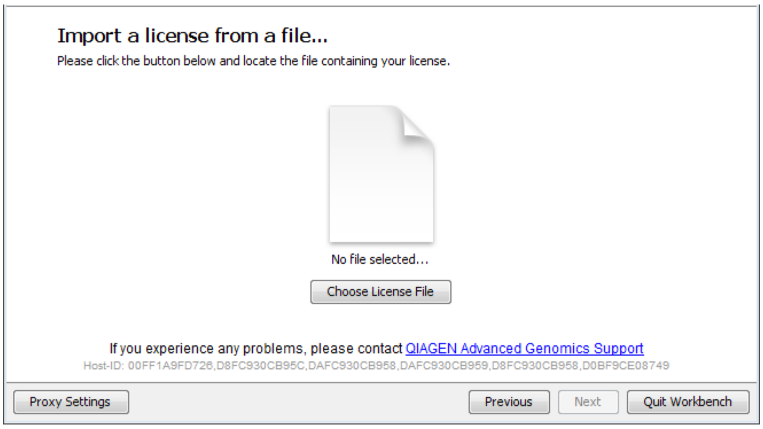
Accepting the license agreement

The End User License Agreement (EULA) must be read and accepted as part of the installation process. Please read the EULA text carefully, and if you agree to it, check the box next to the text **I accept these terms**. If further information is requested from you, please fill this in before clicking on the **Finish** button.

1.4.3 Import a license from a file

If you already have a license file associated with the host ID of your machine, it can be imported using this option.

When you have clicked on the **Next** button, you will see the dialog shown in 1.11.



Import a license from a file...

Please click the button below and locate the file containing your license.

No file selected...

If you experience any problems, please contact QIAGEN Advanced Genomics Support

Host-ID: 00FF1A9FD726,D8FC930CB95C,DAFC930CB958,DAFC930CB959,D8FC930CB958,D0BF9CE08749

Figure 1.11: *Selecting a license file.*

Click on the **Choose License File** button, locate the license file and selected it. Then click on the **Next** button.

Accepting the license agreement

The End User License Agreement (EULA) must be read and accepted as part of the installation process. Please read the EULA text carefully, and if you agree to it, check the box next to the text **I accept these terms**. If further information is requested from you, please fill this in before clicking on the **Finish** button.

1.4.4 Upgrade license

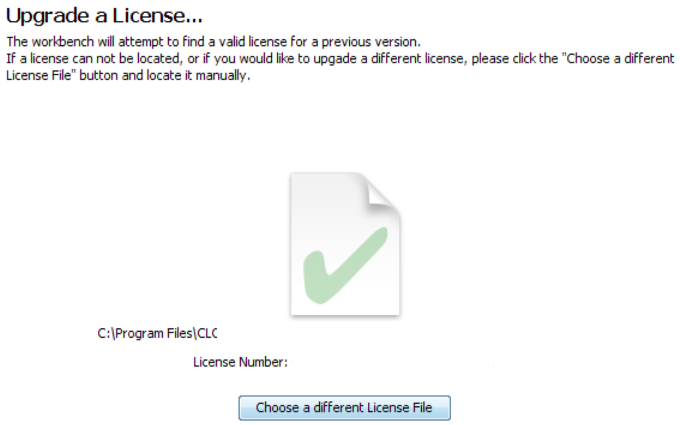
The option "Upgrade from an existing Workbench installation" can be convenient when you have been using another version of a licensed Workbench and the license is covered by our Maintenance, Upgrades and Support (MUS) program. Licenses not covered by MUS cannot be updated to support a new major Workbench release line.

If your license is covered our Maintenance, Upgrades and Support (MUS) program but you experience problems downloading a license for the new version of the software, please contact bioinformaticslicense@qiagen.com.

The Workbench will need direct access to the external network to use this option. If the Workbench cannot connect to the external network directly, please see section 1.4.7.

After selecting the "Upgrade from an existing Workbench installation" option, click on the **Next** button. The Workbench will search for an earlier installation of the same Workbench product you are upgrading to.

If it finds that installation, it will locate the existing license file and show information like that in figure 1.12.



Upgrade a License...

The workbench will attempt to find a valid license for a previous version.
If a license can not be located, or if you would like to upgade a different license, please click the "Choose a different License File" button and locate it manually.

C:\Program Files\CLC

License Number:

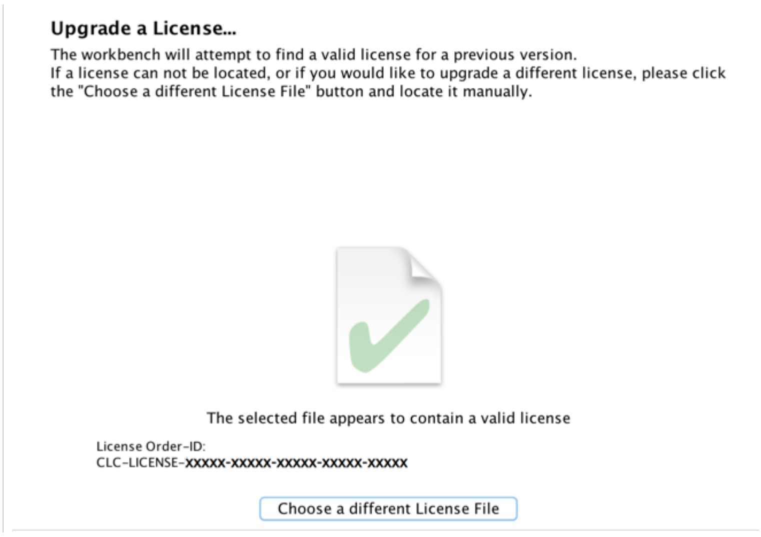
Figure 1.12: *An license from an older installation was found.*

When you click on the **Next** button, the Workbench checks if you are entitled to upgrade your license. This is done by contacting QIAGEN Aarhus servers.

If the earlier Workbench version could not be found, which can be the case if you have installed to a custom location or are upgrading from one Workbench product to another product replacing it[1], then click on the "Choose a different License File" button. Navigate to where the older license file is, which will be in a subfolder called "licenses" within the installation area of the Workbench you are upgrading from. Select the license file and click on the "Open" button.

[1] In November 2018, the Biomedical Genomics Workbench was replaced by the CLC Genomics Workbench and a free plugin, Biomedical Genomics Analysis. Licenses for the Biomedical Genomics Workbench covered by MUS at that time can be used to download a valid license for the CLC Genomics Workbench, but the upgrade functionality is not able to automatically find the older license file.

If the license selected can be updated, a message similar to that shown in figure 1.13 will be displayed. If there is a problem updating the selected license, a dialog will appear indicating this.



Upgrade a License...

The workbench will attempt to find a valid license for a previous version.
If a license can not be located, or if you would like to upgrade a different license, please click the "Choose a different License File" button and locate it manually.

The selected file appears to contain a valid license

License Order-ID:
CLC-LICENSE-XXXXX-XXXXX-XXXXX-XXXXX-XXXXX

Figure 1.13: *A license in an older installation was found.*

Click on the **Next** button and then choose how to proceed to get the updated license file.

In this dialog, there are two options:

- **Direct Download**. Download the license directly. This method requires that the Workbench has access to the external network.
- **Go to CLC License Download web page**. The online license download form will be opened in a web browser. This option is suitable for when downloading a license for use on another machine that does not have access to the external network, and thus cannot access the QIAGEN Aarhus servers.

After selecting your method of choice, click on the button labeled **Next**.

Direct download

After choosing the **Direct Download** option and clicking on the button labeled **Next**, a dialog similar to that shown in figure 1.14 will appear if the license is successfully downloaded and installed.

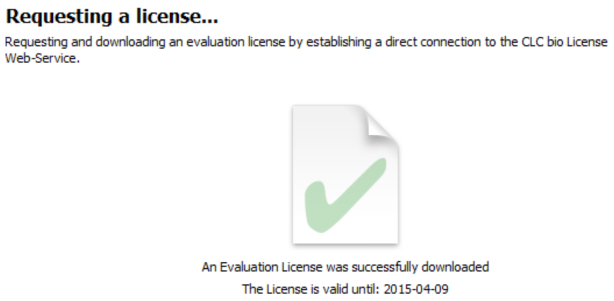
When the license has been downloaded and installed, the **Next** button will be enabled.

If there is a problem, a dialog will appear indicating this.

Go to license download web page

After choosing the **Go to CLC License Download web page** option and clicking on the button labeled **Next**, the license download form will be opened in a web browser, as shown in figure 1.15.

Click on the **Download License** button and then save the license file.



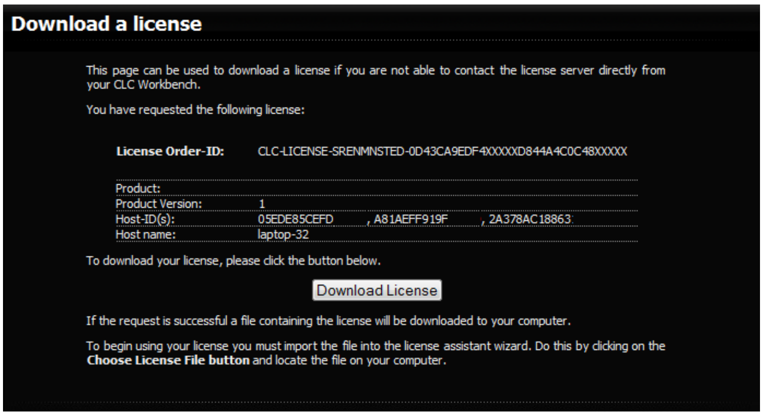
Requesting a license...

Requesting and downloading an evaluation license by establishing a direct connection to the CLC bio License Web-Service.

An Evaluation License was successfully downloaded

The License is valid until: 2015-04-09

Figure 1.14: *A license has been successfully downloaded and installed for use.*



Download a license

This page can be used to download a license if you are not able to contact the license server directly from your CLC Workbench.

You have requested the following license:

License Order-ID:	CLC-LICENSE-SRENMNSTED-0D43CA9EDF4XXXXXD844A4C0C48XXXXX
Product:	
Product Version:	1
Host-ID(s):	05EDE85CEFD , A81AEFF919F , 2A378AC18863
Host name:	laptop-32

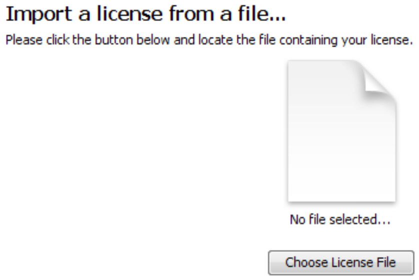
To download your license, please click the button below.

If the request is successful a file containing the license will be downloaded to your computer.

To begin using your license you must import the file into the license assistant wizard. Do this by clicking on the **Choose License File button** and locate the file on your computer.

Figure 1.15: *The license download form opened in a web browser.*

Back in the Workbench window, you will now see the dialog shown in 1.16.



Import a license from a file...

Please click the button below and locate the file containing your license.

No file selected...

Figure 1.16: *Importing the license file downloaded from the web page.*

Click on the **Choose License File** button, find the saved license file and select it. Then click on the **Next** button.

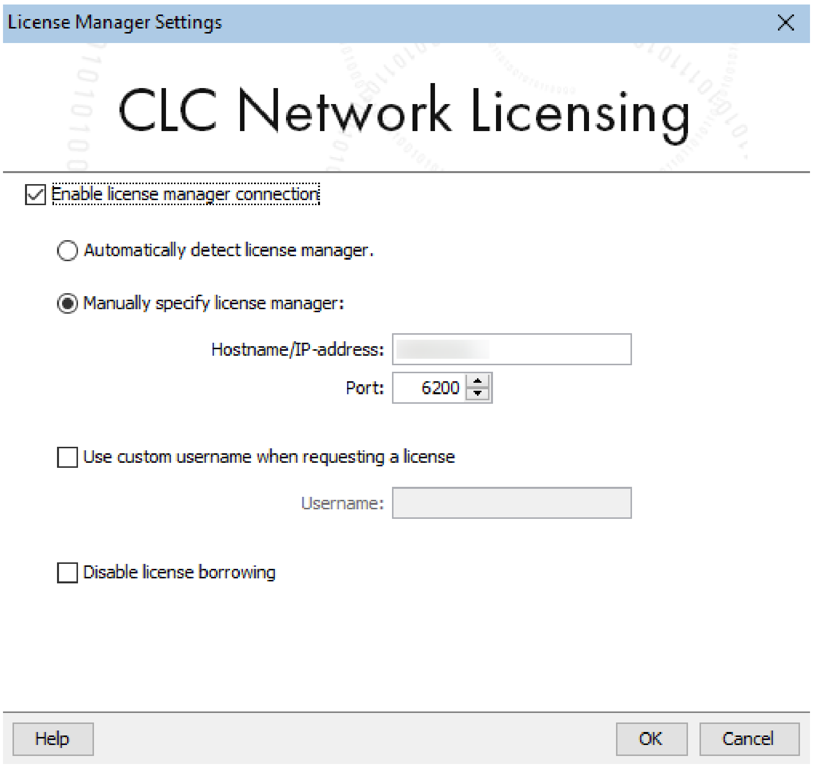
Accepting the license agreement

The End User License Agreement (EULA) must be read and accepted as part of the installation process. Please read the EULA text carefully, and if you agree to it, check the box next to the text **I accept these terms**. If further information is requested from you, please fill this in before clicking on the **Finish** button.

1.4.5 Configure license manager connection

If your organization is running a *CLC Network License Manager*, you can configure your Workbench to connect to it to get a license.

To configure a Workbench to connect to a *CLC Network License Manager*, select the **Configure license manager connection** option and click on the **Next** button. A dialog appears, as shown in figure 1.17.



License Manager Settings

CLC Network Licensing

☑ Enable license manager connection

○ Automatically detect license manager.

● Manually specify license manager:

Hostname/IP-address:

Port: 6200

□ Use custom username when requesting a license

Username:

□ Disable license borrowing

Figure 1.17: *Configuring the connection to a CLC Network License Manager.*

The options in that dialog are:

- **Enable license manager connection.** This box must be checked for the Workbench is to contact the *CLC Network License Manager* to get a license for the *CLC Main Workbench*.
- **Automatically detect license manager.** By checking this option the Workbench will look for a *CLC Network License Manager* accessible from the Workbench. Automatic server discovery sends UDP broadcasts from the Workbench on port 6200. Available license servers respond to the broadcast. The Workbench then uses TCP communication for to get a license, if one is available. Automatic server discovery works only on local networks and will not work on WAN or VPN connections. Automatic server discovery is not guaranteed to work on all networks. If you are working on an enterprise network on where local firewalls or routers cut off UDP broadcast traffic, then you may need to configure the details of the *CLC Network License Manager* using the **Manually specify license manager** option instead.
- **Manually specify license manager.** Select this option to enter the details of the machine the *CLC Network License Manager* is running on, specifically:

  - **Host name**. The address of the machine the *CLC Network License Manager* is running on.
  - **Port**. The port used by the *CLC Network License Manager* to receive requests.

- **Use custom username when requesting a license**. Optional. When unchecked (the default), the username of the account being used to run the Workbench is the username used when contacting the license manager. When this option is checked, a different username can be entered for that purpose. Note that borrowing licenses is not supported with custom usernames.

- **Disable license borrowing on this computer**. Check this box if you do not want users of this Workbench to borrow a license. See section 1.4.5 for further details.

Releasing Workbench network licenses

Once a network license for a *CLC Workbench* has been obtained, the Workbench must be shut down to release that license for others to use. While it is possible for a license to be pulled from a running Workbench, in practice, that Workbench will immediately retrieve the license just released.

Modules and network licenses

A relevant license is needed to run tools delivered by modules, and to submit jobs to be run on the cloud via a *CLC Workbench*.

To maximize availability, module licenses are first checked out when a job requiring the module is undertaken. Module licenses are checked back in (returned) when the *CLC Workbench* is closed, or four hours after the most recent job requiring that license was launched, whichever is shortest.

Job completion does not depend on the *CLC Workbench* having a module license checked out.

A note about borrowing module licenses: If you plan to borrow network module licenses and the *CLC Network License Manager* will not be continuously accessible from the *CLC Workbench*, then while there is still a connection, launch a job that requires the relevant module. If available, the relevant license will be checked out, and can then be borrowed. When a module license is borrowed, the four hour validity period mentioned above is not relevant. The module licenses are borrowed for the time period that you specify.

See section 1.4.5 for further information about borrowing a license.

Borrowing a license

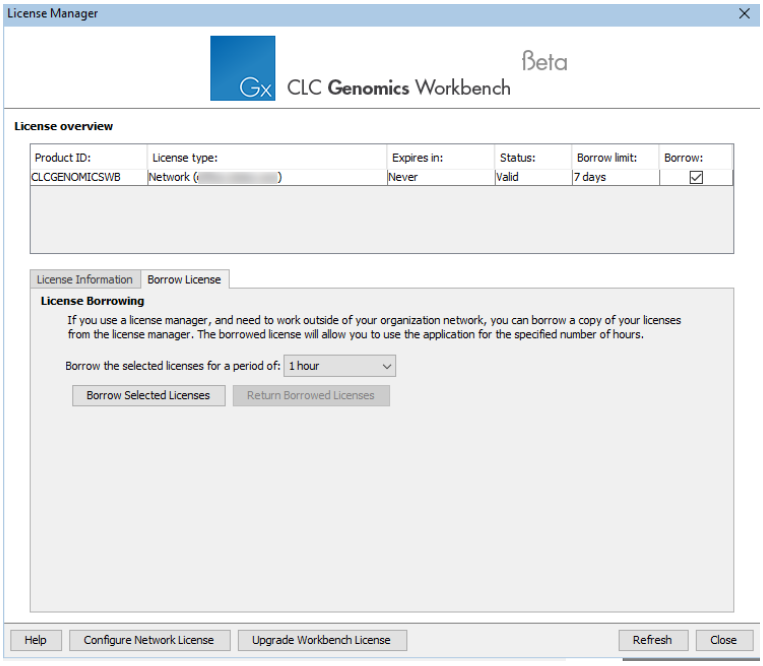
A *CLC Main Workbench* using a network license normally needs to maintain a connection to the *CLC Network License Manager*. However, if allowed by the network license administrator, network licenses can be *borrowed* for offline use for a period of time. While the license is borrowed, there is one less network license available for other users. Borrowed licenses can be returned early.

The Workbench must be connected to the *CLC Network License Manager* at the point when the license is borrowed or returned. The procedure for borrowing a license is:

1. Go to the Workbench menu option:

Help | License Manager...

2. Click on the "Borrow License" tab to display the license borrowing settings (figure 1.18).



License overview

Product ID:	License type:	Expires in:	Status:	Borrow limit:	Borrow:
CLCGENOMICSWB	Network ([redacted])	Never	Valid	7 days	☑

License Borrowing

If you use a license manager, and need to work outside of your organization network, you can borrow a copy of your licenses from the license manager. The borrowed license will allow you to use the application for the specified number of hours.

Borrow the selected licenses for a period of: 1 hour

Figure 1.18: *Borrowing a license from a CLC Network License Manager.*

3. Select the license(s) that you wish to borrow by clicking in the checkboxes in the Borrow column in the License overview panel.

   If you plan to borrow module licenses but they are not listed, start a job that requires that module. This will check out the relevant module license, so that it becomes available to borrow.

4. Choose the length of time you wish to borrow the license(s) for using the drop down list in the Borrow License tab. By default the maximum is 7 days, but network license administrators can specify a lower limit than this.

5. Click **Borrow Selected Licenses**.

6. Close the License Manager when you are done.

You can now go offline and continue working with the *CLC Main Workbench*. When the time period you borrowed the license for has elapsed, the network license will be again made available for other users. To continue using *CLC Main Workbench* with a license, you will need to connect to the network again so the Workbench can request another license.

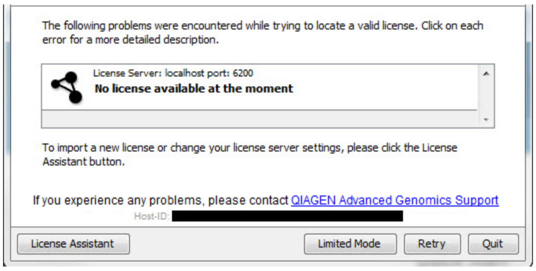
You can return borrowed licenses early opening up the **License Manager**, going to the "Borrow License" tab, and clicking on the **Return Borrowed Licenses** button.

Common issues when using a network license

Some issues that may be experienced when using network licenses are:

- No license is available at that point in time.

  If all licenses for your product are in use, a message saying "No license available at the moment" will appear (figure 1.19).



The following problems were encountered while trying to locate a valid license. Click on each error for a more detailed description.

License Server: localhost port: 6200

No license available at the moment

To import a new license or change your license server settings, please click the License Assistant button.

If you experience any problems, please contact QIAGEN Advanced Genomics Support

Host-ID: [redacted]

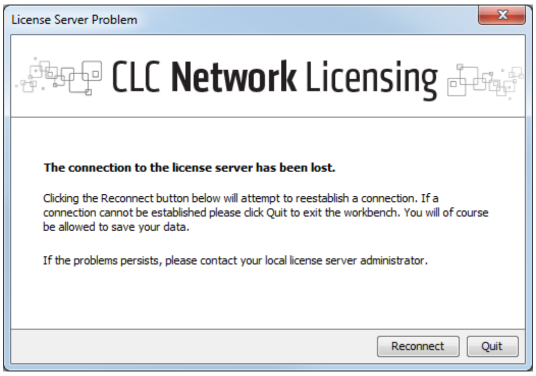
Figure 1.19: *When there are no available network licenses for the software, a message appears to indicate this.*

  After at least one license is returned to the pool, you will be able to run the software and get the necessary license. If running out of licenses is a frequent issue, you may wish to discuss this with your administrator.

  Data can be viewed, imported and exported, and very basic analyses launched, by running the Workbench in Viewing Mode. Click on the **Viewing Mode** button in that dialog to launch the Workbench in this mode.

- The connection to the CLC Network License Manager is lost.

  If the Workbench connection to the *CLC Network License Manager* is lost, you will see a dialog like that shown in figure 1.20.



License Server Problem

CLC Network Licensing

The connection to the license server has been lost.

Clicking the Reconnect button below will attempt to reestablish a connection. If a connection cannot be established please click Quit to exit the workbench. You will of course be allowed to save your data.

If the problems persists, please contact your local license server administrator.

Figure 1.20: *This Workbench was unable to establish a connection to obtain a network license.*

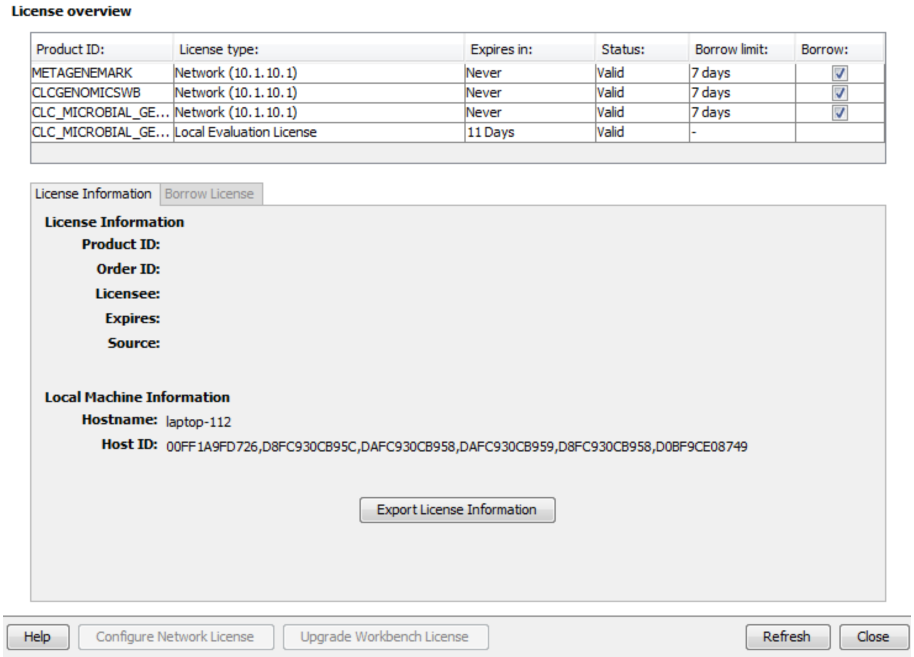
  If you have chosen the option to **Automatically detect license manager** and you have not succeeded in connecting to the *CLC Network License Manager* before, please check with your local IT support that automatic detection is possible at your site. If it is not, you will need to specify the settings, as described earlier in this section.

  If you have successfully contacted the *CLC Network License Manager* from your Workbench previously, please contact your local administrator. Common issues include that the *CLC Network License Manager* is not running or that network details have changed.

1.4.6 Viewing or updating license information

To view or edit information about the n license(s) the Workbench is currently using, open the Workbench's License Manager (figure 1.21) using the menu option:

Help | License Manager (▤)



License overview

Product ID:	License type:	Expires in:	Status:	Borrow limit:	Borrow:
METAGENEMARK	Network (10.1.10.1)	Never	Valid	7 days	☑
CLCGENOMICSWB	Network (10.1.10.1)	Never	Valid	7 days	☑
CLC_MICROBIAL_GE...	Network (10.1.10.1)	Never	Valid	7 days	☑
CLC_MICROBIAL_GE...	Local Evaluation License	11 Days	Valid	-	

License Information

Product ID:
Order ID:
Licensee:
Expires:
Source:

Local Machine Information

Hostname: laptop-112
Host ID: 00FF1A9FD726,D8FC930CB95C,DAFC930CB958,DAFC930CB959,D8FC930CB958,D0BF9CE08749

License Information
Borrow License
Export License Information
Help
Configure Network License
Upgrade Workbench License
Refresh
Close

Figure 1.21: *License information and license-related functionality is available in the Workbench License Manager.*

The Workbench's License Manager can be used to:

- See information about its license (e.g. the license type, when it expires, etc.)
- Configure the connection to a *CLC Network License Manager*. Click on the **Configure Network License** button at the lower left corner to open the dialog seen in figure 1.17.
- Upgrade from an evaluation license. Click on the **Upgrade Workbench License** button to open the dialog shown in figure 1.2.
- Export license information to a text file.
- Borrow a license from a *CLC Network License Manager* when network licenses are in use.

If you wish to switch away from using a network license, click on the button to **Configure Network License** and uncheck the box beside the text **Enable license manager connection** in the dialog. When you restart the Workbench, you can set up the new license as described in section 1.4.

1.4.7 Download a static license on a non-networked machine

To download a static license for a machine that does not have direct access to the external network, you can follow the steps below:

- Install the *CLC Main Workbench* on the machine you wish to run the software on.
- Start up the software as an administrative user and find the host ID of the machine that you will run the CLC Workbench on. You can see the host ID of the machine at the bottom of the License Assistant window in grey text, or, if working in Viewing Mode, by launching the **License Manager** from under the Workbench **Help** menu option.
- Make a copy of this host ID such that you can use it on a machine that has internet access.
- Go to a computer with internet access, open a browser window and go to the network license download web page:

  `https://secure.clcbio.com/LmxWSv3/GetLicenseFile`
- Paste in your license order ID and the host ID that you noted down in the relevant boxes on the web page.
- Click on 'Download License' and save the resulting .lic file.
- Open the Workbench on your non-networked machine. In the Workbench license manager choose 'Import a license from a file'. In the resulting dialog click on the 'Choose License File' button and then locate and selct the .lic file you have just downloaded.

  If the License Manager does not start up by default, you can start it up by going to the menu option:

  Help | **License Manager** ()
- Click on the **Next** button and go through the remaining steps to install the license.

1.4.8 Viewing mode

Using a CLC Workbench in Viewing Mode is a free and easy way to access extensive data viewing capabilities, basic bioinformatics analysis tools, as well as import and export functionality.

Data viewing

Any data type supported by the Workbench being used can be viewed in Viewing Mode. Plugins or modules can also be installed when in Viewing Mode, expanding the range of data types supported.

Viewing Mode of the CLC Workbenches can be particularly useful when sharing data with colleagues or reviewers who wish to view and investigate data you have generated but who do not have access to a Workbench license.

Data import, export and analysis in Viewing Mode

When working in Viewing Mode, the Import and Export buttons in the top Toolbar are enabled, and standard import and export functionality for many bioinformatics data types is supported. Tools available can be seen in the Workbench Tools menu, as illustrated in figure 1.22.

Starting a CLC Workbench in Viewing Mode

A button labeled **Viewing Mode** is presented in the Workbench License Manager when a Workbench is started up without a license installed, as shown in figure 1.23. This button is also

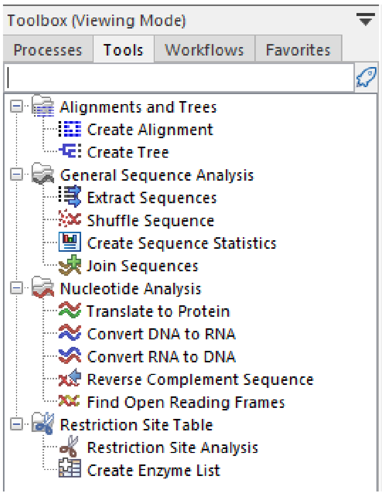


Figure 1.22: *Bioinformatics tools available when using Viewing Mode are found under the Tools menu.*

visible in message windows that appear if a Workbench is started up that has an expired license or that is configured to use a network license but all the available licenses have been checked out by others, as described in section 1.4.5.

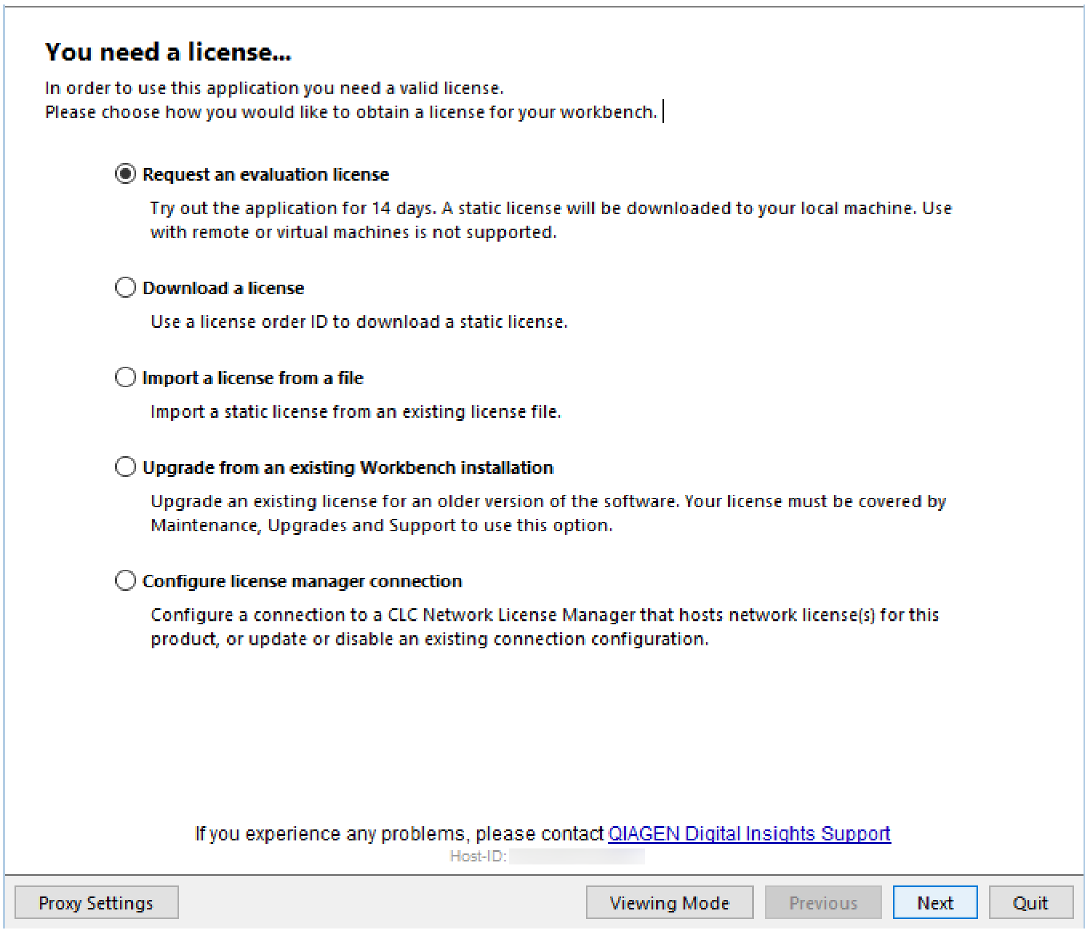
Click on the **Viewing Mode** button to start up the Workbench in Viewing Mode.

To go from running in Viewing Mode to running a Workbench with its full functionality, it just needs to have access to a valid license. This can be done by installing a static license, or when using a network license, by restarting the Workbench when licenses are once again available.

1.4.9 Start in safe mode

If the program becomes unstable on start-up, you can start it in **Safe mode**. This is done by pressing and holding down the Shift button while the program starts.

When starting in safe mode, the user settings (e.g. the settings in the **Side Panel**) are deleted and cannot be restored. Your data stored in the **Navigation Area** is not deleted. When started in safe mode, some of the functionalities are missing, and you will have to restart the *CLC Main Workbench* again (without pressing Shift).



You need a license...

In order to use this application you need a valid license.
Please choose how you would like to obtain a license for your workbench.

● **Request an evaluation license**

Try out the application for 14 days. A static license will be downloaded to your local machine. Use with remote or virtual machines is not supported.

○ **Download a license**

Use a license order ID to download a static license.

○ **Import a license from a file**

Import a static license from an existing license file.

○ **Upgrade from an existing Workbench installation**

Upgrade an existing license for an older version of the software. Your license must be covered by Maintenance, Upgrades and Support to use this option.

○ **Configure license manager connection**

Configure a connection to a CLC Network License Manager that hosts network license(s) for this product, or update or disable an existing connection configuration.

If you experience any problems, please contact QIAGEN Digital Insights Support

Host-ID:

Figure 1.23: *Click on the Viewing Mode button at the bottom of the License Manager window to launch the Workbench in Viewing Mode.*

1.5 Plugins

The functionality of the *CLC Main Workbench* can be extended by installing plugins. The built-in Plugin Manager provides an up-to-date listing of the plugins available.

Alternatively, visit our plugin webpage for a list: `https://digitalinsights.qiagen.com/products-overview/plugins/`.

Plugins are installed and uninstalled using the **Plugin Manager**, which can be opened using the **Manage Plugins ( ) button in the Toolbar**, or by going to the top level menu:

Utilities | Manage Plugins... ( )

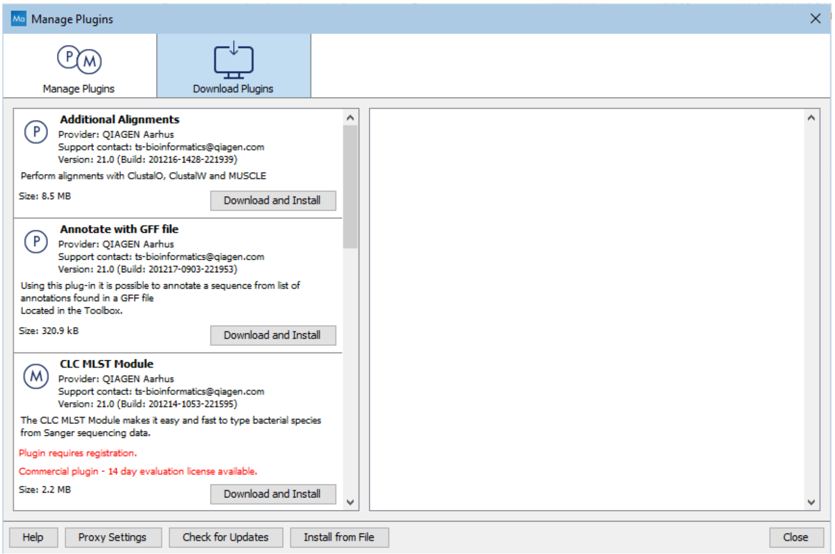
Note: To install plugins and modules using a centrally installed *CLC Workbench*, the software must be run in *administrator mode*. On Windows, right-click on the program shortcut and choose "Run as Administrator". On Linux, this usually means running the software with sudo privileges.

The Plugin Manager has two tabs at the top:

- **Manage Plugins** An overview of plugins that are installed.
- **Download Plugins** An overview of plugins available from QIAGEN that are not yet installed on your Workbench.

1.5.1 Install

To install a plugin, open the Plugin Manager and click on the **Download Plugins** tab. This will display an overview of the plugins available (figure 1.24).



Additional Alignments
Provider: QIAGEN Aarhus
Support contact: ts-bioinformatics@qiagen.com
Version: 21.0 (Build: 201216-1428-221939)
Perform alignments with ClustalO, ClustalW and MUSCLE
Size: 8.5 MB

Annotate with GFF file
Provider: QIAGEN Aarhus
Support contact: ts-bioinformatics@qiagen.com
Version: 21.0 (Build: 201217-0903-221953)
Using this plug-in it is possible to annotate a sequence from list of annotations found in a GFF file
Located in the Toolbox.
Size: 320.9 kB

CLC MLST Module
Provider: QIAGEN Aarhus
Support contact: ts-bioinformatics@qiagen.com
Version: 21.0 (Build: 201214-1053-221595)
The CLC MLST Module makes it easy and fast to type bacterial species from Sanger sequencing data.
Plugin requires registration.
Commercial plugin - 14 day evaluation license available.
Size: 2.2 MB

Figure 1.24: *The plugins that are available for download.*

Select a plugin in the list to display additional information about it in the right hand pane. Click on **Download and Install** to to install the plugin.

Accepting the license agreement

The End User License Agreement (EULA) must be read and accepted as part of the installation process. Please read the EULA text carefully, and if you agree to it, check the box next to the text **I accept these terms**. If further information is requested from you, please fill this in before clicking on the **Finish** button.

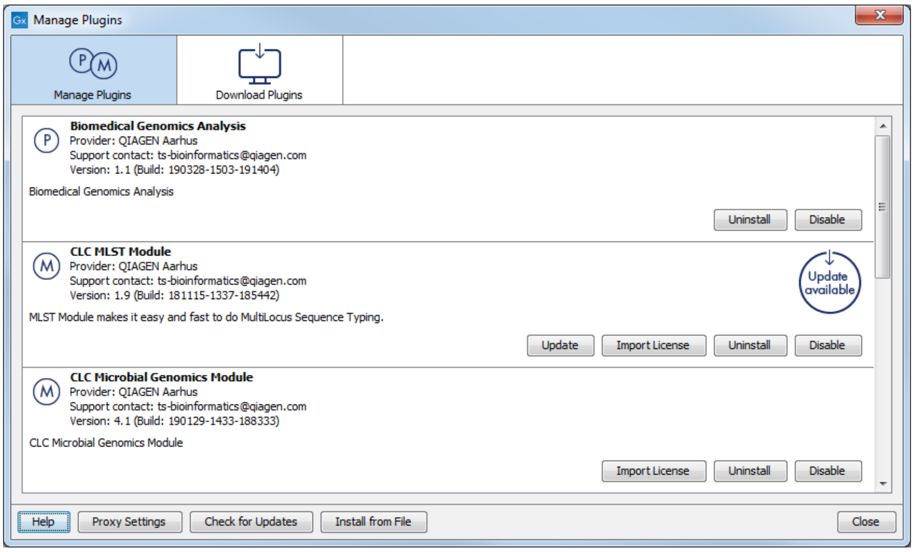
If you have a .cpa plugin installer file on your computer, for example if you have downloaded it from our website, install the plugin by clicking on the **Install from File** button at the bottom of the dialog and specifying the plugin *.cpa file.

When you close the Plugin Manager after making changes, you will be prompted to restart the software. Plugins will not be fully installed, or removed, until the *CLC Workbench* has been restarted.

1.5.2 Uninstall

Plugins are uninstalled using the Plugin Manager (figure 1.25). This can be opened using the **Manage Plugins ( ) button in the Toolbar**, or by going to the top level menu:

Utilities | **Manage Plugins... ( )**



Biomedical Genomics Analysis
Provider: QIAGEN Aarhus
Support contact: ts-bioinformatics@qiagen.com
Version: 1.1 (Build: 190328-1503-191404)
Biomedical Genomics Analysis

CLC MLST Module
Provider: QIAGEN Aarhus
Support contact: ts-bioinformatics@qiagen.com
Version: 1.9 (Build: 181115-1337-185442)
MLST Module makes it easy and fast to do MultiLocus Sequence Typing.

CLC Microbial Genomics Module
Provider: QIAGEN Aarhus
Support contact: ts-bioinformatics@qiagen.com
Version: 4.1 (Build: 190129-1433-188333)
CLC Microbial Genomics Module

Figure 1.25: *The plugin manager with plugins installed.*

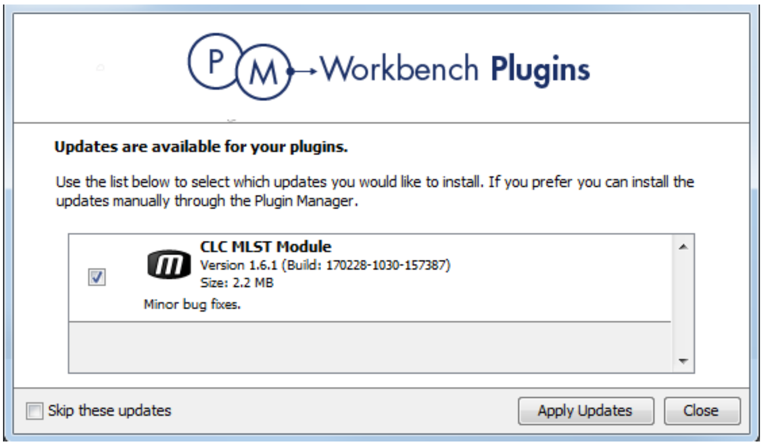
The installed plugins are shown in the **Manage plugins** tab of the plugin manager. To uninstall, select the plugin in the list and click **Uninstall**.

If you do not wish to completely uninstall the plugin, but you do not want it to be used next time you start the Workbench, click the **Disable** button.

When you close the dialog, you will be asked whether you wish to restart the workbench. The plugin will not be uninstalled until the workbench is restarted.

1.5.3 Updating plugins

If a new version of a plugin is available, you will get a notification during start-up (figure 1.26).



Updates are available for your plugins.

Use the list below to select which updates you would like to install. If you prefer you can install the updates manually through the Plugin Manager.

☑ **CLC MLST Module**
Version 1.6.1 (Build: 170228-1030-157387)
Size: 2.2 MB
Minor bug fixes.

□ Skip these updates

Figure 1.26: *Plugin updates.*

In this list, select which plugins you wish to update, and click **Install Updates**. If you press **Cancel** you will be able to install the plugins later by clicking **Check for Updates** in the Plugin manager (see figure 1.25).

1.6 Network configuration

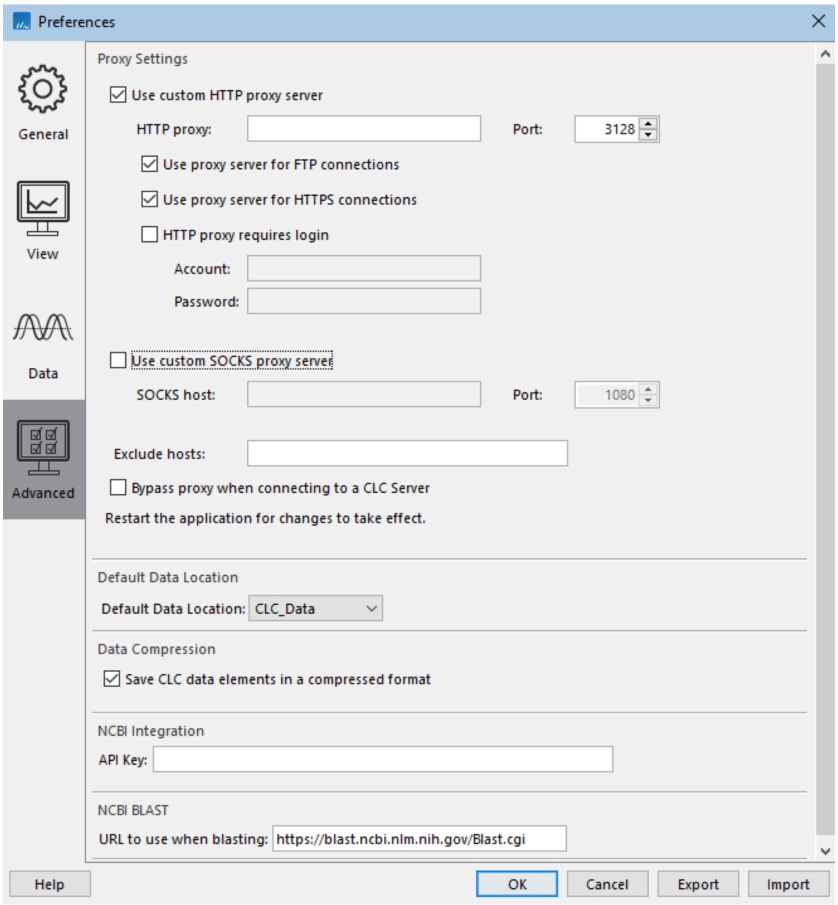
If you use a proxy server to access the Internet you must configure *CLC Main Workbench* to use this. Otherwise you will not be able to perform any online activities.

CLC Main Workbench supports the use of an HTTP-proxy and an anonymous SOCKS-proxy.

To configure your proxy settings, go to **Edit | Preferences** and choose the **Advanced** tab (figure 1.27).

You have the choice between an HTTP-proxy and a SOCKS-proxy. The *CLC Workbench* only supports the use of a SOCKS-proxy that does not require authorization.

You can select whether the proxy should also be used for FTP and HTTPS connections.



Proxy Settings

☑ Use custom HTTP proxy server

HTTP proxy: Port: 3128

☑ Use proxy server for FTP connections

☑ Use proxy server for HTTPS connections

☐ HTTP proxy requires login

Account:

Password:

☐ Use custom SOCKS proxy server

SOCKS host: Port: 1080

Exclude hosts:

☐ Bypass proxy when connecting to a CLC Server

Restart the application for changes to take effect.

Default Data Location

Default Data Location: CLC_Data

Data Compression

☑ Save CLC data elements in a compressed format

NCBI Integration

API Key:

NCBI BLAST

URL to use when blasting: https://blast.ncbi.nlm.nih.gov/Blast.cgi

Figure 1.27: *Advanced preference settings include proxy configuration options.*

List hosts that should be contacted directly, i.e. not via the proxy server, in the **Exclude hosts** field. The value can be a list, with each host separated by a `|` symbol. The wildcard character `*` can also be used. For example: `*.foo.com|localhost`.

The proxy can be bypassed when connecting to a *CLC Server* by checking the box next to **Bypass proxy when connecting to a CLC Server**.

Workbenches can be preconfigured to bypass the proxy settings when connecting to a *CLC Server* by configuring this setting in a `proxy.properties` file, where the IP address (not the host name) of the *CLC Server* is provided in the *proxyexclude* field. See https://resources.qiagenbioinformatics.com/manuals/workbenchdeployment/current/index.php?manual=Per_computer_Workbench_information.html for further details.

If you have problems with these settings, please contact your systems administrator.

Part II

Core Functionalities

Chapter 2

User interface

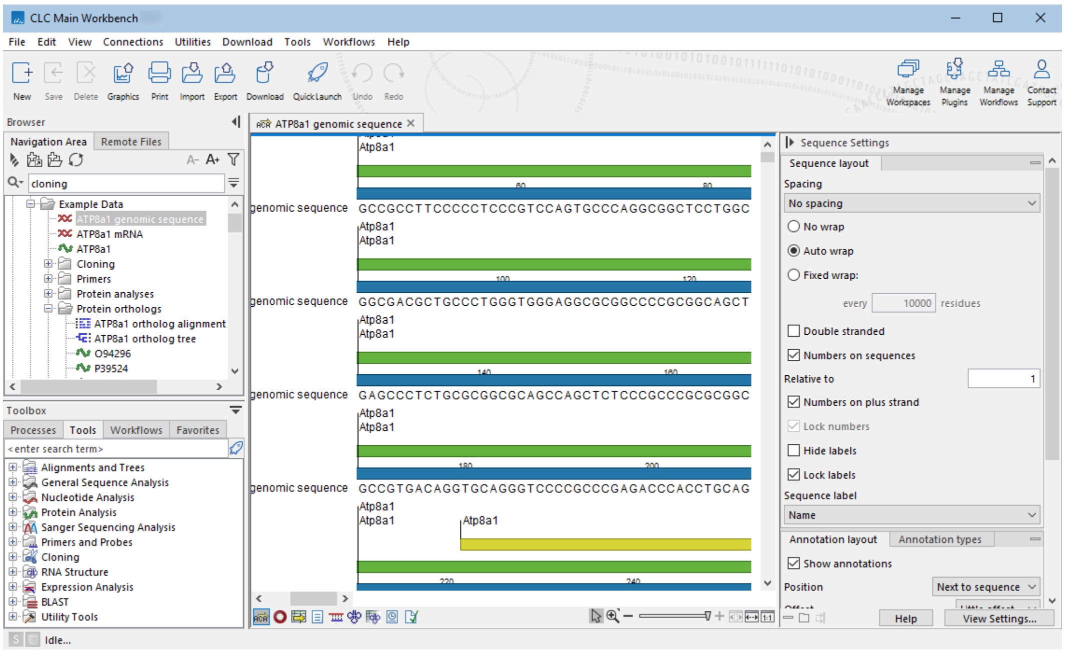
Contents

The user interface of the *CLC Main Workbench* when it is first opened looks like that shown in figure 2.1.

Key areas are listed below with a brief description and links to further information.

- **Navigation Area** Data elements stored in File Locations are listed in the Navigation Area. (Section 3.1).
- **Toolbox Area** This area contains 4 tabs:
  - **Processes** Running and finished processes are listed under this tab. (Section 2.4)
  - **Tools** Analysis tools are available under this tab. (Section 2.3)
  - **Workflows** Template workflows and installed workflows are available under this tab. Apart from running workflows from here, you can right-click on them and choose to open a copy of the workflow. This opens a copy in the Workflow Editor (section 13.1).
  - **Favorites** Tools you use most often are listed here, and you can add tools you want, for quick access. (Section 2.3)

- **View Area** Data and workflows can be opened in this area for viewing and editing. (Section 2.1) A **Side Panel** with configuration options is present on the right hand side when items are open in this area (Section 4.6).
- **Menu bar** and **Toolbar** Many tools and associated actions can be launched using buttons and options in these areas.
- **Status Bar** The Workbench status and its connections to other systems is presented in this area. (Section 2.4).



genomic sequence GCCGCCTTCCCCCTCCCGTCCAGTGCCCAGGCGGCTCCTGGC

genomic sequence GGCGACGCTGCCCTGGGTGGGAGGCGCGGCCCCGCGGCAGCT

genomic sequence GAGCCCTCTGCGCGGCGCAGCCAGCTCTCCCGCCCGCGCGGC

genomic sequence GCCGTGACAGGTGCAGGGTCCCCGCCCGAGACCCACCTGCAG

Sequence Settings

Sequence layout

Spacing: No spacing

○ No wrap

● Auto wrap

○ Fixed wrap: every 10000 residues

☐ Double stranded

☑ Numbers on sequences

Relative to 1

☑ Numbers on plus strand

☑ Lock numbers

☐ Hide labels

☑ Lock labels

Sequence label: Name

Annotation layout Annotation types

☑ Show annotations

Position: Next to sequence

Figure 2.1: *The* **CLC Workbench** *interface includes the Navigation Area in the top left, several tabs in the Toolbox area at the bottom left, a large viewing area on the right, menus and toolbars at the top, and a status bar at the bottom. A sequence has been opened in the View area, and a Side Panel containing settings relevant to viewing sequences is thus present on the far right.*

Different areas of the interface can be hidden or made visible, as desired. Options controlling this are available under the View menu at the top. For example, whether the Toolbox panel should be visible, and which tabs should be visible in the Toolbox area can be configured using the menu options under:

View | Show/Hide Toolbox

You can also collapse the various areas by clicking on buttons like (▼) or (◀|), where they appear. Similar buttons are presented for revealing areas if they are hidden.

2.1 View Area

The **View Area** is where elements are displayed when opened. Each open element is shown in a view, with a tab at the top containing the element's name (figure 2.2). Mouse-over a tab to reveal a tooltip containing summary information about the element.

Tabs can be dragged to put them in the order desired, and to move them to a different area in a split view (section 2.1.4).

Right-clicking on a tab opens a menu with various navigation options, as well as the ability to select tools and viewing options, etc.

Opening and viewing elements in the View Area

There are multiple ways to open an element in the View Area, including:

1. Double click on an element in the Navigation Area.
2. Right-click on an element in the Navigation Area, and choose the **Show** option from the context menu.
3. Drag elements from the Navigation Area into the Viewing Area.
4. Select an element in the Navigation Area and use the keyboard shortcut Ctrl + O (⌘ + O on macs)
5. Choose the option to "Open" results when launching an analysis. (This is only recommended when small numbers of elements will be generated, and where it is not important to save the results directly.)

When opening an element while another element is already open, the newly opened element will become the active tab. Click on any other tab to open it and make it the active view. Alternatively, use the keyboard shortcuts to navigate between tabs: Ctrl + PageUp or PageDown (or ⌘ + PageUp or PageDown on macs).

To provide more space for viewing data, you can hide Navigation Area and Toolbox by clicking the hide icon (◀|) at the top of the Navigation Area. You can also hide the Side Panel using the same icon at the top of the Side Panel.

Tooltips

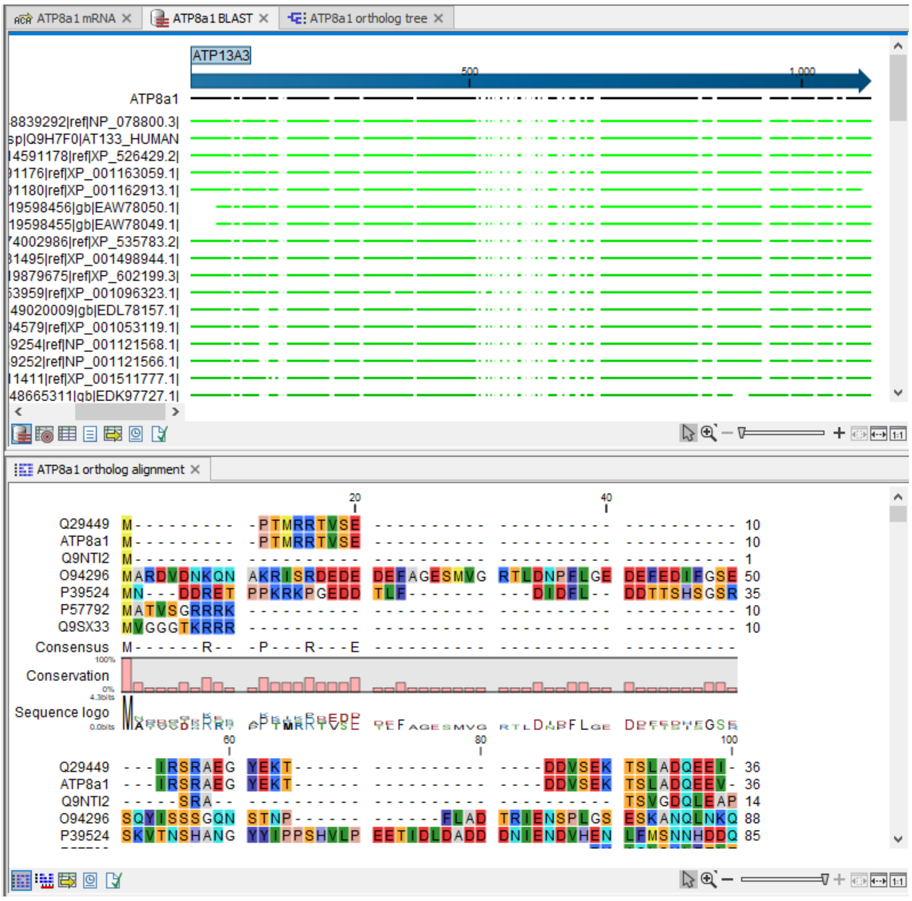
For some data types and some views, tooltips provide additional useful information. Hover the mouse cursor over an area of interest to reveal these. For example, hover over an annotation on a sequence and a tooltip containing details about that annotation is shown. Hover over a variant in a variant track, and information about that variant is shown.

If you wish to hide such tooltips while moving the mouse around in a view, hold down the Ctrl key.

Tooltips can take a moment to appear. To make them show up immediately while moving the mouse around in a view, hold down the Shift key.

Accessing different views of the same data element

Various views of a data element are available. These can be opened by clicking on the icons at the bottom of an open view. The last two icons are always those for opening the **History** (🕘) and **Element Info** (📝) views. These are described in section 2.5.



```
Q29449  M--------- -PTMRRTVSE ---------- ---------- ---------- 10
ATP8a1  M--------- -PTMRRTVSE ---------- ---------- ---------- 10
Q9NTI2  M--------- ---------- ---------- ---------- ---------- 1
O94296  MARDVDNKQN AKRISRDEDE DEFAGESMVG RTLDNPFLGE DEFEDIFGSE 50
P39524  MN---DDRET PPKRKPGEDD TLF------- ---DIDFL-- DDTTSHSGSR 35
P57792  MATVSGRRRK ---------- ---------- ---------- ---------- 10
Q9SX33  MVGGGTKRRR ---------- ---------- ---------- ---------- 10
Consensus M------R-- -P---R---E ---------- ---------- ----------

Q29449  ---IRSRAEG YEKT------ ---------- ----DDVSEK TSLADQEEI- 36
ATP8a1  ---IRSRAEG YEKT------ ---------- ----DDVSEK TSLADQEEV- 36
Q9NTI2  -----SRA-- ---------- ---------- ---------- TSVGDQLEAP 14
O94296  SQYISSSGQN STNP------ ------FLAD TRIENSPLGS ESKANQLNKQ 88
P39524  SKVTNSHANG YYIPPSHVLP EETIDLDADD DNIENDVHEN LFMSNNHDDQ 85
```

Figure 2.2: *Four elements are open in the View Area, organized in 2 areas horizontally - 3 elements in the top area, and one in the bottom. The active view is in the top area, as indicated by the blue bar just under the tabs.*

For illustration, the icons for views available for sequence elements are shown in figure 2.3. Clicking on the **Show As Circular** (○) icon would present the sequence in a circular view. Mouse over any of these icons to see the type of view they represent.

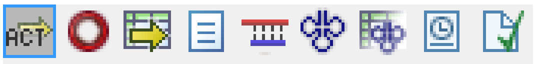


Figure 2.3: *The icons presented at the bottom of an open nucleotide sequence. Clicking on each of these presents a different view of the data.*

Linked views Different views of the same element, or different elements referred to by another element, can be opened in a "linked view". This is particularly useful with multiple viewing areas open, i.e. split views. When views are linked, selecting an item or region in one view brings the relevant item or region into focus in the linked view(s). See figure 2.4, where a region was selected in one view, and that selection is then also shown in the other view.

To open a linked view, keep the Ctrl key (⌘ on macs) depressed and then click on the item to open. E.g. to open a different view of the same element, click on one of the icons at the bottom of the open view. The new view will open, often in a second, horizontal view area. When the View

Area is already split horizontally, the new view is opened in the area not occupied by the original view.

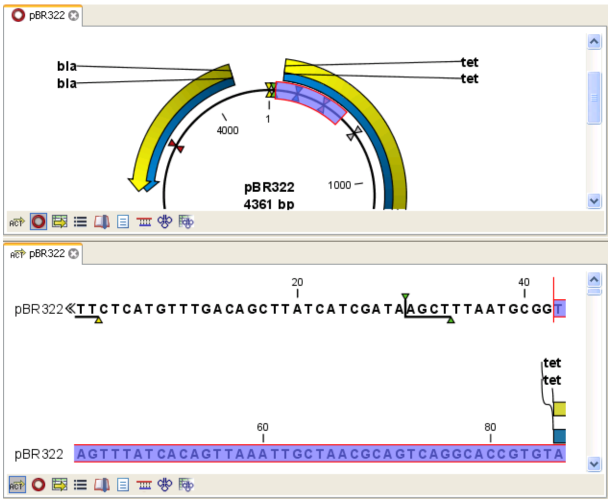

pBR322
bla
bla
tet
tet
4000
1
1000
pBR322
4361 bp
pBR322
20
40
pBR322 TTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGT
tet
tet
60
80
pBR322 AGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGTGTA

Figure 2.4: *A split view of the same sequence element*

Further information about split views is provided in section 2.1.4.

2.1.1 Close views

To close a view, click on the X to the right of its name in the top tab. Alternatively, right-click on the element tab and choose from the following options (figure 2.5):

- **Close Tab** Close just the selected tab.
- **Close Tab Group** When tabs are open in a split view, all tabs in the same area as the selected tab will be closed. When not in split view, this option has the same effect as **Close All Tabs**.
- **Close All Other Tabs** Close all tabs in all tab areas except the selected tab.
- **Close All Tabs** Close all tabs in all tab areas.

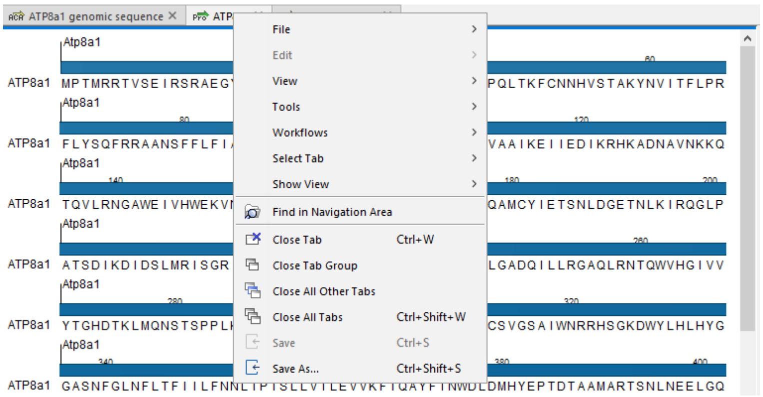
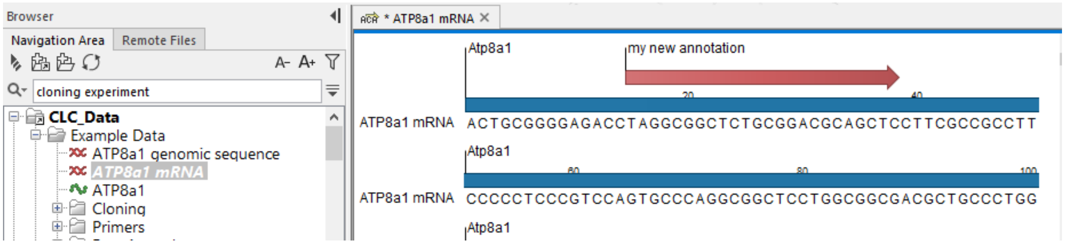


Figure 2.5: *Right-click on the tab for a view, to see the options relating to closing open views.*

2.1.2 Save changes in a view

When a new view is created, an * in front of the name of the view in the tab indicates that the element has not been saved yet. Similarly, when changes to an element are made in a view, an * is added before the element name on the tab and the element name is shown in *bold and italic* in the Navigation Area (figure 2.6).



ATP8a1 mRNA ACTGCGGGGAGACCTAGGCGGCTCTGCGGACGCAGCTCCTTCGCCGCCTT

ATP8a1 mRNA CCCCCTCCCGTCCAGTGCCCAGGCGGCTCCTGGCGGCGACGCTGCCCTGG

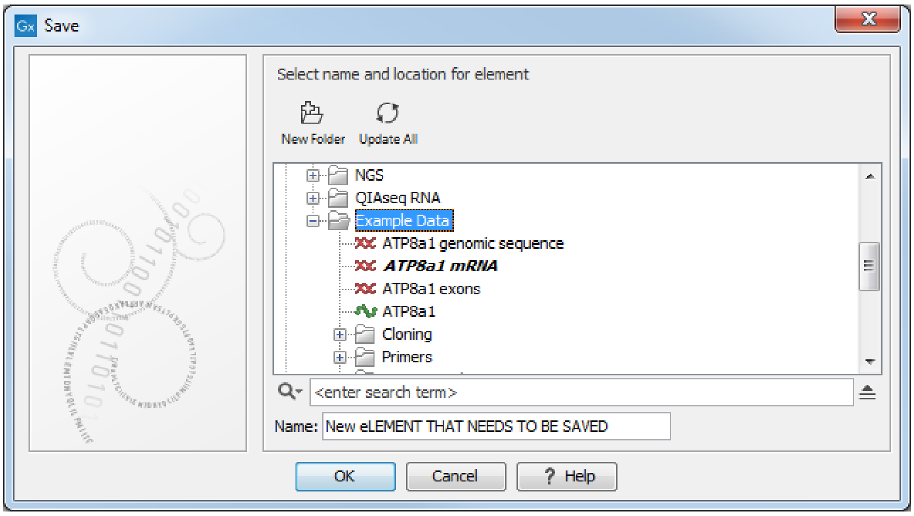
Figure 2.6: *The ATP8a1 mRNA element has been edited, but the changes are not saved yet. This is indicated by an * on the tab name in the View Area, and by the use of bold, italic font for the element's name in the Navigation Area.*

The **Save** function may be activated in two ways: Select the tab of the view you want to save and

Save (**) or Ctrl + S (⌘ + S on Mac)**

If you close a tab of a view containing an element that was edited, you will be asked if you want to save.

When saving an element from a new view that has not been opened from the Navigation Area, a save dialog appears (figure 2.7). In this dialog, you can name the element and select the folder in which you want to save the element.



Name: New eLEMENT THAT NEEDS TO BE SAVED

Figure 2.7: *Save dialog. The new element has been name "New element that needs to be saved" and will be saved in the "Example Data" folder.*

2.1.3 Undo/Redo

If you make a change to an element in a view, e.g. remove an annotation in a sequence or modify a tree, you can undo the action. In general, **Undo** applies to all changes you can make when right-clicking in a view. **Undo** is done by:

Click undo ( ) in the Toolbar or **Ctrl + Z**

If you want to undo several actions, just repeat the steps above.

To reverse the undo action:

Click the redo icon in the Toolbar or **Ctrl + Y**

Note! Actions in the Navigation Area, e.g., renaming and moving elements, cannot be undone. However, you can restore deleted elements (see section 3.1.8).

You can set the number of possible undo actions in the Preferences dialog, see section 4.

2.1.4 Arrange views in View Area

Views are arranged in the **View Area** by their tabs. The order of the views can be changed using drag and drop.

If a tab is dragged into a view, the area where the tab will be placed is highlighted blue. The blue area can be a tab bar in another view, or the bottom of an existing view. In that case, the tab will be moved to a new split view.

You can also split a View Area horizontally or vertically using the menus.

Splitting horizontally may be done this way:

right-click a tab of the view | View | Split Horizontally ( )

This action opens the chosen view below the existing view. When the split is made vertically, the new view opens to the right of the existing view (see figure 2.8).

A blue bar underneath the tab area indicates the active area, i.e. where the focus is at that time.

Splitting the View Area can be undone by dragging the tab of the bottom view to the tab of the top view, or by using the **Maximize/Restore View** function.

Select the view you want to maximize, and click

View | Maximize/restore View ( ) or **Ctrl + M**

right-click the tab | View | Maximize/restore View ( )

or **double-click the tab of view**

The following restores the size of the view:

View | Maximize/restore View ( ) or **Ctrl + M**

or **double-click title of view**

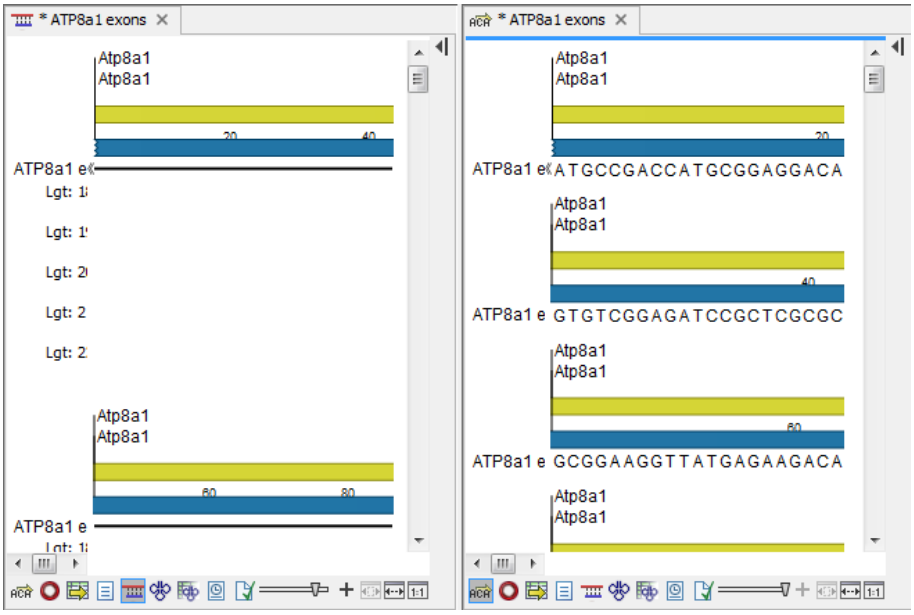

* ATP8a1 exons
Atp8a1
Atp8a1
20
40
ATP8a1 e
Lgt: 1
Lgt: 1
Lgt: 2
Lgt: 2
Lgt: 2
60
80
* ATP8a1 exons
ATP8a1 e ATGCCGACCATGCGGAGGACA
ATP8a1 e GTGTCGGAGATCCGCTCGCGC
ATP8a1 e GCGGAAGGTTATGAGAAGACA

Figure 2.8: *A vertical split screen.*

2.1.5 Moving a view to a different screen

Using multiple screens can be a great benefit when analyzing data with the *CLC Main Workbench*. You can move a view to another screen by dragging the tab of the view and dropping it outside the workbench window. Alternatively, you can right-click in the view area or on the tab itself and select **View | Move to New Window** from the context menu.

An example is shown in figure 2.9, where the main Workbench window shows a table of open reading frames, and the screen to the right is used to display the sequence and annotations.

You can make more detached windows, by dropping tabs outside the open workbench windows, or you can drag more tabs to a detached window. To get a tab back to the main workbench window, just drag the detached tab back, and drop it next to the other tabs in the top of the view area. **Note:** You should not drag the detached window header, just the tab itself.

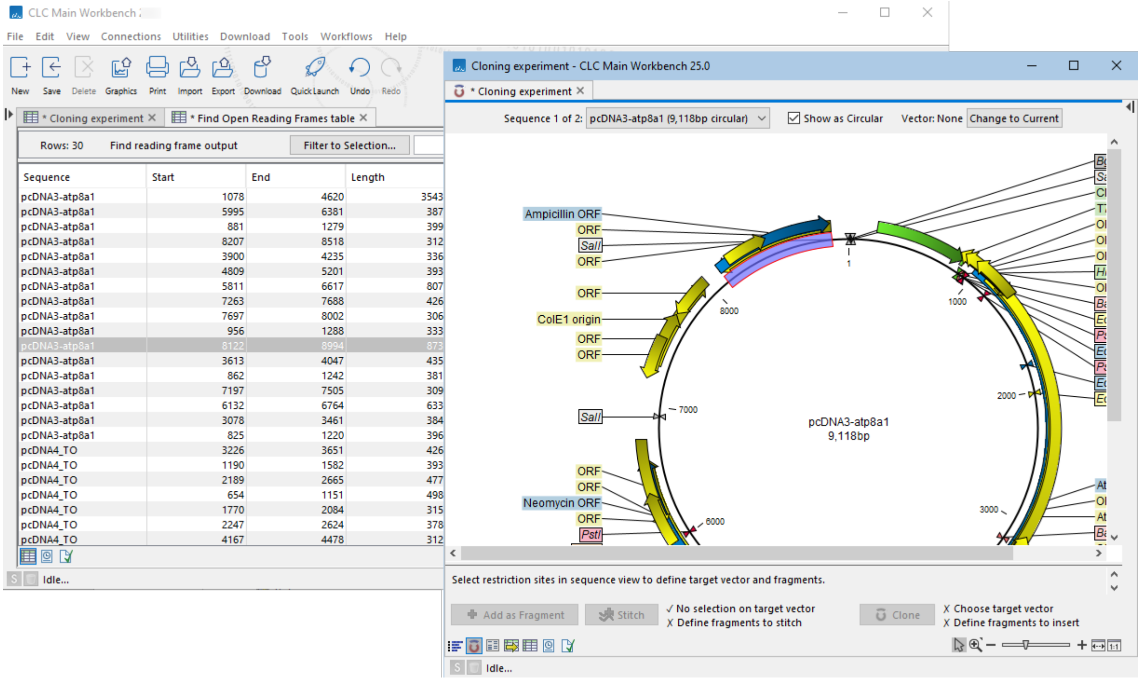
2.1.6 Side Panel

When an element is open for viewing, options for configuring that view are available in the **Side Panel**. The settings for a given view can be saved and re-used, as described in section 4.6.

The **Side Panel** is organized into palettes, where related settings are grouped (figure 2.10). The specific palettes and options depend on the data type and the view that is open. The buttons in the top right side of each palette can be used for expanding the contents for viewing (□), or collapsing (▭). Using the buttons at the bottom left side of the Side Panel, all the palettes can be expanded (□) or collapsed (▭).

Palettes can be placed at a different level in the Side Panel or undocked to be viewed anywhere on the screen (figure 2.11). For this, click on the palette name and while keeping the mouse depressed, drag the palette up and down, or drag it outside the *CLC Workbench*.

A palette can be re-docked by clicking on its tab name and dragging it back into the Side Panel.



CLC Main Workbench
File Edit View Connections Utilities Download Tools Workflows Help
New Save Delete Graphics Print Import Export Download Quick Launch Undo Redo
* Cloning experiment
* Find Open Reading Frames table
Cloning experiment - CLC Main Workbench 25.0
Ampicillin ORF
ORF
SalI
ColE1 origin
Neomycin ORF
PstI
pcDNA3-atp8a1
9,118bp
1000
2000
3000
6000
7000
8000
Add as Fragment
Stitch
Clone
Idle...

Rows: 30 Find reading frame output Filter to Selection...

Sequence	Start	End	Length
pcDNA3-atp8a1	1078	4620	3543
pcDNA3-atp8a1	5995	6381	387
pcDNA3-atp8a1	881	1279	399
pcDNA3-atp8a1	8207	8518	312
pcDNA3-atp8a1	3900	4235	336
pcDNA3-atp8a1	4809	5201	393
pcDNA3-atp8a1	5811	6617	807
pcDNA3-atp8a1	7263	7688	426
pcDNA3-atp8a1	7697	8002	306
pcDNA3-atp8a1	956	1288	333
pcDNA3-atp8a1	8122	8994	873
pcDNA3-atp8a1	3613	4047	435
pcDNA3-atp8a1	862	1242	381
pcDNA3-atp8a1	7197	7505	309
pcDNA3-atp8a1	6132	6764	633
pcDNA3-atp8a1	3078	3461	384
pcDNA3-atp8a1	825	1220	396
pcDNA4_TO	3226	3651	426
pcDNA4_TO	1190	1582	393
pcDNA4_TO	2189	2665	477
pcDNA4_TO	654	1151	498
pcDNA4_TO	1770	2084	315
pcDNA4_TO	2247	2624	378
pcDNA4_TO	4167	4478	312

Sequence 1 of 2: pcDNA3-atp8a1 (9,118bp circular) ☑ Show as Circular Vector: None Change to Current

Select restriction sites in sequence view to define target vector and fragments.

✓ No selection on target vector
X Define fragments to stitch

X Choose target vector
X Define fragments to insert

Figure 2.9: *Showing the table on one screen while the sequence is displayed on another screen. Clicking the table of open reading frames causes the focus to shift to the corresponding region in the linked view, and that region to be selected.*

A red highlight indicates where it will be placed. Alternatively, clicking on the (⇥) button at the top left of the floating palette will place it at the bottom of the Side Panel. All floating palettes can be re-docked by clicking on the (⇥) button at the bottom of the Side Panel.

The whole Side Panel can be hidden or revealed using buttons at the top right: (|▸) to hide the Side Panel and (◂|) to reveal it, if it was hidden. The keyboard shortcut Ctrl + U (⌘ + U on Mac) can also be used for these actions.

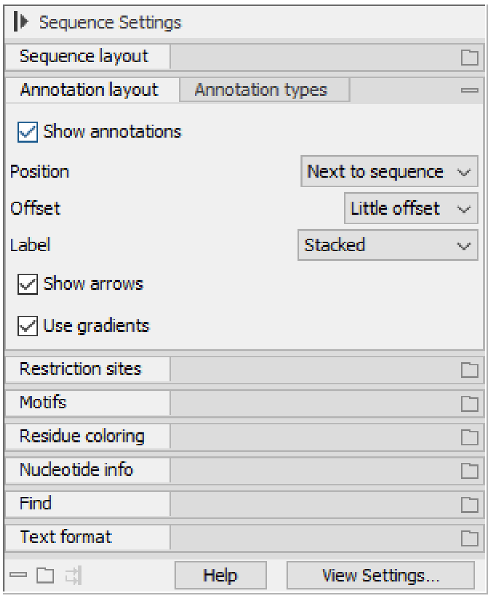
Changing the colors

Where color settings are offered, you have the option to use a fixed color or a gradient. For example, in figure 2.11, each annotation type is displayed using a fixed color, while the quality scores are displayed using a gradient. The color/gradient used can be changed by clicking on it.

A fixed color can be chosen from a predefined set of swatches (figure 2.12) or defined by setting the:

- Hue, saturation, and value (HSV).
- Hue, saturation, and lightness (HSL).
- Red, green, and blue combination (RGB).
- Cyan, magenta, yellow, and black combination (CMYK).

A gradient can be chosen from a predefined set of gradients (figure 2.13) or customized by setting:



☑ Show annotations

Position: Next to sequence

Offset: Little offset

Label: Stacked

☑ Show arrows

☑ Use gradients

Figure 2.10: *Side Panel settings for a nucleotide sequence. The Annotation layout palette is expanded, while the remaining palettes are collapsed. In the bottom left corner of the Side Panel are buttons for expanding, collapsing and re-docking all palettes.*

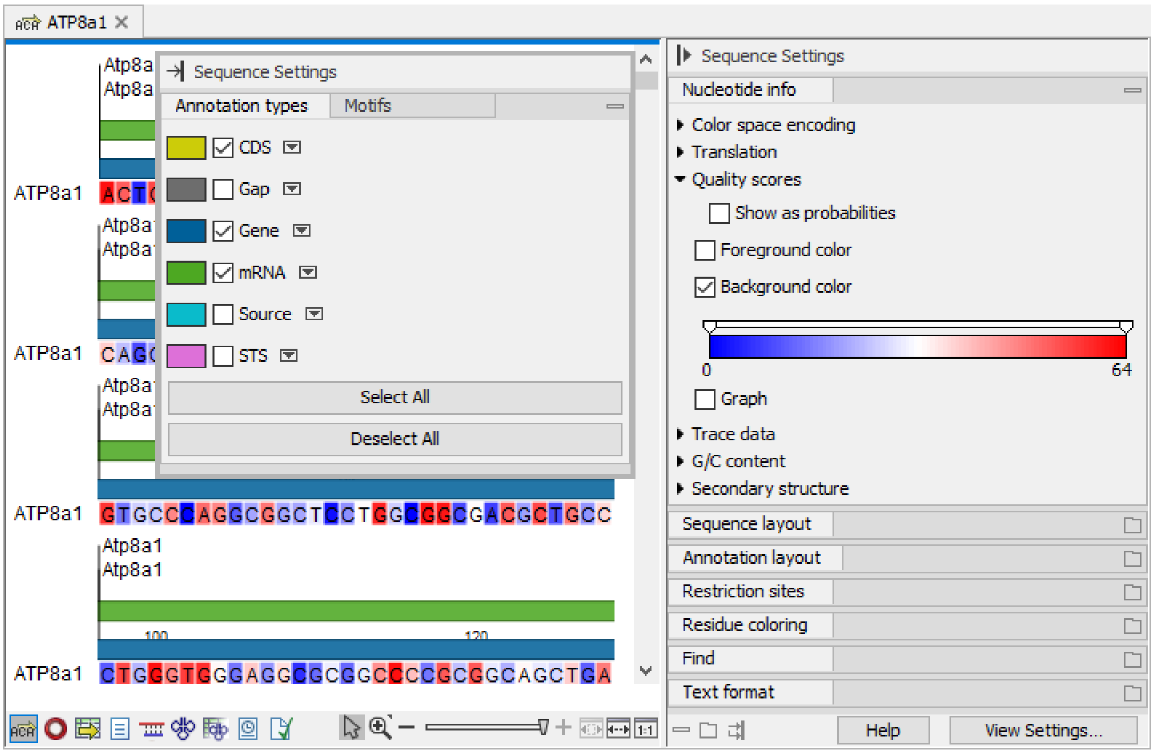
- The type of the gradient.
  - Continuous: the color gradually changes from one set color and location to the next.
  - Discrete: only the set colors are used and they change abruptly at the specified locations.
- Each color in the gradient and its location within the gradient.
  - The location can be provided as a percentage, ranging from 0% to 100%, or as an absolute value, with the minimum and maximum determined by the underlying data.

    For example, the range of the gradient from figure 2.13 is 0 – 64. Setting the location to 50% corresponds to the absolute value of 32 (0 + (64 - 0) * 0.5).

    Add locations by clicking on () or (). Remove intermediate locations by clicking on ().
  - The color can be chosen as described above.

Gradients settings can be reused, making it easy to apply the same gradient consistently across different views. This is done using buttons in the 'Configure gradient' dialog (figure 2.13):

- Click on **Copy All** to copy the gradient configuration. You can paste this into a text file for later use.
- Click on the **Paste** button to apply copied gradient settings. Colors and locations present in the 'Configure gradient' dialog are overwritten by this action.



☑ CDS
□ Gap
☑ Gene
☑ mRNA
□ Source
□ STS

▸ Color space encoding
▸ Translation
▾ Quality scores
□ Show as probabilities
□ Foreground color
☑ Background color
0 — 64
□ Graph
▸ Trace data
▸ G/C content
▸ Secondary structure

Figure 2.11: *The Annotation types and Motifs palettes have been undocked. The Nucleotide info palette has been moved at the top of the Side Panel. The background color of nucleotides reflects the quality scores.*

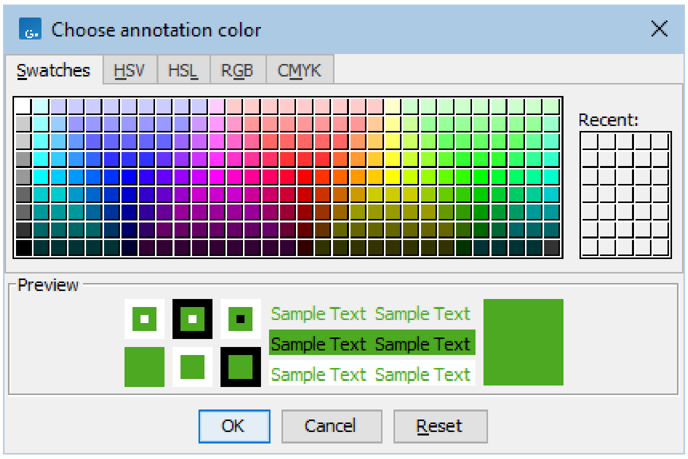
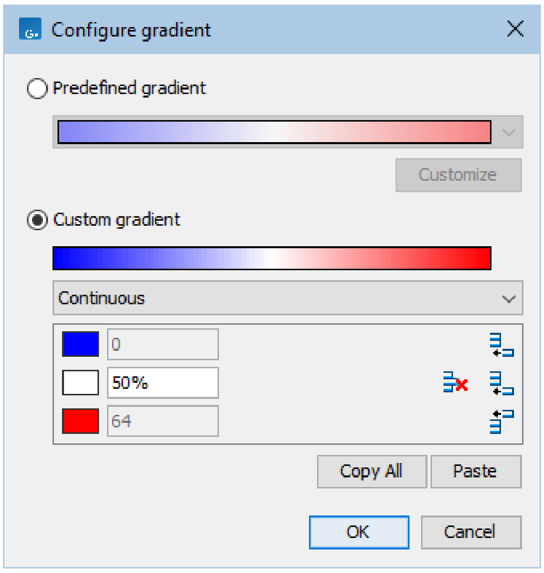


Figure 2.12: *Clicking on the color of the mRNA annotation type opens a dialog where the color can be changed.*



Configure gradient

○ Predefined gradient

● Custom gradient

Continuous

🟦	0
□	50%
🟥	64

Figure 2.13: *Clicking on the gradient of the quality scores opens a dialog where the gradient can be changed.*

2.2 Zoom functionality in the View Area

All views except tabular and text views support zooming in and out. This section describes the most common zoom functionality. For protein 3D structures, see section 15.2.

Zoom tools are located at the bottom right corner of most views (figure 2.14).

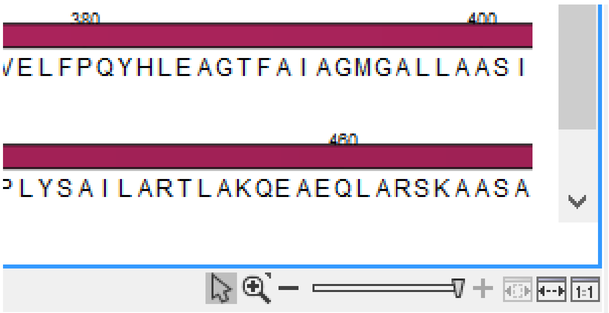


Figure 2.14: *Zoom tools are located at the bottom right corner of the view.*

The zoom tools include:

- Shortcuts for zooming out to fit the width of the view ( ) or zooming in all the way to see details (1:1).
- A shortcut to zoom to a selection ( ). Select a region in the view, and then click this icon to zoom in on the selected region. (Keyboard shortcut Ctrl + 1)
- A slider to zoom in and zoom out to any desired level. The slider position reflects the current zoom level. Move the slider left to zoom out, or right to zoom in. For fine grained control, click on the slider and move the mouse up slightly or down slightly.
- Mouse mode buttons:
  - **Selection mode** ( ). Used when you wish to select data in a view. This is the default.
  - **Zoom in mode** ( ) When selected, whenever you click the view, it zooms in. Alternatively, click on a location in the view, and the view will zoom in, with the focus on that location, or drag a box around an area, and the view will be zoomed to that area. (Keyboard shortcut Ctrl + 2)

    If you press and hold on ( ) or right-click on it, two other modes become available (figure 2.15).
  - **Panning** ( ) When selected, you can pan around in the the view around using the mouse. (Keyboard shortcut Ctrl + 4)
  - **Zoom out** ( ) When selected, whenever you click the view, it zooms out. (Keyboard shortcut Ctrl + 3)

Additional notes:

- If you hold the mouse over the selection and zoom tools, tooltips will appear that provide further information about how to use the tools.
- If you press the **Shift** button on your keyboard while in zoom mode, the zoom function is reversed.

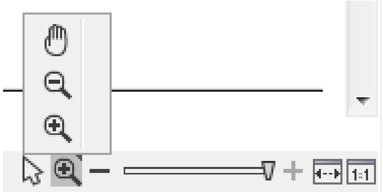


Figure 2.15: *Additional mouse modes can be found in the zoom tools when right-clicking on the magnifying glass.*

- You may have to click in the view before you can use the keyboard or the scroll wheel to zoom.

In many views, you can zoom in by pressing '+' on your keyboard, or zoom out by pressing '-' on your keyboard.

If you have a mouse with a scroll wheel, you can also do the following:

Zoom in: **Press and hold Ctrl (⌘ on Mac) | Move the scroll wheel on your mouse forward**

and

Zoom out: **Press and hold Ctrl (⌘ on Mac) | Move the scroll wheel on your mouse backwards**

2.3 Toolbox panel

The Toolbox panel at the bottom, left side of the Workbench, provides access to tools, installed workflows and template workflows, described in more detail below. The Processes tab, also present here, is described in section 2.4.

The font size in the Toolbox tabs can be increased or decreased using the (A-) or (A+) icons at the top, right hand side of the Navigation Area. Changing the font size is described in more detail in section 3.1.

Launching tools and workflows is described in section 11.1, with further details about launching workflows, individually or in batch mode, covered in chapter 13.

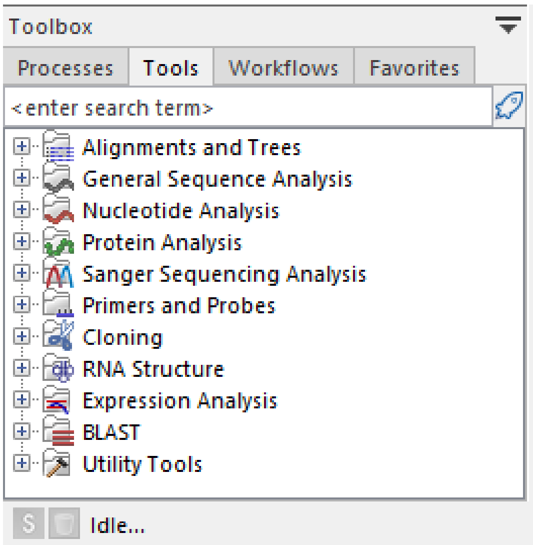
Tools tab

Tools available in the *CLC Workbench* are provided under the Tools menu, which is available in the Toolbox panel and as a menu at the top of the Workbench. The Tools menu is also available, in an extended form, in the Add Elements dialog available in the Workflow Editor (see section 13.1.1).

Tools are organized in folders according to their functionality. Tools provided by plugins may be in a plugin-specific folder (figure 2.16). When connected to a *CLC Genomics Server* with external applications configured and available, a folder for these will also be present in the Tools menu (figure 2.17).

You can search for tools of interest in the Tools tab in the Toolbox by entering a search term into the field at the top of the tab.

Workflows tab



Toolbox

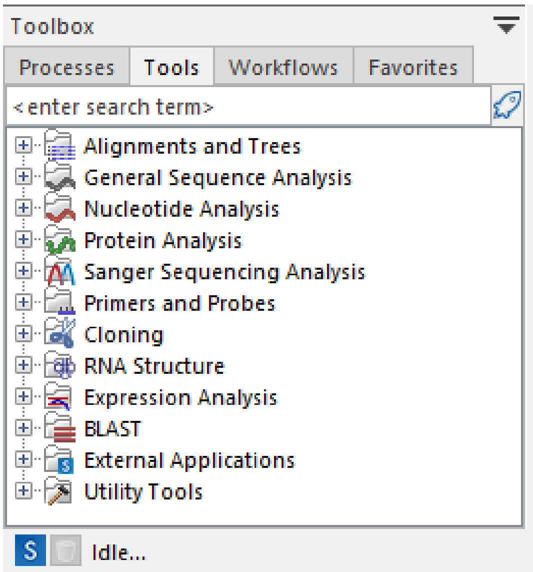
Processes | Tools | Workflows | Favorites

<enter search term>

- Alignments and Trees
- General Sequence Analysis
- Nucleotide Analysis
- Protein Analysis
- Sanger Sequencing Analysis
- Primers and Probes
- Cloning
- RNA Structure
- Expression Analysis
- BLAST
- Utility Tools

Idle...

Figure 2.16: *The Tools tab in the Toolbox contains folders of available tools. This Workbench is not connected to a CLC Server, as indicated by the grey server icon in the status bar.*



Toolbox

Processes | Tools | Workflows | Favorites

<enter search term>

- Alignments and Trees
- General Sequence Analysis
- Nucleotide Analysis
- Protein Analysis
- Sanger Sequencing Analysis
- Primers and Probes
- Cloning
- RNA Structure
- Expression Analysis
- BLAST
- External Applications
- Utility Tools

S Idle...

Figure 2.17: *This Workbench is connected to a CLC Server, as indicated by the blue server icon in the status bar. External applications have been configured and enabled on that CLC Server, so an External Applications folder is listed, which contains those external applications. The server icon within that folder's icon is a reminder that these are only available when logged into the CLC Server.*

Workflows installed on the Workbench and template workflows (see section 13.5) are listed in the Workflows tab in the Toolbox (figure 2.18). The Workflows menu is also available at the top of the Workbench.

When connected to a *CLC Server*, workflows installed on that server will be available from a folder in the Workflows menu called **Installed Workflows (Server)** (figure 2.19).

You can search for workflows of interest in the Workflows tab in the Toolbox by entering a search term into the field at the top of the tab.

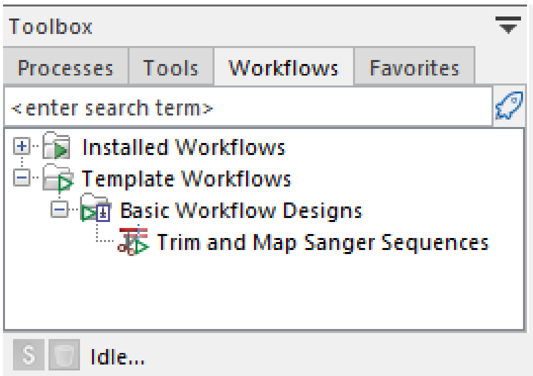


Figure 2.18: *The Workflows tab in the Toolbox contains folders for workflows installed on the Workbench and template workflows. This Workbench is not connected to a CLC Server, as indicated by the grey server icon in the status bar. There are also no active AWS Connections, as indicated by the grey cloud icon in the status bar.*

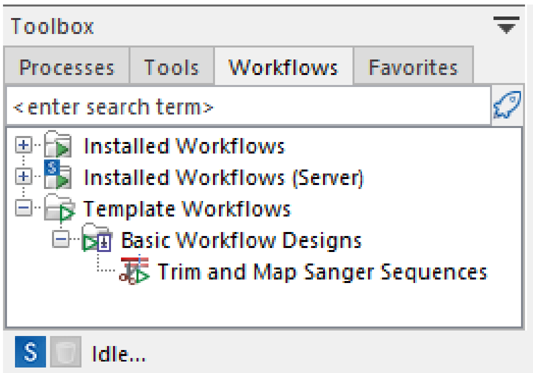


Figure 2.19: *This Workbench is connected to a CLC Server, as indicated by the blue server icon in the status bar. This CLC Server has workflows installed on it, so a folder containing those workflows is present in the Workflows menu. (Installed Workflows (Server)).*

Favorites tab

You can specify tools, or folders of tools, that you want to find quickly as favorites. In addition, the 10 tools you use most frequently are automatically identified as your frequently used tools or workflows. These lists are also made available in the Quick Launch tool (), started using the Launch button in the toolbar, and in the Add Elements dialog available in the Workflow Editor (see section 13.1.1).

Manually adding tools to the Favorites list is done from tabs in the Toolbox panel:

- Right-click on a tool or folder of tools in the Tools tab and choose the option "Add to Favorites" from the menu that appears (figure 2.21), or
- Open the Favorites tab, right-click in the Favorites folder, choose the option "Add tools" or "Add group of tools". Then select the tool or tool group to add.
- From the Favorites tab, click on an item in the Frequently used folder and drag it into the Favorites folder.

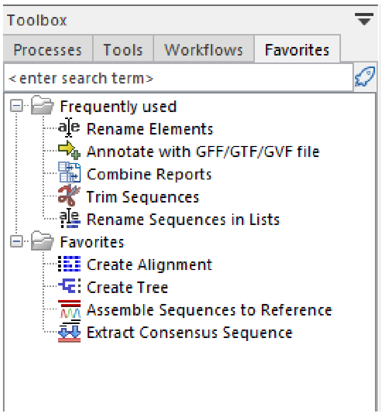


Figure 2.20: *Under the Favorites tab is a folder containing your frequently used tools, which are added automatically, based on usage, and a folder containing tools you have specified as favorites.*

Items within the Favorites folder can be re-ordered by opening the Favorites tab in the Toolbox area in the bottom, left hand side of the Workbench and dragging tools up and down within the list. (Folders cannot be repositioned.)

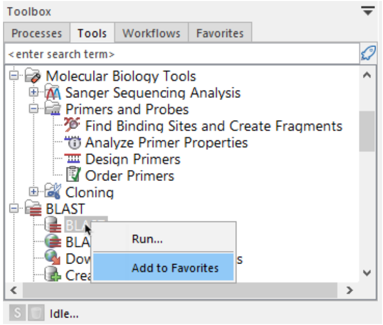


Figure 2.21: *Tools or workflows can be added to the Favorites tab by right-clicking on them in the Toolbox area and choosing the "Add to Favorites" option.*

To remove an item from the Favorites tab, right-click on it and choose the option **Remove from Favorites** from the menu that appears.

You can search for items of interest in the Favorites tab in the Toolbox by entering a search term into the field at the top of the tab.

2.4 Processes tab and Status bar

The Status bar is located at the bottom of the *CLC Main Workbench*. On the left side, information is displayed about whether a process is running or the Workbench is idle. Further to the left is information on the status of connections to other systems, such as a *CLC Server*. On the right hand side, context dependent information is displayed. For example, when selecting part of a sequence, the size of a selected region will be reported, or when mousing over a variant in a variant track, the location of the variant is reported.

Detailed information about running and completed processes for the Workbench session is provided under the Processes tab, found in the lower, left side of the Workbench. When logged into a *CLC Server*, the status of your jobs that are running, completed or queued on the server, are also displayed.

Several options are available after clicking on the small icon (▾) next to a given process, as shown in figure 2.22).

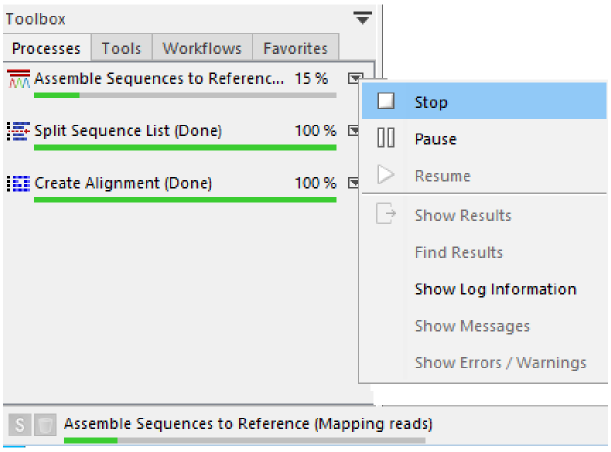

Toolbox
Processes
Tools
Workflows
Favorites
Assemble Sequences to Referenc... 15 %
Split Sequence List (Done) 100 %
Create Alignment (Done) 100 %
Stop
Pause
Resume
Show Results
Find Results
Show Log Information
Show Messages
Show Errors / Warnings
S
Assemble Sequences to Reference (Mapping reads)

Figure 2.22: *Completed jobs run during a Workbench and the progress of running jobs is visible in the Processes tab. The progress of a running job is also visible in the bottom frame of the Workbench. Clicking the small icon next to a process in the Process tab reveals a menu with actions that can be taken.*

For completed jobs, these options provide a convenient way to locate results in the Navigation Area:

- **Show results** Open the results generated by that process in the Viewing Area. (Relevant if results were saved, as described in section 11.2.)
- **Find results** Highlight the results in the Navigation Area. (Relevant if results were saved, as described in section 11.2.)
- **Show Log Information** Opens a log of the progress of the process. This is the same log that opens if the option **Open Log** option is selected when launching a task.
- **Show Messages** Show any messages that were produced during the processing of your data.

Stopped, paused and finished processes are not automatically removed from the Processes tab during a Workbench session. They can, however, be removed by right clicking in the Processes tab and selecting the option "Remove Finished Processes" or by going to the option in the main menu system:

Utilities | Remove Finished Processes (✕) .

If you close the Workbench while jobs are still running on it, a dialog will ask for confirmation before closing. Workbench processes are stopped when the software is closed and these processes are not automatically restarted when you start the Workbench again. Closing the Workbench does *not* interrupt jobs sent to a *CLC Server*, as described below.

Processes submitted to a CLC Server

Processes submitted to a *CLC Server* are listed in the Processes tab when the Workbench is logged into the server. Such processes have a server icon (S) to their left, rather than icons specific to the analysis being run. Processes that are queued or running on a *CLC Server* will reappear in the Workbench processes tab if you restart the Workbench (and log into the server). *CLC Server* processes already finished when you close the Workbench will not be shown again in the processes tab when you restart your Workbench.

Like running Workbench processes, processes running on a *CLC Server* can be stopped, by selecting clicking the small icon (▾) next to the process and selecting the option "Stop". However, unlike jobs running on a Workbench, they cannot be paused or resumed.

Of note when running jobs on a *CLC Server*: If you choose the option "On my local disk or a place I have access to" when launching an import task, then the Workbench must maintain its connection to the *CLC Server* during the first part of the import process, data upload. If you try to close the Workbench during this phase, you will see a warning dialog. You can see what stage tasks are at in the **Processes** tab. Data upload from the Workbench to the server runs as a local, Workbench process. When the upload stage is complete, a new process for the import is started. This import process will have a server icon (S) to the left of it. At this point, you can disconnect or close your Workbench without affecting the import.

2.5 History and Element Info views

History view

The History view shows the log of all operations carried out on the element. This detailed record can be viewed within the *CLC Main Workbench*, as described here, or exported to a pdf format file.

To open the History view, click on the **Show History** ( ) icon under the View area.

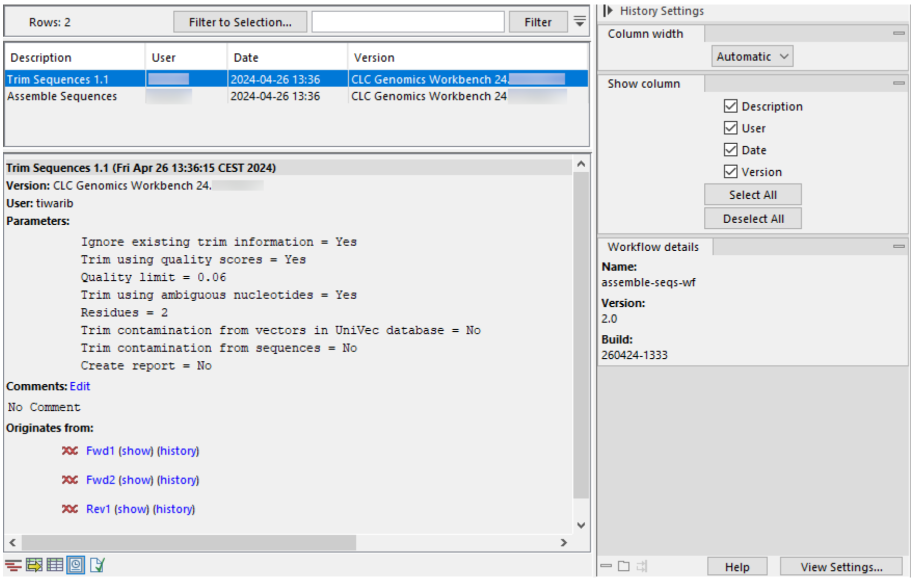
The table at the top of the History view contains a row for each operation that has affected this data element. When rows are selected in the table, full details for those operations are displayed in the bottom panel (figure 2.23).

Information in the table:

Summary information is shown in the table for each operation carried out in the creation of this data element:

- **Description** The operation performed
- **User** The username of the person who performed the operation. If you import data created by another person, that person's username will be shown.
- **Date and time** Date and time the operation was carried out. These are displayed according to your locale settings (see section 4.1).
- **Version** The software name and version used for that operation.

Information in the lower panel:



Description	User	Date	Version
Trim Sequences 1.1	[redacted]	2024-04-26 13:36	CLC Genomics Workbench 24.[redacted]
Assemble Sequences	[redacted]	2024-04-26 13:36	CLC Genomics Workbench 24[redacted]

Trim Sequences 1.1 (Fri Apr 26 13:36:15 CEST 2024)
Version: CLC Genomics Workbench 24.[redacted]
User: tiwarib
Parameters:

```
        Ignore existing trim information = Yes
        Trim using quality scores = Yes
        Quality limit = 0.06
        Trim using ambiguous nucleotides = Yes
        Residues = 2
        Trim contamination from vectors in UniVec database = No
        Trim contamination from sequences = No
        Create report = No
```

Comments: Edit
No Comment
Originates from:
Fwd1 (show) (history)
Fwd2 (show) (history)
Rev1 (show) (history)

Column width: Automatic

Show column:
☑ Description
☑ User
☑ Date
☑ Version

Workflow details
Name: assemble-seqs-wf
Version: 2.0
Build: 260424-1333

Figure 2.23: *The history of an element created by an installed workflow called assemble-seqs-wf.*

- **Parameters** The parameter values used for an analysis.
- **Comments** Additional details added here by tools or details that have been added manually. Click on **Edit** to add information to this field.
- **Originates from** A list of the elements that the current element originated from. Clicking on the name of an originating element selects it in the Navigation Area. Click on the "(show)" link to open the originating element to its default view. Click on "(history)" to open the originating element to its History view.

Information in the side panel:

- **Column width**
- **Show column**
- **Workflow details** Present if the element is an output from a workflow. The name and version of the workflow are listed here, and if the element was generated by an installed workflow (including template workflows), the workflow build id is also reported[1]. If the element is output by a workflow launched from the Workflow Editor, the version is reported, but there will be no build id.

If an installer has never been made for a workflow, then data elements created using that workflow (launched from the Workflow Editor), will have 0.1 reported as the workflow version in their history. Workflows that have been used to make an installer inherit the most recent version assigned when creating the workflow installer. See section 13.6.2 for more on creating workflow installers.

[1] Workflow build ids are included in the history of elements generated using version 24.0 or later.

Element Info view

To open the Element Info view, click on the **Show Element Info** ( ) icon under the View area.

The Element Info view contains information about the element, such as its name, description and other attributes. If the element is associated with metadata, that association is also reported here.

For further details about element information, please see section 14.4. For further information about metadata associations, see section 12.3.2.

2.6 Workspace

Workspaces are useful when working on more than one project. Open views and their arrangement are saved in a given workspace. Switching between workspaces can thus save much time when working on several different sets of data and results.

Initially, there is a single workspace called "Default". When you set up other workspaces, you assign each a name, which is used when re-opening that workspace, and which is displayed in the title bar of the Workbench when it is the active workspace.

The state of each workspace is saved automatically when the Workbench is closed down. The workspace that was open when closing down is the one that will be opened when the Workbench is started up again.

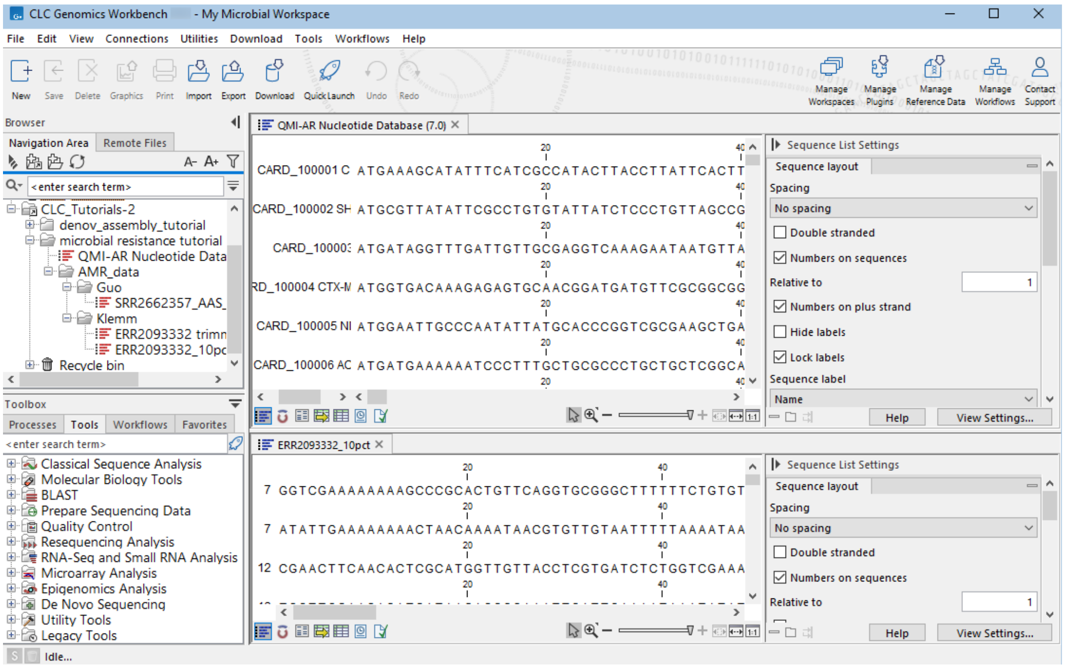

CLC Genomics Workbench - My Microbial Workspace
File Edit View Connections Utilities Download Tools Workflows Help
New Save Delete Graphics Print Import Export Download QuickLaunch Undo Redo
Manage Workspaces Manage Plugins Manage Reference Data Manage Workflows Contact Support
Browser
Navigation Area Remote Files
<enter search term>
CLC_Tutorials-2
denov_assembly_tutorial
microbial resistance tutorial
QMI-AR Nucleotide Data
AMR_data
Guo
SRR2662357_AAS_
Klemm
ERR2093332 trimn
ERR2093332_10pc
Recycle bin
Toolbox
Processes Tools Workflows Favorites
<enter search term>
Classical Sequence Analysis
Molecular Biology Tools
BLAST
Prepare Sequencing Data
Quality Control
Resequencing Analysis
RNA-Seq and Small RNA Analysis
Microarray Analysis
Epigenomics Analysis
De Novo Sequencing
Utility Tools
Legacy Tools
Idle...
QMI-AR Nucleotide Database (7.0)
CARD_100001 C ATGAAAGCATATTTCATCGCCATACTTACCTTATTCACTT
CARD_100002 SH ATGCGTTATATTCGCCTGTGTATTATCTCCCTGTTAGCCG
CARD_10000 ATGATAGGTTTGATTGTTGCGAGGTCAAAGAATAATGTTA
RD_100004 CTX-M ATGGTGACAAAGAGAGTGCAACGGATGATGTTCGCGGCGG
CARD_100005 NI ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGA
CARD_100006 AC ATGATGAAAAAATCCCTTTGCTGCGCCCTGCTGCTCGGCA
ERR2093332_10pct
7 GGTCGAAAAAAAAGCCCGCACTGTTCAGGTGCGGGCTTTTTTCTGTGT
7 ATATTGAAAAAAAACTAACAAAATAACGTGTTGTAATTTTTAAAATAA
12 CGAACTTCAACACTCGCATGGTTGTTACCTCGTGATCTCTGGTCGAAA
Sequence List Settings
Sequence layout
Spacing
No spacing
□ Double stranded
☑ Numbers on sequences
Relative to 1
☑ Numbers on plus strand
□ Hide labels
☑ Lock labels
Sequence label
Name
Help View Settings...

Figure 2.24: *The workspace called "My Microbial Workspace" is open after selecting it from the menu opened by clicking on the Manage Workspaces button in the Toolbar. The name of the workspace is visible in the Workbench title bar.*

Workspaces do not affect the underlying organization of data, so folders and elements remain the same in the Navigation Area.

Workspaces can be created, opened and deleted using the options available under the **Manage Workspaces** button in the top Toolbar, as described below. This functionality is also present under the View menu.

Creating a workspace

Create a new workspace by clicking in the **Manage Workspaces** button in the top Toolbar.

In the drop-down menu that appears, choose the option **Create Workspace**.

In the dialog that appears, enter a name for the new workspace.

When you click on the OK button, the new workspace is created and opened. The name of the workspace will be in the title bar of the Workbench.

Initially, the **Navigation Area** may be collapsed. Open it up again by clicking in the small black triangle at the top right of the Navigation Area.

Opening a workspace

Switch between workspaces by clicking in the **Manage Workspaces** button in the top Toolbar and selecting the desired workspace from the list presented.

The name of the active workspace will be greyed out in the list.

Deleting a workspace

To delete a workspace, click on the **Manage Workspaces** button in the top Toolbar and select the option **Delete Workspace**.

Workspaces that can be deleted are listed in a drop-down menu in the dialog that appears. Select the one to delete.

Deletion of workspaces cannot be undone.

Note: The Default workspace is not offered, as it cannot be deleted.

2.7 List of shortcuts

The keyboard shortcuts available in *CLC Main Workbench* are listed below.

General

Action	Windows/Linux	macOS
Adjust selection	Shift + arrow keys	Shift + arrow keys
Back to Navigation Area	Alt + Home or Alt + fn + left arrow	⌘ + Home or ⌘ + fn + left arrow
BLAST	Ctrl + Shift + L	⌘ + Shift + L
BLAST at NCBI	Ctrl + Shift + B	⌘ + Shift + B
Close	Ctrl + W	⌘ + W
Close all views	Ctrl + Shift + W	⌘ + Shift + W
Copy	Ctrl + C	⌘ + C
Create alignment	Ctrl + Shift + A	⌘ + Shift + A
Create track list	Ctrl + L	⌘ + L
Cut	Ctrl + X	⌘ + X
Delete	Delete	Delete or ⌘ + Backspace
Exit	Alt + F4	⌘ + Q
Export	Ctrl + E	⌘ + E
Export graphics	Ctrl + G	⌘ + G
Find Next Conflict	'.' (dot)	'.' (dot)
Find Previous Conflict	',' (comma)	',' (comma)
Help	F1	F1
Import	Ctrl + I	⌘ + I
Launch tools	Ctrl + Shift + T	⌘ + Shift + T
Maximize/restore View size	Ctrl + M	⌘ + M
Move gaps in alignment	Ctrl + arrow keys	⌘ + arrow keys
New Folder	Ctrl + Shift + N	⌘ + Shift + N
New Sequence	Ctrl + N	⌘ + N
Panning Mode	Ctrl + 4	⌘ + 4
Paste	Ctrl + V	⌘ + V
Print	Ctrl + P	⌘ + P
Redo	Ctrl + Y	⌘ + Y
Rename	F2	F2
Save	Ctrl + S	⌘ + S
Save As	Ctrl + Shift + S	⌘ + Shift + S
Scrolling horizontally	Shift + Scroll wheel	Shift + Scroll wheel
Search local data	Ctrl + Shift + F	⌘ + Shift + F
Search via Side Panel	Ctrl + F	⌘ + F
Search NCBI	Ctrl + B	⌘ + B
Search UniProt	Ctrl + Shift + U	⌘ + Shift + U
Select All	Ctrl + A	⌘ + A
Select Selection Mode	Ctrl + 1 (one)	⌘ + 1 (one)
Show folder content	Ctrl + O	⌘ + O
Show/hide Side Panel	Ctrl + U	⌘ + U
Show tooltip without delay	press and hold Shift	press and hold Shift
Sort folder	Ctrl + Shift + R	⌘ + Shift + R
Split Horizontally	Ctrl + T	⌘ + T
Split Vertically	Ctrl + J	⌘ + J
Suppress tooltip	press and hold Ctrl	press and hold Ctrl
Switch tabs in View Area	Ctrl + PageUp/PageDown or Ctrl + fn + arrow up/down	Ctrl + PageUp/PageDown or Ctrl + fn + arrow up/down
Switch views	Ctrl + Shift + PageUp Ctrl + Shift + PageDown	Ctrl + Shift + PageUp/arrow up Ctrl + Shift + PageDown/arrow down
Translate to Protein	Ctrl + Shift + P	⌘ + Shift + P
Undo	Ctrl + Z	⌘ + Z
Update folder	F5	F5
User Preferences	Ctrl + K	⌘ + ,

Scroll and Zoom shortcuts

Action	Windows/Linux	macOS
Vertical scroll in reads tracks	Alt + Scroll wheel	Alt + Scroll wheel
Vertical scroll in reads tracks, fast	Shift+Alt+Scroll wheel	Shift+Alt+Scroll wheel
Vertical zoom in graph tracks	Ctrl + Scroll wheel	⌘ + Scroll wheel
Zoom	Ctrl + Scroll wheel	⌘ + Scroll wheel
Zoom In Mode	Ctrl + 2	⌘ + 2
Zoom In (without clicking)	’+’ (plus)	’+’ (plus)
Zoom Out Mode	Ctrl + 3	⌘ + 3
Zoom Out (without clicking)	’-’ (minus)	’-’ (minus)
Zoom to base level	Ctrl + 0	⌘ + 0
Zoom to fit screen	Ctrl + 6	⌘ + 6
Zoom to selection	Ctrl + 5	⌘ + 5
Reverse zoom mode	press and hold Shift	press and hold Shift

Workflows related shortcuts

Action	Windows/Linux	macOS
Adjust workflow layout	Shift + Alt + L	⌘ + Shift + Alt + L
Workflow, add element	Alt + Shift + E	Alt + Shift + E
Workflow, collapse if its expanded	Alt + Shift + ’-’ (minus)	Alt + Shift + ’-’
Workflow, create installer	Alt + Shift + I	Alt + Shift + I
Workflow, execute	Ctrl + enter	⌘ + enter
Workflow, expand if its collapsed	Alt + Shift + ’+’ (plus)	Alt + Shift + ’-’
Workflow, highlight used elements	Alt + Shift + U	Alt + Shift + U
Workflow, remove all elements	Alt + Shift + R	Alt + Shift + R

Combinations of keys and mouse movements

Action	Windows/Linux	macOS	Mouse movement
Maximize View			Double-click the tab of the View
Restore View			Double-click the View title
Reverse zoom mode	Shift	Shift	Click in view
Select multiple elements not grouped together	Ctrl	⌘	Click elements
Select multiple elements grouped together	Shift	Shift	Click elements
Select Editor and highlight the corresponding element in the Navigation Area	Alt or Ctrl	⌘	Click tab

"Elements" in this context refers to elements and folders in the **Navigation Area** selections on sequences, and rows in tables.

Chapter 3

Data management and search

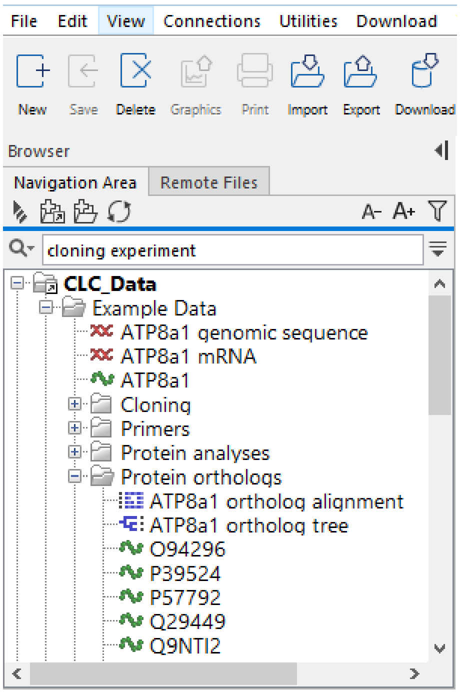
Contents

This chapter explains general data management features of *CLC Main Workbench*. The first section explains the basics of the data organization and the **Navigation Area**. The next section explains how to set up custom attributes for the data that can be used for more advanced data management. Finally, there is a section about how to search for data in your CLC Locations. The use of metadata tables in *CLC Main Workbench* is described separately, in chapter 12.

We recommend that data is only added and removed from CLC Data Locations using CLC software. If files are moved using other methods, the data may not be found when launching an analysis, and searches may not find that data. To address issues where data present in a CLC Data Location cannot be found, re-build the index for that location and try again. Information about rebuilding indexes can be found in section 3.4.

3.1 Navigation Area

The **Navigation Area** (figure 3.1) is used for organizing and navigating data.



cloning experiment

Figure 3.1: *The Navigation Area.*

Each CLC data element has a name and an icon that represents the type of data in the element. A list of many of the icons and the type of data they represent can be found at `https://qiagen.my.salesforce-sites.com/KnowledgeBase/KnowledgeNavigatorPage?id=kA41i000000L5uFCAS`.

Non-CLC files placed into CLC locations will have generic icons beside them, and any suffix in the original file name will be visible in the Navigation Area. (e.g. `.pdf`, `.xml` and so on.)

Elements placed in a folder (e.g. by copy/pasting or dragging) are put at the bottom of the folder listing. If the element is placed on another element, it is put just below that element in the folder listing. If an element of the same name already exists in that folder, a copy is created with the name extension "-1", "-2" etc. See section 3.1.6 for further details.

Elements in a folder can be sorted alphabetically by right-clicking on the folder and choosing the option **Sort Folder** from the menu that appears. When sorting this way on Windows, subfolders are placed at the top of the folder with elements listed below in alphabetical order. On Mac, subfolders and elements are listed together, in alphabetical order.

Opening and viewing CLC data elements is described in section 2.1.

Just above the data listing area is a Quick Search field, which can be used to find elements in your CLC Locations. See section 3.4.1.

Just above the Quick Search field are icons that can be clicked upon. On the left side, from left to right:

- **Collapse all** ( ). Close all the open folders in the Navigation Area.

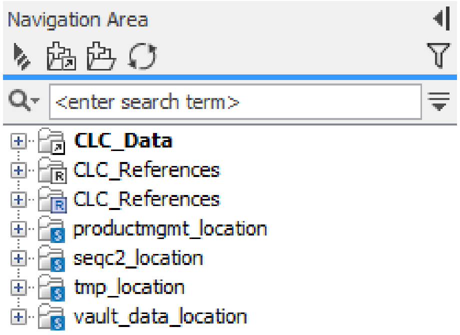
- **Add File Location** (📁). Add a new top level location for storing CLC data. See section 3.1.2 for further details.
- **Create Folder** (📁) Create new folders within an existing CLC Location.
- **Update All** (⟳) Refresh the view of the Navigation Area.

On the right side, from left to right:

- **Decrease font size** (A-) and **increase font size** (A+) Decrease or increase the font size in the Navigation Area, both in the left hand side of the Workbench and other locations, such as launch wizards steps where data elements can be selected. The font size in the Tools, Workflows and Favorites tabs in the Toolbox, just below the Navigation Area, are also adjusted.
- **Restrict data types listed** (▽) Click on this icon to reveal a list of data types. Selecting one of these types will result in only elements of that types, and folders, being shown in the Navigation Area. Click on it again to and select "All Elements" to see all elements listed once more.
- **Hide the Navigation Area and Toolbox** (◀|). This icon is at the top, right hand side. Clicking on it hides the **Navigation Area** and the **Toolbox** panels, providing more area for viewing data. To reveal the panels again, click on the (|▶) icon that is shown when the panels are hidden.

Data held on a CLC Server

If you have logged into a *CLC Server* from your Workbench, then data stored on the *CLC Server* will also be listed in the Navigation Area (figure 3.2).



Navigation Area

<enter search term>

CLC_Data
CLC_References
CLC_References
productmgmt_location
seqc2_location
tmp_location
vault_data_location

Figure 3.2: *Data locations on a CLC Server are highlighted with blue square icons in the Navigation Area.*

Locations on a *CLC Server* are configured by the server administrator.

When you launch a job to run on the *CLC Main Workbench*, you can choose data located in *CLC Main Workbench* File Locations or in *CLC Server* File Locations. When running jobs on a *CLC Server*, you can only select data in locations known to the server (see section 11.1.1).

3.1.1 Data structure

The data in the **Navigation Area** is organized into a number of **Locations**, also known as File Locations or Data Locations. A location represents a folder on the computer: The data shown under a Workbench location in the **Navigation Area** is stored on the computer, in the folder on the file system that the location points to. The full path to the system folder can be seen by mousing over the data location folder icon (figure 3.3).

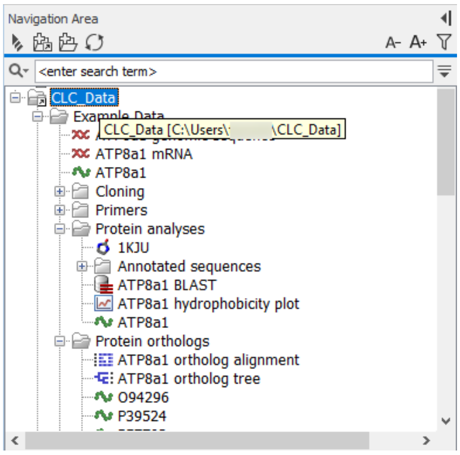


Figure 3.3: *Mousing over the 'CLC_Data' location reveals a tooltip showing the full path to the folder on the underlying file system.*

When the *CLC Main Workbench* is started for the first time, there will be a location called *CLC_Data*, which is the default data location.

Adding more locations and removing locations is described in section 3.1.2. Another location can be specified as the default by right-clicking on the location folder in the Navigation Area and choosing the option **Set as Default Location** from under Location in the menu (figure 3.4). This setting only applies to you. Other people using the same Workbench can set their own default locations.

Administrators can also change the default data location for all users of a Workbench. This is described at https://resources.qiagenbioinformatics.com/manuals/workbenchdeployment/current/index.php?manual=Default_Workbench_data_storage.html.

Note: There will also be a location called *CLC_References*. This location is of relevance primarily if you are working with others using a *CLC Genomics Workbench*, who are sharing their results and reference data with you. It is intended for storing genomic references and associated data, downloaded using the Reference Data Manager, distributed with the *CLC Genomics Workbench*.

3.1.2 Adding and removing locations

When a Workbench is first installed a data location is configured by default. Unless your administrator has set things up otherwise, it will be named `CLC_Data`. It points to the following folder on the underlying system:

- Windows: C:\Users\<your_username>\CLC_Data
- Mac: ~/CLC_Data

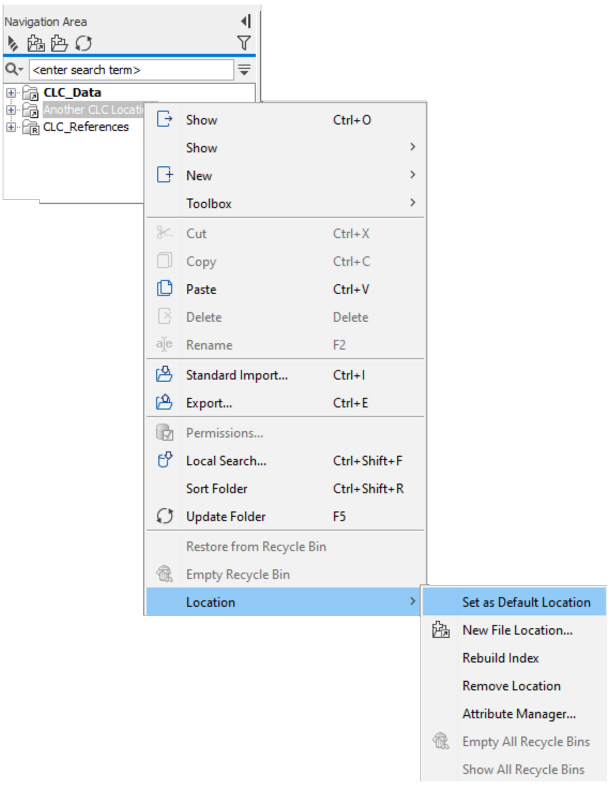


Figure 3.4: *Data location options are available in a right-click context menu. Here, a new data location is being specified as the default.*

- Linux: /homefolder/CLC_Data

Adding locations

To add a new location, click on the ( ) icon at the top of the Navigation Area, or go to:

File | Location | New File Location ( )

Navigate to the folder to add as a CLC data location (see figure 3.5).

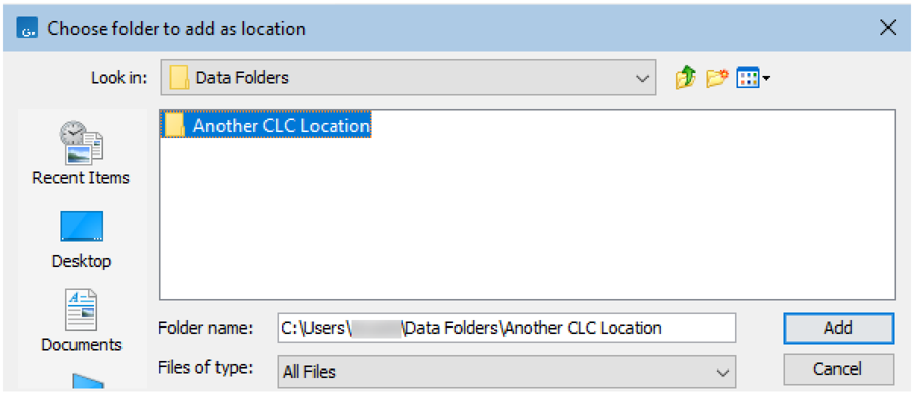
The name of the new location will be the name of the folder selected. To see the full path to the folder on the file system, hover the mouse cursor over the location icon ( ).

The new location will appear in the **Navigation Area** (figure 3.6).

Additional notes about CLC locations

Requirements for folders being used as CLC locations:

- You must have permission to read from that folder, and if you plan to save new data elements or update data elements, you must have permission to write to that folder.
- The folder chosen as a CLC location must not a subfolder of any area already being used as a CLC Workbench or CLC Server location.



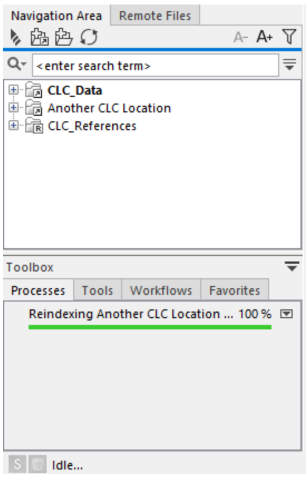
Look in: Data Folders

Another CLC Location

Folder name: C:\Users\[redacted]\Data Folders\Another CLC Location

Files of type: All Files

Figure 3.5: *Navigating to a folder to use as a new CLC location.*



CLC_Data
Another CLC Location
CLC_References

Reindexing Another CLC Location ... 100 %

Idle...

Figure 3.6: *A new CLC location has been added. When the selected folder has not been used as a CLC location before, index files will be built, with the index building process listed in the Processes tab below the Navigation Area.*

Folders on a network drive or a removable drive can act as CLC locations. Please note though that interruptions to file access can lead to problems. For example, if you have set up a CLC location on One Drive, start editing a cloning experiment, and your laptop goes to sleep, unsaved work may be lost, and errors relating to the lost connection may be reported. If your CLC locations are on such systems, enabling offline line access (aka "always available files") can avoid such issues.

Locations appear inactive in the **Navigation Area** if the relevant drive is not available when you start up the Workbench. Once the drive is available, click on the Update All symbol (⟳) at the top of the Navigation area to refresh the view. All available locations will then be shown as active. There can be sometimes be a short delay before the interface update completes.

See sectioN 3.1.3 for information relating to sharing CLC data locations.

Removing locations

To remove a CLC data location, right-click on the location (the top level folder), and select **Location** | **Remove Location**. The Location menu is also available under the top level **File** menu.

CLC data locations that have been removed can simply be re-added if you wish to access the data via the Workbench Navigation Area again.

After removing the CLC location, standard operating system functionality can be used to remove the folder and its contents from the local file system, if desired.

3.1.3 Data sharing information

The same underlying folder, for example on a network drive, can be added as a CLC data location in multiple Workbenches. This allows data to be easily shared. However, it is important to note that *data sharing is not actively supported*. Specifically:

- We do not support concurrent alteration of data. While the software will often detect this situation and handle it appropriately, by for example only allowing read access to all but the one party editing the file, we do not guarantee this.
- Any functionality that involves using the data search indices, (e.g. search functionality, associating metadata with data), will not work properly for shared data locations. Re-indexing a Data Location can help in the short term, but as soon as a new file is created by another piece of software, the index will be out of date.

If you decide to share data via Workbenches this way, it is vital that when adding a CLC location already used by other Workbenches as a CLC location, **the exact same folder in the file system hierarchy as the other Workbenches have used** is the one selected to add as a location. Indicating a folder higher up or lower down in the hierarchy will cause problems with the indexing of the files. This can lead to newly created objects made by Workbench A not being found when searching from Workbench B and vice versa, as well as issues with associations to CLC Metadata Tables.

Sharing the location of a folder or element with others

When working with others with the same CLC Location available via their *CLC Workbench*, or who connect to the same *CLC Server*, you can easily share the location of a particular element or folder, as described under *Searching using CLC URLs* in section 3.4.1.

3.1.4 Create new folders

Creating a new folder can be done in two ways:

Right-click an element in the Navigation Area | New | Folder ( )

or **File | New | Folder ( )**

If a folder is selected in the Navigation Area when adding a new folder, the new folder is added at the bottom of this folder. If an element is selected, the new folder is added right above that element.

You can move the folder manually by selecting it and dragging it to the desired destination.

3.1.5 Multiselecting elements

More than one element can be selected at the same time by:

- Holding down the <Ctrl> key (⌘ on Mac) while clicking on multiple elements selects the elements that have been clicked.
- Selecting one element, and selecting another element while holding down the <Shift> key selects all the elements listed between the two locations (the two end locations included).
- Selecting one element, and moving the cursor with the arrow-keys while holding down the <Shift> key, enables you to increase the number of elements selected.

3.1.6 Copying and moving elements and folders

To copy or move elements or folders in the Navigation Area, start by selecting the elements and/or folders. You can then use explicit actions for cutting, copying and pasting the selections, or you can use drag-and-drop to move them around.

Cut, copy and paste actions

After selecting elements and/or folders, **Cut** (✂) and **Copy** (❐) become available. After cutting or copying, the **Paste** (🗋) action becomes available. There are several ways to access these actions:

- In the menu available if you right-click on a selected element or folder.
- In the top level **Edit** menu.
- As keyboard shortcuts:
  - Ctrl + C (⌘ + C on Mac) to copy
  - Ctrl + X (⌘ + X on Mac) to cut
  - Ctrl + V (⌘ + V on Mac) to paste

  When you cut an element, it will appear "grayed out" until you activate the paste function. You can revert the cut command by copying another element.

Drag and drop for copying or moving

To use drag and drop to copy or move elements and/or folders, select the desired items, keep the mouse button depressed, and drag the selection to the new position.

When elements are moved *between different CLC Locations*, copies of the original elements are made and placed in the new location.

When elements are moved *within the same CLC Location*, no copy is created. The elements are just moved from one folder to another.

To make a copy of an element within the same CLC Location, use the **Copy** (❐) and **Paste** (🗋) actions, or depress the Ctrl / Command key while dragging the data element to the new folder.

Copies of an element open in the View area can also be made by clicking on its tab in the View Area and dragging the tab to the desired location in the Navigation Area. This is *not* a way to save updates to an existing element. Any unsaved changes to the original element (the one open in the View area) remain unsaved until an explicit save action is taken on the original.

Names of copied or moved elements

CLC element names must be unique in a given CLC Location. This means that the same name can be used for different elements in different folders, but not for CLC elements in the same folder.

For non-CLC files available in the Navigation Area, the file must have a name unique within that CLC Location.

If an action results in a file with a name that clashes with an existing file, the name of the new element will have a numeric extension appended to guarantee uniqueness, e.g. "-1", "-2", etc.

3.1.7 Updating element and folder names

Renaming elements and folders

To start editing the name of an element or folder in the **Navigation Area**, do one of the following:

- Slow double-click on the item's name. I.e. Click on the name once, leave a short pause and click on the name again.

  The speed of a slow double-click is usually defined at the system level. Double-clicking quickly on an element's name will open it in the viewing area, and double-clicking quickly on a folder name will open a closed folder or close an open folder.

- Click on the item's name to select it and then click on the function key F2.

- Click on the item's name to select it, and then select the option Rename from the top-level Edit menu.

When you have finished editing the name, click on the **Enter** key or select another element in the **Navigation Area**. To disregard changes before saving them, click on the **Esc** key.

If you update the name of an item you do not have permission to change, the new name will not be kept. The original name will be retained.

Renaming annotations is described in section 14.3.3.

3.1.8 Delete, restore and remove elements

When data in a *CLC Main Workbench* File Location is deleted, it is moved to the recycle bin within that File Location. From the recycle bin, data can be restored, i.e. taken back out of the recycle bin, or it can be deleted from the disk by emptying the recycle bin. Disk space is not freed up until data is deleted from the disk.

To delete an element, or a folder of elements:

1. Move it to the recycle bin by using the **Delete ()** option from the **Edit** menu, the right-click menu of an element, or in the **Toolbar**, or use the **Delete key** on your keyboard.
2. Empty the recycle bin using the **Empty Recycle Bin** command available under the **Edit** menu or in the menu presented when you right-click on a **Recycle Bin** ().

Note! Emptying the recycle bin cannot be undone. Data is not recoverable after it has been removed by emptying the recycle bin.

For deleting annotations from sequences, see section 14.3.5.

To restore items in a recycle bin:

- Drag the items using your mouse into the folder where they used to be, or
- Right-click on the element and choose the option **Restore from Recycle Bin**.

Deleting data held on a CLC Server

Deleting data and folders works the same way for data in *CLC Server* File Locations as described above. The following differences should, however, be noted:

- The contents of your server-based recycle bin can be accessed by you and by your server administrator.
- *CLC Server* settings can affect how you work with server-based recycle bins. For example:
  - Recycle bins can be configured to be automatically emptied periodically.
  - The ability to empty server-based recycle bins can be restricted to something only administrators can do.

3.1.9 Show folder elements in a table

A location or a folder might contain large amounts of elements. It is possible to view their elements in the View Area:

select a folder or location | **Show () in the Toolbar**

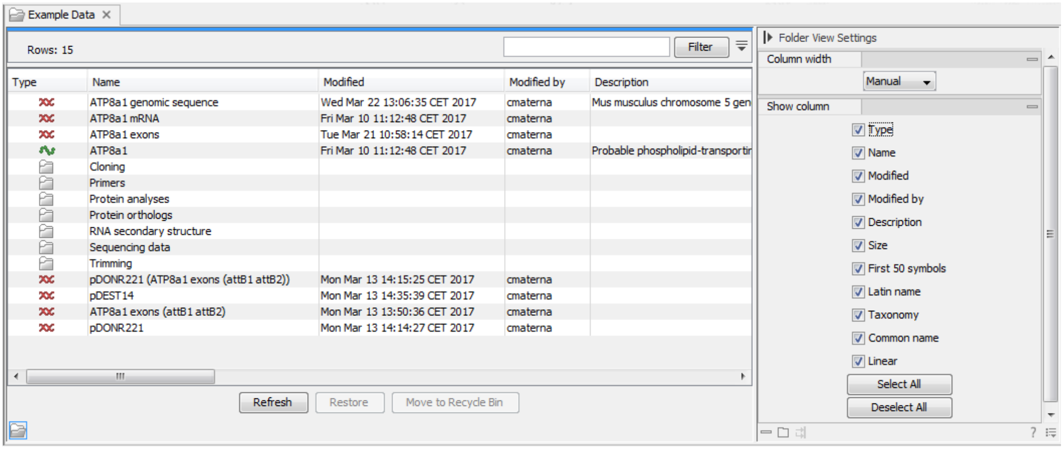
or **select a folder or location** | right click on the folder and select **Show ()** | **Contents ()**

An example is shown in figure 3.7.

When the elements are shown in the view, they can be sorted by clicking the heading of each of the columns. You can further refine the sorting by pressing Ctrl (⌘ on Mac) while clicking the heading of another column.

Sorting the elements in a view does not affect the ordering of the elements in the **Navigation Area**.

Note! The view only displays one "layer" at a time: the content of subfolders is not visible in this view. Also note that only sequences have the full span of information like organism etc.



Example Data

Rows: 15

Type	Name	Modified	Modified by	Description
	ATP8a1 genomic sequence	Wed Mar 22 13:06:35 CET 2017	cmaterna	Mus musculus chromosome 5 gen
	ATP8a1 mRNA	Fri Mar 10 11:12:48 CET 2017	cmaterna	
	ATP8a1 exons	Tue Mar 21 10:58:14 CET 2017	cmaterna	
	ATP8a1	Fri Mar 10 11:12:48 CET 2017	cmaterna	Probable phospholipid-transportir
	Cloning			
	Primers			
	Protein analyses			
	Protein orthologs			
	RNA secondary structure			
	Sequencing data			
	Trimming			
	pDONR221 (ATP8a1 exons (attB1 attB2))	Mon Mar 13 14:15:25 CET 2017	cmaterna	
	pDEST14	Mon Mar 13 14:35:39 CET 2017	cmaterna	
	ATP8a1 exons (attB1 attB2)	Mon Mar 13 13:50:36 CET 2017	cmaterna	
	pDONR221	Mon Mar 13 14:14:27 CET 2017	cmaterna	

Folder View Settings

Column width: Manual

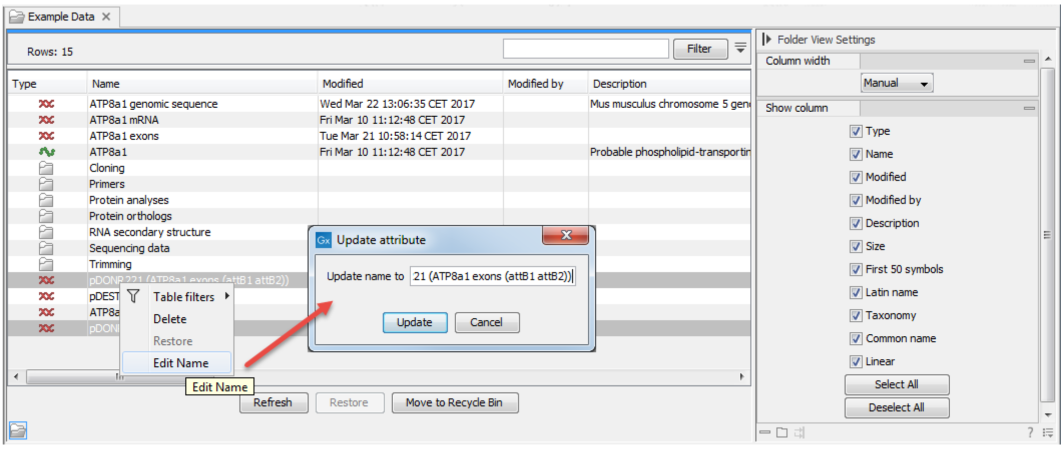
Show column

☑ Type
☑ Name
☑ Modified
☑ Modified by
☑ Description
☑ Size
☑ First 50 symbols
☑ Latin name
☑ Taxonomy
☑ Common name
☑ Linear

Figure 3.7: *Viewing the elements in a folder.*

Batch edit folder elements You can select a number of elements in the table, right-click and choose **Edit** to batch edit the elements. In this way, you can change for example the description or name of several elements in one go.

In figure 3.8 you can see an example where the name of two sequence are renamed in one go. In this example, a dialog with a text field will be shown, letting you enter a new name for these two sequences.



Example Data

Rows: 15

Type	Name	Modified	Modified by	Description
	ATP8a1 genomic sequence	Wed Mar 22 13:06:35 CET 2017		Mus musculus chromosome 5 gen
	ATP8a1 mRNA	Fri Mar 10 11:12:48 CET 2017		
	ATP8a1 exons	Tue Mar 21 10:58:14 CET 2017		
	ATP8a1	Fri Mar 10 11:12:48 CET 2017		Probable phospholipid-transportir
	Cloning			
	Primers			
	Protein analyses			
	Protein orthologs			
	RNA secondary structure			
	Sequencing data			
	Trimming			
	pDONR221 (ATP8a1 exons (attB1 attB2))			
	pDEST			
	ATP8a			
	pDON			

Update attribute

Update name to: 21 (ATP8a1 exons (attB1 attB2))

Folder View Settings

Column width: Manual

Show column

☑ Type
☑ Name
☑ Modified
☑ Modified by
☑ Description
☑ Size
☑ First 50 symbols
☑ Latin name
☑ Taxonomy
☑ Common name
☑ Linear

Figure 3.8: *Changing the common name of two sequences.*

Note! This information is directly saved and you cannot undo.

Drag and drop folder elements You can drag and drop objects from the folder editor to the Navigation area. This will create a copy of the objects at the selected destination. New elements can be included in the folder editor in the view area by dragging and dropping an element from a destination in the Navigation Area to the folder in the Navigation Area that you have open in the view area. It is not possible to drag elements directly from the Navigation Area to the folder editor in the View area.

3.2 Working with non-CLC format files

This section covers working with non-CLC format files stored in a CLC Location. Non-CLC format files can be recognized by their icons (e.g. representing a pdf or Word document), or by having a generic icon, when no specific icon could be assigned. Non-clc format files also usually have the original file extension in their names, such as `.pdf`, `.docx`, `.xml` and so on.

Non-CLC format files can be imported to a CLC Location using the Standard Import tool (section 7.1). Any format not recognized is imported as an external (non-CLC format) file. Such files cannot be opened directly in the *CLC Workbench*, but double clicking on its name in the Navigation Area will open it in a relevant program, if your system supports this. Alternatively, if your system supports it, click on the file in the Navigation Area and, keeping the mouse button depressed, drag it onto a relevant program, for example one represented by an icon in a toolbar, to indicate the file should be opened by that tool.

Copying non-CLC format files to your file system

A copy of non-CLC format files in CLC Locations can be saved to other areas of the file system easily:

1. Select the non-CLC format files in the Navigation Area.
2. Right-click on a selected file and choose the option **Save to disk...** from the menu (figure 3.9).

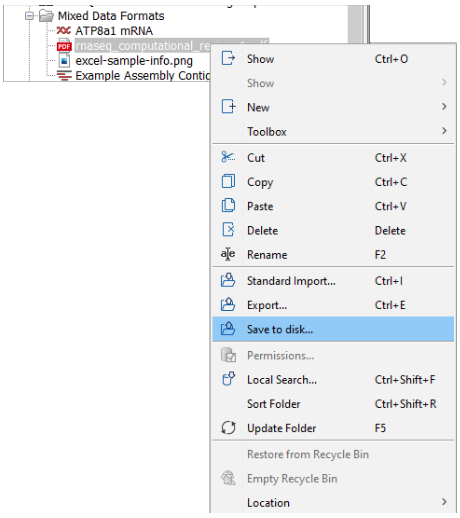


Figure 3.9: *Select one or more non-CLC format files, right-click and choose the option "Save to disk...".*

Using drag and drop for copying or moving non-CLC format files

Non-CLC format files can be saved to another place accessible on your system using drag and drop. To do this:

1. Select the non-CLC format file(s) in the Workbench Navigation Area.

2. Keeping the mouse button depressed, drag the selection to a local file browser.

Note: Dragging to a place on the same file system as the CLC Location results in the file(s) being *moved* from the CLC Location to the new location. Dragging to a location on a different file system results in the file(s) being copied, thus leaving the original file(s) in place in the CLC Location.

The "Save to disk..." functionality described in the section above always makes a copy.

3.3 Customized attributes on data locations

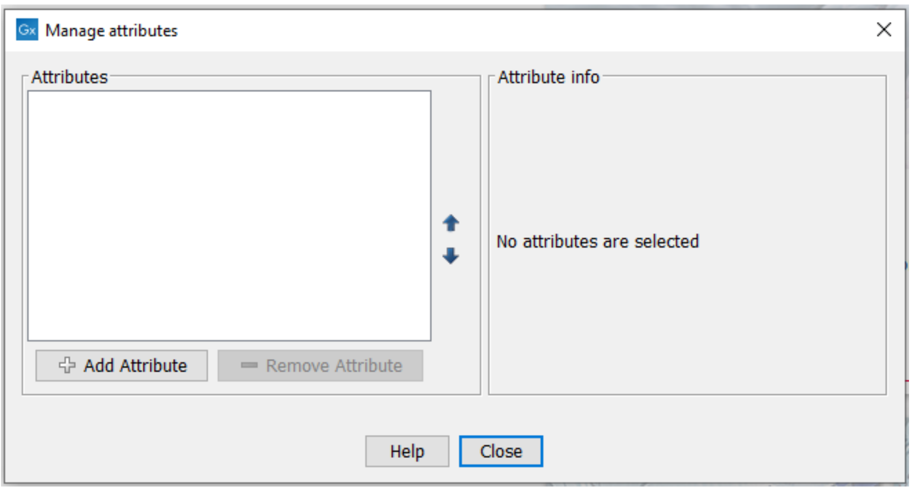
Location-specific attributes can be set on all elements stored in a given data location. Attributes could be things like company-specific information such as LIMS id, freezer position etc. Attributes are set using a CLC Workbench acting as a client to the CLC Server.

Note that the attributes scheme belongs to a particular data location, so if there are multiple data locations, each will have its own set of attributes.

To configure which fields that should be available[1] go to the Workbench:

right-click the data location | Location | Attribute Manager

This will display the dialog shown in figure 3.10.



No attributes are selected

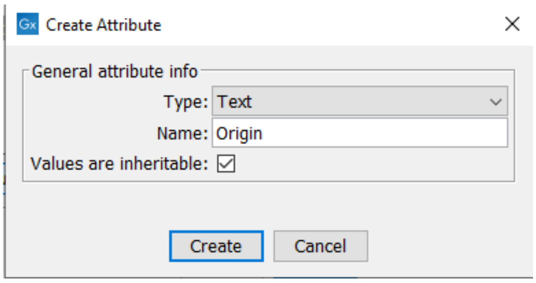
Figure 3.10: *Adding attributes.*

Click the **Add Attribute** (✚) button to create a new attribute. This will display the dialog shown in figure 3.11.

First, select what kind of attribute you wish to create. This affects the type of information that can be entered by the end users, and it also affects the way the data can be searched. The following types are available:

- **Checkbox.** This is used for attributes that are binary (e.g. true/false, checked/unchecked and yes/no).
- **Text.** For simple text with no constraints on what can be entered.

[1] If the data location is a server location, you need to be a server administrator to do this.



Create Attribute

General attribute info

Type: Text

Name: Origin

Values are inheritable: ☑

Figure 3.11: *The list of attribute types.*

- **Hyper Link**. This can be used if the attribute is a reference to a web page. A value of this type will appear to the end user as a hyper link that can be clicked. Note that this attribute can only contain one hyper link. If you need more, you will have to create additional attributes.
- **List**. Lets you define a list of items that can be selected (explained in further detail below).
- **Number**. Any positive or negative integer.
- **Bounded number**. Same as number, but you can define the minimum and maximum values that should be accepted. If you designate some kind of ID to your sequences, you can use the bounded number to define that it should be at least 1 and max 99999 if that is the range of your IDs.
- **Decimal number**. Same as number, but it will also accept decimal numbers.
- **Bounded decimal number**. Same as bounded number, but it will also accept decimal numbers.

When a data element is copied, attribute values are transferred to the copy of the element by default. To prevent the values for an attribute from being copied, uncheck the **Values are inheritable** checkbox.

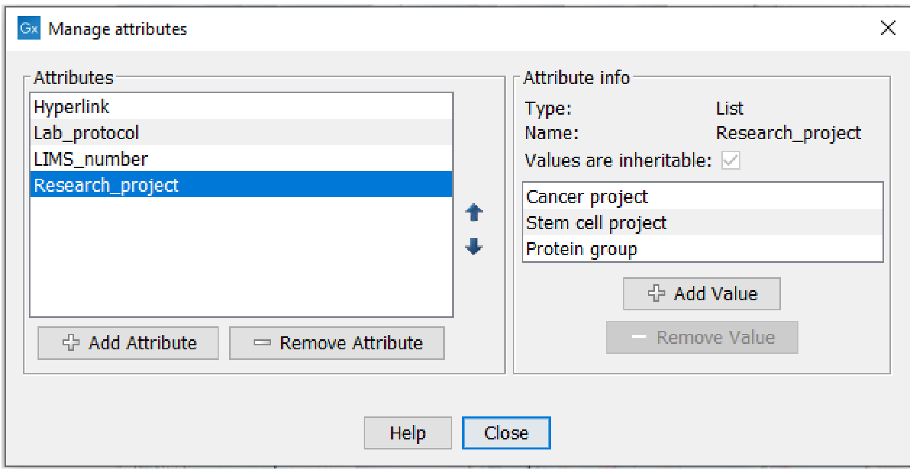
When you click **OK**, the attribute will appear in the list to the left. Clicking the attribute will allow you to see information on its type in the panel to the right.

Lists are a little special, since you have to define the items in the list. When you choose to add the list attribute in the left side of the dialog, you can define the items of the list in the panel to the right by clicking **Add Item** (✚) (see figure 3.12).

Remove items in the list by pressing **Remove Item** (−).

Removing attributes To remove an attribute, select the attribute in the list and click **Remove Attribute** (−). This can be done without any further implications if the attribute has just been created, but if you remove an attribute where values have already been given for elements in the data location, it will have implications for these elements: The values will not be removed, but they will become static, which means that they cannot be edited anymore.

If you accidentally removed an attribute and wish to restore it, this can be done by creating a new attribute of exactly the same name and type as the one you removed. All the "static" values will now become editable again.



Attributes: Hyperlink, Lab_protocol, LIMS_number, Research_project

Type: List
Name: Research_project
Values are inheritable: ☑

Cancer project
Stem cell project
Protein group

Figure 3.12: *Defining items in a list.*

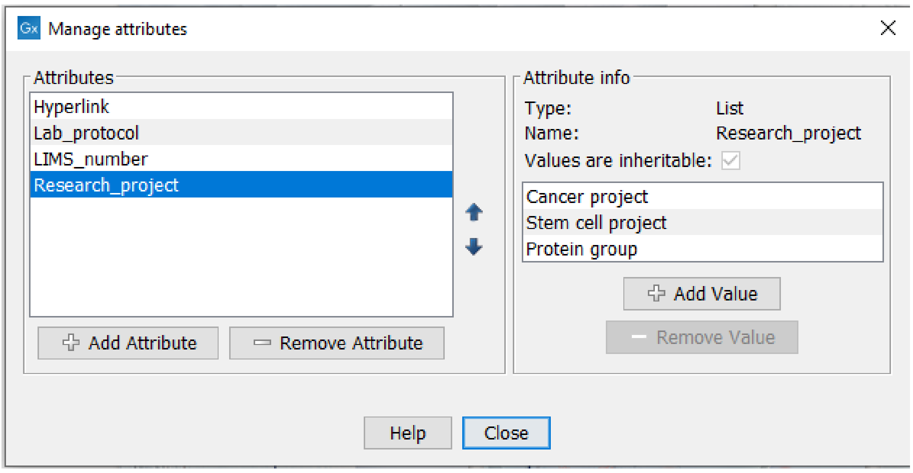
When you remove an attribute, it will no longer be possible to search for it, even if there is "static" information on elements in the data location.

Renaming and changing the type of an attribute is not possible - you will have to create a new one.

Changing the order of the attributes You can change the order of the attributes by selecting an attribute and click the **Up** and **Down** arrows in the dialog. This will affect the way the attributes are presented for the user.

3.3.1 Filling in values

When a set of attributes has been created (as shown in figure 3.13), the end users can start filling in information.



Attributes: Hyperlink, Lab_protocol, LIMS_number, Research_project

Type: List
Name: Research_project
Values are inheritable: ☑

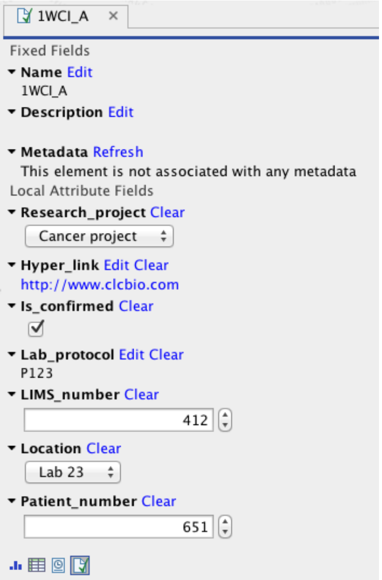
Cancer project
Stem cell project
Protein group

Figure 3.13: *A set of attributes defined in the attribute manager.*

This is done in the element info view:

right-click a sequence or another element in the Navigation Area | Show ( ) | Element info ( )

This will open a view similar to the one shown in figure 3.14.



1WCI_A ×

Fixed Fields

Name Edit
1WCI_A

Description Edit

Metadata Refresh
This element is not associated with any metadata

Local Attribute Fields

Research_project Clear
Cancer project

Hyper_link Edit Clear
http://www.clcbio.com

Is_confirmed Clear
☑

Lab_protocol Edit Clear
P123

LIMS_number Clear
412

Location Clear
Lab 23

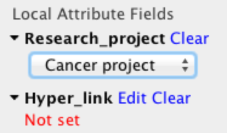
Patient_number Clear
651

Figure 3.14: *Adding values to the attributes.*

You can now enter the appropriate information and **Save**. When you have saved the information, you will be able to search for it (see below).

Note that the element (e.g. sequence) needs to be saved in the data location before you can edit the attribute values.

When nobody has entered information, the attribute will have a "Not set" written in red next to the attribute (see figure 3.15).



Local Attribute Fields

Research_project Clear
Cancer project

Hyper_link Edit Clear
Not set

Figure 3.15: *An attribute which has not been set.*

This is particularly useful for attribute types like checkboxes and lists where you cannot tell, from the displayed value, if it has been set or not. Note that when an attribute has not been set, you cannot search for it, even if it looks like it has a value. In figure 3.15, you will *not* be able to find this sequence if you search for research projects with the value "Cancer project", because it has not been set. To set it, simply click in the list and you will see the red "Not set" disappear.

If you wish to reset the information that has been entered for an attribute, press "Clear" (written in blue next to the attribute). This will return it to the "Not set" state.

The **Folder editor**, invoked by pressing **Show** on a given folder from the context menu, provides a quick way of changing the attributes of many elements in one go. For details, see https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Show_folder_elements_in_table.html.

3.3.2 What happens when a clc object is copied to another data location?

The user supplied information, which has been entered in the **Element info**, is attached to the attributes that have been defined in this particular data location. If you copy the sequence to another data location or to a data location containing another attribute set, the information will become fixed, meaning that it is no longer editable and cannot be searched for. Note that attributes that were "Not set" will disappear when you copy data to another location.

If the element (e.g. sequence) is moved back to the original data location, the information will again be editable and searchable.

If the e.g. Molecule Project or Molecule Table is moved back to the original data location, the information will again be editable and searchable.

3.3.3 Searching custom attributes

When a text-based attribute is created, it becomes available for searching using Quick Search or Local Search.

Quick Search: Precede the name of the attribute with "attrib_" and then add a colon followed by the query term, e.g. "attrib_color:blue".

You must use whole words when searching for elements containing particular values for local attributes.

For searching purposes, **words** are the terms on either side of a space, hyphen or underscore in a name. The names of elements and folders are split into words when indexing.

In the **Local Search** ( ) tool, the local attributes are available to select from a drop down list, in addition to Name and Path.

Read more about search here: https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Local_Search.html.

3.4 Searching for data in CLC Locations

There are two ways of searching for data in your CLC Locations:

- **Quick Search** Available above the **Navigation Area** and described in section 3.4.1. By default, terms entered are used to search for the names of data elements and folders across all available CLC Locations.
- **Local Search** Available under the Utilities menu and described in section 3.4.2. All CLC Locations can be searched, or an individual Location can be searched. Local searches can be saved, so that the same search can be run again easily.

Re-indexing CLC Locations

The *CLC Main Workbench* automatically indexes data added to a CLC Data Location using CLC software tools. These indexes are used when searching in the Navigation Area, to fetch the relevant data elements when launching an analysis and for resolving associations between data elements and CLC Metadata Tables. When problems with any of these are experienced, it may be resolved by re-indexing that CLC Location.

To re-index, right-click on the relevant CLC Location in the Navigation Area and then, from the menu that appears, select the option:

Location | Rebuild Index

Rebuilding the index for locations with a lot of data can take some time.

The index building process can be stopped under the Processes tab, described in section 2.4.

Indexes are updated automatically when data is moved between CLC Locations, but not when data elements are moved within a given CLC Location. When searching based on names, this does not matter. However, for searches based on information in the path, the index may need to be rebuilt before searching.

3.4.1 Quick Search

The search field just above the Navigation Area on the left side of the *CLC Main Workbench* (figure 3.16) can be used to search for data elements and folders in all available CLC Locations. By default matches against names are reported. Searches are case insensitive.

A CLC URL, which specifies a specific element or folder, can also be entered. This is described at the end of this section.

Using Quick Search for searches based on names

The examples below refer to searches where the following elements are present in a CLC Location.

- E. coli reference sequence
- Broccoli sequence
- Coliform set

Search with a single term to look for any element or folder with a name containing that term.

Example 1: A search for *coli* would return all 3 elements listed above.

Search with two or more terms to look for any element or folder with a name containing all of those terms.

Example 2: A search for *coli set* would return "Coliform set" but not the other two entries listed in the earlier example.

Search with two or more words in quotes to look for any element or folder name containing those words, appearing consecutively, in the order provided. Whole words must be used within quotes, rather than partial terms.

For searching purposes, **words** are the terms on either side of a space, hyphen or underscore in a name. The names of elements and folders are split into words when indexing.

Example 3: A search for *"coli reference"* would find an element called "E. coli reference sequence".

Example 4: A search for *"coli sequence"* would not return any of the elements in the example list. In the name "E. coli reference sequence", the words coli and sequence are not placed consecutively, and in "Broccoli sequence", "coli" is a partial term rather than a whole word.

Why only words when searching with quotes? The use of quotes allows quite specific searches to be run quickly, but only using words, as defined by the indexing system.

Tip: Searches with whole words are faster than searching with partial terms. If a term is a word in some names but a partial term in others, the hits found using the complete word are returned first. E.g. searches with the term *cancer* would return elements with names like "cancer reads" and "my cancer sample" before an element with a names like "cancerreads".

Note: Wildcards (`* ? ~`) are ignored in basic searches. If you wish to define a search using wildcards, use the advanced search functionality of Quick Search.

Advanced searching using Quick Search

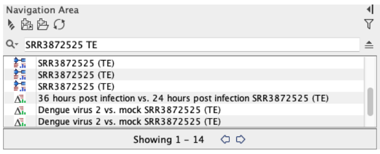
When a search term is preceded by "name:", more specific searches can be defined, using standard wild cards. If multiple query terms are provided, elements and folders containing all terms are returned. Searches are case insensitive.

Terms in the path can also be searched for by preceding the term with "path:".

If you have defined local (custom) attributes for any of your CLC Locations, the contents of these can also be searched with using the name of the attribute preceded by "attrib_", e.g. "attrib_color:blue".

Example 5: A search for **path:tutorials name:cancer** would find all data elements or folders where at least one folder in its path contained "tutorials" and the name of the folder or element included the word "cancer". Word here is as defined earlier in this section. In this example, an element named "cancer tissue reads" in a subfolder under "CLC_Tutorials" would be found, but an element called "cancerreads" in that subfolder would not be.

Example 6: A search for **path:tutorials cancer** would find all data elements or folders where at least one folder in its path contained "tutorials" (with capital or small letters) and the name of the folder or element reported *including* the word "cancer". In this example, an element named "cancer tissue reads" in a subfolder under "CLC_Tutorials" would be found, and so would an element called "cancerreads" in that subfolder.



SRR3872525 TE

SRR3872525 (TE)
SRR3872525 (TE)
SRR3872525 (TE)
36 hours post infection vs. 24 hours post infection SRR3872525 (TE)
Dengue virus 2 vs. mock SRR3872525 (TE)
Dengue virus 2 vs. mock SRR3872525 (TE)

Figure 3.16: *Enter terms in the Quick Search field to look for elements and folders.*

Advanced search expressions

The terms below can only be used when the field being searched has been specified explicitly, e.g. by preceding the search term with "name:" or "path:".

- **Wildcard multiple character search (*).** Appending an asterisk * to the search term find matches that start with that term. E.g. A search for `BRCA*` will find terms like *BRCA1*, *BRCA2*, and *BRCA1gene*.

- **Wildcard single character search (?)**. The ? character represents exactly one character. For example, searching for `BRCA?` would find *BRCA1* and *BRCA2*, but would not find *BRCA1gene*.
- **Search related words (~)**. Appending a tilde to the search term looks for fuzzy matches, that is, terms that almost match the search term, but are not necessarily exact matches. For example, : `ADRAA~` will find terms similar to ADRA1A.

Search results

When there are many hits, only the first 50 are shown initially. To see the next 50, click on the **Next** (➡) arrow just under the list of results.

The number to return initially can be configured in the Workbench Preferences, as described in section 4.

Finding the element or folder in the Navigation Area

You can move the focus from a search result to its location in the Navigation Area by doing one of the following:

- Click on a search result while keeping the Alt key depressed.
- Right-click on a search result and click on **Show Location** in the menu presented.

Quick Search history

You can access the 10 most recent searches by clicking the icon (🔍▾) to the left of the search field (figure 3.17). When one of these is selected, the search is automatically run again.

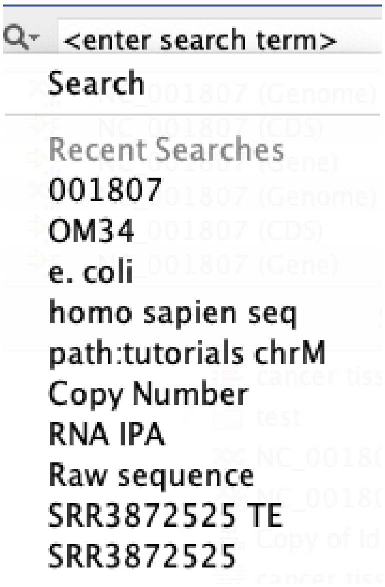
Searching using CLC URLs

A particular data element or folder can be quickly found by entering its CLC URL into the Quick Search box (figure 3.18). An example of when this is useful is when working using a *CLC Server* and sharing the location of particular data elements with another person.

To obtain and use a CLC URL:

1. Copy the element or folder in your Navigation Area (section 3.1.6) .
2. Paste the contents of the clipboard (i.e. the copied information) to a place that expects text. The text that will be pasted is the CLC URL for that element or folder.

   Examples of where text is expected include a text editor, email, messaging system, etc. It also includes the Quick Search field.
3. Paste the CLC URL into the Quick Search field above the Navigation Area to locate the element or folder it refers to.



<enter search term>

Search

Recent Searches

001807

OM34

e. coli

homo sapien seq

path:tutorials chrM

Copy Number

RNA IPA

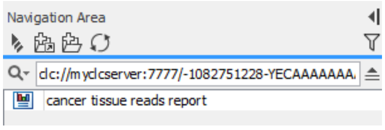
Raw sequence

SRR3872525 TE

SRR3872525

Figure 3.17: *Recent searches are listed and can be selected to be re-run by clicking on the icon to the left of the search field.*

If you move the element or folder within the *same* CLC Location, the CLC URL will continue to work.



clc://myclcserver:7777/-1082751228-YECAAAAAAA

cancer tissue reads report

Figure 3.18: *Data elements can be located using a CLC URL.*

3.4.2 Local Search

The **Local Search** tool is useful when searching large data locations and if you wish to save searches to be run multiple times.

To launch the **Local Search** tool, go to:

Utilities | **Local Search** ( )

or **Ctrl + Shift + F (⌘ + Shift + F on Mac)**

You can choose to search across all CLC Locations or you can specify a single Location to search.

You can search for terms in the names of elements or folders, as well as terms in the path to those elements or folders. If you have defined local (custom) attributes for any of your CLC Locations, the contents of these can also be searched.

More than one search term can be added by clicking on the **Add search parameters** button. To search for terms of the same type (e.g. terms in names), you can just add multiple terms in the same search field, as described below.

Click on the **Search** button to start the search.

Using Local Search for searches based on names

The examples below refer to searches where the following elements are present in a CLC Location.

- E. coli reference sequence
- Broccoli sequence
- Coliform set

Search with a single term to look for any element or folder with a name containing that term.

Example 1: A search for *coli* would return all 3 elements listed above.

Search with two or more terms to look for any element or folder with a name containing all of those terms.

Example 2: A search for *coli set* would return "Coliform set" but not the other two entries listed in the earlier example.

Search with two or more words in quotes to look for any element or folder name containing those words, appearing consecutively, in the order provided. Whole words must be used within quotes, rather than partial terms.

For searching purposes, **words** are the terms on either side of a space, hyphen or underscore in a name. The names of elements and folders are split into words when indexing.

Example 3: A search for *"coli reference"* would find an element called "E. coli reference sequence".

Example 4: A search for *"coli sequence"* would not return any of the elements in the example list. In the name "E. coli reference sequence", the words coli and sequence are not placed consecutively, and in "Broccoli sequence", "coli" is a partial term rather than a whole word.

Why only words when searching with quotes? The use of quotes allows quite specific searches to be run quickly, but only using words, as defined by the indexing system.

Tip: Searches with whole words are faster than searching with partial terms. If a term is a word in some names but a partial term in others, the hits found using the complete word are returned first. E.g. searches with the term *cancer* would return elements with names like "cancer reads" and "my cancer sample" before an element with a names like "cancerreads".

Note: Wildcards (`* ? ~`) are ignored in basic searches. If you wish to define a search using wildcards, use the advanced search functionality of Quick Search.

Saving Local Searches

Local searches can be saved (⇐), so that the same search can be run again easily. Saving a search saves the query, not the results.

The search you just set up can be saved by doing one of the following:

- Choose **Save As...** from under the File menu, or
- Click on the tab of the search view and drag and drop it into a folder in the Navigation Area.

These actions save the search query. (It does not save the search results.)

This can be useful when you run the same searches periodically.

3.5 Backing up data from the CLC Workbench

CLC Workbench data is stored in CLC File Locations. To back up all of your CLC data, back up each of your CLC File Locations (option 1). To back up smaller amounts of data, export selected data elements to a zip file (option 2).

Option 1: Backing up a CLC File Location

Each CLC File Location is associated with a folder on the underlying file system. Hovering the mouse cursor over a CLC File Location ( ), i.e. a top level folder in the Navigation Area, reveals a tooltip containing the location of that file system folder. Backing up that folder equates to backing up a given CLC File Location.

To recover the data later, retrieve the folder from backup and add it as a CLC File Location (see section 3.1.2).

Note: If the original CLC File Location is still present, the copy from backup will *not* be added. If you wish to add the folder from backup as a new CLC File Location, the options are:

- Remove the original CLC File Location, and then add the folder from backup as a new CLC File Location,

  or

- Remove the file called ".clcinfo" from the top level of the folder from backup, and then add the folder as a CLC File Location.

CLC File Location information is stored in an XML file called model_settings_300.xml located in the settings folder in the user home area. Further details about this file and how it pertains to data locations in the Workbench can be found in the Workbench Deployment Manual: https://resources.qiagenbioinformatics.com/manuals/workbenchdeployment/current/index.php?manual=Changing_default_location.html.

Option 2: Export a folder of data or individual data elements to a CLC zip file

This option is for backing up smaller amounts of data, for example, backing up certain results, or backing up a whole CLC File Location, that contains a small amount of data.

To export data, click on the Export ( ) button in the top toolbar, or go to:

File | Export ( )

Choose zip as the format to export to.

The data to export to the zip file can then be selected.

Further details about exporting data this way is provided in section 8.1.4.

To imported the zip file back into a *CLC Workbench*, click on the Import ( ) button in the top toolbar and select **Standard Import**, or go to:

File | **Import** (📥) | **Standard Import**

and select *Automatic import* in the Options area.

3.6 Working with AWS S3 using the Remote Files tab

Browsing data in AWS S3 using the Remote Files tab

Available AWS S3 buckets are listed under the **Remote Files** tab, to the right of the Navigation Area tab (figure 3.19).

If AWS S3 buckets are available from more than one source, those sources are listed in a drop-down menu. Possible sources are AWS Connections or public S3 buckets configured in the *CLC Workbench*, or configured in a *CLC Server* that the Workbench is connected to. A server icon (S) is shown beside sources available via a *CLC Server* (figure 3.20).

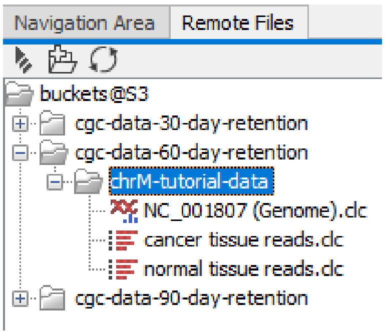


Figure 3.19: *AWS S3 buckets you have access to are available under the Remote Files tab.*

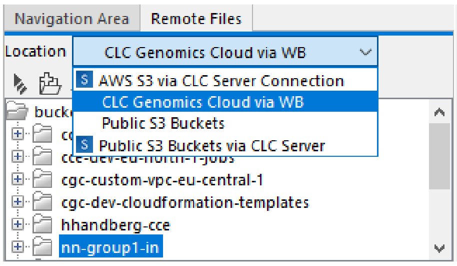

Navigation Area
Remote Files
Location
CLC Genomics Cloud via WB
AWS S3 via CLC Server Connection
CLC Genomics Cloud via WB
Public S3 Buckets
Public S3 Buckets via CLC Server
buck
cgc-custom-vpc-eu-central-1
cgc-dev-cloudformation-templates
hhandberg-cce
nn-group1-in

Figure 3.20: *This Workbench has a valid AWS Connection and is connected to a CLC Server with a valid AWS Connection. At least one public S3 bucket has been configured in the Workbench and in the CLC Server. The S3 buckets available from the selected source are listed in the Remote Files tab.*

Uploading data to AWS S3 using the Remote Files tab

To upload data from your Navigation Area to AWS S3, right-click on a folder in the Remote Files tab and choose the option **Upload to this folder** (figure 3.21).

Upload is sequential. Information about the data upload is shown in the Processes tab, at the bottom left of the Workbench (figure 3.22).

Downloading results via the Remote Files tab

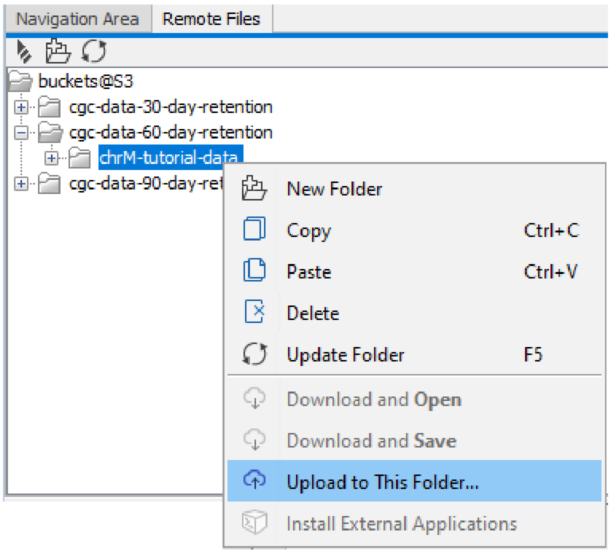


Figure 3.21: *To upload data from your Navigation Area to AWS S3, open the Remote Files tab, right-click on the folder you wish to upload data to, and select the option "Upload to This Folder".*

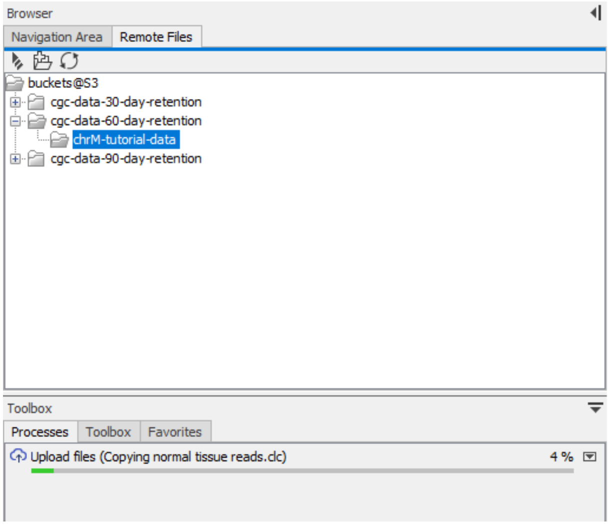

Browser
Navigation Area
Remote Files
buckets@S3
cgc-data-30-day-retention
cgc-data-60-day-retention
chrM-tutorial-data
cgc-data-90-day-retention
Toolbox
Processes
Toolbox
Favorites
Upload files (Copying normal tissue reads.clc)
4 %

Figure 3.22: *After choosing to upload data to S3, the progress of the upload is reported in the Processes tab.*

Right-click on an element or elements under the Remote Files tab to download from AWS S3 (figure 3.23). When only a file or files are selected, options to **Download and Open** and **Download and Save** will be available. When a folder has been selected, only the **Download and Save** is available.

After choosing to download the data, you can choose to download it to a CLC File Location or to another area on your system.

To see all the outputs of a particular job that has been run on a *CLC Genomics Cloud* setup, double-click on a `workflow-result.json` file. All the results can then be downloaded and opened from that list, or individual elements can be selected and downloaded. The Execution Log is also available from this list (see figure 3.24).

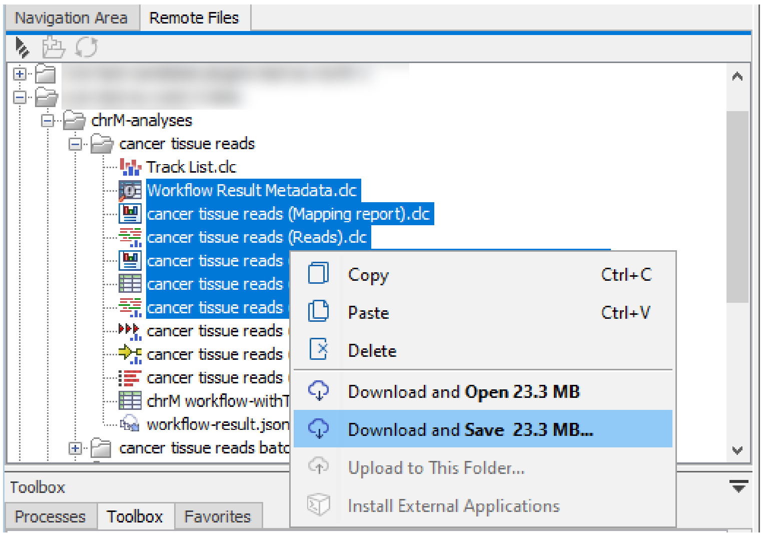


Figure 3.23: *Select folders and/or files in the Remote Files tab and right-click to reveal options for downloading that data.*

If the Navigation Tools plugin is installed, bookmarks for items in the Remote Files tab can be made. Double-clicking on bookmarks for individual results files or folders opens the bookmarked items, as standard. Double-clicking on a bookmark for a workflow-result.json file reveals the same list of options as double-clicking on the workflow-result.json file in the Remote Files tab directly. Further details about bookmarks are provided in the Navigation Tools manual at https://resources.qiagenbioinformatics.com/manuals/navigationtools/current/index.php?manual=Introduction.html.

Note: AWS charges for downloading data from S3. By default, when the download size exceeds 1 GB, you are prompted for confirmation that you wish to proceed. The size required to trigger this warning can be changed in the General section of the Workbench Preferences (figure 3.25).

Downloading data using a URL

Pasting an URL into the Workbench Navigation Area will import the files, or a folder containing files, using Standard Import. File types are automatically detected. Thus, as well as CLC format files being available to view and work further with in the Workbench, other files in formats recognized by Standard Import will be imported as CLC format files, allowing them to be worked with in the Workbench. Further details about Standard Import are provided at: https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Standard_import.html.

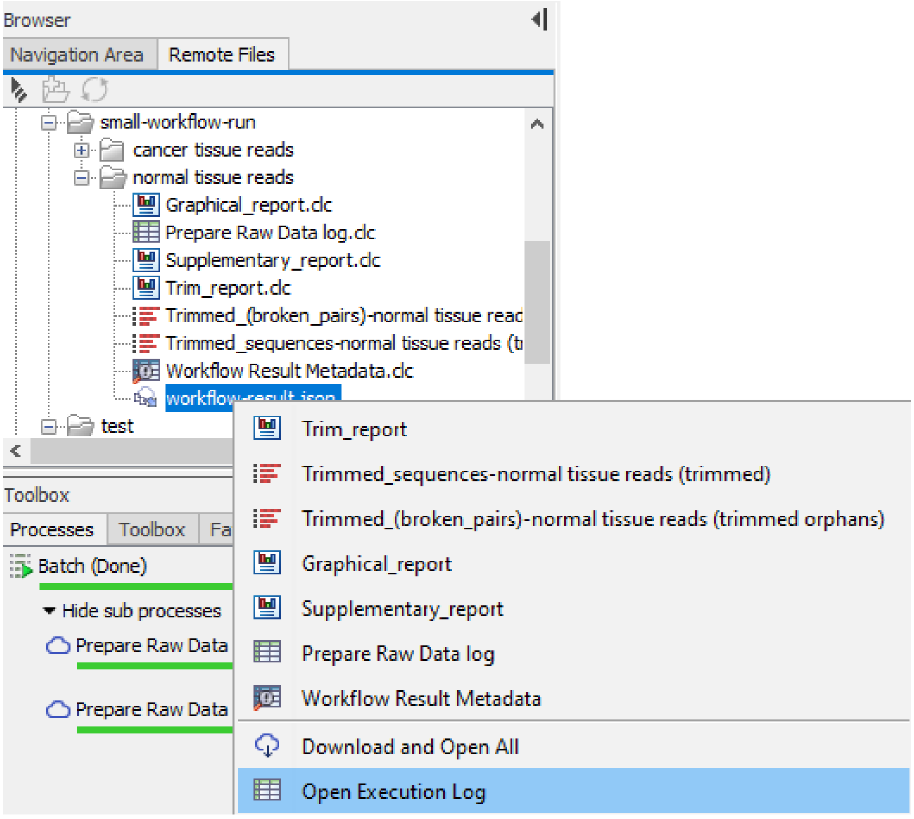
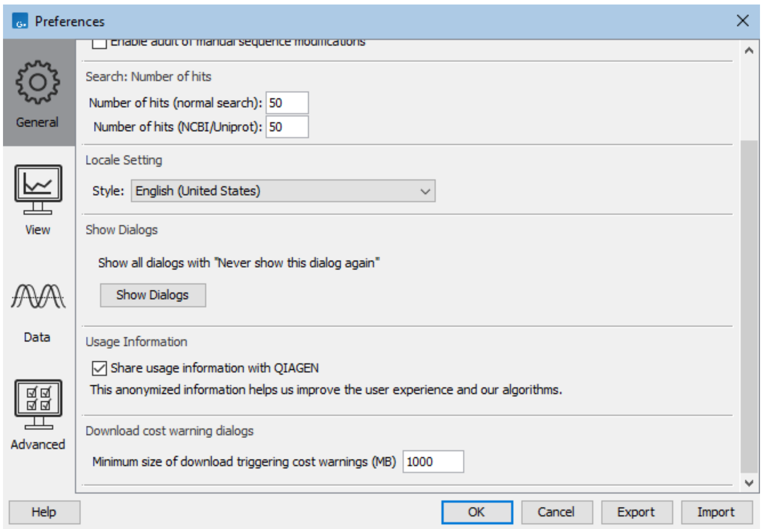


Figure 3.24: *Double-click on a workflow-result.json file in the Remote Files tab in the Workbench to reveal a list of all results from a job run in the cloud, as well as the Execution Log. All items can be downloaded and opened from this menu, or individual items can be selected and downloaded.*



□ Enable audit of manual sequence modifications

Search: Number of hits

Number of hits (normal search): 50

Number of hits (NCBI/Uniprot): 50

Locale Setting

Style: English (United States)

Show Dialogs

Show all dialogs with "Never show this dialog again"

Show Dialogs

Usage Information

☑ Share usage information with QIAGEN

This anonymized information helps us improve the user experience and our algorithms.

Download cost warning dialogs

Minimum size of download triggering cost warnings (MB) 1000

Figure 3.25: *The download size above which a cost warning dialog is shown can be adjusted in the Workbench Preferences. The default value is 1000 MB.*

Chapter 4

User preferences and settings

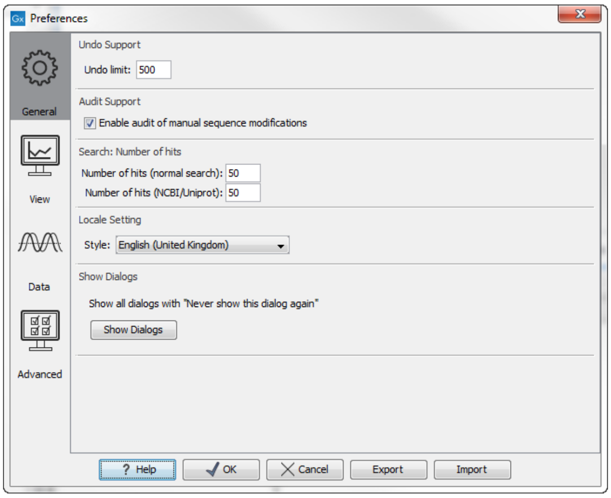
Contents

The **Preferences** dialog (figure 4.1) offers opportunities for changing the default settings for different features of the program. Preference settings are grouped under four tabs, each of which is described in the sections to follow.

The **Preferences** dialog is opened in one of the following ways:

Edit | Preferences ( )

or **Ctrl + K (⌘ + ; on Mac)**



Undo Support

Undo limit: 500

Audit Support

☑ Enable audit of manual sequence modifications

Search: Number of hits

Number of hits (normal search): 50

Number of hits (NCBI/Uniprot): 50

Locale Setting

Style: English (United Kingdom)

Show Dialogs

Show all dialogs with "Never show this dialog again"

Show Dialogs

Figure 4.1: *Preference settings are grouped under the General, View, Data , and Advanced tabs.*

4.1 General preferences

The **General preferences** include:

- **Undo Limit** The default number of undo actions is 500. Undoing and redoing actions is described in section 2.1.3).
- **Audit Support** If this option is checked, all manual editing of sequences will be marked with an annotation on the sequence (figure 4.2). Placing the mouse on the annotation will reveal additional details about the change made to the sequence (see figure 4.3). Note that no matter whether **Audit Support** is checked or not, all changes are recorded in the History log ( ) (see section 2.5).

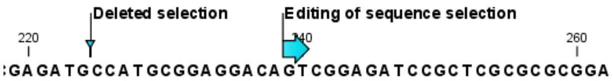
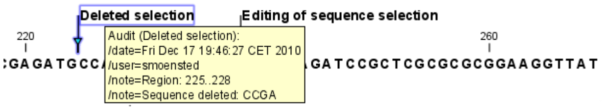

Deleted selection
Editing of sequence selection
220
240
260
:GAGATGCCATGCGGAGGACAGTCGGAGATCCGCTCGCGCGCGGA

Figure 4.2: *Annotations added when the sequence is edited.*



Audit (Deleted selection):
/date=Fri Dec 17 19:46:27 CET 2010
/user=smoensted
/note=Region: 225..228
/note=Sequence deleted: CCGA

Figure 4.3: *Details of the editing.*

- **Number of hits** The number of hits shown in *CLC Main Workbench*, when e.g. searching NCBI. (The sequences shown in the program are not downloaded, until they are opened or dragged/saved into the Navigation Area).
- **Locale Setting** Specify which country you are located in. This determines how punctuation is used in numbers.
- **Show Dialogs** Many information dialogs have a checkbox with the option: "Never show this dialog again". If you have checked such a box, but later decide you wish to see these notifications, click on the **Show Dialogs** button.
- **Usage information** When this item is checked, anonymous information is shared with QIAGEN about how the Workbench is used. This option is enabled by default.

  The information shared with QIAGEN is:

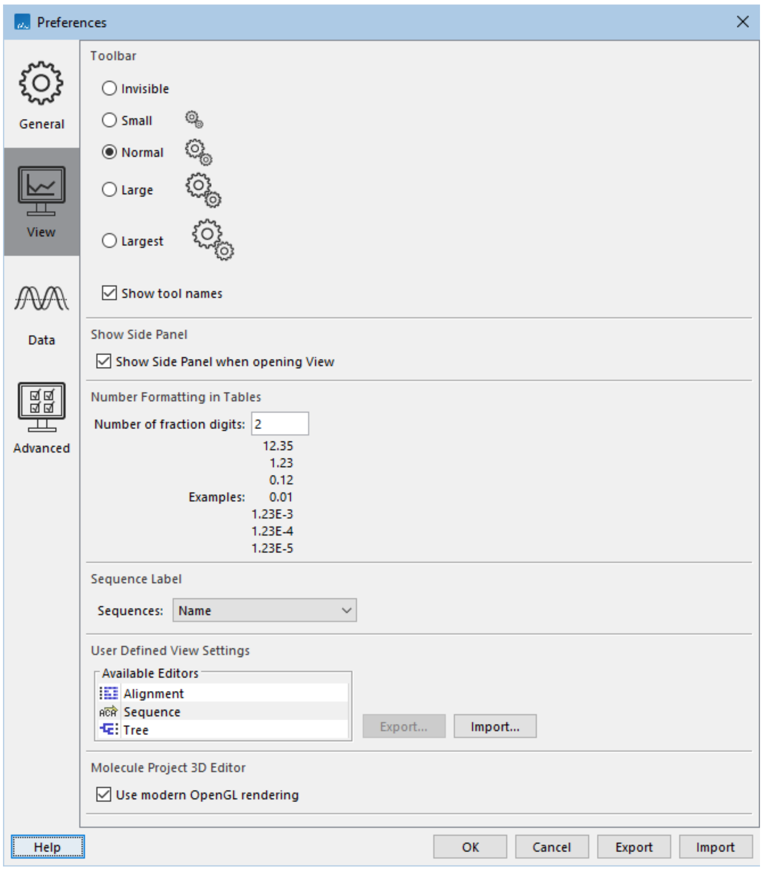
  - Launch information (operating system, product, version, and memory available)
  - The names of the tools and workflows launched (but not the parameters or the data used)
  - Errors (but without any information that could lead to loss of privacy: file names and organisms will not be logged)
  - Installation and removal of plugins and modules

  The following information is also sent:

  - An installation ID. This allows us to group events coming from the same installation. It is not possible to connect this ID to personal or license information.
  - A geographic location. This is predicted based on the IP-address. We do not store IP-addresses after location information has been extracted.
  - A time stamp

4.2 View preferences

There are five groups of default **View** settings (figure 4.4):



Toolbar

○ Invisible

○ Small

● Normal

○ Large

○ Largest

☑ Show tool names

Show Side Panel

☑ Show Side Panel when opening View

Number Formatting in Tables

Number of fraction digits: 2

Examples: 12.35
1.23
0.12
0.01
1.23E-3
1.23E-4
1.23E-5

Sequence Label

Sequences: Name

User Defined View Settings

Available Editors

- Alignment
- Sequence
- Tree

Molecule Project 3D Editor

☑ Use modern OpenGL rendering

Figure 4.4: *Settings relating to views and formating are found under the View tab in Preferences.*

1. **Toolbar** Specify Toolbar icon size, and whether to display names below those icons.

2. **Show Side Panel** Choose whether to display the Side Panel by default when opening a new view.

   For any open view, the Side Panel can be collapsed by clicking on the small triangle at the top left side of the settings area or by using the key combination Ctrl + U (⌘ + U on Mac).

3. **Number formatting in tables** Specify the number formatting to use in tables.

   The examples below the text field are updated to reflect the option selected.

   Open tables must be closed and re-opened for these changes to be applied to them.

4. **Sequence Label** allows you to change the source of the name to use when listing sequence elements in the Navigation Area.

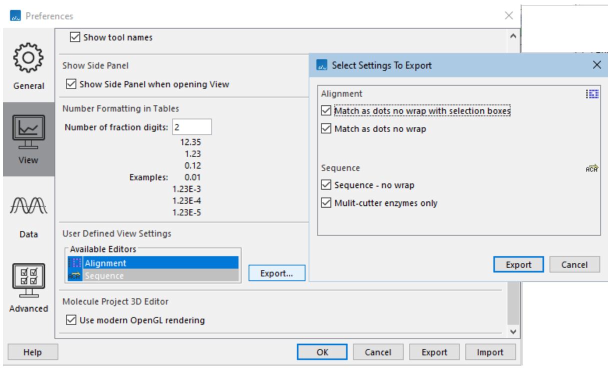
   - Name (the default)
   - Accession (sequences downloaded from databases like GenBank have an accession number).
   - Latin name.

- Latin name (accession).
- Common name.
- Common name (accession).

5. **User Defined View Settings** Data types for which custom view settings have been defined are listed here. The default settings to apply to a given data type can be specified.

   Custom view settings can be exported to a file and imported from a file using the **Export...** and **Import...** button, respectively.

   To export, select items in the "Available Editors" list and then click on the Export button. A `.vsf` file will be saved to the location you specify. You will have the opportunity to deselect any custom view settings you do not wish to export.



Preferences

☑ Show tool names

Show Side Panel

☑ Show Side Panel when opening View

Number Formatting in Tables

Number of fraction digits: 2

Examples: 12.35 1.23 0.12 0.01 1.23E-3 1.23E-4 1.23E-5

User Defined View Settings

Available Editors: Alignment, Sequence

Molecule Project 3D Editor

☑ Use modern OpenGL rendering

Select Settings To Export

Alignment

☑ Match as dots no wrap with selection boxes

☑ Match as dots no wrap

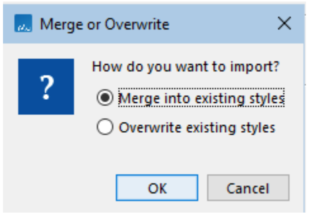
Sequence

☑ Sequence - no wrap

☑ Mulit-cutter enzymes only

Figure 4.5: *Data types for which custom view settings have been defined are listed in the View tab. Settings for multiple views can be exported by selecting them in the list and clicking on the Export... button. Any custom views that should not be included can be delselected before exporting.*

To import view settings, select a `.vsf` file and click on the **Import...** button. Specify whether the new settings should be merged with the existing settings or whether they should overwrite the existing settings (figure 4.6). **Note:** If you choose to overwrite existing settings, all existing custom view settings are deleted.



Merge or Overwrite

How do you want to import?

● Merge into existing styles

○ Overwrite existing styles

Figure 4.6: *When importing view settings, specify whether to merge the new settings with the existing ones or whether to overwrite existing custom settings.*

Note: The Export and Import buttons directly under the list of view settings are for exporting and importing just view settings. The buttons at the bottom of the **Preferences** dialog are for exporting all preferences (see section 4.5).

Specifying default view settings for a given data type can also be done using the **Manage View Settings** dialog, described in section 4.6. Export and import can also be done there.

6. **Molecule Project 3D Editor** gives you the option to turn off the modern OpenGL rendering for **Molecule Projects** (see section 15.2).

4.3 Data preferences

The data preferences contain preferences related to interpretation of data:

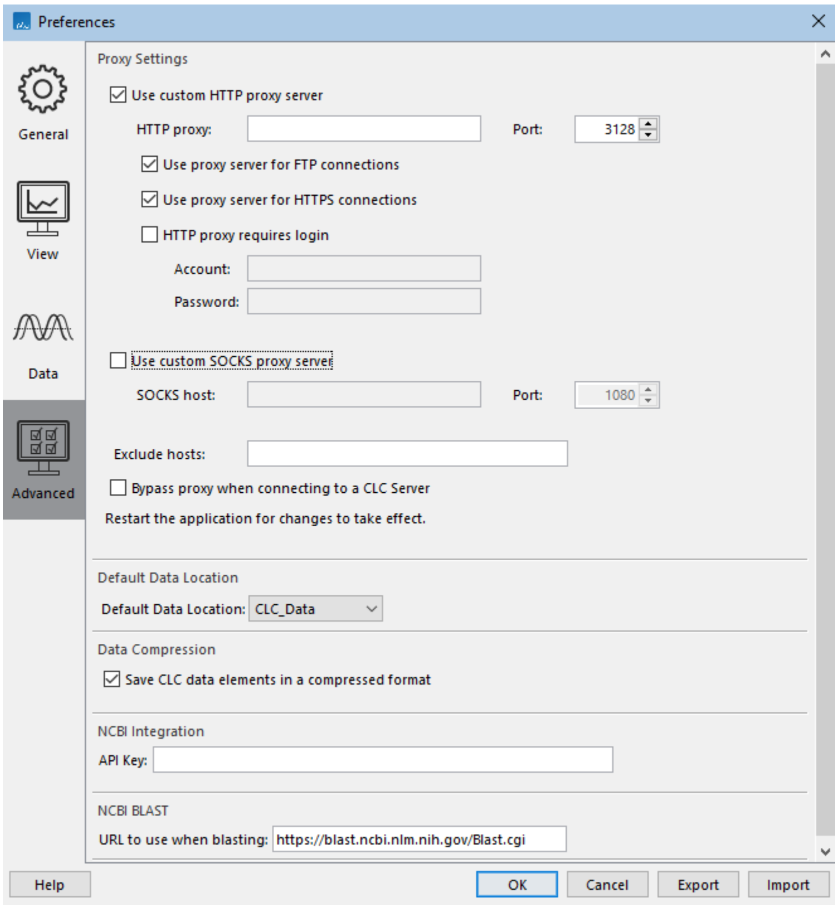
- Multisite Gateway Cloning primer additions, a list of predefined primer additions for Gateway cloning (see section 23.5.1).

4.4 Advanced preferences

Proxy Settings *CLC Main Workbench* supports the use of an HTTP-proxy and an anonymous SOCKS-proxy.

You have the choice between an HTTP-proxy and a SOCKS-proxy. The *CLC Workbench* only supports the use of a SOCKS-proxy that does not require authorization.

You can select whether the proxy should also be used for FTP and HTTPS connections.



Preferences

Proxy Settings

☑ Use custom HTTP proxy server

HTTP proxy: Port: 3128

☑ Use proxy server for FTP connections

☑ Use proxy server for HTTPS connections

☐ HTTP proxy requires login

Account:

Password:

☐ Use custom SOCKS proxy server

SOCKS host: Port: 1080

Exclude hosts:

☐ Bypass proxy when connecting to a CLC Server

Restart the application for changes to take effect.

Default Data Location

Default Data Location: CLC_Data

Data Compression

☑ Save CLC data elements in a compressed format

NCBI Integration

API Key:

NCBI BLAST

URL to use when blasting: https://blast.ncbi.nlm.nih.gov/Blast.cgi

Figure 4.7: *Advanced preference settings include proxy configuration options.*

List hosts that should be contacted directly, i.e. not via the proxy server, in the **Exclude hosts** field. The value can be a list, with each host separated by a | symbol. The wildcard character * can also be used. For example: `*.foo.com|localhost`.

The proxy can be bypassed when connecting to a *CLC Server* by checking the box next to **Bypass proxy when connecting to a CLC Server**.

Workbenches can be preconfigured to bypass the proxy settings when connecting to a *CLC Server* by configuring this setting in a `proxy.properties` file, where the IP address (not the host name) of the *CLC Server* is provided in the *proxyexclude* field. See https://resources.qiagenbioinformatics.com/manuals/workbenchdeployment/current/index.php?manual=Per_computer_Workbench_information.html for further details.

If you have problems with these settings, please contact your systems administrator.

Default data location The default location is used when you import a file without selecting a folder or element in the Navigation Area first. It is set to the folder called CLC_Data in the Navigation Area, but can be changed to another data location using a drop down list of data locations already added (see section 3.1.2). Note that the default location cannot be removed, but only changed to another location.

Data Compression CLC format data is stored in an internally compressed format. The application of internal compression can be disabled by unchecking the option "Save CLC data elements in a compressed format". This option is enabled by default. Turning this option off means that data created may be larger than it otherwise would be.

Enabling data compression may impose a performance penalty depending on the characteristics of the hardware used. However, this penalty is typically small, and we generally recommend that this option remains enabled. Turning this option off is likely to be of interest only at sites running a mix of older and newer CLC software, where the same data is accessed by different versions of the software.

Compatibility information:

- A new compression method was introduced with version 22.0 of the CLC Genomics Workbench, CLC Main Workbench and CLC Genomics Server. Compressed data created using those versions can be read by version 21.0.5 and above, but not earlier versions.
- Internal compression of CLC data was introduced in CLC Genomics Workbench 12.0, CLC Main Workbench 8.1 and CLC Genomics Server 11.0. Compressed data created using these versions is not compatible with older versions of the software. Data created using these versions can be opened by later versions of the software, including versions 22.0 and above.

To share specific data sets for use with software versions that do not support the compression applied by default, we recommend exporting the data to CLC or zip format and turning on the export option "Maximize compatibility with older CLC products". See section 8.1.4.

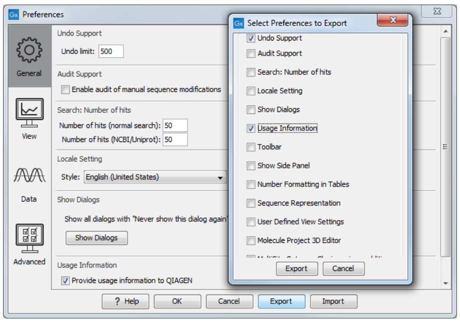
NCBI Integration Without an API key, access to NCBI from asingle IP-address is limited to 3 requests per second; if many workbenches use the same IP address when running the Search for Sequences at NCBI and Search for PDB Structures at NCBI tools they may hit this limit. In this case, you can create an API key for NCBI E-utilities in your NCBI account and enter it here.

NCBI BLAST The standard URL for the BLAST server at NCBI is: https://blast.ncbi.nlm.nih.gov/Blast.cgi, but it is possible to specify an alternate server URL to use for BLAST searches. Be careful to specify a valid URL, otherwise BLAST will not work.

4.5 Export/import of preferences

Your *CLC Main Workbench* preferences can be exported or other sets of preferences imported. This is a way to share the setup of a *CLC Main Workbench* with others, or to back up your own preferences.

To export, open the **Preferences** dialog and click on the Export button at the bottom of the Preferences dialog. Select the relevant preference types and and then proceed with the export (figure 4.8). A `*.cpf` will be exported, which can be imported by other *CLC Main Workbench* users, if desired.



Preferences

Undo Support
Undo limit: 500

Audit Support
☐ Enable audit of manual sequence modifications

Search: Number of hits
Number of hits (normal search): 50
Number of hits (NCBI/Uniprot): 50

Locale Setting
Style: English (United States)

Show Dialogs
Show all dialogs with "Never show this dialog again"

Usage Information
☑ Provide usage information to QIAGEN

Select Preferences to Export

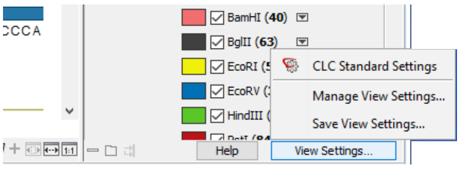
☑ Undo Support
☐ Audit Support
☐ Search: Number of hits
☐ Locale Setting
☐ Show Dialogs
☑ Usage Information
☐ Toolbar
☐ Show Side Panel
☐ Number Formatting in Tables
☐ Sequence Representation
☐ User Defined View Settings
☐ Molecule Project 3D Editor

Figure 4.8: *Select the preference types to export.*

Note: The "User Defined View Settings" option here refers only to information on which view settings to set as the default for each view type. To export the view settings themselves, export a `.vsf` file from the User Defined View Settings section under the View tab of Preferences, as described in section 4.2.

4.6 Side Panel view settings

The **View Settings...** menu at the bottom, right of the **Side Panel** provides options for saving, applying and managing view settings (figure 4.9).



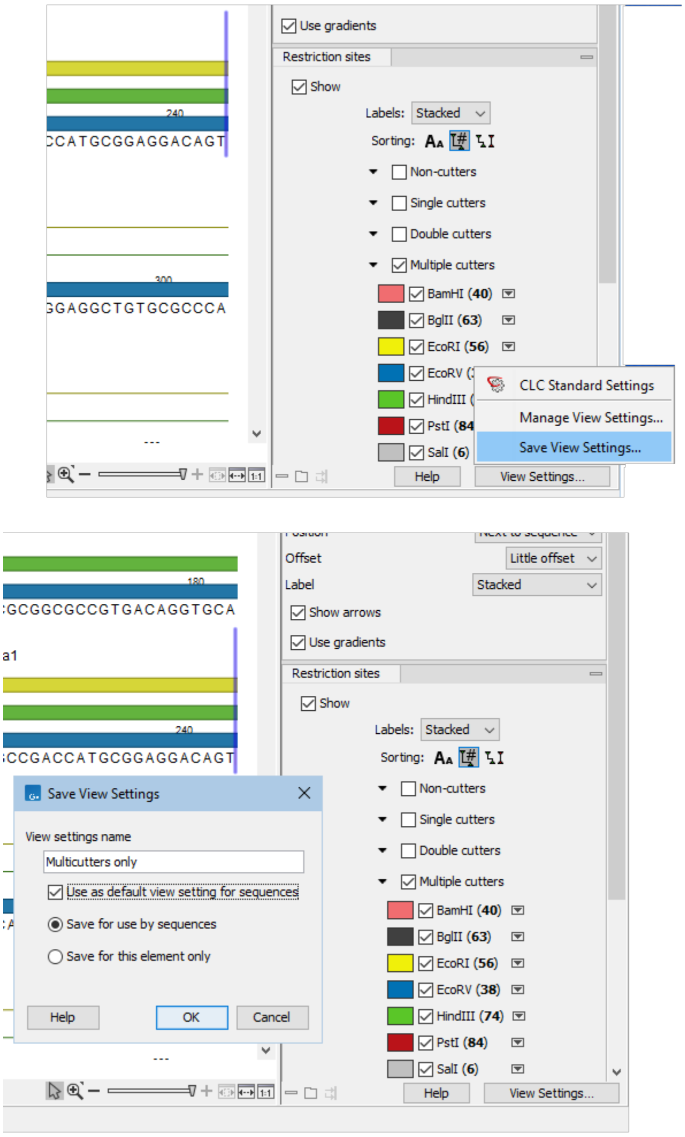
☑ BamHI (40)
☑ BglII (63)
☑ EcoRI (5
☑ EcoRV (
☑ HindIII (

Figure 4.9: *Click on the View Settings button at the bottom of a Side Panel to apply a new view settings or to open dialogs for saving and managing view settings.*

This section focuses on the functionality provided under the **View Settings...** menu for applying and managing view settings. For general information about Side Panel settings, see section 2.1.6. For view settings specific to tables, including column ordering, see section 9.

Saving view settings

To save view settings for later use, select the option **Save View Settings...** under the **View Settings...** menu. In the resulting dialog (figure 4.10), provide a name for this group of settings, and specify whether it should available for use only by this particular data element, or whether it should be made available for other elements. In the latter case, you can specify if this group of settings should be used as the default for this view, thereby affecting all elements with that view.



Save View Settings

View settings name: Multicutters only

☑ Use as default view setting for sequences

● Save for use by sequences

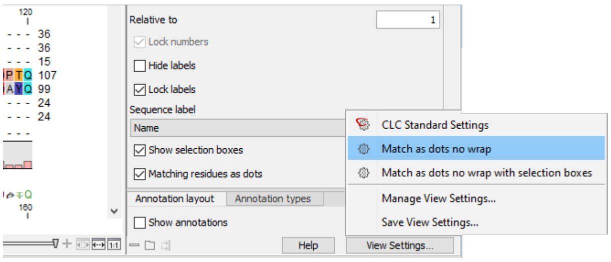
○ Save for this element only

Figure 4.10: *Click on the Save View Settings menu item (top) to open a dialog for saving the settings. A name needs to be supplied for these settings. The settings can be made available only for the data element being used or for all data elements of that type. Here, these settings have been set as the default for all elements of this type (bottom).*

View settings are user-specific. If your *CLC Workbench* is shared by multiple people, you will need to export any custom view settings you wish them to have access to and they will need to import them, as described in the **Sharing view settings** section below.

Applying saved view settings

To apply a new set of saved view settings to an open element, click on the **View Settings...** button and then select from the settings listed (figure 4.11).



Relative to 1

☑ Lock numbers

☐ Hide labels

☑ Lock labels

Sequence label: Name

☑ Show selection boxes

☑ Matching residues as dots

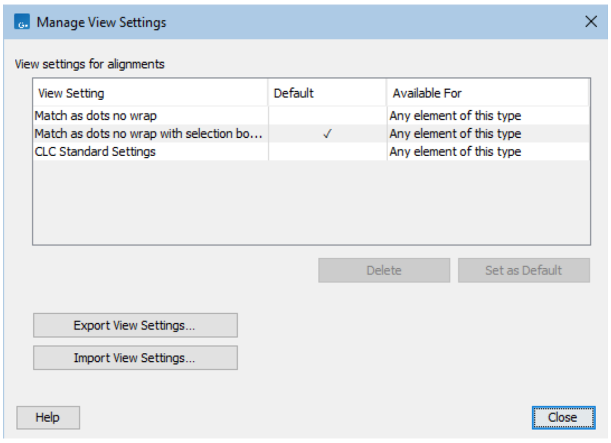
☐ Show annotations

Figure 4.11: *Select from saved view settings for the type of element open by clicking on the View Settings button at the bottom of a Side Panel.*

View settings named *CLC Standard Settings* are available for each data type. Until custom view settings are saved and set as the default for a given data type, the CLC Standard Settings are used.

Managing view settings

Manage view settings for the open view by selecting the option **Manage View Settings...** from the **View Settings...** menu. In this dialog, you can set a new default, delete view settings, as well as export and import view settings (figure 4.12).



View settings for alignments

View Setting	Default	Available For
Match as dots no wrap		Any element of this type
Match as dots no wrap with selection bo...	✓	Any element of this type
CLC Standard Settings		Any element of this type

Figure 4.12: *In the Manage View Settings dialog, you can specify the default for that view, delete saved settings, as well as export and import view settings.*

To browse all custom view settings available in your *CLC Workbench*, open the View tab under **Preferences (⚙)**, as described in section 4.2.

Sharing view settings

To share saved view settings for the open view type with others, click on the **Export...** button in the **Manage View Settings...** dialog. Select the sets to export. When prompted, select a folder to save the exported settings to, and then provide a filename in the field at the top. A file of that name with the suffix `.vsf` will be saved.

To import view settings, open the **Manage View Settings...** dialog, click on the **Import...** button and select a (`.vsf`) file to import.

Note: To export and import view settings for multiple view types, use the functionality under **Preferences (⚙)**, described in section 4.2.

Chapter 5

Printing

Contents

CLC Main Workbench offers different choices of printing the result of your work.

This chapter deals with printing directly from *CLC Main Workbench*. Another option for using the graphical output of your work, is to export graphics (see chapter 8.2) in a graphic format, and then import it into a document or a presentation.

All the kinds of data that you can view in the **View Area** can be printed. The *CLC Main Workbench* uses a WYSIWYG principle: What You See Is What You Get. This means that you should use the options in the Side Panel to change how your data, e.g. a sequence, looks on the screen. When you print it, it will look exactly the same way on print as on the screen.

For some of the views, the layout will be slightly changed in order to be printer-friendly.

It is not possible to print elements directly from the **Navigation Area**. They must first be opened in a view in order to be printed. To print the contents of a view:

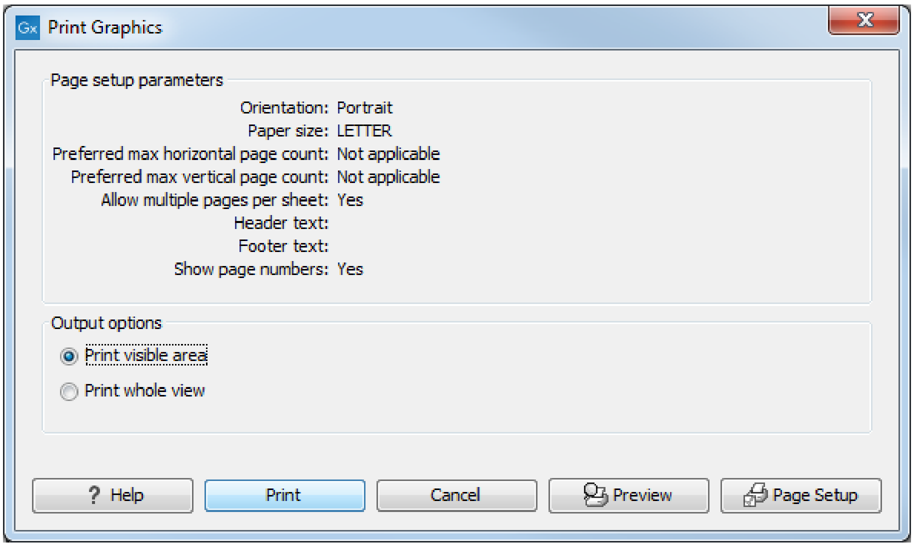
select relevant view | Print (🖶) in the toolbar

This will show a print dialog (see figure 5.1).

In this dialog, you can:

- Select which part of the view you want to print.
- Adjust **Page Setup**.
- See a print **Preview** window.

These three options are described in the three following sections.



Print Graphics

Page setup parameters

Orientation: Portrait
Paper size: LETTER
Preferred max horizontal page count: Not applicable
Preferred max vertical page count: Not applicable
Allow multiple pages per sheet: Yes
Header text:
Footer text:
Show page numbers: Yes

Output options

● Print visible area
○ Print whole view

Figure 5.1: *The Print dialog.*

5.1 Selecting which part of the view to print

In the print dialog you can choose to:

- **Print visible area**, or
- **Print whole view**

These options are available for all views that can be zoomed in and out. In figure 5.2 is a view of a circular sequence which is zoomed in so that you can only see a part of it.

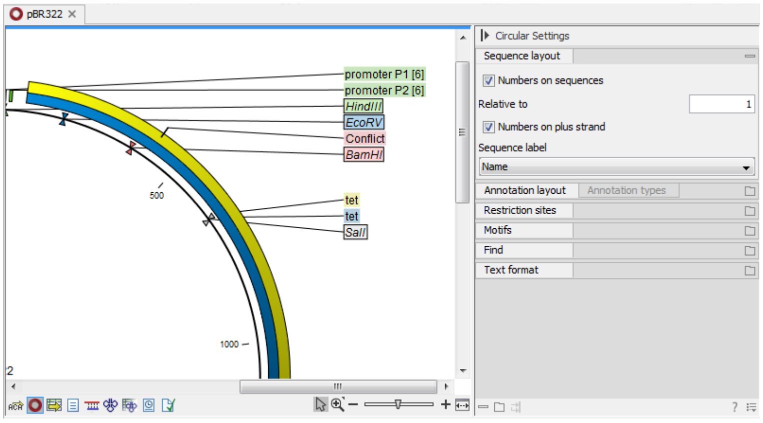


Figure 5.2: *A circular sequence as it looks on the screen.*

When selecting **Print visible area**, your print will reflect the part of the sequence that is *visible* in the view. The result from printing the view from figure 5.2 and choosing **Print visible area** can be seen in figure 5.3.

On the other hand, if you select **Print whole view**, you will get a result that looks like figure 5.4. This means that you also print the part of the sequence which is not visible when you have zoomed in.

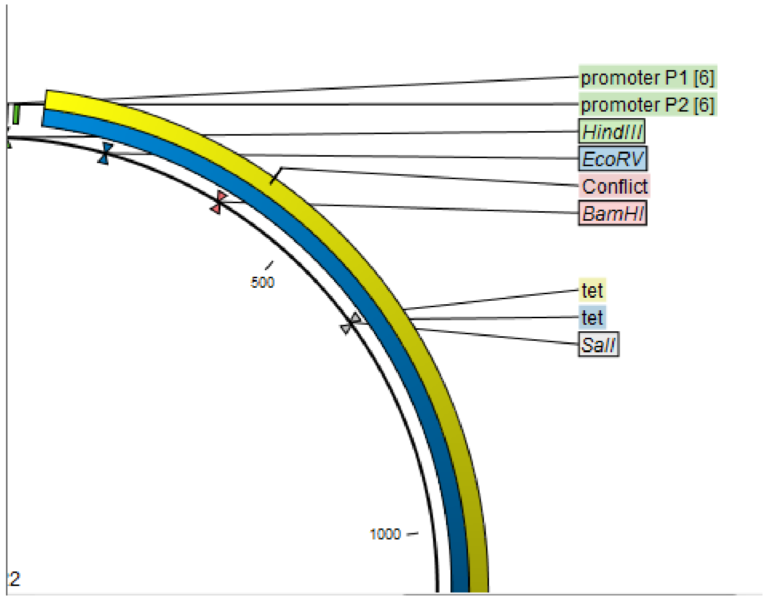


Figure 5.3: *A print of the sequence selecting Print visible area.*

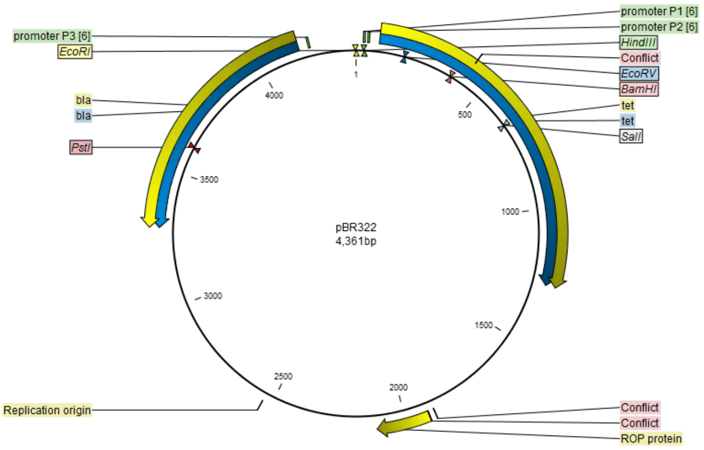


Figure 5.4: *A print of the sequence selecting Print whole view. The whole sequence is shown, even though the view is zoomed in on a part of the sequence.*

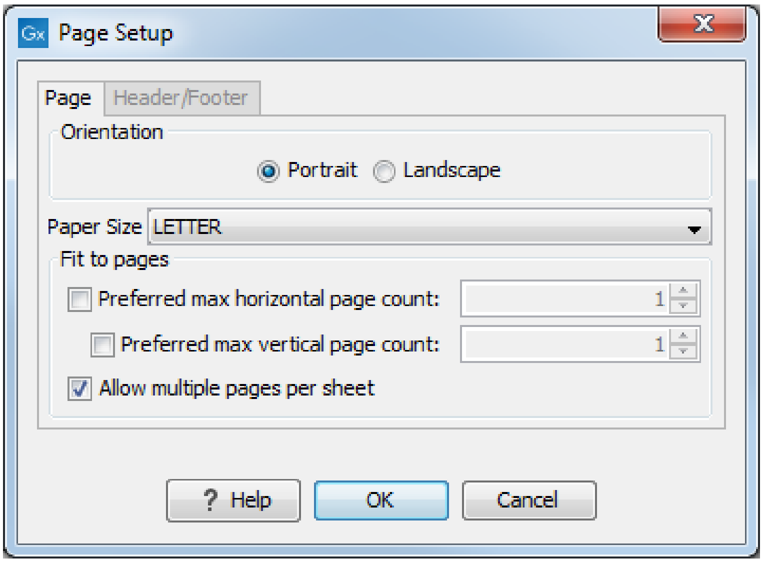
5.2 Page setup

No matter whether you have chosen to print the visible area or the whole view, you can adjust page setup of the print. An example of this can be seen in figure 5.5

In this dialog you can adjust both the setup of the pages and specify a header and a footer by clicking the tab at the top of the dialog.

You can modify the layout of the page using the following options:

- **Orientation**.
  - **Portrait**. Will print with the paper oriented vertically.
  - **Landscape**. Will print with the paper oriented horizontally.
- **Paper size**. Adjust the size to match the paper in your printer.



Orientation

● Portrait ○ Landscape

Paper Size LETTER

Fit to pages

□ Preferred max horizontal page count: 1

□ Preferred max vertical page count: 1

☑ Allow multiple pages per sheet

Figure 5.5: *Page Setup.*

- **Fit to pages**. Can be used to control how the graphics should be split across pages (see figure 5.6 for an example).
  - **Horizontal pages**. If you set the value to e.g. 2, the printed content will be broken up horizontally and split across 2 pages. This is useful for sequences that are not wrapped
  - **Vertical pages**. If you set the value to e.g. 2, the printed content will be broken up vertically and split across 2 pages.

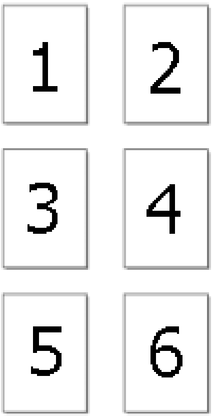


Figure 5.6: *An example where Fit to pages horizontally is set to 2, and Fit to pages vertically is set to 3.*

Note! It is a good idea to consider adjusting view settings (e.g. **Wrap** for sequences), in the **Side Panel** before printing. As explained in the beginning of this chapter, the printed material will look like the view on the screen, and therefore these settings should also be considered when adjusting **Page Setup**.

Header and footer Click the **Header/Footer** tab to edit the header and footer text. By clicking in the text field for either **Custom header text** or **Custom footer text** you can access the auto formats for header/footer text in **Insert a caret position**. Click either **Date**, **View name**, or **User name** to include the auto format in the header/footer text.

Click **OK** when you have adjusted the **Page Setup**. The settings are saved so that you do not have to adjust them again next time you print. You can also change the **Page Setup** from the **File** menu.

5.3 Print preview

The preview is shown in figure 5.7.

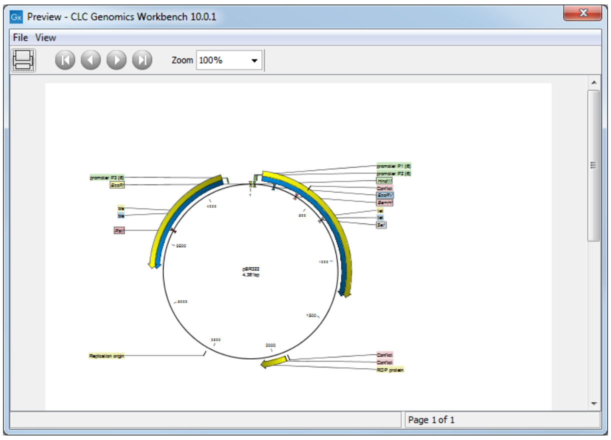


Figure 5.7: *Print preview.*

The **Print preview** window lets you see the layout of the pages that are printed. Use the arrows in the toolbar to navigate between the pages. Click Print (🖶) to show the print dialog, which lets you choose e.g. which pages to print.

The **Print preview** window is for preview only - the layout of the pages must be adjusted in the **Page setup**.

Chapter 6

Connections to other systems

Contents

Under the Connections menu are tools for connecting the *CLC Main Workbench* to other systems.

6.1 CLC Server connection

Using a *CLC Server*, data can be stored centrally and analyses run on a central machine rather than on a personal computer. After logging into the *CLC Server* from a *CLC Workbench*:

- Data in *CLC Server* locations will be listed in the Workbench Navigation Area.
- When launching analyses that can be run on the *CLC Server*, you will be offered the choice of running them using the Workbench or the *CLC Server*.
- Workflows installed on the *CLC Server* will be available to launch.
- External applications configured and enabled on the *CLC Server* will be available to launch and to include in workflows.

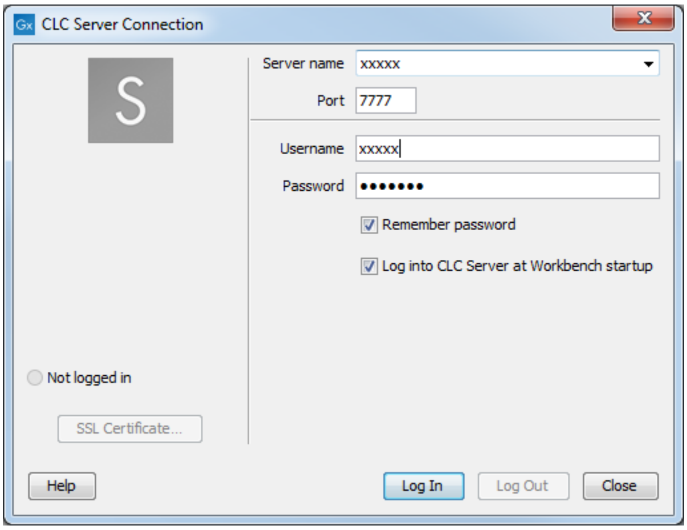
Logging into a CLC Server from a CLC Workbench

To log in to a *CLC Server*, open the **CLC Server Connection** dialog by going to:

File | **CLC Server Connection (S)**

or click on the server icon ((S)) at the bottom left hand side of the Workbench frame. This icon is blue when there is an active server connection or grey when there is not.

Fill in the details requested in the **CLC Server Connection** dialog (figure 6.1) and click on the **Log In** button to connect to the *CLC Server*. Information about logging into an SSL-enabled *CLC Server* are provided below.



CLC Server Connection

Server name: xxxxx

Port: 7777

Username: xxxxx

Password: •••••••

☑ Remember password

☑ Log into CLC Server at Workbench startup

○ Not logged in

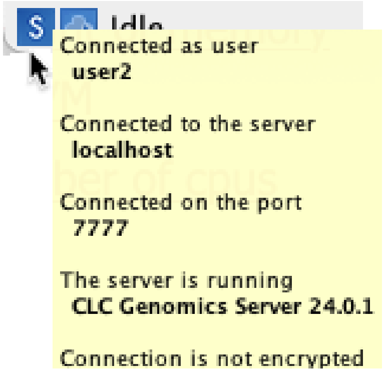
Figure 6.1: *Logging into a CLC Server using the default port, 7777*

Your username and the server details are saved between Workbench sessions. To save your password also, check the **Remember password** box.

To connect to the *CLC Server* automatically when the *CLC Workbench* starts up, check the **Log into CLC Server at Workbench startup** box. This option is only available when the **Remember password** option has been enabled.

Checking the status of a CLC Server connection

The status of an active connection can be seen by opening the **CLC Server Connection** dialog, as described above, or by hovering the mouse cursor over the server icon ((S)) at the bottom left hand side of the Workbench frame (figure 6.2).



Idle

Connected as user
 user2

Connected to the server
 localhost

Connected on the port
 7777

The server is running
 CLC Genomics Server 24.0.1

Connection is not encrypted

Figure 6.2: *Hover the mouse cursor over the Server icon in the bottom left corner of the Workbench frame to quickly view the status of the server connection.*

Key documentation about working with a CLC Server

Further information about working with a *CLC Server* from the *CLC Main Workbench*:

- Launching tasks on a *CLC Server*: section 11.1.1.

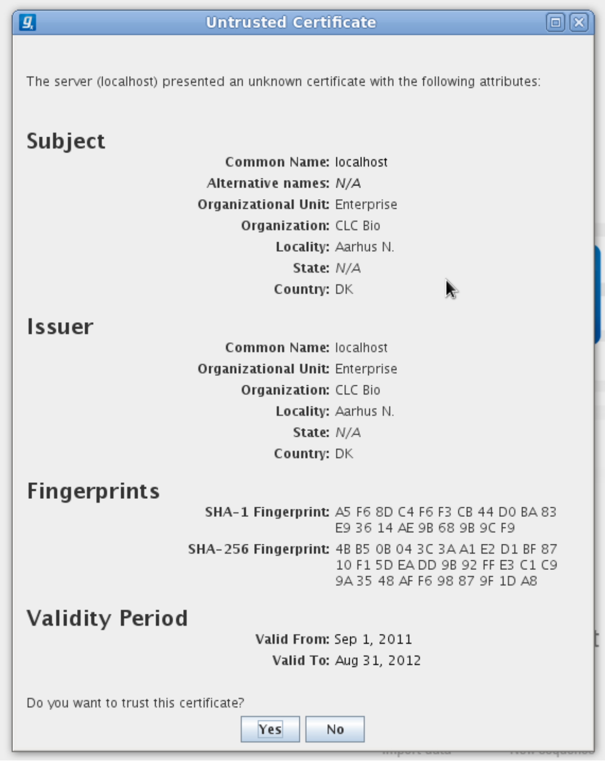
- Monitoring processes sent to the *CLC Server* from a CLC Workbench: section 2.4
- Viewing and working with data held on a *CLC Server*: section 3.1,
- Deleting data held on a *CLC Server*: section 3.1.8.
- Importing data to and exporting data from a *CLC Server* is described in section 6.1.1.

For those logging into the *CLC Server* as a user with administrative privileges, an option called Manage Server Users and Groups... will be available. This is described at `https://resources.qiagenbioinformatics.com/manuals/clcserver/current/admin/index.php?manual=User_authentication_using_Workbench.html`.

Connecting to a CLC Server using SSL

To log into a *CLC Server* with SSL enabled, provide the secure port in the Port field of the **CLC Server Connection** dialog in the Workbench.

If SSL is detected on the port provided, the CLC Server's certificate will be verified before the connection is established. A warning is displayed if the certificate is not signed by a recognized Certificate Authority (CA) (figure 6.3). When such a certificate has been accepted once, the warning will not appear again.



The server (localhost) presented an unknown certificate with the following attributes:

Subject

Common Name: localhost
Alternative names: *N/A*
Organizational Unit: Enterprise
Organization: CLC Bio
Locality: Aarhus N.
State: *N/A*
Country: DK

Issuer

Common Name: localhost
Organizational Unit: Enterprise
Organization: CLC Bio
Locality: Aarhus N.
State: *N/A*
Country: DK

Fingerprints

SHA-1 Fingerprint: A5 F6 8D C4 F6 F3 CB 44 D0 BA 83 E9 36 14 AE 9B 68 9B 9C F9
SHA-256 Fingerprint: 4B B5 0B 04 3C 3A A1 E2 D1 BF 87 10 F1 5D EA DD 9B 92 FF E3 C1 C9 9A 35 48 AF F6 98 87 9F 1D A8

Validity Period

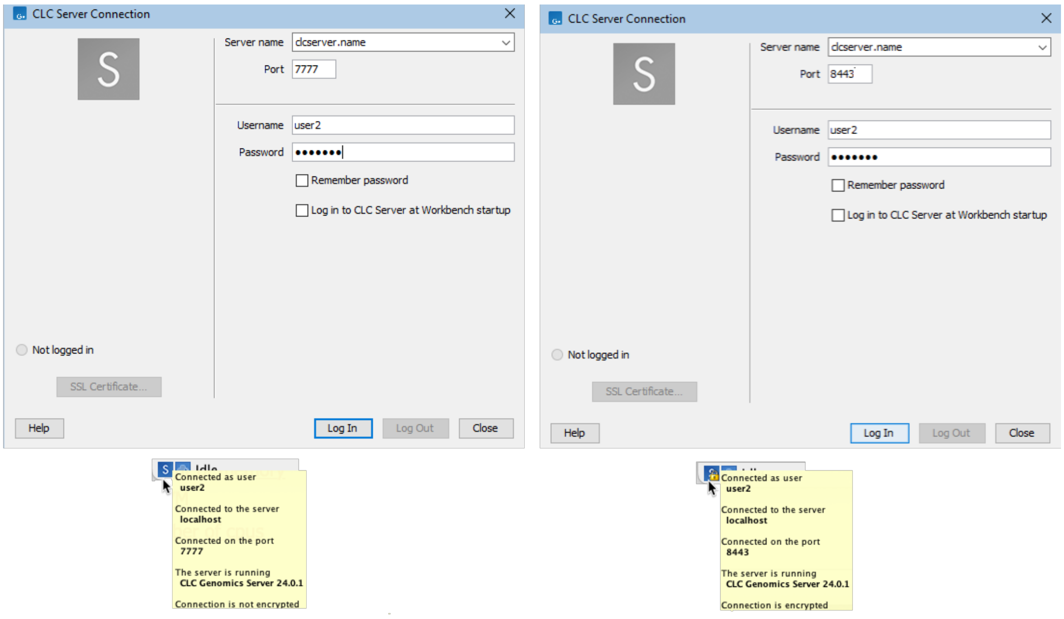
Valid From: Sep 1, 2011
Valid To: Aug 31, 2012

Do you want to trust this certificate?

Figure 6.3: *A warning is shown when the certificate is not signed by a recognized CA.*

The certificate details can be viewed again later by clicking on the **SSL Certificate** button in the **CLC Server Connection** dialog.

The connection status information in the tooltip revealed when hovering over the (S) icon in the bottom left corner of the Workbench frame includes whether the connection is encrypted or not (figure 6.4).



CLC Server Connection

Server name clcserver.name
Port 7777
Username user2
Password •••••••
□ Remember password
□ Log in to CLC Server at Workbench startup
○ Not logged in

Connected as user
user2

Connected to the server
localhost

Connected on the port
7777

The server is running
CLC Genomics Server 24.0.1

Connection is not encrypted

CLC Server Connection

Server name clcserver.name
Port 8443
Username user2
Password •••••••
□ Remember password
□ Log in to CLC Server at Workbench startup
○ Not logged in

Connected as user
user2

Connected to the server
localhost

Connected on the port
8443

The server is running
CLC Genomics Server 24.0.1

Connection is encrypted

Figure 6.4: *Login details and connection status information for an unencrypted connection to a CLC Server (left) and an encrypted connection (right). A padlock on the server icon in the bottom left corner of the Workbench frame also indicates the connection is encrypted.*

6.1.1 CLC Server data import and export

This section covers general points related to importing data to and exporting data from a *CLC Server* when working with the *CLC Main Workbench*. General information about data import and data export is in chapter 7 and chapter 8, respectively.

Detailed information about *CLC Server* configuration is in the server administration manual at: https://resources.qiagenbioinformatics.com/manuals/clcserver/current/admin/index.php?manual=Introduction.html

Data import to a CLC Server

The general location of the data to import is specified from a drop-down list (figure 6.5). If direct data transfer from client systems is enabled on the *CLC Server*, the option "File system" is included in that list, allowing files on the local system to be selected for import.

When the option represented in figure 6.5 as "Server Name (CLC Genomics Server)" is selected, the available import/export directories on that CLC Server will be listed in the "Server files" area. When an AWS location is selected, the available S3 buckets will be listed. When BaseSpace is selected, you will be prompted to log into BaseSpace before proceeding.

When "File system" is selected, the *CLC Main Workbench* must maintain its connection to the *CLC Server* during the first part of the import process, data upload. Further details about this are in section 2.4.

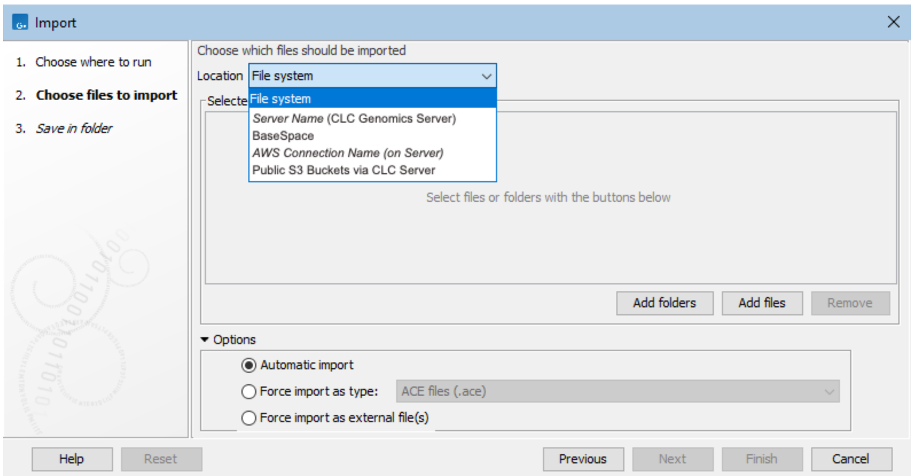

Import
1. Choose where to run
2. Choose files to import
3. Save in folder
Choose which files should be imported
Location File system
Selecte
File system
Server Name (CLC Genomics Server)
BaseSpace
AWS Connection Name (on Server)
Public S3 Buckets via CLC Server
Select files or folders with the buttons below
Add folders
Add files
Remove
Options
● Automatic import
○ Force import as type: ACE files (.ace)
○ Force import as external file(s)
Help
Reset
Previous
Next
Finish
Cancel

Figure 6.5: *When an import is run on a CLC Server, the list of locations that data can imported from reflects the server configuration.*

Note that when importing data from an AWS S3 bucket, the data is first downloaded from AWS, which AWS charges for.

Data export from a CLC Server

When the *CLC Main Workbench* is connected to a *CLC Server*, data in CLC Server File System Locations can be exported. The choice of execution environment, as well as settings on the Workbench and Server, affect the locations data can be selected for export from and where the exported files can be saved to:

- **Running the export on the Workbench:**
  - Data in Workbench or Server CLC File System Locations can be selected for export.
  - Exported files can be saved to areas the *CLC Main Workbench* has access to, including AWS S3 buckets if an AWS S3 Connection has been configured in the *CLC Main Workbench*.
- **Running the export on the CLC Server or Grid via CLC Server:**
  - Data from Server File System Locations can be can be selected for export.
  - Exported files can be saved to Server import/export directories or to an AWS S3 bucket if an AWS Connection has been configured in the *CLC Server*.

6.2 AWS Connections

AWS connections are used when:

- Accessing AWS S3 buckets, to import data from or export data to.
- Submitting analyses to a *CLC Genomics Cloud* setup, if available on that AWS account.

Configuring access to your AWS accounts requires AWS IAM credentials. Configuring access to public S3 buckets requires only the name of the bucket.

Working with stored data in AWS S3 buckets via the Workbench is of particular relevance when submitting jobs to run on a *CLC Genomics Cloud* setup making use of functionality provided by the *CLC Cloud Module*.

When launching workflows to run locally using on-the-fly import and selecting files from AWS S3, the files selected are first downloaded to a temporary folder and are subsequently imported.

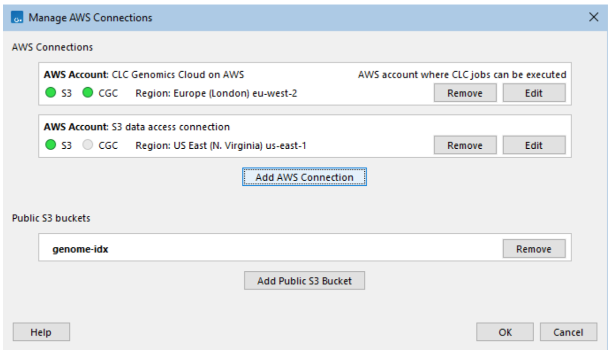
All traffic to and from AWS is encrypted using a minimum of TLS version 1.2.

Configuring access to AWS resources

To configure an AWS Connection or to configure access to public AWS S3 buckets, go to:

> **Connections** | **AWS Connections** ( )

Already configured AWS connections and their status, and public S3 buckets are listed (figure 6.6). Editing or removal of these configurations is done from here.


Manage AWS Connections

AWS Connections

AWS Account: CLC Genomics Cloud on AWS — AWS account where CLC jobs can be executed
● S3 ● CGC Region: Europe (London) eu-west-2

AWS Account: S3 data access connection
● S3 ○ CGC Region: US East (N. Virginia) us-east-1

Public S3 buckets

genome-idx

Figure 6.6: *The configuration dialog for AWS connections. Here, two valid AWS connections, their status, and a public S3 bucket are listed.*

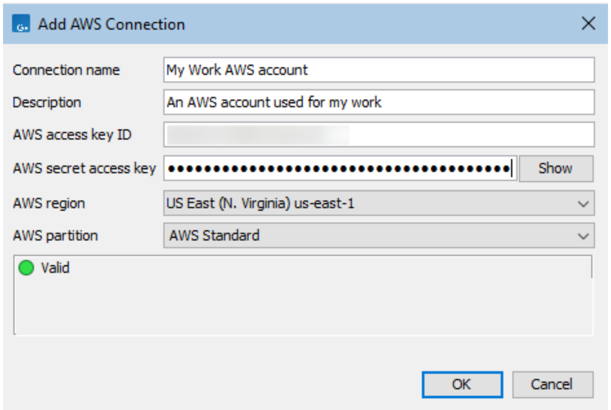
Configuring an AWS Connection

To configure a new AWS Connection, click on the **Add AWS Connection** button and enter the following information in the dialog (figure 6.7):

- **Connection name**: A short name of your choice, identifying the AWS account. This name will be shown as the name of the data location when importing data to or exporting data from Amazon S3.
- **Description**: A description of the AWS account (optional).
- **AWS access key ID**: The access key ID for programmatic access for your AWS IAM user.
- **AWS secret access key**: The secret access key for programmatic access for your AWS IAM user.
- **AWS region**: An AWS region. Select from the drop-down list.

- **AWS partition**: The AWS partition for your account.

The dialog continuously validates the settings entered. When they are valid, the Status box will contain the text "Valid" and a green icon will be shown. Click on **OK** to save the settings.



Add AWS Connection

Connection name: My Work AWS account

Description: An AWS account used for my work

AWS access key ID:

AWS secret access key: ••

AWS region: US East (N. Virginia) us-east-1

AWS partition: AWS Standard

● Valid

Figure 6.7: *Configuration of an AWS Connection in a CLC Workbench*

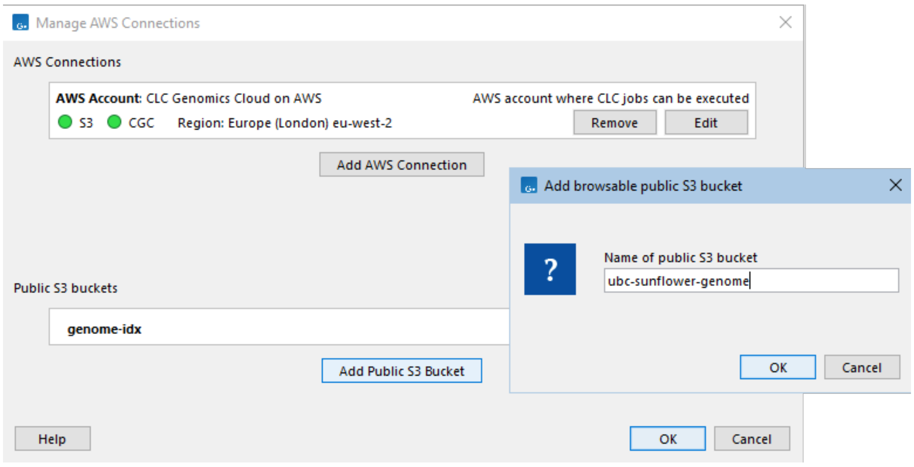
AWS connection status is indicated using colors. Green indicates the connection is valid and ready for use. Connections to a *CLC Genomics Cloud* are indicated in the CGC column (figure 6.6). To submit analyses to the *CLC Genomics Cloud*, the *CLC Cloud Module* must be installed and a license for that module must be available.

AWS credentials entered are stored, obfuscated, in Workbench user configuration files.

Note: Multiple AWS Connections using credentials for the same AWS account cannot be configured.

Adding a public S3 bucket

To add a public bucket, click on the **Add Public S3 button** and provide the public bucket name (figure 6.8).



Manage AWS Connections

AWS Connections

AWS Account: CLC Genomics Cloud on AWS — AWS account where CLC jobs can be executed

● S3 ● CGC Region: Europe (London) eu-west-2

Public S3 buckets

genome-idx

Add browsable public S3 bucket

Name of public S3 bucket: ubc-sunflower-genome

Figure 6.8: *Provide a public AWS S3 bucket name to enable access to data in that public bucket.*

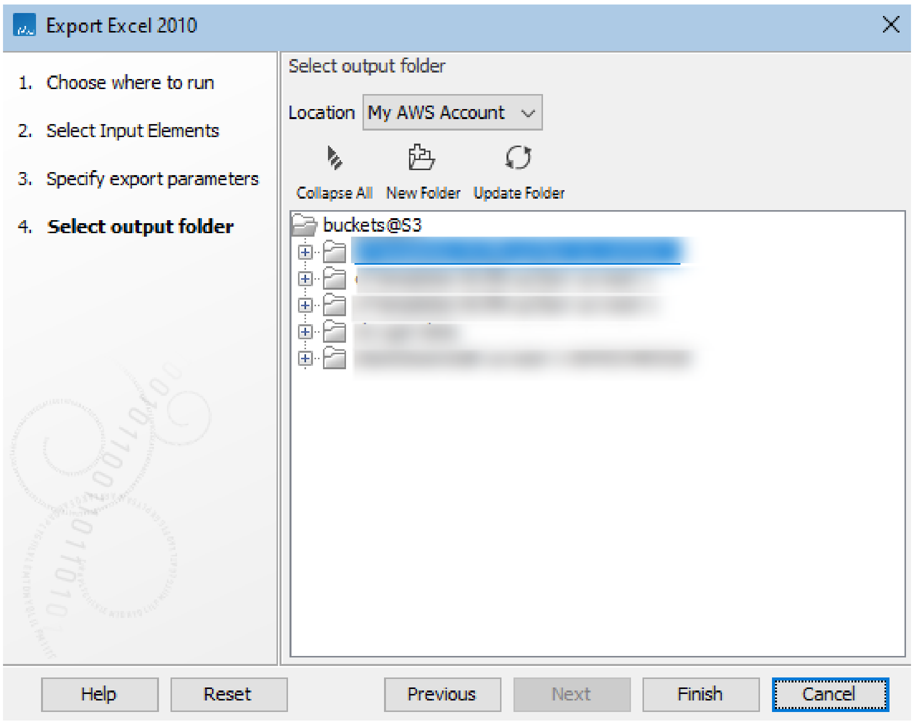
Importing data from AWS S3

AWS S3 buckets for each AWS Connection and public S3 bucket configured are available in relevant import tool wizards and in workflow launch wizards when using on-the-fly import (see Launching workflows individually and in batches (section 13.3)).

AWS S3 buckets can be browsed under the **Remote Files** tab next to the **Navigation Area** tab, in the top left side of the Workbench (see section 3.6).

Exporting data to AWS S3

To export data to an AWS S3 bucket, launch the exporter, and when prompted for an export location, select the relevant option from the drop-down menu (figure 6.9).



Export Excel 2010

1. Choose where to run
2. Select Input Elements
3. Specify export parameters
4. **Select output folder**

Select output folder

Location: My AWS Account

Figure 6.9: *After an AWS connection is selected when exporting, you can select the S3 bucket and location within that bucket to export to.*

Chapter 7

Importing data

Contents

Many data formats are supported for import into the *CLC Main Workbench*. Data types that are not recognized are imported as "external files". Such files are opened in the default application for that file type on your computer (e.g. Word documents will open in Word). This chapter describes import of data, with a focus on import of common bioinformatics data formats.

Where data can be imported from

Data can be imported from any location accessible via the system the *CLC Main Workbench* is installed on, including from AWS S3 if AWS Connections are configured (see section 6.2). Importing data from AWS S3 involves downloading files from AWS, which AWS charges for.

If connected to a *CLC Server*, data can be imported from areas accessible via the *CLC Server* (see section 6.1.1).

7.1 Standard import

CLC Main Workbench has support for a wide range of bioinformatic data formats. See section H.1 for a list.

The Standard Import tool supports imports of many standard bioinformatics and tabular formats, as well as Excel format files. The full list of supported formats is provided in the tool, as described further below. Information about supported formats is also provided in the appendix section H.1.

Standard Import can by launched by clicking on the **Import (📥)** icon in the Toolbar and choosing **Standard Import**.

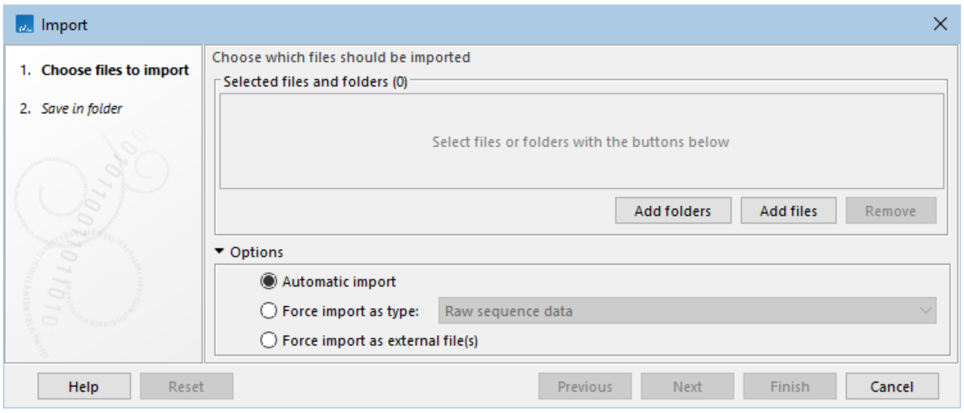
Alternatively, go to **File** | **Import (📥)** | **Standard Import (📥)**.

Select files to import by clicking on the **Add files** button. Alternatively, specify folders containing files to import by clicking on the **Add folders** button (figure 7.1).

The default option is **Automatic import**. The file format is automatically detected based on a combination of the file extension (e.g. .fa for fasta) and detection of file contents specific to particular formats. Based on this, the relevant importer is run. The particular importer used is recorded in the element history. If the format is not supported, the file is imported as an external file, that is, it is imported to the CLC Location in its original format. See section 3.2 for details about working with such files.

You can specify the format explicitly using the option **Force import as type**. When choosing this option, the full list of supported data types is provided in a drop-down list.

The **Force import as external file** option can be useful if you are trying to import a standard format file, such as a text file, but it is being detected as bioinformatics format file, such as sequence data.



Import

1. **Choose files to import**
2. *Save in folder*

Choose which files should be imported

Selected files and folders (0)

Select files or folders with the buttons below

▾ Options

● Automatic import

○ Force import as type: Raw sequence data

○ Force import as external file(s)

Figure 7.1: *The Standard Import tool.*

Standard Import is also used to import files that are dragged from a file browser and dropped into the Navigation Area. In this case, the file format is automatically detected. To force the file type, launch the tool explicitly, as described at the start of this section.

Importing by copy/pasting text

Data can be copied and pasted directly into the Navigation Area.

Copy text | Select a folder in the Navigation Area | Paste ()

Standard import with automatic format detection is run using the pasted content as input.

This is a fast way to import data, but importing files as described above is less error prone, and thus generally recommended instead.

Chapter 8

Exporting data and graphics

Contents

Data and graphics can be exported from the *CLC Main Workbench* using export tools and workflows that contain export elements. Some types of data can also be exported using options available in right-click menus (see section 8.3) and others can be copy/pasted from within the *CLC Main Workbench* to other applications (see section 8.4).

Where data can be exported to

Data can be exported to any location accessible via the system the *CLC Main Workbench* is installed on, or to AWS S3 if an AWS Connection is configured that allows this (see section 6.2).

If connected to a *CLC Server*, data can be exported to areas accessible via the *CLC Server* (see section 6.1.1).

8.1 Data export

Data can be exported to many standard formats. The full list of supported formats in presented when launching the Export tool. Information about supported formats is also provided in the appendix section H.1.

Launch the standard export functionality by clicking on the Export button on the toolbar, or selecting the menu option:

File | **Export** ()

An additional export tool is available from under the File menu:

File | **Export with Dependent Elements**

This tool is described further in section 8.1.5.

The general steps when configuring a standard export job are:

- (Optional) Select data elements or folders to export in the **Navigation Area**.
- Launch the Export tool by clicking on the Export button in the Workbench toolbar or by selecting **Export** under the File menu.
- Select the format to export the data to.
- Select the data elements to export, or confirm elements that had been pre-selected in the **Navigation Area**.
- Configure the export parameters, including whether to output to a single file, whether to compress the outputs and how the output files should be named. Other format-specific options may also be provided.
- Select where the data should be exported to.
- Click **Finish**.

8.1.1 Export formats

Finding and selecting a format to export to When the Export tool is launched, a list of the available data formats is presented (figure 8.1).

You can quickly find a particular format by typing a relevant search term into the text box at the top of the Export window, as shown in figure 8.2. Any formats with that search term in their name or description will be listed in the window. The search term is remembered when the Export tool is next launched. Delete the text from the search box if you wish to have all export formats listed.

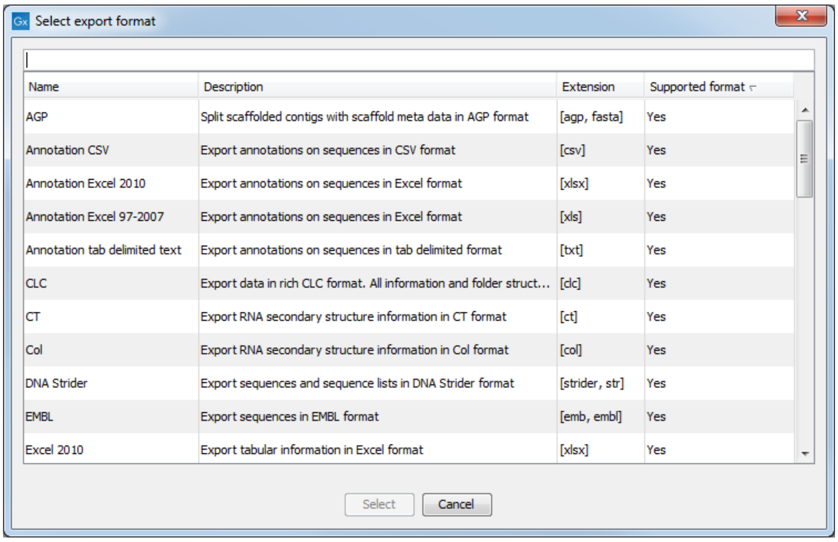
Support for choosing an appropriate export format is provided in 2 ways:

- If data elements are selected in the **Navigation Area** before launching the Export tool, then a "Yes" or a "No" in the **Supported formats** column specifies whether or not the selected data elements can be exported to that format. If you have selected multiple data elements of different types, then formats that some, but not all, selected data elements can be exported to are indicated by the text "For some elements".

By default, supported formats appear at the top of the list.

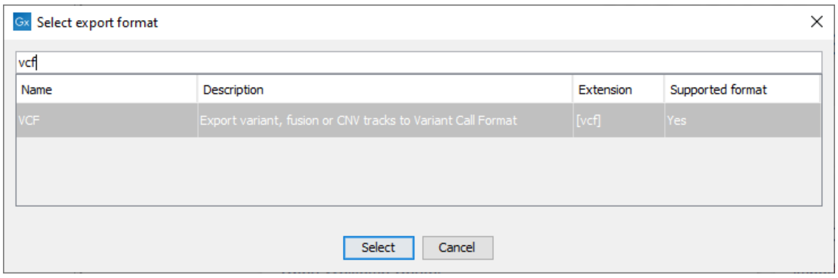
- If no data elements are selected in the **Navigation Area** when the Export tool is launched, then the list of export formats is provided, but each row will have a "Yes" in the **Supported format** column. After an export format has been selected, only the data elements that can be exported to that format will be listed for selection in the next step of the export process.

  Only zip format is supported when a folder, rather than data elements, is selected for export. In this case, all the elements in the folder are exported in CLC format, and a zip file containing these is created. See section 8.1.4.



Name	Description	Extension	Supported format
AGP	Split scaffolded contigs with scaffold meta data in AGP format	[agp, fasta]	Yes
Annotation CSV	Export annotations on sequences in CSV format	[csv]	Yes
Annotation Excel 2010	Export annotations on sequences in Excel format	[xlsx]	Yes
Annotation Excel 97-2007	Export annotations on sequences in Excel format	[xls]	Yes
Annotation tab delimited text	Export annotations on sequences in tab delimited format	[txt]	Yes
CLC	Export data in rich CLC format. All information and folder struct...	[clc]	Yes
CT	Export RNA secondary structure information in CT format	[ct]	Yes
Col	Export RNA secondary structure information in Col format	[col]	Yes
DNA Strider	Export sequences and sequence lists in DNA Strider format	[strider, str]	Yes
EMBL	Export sequences in EMBL format	[emb, embl]	Yes
Excel 2010	Export tabular information in Excel format	[xlsx]	Yes

Figure 8.1: *The Select export format dialog. Here, some sequence lists had been selected in the Navigation Area before the Export tool was launched. The formats that the selected data elements can be exported to contain a "Yes" in the Selected format column. Other export formats are listed below the supported ones, with "No" in the Supported format column.*



vcf

Name	Description	Extension	Supported format
VCF	Export variant, fusion or CNV tracks to Variant Call Format	[vcf]	Yes

Figure 8.2: *The text field has been used to search for the term "VCF" in the export format name or description field in the Select export dialog.*

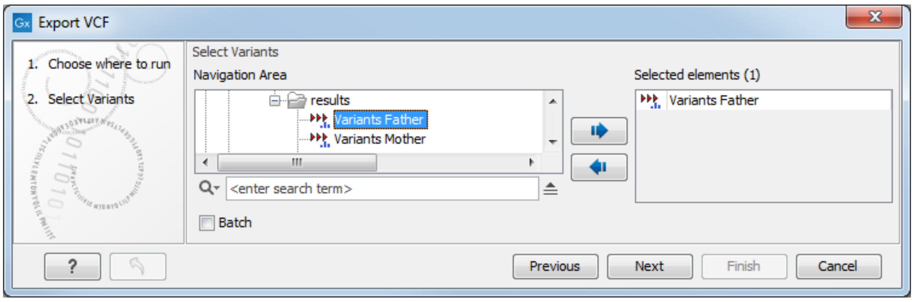
When the desired export format has been selected, click on the button labeled **Select**.

A dialog then appears, with a name reflecting the format you have chosen. For example if the VCF format was selected, the window is labeled "Export VCF".

If you are logged into a CLC Server, you will be asked whether to run the export job using the

Workbench or the Server. After this, you are provided with the opportunity to select or de-select data to be exported.

Selecting data for export In figure 8.3 we show the selection of a variant track for export to VCF format.



Export VCF

1. Choose where to run
2. Select Variants

Select Variants

Navigation Area: results – Variants Father, Variants Mother

Selected elements (1): Variants Father

Figure 8.3: *The Select export dialog. Select the data element(s) to export.*

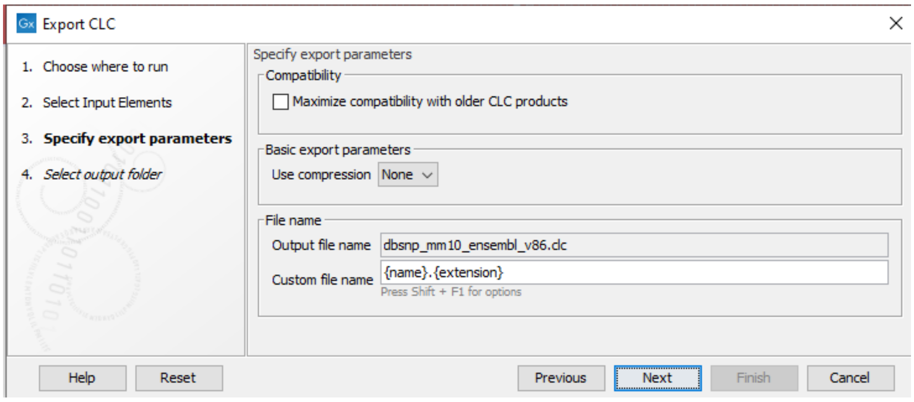
Further information is available about exporting the following types of information:

- Tables: section 8.1.6
- Reports to JSON format: section 8.1.8
- Graphics to a range of formats: section 8.1.9
- Data element history: section 8.1.10

8.1.2 Export parameters

The settings in the areas **Basic export parameters** and **File name** are offered when exporting to any format.

There may also be additional parameters for particular export formats. This is illustrated for the CLC exporter in figure 8.4.



Export CLC

1. Choose where to run
2. Select Input Elements
3. **Specify export parameters**
4. *Select output folder*

Specify export parameters

Compatibility

□ Maximize compatibility with older CLC products

Basic export parameters

Use compression: None

File name

Output file name: dbsnp_mm10_ensembl_v86.clc

Custom file name: {name}.{extension}

Press Shift + F1 for options

Figure 8.4: *Configure the export parameters. When exporting to CLC format, you can choose to maximize compatibility with older CLC products.*

Examples of configuration options:

- **Maximize compatibility with older CLC products** This is described in section 8.1.4.
- **Compression options** Within the **Basic export parameters** section, you can choose to compress the exported files. The options are no compression (None), gzip or zip format. Choosing zip format results in all data files being compressed into a single file. Choosing gzip compresses the exported file for each data element individually.
- **Paired reads settings** In the case of Fastq Export, the option "Export paired sequence lists to two files" is selected by default: it will export paired-end reads to two fastq files rather than a single interleaved file.
- **Exporting multiple files** If you have selected multiple files of the same type, you can choose to export them in one single file (only for certain file formats) by selecting "Output as single file" in the **Basic export parameters** section. If you wish to keep the files separate after export, make sure this box is not ticked. **Note:** Exporting in zip format will export only one zipped file, but the files will be separated again when unzipped.

The name to give exported files is also configured here. This is described in detail in section 8.1.3.

In the final wizard step, you select the location to save the exported files to.

8.1.3 Specifying the exported file name(s)

The names to give exported files can be configured in the export wizard. Names can be specified directly or placeholders can be used. Placeholders specify particular types of information, and thus are a convenient way to apply a consistent naming pattern to many exports.

The default is to use the placeholders `{name}` and `{extension}`, as shown in figure 8.5. Using these, the original data element name is used as the basename of the exported file, and the file format is used as the suffix. The actual filename that would result is shown in the **Output file name** field for the first element being exported.

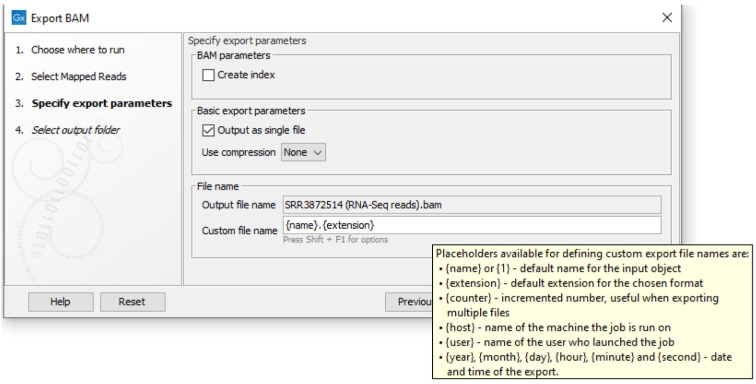
When deciding on an output name, you can choose any combination of the different placeholders, standard text characters and punctuation, as in `{name}({day}-{month}-{year})`. As you add or remove text and terms in the **Custom file name** field, the text in the **Output file name** field will change so you can see what the result of your naming choice will be for your data.

An example where specific text instead of a placeholder might be preferred would be if the extension used for a particular format is not as desired. For example, the extension used for fasta files is `.fa`. To use `.fasta` instead, replace `{extension}` with "`.fasta` in the **Custom file name** field, as shown in figure 8.6.

When exporting a single file, the desired filename can just be typed in the **Custom file name** field. This should not be done when exporting to more than one file, as this would result in every exported file having an identical name.

The following placeholders are available:

- **{name}** or **{1}** - default name of the data element being exported
- **{extension}** - default extension for the chosen export format
- **{counter}** - a number that is incremented per file exported. i.e. If you export more than one file, counter is replaced with 1 for the first file, 2 for the next and so on.



Specify export parameters

BAM parameters

□ Create index

Basic export parameters

☑ Output as single file

Use compression: None

File name

Output file name: SRR3872514 (RNA-Seq reads).bam

Custom file name: {name}.{extension}

Press Shift + F1 for options

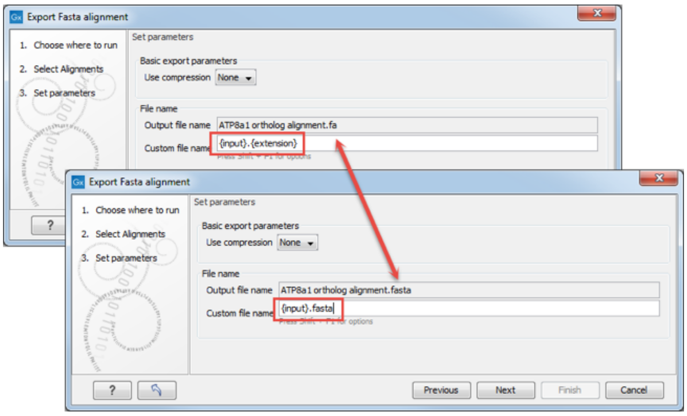
Placeholders available for defining custom export file names are:
- {name} or {1} - default name for the input object
- {extension} - default extension for the chosen format
- {counter} - incremented number, useful when exporting multiple files
- {host} - name of the machine the job is run on
- {user} - name of the user who launched the job
- {year}, {month}, {day}, {hour}, {minute} and {second} - date and time of the export.

Figure 8.5: *The default placeholders, separate by a "." are being used here. The tooltip for the Custom file name field provides information about these and other available placeholders.*

- **{host}** - name of the machine the job is run on
- **{user}** - name of the user who launched the job
- **{year}**, **{month}**, **{day}**, **{hour}**, **{minute}**, and **{second}** - timestamp information based on the time an output is created. Using these placeholders, items generated by a tool at different times can have different filenames.

Note: Placeholders available for Workflow Export elements are different and are described in section 13.2.4.

Exported files can be saved into subfolders by using a forward slash character / at the start of the custom file name definition. When defining subfolders, all later forward slash characters in the configuration, except the last one, are interpreted as further levels of subfolders. For example, a name like `/outputseqs/level2/myoutput.fa` would put a file called `myoutput.fa` into a folder called `level2` within a folder called `outputseqs`, which would be placed within the output folder selected in the final wizard step when launching the export tool. If the folders specified in the configuration do not already exist, they are created. Folder names can also be specified using placeholders.



Set parameters

Basic export parameters

Use compression: None

File name

Output file name: ATP8a1 ortholog alignment.fa

Custom file name: {input}.{extension}

Export Fasta alignment

Set parameters

Basic export parameters

Use compression: None

File name

Output file name: ATP8a1 ortholog alignment.fasta

Custom file name: {input}.fasta

Press Shift + F1 for options

Figure 8.6: *The file name extension can be changed by typing in the preferred file name format.*

8.1.4 Export of folders and data elements in CLC format

The *CLC Main Workbench* stores data in CLC format. A CLC format file holds all the information for a given data element. This means the data itself, as well as information about that data, like history information.

Data can be exported in CLC format by selecting the CLC fomat, or the zip format, from the list of available formats.

If CLC format is chosen, each selected data element can be exported to an individual file. An option is offered later in the export process to apply gzip or zip compression. Choosing gzip compression at this stage will compress each data element individually. Choosing zip produces a single file containing the individual CLC format files. If a single zip file containing one or more CLC format files is the desired outcome, choosing the zip format in the first step of the export process specifies this directly.

If a folder is selected for export, only the zip format is supported. In this case, each data element in that folder will be exported to CLC format, and all these files will be compressed in a single zip file.

CLC format files, or zip files containing CLC format data, can be imported directly into a workbench using the Standard Import tool and selecting "Automatic import" in the Options area.

Backing up and sharing data

If you are backing up data, or plan to share data with colleagues who have a CLC Workbench, exporting to CLC format is usually the best choice. All information associated with that data element will then be available when the data is imported again. CLC format is also recommended when sharing data with the QIAGEN Bioinformatics Support team.

If you are planning to share your data with someone who does not have access to a licensed *CLC Main Workbench* but just wishes to view the data, then you can export to CLC format, and they can run the *CLC Main Workbench* in Viewing Mode, which does not require a license, see Viewing mode 1.4.8 In Viewing Mode, CLC format data can be imported and viewed in the same way it would be using a licensed Workbench.

Compatibility of the CLC data format between Workbench versions

When exporting to CLC or zip format, the option "Maximize compatibility with older CLC products" is presented at the **Specify export parameters** step. With that option checked, data is exported without internal compression applied. This is only relevant when sharing data with someone using a version of the CLC software where the compression applied by default is not supported.

Compatibility information:

- A new compression method was introduced with version 22.0 of the CLC Genomics Workbench, CLC Main Workbench and CLC Genomics Server. Compressed data created using those versions can be read by version 21.0.5 and above, but not earlier versions.

- Internal compression of CLC data was introduced in CLC Genomics Workbench 12.0, CLC Main Workbench 8.1 and CLC Genomics Server 11.0. Compressed data created using these versions is not compatible with older versions of the software. Data created using these versions can be opened by later versions of the software, including versions 22.0 and above.

Information on how to turn off internal data compression entirely is provided in section 4.4. We generally recommend, however, that data compression remains enabled.

8.1.5 Export of dependent elements

Sometimes it can be useful to export the results of an analysis and its dependent elements. That is, the results along with the data that was used in the analysis. For example, one might wish to export an alignment along with all the sequences that were used in generating that alignment.

To export a data element with its dependent elements:

- Select the parent data element (like an alignment) in the **Navigation Area**.
- Start up the exporter tool by going to **File** | **Export with Dependent Elements**.
- Edit the output name if desired and select where the resulting zip format file should be exported to.

The file you export contains compressed CLC format files containing the data element you chose and all its dependent data elements.

A zip file created this way can be imported directly into a CLC workbench by going to

File | **Import ()** | **Standard Import**

and selecting "Automatic import" in the Options area.

Compatibility of the CLC data format between Workbench versions Internal compression of CLC data was introduced in CLC Genomics Workbench 12.0, CLC Main Workbench 8.1 and CLC Genomics Server 11.0. If you are sharing data for use in software versions older than these, then please use the standard Export functionality, selecting all the data elements, or folders of elements, to export and choosing either CLC or zip format as the export format. Further information about this is provided in section 8.1.4.

8.1.6 Export of tables

Data in tables can be exported to CSV, tab-delimited, Excel, or HTML format files.

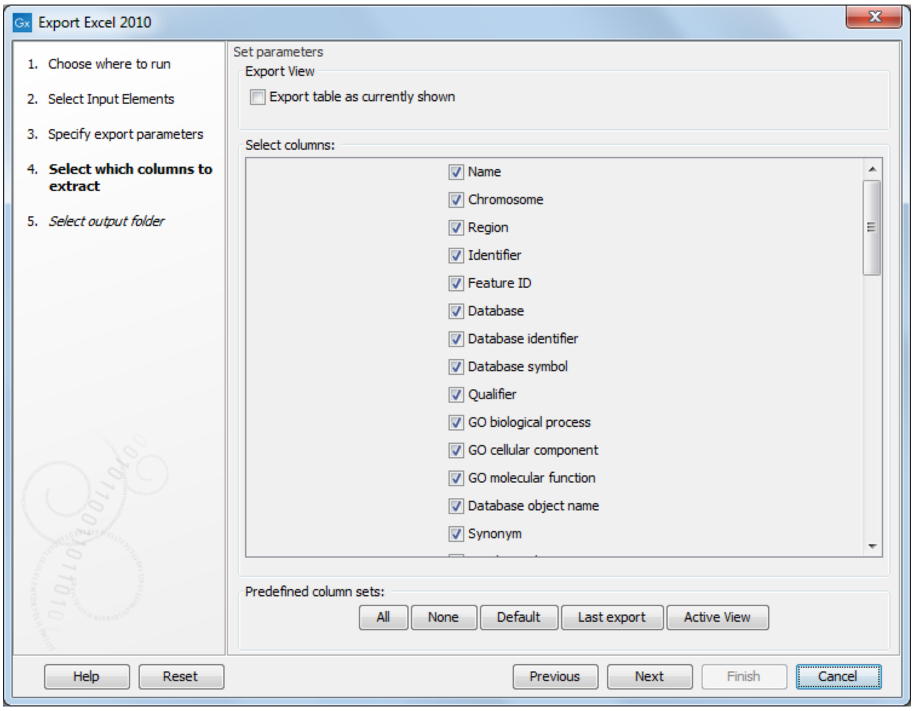
The "Export all columns" option is selected by default. When it is deselected, options for selecting the columns to export are presented in the next wizard step.

When selecting specific columns for export, the option "Export the table as currently shown" is particularly useful if you have filtered, sorted, or selected particular columns in a table that is open in a View. In this case, the effects of these manipulations are preserved in the exported file. This option is not available for all data types.

When the "Export the table as currently shown" is unchecked or disabled, checkboxes for each column to be exported are available to select or deselect. The buttons below that section can help speed up the process of column selection:

- **All** Select all possible columns.
- **None** Clear the existing selection.
- **Default** Select a standard set of columns, as defined by the software for this data type.
- **Last export** Select the columns that were selected during the most recent previous export.
- **Active View** Select the same set of columns as those selected in the Side Panel of the open data element. This button is only visible if the element being exported is in an open View.

In the final wizard step, select the location where the exported elements should be saved.



Export Excel 2010

1. Choose where to run
2. Select Input Elements
3. Specify export parameters
4. **Select which columns to extract**
5. *Select output folder*

Set parameters

Export View

☐ Export table as currently shown

Select columns:

☑ Name
☑ Chromosome
☑ Region
☑ Identifier
☑ Feature ID
☑ Database
☑ Database identifier
☑ Database symbol
☑ Qualifier
☑ GO biological process
☑ GO cellular component
☑ GO molecular function
☑ Database object name
☑ Synonym

Predefined column sets:

Figure 8.7: *Selecting table columns to be exported.*

The data exported will reflect any filtering and sorting applied.

Considerations when exporting tables

- **Row limits** Excel limits the number of hyperlinks in a worksheet to 66,530. When exporting a table of more than 66,530 rows, Excel will "repair" the file by removing all hyperlinks. If you want to keep the hyperlinks valid, you will need to subset your data and then export it to several worksheets, where each would have fewer than 66,530 rows.
- **Decimal places** When exporting to CSV, tab-separated, or Excel formats, numbers with many decimals are exported with 10 decimal places, or in scientific notation (e.g. 1.123E-5) when the number is close to zero.

When exporting a table in HTML format, data are exported with the number of decimals that have been defined in the *CLC Main Workbench* preference settings. When tables are exported in HTML format from a *CLC Server* the default number of decimal places is 3.

- **Decimal notation** When exporting to CSV and tab delimited files, decimal numbers are formatted according to the Locale setting of the *CLC Main Workbench* (see General preferences 4.1. If you open the CSV or tab delimited file with software like Excel, that software and the *CLC Workbench* should be configured with the same Locale.

8.1.7 GFF3 export

All the annotations on sequences (individual elements or in sequence lists) can be exported to a GFF3 format file using the dedicated GFF3 exporter.

A subset of annotations on a sequence or sequence list can be exported from the Annotation Table (▤) view: select the annotations to export in the table, right-click, and choose **Export Selection to GFF3 File** from the menu (figure 14.20). Further information about sequence annotations is in section 14.3.

8.1.8 JSON export

Reports can be exported in JSON format. An exported JSON file contains 4 main elements:

- **header**. Contains information about the version of the JSON exporter and front page elements included in the report (the front page elements are visible in the PDF export of the report).
- **data**. Contains the actual data found in the report (sections, subsections, figures, tables, text).
- **metadata**. Contains information about metadata files the report referenced to.
- **history**. Contains information about the history of the report (as seen in the "Show history" view).

The data section contains nested elements following the structure of the report:

- The keys of sections (and subsections, etc) are formed from the section (and subsection, etc) title, with special characters replaced. For example, the section "Counted fragment by type (total)" is exported to an element with the key "counted_fragments_by_type_total".
- A section is made of the section title, the section number, and all other elements that are nested in it (e.g., other subsections, figures, tables, text).
- Figures, tables and text are exported to elements with keys "figure_n", "table_n" and "text_n", n being the number of the elements of that type in the report.
- Figures contain information about the titles of the figure, x axis, and y axis, as well as legend and data. This data is originally available in the Workbench by double clicking on a figure in a report and using the "Show Table" view.

- The names of table columns are transformed to keys in a similar way to section titles.

Once exported, the JSON file can be parsed and further processed. For example, using R and the package jsonlite, reports from different samples can be jointly analyzed. This enables easy comparison of any information present in the original reports across samples.

Example of R script to generate a combined RNA-Seq report

R scripts can be used to process reports in JSON format. The script below is an example of how RNA-Seq reports generated by the CLC Genomics Workbench could be processed to compare information across samples. The same idea could be used for processing other sorts of reports.

The script uses jsonlite to parse all the JSON reports. Plots are produced using the package ggplot2. This script is intended for inspiration only and is not supported.

```
library(jsonlite)
library(tools)
library(ggplot2)
```

The script relies on the following functions to extract the data from the parsed JSON files.

```
# Get the read count statistics from a parsed JSON report.
get_read_count_stats <- function(parsed_report) {
    mapping_statistics <- parsed_report$data$mapping_statistics
    total_reads <- 0
    stats <- c()
    if ("single_reads" %in% names(mapping_statistics)) {
        table <- mapping_statistics$single_reads$table_1
        # use the id column to give names to the rows
        row.names(table) <- table$id
        stats <- c(table["Reads mapped", "percent"],
                   table["Reads not mapped", "percent"])
        total_reads <- total_reads + table["Total", "number_of_sequences"]
    }
    else {
        stats <- c(stats, rep(NA, 2))
    }
    if ("paired_reads" %in% names(mapping_statistics)) {
        table <- mapping_statistics$paired_reads$table_1
        # use the id column to give names to the rows
        row.names(table) <- table$id
        stats <- c(stats,
                   table["Reads mapped in pairs", "percent"],
                   table["Reads mapped in broken pairs", "percent"],
                   table["Reads not mapped", "percent"])
        total_reads <- total_reads + table["Total", "number_of_sequences"]
    }
    else {
        stats <- c(stats, rep(NA, 3))
    }
    stats <- c(total_reads, stats)
    names(stats) <- c("reads_count", "single_mapped", "single_not_mapped",
                      "paired_mapped_pairs", "paired_broken_pairs",
                      "paired_not_mapped")
    return(data.frame(sample = basename(file_path_sans_ext(report)),
                      t(stats)))
}
```

```
#' Get the paired distance from a parsed report. Returns null if the reads were
#' unpaired.
get_paired_distance <- function(parsed_report) {
    section <- parsed_report$data$read_quality_control
    if (!("paired_distance" %in% names(section))) {
        return(NULL)
    } else {
        figure <- section$paired_distance$figure_1
        return(data.frame(sample = basename(file_path_sans_ext(report)),
                          figure$data))
    }
}
```

```
#' Get the figure, x axis, and y axis titles from the paired distance figure
#' from a parsed report. Returns null if the reads were unpaired.
get_paired_distance_titles <- function(parsed_report) {
    section <- parsed_report$data$read_quality_control
    if (!("paired_distance" %in% names(section))) {
        return(NULL)
    } else {
        figure <- section$paired_distance$figure_1
        return(c("title" = figure$figure_title,
                 "x" = figure$x_axis_title,
                 "y" = figure$y_axis_title))
    }
}
```

```
#' Re-order the intervals for the paired distances by using the starting value of the interval.
order_paired_distances <- function(paired_distance) {
    distances <- unique(paired_distance$distance)
    starting <- as.numeric(sapply(strsplit(distances, split = " - "), function(l) l[1]))
    distances <- distances[sort.int(starting, index.return = TRUE)$ix]
    paired_distance$distance <- factor(paired_distance$distance, levels = distances)
    # calculate the breaks used on the x axis for the paired distances
    breaks <- distances[round(seq(from = 1, to = length(distances), length.out = 15))]
    return(list(data = paired_distance, breaks = breaks))
}
```

Using the above functions, the script below parses all the JSON reports found in the "exported reports" folder, to build a read count statistics table (read_count_statistics), and a paired distance histogram.

```
reports <- list.files("exported reports/", full.names = TRUE)

read_count_statistics <- data.frame()
paired_distance <- data.frame()
titles <- c(NA, NA, NA)

for (report in reports) {
    parsed_report <- fromJSON(report)

    read_count_statistics <- rbind(read_count_statistics,
                                   get_read_count_stats(parsed_report))
    paired_distance <- rbind(paired_distance,
                             get_paired_distance(parsed_report))
    titles <- get_paired_distance_titles(parsed_report)
}
```

```
paired_distance <- order_paired_distances(paired_distance)

ggplot(paired_distance$data, aes(x = distance, y = number_of_reads, fill = sample)) +
    geom_bar(stat = "identity", position = "dodge") +
    scale_x_discrete(breaks = paired_distance$breaks, labels = paired_distance$breaks) +
    labs(title = titles["title"], x = titles["x"],  y = titles["y"]) +
    theme(legend.position = "bottom")
```

8.1.9 Graphics export

CLC Main Workbench supports two ways of exporting graphics:

- You can export the current view, either the visible area or the entire view, by clicking on the **Graphics** button () in the top Toolbar. This is the generally recommended route for exporting graphics for individual data elements, and is described in section 8.2.
- For some data types, graphics export tools are available from the main **Export** menu, which can be opened by clicking on the **Export ()** button in the top Toolbar. These are useful if you wish to export different data using the same view in an automated fashion, for example by running the export tool in batch mode or in a workflow context. This functionality is described below.

Using export tools to export graphics

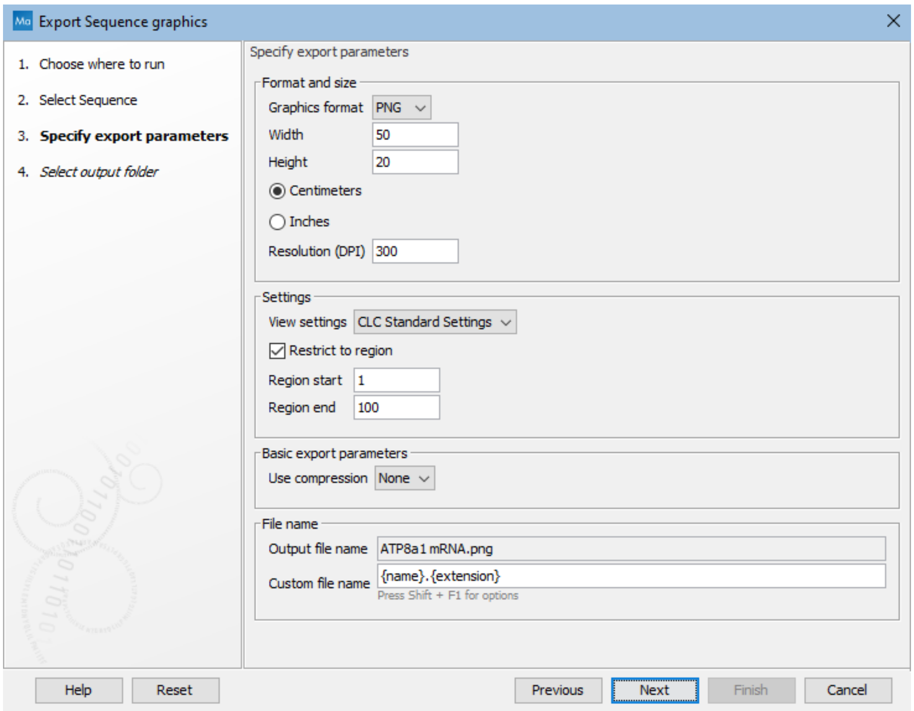
The following types of data can be exported using dedicated export tools:

- Alignments
- Heat maps
- Read mappings
- Sequences
- Tracks
- Track lists

The general actions taken are:

- Click on the **Export ()** button in the top Toolbar or choose the **Export** option under the File menu.
- Type "graphics" in the top field to see just a list of graphics exporters, and then select the one you wish to use. For example, if you wish to export an alignment as graphics, select "Alignment graphics" in the list.
- Select the data elements to be exported.
- Configure any relevant options. Detailed descriptions of these are provided below.
- Select where the data should be exported to.

Options available when exporting sequences, alignments and read mappings to graphics format files are shown in figure 8.8.



Export Sequence graphics

1. Choose where to run
2. Select Sequence
3. **Specify export parameters**
4. *Select output folder*

Specify export parameters

Format and size

Graphics format: PNG

Width: 50

Height: 20

● Centimeters

○ Inches

Resolution (DPI): 300

Settings

View settings: CLC Standard Settings

☑ Restrict to region

Region start: 1

Region end: 100

Basic export parameters

Use compression: None

File name

Output file name: ATP8a1 mRNA.png

Custom file name: {name}.{extension}

Press Shift + F1 for options

Figure 8.8: *Options available when exporting sequences, alignments and read mappings to graphics format files.*

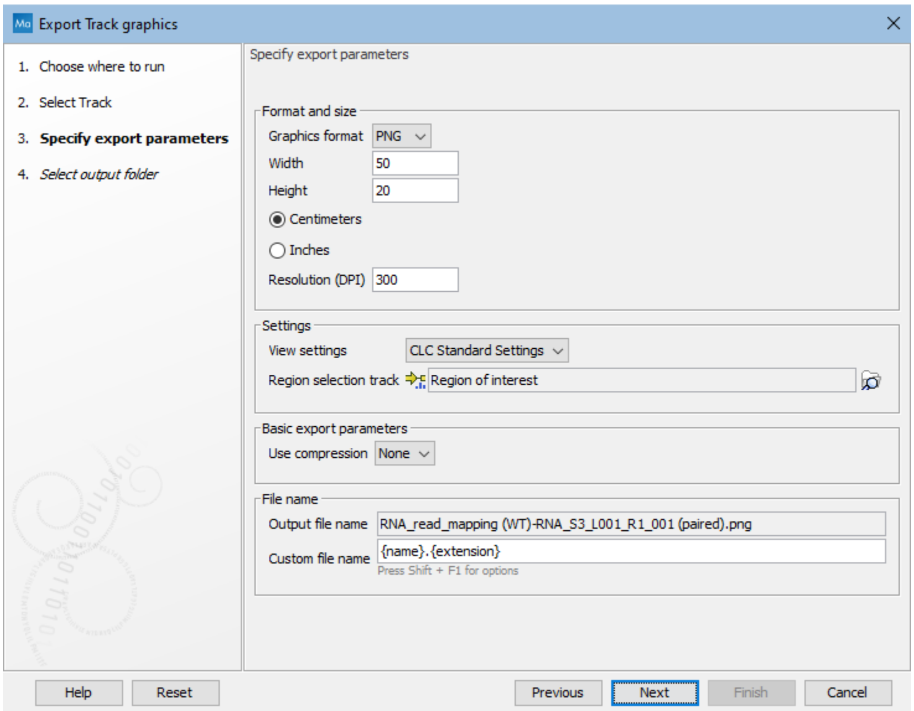
The options available when exporting tracks and track lists to graphics format files are shown in figure 8.9.

The format and size of the exported graphics can be configured using:

- **Graphics format**: Several export formats are available, including bitmap formats (such as .png, .jpg) and vector graphics (.svg, .ps, .eps).
- **Width and height**: The desired width and height of the exported image. This can be specified in centimeters or inches.
- **Resolution**: The resolution, specified in the units of "dpi" (dots per inch).

The appearance of the exported graphics can be configured using:

- **View settings**: The view settings available for the data type being exported. To determine how the data will look when a particular view is used, open a data element of the type you wish to export, click on the **Save View** button visible at the bottom of the Side Panel, and apply the view settings in the dialog that appears. View settings are described in section 4.6. Custom view settings will be available to choose from when exporting if the "Save for all <data type> views" option was checked when the view was saved.
- **Region restriction**: The region to be exported. For sequences, alignments and read mappings, the region is specified using start and end coordinates. For tracks and track lists, you provide an annotation track, where the region corresponding to the full span of the *first* annotation is exported. The rest of the annotations in the track have no effect.



Specify export parameters

Format and size

Graphics format: PNG

Width: 50

Height: 20

● Centimeters

○ Inches

Resolution (DPI): 300

Settings

View settings: CLC Standard Settings

Region selection track: Region of interest

Basic export parameters

Use compression: None

File name

Output file name: RNA_read_mapping (WT)-RNA_S3_L001_R1_001 (paired).png

Custom file name: {name}.{extension}

Press Shift + F1 for options

Figure 8.9: *Options available when exporting tracks and track lists to graphics format files.*

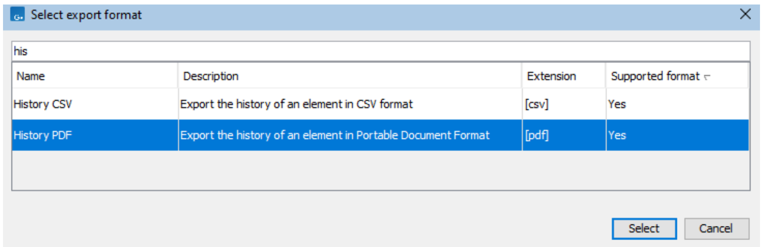
8.1.10 Export history

The history for a data element can be exported to PDF or to CSV format files. This includes information like the date and time data was imported or an analysis was run, the parameters and values set, and where the data came from. For elements created by a workflow, the name and version of that workflow is included in the PDF export. If created using an installed workflow, the workflow build-id is also included (figure 8.12).

The history information for an element can be seen in the *CLC Main Workbench* by clicking on the Show History view ( ) at the bottom of the viewing area when a data element is open (see section 2.5).

To export the history of a data element, click on the **Export ( )** in the Toolbar and select **History PDF** or **History CSV** (figure 8.10).

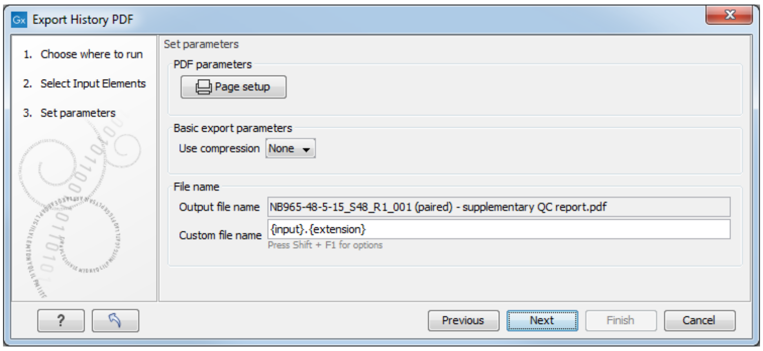
After selecting the data to export the history for, you can configure standard export parameters for the type of format you are exporting to (figure 8.11).



his

Name	Description	Extension	Supported format
History CSV	Export the history of an element in CSV format	[csv]	Yes
History PDF	Export the history of an element in Portable Document Format	[pdf]	Yes

Figure 8.10: *Select "History PDF" for exporting the history of an element as a PDF file.*

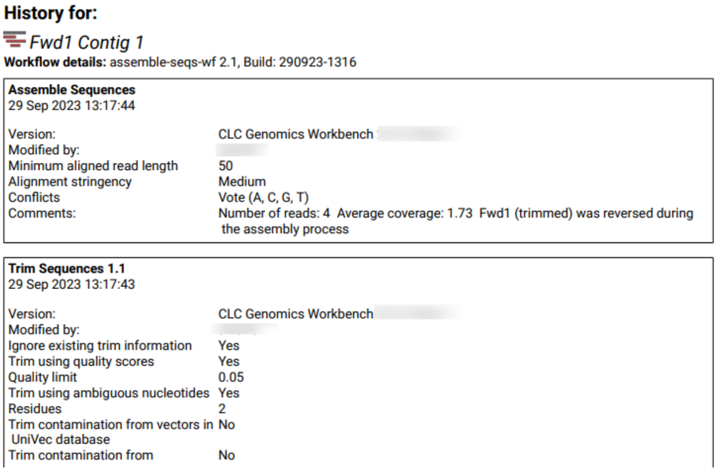


Use compression: None

Output file name: NB965-48-5-15_S48_R1_001 (paired) - supplementary QC report.pdf

Custom file name: {input}.{extension}

Figure 8.11: *When exporting the history in PDF, it is possible to adjust the page setup.*



History for:

Fwd1 Contig 1

Workflow details: assemble-seqs-wf 2.1, Build: 290923-1316

Assemble Sequences
29 Sep 2023 13:17:44

Version:	CLC Genomics Workbench [redacted]
Modified by:	[redacted]
Minimum aligned read length	50
Alignment stringency	Medium
Conflicts	Vote (A, C, G, T)
Comments:	Number of reads: 4 Average coverage: 1.73 Fwd1 (trimmed) was reversed during the assembly process

Trim Sequences 1.1
29 Sep 2023 13:17:43

Version:	CLC Genomics Workbench [redacted]
Modified by:	[redacted]
Ignore existing trim information	Yes
Trim using quality scores	Yes
Quality limit	0.05
Trim using ambiguous nucleotides	Yes
Residues	2
Trim contamination from vectors in UniVec database	No
Trim contamination from	No

Figure 8.12: *An example of the top of the exported PDF containing the history of an element generated using an installed workflow.*

8.2 Export graphics to files

CLC Main Workbench supports two ways of exporting graphics:

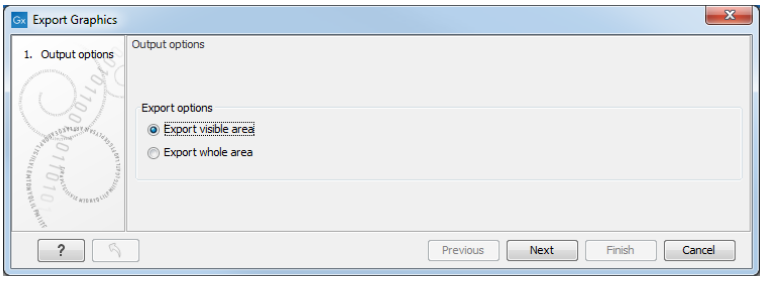
- You can export the current view, either the visible area or the entire view, by clicking on the **Graphics** button ( ) in the top Toolbar. This is the generally recommended route for exporting graphics for individual data elements, and is described below.
- For some data types, graphics export tools are available in the main **Export** menu, which can be opened by clicking on the **Export ( )** button in the top Toolbar. These are useful if you wish to export different data using the same view in an automated fashion, for example by running the export tool in batch mode or in a workflow context. That functionality is described in section 8.1.9.

Exporting a view of data element to a graphics format file

To export a view of an open data element to a graphics file, click on the **Graphics** button ( ) in the top Toolbar, or choose **Export Graphics** from under the File menu.

How the data looks on the screen is how it will look in the exported file. Before exporting, the options in the Side Panel can be used to make adjustments as necessary.

For views that can be zoomed into or out of, you will be offered the choice of exporting the whole view or just the visible area (figure 8.13). For 3D structures, the section visible will always be exported, i.e. the equivalent to selecting to export just the visible area.



Export Graphics

1. Output options

Output options

Export options

● Export visible area

○ Export whole area

Figure 8.13: *The whole view or just the visible area can be selected for export.*

A view of a circular sequence, zoomed in so that you can only see a part of it, is shown in figure 8.14.

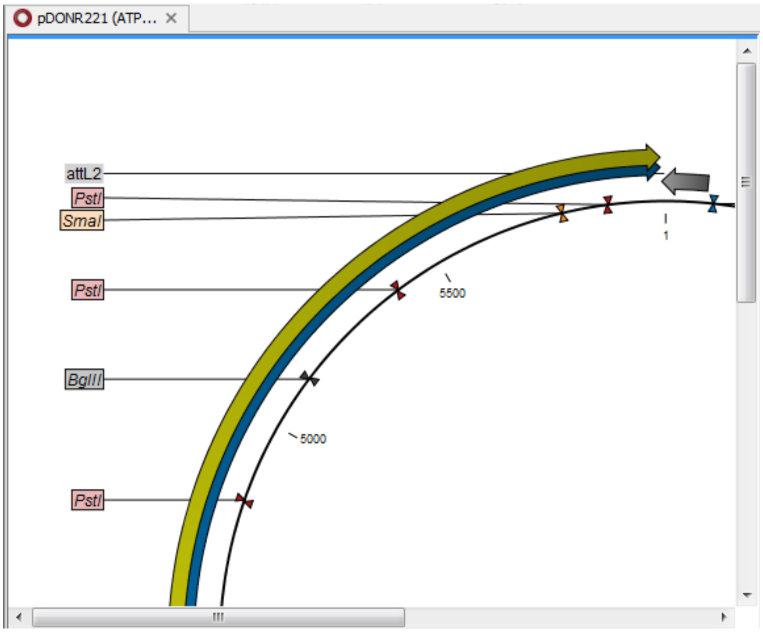


Figure 8.14: *A circular sequence, as it looks on the screen when zoomed in.*

When the option **Export visible area** is selected, the exported file will only contain the part of the sequence that is *visible* in the view. The result of doing this for the view shown in figure 8.14 is shown in figure 8.15.

If you select **Export whole view**, the result would look like that shown in figure 8.16, where the part of the sequence not visible on screen is also exported.

You are prompted for the file format to use, and the name and location to save the output to.

For vector-based image formats, click on **Finish** to start the export. For bitmap-basd image formats, click on **Next** to select a resolution level. You can click on **Finish** directly instead, in which case the image will be exported using the setting used the last time this functionality was run. If it has not been run before the default settings are used.

The available export formats are described in section 8.2.1.

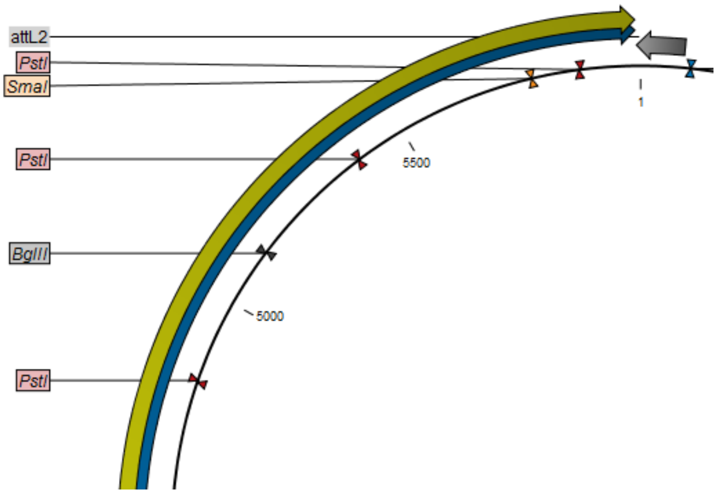


Figure 8.15: *The exported graphics file when Export visible area was selected.*

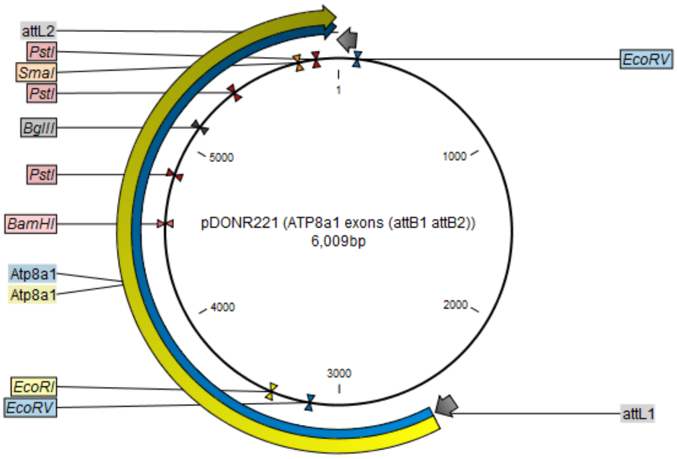


Figure 8.16: *The exported graphics file when Export whole view was selected. The whole sequence is shown, not just the part visible on screen when the view was exported.*

8.2.1 File formats

CLC Main Workbench supports the following file formats for graphics export:

Format	Suffix	Type
Portable Network Graphics	.png	bitmap
JPEG	.jpg	bitmap
Tagged Image File	.tif	bitmap
PostScript	.ps	vector graphics
Encapsulated PostScript	.eps	vector graphics
Portable Document Format	.pdf	vector graphics
Scalable Vector Graphics	.svg	vector graphics

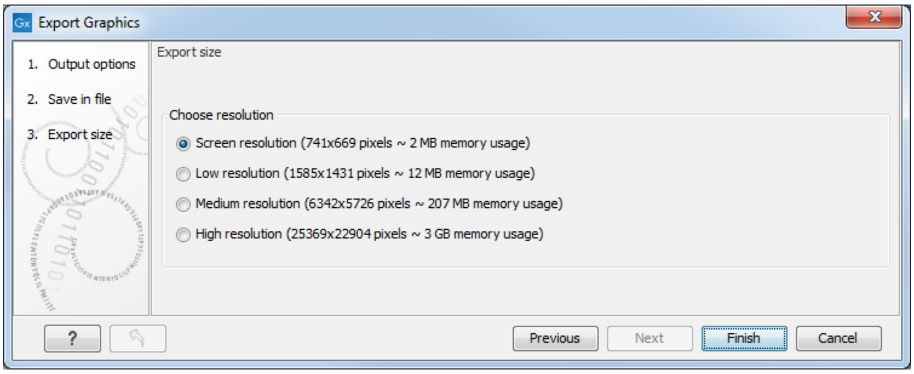
These formats can be divided into bitmap and vector graphics. The difference between these two categories is described below:

Bitmap images In a bitmap image, each dot in the image has a specified color. This implies, that if you zoom in on the image there will not be enough dots, and if you zoom out there will be too many. In these cases the image viewer has to interpolate the colors to fit what is actually looked at. A bitmap image needs to have a high resolution if you want to zoom in. This format is a good choice for storing images without large shapes (e.g. dot plots). It is also appropriate if you don't have the need for resizing and editing the image after export.

To produce a high resolution image with all the details of a large element visible, e.g. a large phylogenetic tree or a read mapping, we recommend exporting to a vector based format.

If Screen resolution and High resolution settings show the same pixel dimensions, this can be because the maximum supported number of pixels has been exceeded.

Parameters for bitmap formats For bitmap files, clicking **Next** will display the dialog shown in figure 8.17.



Export size

Choose resolution

● Screen resolution (741x669 pixels ~ 2 MB memory usage)
○ Low resolution (1585x1431 pixels ~ 12 MB memory usage)
○ Medium resolution (6342x5726 pixels ~ 207 MB memory usage)
○ High resolution (25369x22904 pixels ~ 3 GB memory usage)

Figure 8.17: *Parameters for bitmap formats: size of the graphics file.*

You can adjust the size (the resolution) of the file to four standard sizes:

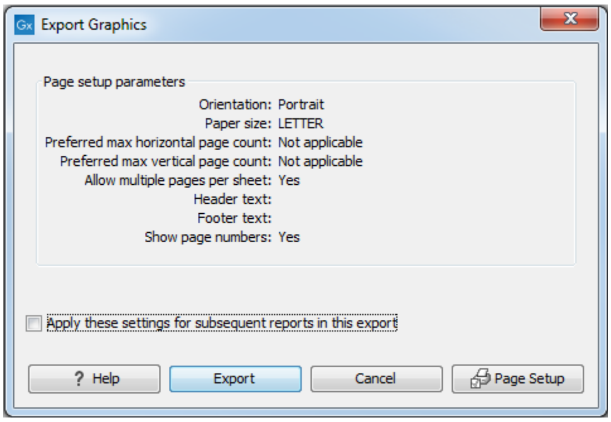
- Screen resolution
- Low resolution
- Medium resolution
- High resolution

The actual size in pixels is displayed in parentheses. An estimate of the memory usage for exporting the file is also shown. If the image is to be used on computer screens only, a low resolution is sufficient. If the image is going to be used on printed material, a higher resolution is necessary to produce a good result.

Vector graphics Vector graphic is a collection of shapes. Thus what is stored is information about where a line starts and ends, and the color of the line and its width. This enables a given viewer to decide how to draw the line, no matter what the zoom factor is, thereby always giving a correct image. This format is good for graphs and reports, but less usable for dot plots. If the image is to be resized or edited, vector graphics are by far the best format to store graphics. If you open a vector graphics file in an application such as Adobe Illustrator, you will be able to manipulate the image in great detail.

Graphics files can also be imported into the **Navigation Area**. However, no kinds of graphics files can be displayed in *CLC Main Workbench*. See section 3.2 for more about importing external files into *CLC Main Workbench*.

Parameters for vector formats For PDF format, the dialog shown in figure 8.18 will sometimes appear after you have clicked finished (for example when the graphics use more than one page, or there is more than one PDF to export).



Orientation: Portrait
Paper size: LETTER
Preferred max horizontal page count: Not applicable
Preferred max vertical page count: Not applicable
Allow multiple pages per sheet: Yes
Header text:
Footer text:
Show page numbers: Yes

☐ Apply these settings for subsequent reports in this export

Figure 8.18: *Page setup parameters for vector formats.*

The settings for the page setup are shown. Clicking the **Page Setup** button will display a dialog where these settings can ba adjusted. This dialog is described in section 5.2.

It is then possible to click the option "Apply these settings for subsequent reports in this export" to apply the chosen settings to all the PDFs included in the export for example.

The page setup is only available if you have selected to export the whole view - if you have chosen to export the visible area only, the graphics file will be on one page with no headers or footers.

Exporting protein reports It is possible to export a protein report using the normal **Export** function () which will generate a pdf file with a table of contents:

Click the report in the Navigation Area | Export () in the Toolbar | select pdf

You can also choose to export a protein report using the **Export graphics** function (), but in this way you will not get the table of contents.

8.3 Export graph data points to a file

Data points for graphs displayed along the sequence or along an alignment or mapping can be exported to a semicolon-separated text file (csv format). An example of such a graph is shown in figure 8.19, showing the conservation score of reads in a read mapping.

To export the data points for the graph, right-click the graph and choose **Export Graph to Comma-separated File**. Depending on what kind of graph you have selected, different options

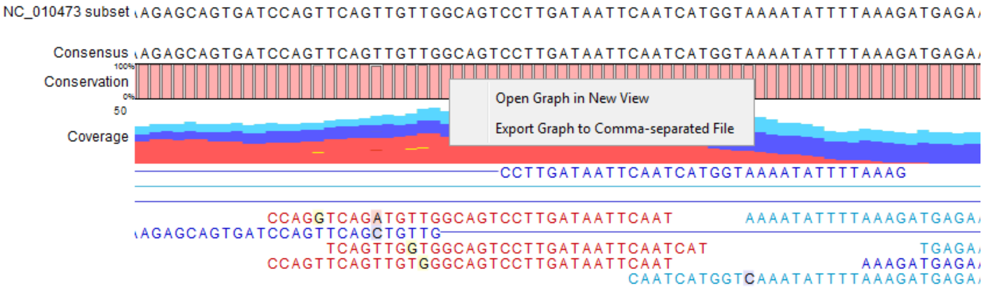
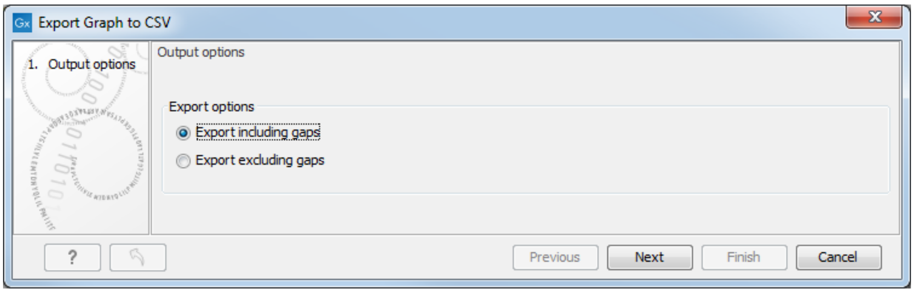


Figure 8.19: *A conservation graph displayed along mapped reads. Right-click the graph to export the data points to a file.*

will be shown: If the graph is covering a set of aligned sequences with a main sequence, such as read mappings and BLAST results, the dialog shown in figure 8.20 will be displayed. These kinds of graphs are located under **Alignment info** in the Side Panel. In all other cases, a normal file dialog will be shown letting you specify name and location for the file.



Output options

Export options

● Export including gaps

○ Export excluding gaps

Figure 8.20: *Choosing to include data points with gaps*

In this dialog, select whether you wish to include positions where the main sequence (the reference sequence for read mappings and the query sequence for BLAST results) has gaps. If you are exporting e.g. coverage information from a read mapping, you would probably want to exclude gaps, if you want the positions in the exported file to match the reference (i.e. chromosome) coordinates. If you export including gaps, the data points in the file no longer corresponds to the reference coordinates, because each gap will shift the coordinates.

Clicking **Next** will present a file dialog letting you specify name and location for the file.

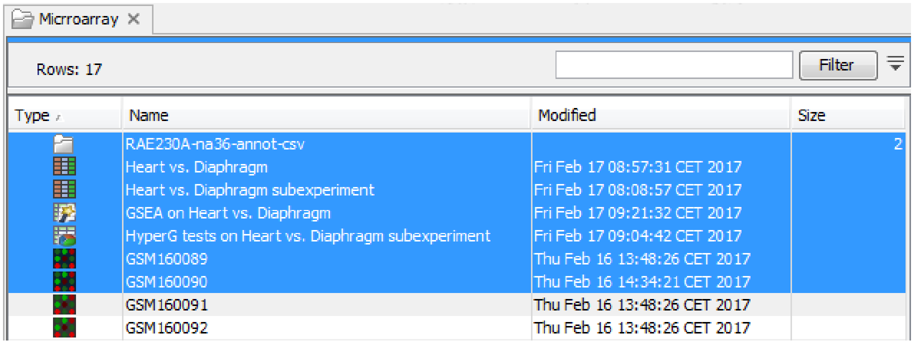
The output format of the file is like this:

```
"Position";"Value";
"1";"13";
"2";"16";
"3";"23";
"4";"17";
...
```

8.4 Copy/paste view output

The contents of various element types can be copied and then pasted into programs outside of the Workbench. For example, tables from reports, table views of sequence lists, and tabular listings of folders, can be copied and then pasted into text editors or programs like Excel.

Example: Right click a folder in the Navigation Area and choose **Show** | **Content**. The folder contents are shown as a table in the viewing area. Select one or more of the rows and then copy them (Ctrl + C). That information can then be pasted into other programs (figure 8.21)



Rows: 17

Type	Name	Modified	Size
	RAE230A-na36-annot-csv		2
	Heart vs. Diaphragm	Fri Feb 17 08:57:31 CET 2017	
	Heart vs. Diaphragm subexperiment	Fri Feb 17 08:08:57 CET 2017	
	GSEA on Heart vs. Diaphragm	Fri Feb 17 09:21:32 CET 2017	
	HyperG tests on Heart vs. Diaphragm subexperiment	Fri Feb 17 09:04:42 CET 2017	
	GSM160089	Thu Feb 16 13:48:26 CET 2017	
	GSM160090	Thu Feb 16 14:34:21 CET 2017	
	GSM160091	Thu Feb 16 13:48:26 CET 2017	
	GSM160092	Thu Feb 16 13:48:26 CET 2017	

Figure 8.21: *Selected elements in a Folder Content view.*

Workflow designs can be copied as an image. To do this, select the elements in the workflow design (click in the workflow editor and then press keys Ctrl + A), then copy (Ctrl + C), and then paste where you wish the image to be placed, for example, in an email or presentation program. Workflows are described in detail in chapter 13.

Chapter 9

Working with tables

Contents

General features relevant to many table types are described in this section. For functionality associated with specific table types, please refer to the manual section describing that particular data type.

Key functionality available for tables includes:

- **Sorting** A table can be sorted according to the values of a particular column by clicking a column header. Clicking once will sort in ascending order. A second click will change the order to descending. A third click will set the order back its original order.

  Pressing Ctrl - ⌘ on Mac - while you click other columns will refine the existing sorting with the values of the additional columns, in the order in which you clicked them.

- **Configuring the view** This includes specifying which columns should be visible and defining the column order (see section 9.1). View settings can be saved for later use, with a specific table or for any similar table. (see section or chapter 4.6).

- **Displaying only the selected rows** Click on the **Filter to Selection...** button above a table to update the view to show only the selected rows.

  Rows can be selected manually, or by using the "Select in other views" option, which is available for some tables, generally those with an associated graphical view such as a Venn diagram, or a volcano plot.

  To view the full table again, click on the **Filter to Selection...** button and choosing the option **Clear selection filter**.

- **Displaying only rows with content of interest** Tables can be interactively filtered using simple or complex search criteria such that only rows containing content of interest are shown. Sets of table filters can be saved for re-use. See section or chapter 9.2 for details.

Scroll bars appear at the bottom and at the right of a table when table contents exceed the size of the viewing area.

Table-specific right-click menu options

Right-clicking within a table reveals standard right-click menu options, as well as the following table-specific options:

- **File |Export Table** Export the table to CSV, TSV, HTML or Excel format. Filtering, sorting, column selection and column order are respected when exporting the table this way.
- **Edit | Copy Cell** Right-click on a cell and choose this option to copy the contents of that cell to the clipboard.

The option call **Table filters**, also available in the right-click menu, is explained in section or chapter 9.2.

9.1 Table view settings and column ordering

View settings for tables are configurable in the Side Panel (figure 9.1). These settings include how column widths should be determined, as well as which columns should be visible and the column order.

Saving view settings

View settings can be saved for continued use with this table, or later use with this table or other similar tables. Saving view settings is described in section or chapter 4.6. Table specific considerations when saving view settings include:

- If saved view settings are applied to a table that contains columns not defined in those view settings, those columns will be placed at the far right of the table.
- Saved view settings referring to columns not present in the table that they are being applied to are ignored.

Column width view settings

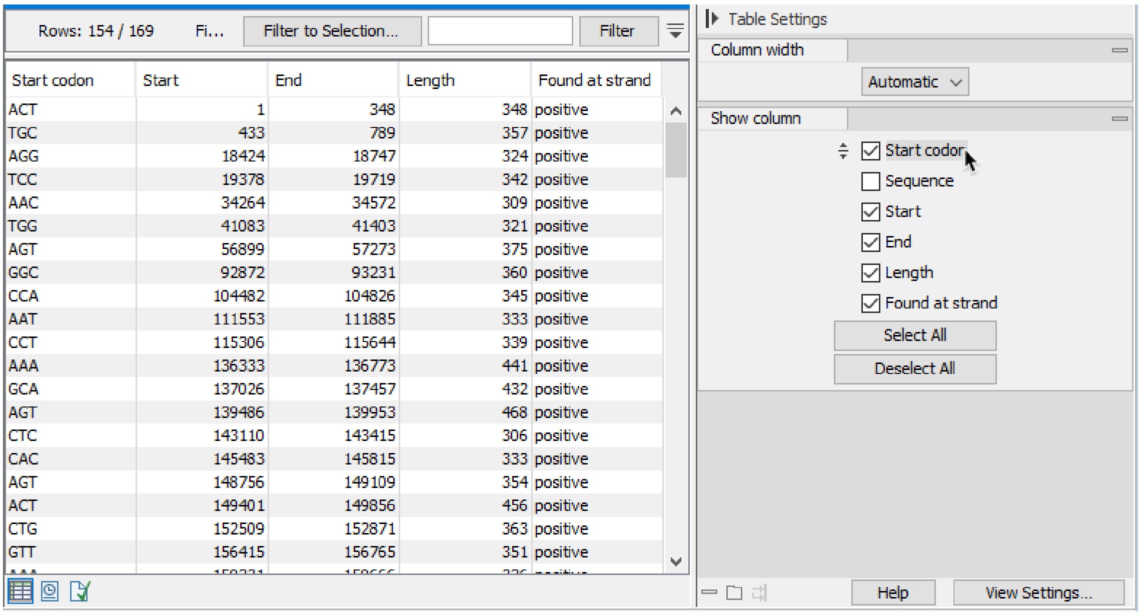
Column width options in the Side Panel are:

- **Automatic** Columns are sized to fit the width of the viewing area.
- **Manual** The width of each column can be adjusted manually.

Columns to show in the table

The columns available for that table are listed. Checking the box beside a column name makes that column visible in the viewing area. Unchecking that box hides the column.

Select All and **Deselect All** buttons allow you to make all columns visible in the viewing area, or hide all columns, in a single click.



Start codon	Start	End	Length	Found at strand
ACT	1	348	348	positive
TGC	433	789	357	positive
AGG	18424	18747	324	positive
TCC	19378	19719	342	positive
AAC	34264	34572	309	positive
TGG	41083	41403	321	positive
AGT	56899	57273	375	positive
GGC	92872	93231	360	positive
CCA	104482	104826	345	positive
AAT	111553	111885	333	positive
CCT	115306	115644	339	positive
AAA	136333	136773	441	positive
GCA	137026	137457	432	positive
AGT	139486	139953	468	positive
CTC	143110	143415	306	positive
CAC	145483	145815	333	positive
AGT	148756	149109	354	positive
ACT	149401	149856	456	positive
CTG	152509	152871	363	positive
GTT	156415	156765	351	positive

Column width

Automatic

Show column

☑ Start codon
□ Sequence
☑ Start
☑ End
☑ Length
☑ Found at strand

Select All

Deselect All

Figure 9.1: *A table with all but one available columns visible, and the "Start codon" column moved to the start of the table from its original location, which was at the end of the table.*

Changing the column order

The column order in tables can be changed. This affects the order in the view as well as column order in files exported from the table.

Ways to change column order in a table:

1. **Drag the column to the desired location in the viewing area.**

   Click on the column heading in the viewing area and, keeping the mouse button depressed, drag it to the desired location in the table.

   The order of the columns in the Side Panel is updated automatically. Check the Side Panel listing to ensure the column is positioned as desired relative to any hidden (unchecked) columns.

2. **Move the column to the desired location in the Show columns palette in the Side Panel.**

   Hover over the column name in the Side Panel, revealing the (≑) icon, then depress the mouse button and drag the column to the position desired.

   The order of the columns in the viewing area is updated automatically.

3. **Apply saved view settings where a relevant column order has been defined.** See section or chapter 4.6 for details about applying saved view settings.

Files exported from a table open for viewing, such as .csv files, can be exported using this custom column order. See section 8.1.6 for details.

9.2 Filtering tables

The rows shown in a table view can be limited to just those of interest by simple or advanced filtering, defined using functionality just above the table. Saving and reusing sets of table filters is described at the end of this section.

Filtering the rows of a table interactively does *not* change the underlying content of the table. Some table types have a button allowing the creating of a new table containing only the visible rows.

Simple filtering

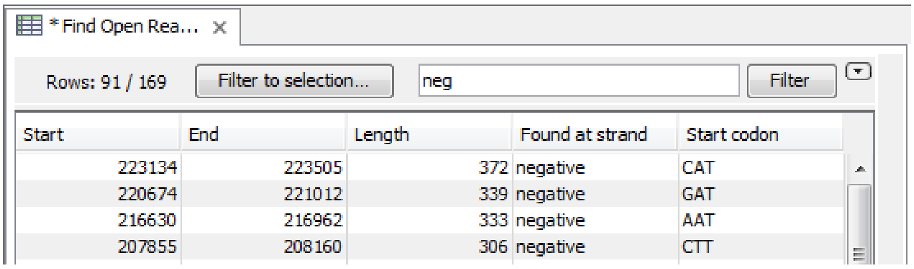
The default view of a table supports simple filtering, where rows containing a particular search term can be entered into a field to the left of the **Filter** button (figure 9.2). Simple filtering is enabled when there is an upwards pointing arrow at the top right of the table view. The keyboard shortcut Ctrl + F (mac: ⌘ + F) jumps the cursor into the simple filter field. (Clicking on the arrow beside that field reveals advanced filtering options, which are described later in this section.)

Simple filtering starts automatically, as you type, unless the table has more than 10,000 rows. In that case, click on the **Filter** button after typing the term to filter for.

The number of rows with a match to the term is reported in the top left of the table.

The following characters have special meanings when used in the simple filtering field:

- **Space** Terms separated by spaces are treated as individual search terms unless the terms are placed within quotes. E.g. the term `cat dog` would return all rows with the term cat and/or the term dog in them, in any order.
- **Single and double quotes ' and "** Enclose a term containing spaces in quotes to search for exactly that term. E.g. `"cat dog"` would return rows containing the single term `cat dog`.
- **Backslash** Use this term to escape special characters. For example, to search for the term term `"cat"` including the quotation marks, enter `\"cat\"`.
- **Minus -** Please a minus symbol before a termm to exclude rows containing that term. e.g. `-cat -dog` would exclude all rows containing either cat or dog.
- **Colon :** Specify the name of a column to be searched for the term. E.g. `Animal:cat` would search for the term `cat` only in a column called `Animal`. For this sort of filtering, please also refer to the advanced filtering information, below.

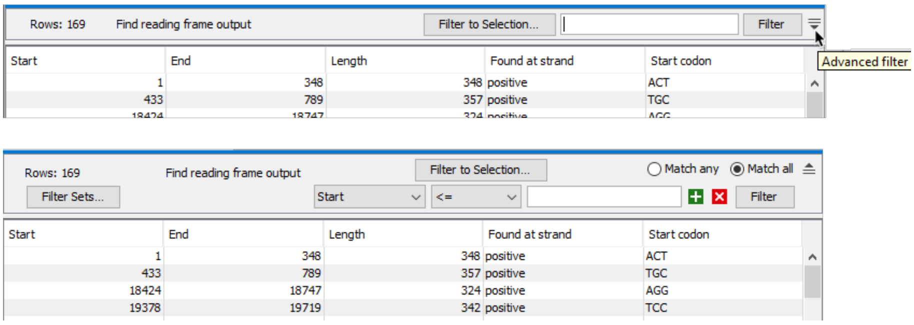


Start	End	Length	Found at strand	Start codon
223134	223505	372	negative	CAT
220674	221012	339	negative	GAT
216630	216962	333	negative	AAT
207855	208160	306	negative	CTT

Figure 9.2: *Filtering for rows that contain the term "neg" using the Filter button*

Advanced filtering

Functionality to define sets of filter criteria is revealed by clicking on the downwards-pointing arrow at the top right of the table view, (figure 9.3).



Start	End	Length	Found at strand	Start codon
1	348	348	positive	ACT
433	789	357	positive	TGC
18424	18747	324	positive	AGG

Start	End	Length	Found at strand	Start codon
1	348	348	positive	ACT
433	789	357	positive	TGC
18424	18747	324	positive	AGG
19378	19719	342	positive	TCC

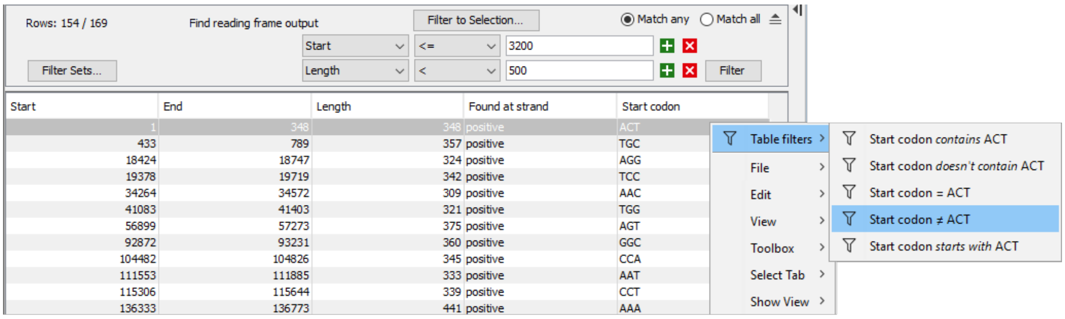
Figure 9.3: *When the Advanced filter icon is clicked on (top), Advanced filtering fields are revealed (bottom)*

Each filter criterion consists of a column name, an operator and a value. Examples are described below.

Filter criteria can be added by:

- Clicking the **Add** (🟩) icon.
- Right-clicking on a value in the table and selecting the **Table filters** option from the menu that appears. Predefined criteria for that column and value combination will be listed (figure 9.4). Selecting one of these adds it to the list of filters at the top of the table.

Filter criteria can be removed by clicking on the (☒) icons.



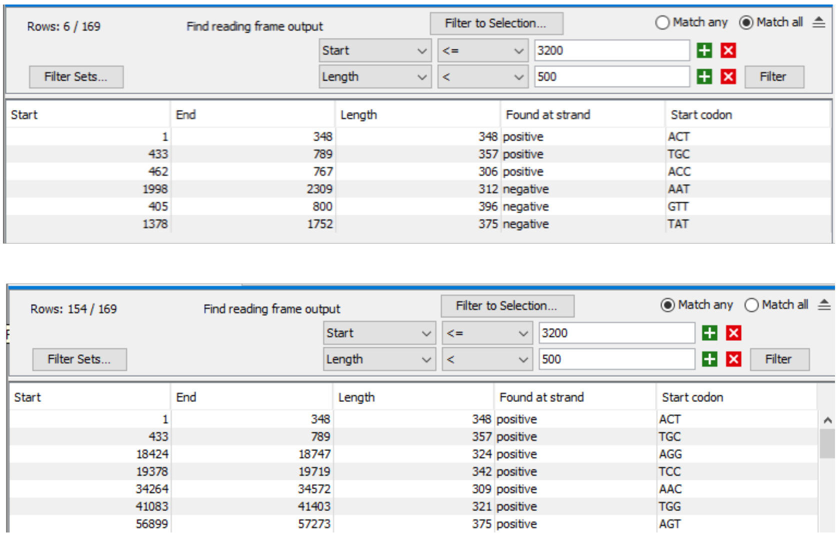
Start	End	Length	Found at strand	Start codon
1	348	348	positive	ACT
433	789	357	positive	TGC
18424	18747	324	positive	AGG
19378	19719	342	positive	TCC
34264	34572	309	positive	AAC
41083	41403	321	positive	TGG
56899	57273	375	positive	AGT
92872	93231	360	positive	GGC
104482	104826	345	positive	CCA
111553	111885	333	positive	AAT
115306	115644	339	positive	CCT
136333	136773	441	positive	AAA

Figure 9.4: *Right-click on a cell value and choose Table filters to reveal predefined criteria that can be added to the list of filters for this table.*

Match all and **Match any** options allow you to specify, respectively, whether all criteria must be met for a row to shown, or whether matching a single criteria is enough for a row to be shown (figure 9.5).

The number of rows with a match to the term is reported in the top left of the table.

Operators available for columns containing text are listed below. Tests for matches are not case specific.



Rows: 6 / 169 Find reading frame output

Filter to Selection... ○ Match any ● Match all

Start <= 3200

Filter Sets... Length < 500 Filter

Start	End	Length	Found at strand	Start codon
1	348	348	positive	ACT
433	789	357	positive	TGC
462	767	306	positive	ACC
1998	2309	312	negative	AAT
405	800	396	negative	GTT
1378	1752	375	negative	TAT

Rows: 154 / 169 Find reading frame output

Filter to Selection... ● Match any ○ Match all

Start <= 3200

Filter Sets... Length < 500 Filter

Start	End	Length	Found at strand	Start codon
1	348	348	positive	ACT
433	789	357	positive	TGC
18424	18747	324	positive	AGG
19378	19719	342	positive	TCC
34264	34572	309	positive	AAC
41083	41403	321	positive	TGG
56899	57273	375	positive	AGT

Figure 9.5: *The same two criteria are defined, but with "Match all" selected in the top image, and "Match any" selected in the bottom image.. Six rows out of 169 match all the criteria, while 154 rows match one or both criteria.*

- **contains**
- **doesn't contain**
- **=** Matches exactly
- $\neq$ Does not match
- **starts with**Start with the term provided.
- **is in list** Matches at least one of the entries in the list.
- **is not in list** Does not match any entry in the list.

  Terms in lists can be comma, semicolon, or space separated.

Operators available for columns containing numerical values are:

- **<=** Smaller than or equal to
- **<** Smaller than
- **>=** Greater than or equal to
- **>** Greater than
- **abs. value <** Absolute value smaller than.
- **abs. value >** Absolute value greater than.
- **=** Equal to
- $\neq$ Not equal to

- **is in list** Matches at least one of the entries in the list.
- **is not in list** Does not match any entry in the list.

  Terms in lists can be comma, semicolon, or space separated.

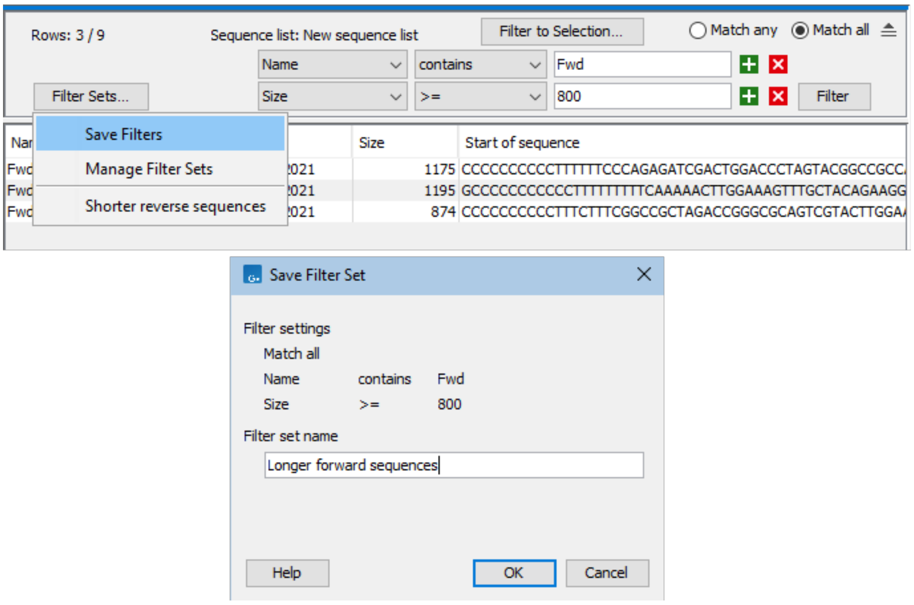
Number formatting and filter criterion: The number of digits to display after the decimal separator (fractional digits) can be set in the *CLC Main Workbench* Preferences. Thus, there may be more digits in a number stored in a table than are shown in a view of that table. For this reason, we recommend using operators that do not require exact matches, such as **=**, when filtering on non-integer values.

Saving and reusing table filter sets

Sets of filter criteria defined in the advanced filtering area at the top of a table can be saved for re-use. Filter sets can also be exported and imported, supporting sharing of filter sets with others.

Options for saving and managing filter sets are provided in a menu revealed when you click on the **Filter Sets...** button (figures 9.6 and 9.8). Saved filter sets are also listed here (figure 9.7). Selecting a saved set from this menu will add those conditions to the top of the table, and apply the filtering.

Saved filter sets can also be applied from the Manage Filters dialog (figure 9.8).



Rows: 3 / 9

Sequence list: New sequence list

○ Match any ● Match all

Name contains Fwd

Size >= 800

Nar		Size	Start of sequence
Fwd	021	1175	CCCCCCCCCCTTTTTTCCCAGAGATCGACTGGACCCTAGTACGGCCGCC
Fwd	021	1195	GCCCCCCCCCCCTTTTTTTTTCAAAAACTTGGAAAGTTTGCTACAGAAGG
Fwd	021	874	CCCCCCCCCCTTTCTTTCGGCCGCTAGACCGGGCGCAGTCGTACTTGGA

Save Filter Set

Filter settings

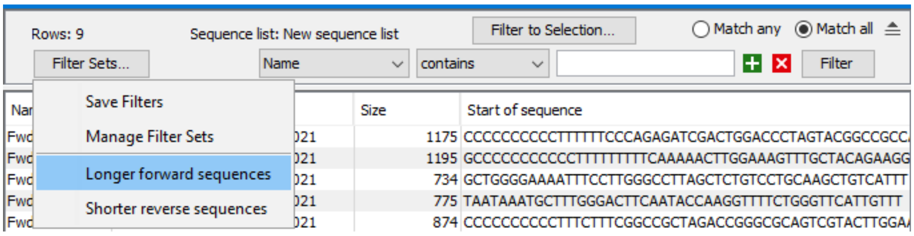
Match all

Name	contains	Fwd
Size	>=	800

Filter set name

Longer forward sequences

Figure 9.6: *Selecting Save Filters from the menu under the Filter Sets... button (top) opens a dialog showing the filter criteria and prompting for a name for the filter set (bottom).*

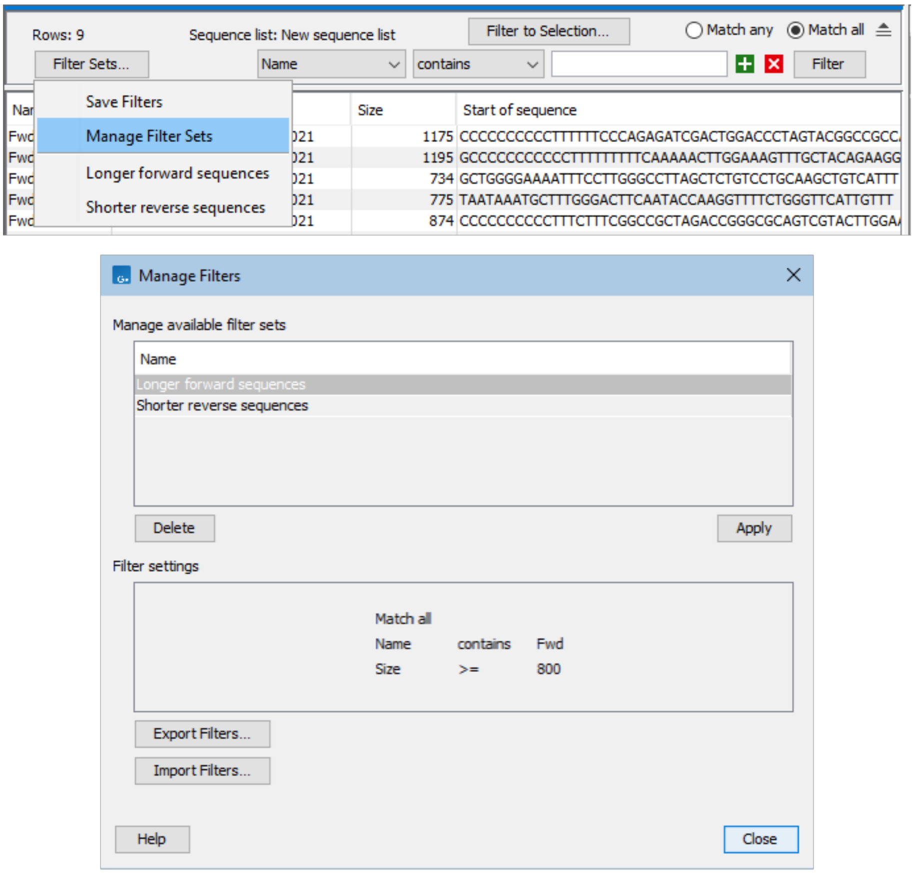


Rows: 9

Sequence list: New sequence list

Nar		Size	Start of sequence
Fwd	021	1175	CCCCCCCCCCTTTTTTCCCAGAGATCGACTGGACCCTAGTACGGCCGCC
Fwd	021	1195	GCCCCCCCCCCCTTTTTTTTTCAAAAACTTGGAAAGTTTGCTACAGAAGG
Fwd	021	734	GCTGGGGAAAATTTCCTTGGGCCTTAGCTCTGTCCTGCAAGCTGTCATTT
Fwd	021	775	TAATAAATGCTTTGGGACTTCAATACCAAGGTTTTCTGGGTTCATTGTTT
Fwd	021	874	CCCCCCCCCCTTTCTTTCGGCCGCTAGACCGGGCGCAGTCGTACTTGGA

Figure 9.7: *Saved filter sets are listed at the bottom of the drop-down menu revealed when you click on the Filter Sets... button.*



Rows: 9

Sequence list: New sequence list

Nar		Size	Start of sequence
Fwd	021	1175	CCCCCCCCCCTTTTTTCCCAGAGATCGACTGGACCCTAGTACGGCCGCC
Fwd	021	1195	GCCCCCCCCCCCTTTTTTTTTCAAAAACTTGGAAAGTTTGCTACAGAAGG
Fwd	021	734	GCTGGGGAAAATTTCCTTGGGCCTTAGCTCTGTCCTGCAAGCTGTCATTT
Fwd	021	775	TAATAAATGCTTTGGGACTTCAATACCAAGGTTTTCTGGGTTCATTGTTT
Fwd	021	874	CCCCCCCCCCTTTCTTTCGGCCGCTAGACCGGGCGCAGTCGTACTTGGA

Manage available filter sets

Name
Longer forward sequences
Shorter reverse sequences

Filter settings

Match all

Name	contains	Fwd
Size	>=	800

Figure 9.8: *Selecting Manage Filters from the menu under the Filter Sets... button (top) opens the Manage Filters dialog, where saved filter sets can be applied to the open table, or deleted. Functionality to export and import filter sets is also provided here (bottom).*

Chapter 10

Data download

Contents

CLC Main Workbench offers different ways of searching and downloading online data. You must be online when initiating and performing the following searches.

10.1 Search for Sequences at NCBI

Search for Sequences at NCBI supports searching the Entrez nucleotide, peptide and EST databases (`https://www.ncbi.nlm.nih.gov/books/NBK44864/`).

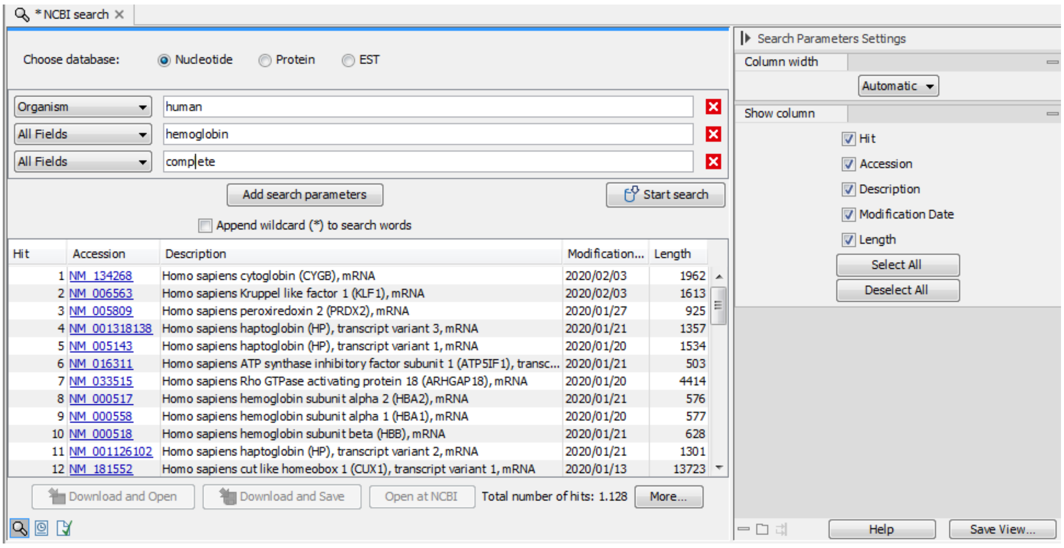
To start this tool, go to:

Download | Search for Sequences at NCBI (**) or **Ctrl + B (⌘ + B on Mac)

Select the nucleotide, protein or EST database using options at the top of the view (figure 10.1).

The search you just set up can be saved by doing one of the following:

- Choose **Save As...** from under the File menu, or
- Click on the tab of the search view and drag and drop it into a folder in the Navigation Area.



Choose database: ● Nucleotide ○ Protein ○ EST

Organism	human
All Fields	hemoglobin
All Fields	complete

□ Append wildcard (*) to search words

Hit	Accession	Description	Modification...	Length
1	NM_134268	Homo sapiens cytoglobin (CYGB), mRNA	2020/02/03	1962
2	NM_006563	Homo sapiens Kruppel like factor 1 (KLF1), mRNA	2020/02/03	1613
3	NM_005809	Homo sapiens peroxiredoxin 2 (PRDX2), mRNA	2020/01/27	925
4	NM_001318138	Homo sapiens haptoglobin (HP), transcript variant 3, mRNA	2020/01/21	1357
5	NM_005143	Homo sapiens haptoglobin (HP), transcript variant 1, mRNA	2020/01/20	1534
6	NM_016311	Homo sapiens ATP synthase inhibitory factor subunit 1 (ATP5IF1), transc...	2020/01/21	503
7	NM_033515	Homo sapiens Rho GTPase activating protein 18 (ARHGAP18), mRNA	2020/01/20	4414
8	NM_000517	Homo sapiens hemoglobin subunit alpha 2 (HBA2), mRNA	2020/01/21	576
9	NM_000558	Homo sapiens hemoglobin subunit alpha 1 (HBA1), mRNA	2020/01/20	577
10	NM_000518	Homo sapiens hemoglobin subunit beta (HBB), mRNA	2020/01/21	628
11	NM_001126102	Homo sapiens haptoglobin (HP), transcript variant 2, mRNA	2020/01/21	1301
12	NM_181552	Homo sapiens cut like homeobox 1 (CUX1), transcript variant 1, mRNA	2020/01/13	13723

Total number of hits: 1.128

Figure 10.1: *The GenBank search view.*

These actions save the search query. (It does not save the search results.)

This can be useful when you run the same searches periodically.

10.1.1 NCBI search options

Conducting a search using **Search for Sequences at NCBI** corresponds to using the same query terms on the NCBI website. For example, searching the nucleotide database corresponds to a search at https://www.ncbi.nlm.nih.gov/nucleotide/. A list of the entries matching the search terms is returned. Those of interest can then be selected and then downloaded, ready for downstream use in the Workbench.

Click on **Add search parameters** to add parameters to your search. The following are available:

- **All Fields** Searches for the terms provided in all fields of the NCBI database.
- **Organism**
- **Definition/Title**
- **Modified** Search for entries modified within the period specified from a drop-down list.
- **Gene Location** Choose from Genomic DNA/RNA, Mitochondrion, or Chloroplast.
- **Molecule** Choose from Genomic DNA/RNA, mRNA or rRNA.
- **Sequence Length** Enter a number for a maximum or minimum length of the sequence.
- **Gene Name**
- **Accession**

Check the "Append wildcard (*) to search words" checkbox to indicate that the term entered should be interpreted as the first part of the term only. E.g. searching for "genom" with that box checked would find entries starting with that term, such as "genomic" and "genome".

When you are satisfied with the parameters you have entered, click on the **Start search** button.

Additional search tips

Feature keys can be added when searching for nucleotide sequences. Writing
`gene[Feature key] AND mouse`
searches for one or more genes and where 'mouse' appears somewhere in the entry. For more information about how to use this syntax, see https://www.ncbi.nlm.nih.gov/books/NBK3837/

Where multiple terms are entered into a single field, the terms are usually treated as if they have an AND between them, which means that entries returned must contain all the specified terms. A notable exception is if multiple accessions are listed in an "All Fields" search field, in which case a results for each valid accession listed is returned.

You can add the words "AND", "OR" or "NOT" to the terms in a given field to further customize your search. Further details about searching Entrez sequence databases can be found at https://www.ncbi.nlm.nih.gov/books/NBK44864/.

10.1.2 Handling of NCBI search results

A list of entries matching the search terms provided is returned. By default, the initial 50 results are returned, with 50 additional results loaded each time the **More...** button is clicked. This number can be configured in the Workbench Preferences (section 4).

Five pieces of information are presented for each entry:

- **Hit** The rank of the entry in the search result
- **Accession** The accession for that entry. Click on the link to open that entry's page at the NCBI in a web browser.
- **Description** The definition line of the entry
- **Modification date** The date the entry was last updated in the database searched
- **Length** The length of the sequence

The columns to display can be configured in "Show column" tab of right hand, side panel settings.

Select one or more rows of the table and use buttons at the bottom of the view to:

- **Download and Open** Sequences are opened in a new view after download is complete.

  You can also download and open sequences by dragging selected rows to a new tab area or by double-clicking on a row.
- **Download and Save** Sequences are downloaded and saved to a location you specify.

  You can also download and save sequences by selecting rows and copying them (e.g. using Ctrl + C), and then selecting a folder in the **Navigation Area** and pasting (e.g. using Ctrl + V).
- **Open at NCBI** The sequence entry page(s) at the NCBI are opened in a web browser.

The functions offered by these buttons are also available in the menu that appears if you right-click over selected rows.

Note: The modification date on sequences downloaded can be more recent than those reported in the results table. This depends on the database versions made available for searching at the NCBI.

Downloading and saving sequences can take some time. This process runs in the background, so you can continue working on other tasks. The download process can be seen in the **Status bar** and it can be stopped, if desired, as described in section 2.4.

10.2 Search for PDB Structures at NCBI

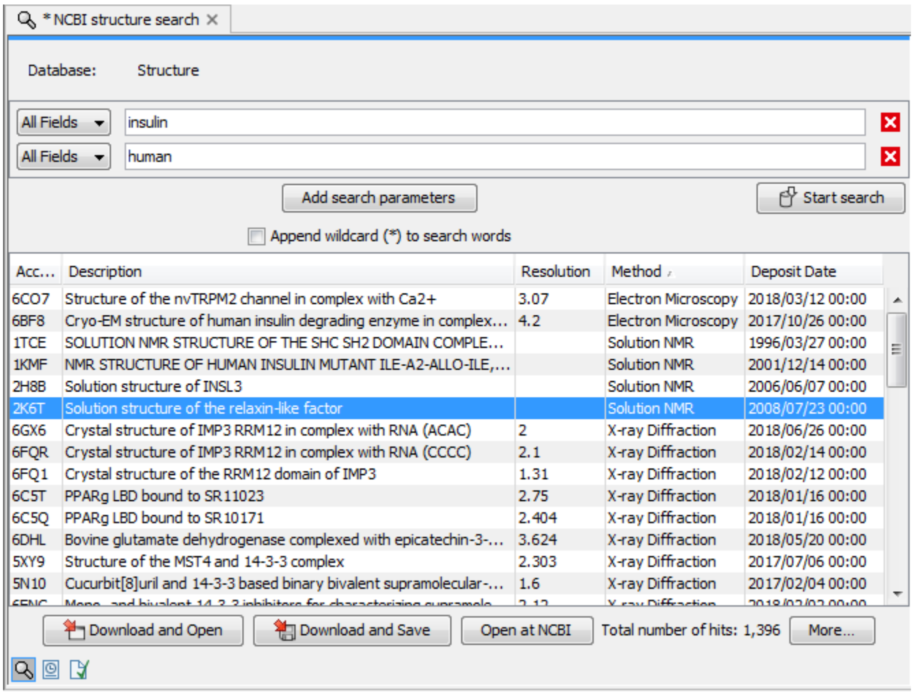
This section describes searches for three dimensional structures from the NCBI structure database `https://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml`. For manipulating and visualization of the downloaded structures see section 15.2.

The NCBI search view is opened in this way:

Download | **Search for PBD Structures at NCBI** ()

or **Ctrl + B (⌘ + B on Mac)**

This opens the view shown in figure 10.2:



Database: Structure

All Fields: insulin

All Fields: human

□ Append wildcard (*) to search words

Acc...	Description	Resolution	Method	Deposit Date
6CO7	Structure of the nvTRPM2 channel in complex with Ca2+	3.07	Electron Microscopy	2018/03/12 00:00
6BF8	Cryo-EM structure of human insulin degrading enzyme in complex...	4.2	Electron Microscopy	2017/10/26 00:00
1TCE	SOLUTION NMR STRUCTURE OF THE SHC SH2 DOMAIN COMPLE...		Solution NMR	1996/03/27 00:00
1KMF	NMR STRUCTURE OF HUMAN INSULIN MUTANT ILE-A2-ALLO-ILE,...		Solution NMR	2001/12/14 00:00
2H8B	Solution structure of INSL3		Solution NMR	2006/06/07 00:00
2K6T	Solution structure of the relaxin-like factor		Solution NMR	2008/07/23 00:00
6GX6	Crystal structure of IMP3 RRM12 in complex with RNA (ACAC)	2	X-ray Diffraction	2018/06/26 00:00
6FQR	Crystal structure of IMP3 RRM12 in complex with RNA (CCCC)	2.1	X-ray Diffraction	2018/02/14 00:00
6FQ1	Crystal structure of the RRM12 domain of IMP3	1.31	X-ray Diffraction	2018/02/12 00:00
6C5T	PPARg LBD bound to SR11023	2.75	X-ray Diffraction	2018/01/16 00:00
6C5Q	PPARg LBD bound to SR10171	2.404	X-ray Diffraction	2018/01/16 00:00
6DHL	Bovine glutamate dehydrogenase complexed with epicatechin-3-...	3.624	X-ray Diffraction	2018/05/20 00:00
5XY9	Structure of the MST4 and 14-3-3 complex	2.303	X-ray Diffraction	2017/07/06 00:00
5N10	Cucurbit[8]uril and 14-3-3 based binary bivalent supramolecular-...	1.6	X-ray Diffraction	2017/02/04 00:00

Total number of hits: 1,396

Figure 10.2: *The structure search view.*

10.2.1 Structure search options

Conducting a search in the **NCBI Database** from *CLC Main Workbench* corresponds to conducting search for structures on the NCBI's Entrez website. When conducting the search from *CLC Main Workbench*, the results are available and ready to work with straight away.

As default, *CLC Main Workbench* offers one text field where the search parameters can be entered. Click **Add search parameters** to add more parameters to your search.

Note! The search is a "AND" search, meaning that when adding search parameters to your search, you search for both (or all) text strings rather than "any" of the text strings.

You can append a wildcard character by clicking the checkbox at the bottom. This means that you only have to enter the first part of the search text, e.g. searching for "prot" will find both "protein" and "protease".

The following parameters can be added to the search:

- **All fields**. Text, searches in all parameters in the NCBI structure database at the same time.
- **Organism**. Text.
- **Author**. Text.
- **PdbAcc**. The accession number of the structure in the PDB database.

The search parameters are the most recently used. The **All fields** allows searches in all parameters in the database at the same time.

All fields also provide an opportunity to restrict a search to parameters which are not listed in the dialog. E.g. writing 'gene[Feature key] AND mouse' in **All fields** generates hits in the GenBank database which contains one or more genes and where 'mouse' appears somewhere in GenBank file. NB: the 'Feature Key' option is only available in GenBank when searching for nucleotide structures. For more information about how to use this syntax, see http://www.ncbi.nlm.nih.gov/entrez/query/static/help/Summary_Matrices.html#Search_Fields_and_Qualifiers

When you are satisfied with the parameters you have entered click **Start search**.

Note! When conducting a search, no files are downloaded. Instead, the program produces a list of links to the files in the NCBI database. This ensures a much faster search.

10.2.2 Handling of NCBI structure search results

The search result is presented as a list of links to the files in the NCBI database. The **View** displays 50 hits at a time (can be changed in the **Preferences** (see chapter 4). More hits can be displayed by clicking the **More...** button at the bottom right of the **View**.

Each structure hit is represented by text in three columns:

- Accession.
- Description.
- Resolution.
- Method.

- Protein chains
- Release date.

It is possible to exclude one or more of these columns by adjust the View preferences for the database search view. Furthermore, your changes in the View preferences can be saved. See section 4.6.

Several structures can be selected, and by clicking the buttons in the bottom of the search view, you can do the following:

- **Download and open.** Download and open immediately.
- **Download and save.** Download and save lets you choose location for saving structure.
- **Open at NCBI.** Open additional information on the selected structure at NCBI's web page.

Double-clicking a hit will download and open the structure. The hits can also be copied into the **View Area** or the **Navigation Area** from the search results by drag and drop, copy/paste or by using the right-click menu as described below.

Drag and drop from structure search results

The structures from the search results can be opened by dragging them into a position in the **View Area**.

Note! A structure is not saved until the **View** displaying the structure is closed. When that happens, a dialog opens: Save changes of structure x? (Yes or No).

The structure can also be saved by dragging it into the **Navigation Area**. It is possible to select more structures and drag all of them into the **Navigation Area** at the same time.

Download structure search results using right-click menu

You may also select one or more structures from the list and download using the right-click menu (see figure 10.3). Choosing **Download and Save** lets you select a folder or location where the structures are saved when they are downloaded. Choosing **Download and Open** opens a new view for each of the selected structures.

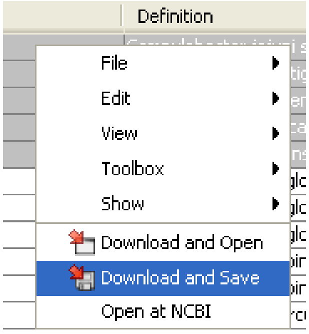


Figure 10.3: *By right-clicking a search result, it is possible to choose how to handle the relevant structure.*

The selected structures are not downloaded from the NCBI website but is downloaded from the RCSB Protein Data Bank `http://www.rcsb.org/pdb/home/home.do` in PDB format.

Copy/paste from structure search results

When using copy/paste to bring the search results into the **Navigation Area**, the actual files are downloaded.

To copy/paste files into the **Navigation Area**:

> **select one or more of the search results | Ctrl + C (⌘ + C on Mac) | select location or folder in the Navigation Area | Ctrl + V**

Note! Search results are downloaded before they are saved. Downloading and saving several files may take some time. However, since the process runs in the background (displayed in the **Status bar**) it is possible to continue other tasks in the program. Like the search process, the download process can be stopped. This is done in the in the **Processes** tab, available in the Toolbox area in the bottom left side of the Workbench.

10.2.3 Save structure search parameters

The search you just set up can be saved by doing one of the following:

- Choose **Save As...** from under the File menu, or
- Click on the tab of the search view and drag and drop it into a folder in the Navigation Area.

These actions save the search query. (It does not save the search results.)

This can be useful when you run the same searches periodically.

10.3 Search for Sequences in UniProt (Swiss-Prot/TrEMBL)

Search for Sequences in UniProt searches the UniProt Knowledgebase (UniProtKB). UniProtKB contains UniProtKB/Swiss-Prot entries, which are manually curated, and UniProtKB/TrEMBL entries, which are annotated by automated systems.

To open the tool, go to:

> **Download | Search for Sequences in UniProt ( )**

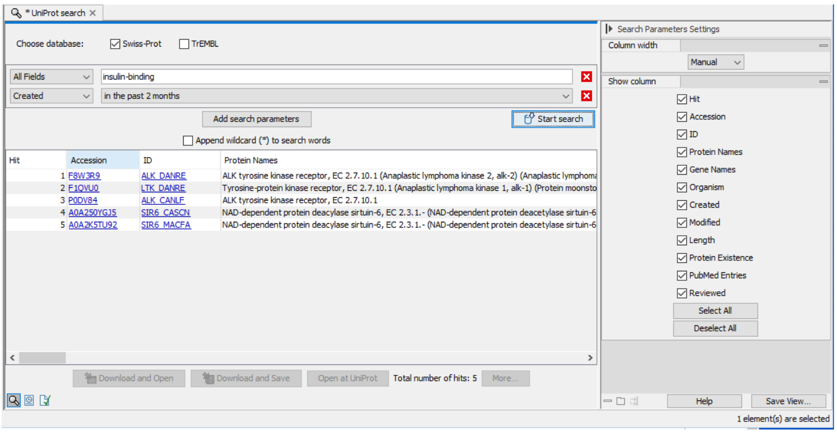
One or more search terms can be entered, and a table of entries matching the search terms is returned. Rows in that table can be selected and the corresponding UniProtKB entries downloaded and opened, or downloaded and saved (figure 10.4).

Searching is described in more detail in section 10.3.1.

Working with results returned by a search is described in section 10.3.2.

10.3.1 UniProt search options

Database choice



Choose database: ☑ Swiss-Prot □ TrEMBL

All Fields: insulin-binding

Created: in the past 2 months

□ Append wildcard (*) to search words

Hit	Accession	ID	Protein Names
1	F8W3R9	ALK_DANRE	ALK tyrosine kinase receptor, EC 2.7.10.1 (Anaplastic lymphoma kinase 2, alk-2) (Anaplastic lymphoma
2	F1QVU0	LTK_DANRE	Tyrosine-protein kinase receptor, EC 2.7.10.1 (Anaplastic lymphoma kinase 1, alk-1) (Protein moonsto
3	P0DV84	ALK_CANLF	ALK tyrosine kinase receptor, EC 2.7.10.1
4	A0A250YGJ5	SIR6_CASCN	NAD-dependent protein deacylase sirtuin-6, EC 2.3.1.- (NAD-dependent protein deacetylase sirtuin-6
5	A0A2K5TU92	SIR6_MACFA	NAD-dependent protein deacylase sirtuin-6, EC 2.3.1.- (NAD-dependent protein deacetylase sirtuin-6

Total number of hits: 5

Search Parameters Settings

Column width: Manual

Show column: ☑ Hit ☑ Accession ☑ ID ☑ Protein Names ☑ Gene Names ☑ Organism ☑ Created ☑ Modified ☑ Length ☑ Protein Existence ☑ PubMed Entries ☑ Reviewed

1 element(s) are selected

Figure 10.4: *Search in UniProtKB by entering search terms and clicking on the "Start search" button. A table containing information about entries matching the query terms is returned.*

Select one of the 2 subsections of UniProtKB to search in, or select both to search all of UniProtKB.

- **Swiss-Prot** Searches among manually curated entries. These are the entries marked as "reviewed" in UniprotKB.
- **TrEMBL** Searches among computationally analyzed entries that have been annotated using automated systems. These are the entries marked "unreviewed" in UniprotKB.

Search fields

A single search field is presented by default. Click on "Add search parameters" to add more.

The following options are available:

- **All fields** Search for the term provided in all fields available at the UniProtKB website https://www.uniprot.org/.
- **Accession** Search for entry accessions.
- **Protein Names** Search for terms in protein names.
- **Gene Names** Search for terms in gene names.
- **Organism** Search for terms in organism names.
- **Created** Search for entries created within the period specified from a drop-down list.
- **Modified** Search for entries modified within the period specified from a drop-down list.
- **Protein existence**. Search for entries with the evidence level specified from a drop-down list.

When the **Append wildcard (*) to search words** is checked, the search is broadened to include entries containing terms starting with text you provided.

Click on the **Start search** button to run the search.

Information about entries meeting all the conditions specified is returned in a table. No data is downloaded at this point. Working with these results, including downloading entries, is described in section 10.3.2.

Saving a configured UniProt search

The search you just set up can be saved by doing one of the following:

- Choose **Save As...** from under the File menu, or
- Click on the tab of the search view and drag and drop it into a folder in the Navigation Area.

These actions save the search query. (It does not save the search results.)

This can be useful when you run the same searches periodically.

10.3.2 Handling of UniProt search results

The results table

By default, the first 50 entries found in UniProtKB that met all the search conditions are returned in the results table. Clicking on the **More...** button at the bottom right loads further results.

The default value of 50 can be changed. See chapter 4 for details.

For each entry, the following information is provided:

- **Hit** The position of the entry in the results. E.g. 1 for the first entry in the list returned, 2 for the second, and so on.
- **Accession** The accession of the entry. Clicking on the link opens the entry's page at the UniprotKB website.
- **ID** The ID of the entry. Clicking on the link opens the entry's page at the UniprotKB website.
- **Protein Names** The protein names and synonyms.
- **Gene Names** The names of the gene(s) encoding the protein.
- **Organism** The scientific name and synonyms of the source organism.
- **Created** The date the entry was created.
- **Modified** The date of the latest annotation update.
- **Length** The length of the canonical sequence.
- **Protein Existence** The level of evidence supporting the existence of the protein.
- **Pubmed Entries** The list of Pubmed IDs mapped to the entry. Clicking on the link opens a page listing these Pubmed entries.
- **Reviewed** Either "reviewed" for entries in Swiss-Prot, or "unreviewed" for entries in TrEMBL.

The columns displayed can be customized using in the side panel settings. See section 4.6 for details.

If you wish to open webpages for several entries at once, highlight the rows of interest and click on the **Open at UniProt** button.

Downloading results from UniprotKB

Entries from UniprotKB can be downloaded and saved, or downloaded and opened, directly in the viewing area. When opened directly, the entries are not saved until you take explicit action to do so (see section 2.1.2).

Downloading many large files may take some time. The download process can be stopped from under the Processes tab (see section 2.4).

To download and save entries, select the rows of interest and then do one of the following:

- Click on the **Download and Save** button. You will be prompted for a location to save the entries to.
- Right-click over a selected area and choose the option **Download and Save** from the menu presented.
- Drag the row(s) to a folder in the Navigation Area.
- Copy (Ctrl-C) to copy the entry information. Click on a folder in the Navigation Area and then paste (Ctrl-V).

The selected entries are downloaded from UniprotKB. Multiple entries selected at the same time are saved to a single protein sequence list.

To download and open entries directly in the viewing area, select the rows of interest and then do one of the following:

- Click on the **Download and Open** button.
- Right-click over a selected area and choose the option **Download and Open** from the menu presented.
- Drag the row(s) until the mouse cursor is next to an existing tab in the view area. When the mouse button is released, a new tab is opened, and the selected entries are downloaded and opened in that tab.

Double-clicking on a single row will download and open that entry.

10.4 Sequence web info

CLC Main Workbench provides direct access to web-based search in various databases and on the Internet using your computer's default browser. You can look up a sequence in the databases of NCBI and UniProt, search for a sequence on the Internet using Google and search for Pubmed references at NCBI. This is useful for quickly obtaining updated and additional information about a sequence.

The functionality of these search functions depends on the information that the sequence contains. You can see this information by viewing the sequence as text (see section 14.5). In the following sections, we will explain this in further detail.

The procedure for searching is identical for all four search options (see also figure 10.5):

> **Open a sequence or a sequence list | Right-click the name of the sequence | Web Info (🌐) | select the desired search function**

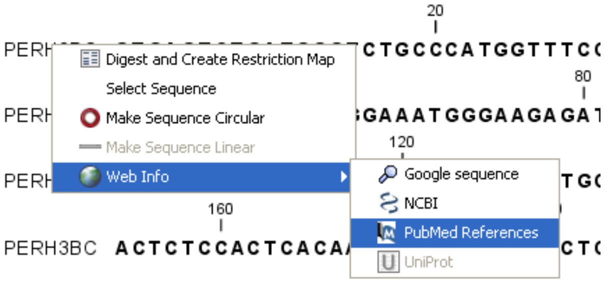


Figure 10.5: *Open webpages with information about this sequence.*

This will open your computer's default browser searching for the sequence that you selected.

Google sequence The Google search function uses the accession number of the sequence which is used as search term on https://www.google.com. The resulting web page is equivalent to typing the accession number of the sequence into the search field on https://www.google.com.

NCBI The NCBI search function searches in GenBank at NCBI (https://www.ncbi.nlm.nih.gov) using an identification number (when you view the sequence as text it is the "GI" number). Therefore, the sequence file must contain this number in order to look it up at NCBI. All sequences downloaded from NCBI have this number.

PubMed References The PubMed references search option lets you look up Pubmed articles based on references contained in the sequence file (when you view the sequence as text it contains a number of "PUBMED" lines). Not all sequence have these PubMed references, but in this case you will se a dialog and the browser will not open.

UniProt The UniProt search function searches in the UniProt database (https://www.uniprot.org/) using the accession number. Furthermore, it checks whether the sequence was indeed downloaded from UniProt.

Additional annotation information When sequences are downloaded from GenBank they often link to additional information on taxonomy, conserved domains etc. If such information is available for a sequence it is possible to access additional accurate online information. If the db_xref identifier line is found as part of the annotation information in the downloaded GenBank file, it is possible to easily look up additional information on the NCBI web-site.

To access this feature, simply right click an annotation and see which databases are available. For tracks, these links are also available in the track table.

Chapter 11

Running tools, handling results and batching

Contents

This section describes how to run tools, and how to handle and inspect results. We cover launching tools for individual runs, as well as launching them in batch mode, where the tool is run multiple times in a hands-off manner, using different input data for each run.

Launching workflows, individually or in batch mode, as well as running sections of workflows in batch mode, are covered in chapter 13.

11.1 Running tools

Launching tools and workflows involves the same series of general actions:

- Data elements to be used in the analysis are selected.
- Any settings necessary for the tool/workflow to run are configured.
- The job is launched.
- Results are opened or saved when the job completes.

There are several ways to launch a tool or installed or template workflow:

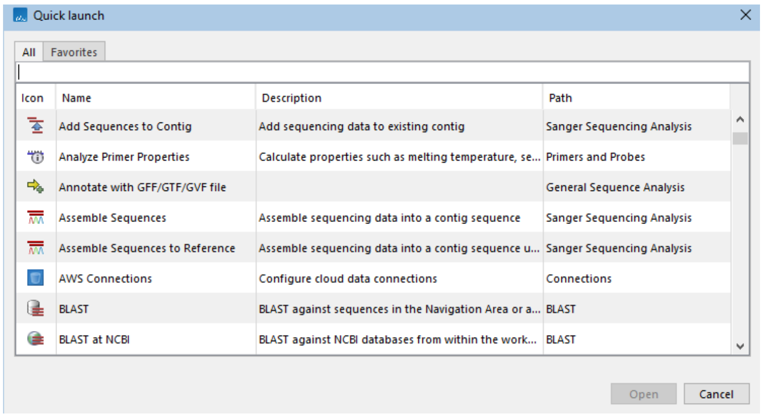
- Double click on its name in a tab the Toolbox panel in the bottom, left side of the Workbench.
- Select it from the Tools or Workflows menu at the top of the Workbench.

- Use the Quick Launch ( ) tool, described below.
- Select the element(s) to analyze and drag them from the Navigation Area onto the name of a tool or workflow in the Toolbox panel.

Using the Quick Launch tool to start jobs

The Quick Launch tool (figure 11.1) can be used to search for, and to launch tools, installed workflows or template workflows. It can be launched in several ways:

- Click on the **Launch** ( ) button in the Workbench toolbar.
- Use the keyboard shortcut Ctrl + Shift + T (⌘ + Shift + T on Mac).
- Click on the **Quick Launch** ( ) option at the top of the Tools menu or Workflows menu.
- Click on the ( ) icon beside the search field at the top of the Tools tab or Workflows tab in the Toolbox panel at the bottom, left of the Workbench.



Icon	Name	Description	Path
	Add Sequences to Contig	Add sequencing data to existing contig	Sanger Sequencing Analysis
	Analyze Primer Properties	Calculate properties such as melting temperature, se...	Primers and Probes
	Annotate with GFF/GTF/GVF file		General Sequence Analysis
	Assemble Sequences	Assemble sequencing data into a contig sequence	Sanger Sequencing Analysis
	Assemble Sequences to Reference	Assemble sequencing data into a contig sequence u...	Sanger Sequencing Analysis
	AWS Connections	Configure cloud data connections	Connections
	BLAST	BLAST against sequences in the Navigation Area or a...	BLAST
	BLAST at NCBI	BLAST against NCBI databases from within the work...	BLAST

Figure 11.1: *Tools, installed workflows and template workflows can be quickly found and launched using the Quick Launch tool.*

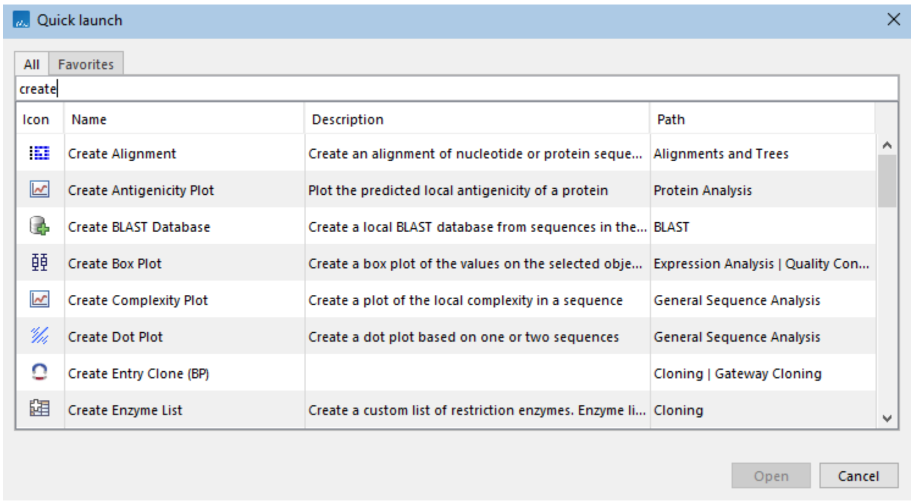
Double-click on a row to launch a tool or workflow from the Quick Launch dialog. Alternatively, select a row and click on the **Open** button.

When terms are entered into the text field at the top of the Quick Launch dialog, only tools and workflows with matches to those terms in their name, description or path will be listed (figure 11.2). Surround terms with single or double quotes to search for specific terms with spaces in them, for example `"sequence list"`.

The Path column contains the location of tools and workflows relative to the Tools or Workflows menu, respectively. Functionality available under other menus includes the relevant menu name in the path.

Click on the Favorites tab to see the subset of tools that are frequently used or have been selected as favorites (see section 2.3).

For tools where names have changed between Workbench versions, searches using terms in the older name will still find the relevant tool.



create

Icon	Name	Description	Path
	Create Alignment	Create an alignment of nucleotide or protein seque...	Alignments and Trees
	Create Antigenicity Plot	Plot the predicted local antigenicity of a protein	Protein Analysis
	Create BLAST Database	Create a local BLAST database from sequences in the...	BLAST
	Create Box Plot	Create a box plot of the values on the selected obje...	Expression Analysis | Quality Con...
	Create Complexity Plot	Create a plot of the local complexity in a sequence	General Sequence Analysis
	Create Dot Plot	Create a dot plot based on one or two sequences	General Sequence Analysis
	Create Entry Clone (BP)		Cloning | Gateway Cloning
	Create Enzyme List	Create a custom list of restriction enzymes. Enzyme li...	Cloning

Figure 11.2: *Typing a term in the search field limits the list of tools and workflows to those with that term in their name, description or path.*

Configuring and submitting jobs

When you launch a tool or an installed workflow, a wizard pops up. The general order of the steps presented via launch wizards are:

1. Specify the execution environment.
2. Select the input data.
3. Configure the available options for the tool.
4. Specify how the results should be handled.

You can move forward and back through the wizard steps by clicking the buttons **Next** and **Previous**, respectively, which are present at the bottom of the wizard. Clicking on the **Help** button in the bottom left corner of the launch wizard opens the documentation for the tool being launched.

The rest of this section covers the general launch wizard steps in more detail.

Specify the execution environment

If more than one execution environment is available, and a default selection has not already been set, the first wizard step will offer a list of the available environments.

For example, if you are logged into a *CLC Server*, or if you have the *CLC Cloud Module* installed and an AWS Connection has been configured with credentials giving access to a *CLC Genomics Cloud* setup, you are offered the option of running the job in different execution environments (figure 11.3).

Information on about launching jobs on a *CLC Server* is provided in section 11.1.1.

Select the input data for analysis tools

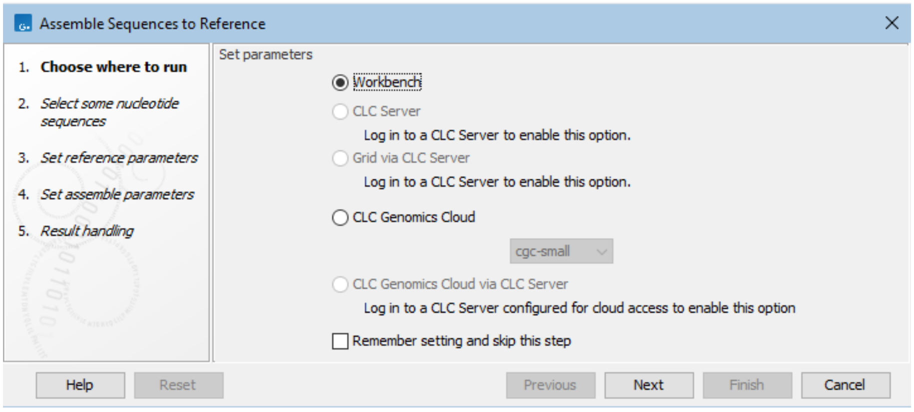

Assemble Sequences to Reference
1. Choose where to run
2. Select some nucleotide sequences
3. Set reference parameters
4. Set assemble parameters
5. Result handling
Set parameters
● Workbench
○ CLC Server
Log in to a CLC Server to enable this option.
○ Grid via CLC Server
Log in to a CLC Server to enable this option.
○ CLC Genomics Cloud
cgc-small
○ CLC Genomics Cloud via CLC Server
Log in to a CLC Server configured for cloud access to enable this option
□ Remember setting and skip this step
Help
Reset
Previous
Next
Finish
Cancel

Figure 11.3: *This Workbench has the CLC Cloud Module installed and has an active AWS Connection to a CLC Genomics Cloud setup. Thus, this job could be run on the Workbench, or run on AWS by selecting the option CLC Genomics Cloud.*

When selecting data to use as input to a tool, a view of the Navigation Area is presented, listing the elements that could be selected as input, as well as folders (figure 11.4). The data types that can be used as input for a given tool are described in the manual section about that tool.

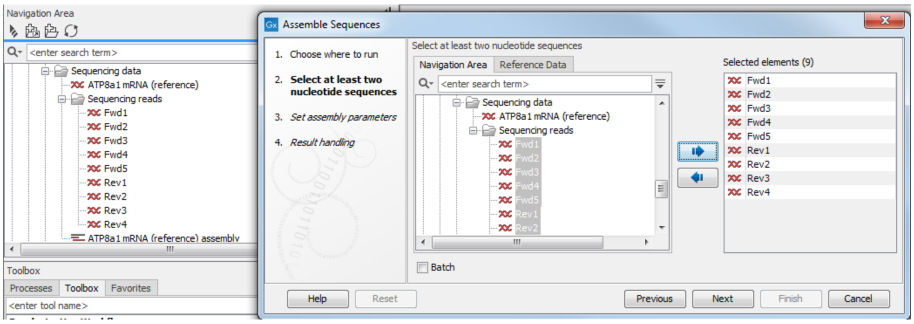


Figure 11.4: *You can select input files for the tool from the Navigation Area view presented on the left hand side of the wizard window.*

Selected elements will be listed in the right hand pane. To select the inputs, you can:

- Double click on them in the Navigation Area view in the launch wizard, or
- Select them with a single click in the Navigation Area view in the launch wizard and then click on the right hand arrow.
- Before opening the launch wizard, pre-select data elements in the main Navigation Area of the Workbench. When the tool is launched, these elements will automatically be placed in the "Selected elements" list.

To remove entries from the "Selected elements" list, double-click on them or select them with a single click and then click on the left hand arrow.

When multiple elements are selected, most analysis tools will analyze them together, as a single input, unless the "Batch" option at the bottom is checked. With the "Batch option checked, the tool is run multiple times, once for each "batch unit", which may be a data element, or a folder containing data elements or containing folders of elements. Batch processing is described in section 11.3.

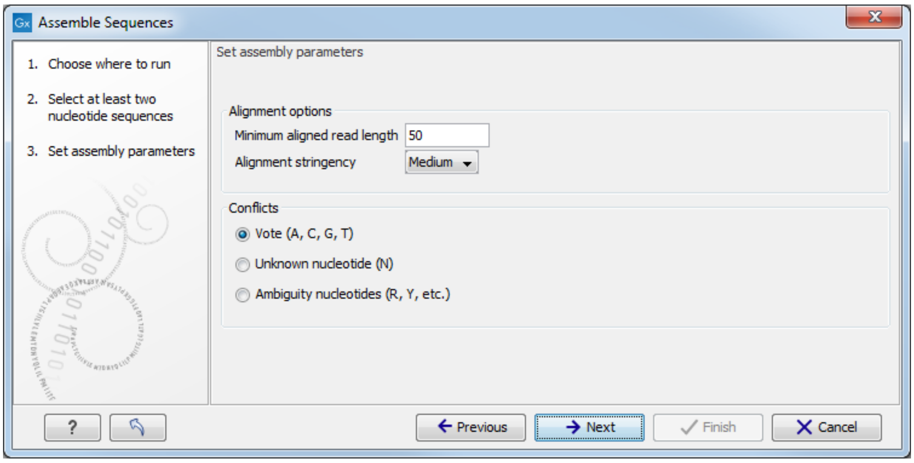
Select the input data for import tools

Selecting files for import is described in chapter 7. It is generally similar to selecting input for analysis tools, but involves selecting files from a file system or remote location. Many import selection wizards also support drag-and-drop for selecting files to import.

Configure the available options for the tool

Depending on the tool, there may be one or more wizard steps containing options affecting how the tool behaves (figure 11.5).

Clicking on the **Reset** button resets the values for the options in that wizard step to their default values.



Assemble Sequences

1. Choose where to run
2. Select at least two nucleotide sequences
3. Set assembly parameters

Set assembly parameters

Alignment options

Minimum aligned read length: 50

Alignment stringency: Medium

Conflicts

● Vote (A, C, G, T)

○ Unknown nucleotide (N)

○ Ambiguity nucleotides (R, Y, etc.)

Figure 11.5: *An example of a "Set parameters" wizard step.*

Specify how the results should be handled

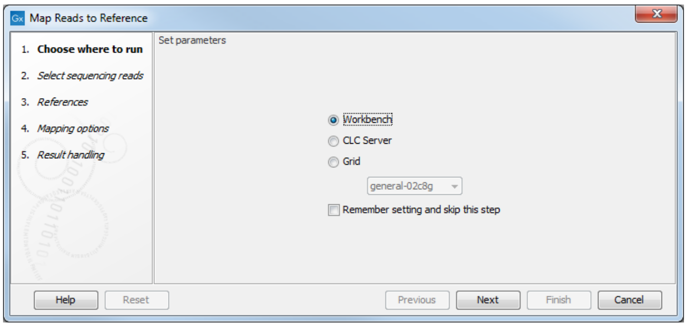
Handling results is described in section 11.2.

11.1.1 Running a tool on a CLC Server

When you launch an analysis from a Workbench that is logged into a *CLC Server*, you are offered the choice of where the analysis should be run (figure 11.6).

- **Workbench**. Run the analysis on the computer the CLC Workbench is running on.
- **Server**. Run the analysis using the *CLC Server*. For job node setups, analyses will be run on the job nodes.
- **Grid**. Only offered if the *CLC Server* setup has grid nodes. Here, jobs are sent from the master *CLC Server* to be run on grid nodes. The grid queue to submit to can be selected from the drop down list under the Grid option.

You can check the **Remember setting and skip this step** option if you wish to always use the selected option when submitting analyses. If you select this option but later change your mind, just start up an analysis and click on the **Previous** button to open these options again.



Map Reads to Reference

1. Choose where to run
2. Select sequencing reads
3. References
4. Mapping options
5. Result handling

Set parameters

● Workbench
○ CLC Server
○ Grid

general-02c8g

□ Remember setting and skip this step

Figure 11.6: *When logged into the CLC Server, you can select where a job should be run.*

Most wizard steps for launching a job on a *CLC Workbench* or on a *CLC Server* are the same. There are two minor differences when launching jobs to run on a *CLC Server*: results are always saved, and a log of the job is always created and saved alongside the results.

Data access: When you run a job on a *CLC Server*, you will generally only be able to select data from and save results to areas known to the *CLC Server*. With default server settings, you will not be able to upload data from your local system. Your server administrator can enabled this if they wish. See https://resources.qiagenbioinformatics.com/manuals/clcserver/current/admin/index.php?manual=Direct_data_transfer_from_client_systems.html.

Disconnecting from the CLC Server: Once the job has been submitted, you can disconnect from the *CLC Server* if you wish, or close the *CLC Workbench* entirely. *Exception:* If you are importing data from the local file system, you must wait until the data has been imported before disconnecting. A notification about server jobs that finished is presented the next time you log in to the *CLC Server*. See section 2.4.

11.2 Handling results

Some tools can generate several outputs. If there is a choice of which ones to generate, you will be able to configure this in the final wizard step, called "Result handling". The kind of output files generated by a tool are described in the tool specific sections of the manual.

For tasks run on a Workbench (as opposed to a *CLC Server*) the "Result handling" window also allows you to decide whether you want to **Open** or **Save** your results.

- **Open.** This will open the result of the analysis in a view. This is the default setting.
- **Save** The results will be saved rather than opened. You will be prompted for where you wish the results to be saved (figure 11.7). You can save to an existing area or create a new folder to save the results into.

You may also have an option called "Open log". If checked, a window will open in the View area after the analysis has started and the progress of the job will be reported there line by line.

Click **Finish** to start the analysis.

If you chose the option to open the results, they will open automatically in one or several tabs in the View Area. The data will not have been saved at this point. The name of each tab is in bold,

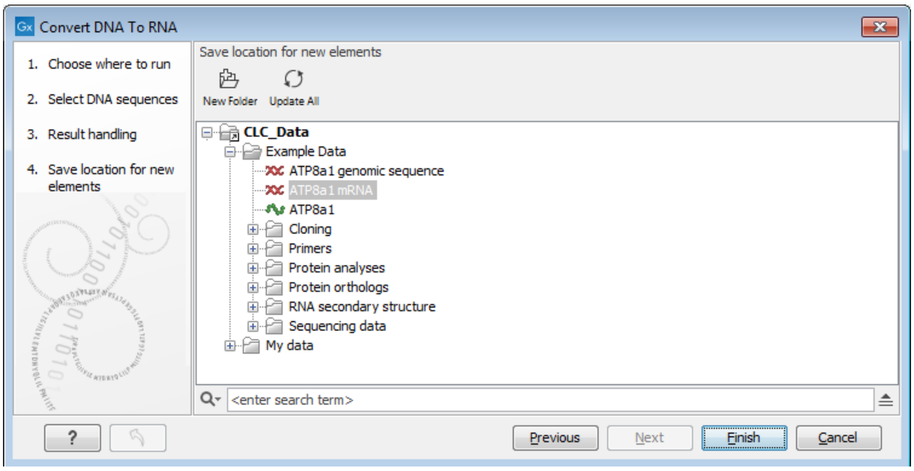


Figure 11.7: *Specify where to save the results of an analysis.*

appended with an asterisk to indicate this. There are several ways to save the results you wish to keep:

- Drag the tab to the relevant location in the Navigation Area.
- Select the tab and then use the key combination Ctrl + S (or ⌘ + S on macOS).
- Right click on the tab and choose "Save" from the context menu.
- Use the "Save" button in the Workbench toolbar.
- Go to the File menu and select the option "Save" or "Save As...".

If you chose to save the results, they will have been saved in the location specified. You can open the results in the Navigation Area directly after the analysis is finished. A quick way to find the results is to click on the little arrow to the right of the analysis name in the Processes tab and choose the option "Show results" or "Find Results", as shown in figure 11.8.

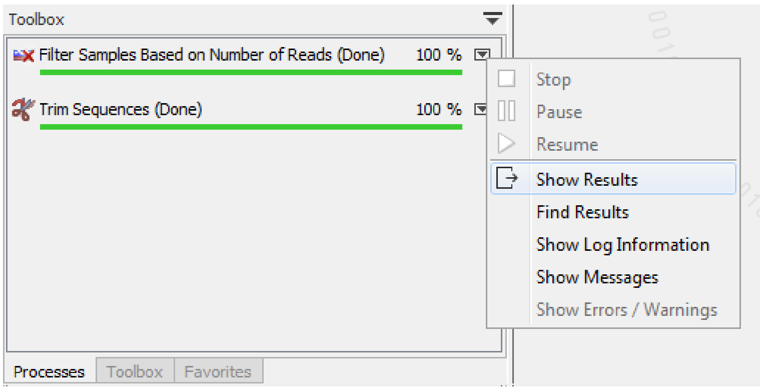


Figure 11.8: *Find or open the analysis results by clicking on the little arrow to the right of the analysis name in the Processes tab and choosing the relevant item from the menu.*

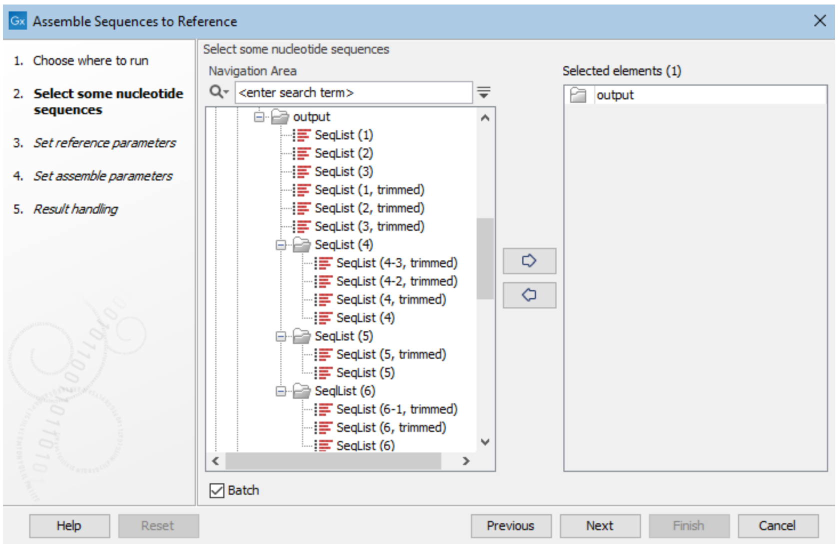
11.3 Batch processing

Batch processing refers to running an analysis multiple times, once per batch unit. For example, if you have 10 sequence lists and wish to run 10 mapping analyses, one per sequence list, you could launch all 10 analyses by setting up one batch job. Here, each sequence list would be a "batch unit".

This section focuses on batch processing when using **individual tools**. Further details about batch processing of workflows is provided in section 13.3 and section 13.3.3.

Batch mode

Batch mode is activated by clicking the **Batch** checkbox in the dialog where the input data is selected (figure 11.9).



Assemble Sequences to Reference

1. Choose where to run
2. **Select some nucleotide sequences**
3. *Set reference parameters*
4. *Set assemble parameters*
5. *Result handling*

Select some nucleotide sequences

Navigation Area

Selected elements (1)

output

☑ Batch

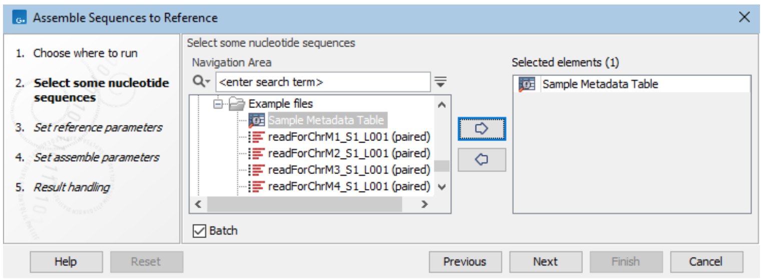
Figure 11.9: *When launching an analysis in Batch mode, individual elements and/or folders can be selected. Here, a single folder that contains both elements and subfolders of elements has been selected.*

In Batch mode, the analysis is run once per batch unit. A batch unit consists of the data elements to be analyzed together. A batch unit can be a single data element, or can consist of multiple data elements.

Batch units are made up of:

- Each data element selected in the launch wizard.
- Elements and folders within a folder selected in the launch wizard, where:
  - Each data element contained directly within that selected folder is a batch unit.
  - Each subfolder directly under the selected folder is a batch unit. I.e. all elements within that subfolder are analyzed together.
  - Elements within more deeply nested subfolders (e.g. subfolders of subfolders of the originally selected folder) are not used in the analysis.
- Elements with associations to a CLC Metadata Table selected in the launch wizard. Each row in the CLC Metadata Table is a batch unit. Data elements associated with a row, of

a type compatible as input to the analysis, are the default contents of a batch unit. See figure 11.10 and figure 11.11.



Assemble Sequences to Reference

1. Choose where to run
2. **Select some nucleotide sequences**
3. *Set reference parameters*
4. *Set assemble parameters*
5. *Result handling*

Select some nucleotide sequences

Navigation Area

<enter search term>

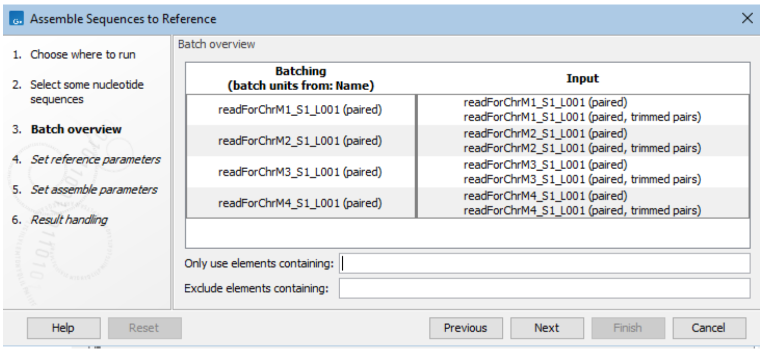
- Example files
  - Sample Metadata Table
  - readForChrM1_S1_L001 (paired)
  - readForChrM2_S1_L001 (paired)
  - readForChrM3_S1_L001 (paired)
  - readForChrM4_S1_L001 (paired)

Selected elements (1)

Sample Metadata Table

☑ Batch

Figure 11.10: *When the Batch box is checked, a CLC Metadata Table can be selected as input.*



Assemble Sequences to Reference

1. Choose where to run
2. Select some nucleotide sequences
3. **Batch overview**
4. *Set reference parameters*
5. *Set assemble parameters*
6. *Result handling*

Batch overview

Batching (batch units from: Name)	Input
readForChrM1_S1_L001 (paired)	readForChrM1_S1_L001 (paired) readForChrM1_S1_L001 (paired, trimmed pairs)
readForChrM2_S1_L001 (paired)	readForChrM2_S1_L001 (paired) readForChrM2_S1_L001 (paired, trimmed pairs)
readForChrM3_S1_L001 (paired)	readForChrM3_S1_L001 (paired) readForChrM3_S1_L001 (paired, trimmed pairs)
readForChrM4_S1_L001 (paired)	readForChrM4_S1_L001 (paired) readForChrM4_S1_L001 (paired, trimmed pairs)

Only use elements containing:

Exclude elements containing:

Figure 11.11: *Data associated with each row in a CLC Metadata Table, of a type compatible with that analysis, make up the default content of batch units.*

Batch overview

In the batch overview step, the elements in each batch unit can be reviewed, and refined based on their names using the fields **Only use elements containing** and **Exclude elements containing**.

In figure 11.12, the batch units, i.e. those elements and folders directly under the folder selected in figure 11.9, are shown. In each batch unit, data elements that could be used in the analysis are listed on the right hand side. Some batch units contain more than one data element. Those data elements would be analyzed together. To limit the analysis to just sequence lists containing trimmed sequences, the term "trim" has been entered into a filter field near the bottom.

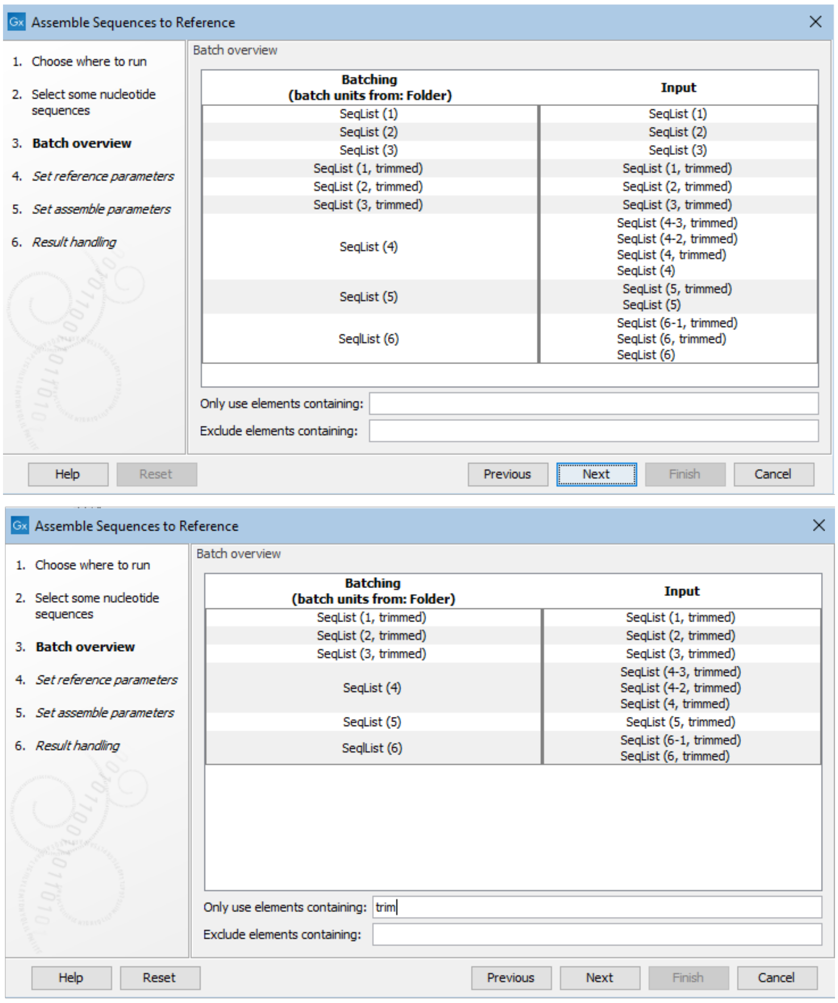
Folders that do not contain any elements compatible with the analysis are not shown in the batch overview.

Organization of the results

The options for where to save analysis outputs are shown in figure 11.13.

The available options are:

- **Save in input folder** Save all outputs into the same folder as the input data. For batch units defined by folders, the results of each analysis are saved into the folder with the input



1. Choose where to run
2. Select some nucleotide sequences
3. **Batch overview**
4. *Set reference parameters*
5. *Set assemble parameters*
6. *Result handling*

Batch overview

Batching (batch units from: Folder)	**Input**
SeqList (1)	SeqList (1)
SeqList (2)	SeqList (2)
SeqList (3)	SeqList (3)
SeqList (1, trimmed)	SeqList (1, trimmed)
SeqList (2, trimmed)	SeqList (2, trimmed)
SeqList (3, trimmed)	SeqList (3, trimmed)
SeqList (4)	SeqList (4-3, trimmed) SeqList (4-2, trimmed) SeqList (4, trimmed) SeqList (4)
SeqList (5)	SeqList (5, trimmed) SeqList (5)
SeqList (6)	SeqList (6-1, trimmed) SeqList (6, trimmed) SeqList (6)

Only use elements containing:

Exclude elements containing:

1. Choose where to run
2. Select some nucleotide sequences
3. **Batch overview**
4. *Set reference parameters*
5. *Set assemble parameters*
6. *Result handling*

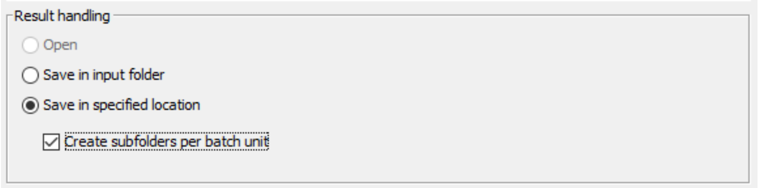
Batch overview

Batching (batch units from: Folder)	**Input**
SeqList (1, trimmed)	SeqList (1, trimmed)
SeqList (2, trimmed)	SeqList (2, trimmed)
SeqList (3, trimmed)	SeqList (3, trimmed)
SeqList (4)	SeqList (4-3, trimmed) SeqList (4-2, trimmed) SeqList (4, trimmed)
SeqList (5)	SeqList (5, trimmed)
SeqList (6)	SeqList (6-1, trimmed) SeqList (6, trimmed)

Only use elements containing: trim

Exclude elements containing:

Figure 11.12: *Overview of the batch units (left) and the input elements defined by each batch unit (right). By default, all elements that can be used as inputs are listed on the right (top). By entering terms in the filter fields, the list of elements in the batch units can be refined. Here, only sequence lists including trimmed sequences will be included (bottom) .*



Result handling

○ Open
○ Save in input folder
● Save in specified location
☑ Create subfolders per batch unit

Figure 11.13: *Options for saving results when an analysis is runin Batch mode.*

data. If the batch units were individual data elements, results are put into the same folder as the input elements.

- **Save in specified location** You will be prompted in the next step to select a folder where the outputs should be saved to. The **Create subfolders per batch unit** checkbox allows you to specify whether subfolders should be created to store the results from each batch unit:
  - When **checked** results for each batch unit are written to a newly created subfolder under the folder you select in the next step. A subfolder is created for each batch unit. (This is the default option.)

  - When **unchecked**, results from all batch units are written to the folder you select in the next step.

The log file

In the final wizard step there is an option to **Create a log**. When checked, a log containing information about all the batch units will be created. This log includes the term "combined log" in its name. A log is also created for each individual batch unit.

Batch unit processes

When the job is running, there is one "master" process representing the overall batch job, and a separate process for each batch unit.

On a *CLC Workbench*, the batch units are executed sequentially - one batch unit at a time. This avoids overloading the computer.

On a *CLC Server*, all the processes are placed in the queue, and the queue takes care of distributing the jobs. If there are multiple job nodes or grid nodes, batch units may be processed in parallel.

Stopping a batch run

To stop the whole batch run, stop the "master" process.

On a *CLC Workbench*, find the master process in the Processes tab in the bottom left side. Click on the little triangle on the right hand side of the master process and choose the option **Stop**.

Chapter 12

Metadata

Contents

Metadata refers to information about data. In the context of the *CLC Main Workbench*, this usually means information about samples. For example a set of reads could come from a particular specimen at a particular time point with particular characteristics. The specimen, time and characteristics would be metadata for that set of reads.

Examples in this chapter refer to tools present in the CLC Genomics Workbench, but the principles apply to other CLC Workbenches.

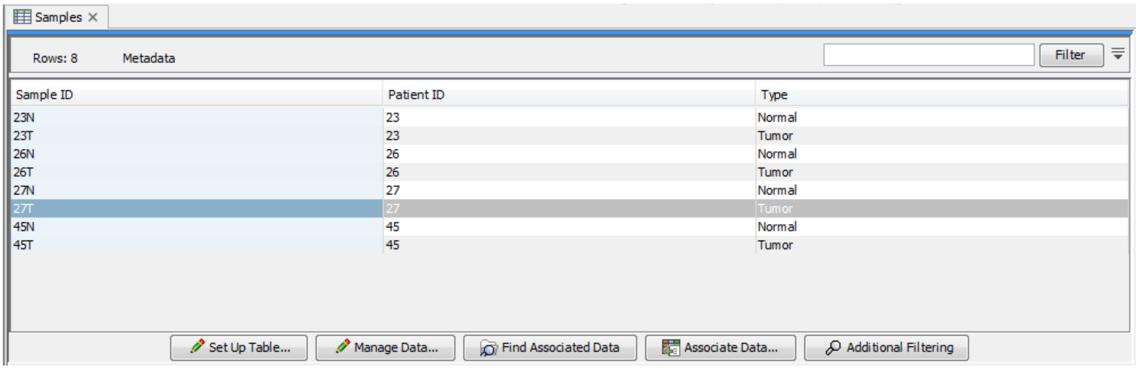
What is metadata used for? Core uses of metadata in CLC software include:

- Defining batch units when launching workflows in batch mode, described in section 13.3.2.
- Distributing data to the relevant input channels in a workflow when using Collect and Distribute elements, described in section 13.2.5.
- Finding and selecting data elements based on sample information (in a CLC Metadata Table). Workflow Result Metadata tables are of particular use when reviewing results generated by workflows run in batch mode and are described in section 13.3.1.

- Running tools where characteristics of the data elements are relevant. An example is Differential Expression for RNA-Seq in the CLC Genomics Workbench, described at https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Differential_Expression_RNA_Seq.html.

Metadata tables

An example of a CLC Metadata Table in the *CLC Main Workbench* is shown in figure 12.1. Each column represents a property of a sample (e.g., identifier, height, age, treatment) and each row contains information relevant to a sample. A single column can be designated the key column. That column must contain unique entries.



Sample ID	Patient ID	Type
23N	23	Normal
23T	23	Tumor
26N	26	Normal
26T	26	Tumor
27N	27	Normal
27T	27	Tumor
45N	45	Normal
45T	45	Tumor

Figure 12.1: *A simple metadata table, with the key column highlighted in blue.*

Each row can have associations with one or more data elements, such as sequence lists, expression tracks, variant tracks, etc. Associating data elements with relevant metadata rows, automatically or manually, is covered in section 12.2

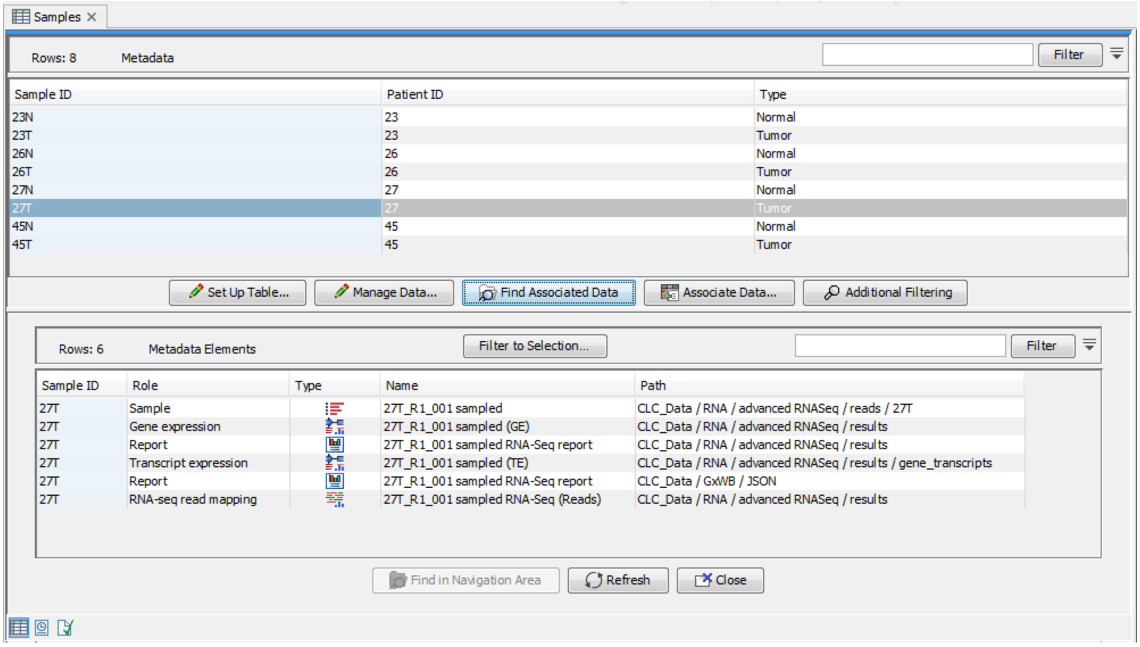
Information from an Excel, CSV or TSV format file can be imported into a CLC Metadata Table, as described in section 12.1.1. CLC Metadata Tables are also generated by workflows, as described in section 13.3.1.

Metadata Elements table

Data elements with an association to a row in a CLC Metadata Table can be listed by selecting the rows of interest and clicking on the **Find Associated Data** button. The Metadata Elements table opens, with a table of information about elements with associations to the selected rows (figure 12.2).

When a data element is associated with a metadata row, the outputs of analyses involving that data usually inherit the metadata association automatically. For example, if a sequence list with an association to a CLC Metadata Table row is used as input to analyses, results of these analyses may also be associated with that row (figure 12.2).

Inheritance of associations to metadata requires that a single association can be unambiguously identified for an output when a tool is run. If an output is derived from two ore more inputs with different metadata associations, then no association will be inherited.



Rows: 8 Metadata

Sample ID	Patient ID	Type
23N	23	Normal
23T	23	Tumor
26N	26	Normal
26T	26	Tumor
27N	27	Normal
27T	27	Tumor
45N	45	Normal
45T	45	Tumor

Rows: 6 Metadata Elements

Sample ID	Role	Type	Name	Path
27T	Sample		27T_R1_001 sampled	CLC_Data / RNA / advanced RNASeq / reads / 27T
27T	Gene expression		27T_R1_001 sampled (GE)	CLC_Data / RNA / advanced RNASeq / results
27T	Report		27T_R1_001 sampled RNA-Seq report	CLC_Data / RNA / advanced RNASeq / results
27T	Transcript expression		27T_R1_001 sampled (TE)	CLC_Data / RNA / advanced RNASeq / results / gene_transcripts
27T	Report		27T_R1_001 sampled RNA-Seq report	CLC_Data / GxWB / JSON
27T	RNA-seq read mapping		27T_R1_001 sampled RNA-Seq (Reads)	CLC_Data / RNA / advanced RNASeq / results

Figure 12.2: *A CLC Metadata Table and corresponding Metadata Elements table showing elements associated with sample 27T.*

12.1 Creating metadata tables

CLC Metadata tables are created in several ways:

- Import metadata from an Excel, CSV or TSV format file.

  To do this, use the **Import Metadata** ( ) tool, described in section 12.1.1.

- Create a new CLC Metadata Table containing a subset of the rows in another CLC Metadata Table.

  To do this, open an existing CLC Metadata Table, select the rows of interest and click on the **Create New Metadata Table...** ( ) button at the bottom of the editor. This option is also available in the menu that opens when you right-click on the selection (figure 12.3).

  Data elements with associations to the selected rows aquire an association with the new CLC Metadata Table also.

- Create a new CLC Metadata Table from scratch.

  This is described in section 12.1.2.

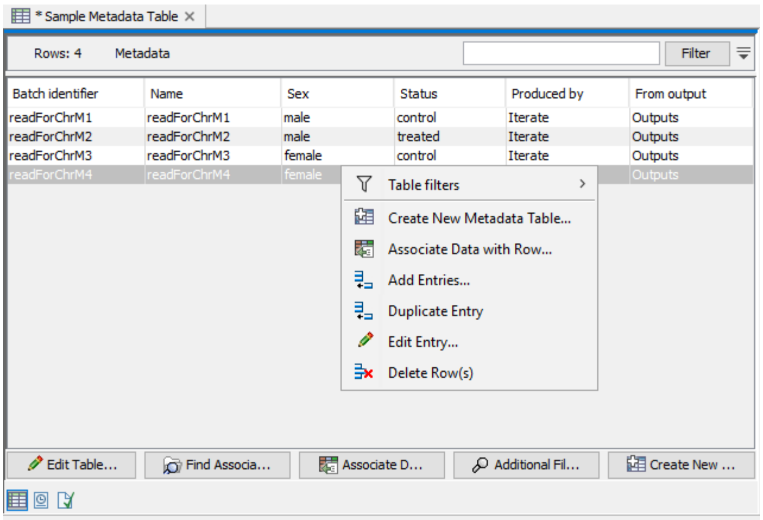
Workflow Result Metadata tables, created when a workflow is run, are also CLC Metadata Tables. These are described in section 13.3.1.

12.1.1 Importing metadata

The Import Metadata tool

To import metadata into a CLC Metadata Table, go to:

File | Import ( ) | **Import Metadata** ( )



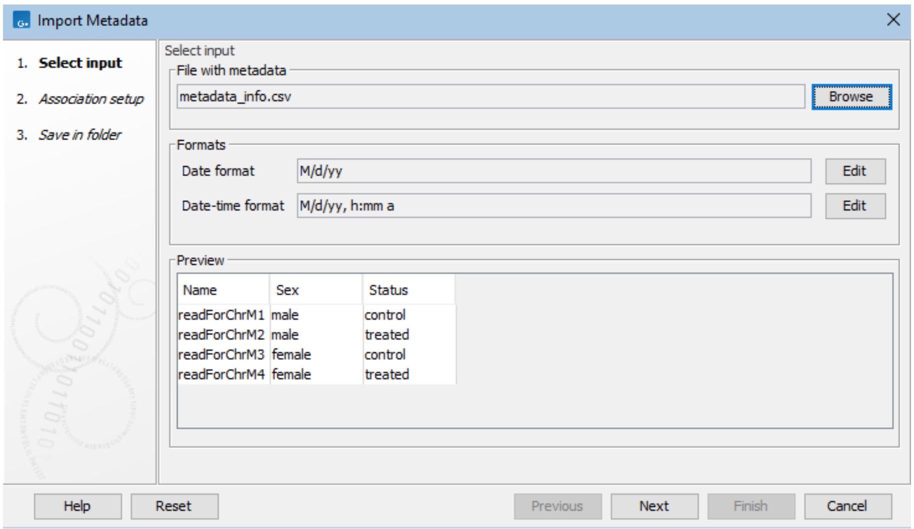
Batch identifier	Name	Sex	Status	Produced by	From output
readForChrM1	readForChrM1	male	control	Iterate	Outputs
readForChrM2	readForChrM2	male	treated	Iterate	Outputs
readForChrM3	readForChrM3	female	control	Iterate	Outputs
readForChrM4	readForChrM4	female			Outputs

Figure 12.3: *Selected rows in a CLC Metadata table can be put into a new CLC Metadata Table using the option "Create New Metadata Table..."*

The first column in the selected file must have unique entries. That column will be designated as the key column. A different column can be specified as the key column later. For the optional step for association of data elements to work, the first column must contain entries that can be matched with the relevant data element names.

Select the Excel, CSV or TSV format file with metadata to be imported. The rows in that file are displayed in the Metadata preview window (figure 12.4).

The format of the columns should reflect the column contents. The designated format can be changed from within the Metadata Table editor, as described in section 12.3.5. There, you can change the column data types (e.g. to types of numbers, dates, true/false) and you can designate a new key column.



1. **Select input**
2. *Association setup*
3. *Save in folder*

Select input

File with metadata: metadata_info.csv

Formats

Date format: M/d/yy

Date-time format: M/d/yy, h:mm a

Preview

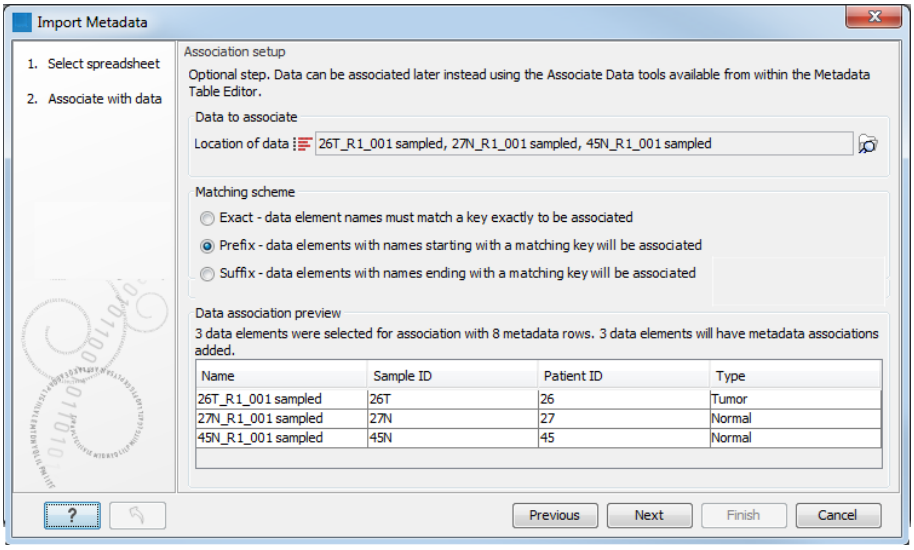
Name	Sex	Status
readForChrM1	male	control
readForChrM2	male	treated
readForChrM3	female	control
readForChrM4	female	treated

Figure 12.4: *Rows being imported from a file containing metadata are shown in the Metadata preview table.*

Associating metadata with data (optional) The "Associate with data" wizard step (figure 12.5), is optional. To proceed without associating data to metadata, click on the **Next** button. Associating data with metadata can be done later, as described in section 12.2.

To associate data with the metadata:

- Click on the file browser button to the right of the **Location of data** field
- Select the data elements to be associated.
- Select the matching scheme to use: Exact, Prefix or Suffix. These options are described in section 12.2.1.



Association setup

Optional step. Data can be associated later instead using the Associate Data tools available from within the Metadata Table Editor.

Data to associate

Location of data: 26T_R1_001 sampled, 27N_R1_001 sampled, 45N_R1_001 sampled

Matching scheme

○ Exact - data element names must match a key exactly to be associated

● Prefix - data elements with names starting with a matching key will be associated

○ Suffix - data elements with names ending with a matching key will be associated

Data association preview

3 data elements were selected for association with 8 metadata rows. 3 data elements will have metadata associations added.

Name	Sample ID	Patient ID	Type
26T_R1_001 sampled	26T	26	Tumor
27N_R1_001 sampled	27N	27	Normal
45N_R1_001 sampled	45N	45	Normal

Figure 12.5: *Three data elements are selected for association. The "Prefix" partial matching scheme is selected for matching data element names with the appropriate metadata row, based on the information in the Sample ID column in this case.*

The Data association preview area shows data elements that will have associations created, along with information from the metadata row they are being linked with. This gives the opportunity to check that the matching is leading to the expected links between data and metadata.

You can then select where you wish the metadata table to be saved and click on **Finish**.

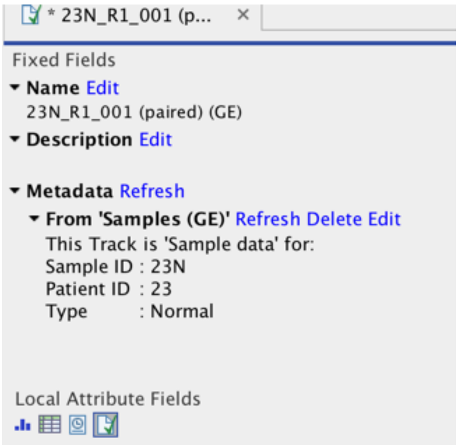
The associated information can be viewed for a given data element in the Show Element Info view (figure 12.6).

12.1.2 Creating a metadata table directly in the Workbench

Creating CLC Metadata tables from scratch within the *CLC Main Workbench* is described in this section.

See also section 12.1.1 for importing information from Excel to create a CLC Metadata Table. If your analysis is contained in a workflow, a CLC Metadata Table can be created automatically. This is described in section 13.3.1.

To create a CLC Metadata Table manually, go to:



* 23N_R1_001 (p...

Fixed Fields

Name Edit
23N_R1_001 (paired) (GE)

Description Edit

Metadata Refresh

From 'Samples (GE)' Refresh Delete Edit
This Track is 'Sample data' for:
Sample ID : 23N
Patient ID : 23
Type : Normal

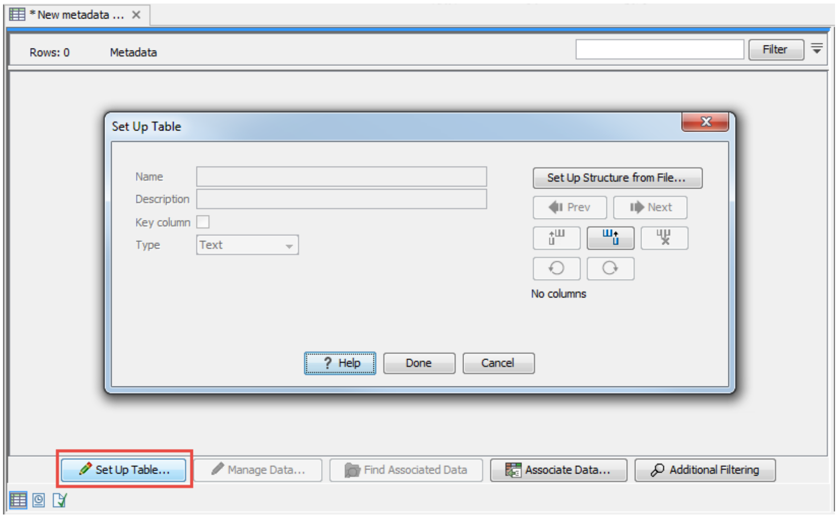
Local Attribute Fields

Figure 12.6: *Metadata associations can be seen, edited, refreshed or deleted via the Show Element Info view.*

File | **New** | **Metadata Table** (▦)

This opens a new metadata table with no columns and no rows. Importing metadata using the Metadata Table Editor requires that the **table structure** is defined first.

Defining the table structure Click **Setup Table** at the bottom of the view (figure 12.7).



* New metadata ...

Rows: 0 Metadata

Set Up Table

Name
Description
Key column □
Type Text

No columns

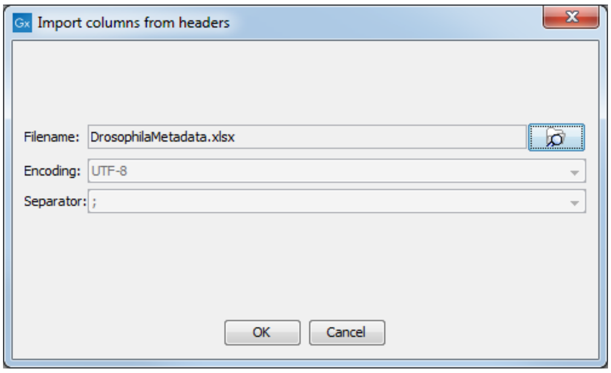
Figure 12.7: *Dialog used to add columns to an empty Metadata Table.*

To create a metadata table from scratch, use the "Add column right" or "Add column left" buttons (▥) to define the table structure with the amount of columns you will need, and edit the fields of each column as needed.

To import the table from a file, click on **Setup Structure from File**. In the dialog that appears (figure 12.8), you need to provide the following information:

- **Filename** The EXCEL or delimited TEXT file to import. Column names should be in the first row of this file.
- **Encoding** For text files only: the encoding used to create the file. The default is UTF-8.

- **Separator** For text files only: The character used to separate the columns. The default is semicolon (;).



Import columns from headers

Filename: DrosophilaMetadata.xlsx

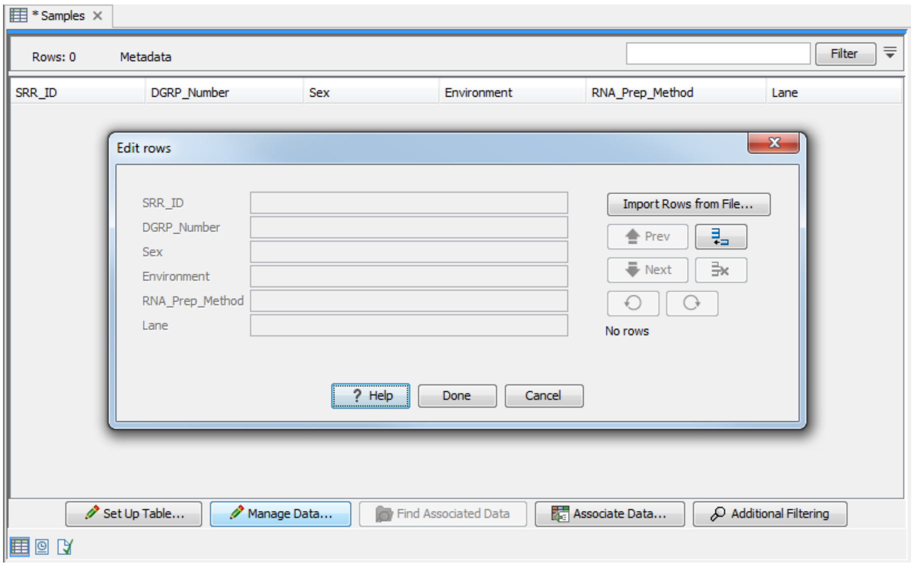
Encoding: UTF-8

Separator: ;

Figure 12.8: *Creating a metadata table structure based on an external file.*

For each column in the external file, a column will be created in the new metadata table. By default the type of these imported columns is "Text". You will see a reminder to set the column type for each column and to designate one of the columns as the key column.

Populating the table Click on **Manage Data** button at the bottom of the view (figure 12.9).



Rows: 0

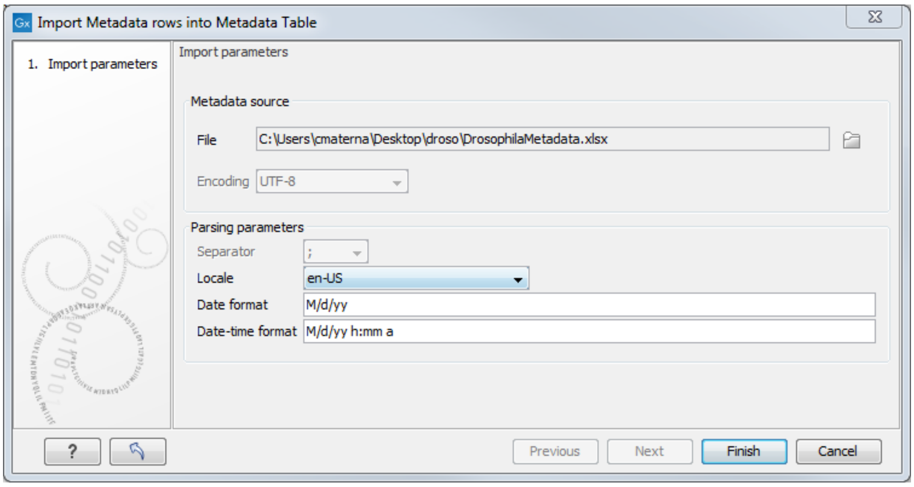
No rows

Figure 12.9: *Tool for managing the metadata itself. Notice the button labeled Import Rows from File.*

The metadata table can then be populated by editing each column manually. Row information is added manually by clicking on the (⊞) button and typing in the information for each column.

It is also possible to import information from an external file. In that case, the column names in the metadata table in the workbench will be matched with those in the external file to determine which values go into which cell. Only cell values in columns with an exact name match will be imported. If the file used contains columns not in the metadata table, the values in those columns will be ignored. Conversely, if the metadata table contains columns not present in the file, imported rows will have no values for those columns.

Click on **Import Rows from File** and select the external file of metadata. This brings up the window shown in figure 12.10.



Import Metadata rows into Metadata Table

1. Import parameters

Import parameters

Metadata source

File C:\Users\cmaterna\Desktop\droso\DrosophilaMetadata.xlsx

Encoding UTF-8

Parsing parameters

Separator ;

Locale en-US

Date format M/d/yy

Date-time format M/d/yy h:mm a

Figure 12.10: *Tool to import rows into a Metadata Table.*

When working with an existing metadata table and adding extra rows, it is generally recommended that a key column be designated first. If a key column is not present, then all rows in the file will be imported. With no key column designated, if any rows from that file were imported into the same metadata table earlier, a duplicate row will be created. With a key column, rows with a new, unique entry for that column are added to the table and existing rows with a key entry in the file will be updated, incorporating any changes present in the file. Duplicate rows will not be created.

The options presented in the Import Metadata Rows into Metadata Table are:

- **File**. The file containing the metadata to import. This can be Excel (.xlsx/.xls) format or a delimited text file.
- **Encoding**. For text files only: The text encoding of the seledcted file. Specifying the correct encoding is important to ensure that the file is correctly interpreted.
- **Separator**. For text files only: the character used to separate columns in the file.
- **Locale**. For text files only: the locale used to format numbers and dates within the file.
- **Date format**. For text files only: the date format used in the imported file.
- **Date-time format**. For text files only: the date-time format used in the imported file.
  The date and date-time templates uses the Java patterns for date and time formatting.
  Meaning of some of the symbols:

Symbol	Meaning	Example
y	Year	2004; 04
d	Day	10
M/L	Month	7; 07; Jul; July; J
a	am-pm	PM
h	Hour (0-12 am pm)	12
H	Hour (0-23)	0
m	Minute	30
s	Second	55

Examples of using this:

Format	Meaning	Example
dd-MM-yy	Short date	31-12-15
yyyy-MM-dd HH:mm	Date and Time	2015-11-23 23:35
yyyy-MM-dd'T'HH:mm	ISO 8601 (standard) format	2015-11-23T23:35

With a short year format (YY), 2000 will be added when imported as, or converted to, Date or Date and time format. Thus, when working with dates before the year 2000 or after 2099, please use a four digit format for the year (YYYY).

Click the button labeled **Finish** button when the necessary fields have been filled in.

The progress and status of the row import can be seen in the Processes tab in the Toolbox area, at the bottom, left side of the Workbench. Any errors resulting from an import that failed can be reviewed here. The most frequent errors are associated with selecting the wrong separator or encoding, or wrong date/time formats when importing rows from delimited text files.

Once the rows are imported, The metadata table can be saved.

12.2 Associating data elements with metadata

Each row in a metadata table can be associated with one or more data elements, such as sequence lists, expression tracks, variant tracks, etc. Each data element can be associated with one row of a given metadata table. Once data elements are associated with rows of a metadata table, it is then possible to use that metadata table to find data elements that share particular attributes, launch analyses like expression analyses where sample attributes are key, define batch units such that an analysis runs once per sample, or to group samples together according to their attributes when running certain types of workflows.

Each association has a "Role" label assigned to the associated element, which can be used to indicate the nature of the data element. For example, a newly imported sequence list could be given a role like "Sample data", or "NGS reads".

Associating data with metadata rows can happen in several ways, depending on the circumstances:

- By default, when input data for an analysis is associated with metadata, the results will inherit any unambiguous association. Appropriate role labels are assigned by the analysis tool. For example, a read mapping tool will assign the role "Unmapped reads" to a sequence list of unmapped reads that it produces.

- By default outputs from a workflow are associated with the relevant metadata rows in workflow results metadata tables. In these tables, the role assigned is always "Result data".
- Manually triggering data associations, either through matching the metadata key column entries with data element names, or by specifying the data element to associate with a given row. Here, roles to apply are chosen by you when triggering the associations.

The rest of this section describes this last point, where you associate data elements to metadata.

To do this, open a metadata table, and then click on the **Associate Data** button at the bottom of the Metadata Table view. Two options are available:

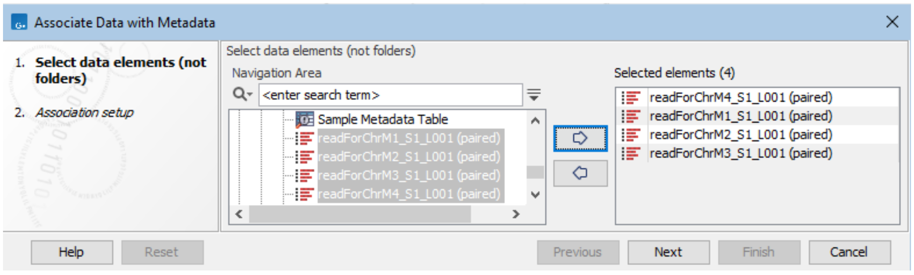
- **Association Data Automatically** Associations are set up based on matches between metadata key column entries and data elements names in a specified location of the Navigation Area. This option is only available if a key column has been specified. See section section 12.2.1.
- **Associate Data with Row** Manually make associations row by row, by selecting a row of the metadata and a particular data element in the Navigation Area. Here, information in the metadata table does not need to match data element names. This option is also available when right-clicking a row in the table. section 12.2.2.

12.2.1 Associate Data Automatically

When using the **Associate Data Automatically** option, associations are created based on matching the name of the selected data elements with the information in the **key column** of a metadata table previously saved in the Navigation Area. Matching is done according to three possible schemes: Exact, Prefix and Suffix (see section 12.2.1).

To associate data automatically, click the **Associate Data** button at the bottom of the Metadata Table view, and select **Associate Data Automatically**.

Select the data the tool should consider when setting up metadata associations (figure 12.11).


Associate Data with Metadata

1. **Select data elements (not folders)**
2. *Association setup*

Select data elements (not folders)

Navigation Area

<enter search term>

Sample Metadata Table
readForChrM1_S1_L001 (paired)
readForChrM2_S1_L001 (paired)
readForChrM3_S1_L001 (paired)
readForChrM4_S1_L001 (paired)

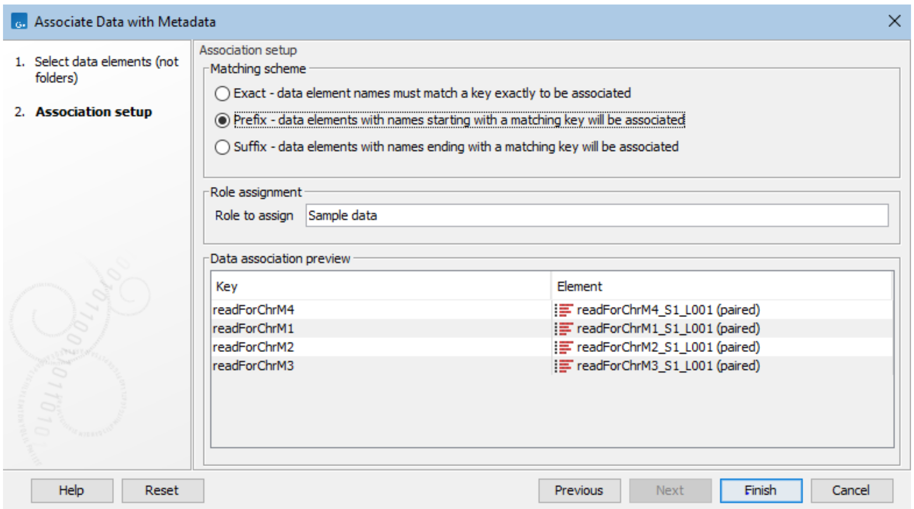
Selected elements (4)

readForChrM4_S1_L001 (paired)
readForChrM1_S1_L001 (paired)
readForChrM2_S1_L001 (paired)
readForChrM3_S1_L001 (paired)

Figure 12.11: *Select data elements to be associated to a CLC Metadata Table.*

In the Association setup step, you specify whether the matching of the data element names to the entries in the key column should be based on exact or partial matching (described below). A preview showing how elements are matched to metadata rows using the selected matching scheme is shown in the wizard (figure 12.12).

You also specify a role for each element. The default role provided is "Sample data". You can specify any term you wish.



1. Select data elements (not folders)
2. **Association setup**

Association setup

Matching scheme

○ Exact - data element names must match a key exactly to be associated

● Prefix - data elements with names starting with a matching key will be associated

○ Suffix - data elements with names ending with a matching key will be associated

Role assignment

Role to assign: Sample data

Data association preview

Key	Element
readForChrM4	readForChrM4_S1_L001 (paired)
readForChrM1	readForChrM1_S1_L001 (paired)
readForChrM2	readForChrM2_S1_L001 (paired)
readForChrM3	readForChrM3_S1_L001 (paired)

Figure 12.12: *Data element names can be matched either exactly or partially to the entries in the key column. Here, the Prefix matching scheme has been selected. A preview showing how elements are matched to metadata rows using that scheme is shown in the Data association preview area, at the bottom.*

After the job has run, data associations and roles are saved for all the selected data elements where the name matches a key column entry according to the selected matching scheme.

Note: Data elements selected that already have associations with the CLC Metadata Table will have their associations *updated* to reflect the current information in the CLC Metadata Table. This means associations will be *deleted* for a selected data element if there are no rows in the metadata table that match the name of that data element. This could happen if, for example, you changed the name of a data element with a metadata association, and did not change the corresponding key entry in the metadata table.

Matching schemes A data element name must match an entry in the key column of a metadata table for an association to be set up between that data element at the corresponding row of the metadata table. Two schemes are available in the **Association Data Automatically** for matching up names with key entries:

- Exact - data element names must match a key exactly to be associated. If any aspect of the key entry differs from the name of a selected data element, no association will be created.
- Prefix - data elements with names partially matching a key will be associated: here the first whole part(s) of a name must match a key entry in the metadata table for an association to be established. This option is explained in more detail below.
- Suffix - data elements with names partially matching a key will be associated: here the last whole part(s) of a name must match a key entry in the metadata table for an association to be established. This option is explained in more detail below.

Partial matching rules For each data element being considered, the partial matching scheme involves breaking a data element name into components and searching for the best match from the key entries in the metadata table. In general terms, the best match means the longest key that matches entire components of the name.

The following describes the matching process in detail:

- Break the data element name into its component parts based on the presence of delimiters. It is these parts that are used for matching to the key entries of the metadata table.

  Delimiters are any non-alphanumeric characters. That is, anything that is not a letter (a-z or A-Z) or number (0-9). So, for example, characters like hyphens (-), plus symbols (+), spaces, brackets, and so on, would be used as delimiters.

  If partial matching was chosen with a data element called `Sample234-1 (mapped) (trimmed)` would be split into 4 parts: `Sample234`, `-1`, `(mapped)` and `(trimmed)`.

- Matches are made at the component level. A whole key entry must match perfectly to at least the first (with the Prefix option) or the last (with the Suffix option) complete component of a data element name.

  For example, a key entry `Sample234` would be a match to the data element with name `Sample234-1 (mapped) (trimmed)` because the whole key entry matches the whole of the first component of the data element name. Conversely, if they key entry had been `Sample23`, no match would be identified, because they whole key entry does not match to at least the whole of the first component of the data element name.

  In cases where a data element could be matched to more than one key, the longest key matched determines the metadata row the data will be associated with.

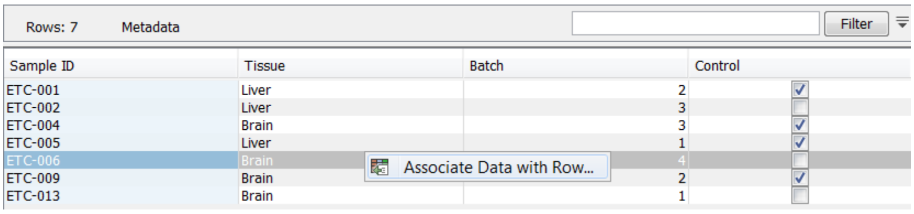
  The table below provides examples to illustrate the partial matching system, on a table that has the keys with sample IDs like in figure 12.13) (i.e., `ETC-001`, `ETC-002`, . . . , `ETC-013`),

Data Element	Key	Reason for association
ETC-001 (Reads)	ETC-001	Key `ETC-001` matches the first part of the name
ETC-001 un-m. . . (single)	ETC-001	”
ETC-001 un-m. . . (paired)	ETC-001	”
ETC-002	ETC-002	Key `ETC-002` matches the whole name
ETC-003	None	No keys match this data element name
ETC-005	ETC-005	Key `ETC-005` matches the whole name
ETC-005-1	ETC-005	Key `ETC-005` matches the first part of the name
ETC-006-5	ETC-006	Key `ETC-006` matches the first part of the name
ETC-007	None	No keys match this data element name
ETC-007 (mapped)	None	”
ETC-008	None	”
ETC-008 (report)	None	”
ETC-009	ETC-009	Key `ETC-009` matches the whole name

12.2.2 Associate Data with Row

The **Associate Data with Row** option is best suited for association of a few metadata tables to a few data elements. This type of association does not require a key column in the metadata table, nor a particular relationship between the name of the data element and the metadata to associate it with.

To associate data elements with a particular row in the metadata table, select the desired row in the metadata table by clicking on it. Then either click the **Associate Data** button at the bottom of the Metadata Table view, or right-click on the selected metadata row and choose the **Associate Data with Row** option (as seen in figure 12.13).



Sample ID	Tissue	Batch	Control
ETC-001	Liver	2	☑
ETC-002	Liver	3	□
ETC-004	Brain	3	☑
ETC-005	Liver	1	☑
ETC-006	Brain	4	□
ETC-009	Brain	2	☑
ETC-013	Brain	1	□

Figure 12.13: *Manual association of data elements to a metadata row.*

A window will open within which you can select the data elements that should have an association with the metadata row.

If a selected data element already has an association with this particular metadata table, that association will be updated. Associations with any other metadata tables will be left as they are.

Enter a role for the data elements that have been chosen and click **Next** until you can choose to **Save** the outputs. Data associations and roles will be saved for the selected data elements.

12.3 Working with data and metadata

12.3.1 Finding data elements based on metadata

Data elements associated with rows of the metadata table can also be found from within a Metadata Table view. From there, it is possible to highlight elements in the Navigation Area and launch analyses on selected data.

Relevant metadata tables can be found using the Quick Search box, described in section 3.4.1.

To find data elements associated with selected metadata rows in a metadata table:

- Select one or more rows of interest in the metadata table.
- Click on the **Find Associated Data** button at the bottom of the view.

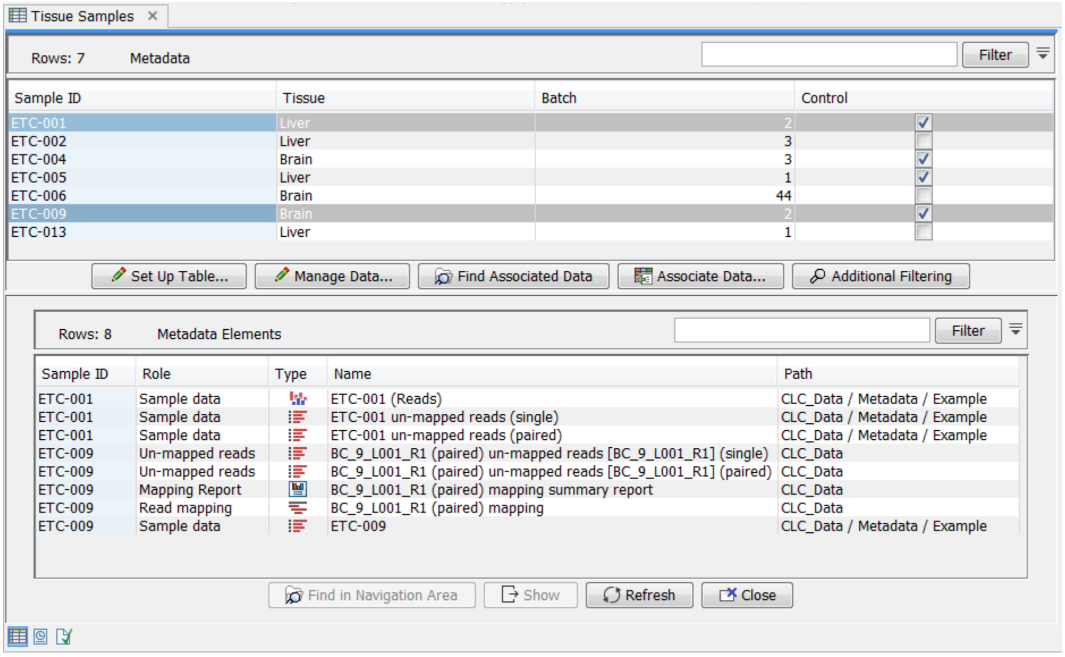
  A table with a listing of the data elements associated to the selected metadata row(s) will appear (figure 12.14).

The search results table shows the type, name, and navigation area path for each data element found. It also shows the key entry of the metadata table row with which the element is associated and the role of the data element for this metadata association. In figure 12.14, there are five data elements associated with sample ETC-009. Three are Sequence Lists, two of which have a role that tells us that they are unmapped reads resulting from the Map Reads to Reference tool.

Clicking the **Refresh** button will re-run the search and refresh the search results table.

Click the button labeled **Close** to close the search table view.

Data elements listed in the search result table can be opened by clicking on the button labeled **Show** at the bottom of the view.



Rows: 7 Metadata

Sample ID	Tissue	Batch	Control
ETC-001	Liver	2	☑
ETC-002	Liver	3	☐
ETC-004	Brain	3	☑
ETC-005	Liver	1	☑
ETC-006	Brain	44	☐
ETC-009	Brain	2	☑
ETC-013	Liver	1	☐

Rows: 8 Metadata Elements

Sample ID	Role	Type	Name	Path
ETC-001	Sample data		ETC-001 (Reads)	CLC_Data / Metadata / Example
ETC-001	Sample data	≡	ETC-001 un-mapped reads (single)	CLC_Data / Metadata / Example
ETC-001	Sample data	≡	ETC-001 un-mapped reads (paired)	CLC_Data / Metadata / Example
ETC-009	Un-mapped reads	≡	BC_9_L001_R1 (paired) un-mapped reads [BC_9_L001_R1] (single)	CLC_Data
ETC-009	Un-mapped reads	≡	BC_9_L001_R1 (paired) un-mapped reads [BC_9_L001_R1] (paired)	CLC_Data
ETC-009	Mapping Report		BC_9_L001_R1 (paired) mapping summary report	CLC_Data
ETC-009	Read mapping	≡	BC_9_L001_R1 (paired) mapping	CLC_Data
ETC-009	Sample data	≡	ETC-009	CLC_Data / Metadata / Example

Figure 12.14: *Metadata Table with search results*

Alternatively, they can be highlighted in the Navigation Area by clicking the **Find in Navigation Area** button.

Analyses can be launched on the selected data elements:

- Directly. Right click on one of the selected elements, choose the menu option Tools, and navigate to the tool of interest. The data selected in the search results table will be listed as selected elements in the launch wizard.
- Via the Navigation area selection. Use the **Find in Navigation Area** button and then launch a tool or workflow. The items that were selected in the Navigation area will be pre-selected in the launch wizard.

If no data elements with associations are found and this is unexpected, please re-index the locations your data are stored in. This is described in section 3.4. For data held in a CLC Server location, an administrator will need to run the re-indexing. Information on this can be found in the CLC Server admin manual at https://resources.qiagenbioinformatics.com/manuals/clcserver/current/admin/index.php?manual=Rebuilding_index.html.

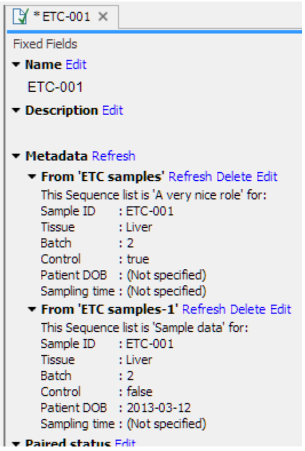
12.3.2 Viewing metadata associations

Metadata associations for a data element are shown using the Element info view, as in figure 12.15.

To show Element Info,

right-click an element in the Navigation Area | **Show** | **Element Info** ( )

The Element Info view contains the details of each metadata association for the data element. The following operations are available:



```
* ETC-001
Fixed Fields
▾ Name Edit
  ETC-001
▾ Description Edit

▾ Metadata Refresh
  ▾ From 'ETC samples' Refresh Delete Edit
    This Sequence list is 'A very nice role' for:
    Sample ID     : ETC-001
    Tissue        : Liver
    Batch         : 2
    Control       : true
    Patient DOB   : (Not specified)
    Sampling time : (Not specified)
  ▾ From 'ETC samples-1' Refresh Delete Edit
    This Sequence list is 'Sample data' for:
    Sample ID     : ETC-001
    Tissue        : Liver
    Batch         : 2
    Control       : false
    Patient DOB   : 2013-03-12
    Sampling time : (Not specified)
▾ Paired status Edit
```

Figure 12.15: *Element Info view with a metadata association*

- **Delete** will remove an association.
- **Edit** will allow you to change the role of the metadata association.
- **Refresh** will reload the metadata details from the Metadata Table; this functionality may be used to attempt to re-fetch metadata that was previously unavailable, e.g. due to server connectivity.

Read more about Element Info view in section 14.4.

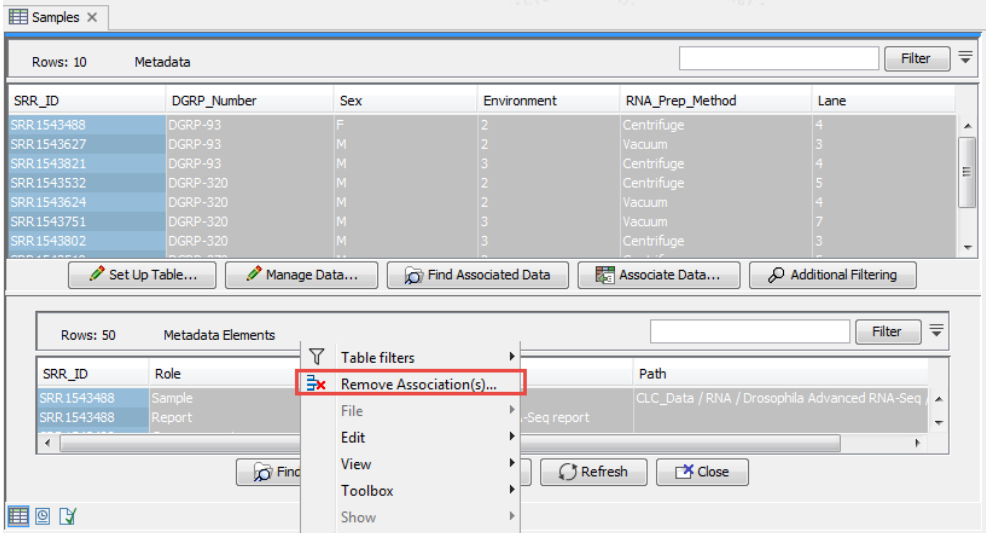
12.3.3 Removing metadata associations

Any or all associations to data elements from rows of a metadata table can be removed by taking the following steps:

1. Open the metadata table containing the rows of interest.
2. Highlight the relevant rows of the metadata table.
3. Click **Find Associated Data**.
4. In the Metadata Elements table that opens, highlight the rows for the data elements the metadata associations should be removed from.
5. Right-click over the highlighted area and choose the option **Remove Association(s)** (figure 12.16). Alternatively, use the Delete key on the keyboard, or on a Mac, the fn and backspace keys at the same time.

Metadata associations can also be removed from within the Element info view for individual data elements, as described in section 12.3.2.

When an metadata association is removed from a data element, this update to the data element is automatically saved.



SRR_ID	DGRP_Number	Sex	Environment	RNA_Prep_Method	Lane
SRR1543488	DGRP-93	F	2	Centrifuge	4
SRR1543627	DGRP-93	M	2	Vacuum	3
SRR1543821	DGRP-93	M	3	Centrifuge	4
SRR1543532	DGRP-320	M	2	Centrifuge	5
SRR1543624	DGRP-320	M	2	Vacuum	4
SRR1543751	DGRP-320	M	3	Vacuum	7
SRR1543802	DGRP-320	M	3	Centrifuge	3

SRR_ID	Role		Path
SRR1543488	Sample		CLC_Data / RNA / Drosophila Advanced RNA-Seq /
SRR1543488	Report	-Seq report	

Figure 12.16: *Removing metadata associations to two data elements via the Metadata Elements table.*

12.3.4 Identifying metadata rows without associated data

Using the Metadata Table view you can apply filters using the standard filtering tools shown at the top of the view as well as by using special metadata filtering in the **Additional Filtering** shown at the bottom. Using the special metadata filtering option **Show only Unassociated Rows**, you can filter the rows visible in the Metadata Table view so only the rows to which no data elements are associated are shown. If desired, these rows could then be used to launch one of the tools for associating data, described in section 12.2.

This filter can be cancelled by clicking on the **Additional Filtering** button again and clicking on the **Show only Unassociated Rows** again. When the filter is active, it has a checkmark beside it. When it is inactive, it does not.

This filter can take a long time if many rows are shown in the table. When working with many rows, it can help if the full table is filtered using the general filters in advance, using the standard filters at the top of the table view. Alternatively you can pre-select some rows and filtering with the Additional filtering option **Filter to Selected Rows**. This filter can be applied multiple times. If the search takes too long, you can cancel it by unselecting the filter from the menu.

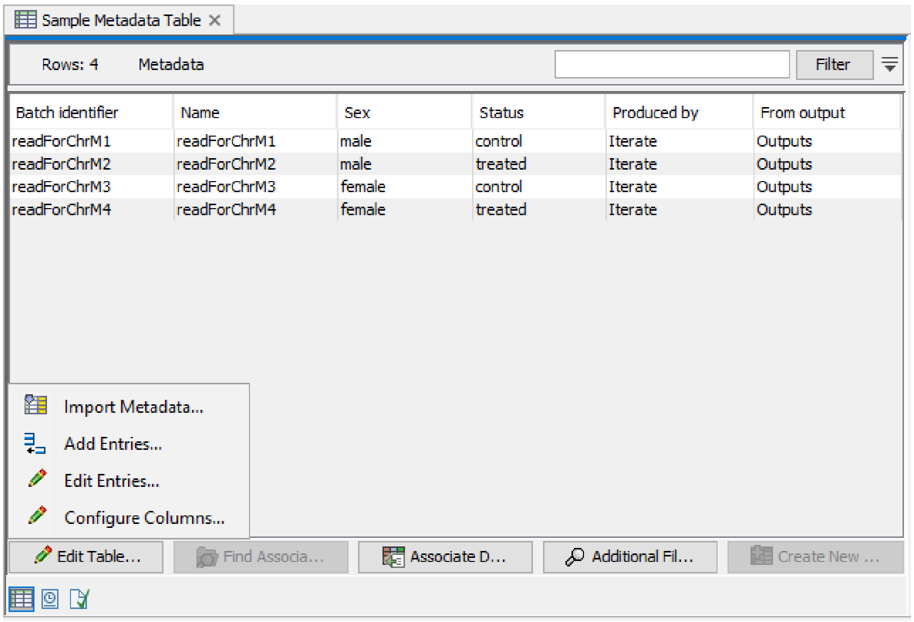
This filter can be cancelled by clicking on the **Additional Filtering** button again and clicking on the **Clear Selection Filter** option.

12.3.5 Editing Metadata tables

CLC Metadata Tables can be edited using functionality under the **Edit Table...** button at the bottom of the Metadata Table editor (figure 12.17) and by right-clicking on a row or rows and selecting from the options presented (figure 12.18). Information can be added directly, or can be take from another CLC Metadata Table or an Excel, CSV or TSV format file.

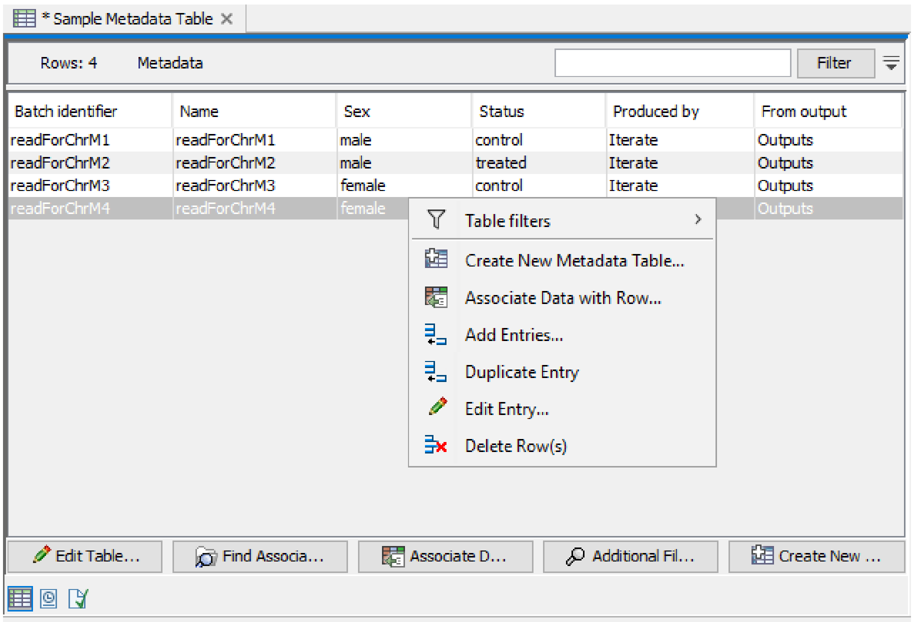
Edit existing rows in a CLC Metadata Table

A dialog to edit entries in a CLC Metadata Table can be opened by:



Batch identifier	Name	Sex	Status	Produced by	From output
readForChrM1	readForChrM1	male	control	Iterate	Outputs
readForChrM2	readForChrM2	male	treated	Iterate	Outputs
readForChrM3	readForChrM3	female	control	Iterate	Outputs
readForChrM4	readForChrM4	female	treated	Iterate	Outputs

Figure 12.17: *Click on the Edit Table... button to open a menu with options for adding, editing or removing information in a CLC Metadata table.*



Batch identifier	Name	Sex	Status	Produced by	From output
readForChrM1	readForChrM1	male	control	Iterate	Outputs
readForChrM2	readForChrM2	male	treated	Iterate	Outputs
readForChrM3	readForChrM3	female	control	Iterate	Outputs
readForChrM4	readForChrM4	female			Outputs

Figure 12.18: *Right-click on selected rows of a CLC Metadata Table to open a menu actions that can be taken.*

- Clicking on the **Edit Table...** button and selecting **Edit Entries.. (✎)**.
- Double clicking on an entry.
- Selecting an entry and hitting the Return key.

Navigate between entries using the buttons on the right. Modifications made take effect as you navigate to another row, or if you close the dialog using **Done**.

Right-click on an individual row in the table and select the **Edit Entry.. ( )** option to edit just that entry. An option to delete rows is also in this menu: **Delete Row(s)** (figure 12.18).

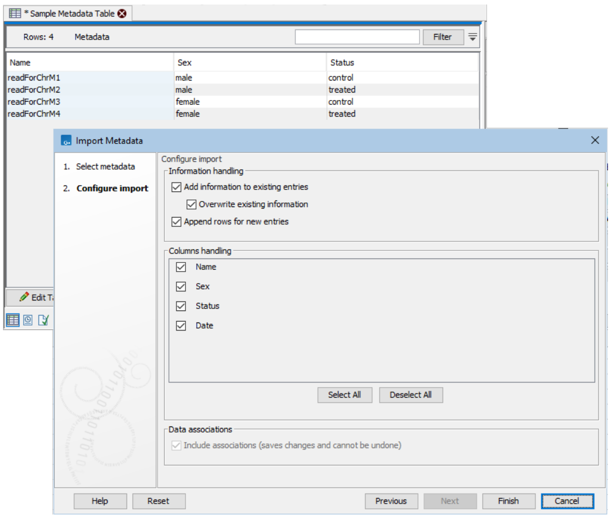
Import further rows into a CLC Metadata Table

More information can be imported into an existing CLC Metadata table by clicking on the **Edit Table...** button and selecting **Import Metadata... ( )**.

Information can be supplied in an Excel, CSV or TSV file, by clicking on the standard folder icon ( ), or from another CLC Metadata Table, by clicking on the browse folder ( ) icon.

For CLC Metadata Tables with a key column, you choose whether new information should be added to existing entries, and whether rows should be added for new entries (figure 12.19). Matching is based on column names.

If there is no key column designated in the original CLC Metadata Table, as is the case in Workflow Result Metadata tables, then data from the new source can only be entered into new rows. A key column can be specified using the **Configure Columns** functionality described in the **Add or edit columns in a CLC Metadata Table** section below.



Name	Sex	Status
readForChrM1	male	control
readForChrM2	male	treated
readForChrM3	female	control
readForChrM4	female	treated

Configure import

Information handling

☑ Add information to existing entries

☑ Overwrite existing information

☑ Append rows for new entries

Columns handling

☑ Name

☑ Sex

☑ Status

☑ Date

Data associations

☑ Include associations (saves changes and cannot be undone)

Figure 12.19: *Additional information can be imported to an existing CLC Metadata table. You can choose whether new information should be added to existing entries, and whether rows should be added for new entries. The columns to import can also be specified.*

Add and delete rows manually

Rows can be added to the end of the CLC Metadata table by clicking on the **Edit Table...** button and selecting **Add Entries... ( )**. This option is also available in the menu that opens when you right-click on a row in the table (figure 12.18).

The option **Duplicate Entry**, available when you right-click on a row in the table, will duplicate that row and put it at the bottom of the table. When a key column has been designated, you will be prompted for a new value for that column for the new row. Data with an association to the original row will *not* automatically have an association to the new row. (A given data element can only have one association to a given CLC Metadata Table.)

Individual rows can also be added using the ( ) button, which inserts a new row after the current one.

Rows may be deleted using the ( ) button.

The (⟲) and (⟳) buttons are used to undo and redo changes respectively.

Add or edit columns in a CLC Metadata Table

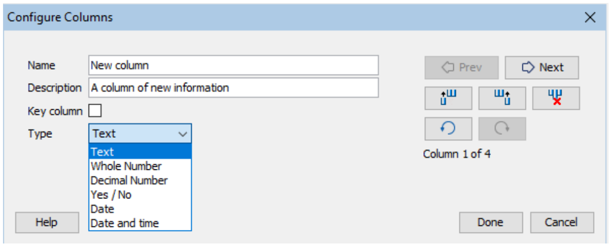
Existing columns can be edited, and new columns added, by clicking on the **Edit Table...** button and selecting **Configure columns...**.

A dialog opens with information about the left-most column in the CLC Metadata Table (figure 12.20). Use the buttons on the right to navigate to other columns and to add or delete columns.

Navigate between the columns using the (◀) **Prev** and (▶) **Next** buttons, or by using left/right arrow keys with Alt key held down. Modifications made to a particular column take effect as you navigate to another column, or if you close the dialog using **Done**.

Individual columns can be added using the ( ) and ( ) buttons, which insert new columns before and after the current column respectively. Columns may be deleted using the ( ) button.

The (⟲) and (⟳) buttons are used to undo and redo changes respectively.



Configure Columns

Name: New column

Description: A column of new information

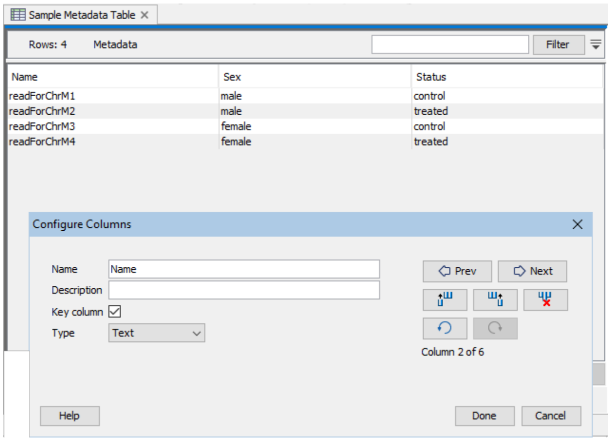
Key column ☐

Type: Text

Text
Whole Number
Decimal Number
Yes / No
Date
Date and time

Column 1 of 4

Figure 12.20: *When adding a new column, a name, description and data type is specified. If it should become the key column, the Key column box should be checked. Use the buttons on the right to navigate to other columns or add further new columns.*



Sample Metadata Table

Rows: 4 Metadata

Name	Sex	Status
readForChrM1	male	control
readForChrM2	male	treated
readForChrM3	female	control
readForChrM4	female	treated

Configure Columns

Name: Name

Description:

Key column ☑

Type: Text

Column 2 of 6

Figure 12.21: *The Name column has been designated as the key column.*

For each column, the following can be configured:

- **Name**. A mandatory header name or title for the column.
- **Description**. An optional description of the information that will be held in the column. The description will appear as a tool tip, visible when you hover the mouse cursor over the column name in the metadata table.
- **Key column**. Any column containing only unique values can be designated as the key column. If a table already has a key column, this option is disabled for other columns. Information in the key column is used when automatically creating associations from data elements, described in (section section 12.2.1).
- **Type**. The type of value allowed. The default data type for columns on import is text, but this can be edited to the following types:
  - **Text** Simple text.
  - **Whole number** Integer values, like `42` or `-7`.
  - **Decimal number** Decimal values, like `3.14` or `1.72e13`.
  - **Yes / No** `Yes/No` or `True/False` values are accepted. Capitalization is not necessary.
  - **Date** Local dates such as `2015-04-23` for April 23rd, 2015.
  - **Date and time** Local date and time such as `2015-04-23 13:37` for 1:37pm on April 23rd, 2015. Note the use of 24-hour clock and that no time zone information is present.

12.4 Moving, copying and exporting metadata

Moving and copying metadata

This section focuses on what happens to data associations when copying metadata tables. This information also pertains to moving metadata tables *between* File Locations, as this is equivalent to a copy action.

To copy a metadata table and the data associated with it, such that the new data element copies are associated with the copy of the metadata table, *select both the metadata table and the data elements and copy them in a single operation*. Of note here:

- The data element copies will have associations with the new copy of the metadata table. The original elements keep their associations with the original metadata table.
- If a metadata table is copied but data elements with associations to it are not also copied in that action, those data elements will be associated with both the copy and the original metadata table.
- If data elements with associations to metadata are copied, but no metadata table is involved in the same copy action, each data element copy will be associated to the same metadata as the original element.

If a metadata table and some, but not all, data elements with associations to it, are copied in a single action, then:

- The data element copies will have associations to the copy of the metadata table, while the original elements (that were copied) remain associated with the original metadata table.

- Elements with associations to the original metadata table that were not copied will have associations to both the original metadata table and the copy. However, if these data elements are later copied (in a separate copy operation), those copies will only be associated with the original metadata table. If they should be associated with the copy of the metadata table, those association must be added as described in section 12.2.

Exporting metadata

The standard Workbench export functionality can be used to export metadata tables to various formats. The system's default locale will be used for the export, which will affect the formatting of numbers and dates in the exported file.

See section 8.1 for more information.

Chapter 13

Workflows

Contents

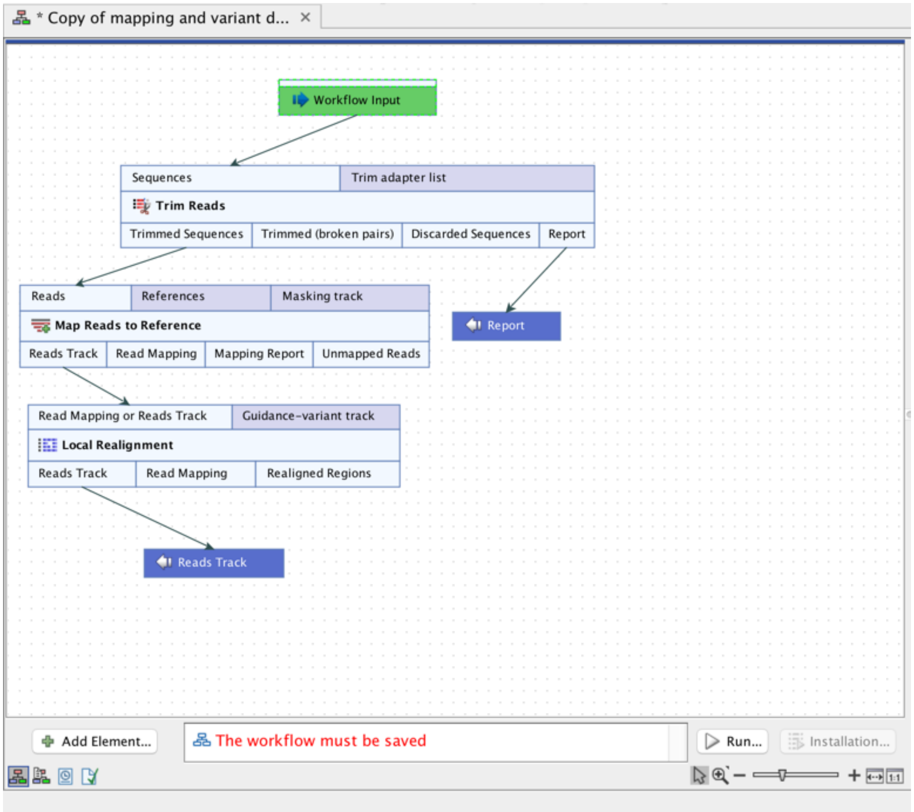
Workflows contain tools linked together, making up an analysis pipeline.

Data from CLC Data Locations can be selected as input, as can raw data in supported standard formats. Results can be saved to CLC locations for viewing and downstream analysis, or exported to standard formats and saved to a location of your choice.

The flow of data through a workflow can be defined in various ways, making it simple to execute complex analyses consistently. Launching workflows in Batch mode, the same analysis is run on many samples using a single launch action.

Workflows are created and customized using the Workflow Editor (figure 13.1). Workflows can be saved and re-opened, just like data elements. You can run workflows from the Workflow Editor, or install them and run them from under the Installed Workflows folder under the Workflows menu (see section 13.6.2).

Template workflows are pre-installed in the *CLC Main Workbench*, and can be referred to as examples, or copied and edited. This is described further in section 13.5.



The workflow must be saved

Figure 13.1: *A workflow open in the Workflow Editor. Workflows consist of connected tools, where the output of one tool is used as input for another tool.*

Examples in this chapter use tools from the *CLC Genomics Workbench*. Some of these are not available in the *CLC Main Workbench*. However, the principles described apply equally to tools in *CLC Main Workbench*.

13.1 Creating and editing workflows

Workflows are created and edited using the Workflow Editor.

To create a new workflow, click on the **Workflows** button ( ) in the Toolbar and then select "New Workflow" ( ).

Alternatively, use the menu option:

File | New | Workflow ( )

To open a copy of an installed workflow in the Workflow Editor, right-click on the workflow name under the Workflows menu in the Toolbox at the bottom, left side of the Workbench, and choose the option **Open Copy of Workflow**. Customizing existing workflows rather than building new workflows from scratch, can save much time.

Template workflows are provided with the *CLC Main Workbench* (figure 13.2).

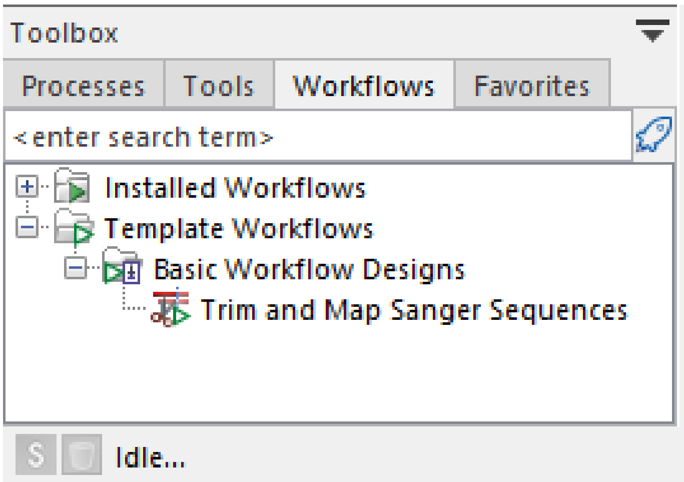


Figure 13.2: *Template workflows are available under the Workflows menu.*

To copy the image of a workflow design, select the elements in the workflow design (click in the workflow editor and then press keys Ctrl + A), then copy (Ctrl + C), and then paste where you wish the image to be placed, for example, in an email or presentation program.

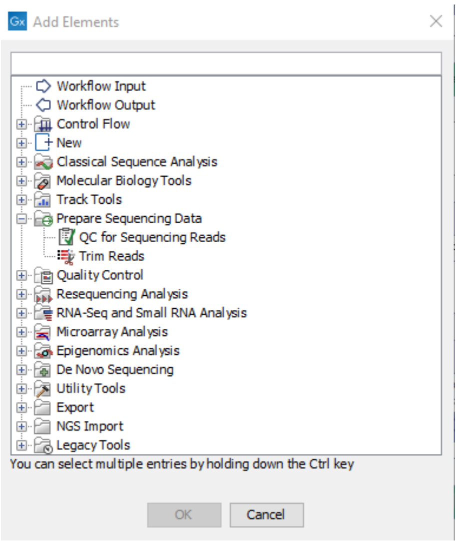
13.1.1 Adding elements to a workflow

Elements are the building blocks of workflows. They are used to define the inputs, the outputs, all the analysis steps to be done, and can be used to control the way data flows through the workflow. Workflow elements are defined in detail in section 13.2. Here we focus on adding them and connecting them to each other within a workflow.

To add elements to a workflow:

- Drag tools from the **Tools** tab in the Toolbox panel in the bottom, left side of the Workbench into the canvas area of the Workflow Editor, or
- Use the **Add Element** dialog (figure 13.3). The following methods can be used to open this dialog:

- Click on the **Add Element** (✚) button at the bottom of the Workflow Editor.
- Right-click on an empty area of the canvas and select the **Add Element** (✚) option.
- Use the keyboard shortcut Shift + Alt + E.



You can select multiple entries by holding down the Ctrl key

Figure 13.3: *Adding elements to a workflow.*

Select one or more elements and click on **OK**. Multiple elements can be selected by keeping the Ctrl key (⌘ on Mac) depressed while selecting them.

- Use one of the relevant options offered when right-clicking on an input or output channel of a workflow element, as shown in figure 13.4 and figure 13.5.

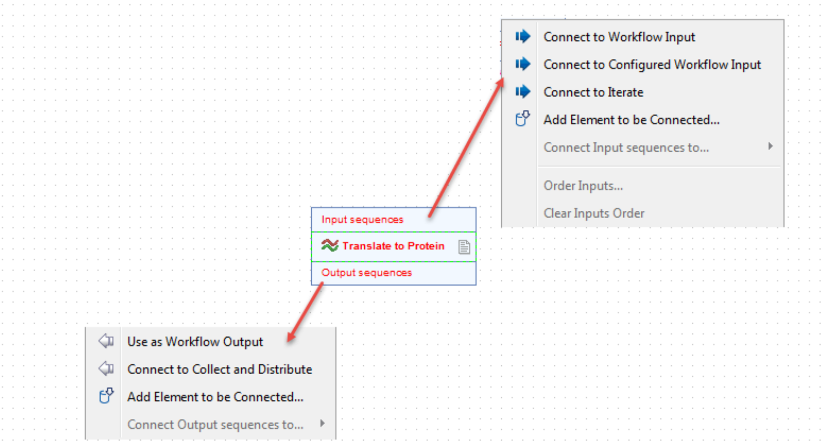

Connect to Workflow Input
Connect to Configured Workflow Input
Connect to Iterate
Add Element to be Connected...
Connect Input sequences to...
Order Inputs...
Clear Inputs Order
Input sequences
Translate to Protein
Output sequences
Use as Workflow Output
Connect to Collect and Distribute
Add Element to be Connected...
Connect Output sequences to...

Figure 13.4: *Connection options are shown in menus when you right click on an input or output channel of a workflow element.*

- **Connect to Workflow Input** or **Connect to Configured Workflow Input** and
- **Use as Workflow Output** to add data to be processed or saved.
- **Add Element to be Connected...** to open the Add Elements pop-up dialog described above.
- **Connect to Iterate** and **Connect to Collect and Distribute** to create an iterative process within a workflow.

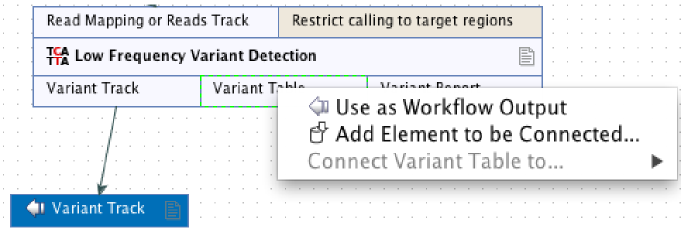

Read Mapping or Reads Track
Restrict calling to target regions
Low Frequency Variant Detection
Variant Track
Variant Table
Variant Report
Use as Workflow Output
Add Element to be Connected...
Connect Variant Table to...
Variant Track

Figure 13.5: *Right clicking on an output channel brings up a menu with relevant connection options.*

Once added, workflow elements can be moved around on the canvas using the 4 arrows icon (✥) that appears when hovering on an element.

Workflow elements can be removed by selecting them and pressed the delete key, or by right-clicking on the element name and choosing **Remove** from the context specific menu, as shown in figure 13.6.

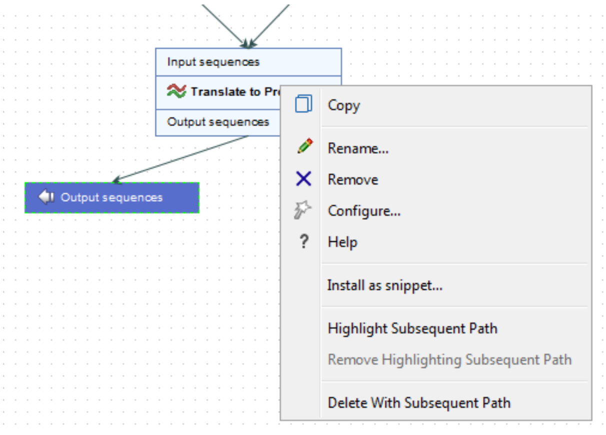


Figure 13.6: *Right clicking on an element name brings up a context specific menu that includes options for renaming or removing elements.*

13.1.2 Connecting workflow elements

Connections between workflow element output channels and input channels define where data flows from and to.

The names of output channels usually indicate the type of data generated and the names of input channels usually indicate the type of data expected. Connections can only be made between compatible output and input channels.

An output channel can be connected to more than one input channel and an input channel can accept data from more than one output channel (figure 13.7).

Connecting output and input channels

Two ways that compatible input and output channels can be connected are:

- Click on an output channel and, keeping the mouse button depressed, drag the cursor to the desired input channel. A green border around the input channel name indicates when

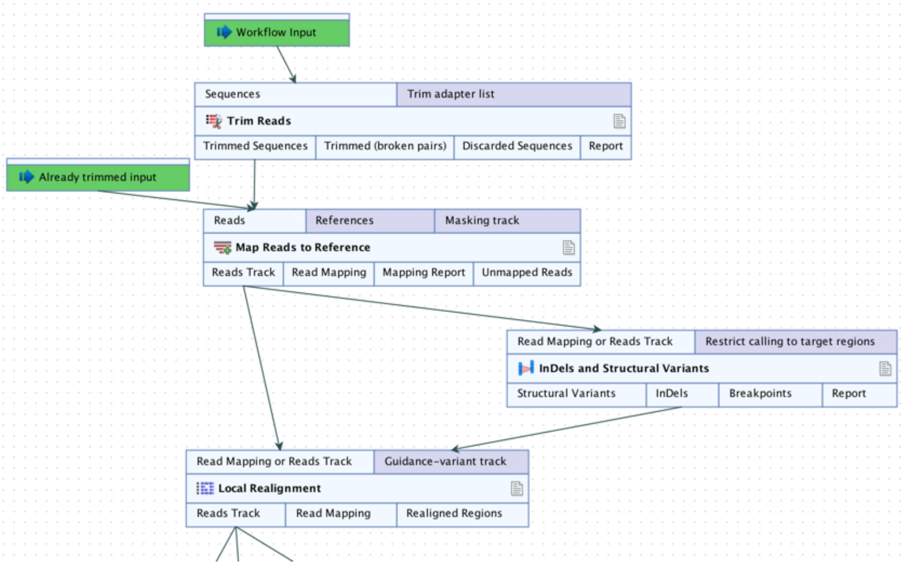


Figure 13.7: *In this workflow, two elements are supplying data to the Reads input channel of the Map Reads to Reference element, while data from the Reads Track output channel of Map Reads to Reference is being used as input to two elements.*

the connection has been made and the mouse button can be released. An arrow is drawn, linking the channels (figure 13.8).

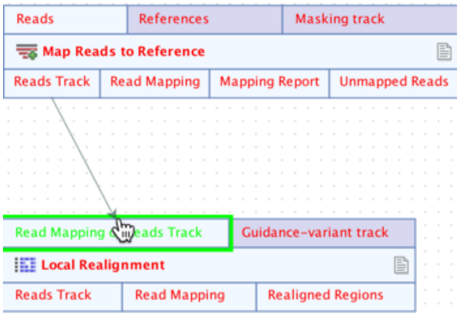


Figure 13.8: *Connecting the "Reads Track" output channel from a Map Reads to Reference element to the "Read Mapping or Reads" input channel of a Local Realignment element.*

- Use the **Connect <channel name> to...** option in the right-click menu of an output or input channel. Hover the cursor over this option to see a list of elements in the workflow with compatible channels. Hovering the cursor over any of these items then shows the particular channels that can be connected to (figure 13.9).

Information about what elements and channels are connected In a small workflow, it is easy to see which elements are connected and how they are connected. In large workflows, the following methods can be helpful:

- Mouse-over the connection line. A tooltip is revealed showing the elements and channels that are connected (figure 13.10).

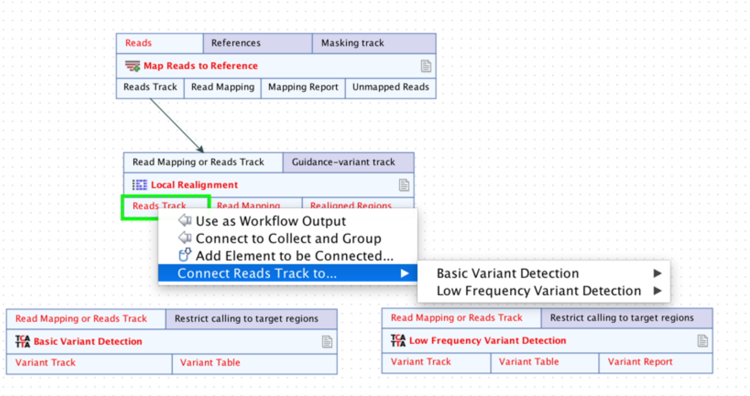


Figure 13.9: *Right-clicking on an output channel displays a context specific menu, with options supporting the connection of this channel to input channels of other workflow elements.*

- Right-click on a connection line and choose the option **Jump to Source** to see the upstream element or **Jump to Destination** to see the downstream element (figure 13.11).

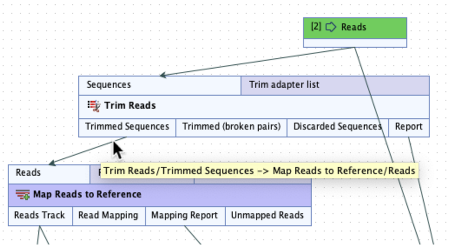


Figure 13.10: *Hover the mouse cursor over a connection to reveal a tooltip containing the names of the elements and channels connected.*

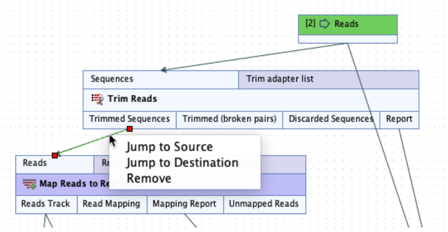


Figure 13.11: *Right-click on a connection to reveal options to jump to the source element or the destination element of that connection.*

Removing connections

To remove a connection, right-click on the connection and select the **Remove** option (figure 13.11).

13.1.3 Ordering inputs

Workflow inputs can be re-ordered when editing a workflow. This can affect the user experience or can have an effect on the contents of outputs generated by the workflow.

1. **Improve the user experience by changing the order that launch wizard steps are presented.**

   By default, the order of the launch wizard steps reflects the order that Input elements were added to the workflow when it was created.

   To make changes to this order, right-click on an empty area of the canvas and choose **Order Workflow Inputs...** from the menu that appears.

   An **Order Inputs** dialog appears (figure 13.12). Select an input and move it up or down in the list by clicking on the up arrow ((⬆)) or down arrow ((⬇)), respectively.

   A number next to an Input element's name indicates its position in the order. These numbers are updated when the ordering is updated.

   These same numbers can be used in Output element naming patterns (see section 13.2.4). If output names using such patterns have already been configured, they may need to be updated.

2. **Influence the content of outputs in cases where input processing order has an effect.**

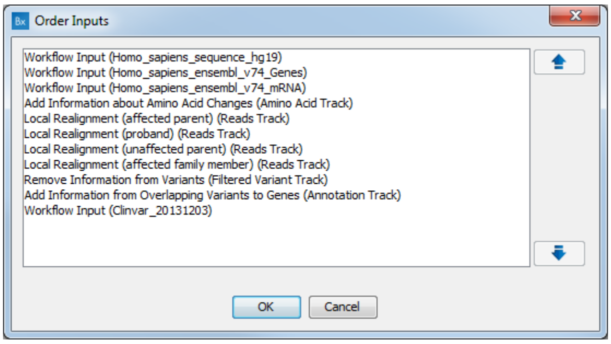
   For example, the order of the sections in a report generated by **Combine Reports** reflects the order that inputs to that tool are processed.

   By default, the main inputs to a tool are processed in the order that the connections to that input channel were added when the workflow was created.

   To make changes to this order, right-click on the relevant input channel and choose the option **Order Inputs...** from the menu that appears.

   An **Order Inputs** dialog appears (figure 13.12). Select an input and move it up or down in the list by clicking on the up arrow ((⬆)) or down arrow ((⬇)), respectively.

   Numbers on the connection arrows are added or updated if any changes are made in this **Order Inputs...** dialog.



Workflow Input (Homo_sapiens_sequence_hg19)
Workflow Input (Homo_sapiens_ensembl_v74_Genes)
Workflow Input (Homo_sapiens_ensembl_v74_mRNA)
Add Information about Amino Acid Changes (Amino Acid Track)
Local Realignment (affected parent) (Reads Track)
Local Realignment (proband) (Reads Track)
Local Realignment (unaffected parent) (Reads Track)
Local Realignment (affected family member) (Reads Track)
Remove Information from Variants (Filtered Variant Track)
Add Information from Overlapping Variants to Genes (Annotation Track)
Workflow Input (Clinvar_20131203)

Figure 13.12: *The order of inputs is displayed and can be updated in an Order Inputs dialog.*

13.1.4 Validating a workflow

A workflow must be valid before it can be run or saved.

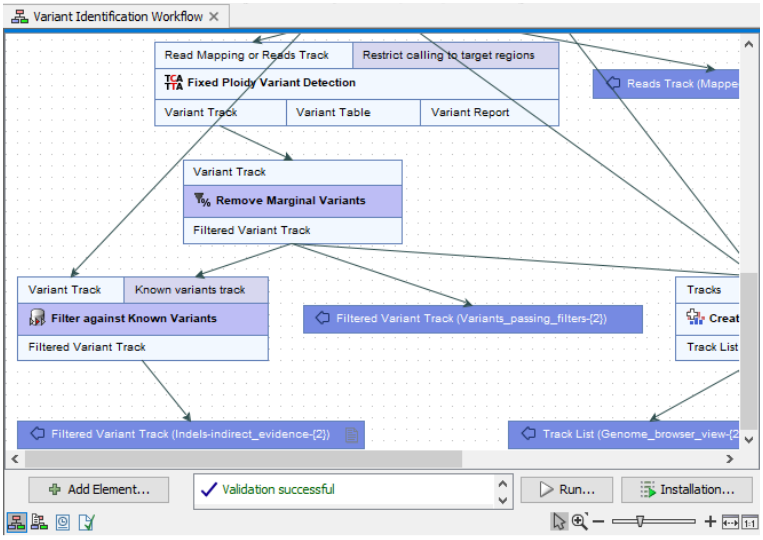
In general terms, a valid workflow has at least one route for data to flow into it, at least one result saved from it, and it contains no unconnected elements. In more detail:

- There must be at least one Input element connected to the main input channel of the element where data starts its flow through the workflow. Where there are multiple independent arms in the workflow, this requirement pertains to each of those arms.
- There must be at least one result saved from the end of each branch within a workflow. In practice this means that at least one Output or Export element must be connected to each terminal element with an output channel.
- All elements must have at least one connection to another element in the workflow.

Validation status is continuously monitored, with messages relating to this reported at the bottom of the editor.

The validation status of a workflow will fall into one of three categories:

1. **Valid and saved** When a workflow is valid and has been saved, the message "Validation successful" is displayed in green text at the bottom of the editor (figure 13.13).


Variant Identification Workflow
Read Mapping or Reads Track
Restrict calling to target regions
Fixed Ploidy Variant Detection
Variant Track
Variant Table
Variant Report
Reads Track (Mappe
Variant Track
Remove Marginal Variants
Filtered Variant Track
Variant Track
Known variants track
Filter against Known Variants
Filtered Variant Track
Filtered Variant Track (Variants_passing_filters-{2})
Tracks
Creat
Track List
Filtered Variant Track (Indels-indirect_evidence-{2})
Track List (Genome_browser_view-{2
Add Element...
Run...
Installation...

✓ Validation successful

Figure 13.13: *The "Validation successful" message indicates that this workflow is valid and has been saved.*

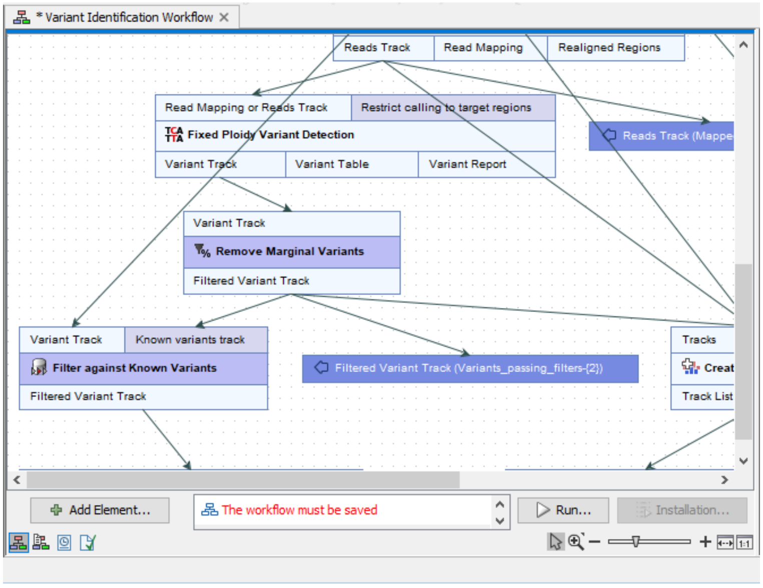
2. **Valid, with changes not yet saved** When a workflow is valid but there are unsaved changes, a single message is displayed at the bottom of the editor saying "The workflow must be saved". The unsaved state is also represented by the asterisk in the name of the tab (figure 13.14).

   Valid workflows can be run before they are saved, allowing changes to be tested before overwriting any previously saved version.

   The **Installation...** button is enabled when a workflow is valid and has been saved. See section or chapter 13.6.2 for information about workflow installation.

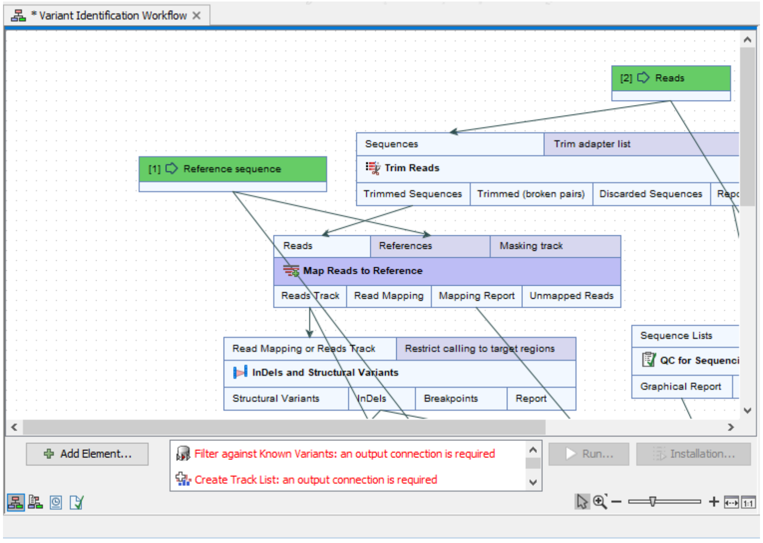
3. **Invalid** Each problem in a workflow is reported at the bottom of the editor (figure 13.15).

   Clicking on a message about a specific element redirects the focus within the editor to that element (figure 13.16).



The workflow must be saved

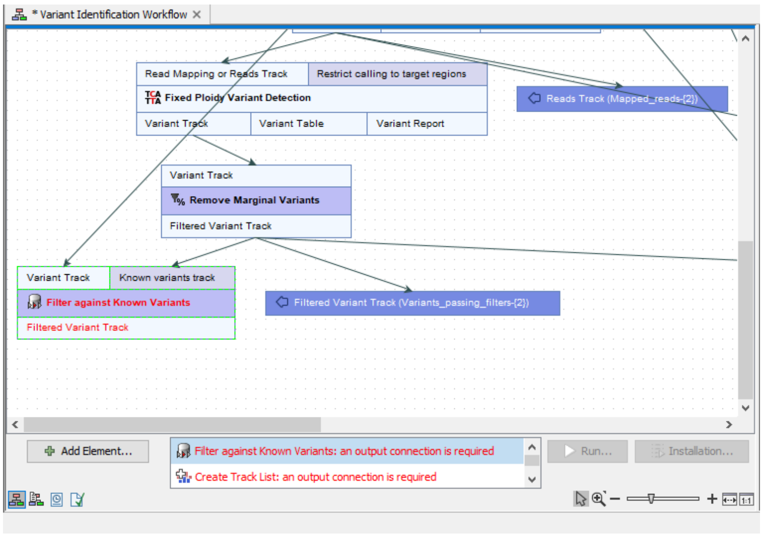
Figure 13.14: *This workflow has changes that have not yet been saved, as indicated by the message at the bottom of the editor and the asterisk beside the workflow name in the tab at the top.*



Filter against Known Variants: an output connection is required

Create Track List: an output connection is required

Figure 13.15: *Problems are reported at the bottom of the workflow editor.*


* Variant Identification Workflow
Read Mapping or Reads Track
Restrict calling to target regions
Fixed Ploidy Variant Detection
Variant Track
Variant Table
Variant Report
Reads Track (Mapped_reads-{2})
Variant Track
Remove Marginal Variants
Filtered Variant Track
Variant Track
Known variants track
Filter against Known Variants
Filtered Variant Track
Filtered Variant Track (Variants_passing_filters-{2})
Add Element...
Run...
Installation...

Filter against Known Variants: an output connection is required

Create Track List: an output connection is required

Figure 13.16: *Clicking on the error message about Filter against Known Variants at the bottom of the editor moved the focus in the editor to that element.*

13.1.5 Viewing the flow of elements in a workflow

Following the path through a workflow from a particular element can help when authoring complex workflows. To highlight the path through the workflow from a particular element, right-click on the element name and select the **Highlight Subsequent Path** option from the context specific menu (figure 13.17). Select **Remove Highlighting Subsequent Path** to remove the highlighting.

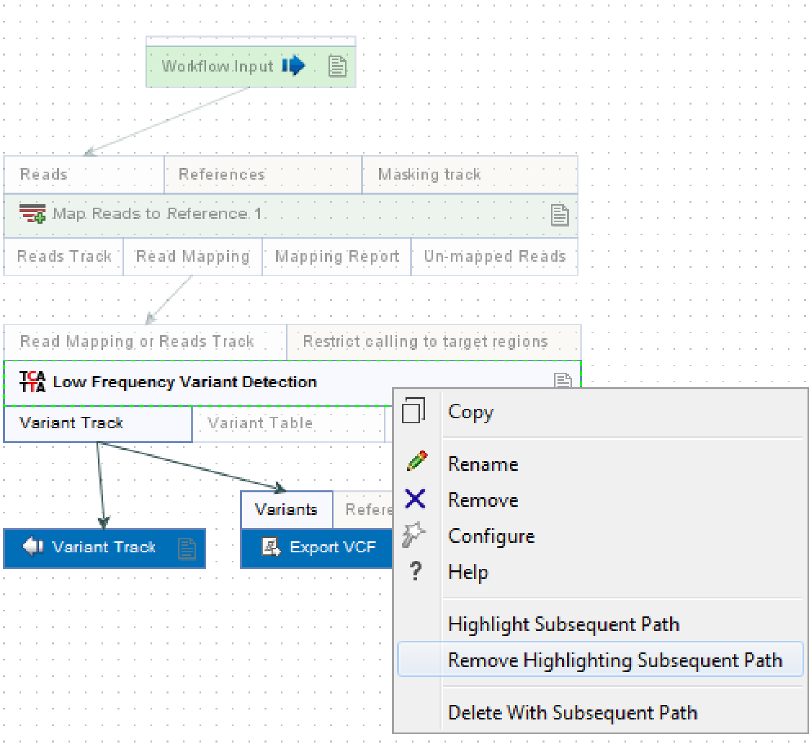


Figure 13.17: *All elements connected downstream of a selected element are highlighted after selecting the Highlight Subsequent Path menu option.*

13.1.6 Adjusting the workflow layout

The layout of elements within a workflow can be adjusted manually or automatically.

- **Manually:** Select one or more workflow elements and then, with the left mouse button depressed, drag these elements to where you want them to be on the canvas.
- **Automatically:** Right-click anywhere on the canvas and choose the option "Layout" (figure 13.18), or use the quick command Shift + Alt + L. The layout of all connected elements in the workflow will be adjusted.

See also section 13.1.9 for information about the **Auto Layout** setting. When enabled that setting causes the layout to be adjusted automatically every time an element is added and connected.

13.1.7 The Configuration Editor view

The **Configuration Editor** view allows the configuration of all of the settings in a workflow through a single window (figure 13.19). This can be more convenient than configuring each workflow

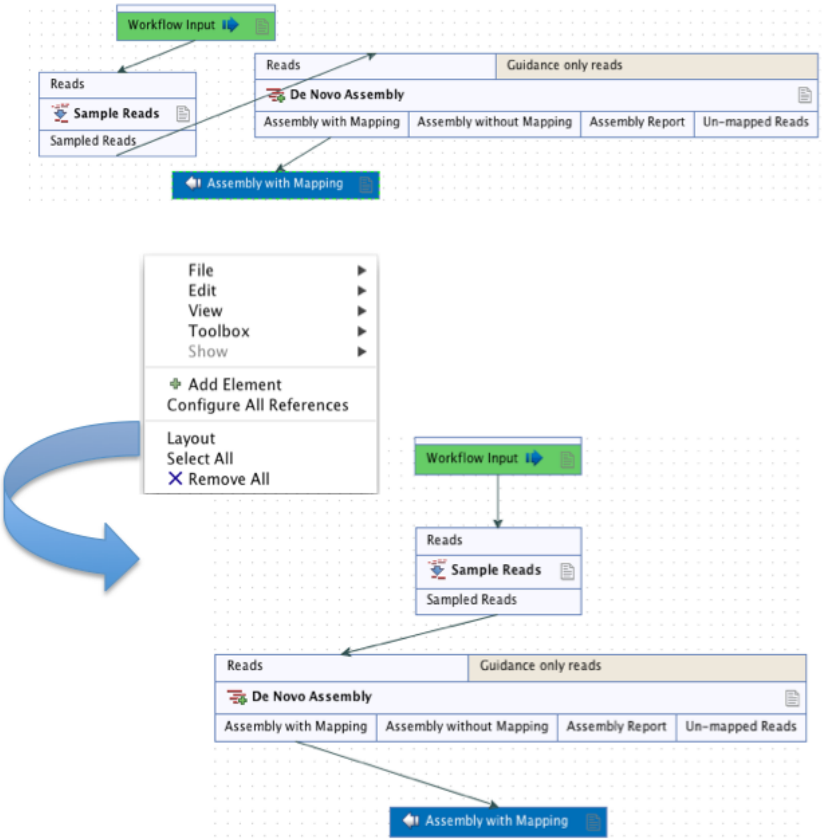


Figure 13.18: *The alignment of workflow elements can be improved using the "Layout" function.*

element individually, especially for experienced workflow authors.

Click on the ( ) icon located in the lower left corner of the Workflow Editor to open this view.

13.1.8 Snippets in workflows

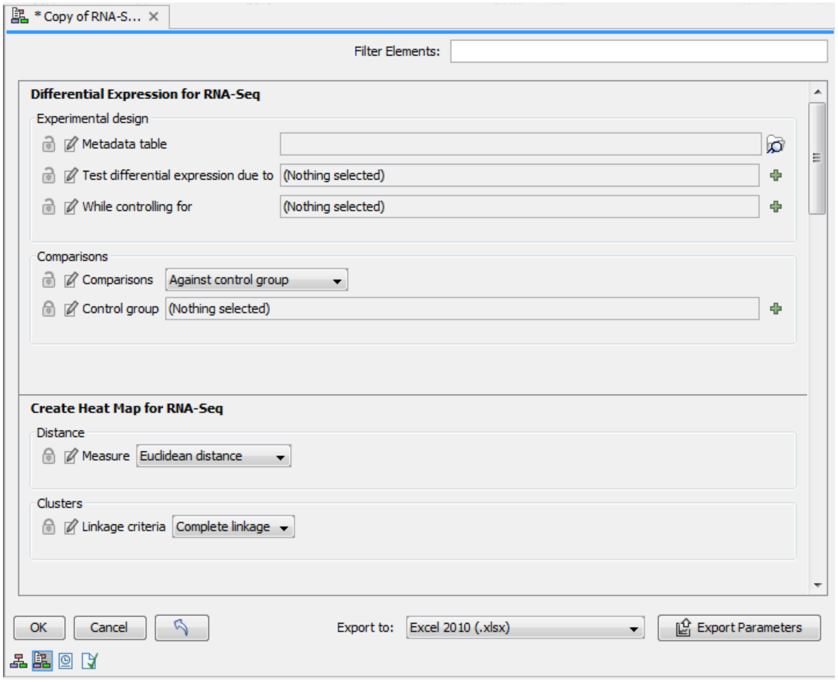
When creating a new workflow, you will often have a number of connected elements that are shared between workflows. These components are called snippets. Instead of building workflows from scratch it is possible to reuse components of an existing workflow.

Snippets can be created from an existing workflow by selecting the elements and the arrows connecting the selected elements. Next, you must right-click in the center of one of the selected elements. This will bring up the menu shown in figure 13.20.

When you have clicked on "Install as snippet" the dialog shown in figure 13.21 will appear. The dialog allows you to name the snippet and view the selected elements that are included in the snippet. You are also asked to specify whether or not you want to include the configuration of the selected elements and save it in the snippet or to only save the elements in their default configuration.

Click **OK**. This will install your snippet and the installed snippet will now appear in the **Side Panel** under the "Snippets" tab (see figure 13.22)

Right-clicking on the installed snippet in the **Side Panel** will bring up the following options (figure 13.23):



Filter Elements:

Differential Expression for RNA-Seq

Experimental design

Metadata table

Test differential expression due to: (Nothing selected)

While controlling for: (Nothing selected)

Comparisons

Comparisons: Against control group

Control group: (Nothing selected)

Create Heat Map for RNA-Seq

Distance

Measure: Euclidean distance

Clusters

Linkage criteria: Complete linkage

Export to: Excel 2010 (.xlsx)

Figure 13.19: *Use the Configuration Editor to edit configurable parameters for all the tools in a given Workflow.*

- **Add**. Adds the snippet to the current open workflow
- **View**. Opens a dialog showing the snippet, which allows you to see the structure
- **Rename**. Allows renaming of the snippet.
- **Configure**. Allows to change the configuration of the installed snippet.
- **Uninstall**. Removes the snippet.
- **Export**. Exports the snippet to ones computer, allowing to share it.
- **Migrate**. Updates the snippet (if required).

If you right-click on the top-level folder you get the options shown in figure 13.24:

- **Create new group**. Creates a new folder under the selected folder.
- **Remove group**. Removes the selected group (not available for the top-level folder)
- **Rename group**. Renames the selected group (not available for the top-level folder)

In the **Side Panel** it is possible to drag and drop a snippet between groups to be able to rearrange and order the snippets as desired. An exported snippet can either be installed by clicking on the 'Install from file' button or by dragging and dropping the exported file directly into the folder where it should be installed.

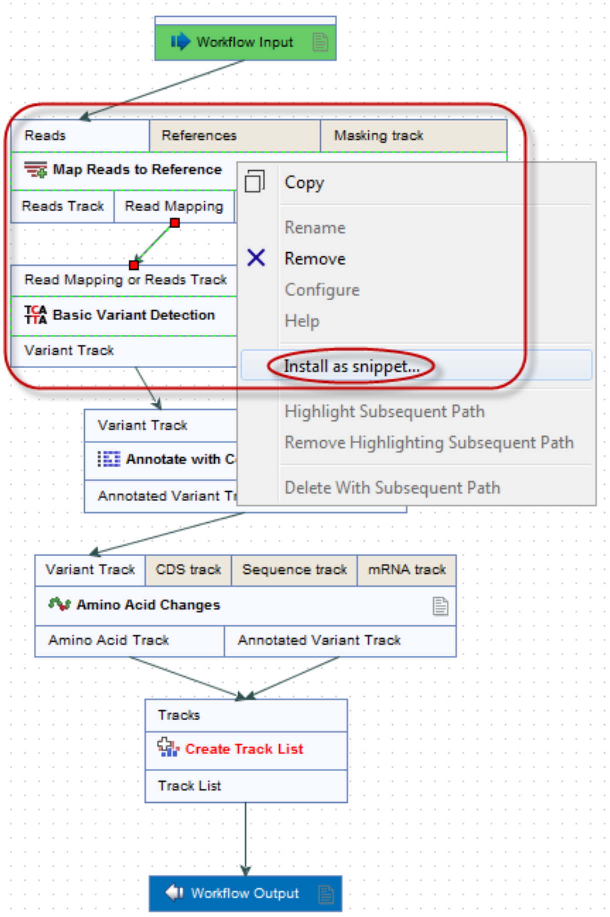
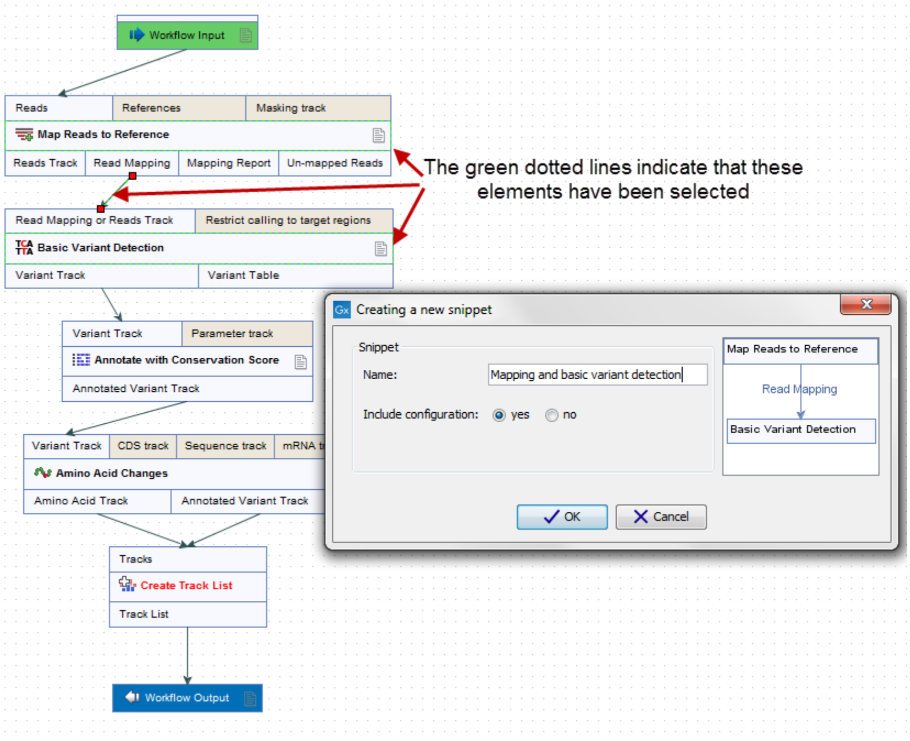


Figure 13.20: *The selected elements are highlighted with a red box in this figure. Select "Install as snippet".*

Add a snippet to a workflow Snippets can be added to a workflow in two different ways; It can either be added by dragging and dropping the snippet from the **Side Panel** into the workflow editor, or it can be added by using the "Add element" option that is shown in figure 13.25.



The green dotted lines indicate that these elements have been selected

Name: Mapping and basic variant detection

Include configuration: ● yes ○ no

Figure 13.21: *In the "Create a new snippet" dialog you can name the snippet and select whether or not you would like to include the configuration. In the right-hand side of the dialog you can see the elements that are included in the snippet.*

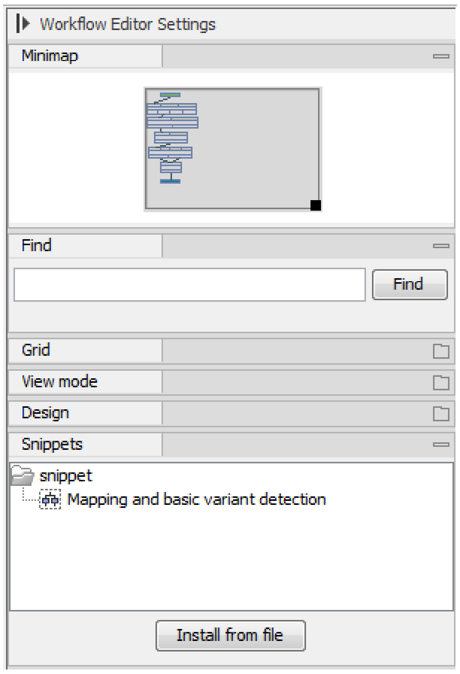


Figure 13.22: *When a snippet is installed, it appears in the Side Panel under the "Snippets" tab.*

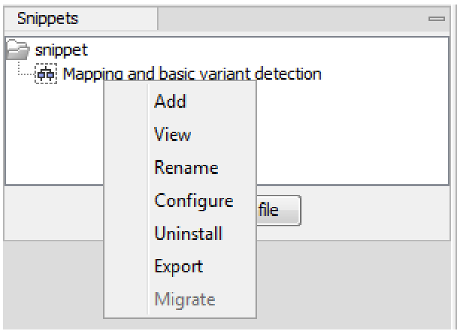


Figure 13.23: *Right-clicking on an installed snippet brings up a range of different options.*

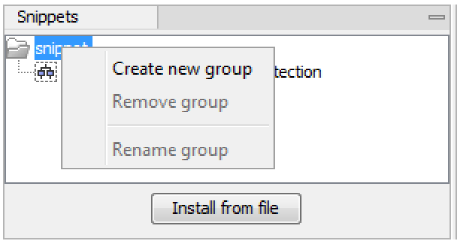
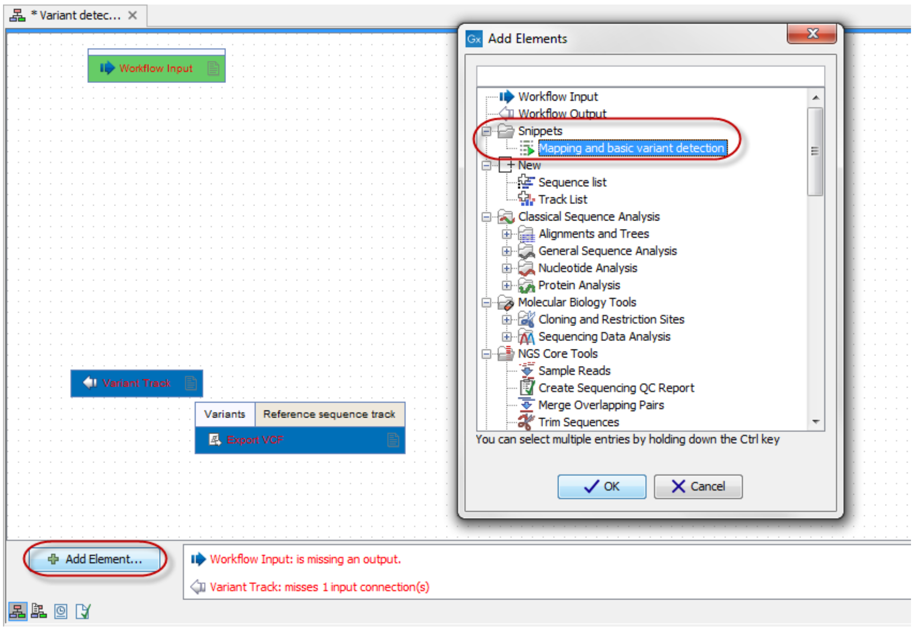


Figure 13.24: *Right-clicking on the snippet top-level folder makes it possible to manipulate the groups.*



You can select multiple entries by holding down the Ctrl key

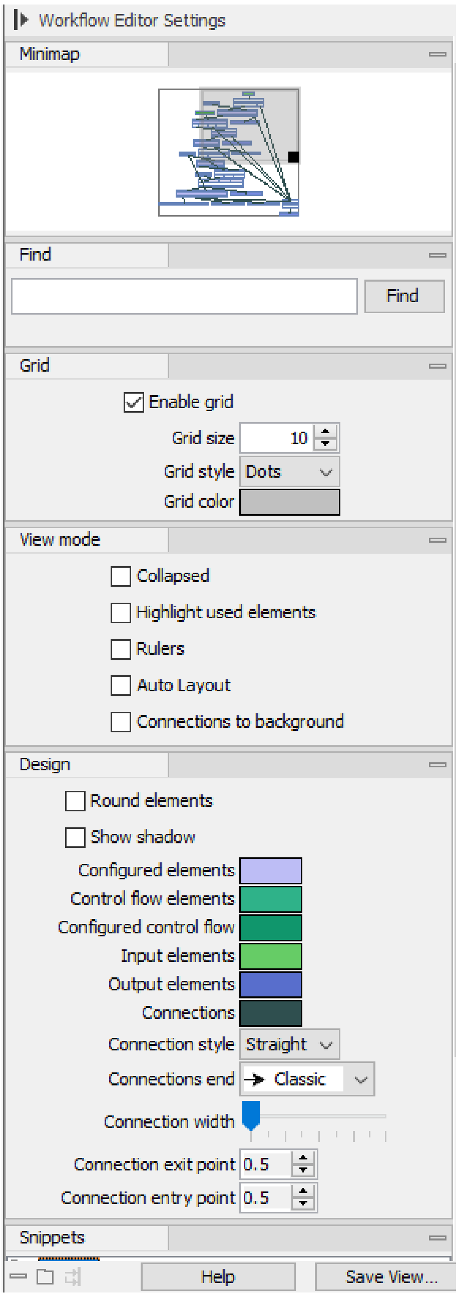
Workflow Input: is missing an output.

Variant Track: misses 1 input connection(s)

Figure 13.25: *Snippets can be added to a workflow in the workflow editor using the 'Add Element' button found in the lower left corner.*

13.1.9 Customizing the Workflow Editor

The Workflow Editor side panel (figure 13.26) contains settings that can make navigating through and editing workflows easier. They can be particularly useful when working with large workflows.



☑ Enable grid
Grid size 10
Grid style Dots
Grid color

□ Collapsed
□ Highlight used elements
□ Rulers
□ Auto Layout
□ Connections to background

□ Round elements
□ Show shadow
Configured elements
Control flow elements
Configured control flow
Input elements
Output elements
Connections
Connection style Straight
Connections end Classic
Connection width
Connection exit point 0.5
Connection entry point 0.5

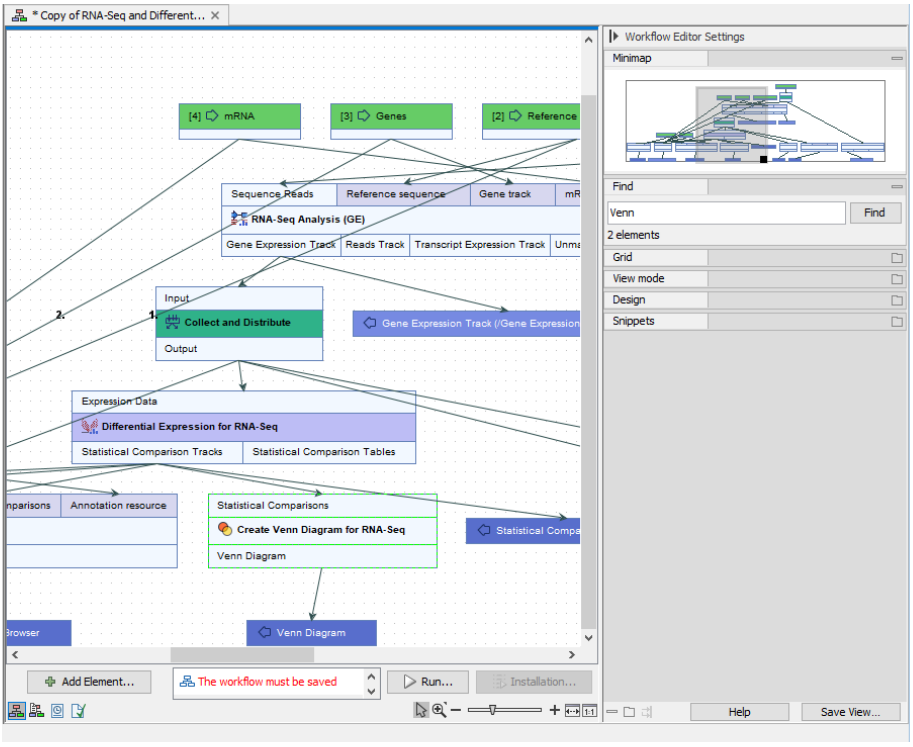
Figure 13.26: *The Workflow Editor side panel*

The Workflow Editor side panel contains the following:

Minimap A zoomed-out overview of the workflow. The darker grey box in the minimap highlights the area of the workflow visible in the editor. Drag that box within the minimap to quickly navigate to a specific area in the editor. The location of this dark grey box is updated when you navigate to another area of the workflow.

Find Search for workflow elements based on names.

After you enter a term and click on the Find button, the number of elements found with the term in their name is reported below the search field. The elements identified are highlighted in the workflow editor (figure 13.27). When multiple elements were found, the view will update so the first element found is visible in the editor. Click on the Find button again to bring the second element into view. Click on Find again to bring the third element into view, and so on.



Find: Venn

2 elements

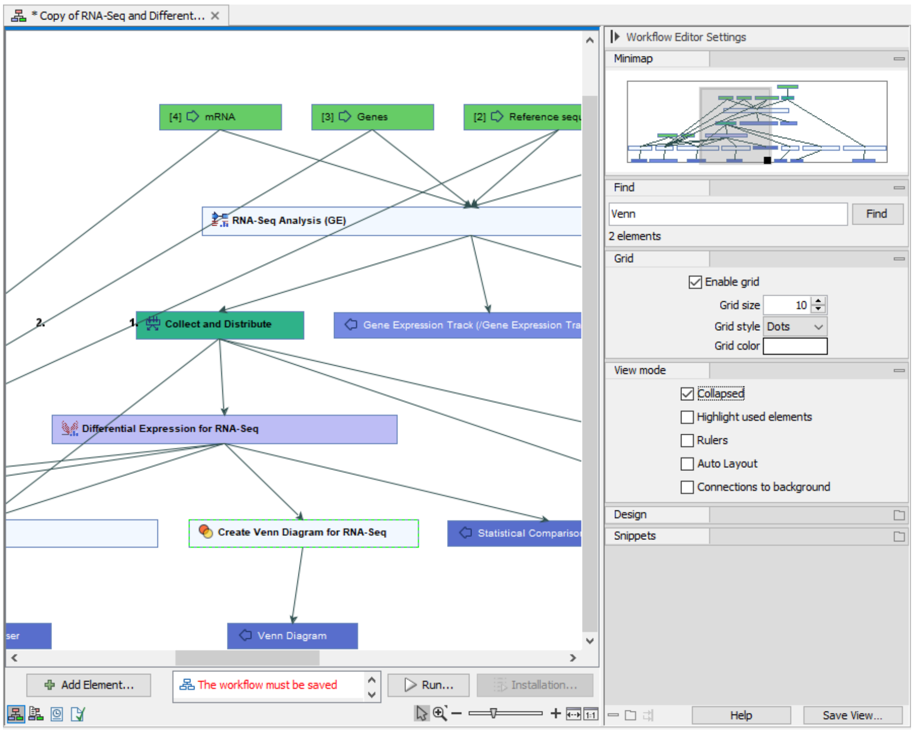
The workflow must be saved

Figure 13.27: *Two elements with names including the term "venn" were found using the Find tool in the side panel. Both are visible in this view, with the first element found highlighted.*

Grid Customize the spacing, style and color of the symbols used in the grid on the canvas, or choose not to display a grid. Workflow elements snap to the grid when they are added or moved around.

View mode Settings under the View tab are particularly useful when working with large workflows, as they can be used to remove aspects of the design that are not of immediate interest.

- **Collapsed** Enable this to hide the input and output channels of workflow elements (figure 13.28).



Find: Venn

2 elements

Grid

☑ Enable grid

Grid size 10

Grid style Dots

Grid color

View mode

☑ Collapsed

□ Highlight used elements

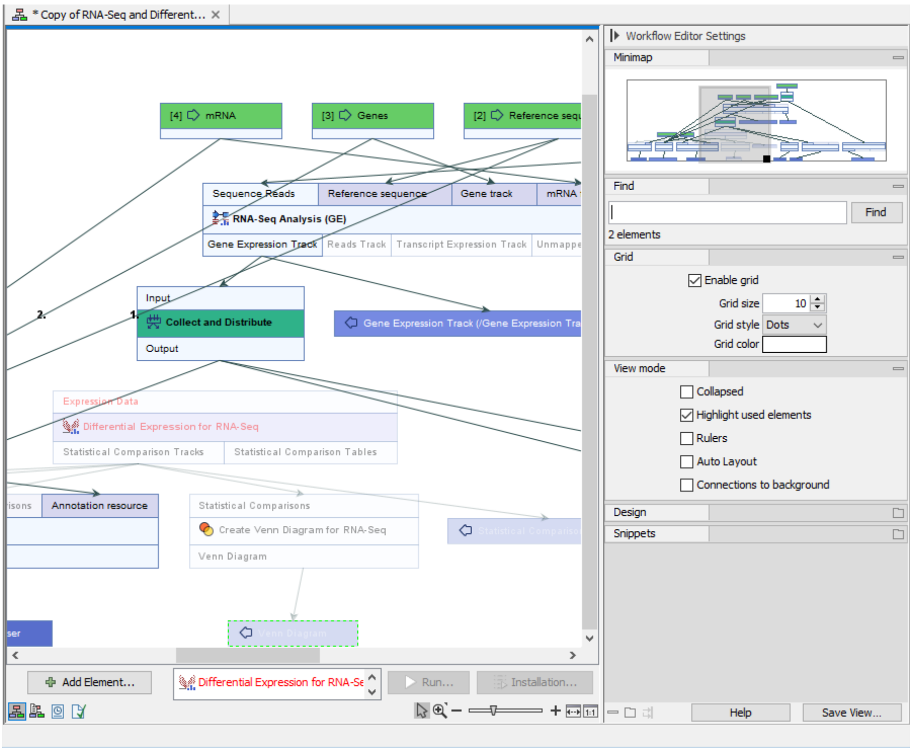
□ Rulers

□ Auto Layout

□ Connections to background

Figure 13.28: *The same workflow as above but with the "Collapse" option in the View mode settings enabled.*

- **Highlight used elements**. Enabling this option results in elements without at least one input and one output connection to appear faded. Elements connected to those missing connections are also faded (figure 13.29). (Shortcut: Alt + Shift + U)



Find: 2 elements

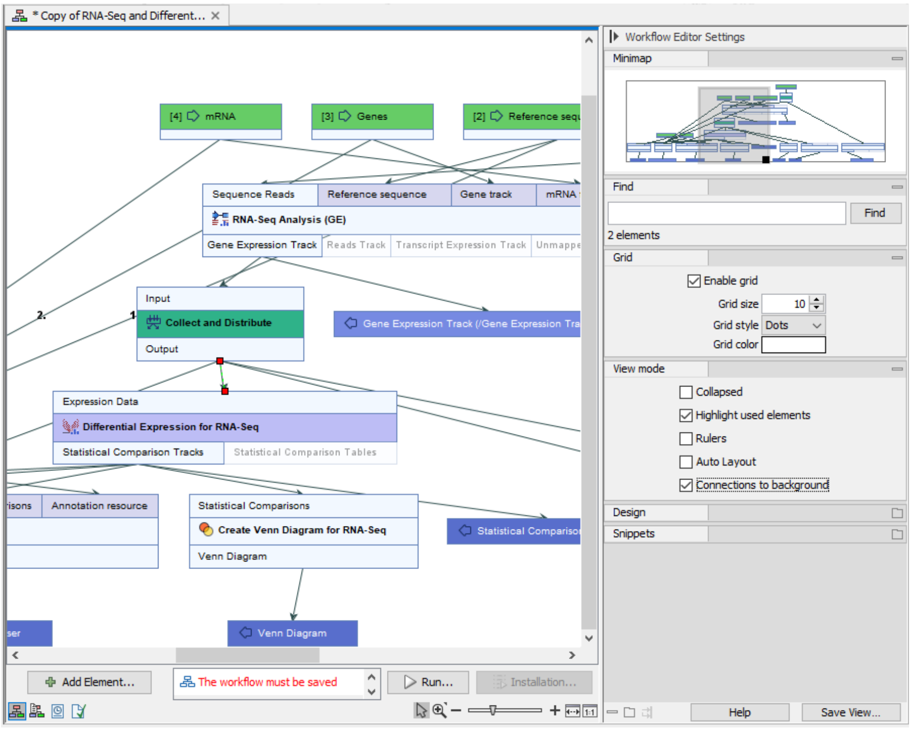
Grid:
☑ Enable grid
Grid size 10
Grid style Dots
Grid color

View mode:
☐ Collapsed
☑ Highlight used elements
☐ Rulers
☐ Auto Layout
☐ Connections to background

Figure 13.29: *A similar workflow to those above but with the "Highlight used elements" option in the View mode settings enabled. The faded coloring makes it easy to spot that the workflow arm starting with Differential Expression for RNA-Seq is not connected to the rest of the workflow.*

- **Rulers** Adds rules along the left vertical and top horizontal edges of the canvas.
- **Auto Layout** Enable this option to adjust the layout automatically every time an element is added and connected. Depending on the workflow design, using the "Layout" option in the right-click menu over the canvas can be preferable (see section 13.1.6).
- **Connections to background** Enable this to put connection lines behind workflow elements (figure 13.30).

  See also the Design options, described below, where you can change the color and design of connections.



The workflow must be saved

Find: 2 elements

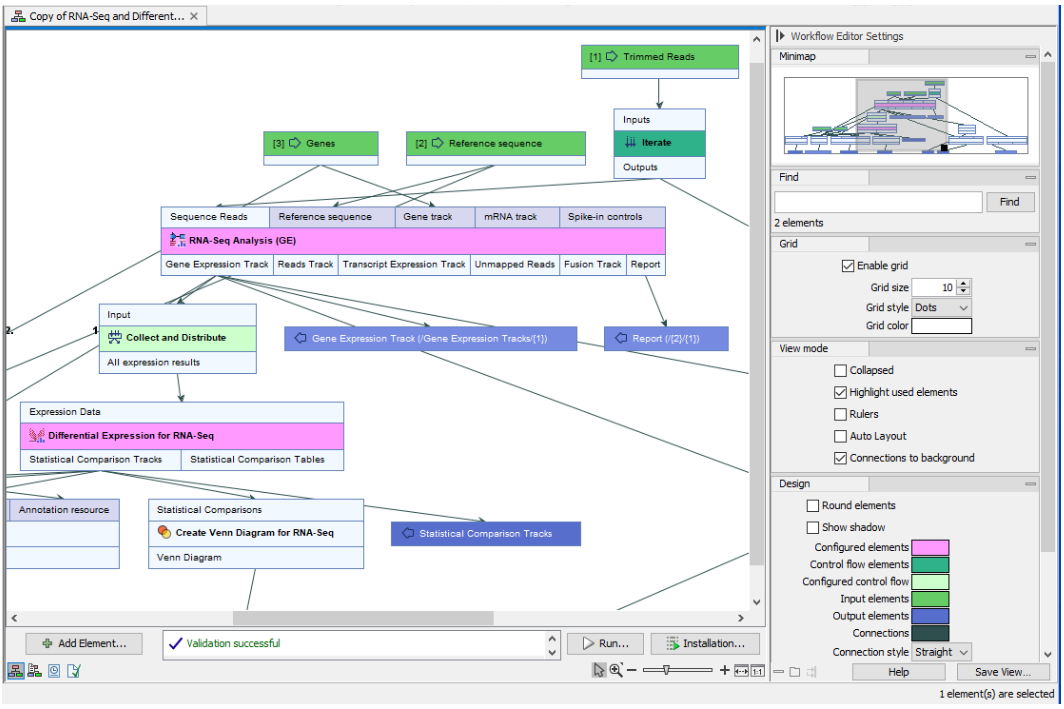
Grid
- ☑ Enable grid
- Grid size: 10
- Grid style: Dots
- Grid color:

View mode
- □ Collapsed
- ☑ Highlight used elements
- □ Rulers
- □ Auto Layout
- ☑ Connections to background

Figure 13.30: *A similar workflow to those above but with the "Connections to background" option in the View mode settings enabled.*

Design Options under the Design tab allow the shapes and colors of element and connections to be defined. Of particular note is the ability to color elements with non-default configurations differently to those with default settings.

- **Round elements** Round the corners of elements.
- **Show shadow** Add shadows under elements.
- **Coloring** Customize element background colors. Default colors assigned to different types of elements can be specified, and elements with non-default configurations can be assigned a different color to elements with default configurations (figure 13.31). See section 13.2.1) for information on element categories.
- **Connection related options** Customize connection lines between elements, including whether they should be straight or elbow lines, their width and where they should enter and exit elements.



✓ Validation successful

Find

2 elements

Grid

☑ Enable grid

Grid size 10

Grid style Dots

Grid color

View mode

☐ Collapsed

☑ Highlight used elements

☐ Rulers

☐ Auto Layout

☑ Connections to background

Design

☐ Round elements

☐ Show shadow

Configured elements 🟪

Control flow elements 🟩

Configured control flow 🟩

Input elements 🟩

Output elements 🟦

Connections ⬛

Connection style Straight

1 element(s) are selected

Figure 13.31: *A similar workflow to those above, but where standard elements with non-default configuration have been assigned the color pink and control flow elements with non-default configurations have been assigned a pale green color, making them easy to spot.*

Snippets Snippets are sections of workflows, which have been saved and can be easily added to a new workflow. These are described in section 13.1.8.

13.2 Workflow elements

Workflow elements are the building blocks of workflows. Detailed customization of how the workflow will behave can be done by adding and connecting relevant elements, and also by setting individual parameter values, and choosing which parameter values can be edited when launching the workflow. Customization of how the workflow will look when used is also possible by editing workflow element names and the names of their parameter values. These names are used in the wizard generated when the workflow is launched.

In this section, we describe general aspects of workflow elements and then focus on particular element types, the role they play in workflows, and their configuration.

13.2.1 Anatomy of workflow elements

Workflow elements can pass data to other elements, accept data from other elements, or do both of these. Elements that data can enter into and flow out of consist of 3 regions: input channels at the top, output channels at the bottom, and the core section in the middle where the element name is (figure 13.32). A page symbol on the right hand side of the middle section of a workflow element indicates the element can be configured.

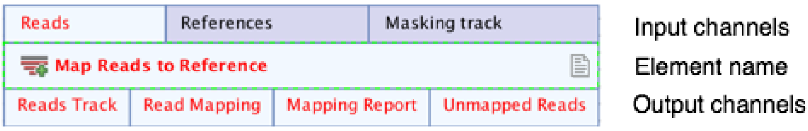


Figure 13.32: *Anatomy of a workflow element.*

Workflow element coloring

Workflow elements are colored according to their category (figure 13.33):

Light green Input elements. Elements for taking in the data to be analyzed.

Dark blue Output and Export elements. Elements that indicate data should be saved to disk, either in a CLC location (Output elements) or any other accessible file location (Export elements).

Light grey An analysis element where the default values are used for all settings.

Purple A configured analysis element, i.e. one or more values in that element have been changed from the defaults.

Emerald green Control flow elements with default settings.

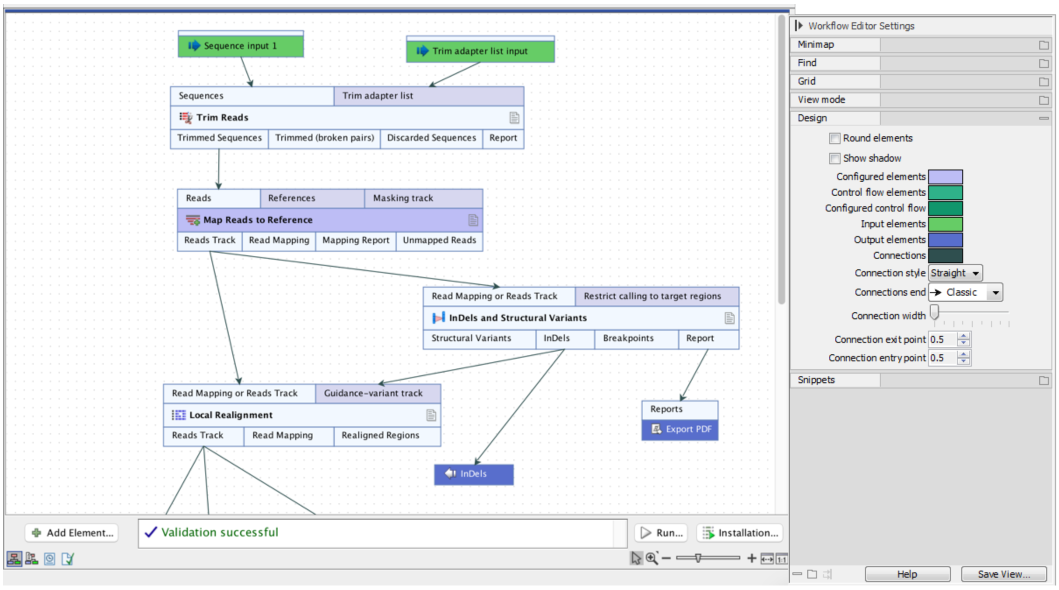
Forest green Configured control flow elements. i.e. one or more values in that element have been changed from the defaults.

Background colors can be changed under the Design tab in the side panel settings of the Workflow editor.

The name of a new element added to a workflow is shown in red text until it is properly connected to other elements.

Configuring Input and Output elements is described insection 13.2.3 and section 13.2.4.

Control flow elements, used to fine tune control of the execution of whole workflows or sections of workflows, are described in section 13.2.5.



□ Round elements

□ Show shadow

Configured elements
Control flow elements
Configured control flow
Input elements
Output elements
Connections
Connection style: Straight
Connections end: Classic
Connection width
Connection exit point: 0.5
Connection entry point: 0.5

Figure 13.33: *An element's color indicates the role it plays and its state. Here, Trim Reads means is using only default parameter values, whereas the purple background for Map Reads to Reference indicates that one or more of its parameter values have been changed. The green elements are Input elements. The blue color of the InDels element and parts of the Export PDF element indicate that data sent to these elements will be saved to disk.*

13.2.2 Basic configuration of workflow elements

Workflow elements can be renamed, and options within the elements can be customized, affecting what is seen when editing the workflow, what is seen when launching the workflow, and the settings used by default when the workflow is run.

Renaming workflow elements

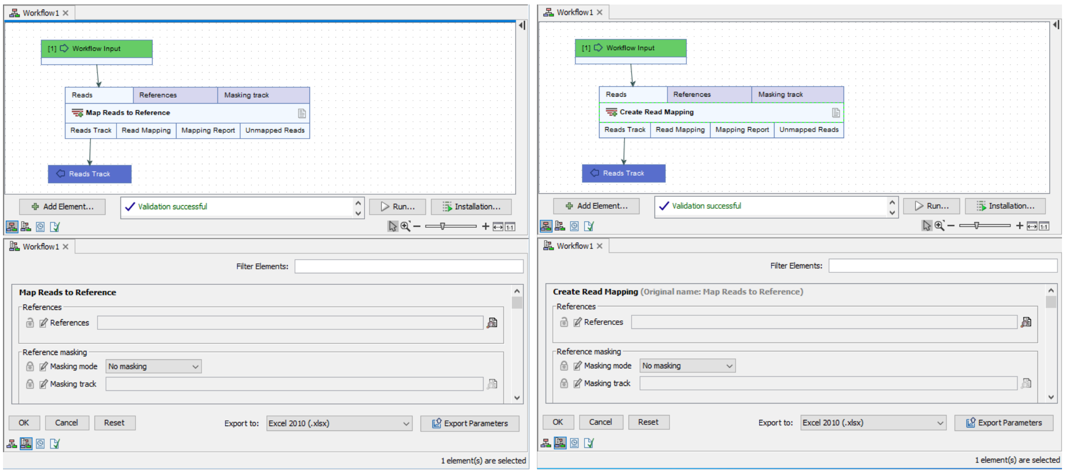
Renaming workflow elements can aid understanding of a workflow design, especially when there are multiple elements for the same tool. Updated element names are reflected in the workflow launch wizard.

To rename an element, right click on the element name and choose the option "Rename...", or select the element in the Workflow Editor and press the F2 key.

The **Find** functionality in the side panel finds elements based the new name.

Both the new name and the original, default name of elements are provided in the Workflow Configuration view (figure 13.34). Terms in the new name or the original, default name can be used in the **Filter Elements** functionality of this view to find settings for configurable elements. This view is described in section 13.1.7.

Note: The Workflow Configuration view can be opened linked to the Workflow view. Linked views are described further in section 2.1.



Map Reads to Reference

References

References

Reference masking

Masking mode: No masking

Masking track

Create Read Mapping (Original name: Map Reads to Reference)

References

References

Reference masking

Masking mode: No masking

Masking track

Figure 13.34: *A workflow before (left) and after (right) the Map Reads to Reference element was renamed. In the linked Workflow Configuration view at the bottom right, both the original and updated element names are listed.*

Configuring options in workflow elements

Configuring options can be done in a workflow element's configuration dialog by:

- Double-clicking on an element name in the Workflow view, or
- Right-clicking on an element name and choosing the Configure... option from the menu that appears.

Options can also be edited in the Workflow Configuration view (figure 13.34).

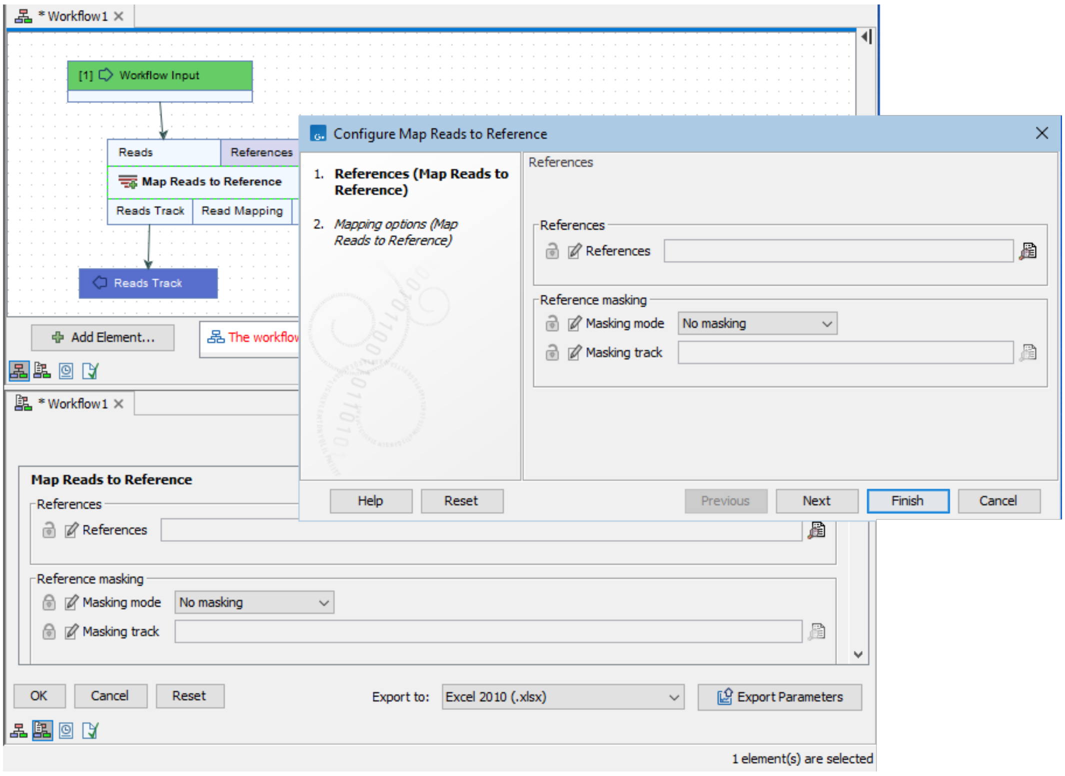
Workflow element customization can include:

1. **Specifying which values can be changed when launching the workflow**

   Beside each option is a lock symbol. Values of unlocked (🔓) options can be changed when launching the workflow. Values of locked (🔒) options cannot be changed when launching the workflow.

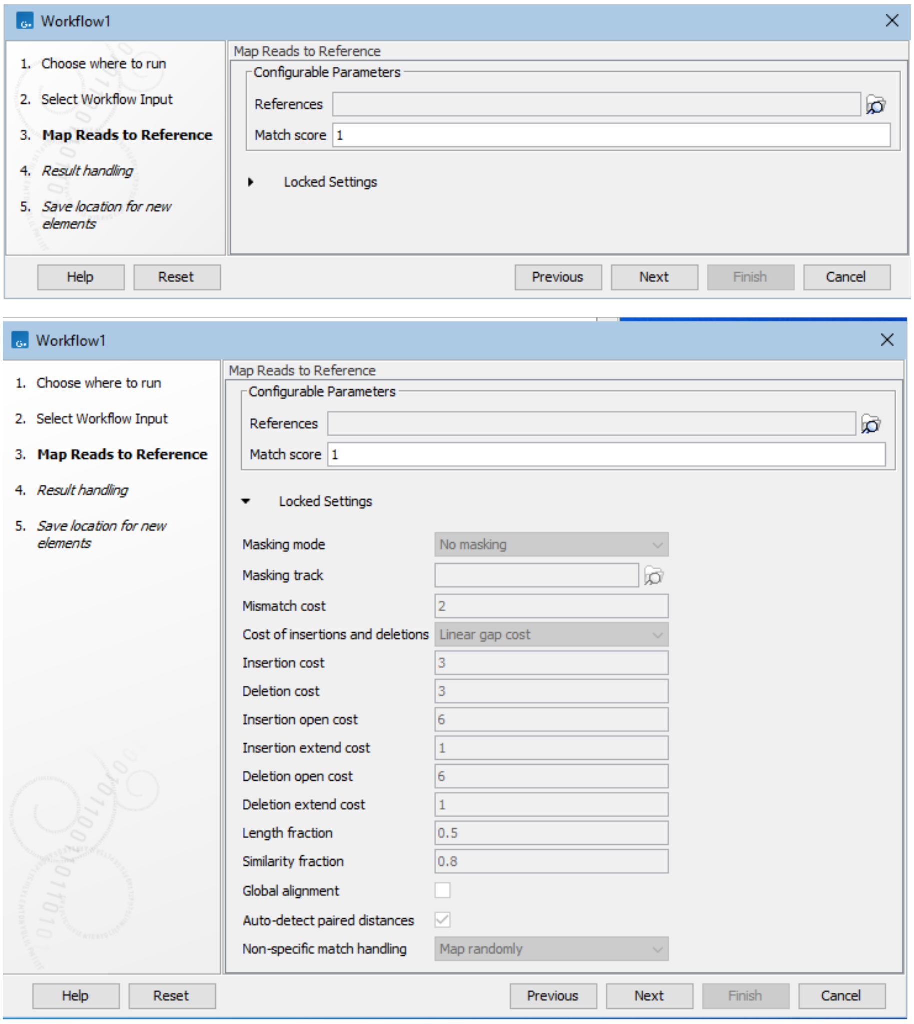
   Click on a lock icon to change it from locked (🔒) to unlocked (🔓), or vice versa.

   The values of all locked settings can been seen in the workflow launch wizard, but they are not presented by default (figure 13.36).



References: References
Reference masking: Masking mode: No masking; Masking track
Export to: Excel 2010 (.xlsx)

Figure 13.35: *The Workflow view (top) and Workflow Configuration view (bottom) have been opened as linked views. The Map Reads to Reference element has been opened for configuration in the Workflow view and the Masking mode and Masking track options have been unlocked. They will correspondingly appear unlocked in the Workflow Configuration view after the Finish button is clicked.*



Workflow1

1. Choose where to run
2. Select Workflow Input
3. **Map Reads to Reference**
4. *Result handling*
5. *Save location for new elements*

Map Reads to Reference

Configurable Parameters

References

Match score 1

▸ Locked Settings

Workflow1

1. Choose where to run
2. Select Workflow Input
3. **Map Reads to Reference**
4. *Result handling*
5. *Save location for new elements*

Map Reads to Reference

Configurable Parameters

References

Match score 1

▾ Locked Settings

Masking mode	No masking
Masking track	
Mismatch cost	2
Cost of insertions and deletions	Linear gap cost
Insertion cost	3
Deletion cost	3
Insertion open cost	6
Insertion extend cost	1
Deletion open cost	6
Deletion extend cost	1
Length fraction	0.5
Similarity fraction	0.8
Global alignment	□
Auto-detect paired distances	☑
Non-specific match handling	Map randomly

Figure 13.36: *A workflow launch wizard step showing the configurable (unlocked) options at the top, with a heading for the locked settings (top). Clicking on the Locked Settings heading reveals a list of the locked options and their values (bottom)*

2. **Changing default values**

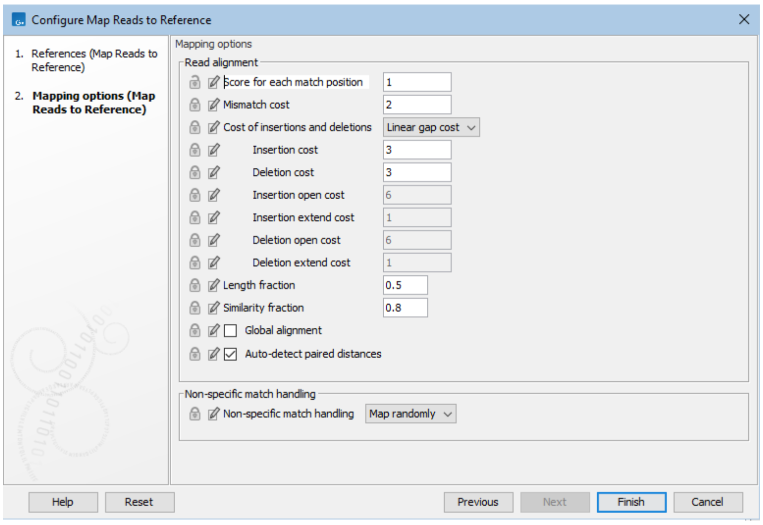
   When elements are added to a workflow, the default values assigned to options are the same as the defaults for the corresponding tool when run from the Tools menu. Values for options can be updated in the workflow design. When this is done, the new values become the defaults used when the workflow is run.

3. **Changing option names**

   When an option name is changed, the new name is used in the workflow launch wizard.

   Renaming options can be useful when only a few options can be configured when launching the workflow and their default names are quite generic.

   Click on the edit icon (📝) beside an option to edit the name (figure 13.37).


Configure Map Reads to Reference
1. References (Map Reads to Reference)
2. Mapping options (Map Reads to Reference)
Help
Reset
Previous
Next
Finish
Cancel

Mapping options

Read alignment

Score for each match position	1
Mismatch cost	2
Cost of insertions and deletions	Linear gap cost
Insertion cost	3
Deletion cost	3
Insertion open cost	6
Insertion extend cost	1
Deletion open cost	6
Deletion extend cost	1
Length fraction	0.5
Similarity fraction	0.8

□ Global alignment

☑ Auto-detect paired distances

Non-specific match handling

Non-specific match handling: Map randomly

Figure 13.37: *An option originally called "Match score" has been renamed "Score for each match position" in the element configuration dialog. It has also been unlocked so the value for this option will be configurable when launching the workflow.*

Note: Clicking on the **Reset** button in a workflow element configuration dialog will reset all changes *in that particular configuration step* to the defaults, including any updated option names.

13.2.3 Configuring Workflow Input elements

Workflow Input elements are used to provide data to a workflow. At least one such element is required in a workflow for supplying the data for analysis. Input elements can be connected to any input channel expecting data.

When there is more than one Input element in a workflow, the order that data is prompted for in the launch wizard can be configured (see Ordering inputs (section 13.1.3)).

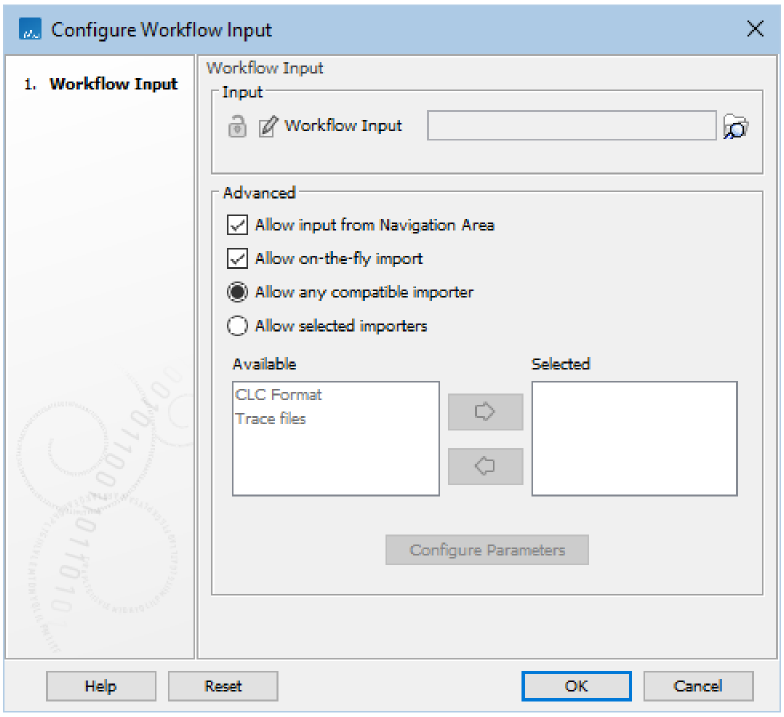
Configuring Input element options

By default, Input elements are configured to allow data to be selected from CLC locations listed in the Navigation Area, or from elsewhere, in which case, the data will be imported using on-the-fly import. The workflow author can limit these options (figure 13.38), as well as configure import options with non-default values. Settings can be locked if they should not be configurable when launching the workflow (see Basic configuration of workflow elements (section 13.2.2)).

On-the-fly import options that can be configured in Input elements are:

- **Allow any compatible importer** All compatible importers will be available when launching the workflow and all the options for each importer will be configurable.
- **Allow selected importers** Specify particular importers to be available when launching the workflow. Options for each selected importer can be configured by clicking on the **Configure Parameters** button.

Note: To specify CLC data not stored in a CLC location as input to a workflow, on-the-fly import must be allowed, and CLC Format must be one of the allowed importers. See also Launching workflows individually and in batches (section 13.3).



☑ Allow input from Navigation Area
☑ Allow on-the-fly import
● Allow any compatible importer
○ Allow selected importers

Available: CLC Format, Trace files

Figure 13.38: *The allowed data sources are configured in Input elements. By default, both checkboxes in the Advanced section are enabled, allowing data to be selected from a CLC location (input from the Navigation Area), or from another location (on-the-fly import). For on-the-fly import, any available importer can be used by default. When only specific importers are allowed, those importers can be configured by selecting each in turn and clicking on the "Configure Parameters" button. In this case, only the data types supported by the specified importers, can be selected as input for on-the-fly import when launching the workflow.*

The "Workflow role" field visible when configuring Workflow Input elements connected to parameter input channels is relevant when working with the *CLC Genomics Workbench*. Further information about this can be found at https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=QIAGEN_Sets.html and https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Custom_Sets.html

Saving data imported on-the-fly

To save data elements created using on-the-fly import, connect a **Save On-the-Fly Imports** ( ) element to the relevant Input element and connect an Output element to it (figure 13.39).

The **Save On-the-Fly Imports** element only acts on data imported using on-the-fly import. If input data is selected from a CLC File Location, this element has no effect.

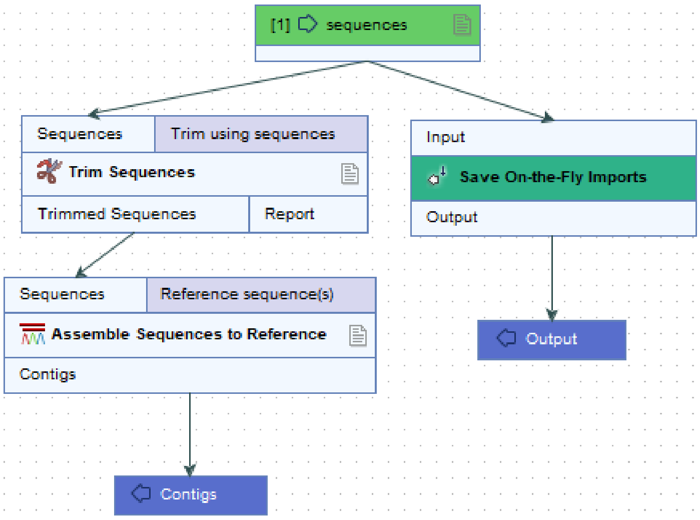


Figure 13.39: *Using this workflow, data imported on-the-fly would be saved as an output from the Save On-the-Fly Imports element.*

A **Save On-the-Fly Imports** element is not needed when an Iterate element is connected to the Input element being used for on-the-fly import. In this situation, an Output element can be connected directly to the Iterate element (figure 13.40).

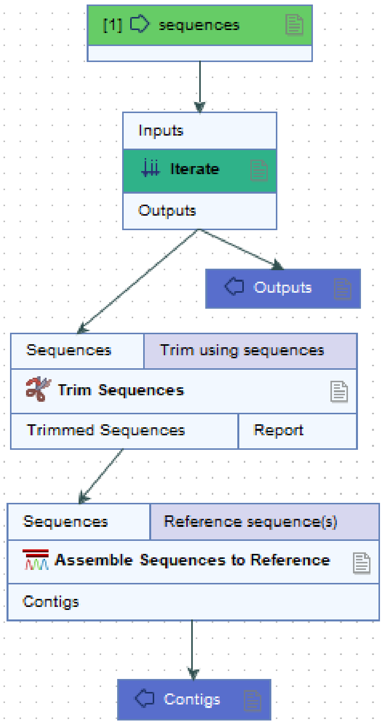


Figure 13.40: *When an Iterate element is connected to an Input element, data imported on-the-fly can be saved by connecting an Output element to the Iterate element.*

13.2.4 Configuring Workflow Output and Export elements

> Results generated by a workflow are only saved if the relevant output channel of a workflow element is connected to a Workflow Output element or an Export element. *Data sent to output channels without an Output or Export element attached are not saved*.
>
> Terminal workflow elements with output channels must have at least one Workflow Output element or Export element connected.

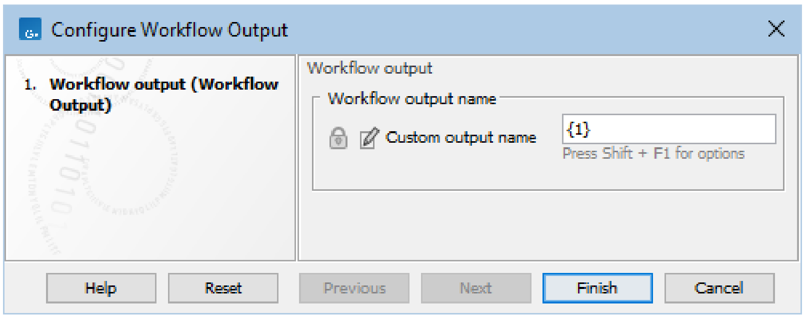
Configuring custom names for workflow results

The names to assign to outputs and exported files from workflows can be configured to include specific text as well as information taken from a workflow run, for example, the names of inputs to the analysis, dates and times the results were generated, etc.

To configure the naming pattern for a Output or Export workflow element, double-click on it, or right-click on it and then select the option **Configure...** from the menu. The naming pattern in Output elements is defined in the **Custom output name** field (figure 13.41). In Export elements, it is defined in the **Custom file name** field.

Hover the mouse cursor over the configuration field to reveal a tooltip containing a list of available *placeholders* (figure 13.42). Placeholders are terms within curly brackets used to indicate that particular information from a workflow run should be included in the output name or exported file name. Terms in placeholders are not case specific.

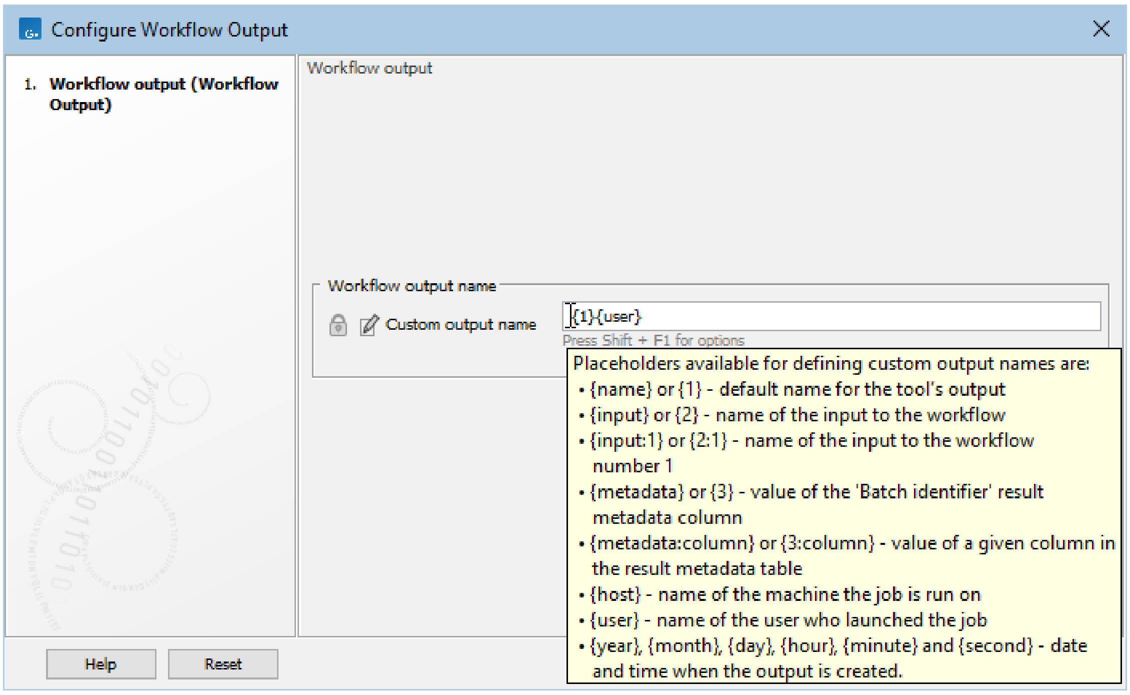
Note: Placeholders used by export tools run directly (not via a workflow) are described in section 8.1.3. Other settings relating to export, relevant both for exports run directly or in a workflow context, are described in section 8.1.2.



Custom output name: {1}

Press Shift + F1 for options

Figure 13.41: *Defining the name to assign to an output from a workflow. The default naming pattern for Output elements uses the placeholder {1}, which is a synonym for the placeholder {name}.*


Configure Workflow Output
1. Workflow output (Workflow Output)
Workflow output
Workflow output name
Help
Reset

Custom output name: {1}{user}

Press Shift + F1 for options

Placeholders available for defining custom output names are:
- {name} or {1} - default name for the tool's output
- {input} or {2} - name of the input to the workflow
- {input:1} or {2:1} - name of the input to the workflow number 1
- {metadata} or {3} - value of the 'Batch identifier' result metadata column
- {metadata:column} or {3:column} - value of a given column in the result metadata table
- {host} - name of the machine the job is run on
- {user} - name of the user who launched the job
- {year}, {month}, {day}, {hour}, {minute} and {second} - date and time when the output is created.

Figure 13.42: *Hover the mouse cursor over the field where a custom name can be configured to reveal a tooltip with a list of available placeholders.*

Placeholders available for Output and Export workflow elements are:

- **{name}** or **{1}** The default name for that output from that tool, i.e. the name that would be used if the tool was run outside a workflow context.
- **{input}** or **{2}** The name of the primary workflow input(s) for the path of the workflow being traversed.

  "Primary workflow input" generally refers to the data being analyzed, i.e. inputs expecting sample data, as opposed to inputs expecting reference data.

For a workflow with multiple primary inputs to an arm of the workflow, **{input}**, or its equivalent **{2}**, would result in the name of each of these primary inputs being included in the names of the outputs from that workflow arm (figure 13.43).

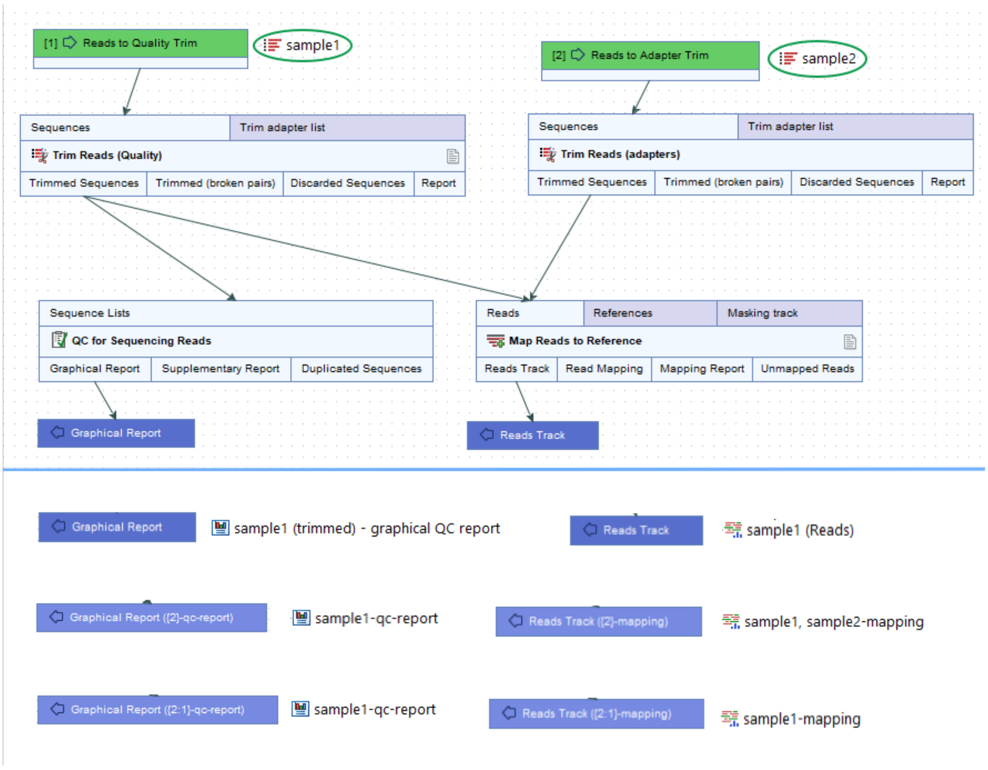


Figure 13.43: *Top: A contrived workflow with two primary inputs (green boxes). The QC for Sequencing Reads step receives data only from the first input, "Reads to Quality Trim". The Map Reads to Reference step receives data originating from both primary inputs. Bottom: The effect of different naming patterns on result names when a sequence list called "sample1" was supplied for the first input and a sequence list called "sample2" was supplied for second input. The first row shows the Output elements and results using the default naming pattern, {1}. The middle row shows the Output elements and results when the naming pattern included the placeholder {2}, and the last row shows them when the naming pattern included the placeholder {2:1}.*

- **{input:N}** or **{2:N}** The name of the Nth input to the workflow. E.g. **{2:1}** specifies the first input to the workflow, while **{2:2}** specifies the second input (figure 13.43).

  Unlike the general form described above, i.e.**{input}** or **{2}**, reference data inputs can be included in names using this placeholder form (figure 13.44).

  For a workflow with only one primary input, **{input}** or **{2}** is equivalent to the more specific form **{input:1}** or **{2:1}**.

  For workflows containing control flow elements, the specific placeholder form, `{2:N}`, is recommended.

  See section 13.1.3 for information about workflow input ordering, and section 13.2.5 for information about control flow elements.

- **{metadata}** or **{3}** The batch unit identifier for workflows executed in batch mode. Depending on how the workflow was configured at launch, this value may be obtained from metadata.

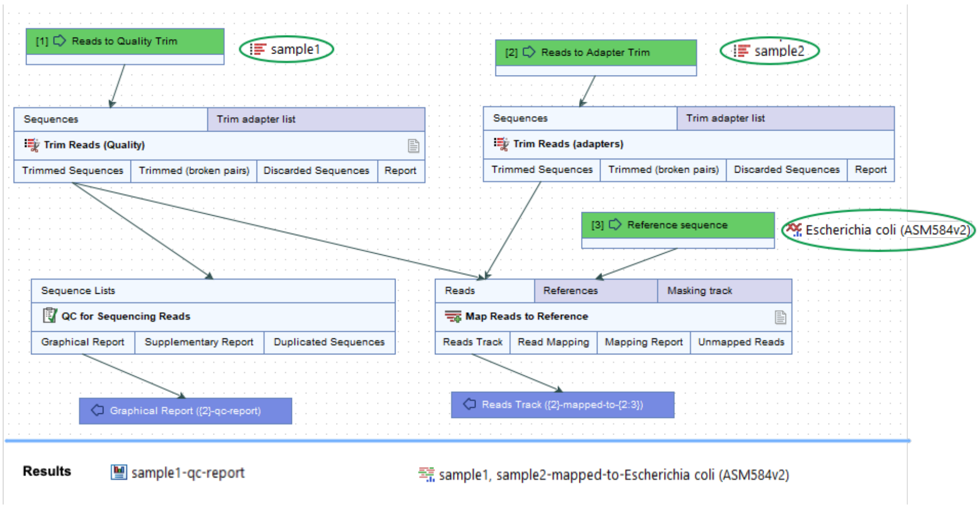


Figure 13.44: *Top: A contrived workflow with two primary inputs and a reference data input (green boxes). Bottom: The names of the results generated in a given workflow run. The naming pattern for the Reads Track output includes {2}, which adds the names of all primary inputs to that analysis step, (sample1, sample2), and {2:3}, which adds the name of the third input, whatever the role that input has. In this case, it is a reference data input and an element called Escherichia coli (ASM584v2) was supplied .*

For workflows not executed in batch mode or without Iterate elements, the value will be identical to that substituted using **{input}** or **{2}**.

Note: For workflows containing control flow elements, the more specific form of placeholder, i.e. the `metadata:columnname` or `{3:columnname}` form, described below, is recommended.

- **{metadata:columnname}** or **{3:columnname}** The value for the batch unit in the column named "columnname" of the metadata selected when launching the workflow. Pertinent for workflows executed in batch mode or workflows that contain Iterate elements. If a column of this name is not found, or a metadata table was not provided when launching the workflow, then the value will be identical to that substituted using **{input}** or **{2}**.
- **{user}** The username of the person who launched the job
- **{host}** The name of the machine the job is run on
- **{year}**, **{month}**, **{day}**, **{hour}**, **{minute}**, and **{second}** Timestamp information based on the time an output is created. Using these placeholders, items generated by a workflow at different times can have different file names.

In addition to the placeholders above, the placeholder **{extension}** is available for exported file names. This is replaced by the default file extension for the exported file's format, e.g. .pdf, .txt.

Saving results to subfolders

Workflow outputs and exported files can be saved into subfolders by adding a forward slash / at the start of the custom name definition.

For example, with an Output element configured with `/variants/{name}`, the resulting output would be saved to a subfolder called `variants`, placed within the folder selected for outputs when the workflow is launched. If a specified subfolder does not already exist, it is created when the outputs are saved.

When defining subfolders for outputs or exported files, terms between all forward slash characters are interpreted as subfolders. For example, a name like `/variants/level2/level3/myoutput` would put the data item called `myoutput` into a folder called `level3` within a folder called `level2`, which itself is inside a folder called `variants`. The `variants` folder would be placed under the location selected for storing the workflow outputs.

Temporary, intermediate workflow results

During a workflow run, temporary, intermediate results may be generated, including for output channels that aren't connected to an Output or Export element.

Such intermediate results are normally deleted automatically after the workflow run completes. If a problem arises such that the workflow does not complete normally, intermediate results may not be deleted and will be in a folder named after the workflow with the word "intermediate" in its name.

13.2.5 Control flow elements

Control flow elements control the flow of data through a workflow. They can be found in the Control Flow folder of the Add Elements wizard (figure 13.45).

Elements are available for:

1. Controlling how data is grouped for analysis. These include **Iterate** and **Collect and Distribute**, described in section 13.2.5.
2. Controlling the flow of the workflow based on its configuration when launched. These include **Fork**, described in section 13.2.5, and **Save On-the-Fly Imports**, described in section 13.2.3.
3. Controlling the flow through the workflow based on aspects of the data. There are several such branching elements, described in section 13.2.5.

Iterate and Collect and Distribute elements

Iterate ( ) and **Collect and Distribute** ( ) elements are used to control how data is grouped for analysis.

- Iterate elements are placed at the top of a branch of a workflow that should be run multiple times, using different inputs in each run. The sets of data to use in each run are referred to as "batch units" or, sometimes, "iteration units".
- Collect and Distribute elements are, optionally, placed downstream of an Iterate element, where they collect outputs from the upstream iteration block (see below) and distribute them as inputs to downstream analyses.

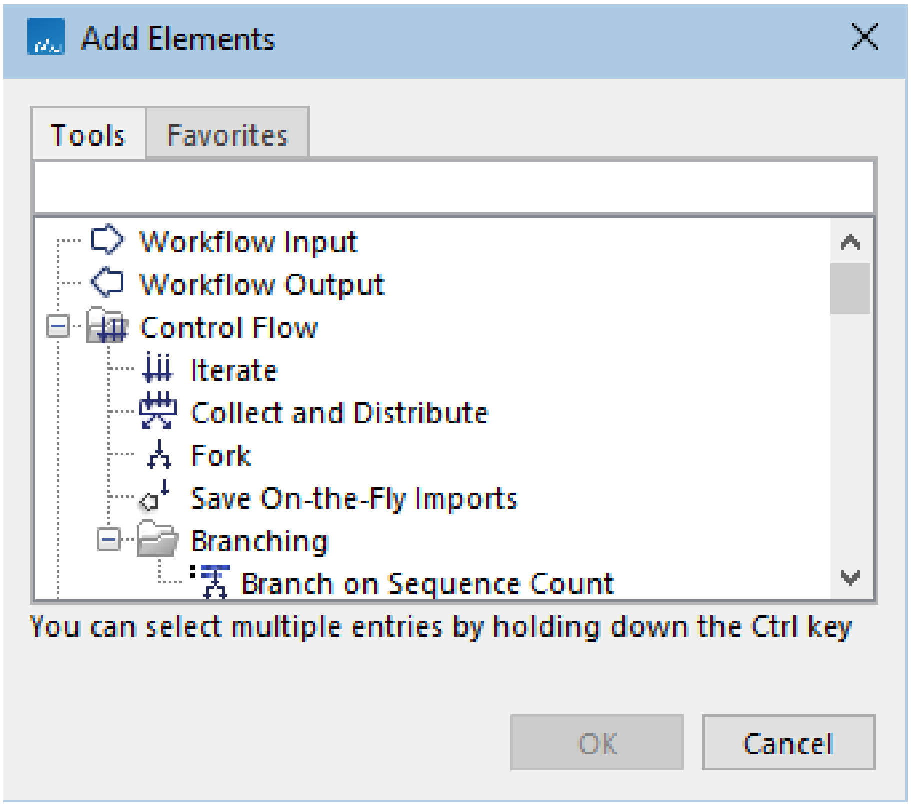


Figure 13.45: *Control flow elements are found under the Control Flow folder in the Add Elements wizard.*

The **RNA-Seq and Differential Gene Expression Analysis** template workflow, distributed with the *CLC Genomics Workbench* (https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=RNA_Seq_Differential_Gene_Expression_Analysis_workflow.html) is an example of a workflow that includes each of these control flow elements.

The steps between an Iterate element and a Collect and Distribute element are referred to as an "iteration block". The workflow in figure 13.46 contains a single iteration block (shaded in turquoise), where steps within that block are run once per batch unit. The Collect and Distribute element (renamed to Collect Expressions) collects all the results from the iteration block and sends it as input to the next stage of the analysis (shaded in purple).

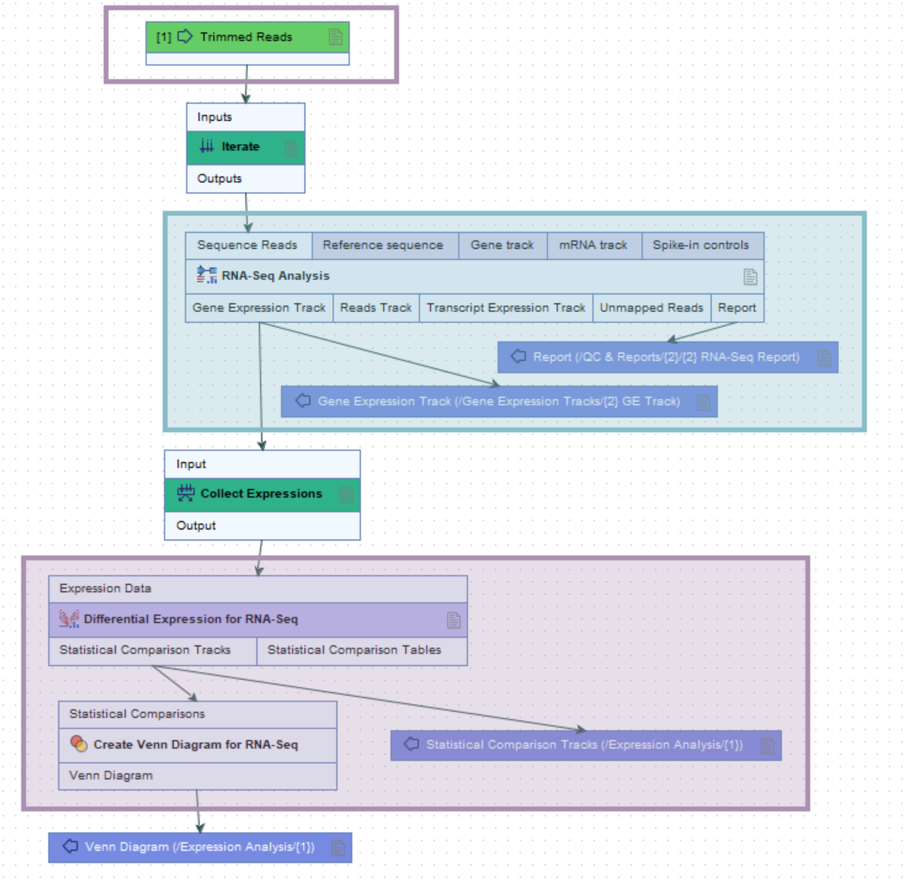

[1] Trimmed Reads
Inputs
Iterate
Outputs
Sequence Reads
Reference sequence
Gene track
mRNA track
Spike-in controls
RNA-Seq Analysis
Gene Expression Track
Reads Track
Transcript Expression Track
Unmapped Reads
Report
Report (/QC & Reports/{2}/{2} RNA-Seq Report)
Gene Expression Track (/Gene Expression Tracks/{2} GE Track)
Input
Collect Expressions
Output
Expression Data
Differential Expression for RNA-Seq
Statistical Comparison Tracks
Statistical Comparison Tables
Statistical Comparisons
Create Venn Diagram for RNA-Seq
Venn Diagram
Statistical Comparison Tracks (/Expression Analysis/{1})
Venn Diagram (/Expression Analysis/{1})

Figure 13.46: *The roles of the Iterate and Collect and Distribute control flow elements are highlighted in the context of RNA-Seq and differential expression analyses. RNA-Seq Analysis lies downstream of an Iterate element, within an iteration block (shaded in turquoise). It will thus be run once per batch unit. Differential Expression for RNA-Seq lies immediately downstream of a Collect and Distribute element (renamed to Collect Expressions), and is sent all the expression results from the iteration block as input for a single analysis.*

Defining batch units for workflows with Iterate elements

Workflow elements downstream of an Iterate element are run once for each batch unit. Details about defining batch units when launching workflows is described at section 13.3.2.

Running a workflow with a single Iterate element at the top of a workflow, no downstream Collect and Distribute element, and a single Input element is equivalent to running a similar workflow without the Iterate element in Batch mode. Setting up batch units in this situation is described in section 11.3.

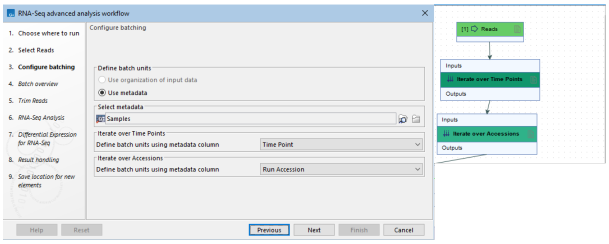
Renaming Iterate elements

Providing meaningful names to Iterate elements can help at both the workflow design stage and also when launching the workflow.

The **Rename** option is available in the menu that appears when you right-click on a workflow element.

Iterate element names are included in the workflow launch wizard in the following steps:

- Configure batching: The name of Iterate elements are provided in association with the drop-down list of column names in the metadata provided. A meaningful Iterate element name can thus help guide the choice of relevant metadata to group the inputs into batch units (figure 13.47).
- Batch overview: There is a column for each Iterate element (figure 13.48). Meaningful names can thus make it easier to review batch unit organization critically when launching the workflow.



Configure batching

Define batch units

○ Use organization of input data

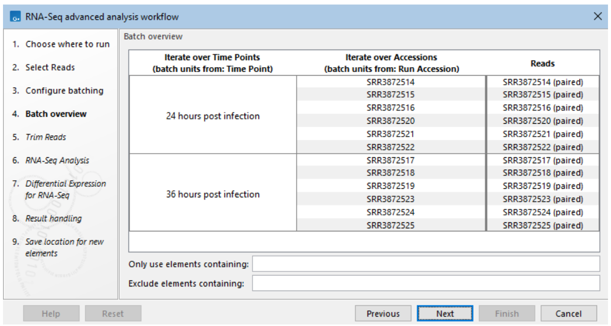
● Use metadata

Select metadata: Samples

Iterate over Time Points: Define batch units using metadata column: Time Point

Iterate over Accessions: Define batch units using metadata column: Run Accession

Figure 13.47: *The two Iterate elements in this workflow (right) have been renamed. Their names are included in the "Configure batching" wizard step in the launch wizard (left).*



Batch overview

Iterate over Time Points (batch units from: Time Point)	Iterate over Accessions (batch units from: Run Accession)	Reads
24 hours post infection	SRR3872514	SRR3872514 (paired)
	SRR3872515	SRR3872515 (paired)
	SRR3872516	SRR3872516 (paired)
	SRR3872520	SRR3872520 (paired)
	SRR3872521	SRR3872521 (paired)
	SRR3872522	SRR3872522 (paired)
36 hours post infection	SRR3872517	SRR3872517 (paired)
	SRR3872518	SRR3872518 (paired)
	SRR3872519	SRR3872519 (paired)
	SRR3872523	SRR3872523 (paired)
	SRR3872524	SRR3872524 (paired)
	SRR3872525	SRR3872525 (paired)

Only use elements containing:

Exclude elements containing:

Figure 13.48: *The batch overview for a workflow with two Iterate elements. The names assigned to the two columns containing the batch unit organization are the names of the corresponding Iterate elements.*

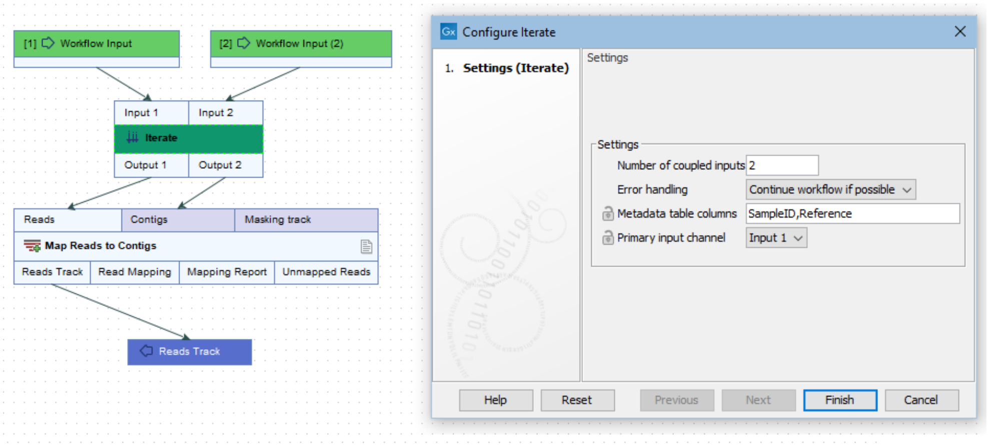
Further configuring Iterate elements

Double-clicking on an Iterate element opens the configuration dialog, which contains the options listed below (figure 13.49). The default settings are relevant for most uses of the Iterate element.

1. **Number of coupled inputs** The number of separate inputs for each given iteration. These inputs are "coupled" in the sense that, for a given iteration, particular inputs are used together. For example, when sets of sample reads should be mapped in the same way, but each set should be mapped to a particular reference (figure 13.50).
2. **Error handling** Specify what should happen if an error is encountered. The default is that the workflow should stop on any error. The alternative is to continue running the workflow if possible, potentially allowing later batches to be analyzed even if an earlier one fails.

3. **Metadata table columns** If the workflow is always run with metadata tables that have the same column structure, then it can be useful to set the value of the column titles here, so the workflow wizard will preselect them. The column titles must be specified in the same order as shown in the workflow wizard when running the workflow. Locking this parameter to a fixed value (i.e. not blank) will require the definition of batch units to be based on metadata. Locking this parameter to a blank value requires the definition of batch units to be based on the organization of input data (and not metadata).

4. **Primary input** If the number of coupled inputs is two or more, then the primary input (used to define the batch units) can be configured using this parameter.



Settings

Number of coupled inputs 2

Error handling Continue workflow if possible

Metadata table columns SampleID,Reference

Primary input channel Input 1

Figure 13.49: *The number of coupled inputs in this simple example is 2, allowing each set of sample reads to be mapped to a paticular reference, rather than using the same reference for all iterations.*

Configuring Collect and Distribute elements

By default, a Collect and Distribute element has one output channel. In this case, all results from the iteration block are collected and passed to downstream steps of the workflow.

More than one output channel can be configured by entering terms in a comma separated list in the **Outputs** field (figure 13.51). The number of terms determines the number of output channels. Connections between these output channels and input channels of downstream elements determine how data should be distributed in the following stage of the workflow.

If the Collect and Distribute element has more than one output channel, the path taken by a given element is determined by the value in the metadata column specified when launching the workflow. This column can be preconfigured in the **Group by metadata column** setting.

For example, when launching the workflow in figure 13.52, a metadata column called "Type" was specified for defining which samples were cases and which were controls. The iteration units were defined by the contents of the "ID" column (figure 13.53).

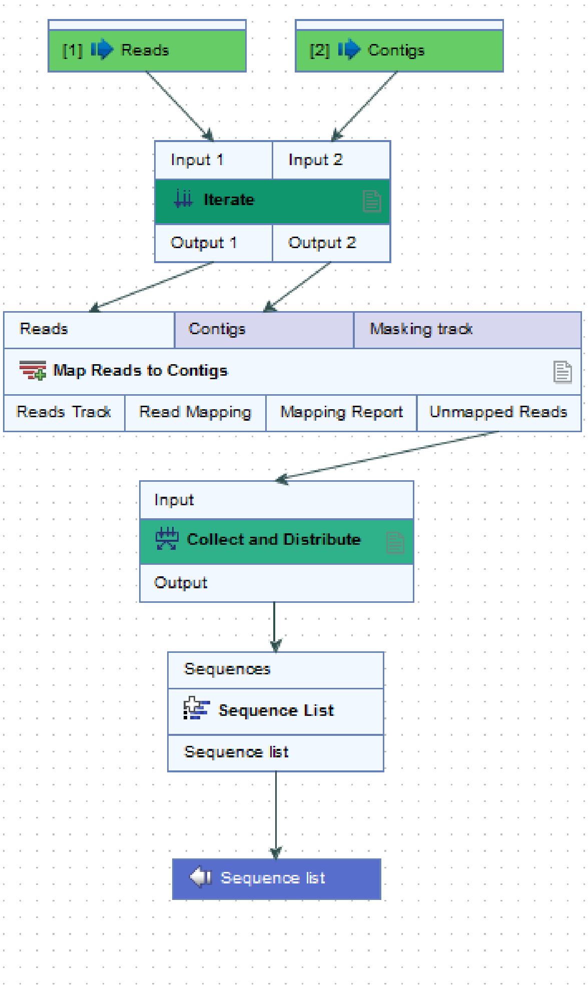
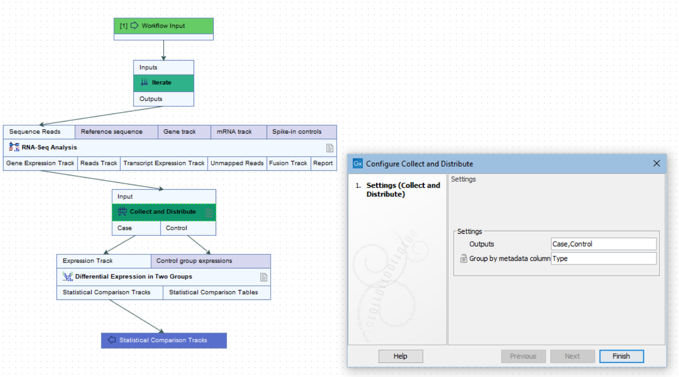


Figure 13.50: *Reads can be mapped to specified contigs due to the 2 input channels of the Iterate element. Using this design, a single sequence list containing all the unmapped reads from all the initial inputs is generated. That would not be possible without the inclusion of the Iterate and Collect and Distribute elements.*



1. Settings (Collect and Distribute)

Settings

Settings

Outputs: Case,Control

Group by metadata column: Type

Figure 13.51: *A comma separated list of terms in the Outputs field of the Collect and Distribute element defines the number of output channels and their names.*

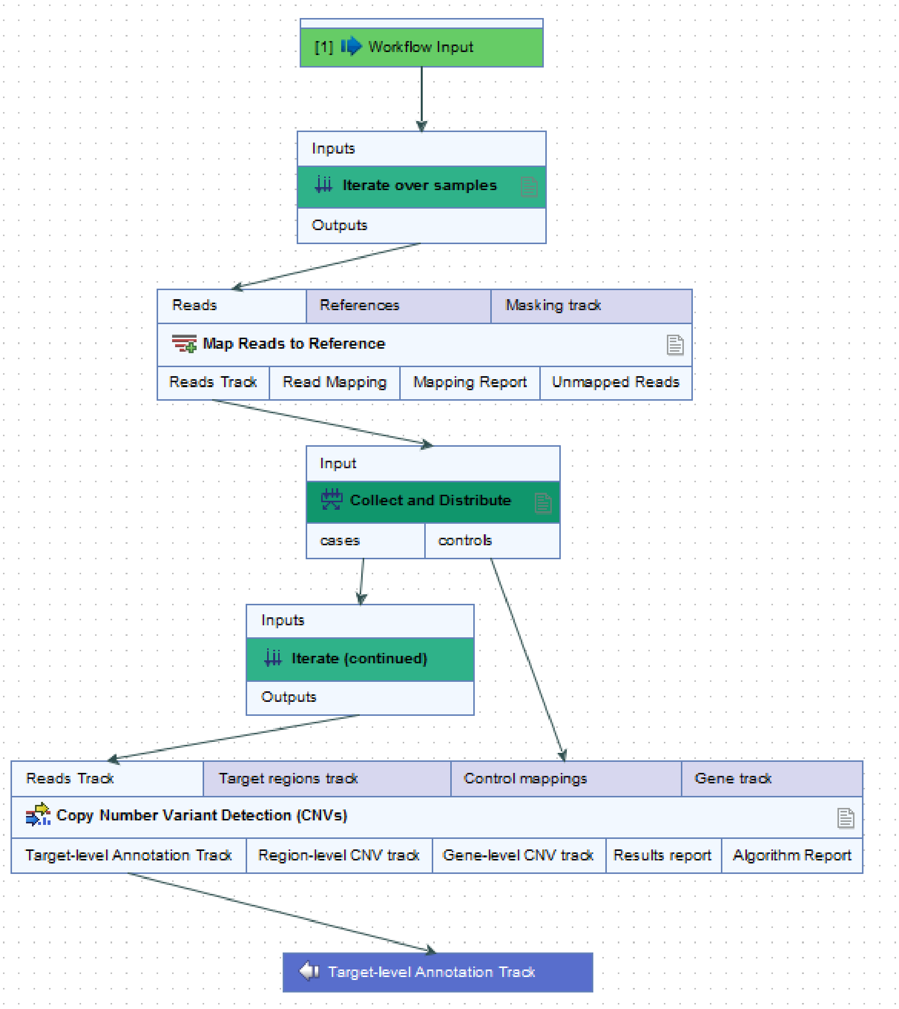
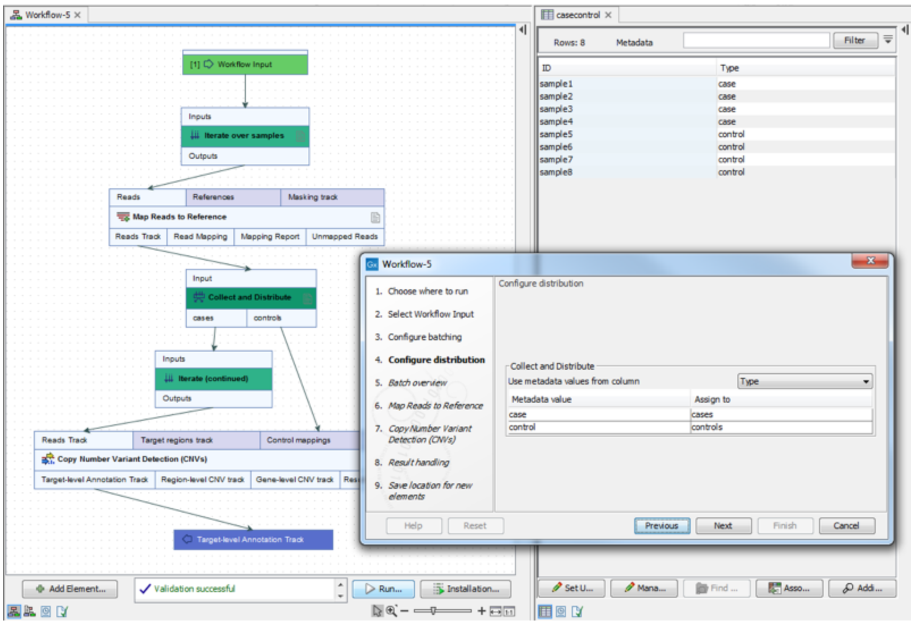


Figure 13.52: *In this workflow, each case sample is analyzed against all of the control samples.*



ID	Type
sample1	case
sample2	case
sample3	case
sample4	case
sample5	control
sample6	control
sample7	control
sample8	control

1. Choose where to run
2. Select Workflow Input
3. Configure batching
4. **Configure distribution**
5. *Batch overview*
6. *Map Reads to Reference*
7. *Copy Number Variant Detection (CNVs)*
8. *Result handling*
9. *Save location for new elements*

Configure distribution

Collect and Distribute

Use metadata values from column: Type

Metadata value	Assign to
case	cases
control	controls

Figure 13.53: *Contents of the metadata column "Type" define which samples are cases and which are controls. Iteration units are defined by the contents of the "ID" column.*

Fork

A choice between running particular parts of an analysis can be offered by including one or more **Fork** (⅄) elements in a workflow.

Including Fork elements in workflows can also help decrease the number of workflows that need to be maintained, as multiple analysis paths can be included in a single workflow, with only one or some of those paths being taken when the workflow is run.

For example, when the workflow shown in figure 13.54 is launched, a choice between "Quality" and "Quality and Vector" is offered in the launch wizard. Choosing "Quality" means the data will flow down the path containing the "Trim on Quality" element, while choosing "Quality and Vector" means the data will flow down the path containing the "Trim Vector and on Quality" element.

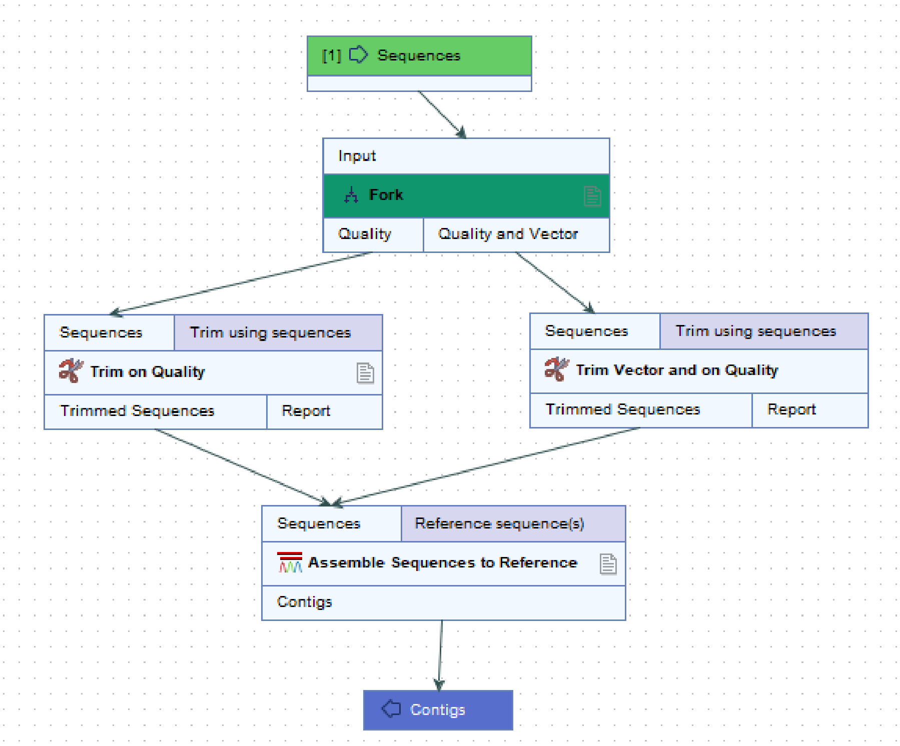


Figure 13.54: *A simple workflow with a Fork element. When launched, a choice is offered in the launch wizard for which path the the analysis should follow.*

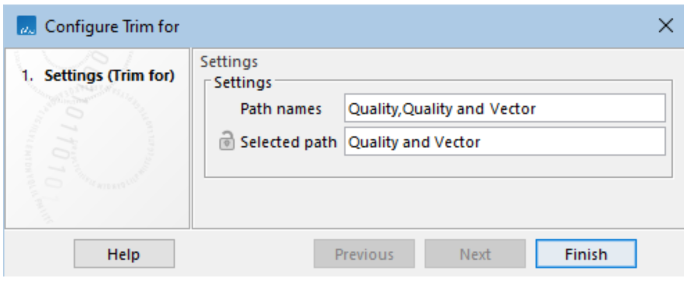
Configuring Fork elements

The configuration of Fork elements determines what is shown in the "Specify workflow paths" launch wizard step (figure 13.55 and figure 13.56). Specifically:

- **The name of the Fork element** The element name is used in the launch wizard to describe the choice being made.
- **The configurable fields in the Fork element** The configurable fields are used to define the available options and, if desired, specify a default:

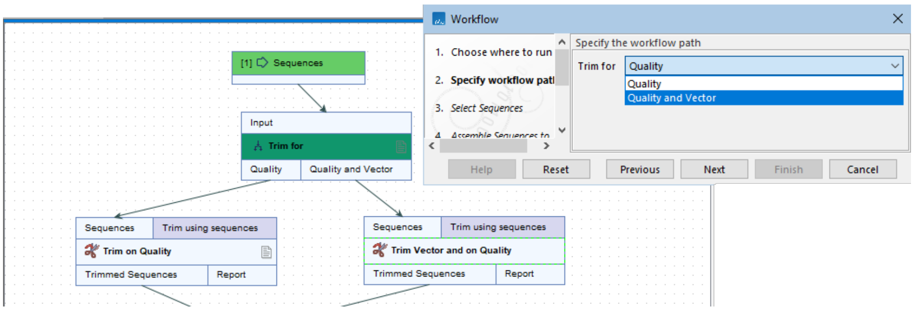
- **Path names** The list of choices of downstream paths from this Fork element, entered as a comma delimited, text list. These are presented in a drop-down list in the launch wizard. An output channel is added to the Fork element for each path name.
- **Selected path** One of the configured path names, which then will be used as the default. If this field is left blank, the first of the paths in the "Path names" list will be pre-selected in the wizard when launching the workflow from a *CLC Workbench*.

Renaming and configuring workflow elements is described in section 13.2.2.



Path names: Quality,Quality and Vector
Selected path: Quality and Vector

Figure 13.55: *A Fork element renamed as "Trim for" is open for configuration. The wizard step seen when launching a workflow with this Fork element is shown in figure 13.56.*



Trim for: Quality

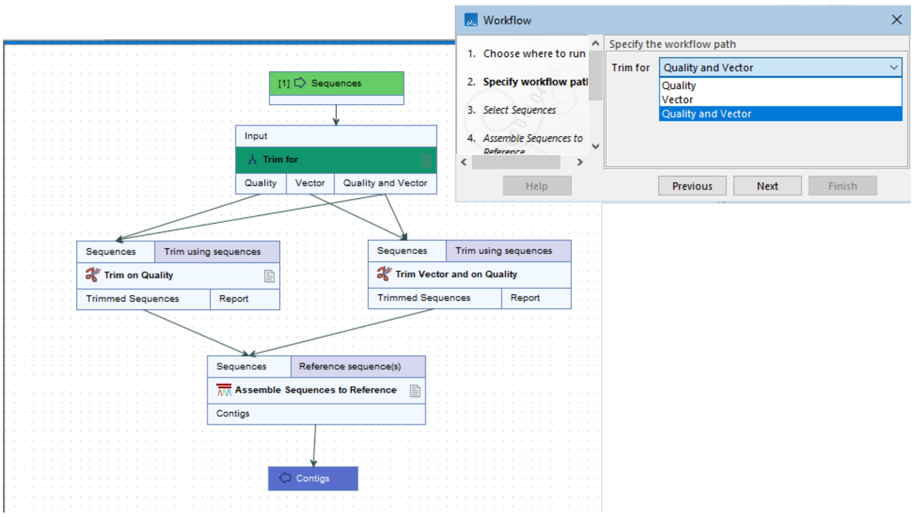
Figure 13.56: *This workflow has a single Fork element, renamed as "Trim for". The path names, "Quality" and "Quality and Vector", (see figure13.55) are listed in the "Specify workflow path" launch wizard step (top right)*

Flexibility potential using Fork elements

Fork elements provide a great deal of flexibility in the types of choices that can be offered when launching workflows. Examples include:

- Providing the choice of following one of several possible analysis paths (figure 13.56).
- Providing the choice of following one of several possible analysis paths or following multiple analysis paths (figure 13.57).
- Providing the choice of whether or not to run a particular part of the analysis (figure 13.58).

When a workflow contains multiple Fork elements, all the corresponding choices are presented in a single "Specify workflow paths" launch wizard step (figure 13.58).



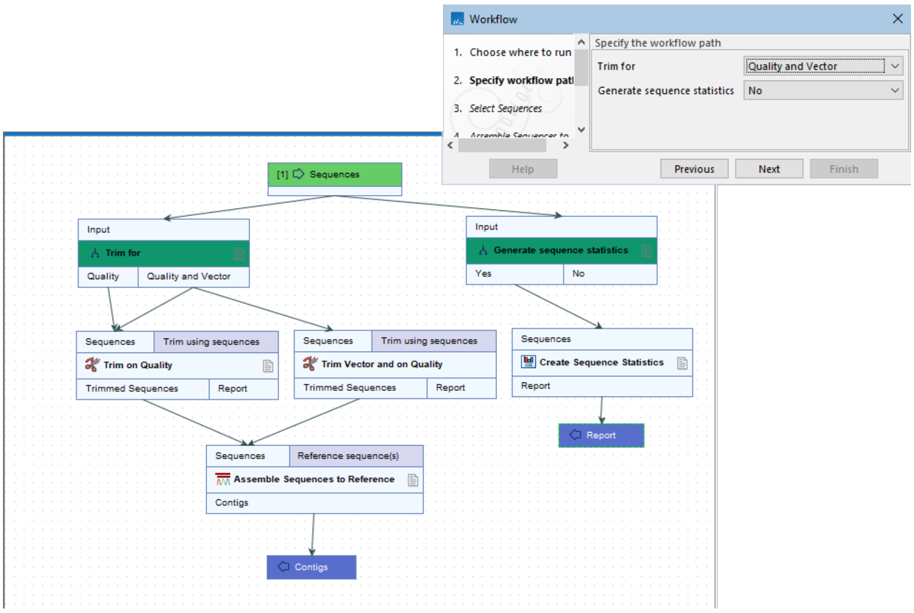
Workflow

1. Choose where to run
2. Specify workflow pati
3. Select Sequences
4. Assemble Sequences to Reference

Specify the workflow path

Trim for: Quality and Vector

Figure 13.57: *When launching this workflow, the choice can be made to trim sequences based on quality, trim for vector sequence, or trim for both.*



Workflow

1. Choose where to run
2. Specify workflow pati
3. Select Sequences
4. Assemble Sequences to

Specify the workflow path

Trim for: Quality and Vector

Generate sequence statistics: No

Figure 13.58: *There are two Fork elements in this workflow, and thus two choices will need to be made when launching it. Both are presented in the "Specify workflow path" launch wizard step. The Yes/No choice for the "Generate sequence statistics" option determines whether or not the Create Sequence Statistics analysis will be run.*

Branching elements

Branching elements control the path that data takes through a workflow.

Branch on Sequence Count

Branch on Sequence Count ( ) elements are used when downstream handling of sequence lists should depend on the number of sequences in the list.

The sequence list provided as input will flow through the Pass or the Fail output channel depending on whether the number of sequences meets the condition specified in the branching element (figure 13.59).

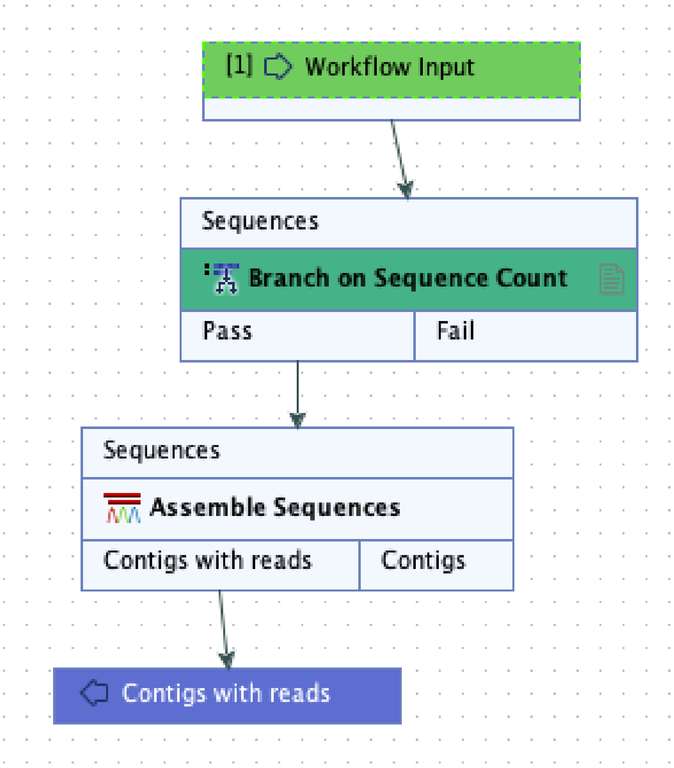
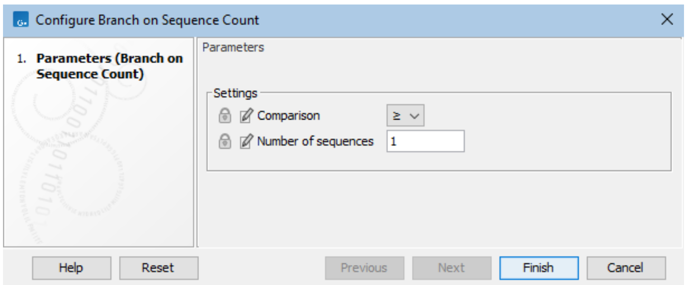


Figure 13.59: *If the sequence list provided as input meets the condition specified in a Branch on Sequence Count element, it will flow through the Pass output channel and be used in the Assemble Sequences step. Otherwise, it will flow through the Fail output channel, where here, it would not be processed further.*

In the Branch on Sequence Count configuration dialog (figure 13.60), the configuration options are:

- **Comparison** The operator to use for the comparison: >=, = or <=, offered in a drop-down list.
- **Number of sequences** The number of sequences to use in the comparison.



Configure Branch on Sequence Count

1. Parameters (Branch on Sequence Count)

Parameters

Settings

Comparison: ≥

Number of sequences: 1

Figure 13.60: *Configuration of a Branch on Sequence Count element*

13.2.6 Input modifying tools

A few tools in the *CLC Workbench* are input modifying, meaning that when the tool is run from the Tools menu, it directly manipulates the input provided, instead of generating a new element as output. However, when such tools are used in a workflow context, they do generate a new element as output[1].

Examples of such tools are:

- Find Open Reading Frames
- Gaussian Statistical Analysis
- Pfam Domain Search
- Proportion-based Statistical Analysis

13.3 Launching workflows individually and in batches

When a workflow is launched, a wizard opens that will take takes you step by step through launching the workflow, including supplying the data to be analyzed, configuring any available options, specifying where outputs should be saved, etc.

Workflows stored in a CLC data location, i.e. available from the Navigation Area, can be launched by opening them, and then clicking on the **Run Workflow** button at the bottom, right hand side of the Workflow Editor.

Installed workflows and template workflows can be launched in the following ways:

- Double click on the workflow name in the Workflows tab in the Toolbox panel, which is in the bottom, left side of the Workbench.
- Select the workflow from the Workflows menu at the top of the Workbench.
- Use the Quick Launch () tool (see section 11.1).

[1] Prior to version 22.0, input modifying tools behaved the same when run directly or in a workflow context. Workflow elements for such tools were marked in the Input channel and affected output channel by an M in a circle. There were restrictions for such tools in workflows in these older versions. Please see the manual for the version you are running for full details

Workflow inputs

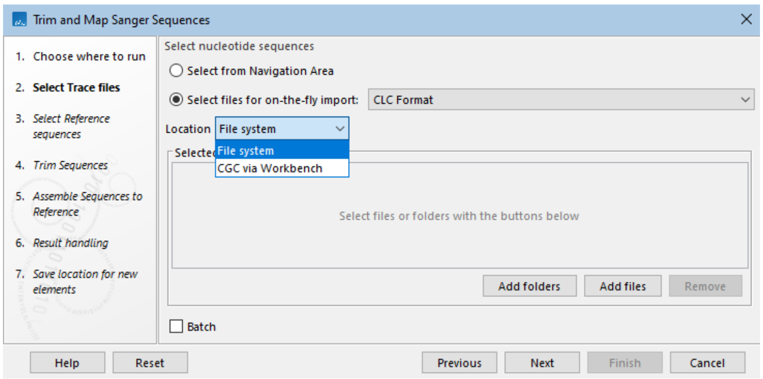
Data to be used in a workflow analysis can be selected from the Navigation Area or can be imported on-the-fly from files stored elsewhere. The specific options available depend on how the workflow was configured by the author. Using on-the-fly import, the first action taken when the workflow is run is to import the specified data.

When **Select files for on-the-fly import** is selected, the format of the data files must be specified using the drop-down menu beside this option. If configuration options are available for the selected importer, they will be shown in the lower part of the dialog.

If remote locations are available, such as *CLC Server* import/export directories, or AWS S3 buckets, a **Location** drop-down menu will be visible above the file selection area, either in the launch wizard (figure 13.61) or in the **Select files** dialog that opens when a **Browse** button is clicked on.

Note:

- To use CLC data stored in an AWS S3 bucket in a workflow analysis, you must choose the option **Select files for on-the-fly import** and choose the format **CLC Format**.
- If you select data from an AWS S3 bucket for an analysis that will be run on your *CLC Workbench* or *CLC Server*, the data will be downloaded from AWS before the analysis begins. For large datasets, this may take some time. Downloading from AWS S3 to a local file system may incur charges from AWS. See `AWS S3 pricing`.



Trim and Map Sanger Sequences

1. Choose where to run
2. **Select Trace files**
3. *Select Reference sequences*
4. *Trim Sequences*
5. *Assemble Sequences to Reference*
6. *Result handling*
7. *Save location for new elements*

Select nucleotide sequences

○ Select from Navigation Area

● Select files for on-the-fly import: CLC Format

Location: File system

Select files or folders with the buttons below

□ Batch

Figure 13.61: *Input data is specified when launching a workflow. CLC data can be selected from the Navigation Area. Data stored elsewhere can be selected after choosing the option "Select files for on-the-fly import" and specifying the format of that data.*

For information about configuring workflow Input elements when creating or editing a workflow, see section 13.2.3.

Workflow outputs

Output and Export elements in workflows specify the analysis results to be saved. In addition to analysis results, a Workflow Result Metadata table can be output. This contains a record of the workflow outputs, as described in section 13.3.1.

The history of data elements generated as workflow outputs contains the name and version the workflow that created it. When an installed workflow was used, the workflow build id is also included (see section 2.5).

Launching batches of analyses

When multiple inputs are provided, you can choose to run the workflow multiple times, one time for each input, or for defined sets of inputs that should be analyzed together. This is referred to as running in **Batch mode**, with the inputs to be analyzed together being referred to as **batch units**. This is described in section 13.3.2.

In addition, using Iterate and Collect and Distribute control flow elements within a workflow allows for part of a workflow being run once per batch unit, while other parts are run on all the data together. This is described in section 13.3.3.

Workflow intermediate results

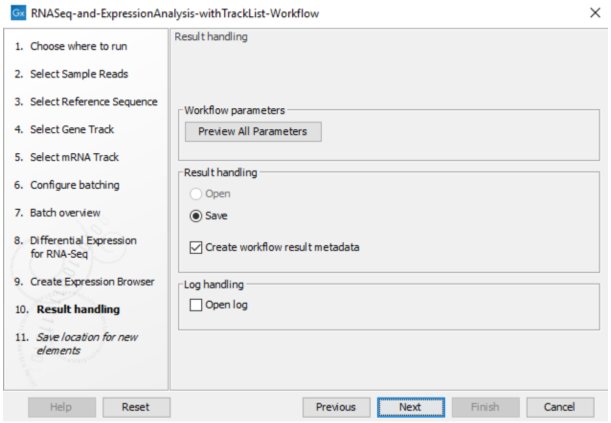
Workflow intermediate results are generated during the workflow execution. These data elements are needed for use by downstream steps but are then deleted when the analysis successfully completes. While they exist, workflow intermediate results are stored in a dedicated subfolder of the folder that is selected to store outputs when the workflow is launched.

Where intermediate results are stored for jobs run on a *CLC Server* depends on settings on the server.

13.3.1 Workflow Result Metadata tables

A Workflow Result Metadata table contains a row describing each output generated by the workflow. Each element generated by the workflow has an association to the relevant row.

Creating a Workflow Result Metadata table is enabled by default (figure 13.62).



RNASeq-and-ExpressionAnalysis-withTrackList-Workflow

1. Choose where to run
2. Select Sample Reads
3. Select Reference Sequence
4. Select Gene Track
5. Select mRNA Track
6. Configure batching
7. Batch overview
8. Differential Expression for RNA-Seq
9. Create Expression Browser
10. **Result handling**
11. *Save location for new elements*

Result handling

Workflow parameters

Result handling
○ Open
● Save
☑ Create workflow result metadata

Log handling
□ Open log

Figure 13.62: *The final step when launching a workflow includes an option to create a workflow result metdata table.*

See section 12.3.1 for information on finding and working with data associated with metadata rows.

Workflow Result Metadata tables from batch runs

Workflow Result Metadata tables can be useful when finding results of workflows run in Batch mode due to the large number of outputs that can be produced.

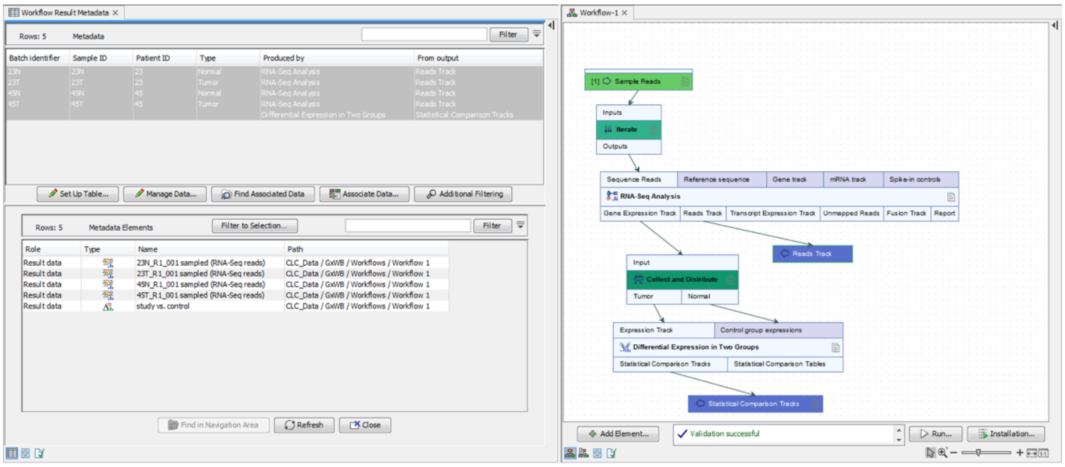
A single table is generated per batch run, containing information about all the results[2].

A *Batch identifier* column is included for any outputs that were generated as part of a batch run. This includes outputs from workflows run in Batch mode, and also outputs from steps in an iteration loop, i.e. downstream of an **Iterate** control flow element (figure 13.63).

Where batch units are defined using metadata, the Workflow Result Metadata table includes original metadata information, where possible.

See section 13.3.2 for more information on launching workflows in batch mode.

See section 13.2.5 for details about control flow elements and section 13.3.3 for more information on launching workflows containing control flow elements.



Batch identifier	Sample ID	Patient ID	Type	Produced by	From output
23N	23N	23	Normal	RNA-Seq Analysis	Reads Track
23T	23T	23	Tumor	RNA-Seq Analysis	Reads Track
45N	45N	45	Normal	RNA-Seq Analysis	Reads Track
45T	45T	45	Tumor	RNA-Seq Analysis	Reads Track
				Differential Expression in Two Groups	Statistical Comparison Tracks

Role	Type	Name	Path
Result data		23N_R1_001 sampled (RNA-Seq reads)	CLC_Data / GxWB / Workflows / Workflow 1
Result data		23T_R1_001 sampled (RNA-Seq reads)	CLC_Data / GxWB / Workflows / Workflow 1
Result data		45N_R1_001 sampled (RNA-Seq reads)	CLC_Data / GxWB / Workflows / Workflow 1
Result data		45T_R1_001 sampled (RNA-Seq reads)	CLC_Data / GxWB / Workflows / Workflow 1
Result data		study vs. control	CLC_Data / GxWB / Workflows / Workflow 1

Figure 13.63: *The Workflow Result Metadata table, top left, was generated from a run of the workflow on the right. Here, 4 RNA-Seq Anaylysis runs occurred within the iteration loop (between the Iterate and the Collect and Distribute elements). Those results were then supplied to Differential Expression in Two Groups, which was run once. There are thus 5 rows in the Workflow Metadata Result table. The RNA-Seq Analysis results each have a batch identifier, while the statistical comparison output does not.*

13.3.2 Running workflows in batch mode

Running analyses in batches occurs when:

- The Batch checkbox at the bottom of input steps in the launch wizard has been checked, and/or
- The workflow contains one or more **Iterate** control flow elements. Steps downstream of **Iterate** elements and upstream of **Collect and Distribute** elements, if present, are run

[2] There is one exception to this. Where batch units have been defined by the organization of the input data and the outputs are to be saved in the same folders as the inputs, one workflow result metadata table is generated per analysis.

once for each batch unit (see section 13.2.5).

A batch unit consists of the data that should be analyzed together. The grouping of data into batch units is defined after the inputs for analysis have been selected.

Defining batch units based on the organization of the input data

For simple workflows, batch units can be defined based on how the input elements or files are organized. This is identical to defining batch units when launching a tool, as described in section 11.3.

Here "simple workflows" means workflows with just one analysis input that changes per batch, for example, the sets of sequencing reads to be mapped, where the same reference sequence is used for every mapping. This equates to a workflow without any Iterate elements being run in Batch mode, or a workflow with just one Iterate element being run (not in Batch mode).

Defining batch units based on metadata

When launching any workflow, batch units can be defined using metadata. For more complex scenarios, this will be the only option. Such scenarios include:

- Where there is more than one level of batch units. This could be:
  - A workflow with more than one Input element, where the inputs to both of these should be grouped into batch units. An example of such a workflow is described in section 13.4.
  - A workflow containing more than one Iterate element.
  - A workflow containing containing an Iterate element that will be run in Batch mode. An example of this is described in the "RNA-Seq and differential gene expression analysis" tutorial, available from https://resources.qiagenbioinformatics.com/tutorials/RNASeq-DGE-analysis.pdf.
- Where Iterate or Collect and Distribute elements in the workflow have been configured to require metadata.

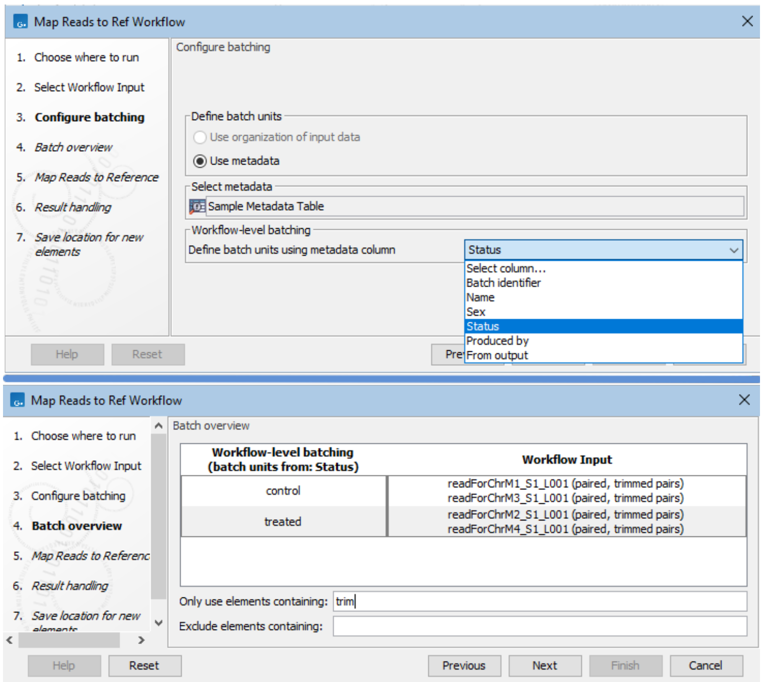
Note: When launching a workflow containing analysis steps that require metadata, the metadata provided to define batch units is also used for those analysis steps. For example, in the **RNA-Seq and Differential Gene Expression Analysis** template workflow, metadata provided to define batch units is also used for the Differential Expression for RNA-Seq step.

There are two ways metadata defining batch units can be provided:

1. **Using a CLC Metadata Table** In this case, the data elements selected as inputs must already have associations to this CLC Metadata Table.

   If a CLC Metadata Table with data associated to it has been selected in the "Select Workflow Input" step of a workflow, that table will be pre-selected in the "Configure batching" step of the launch wizard. You can specify the column that batch units will be based on. Data associated with the table rows for each unique value in that column make up the contents of the batch units. The contents can be refined using the fields below the preview pane (figure 13.64).

Outputs from the workflow that can be unambiguously identified with a single row of the CLC Metadata Table will have an association to that row added. Outputs derived from two or more inputs with different metadata associations will not have associations to this CLC Metadata Table.


Map Reads to Ref Workflow

1. Choose where to run
2. Select Workflow Input
3. **Configure batching**
4. *Batch overview*
5. *Map Reads to Reference*
6. *Result handling*
7. *Save location for new elements*

Configure batching

Define batch units

○ Use organization of input data

● Use metadata

Select metadata: Sample Metadata Table

Workflow-level batching

Define batch units using metadata column: Status

Map Reads to Ref Workflow

1. Choose where to run
2. Select Workflow Input
3. Configure batching
4. **Batch overview**
5. *Map Reads to Referenc*
6. *Result handling*
7. *Save location for new elements*

Batch overview

Workflow-level batching (batch units from: Status)	Workflow Input
control	readForChrM1_S1_L001 (paired, trimmed pairs) readForChrM3_S1_L001 (paired, trimmed pairs)
treated	readForChrM2_S1_L001 (paired, trimmed pairs) readForChrM4_S1_L001 (paired, trimmed pairs)

Only use elements containing: trim

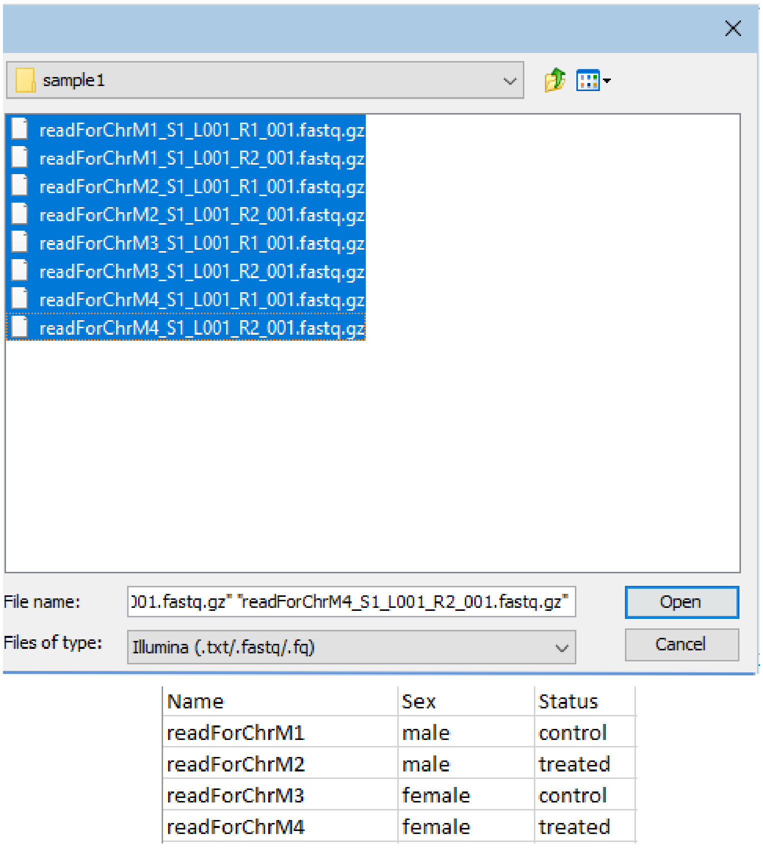
Exclude elements containing:

Figure 13.64: *A CLC Metadata Table with data associated to it was selected as input to a workflow being launched in Batch mode. In the Configure batching wizard step, the metadata source is pre-configured. The column to base batch units on can be selected (top). The Batch overview step shows the data elements in each batch unit. Here "trim" has been entered in the "Only use elements containing" field, so only elements containing the term "trim" in their names are included in the batch units (bottom).*

2. **Using an Excel, CSV or TSV format file**. The metadata in the file is imported into the CLC software at the start of the workflow run. Requirements for this file are:

   - The first row must contain column headers.
   - The first column must contain either the exact names of the files selected or at least enough of the first part of the name to uniquely identify each file with the relevant row of the metadata file. If data is being imported on-the-fly, the file name can include file extensions, but not the path to the data.
   - A column containing information that defines how the data should be grouped for the analysis, i.e. the information that defines the batch units. In many cases, this column contains sample identifiers. This may be the first column if there are as many batch units as input files.

     When the data being imported is paired sequence reads, the first column would contain the names of each input file, and another column would uniquely identify each pair of files (figure 13.65).



readForChrM1_S1_L001_R1_001.fastq.gz
readForChrM1_S1_L001_R2_001.fastq.gz
readForChrM2_S1_L001_R1_001.fastq.gz
readForChrM2_S1_L001_R2_001.fastq.gz
readForChrM3_S1_L001_R1_001.fastq.gz
readForChrM3_S1_L001_R2_001.fastq.gz
readForChrM4_S1_L001_R1_001.fastq.gz
readForChrM4_S1_L001_R2_001.fastq.gz

File name:)01.fastq.gz" "readForChrM4_S1_L001_R2_001.fastq.gz"

Files of type: Illumina (.txt/.fastq/.fq)

Name	Sex	Status
readForChrM1	male	control
readForChrM2	male	treated
readForChrM3	female	control
readForChrM4	female	treated

Figure 13.65: *Paired fastq files from two samples were selected for import (top). The Excel file with information about this data set contains a header row and 4 rows of information, one row per input file. The contents of the first column contain enough of each file name to uniquely identify each input file. The second column contains sample IDs.*

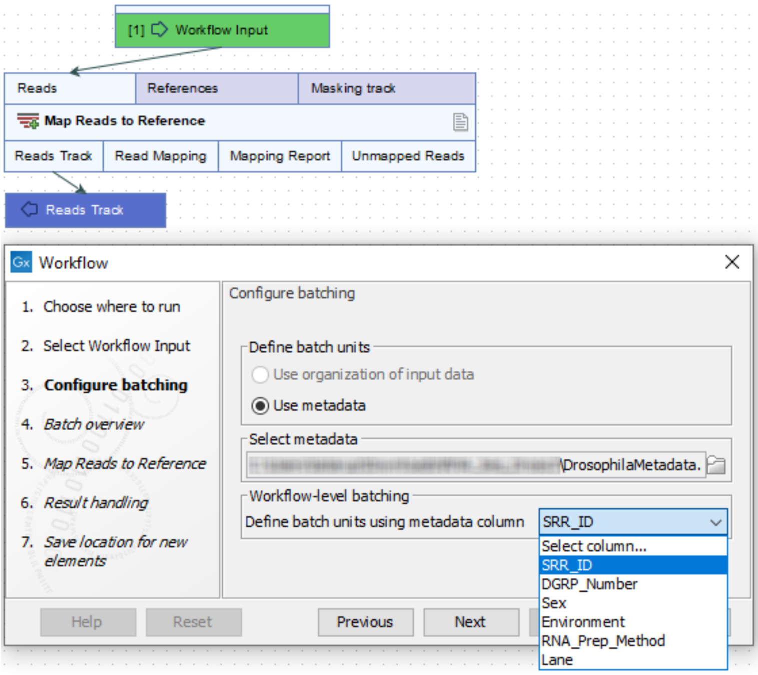
If there is a tool in the workflow that requires descriptive information, for example, factors for statistical testing in **Differential Expression for RNA-Seq**, then the file should also contain columns with this information.

For example, if a data element selected in the Navigation Area has the name `atp8a_1_sample1_day3`, then the first column could contain that name in full, or just enough of the first part of the name to uniquely identify it. This could be, for example, `atp8a_1_sample1`. Similarly, if a data file selected for on-the-fly import is at: `C:\Users\username\My Data\atp8a_1 sample1_day3.clc`, the first column of the Excel spreadsheet could contain `atp8a_1_sample1_day3.clc`, or a prefix long enough to uniquely identify the file, e.g. `atp8a_1_sample1`.

Example: On-the-fly import of single end reads based on metadata

In figure 13.66, a workflow with a single input is being launched in batch mode. The eight files selected contain Illumina single end reads. This raw data will be imported on the fly using metadata to define the batch units. The metadata column in the Excel file that contains information defining the batch units has been specified. Here, files with the same value in the SRR_ID column will be imported and analyzed together.

Each row in the SRR_ID column has a unique entry, so 8 batch units will be made, with one sequence file in each batch unit. If a column containing fewer unique values was selected, one or more batch units would consist of several files. This is illustrated in figure 13.66.



1. Choose where to run
2. Select Workflow Input
3. **Configure batching**
4. *Batch overview*
5. *Map Reads to Reference*
6. *Result handling*
7. *Save location for new elements*

Configure batching

Define batch units
○ Use organization of input data
● Use metadata

Select metadata
\DrosophilaMetadata.

Workflow-level batching
Define batch units using metadata column: SRR_ID

Select column...
SRR_ID
DGRP_Number
Sex
Environment
RNA_Prep_Method
Lane

Figure 13.66: *Batch units are defined according to the values in the SRR_ID column of the Excel file that was selected.*

In the next step, a preview of the batch units is shown. The workflow will be run once for each entry in the left hand column, with the input data grouped as shown in the right hand column (figure 13.67).

Example: On-the-fly import of paired end reads based on metadata

When importing data on-the-fly and organizing batch units based on metadata, the metadata must have a row per file being imported. For paired data, this means at least 2 rows per sample. One column in the file must contain information about which files belong together. This allows the sequence list created to be associated with the relevant information.

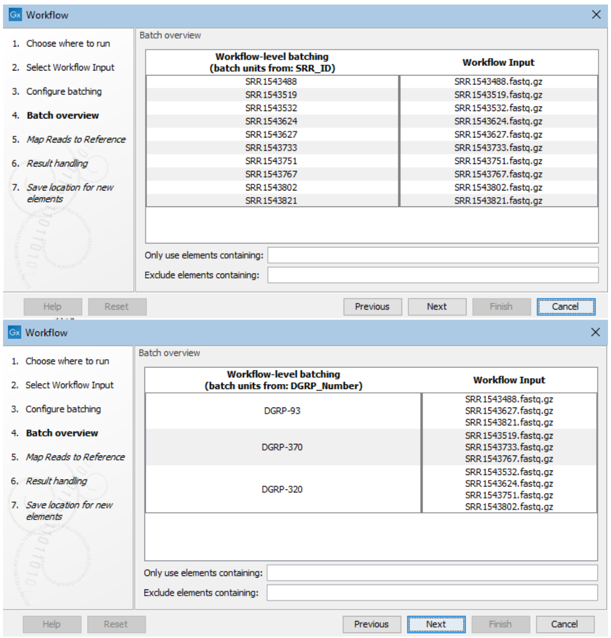
The contents of an Excel file with information about 2 sets of paired files containing Sanger data are shown in figure 13.68.

Each row for data that is in the same batch unit must contain the same descriptive information. Where there is conflicting information for a given batch unit, the value for that column will be ignored. If all entries for a given column are conflicting, the column will not appear in the resulting CLC Metadata Table.

Saving results from workflows run in batch mode

When a workflow is run in batch mode, options are presented in the last step of the wizard for specifying where to save results of individual batches (see section 11.3).

If the workflow contains **Export** elements, an additional option is presented, **Export to separate**



Workflow-level batching (batch units from: SRR_ID)	Workflow Input
SRR1543488	SRR1543488.fastq.gz
SRR1543519	SRR1543519.fastq.gz
SRR1543532	SRR1543532.fastq.gz
SRR1543624	SRR1543624.fastq.gz
SRR1543627	SRR1543627.fastq.gz
SRR1543733	SRR1543733.fastq.gz
SRR1543751	SRR1543751.fastq.gz
SRR1543767	SRR1543767.fastq.gz
SRR1543802	SRR1543802.fastq.gz
SRR1543821	SRR1543821.fastq.gz

Only use elements containing:
Exclude elements containing:

Workflow-level batching (batch units from: DGRP_Number)	Workflow Input
DGRP-93	SRR1543488.fastq.gz SRR1543627.fastq.gz SRR1543821.fastq.gz
DGRP-370	SRR1543519.fastq.gz SRR1543733.fastq.gz SRR1543767.fastq.gz
DGRP-320	SRR1543532.fastq.gz SRR1543624.fastq.gz SRR1543751.fastq.gz SRR1543802.fastq.gz

Only use elements containing:
Exclude elements containing:

Figure 13.67: *The Batch overview step allows you to review the batch units. In the top image, a column called SRR_ID had been selected, resulting in 8 batch units, so 8 workflow runs, with the data from one input file to be used in each batch. In the lower image, a different column was selected to define the batch units. There, the workflow would be run 3 times with the input data grouped as shown.*

directories per batch unit (figure 13.69). When that option is checked, files exported are placed into separate subfolders under the the export folder selected for each export step.

13.3.3 Running part of a workflow multiple times

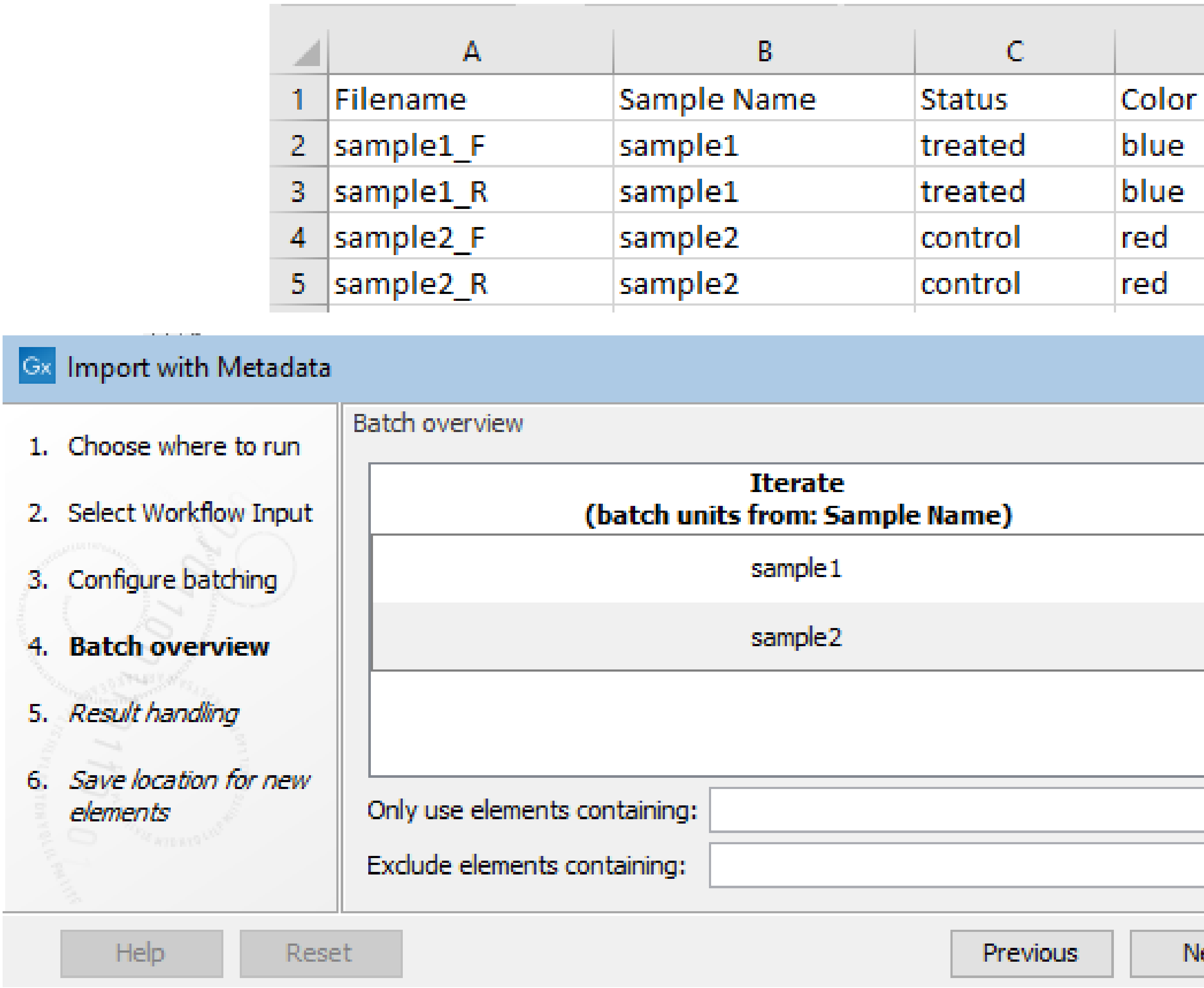
To run a part of the workflow multiple times, once for each batch unit, add an *Iterate* control flow element to the workflow. All elements downstream of an Iterate element are run on each batch unit individually, until a *Collect and Distribute* element is encountered. The parts of the workflow downstream of the Collect and Distribute element are run on all the data together, or in subsets of the data, as configured in the Collect and Distribute element (see section 13.2.5).

For example, the workflow in figure 13.70 would run an RNA-Seq Analysis for each sample separately, and then create a single combined report for the set of samples.

When running on a *CLC Server* the iterating parts of the workflow is run as separate jobs. For parallel execution of these iterations, job nodes or grid nodes must be available.

When using metadata table to specify the batch units, you are prompted to specify the column that defines how the samples should be grouped for execution. In figure 13.71, grouping by the column "ID" results in the RNA-Seq Analysis tool being run 8 times, once for each sample. Selecting the "Gender" column instead results in the RNA-Seq Analysis tool being run 2 times, once for each value in that column, male and female. The Combine Reports tool runs once, using information from all samples.

The name of workflow elements can be changed. This changes the text displayed in the wizard when the workflow is run. This can be useful if a workflow contains multiple identical elements



	A	B	C	
1	Filename	Sample Name	Status	Color
2	sample1_F	sample1	treated	blue
3	sample1_R	sample1	treated	blue
4	sample2_F	sample2	control	red
5	sample2_R	sample2	control	red

Import with Metadata

1. Choose where to run
2. Select Workflow Input
3. Configure batching
4. **Batch overview**
5. *Result handling*
6. *Save location for new elements*

Batch overview

Iterate
(batch units from: Sample Name)

sample1

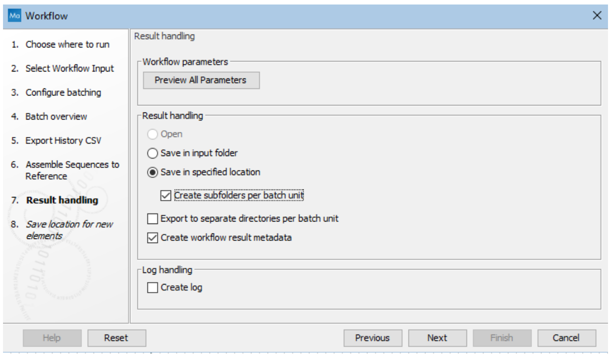
sample2

Only use elements containing:

Exclude elements containing:

Figure 13.68: *An Excel file at the top describes 4 Sanger files that make up two pairs of reads. The "Sample Name" column was identified as the one indicating the group the file belongs to. Information about the relevant sample appears in each row. At the Batch overview step, shown at the bottom, you can check the batch units are as intended.*

(figure 13.72).



Workflow

1. Choose where to run
2. Select Workflow Input
3. Configure batching
4. Batch overview
5. Export History CSV
6. Assemble Sequences to Reference
7. **Result handling**
8. *Save location for new elements*

Result handling

Workflow parameters

Preview All Parameters

Result handling

○ Open

○ Save in input folder

● Save in specified location

☑ Create subfolders per batch unit

□ Export to separate directories per batch unit

☑ Create workflow result metadata

Log handling

□ Create log

Figure 13.69: *Options are presented in the final wizard step for configuring where outputs and exported files from each batch run should be saved.*

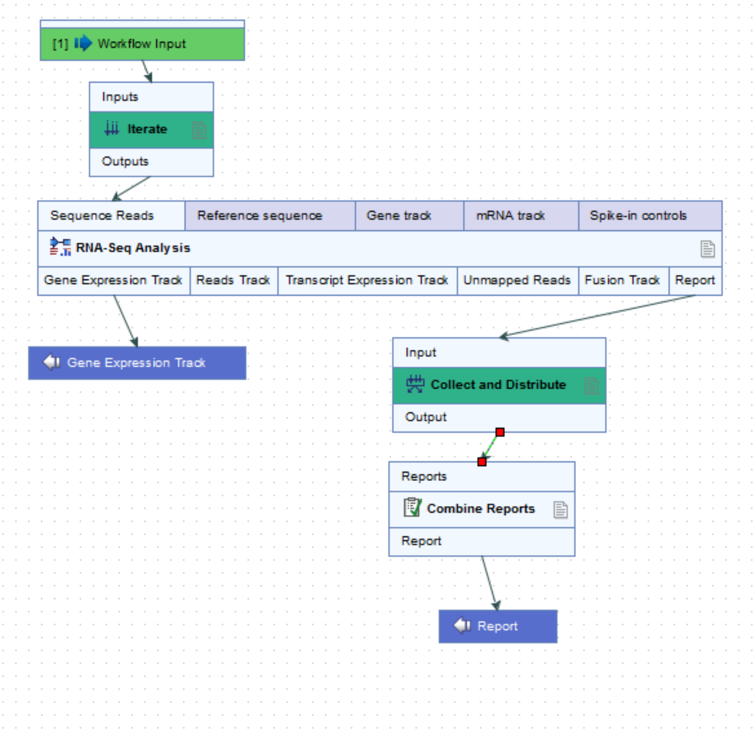
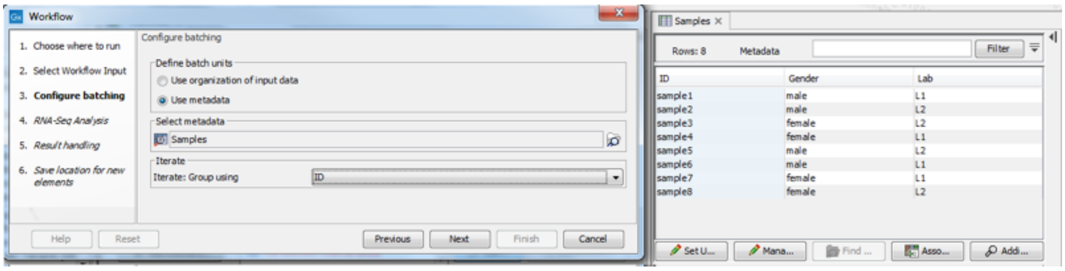


Figure 13.70: *The RNA-Seq analysis tool is run once per sample and a single combined report is then generated for the full set of samples.*



Workflow

1. Choose where to run
2. Select Workflow Input
3. **Configure batching**
4. *RNA-Seq Analysis*
5. *Result handling*
6. *Save location for new elements*

Configure batching

Define batch units

○ Use organization of input data

● Use metadata

Select metadata: Samples

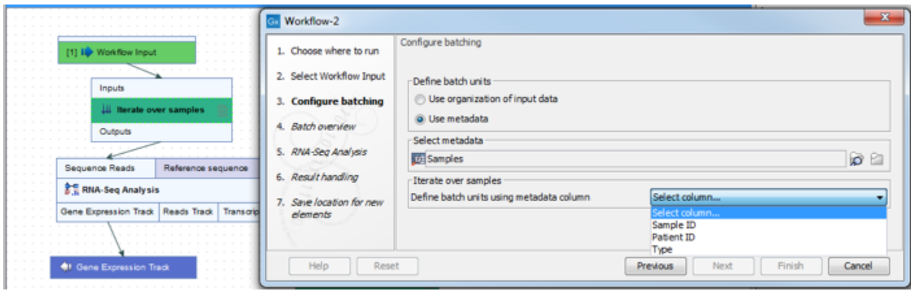
Iterate

Iterate: Group using: ID

Rows: 8 Metadata

ID	Gender	Lab
sample1	male	L1
sample2	male	L2
sample3	female	L2
sample4	female	L1
sample5	male	L2
sample6	male	L1
sample7	female	L1
sample8	female	L2

Figure 13.71: *With the current selection in the wizard, the RNA-Seq Analysis tool will run 8 times, once for each sample. The Combine Reports tool will run once.*



1. Choose where to run
2. Select Workflow Input
3. **Configure batching**
4. *Batch overview*
5. *RNA-Seq Analysis*
6. *Result handling*
7. *Save location for new elements*

Configure batching

Define batch units

○ Use organization of input data

● Use metadata

Select metadata

Samples

Iterate over samples

Define batch units using metadata column: Select column...

Select column...
Sample ID
Patient ID
Type

Figure 13.72: *The Iterate element can be renamed to change the text that is displayed in the wizard when running the workflow.*

13.4 Advanced workflow batching

Fine-tuned control of the execution of whole workflows or sections of workflows can be achieved using metadata describing the relationships between particular samples and using control flow elements in a workflow design. Complex analysis goals that can be met in a straightforward manner include:

- **Grouping the data into different subsets to be analyzed together in particular sections of a workflow.** Groupings of data can be used in the following ways:
  - **Different groupings of data are used as inputs to different sections of the same workflow**. For details, see section 13.3.3 and section 13.4.2.
  - **Different workflow inputs follow different paths through parts of a workflow**. Based on metadata, samples can be distributed into groups to follow different analysis paths in some workflow sections, at the same time as processing them individually and identically through other sections of the same workflow.

    Configuring Collect and Distribute elements is central to the design of this workflow. This is described in section 13.2.5. Running such workflows is described in section 13.3.3.
- **Matching particular workflow inputs for each workflow run**. Where more than one input to a workflow changes per run, the particular input data to use for each run can be defined using metadata. The simplest case is as described in section 13.4.1. However, more complex scenarios, such as when intermediate results should be merged or parts of the workflow should be run multiple times, can also be catered for using control flow elements (see section 13.2.5).

  Examples in this section make reference to CLC Genomics Workbench tools and data types commonly analyzed using that software. However, the principles apply equally to workflows created in the *CLC Main Workbench*.

13.4.1 Batching workflows with more than one input changing per run

When a workflow contains multiple **Input** elements (multiple light green boxes),

A Batch checkbox is available in the launch wizard for each **Input** element attached to a main input channel.

Checking that box indicates that the data supplied for that input should change in each batch run.

By contrast, if multiple elements are selected, and the Batch option is not checked, all elements will be treated a single set, to be used in a single analysis run.

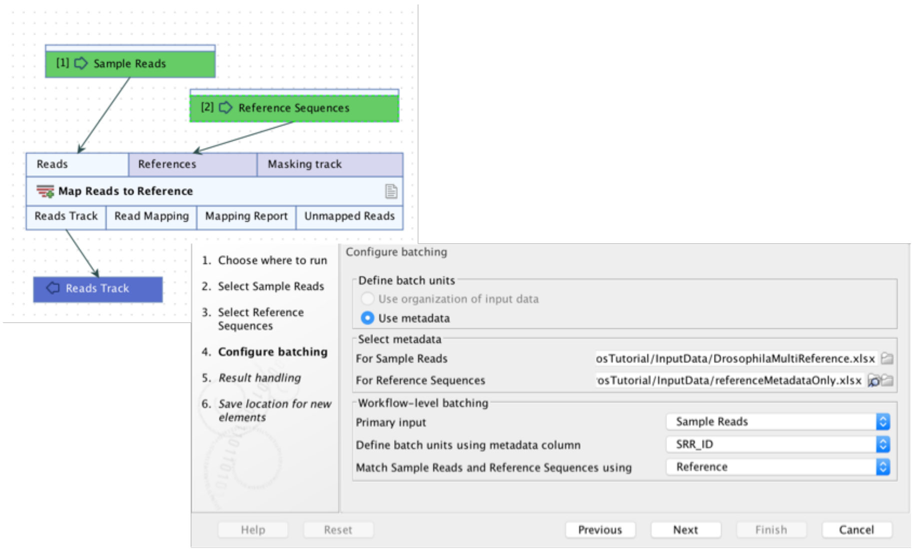
Most commonly, one input is changed per run. For example, in a batch analysis involving read mappings, usually each batch unit would include a different set of reads, but the same reference sequence.

However, it is possible to have two or more inputs that are different in each batch unit. For example, an analysis involving a read mapping, where each set of reads should be mapped to a different reference sequence. In cases like this, batch units must be defined using metadata.

Figure 13.73 shown an example where the aim is to do just this. The workflow contains a Map Reads to Contigs element and two workflow input elements, Sample Reads and Reference Sequences. The information to define the batch units is provided by two Excel files, one containing information about the Sample Reads input and the other with information about the Reference Sequences input. The contents of files that would work for this example are shown in figure 13.74.

Of particular note are:

- The first column of file contains the exact file names for all data for that input, across all of the batch runs.
- At least one column in each file has the same name as a column in the other file. That column should contain the information needed to match the input data, in this case, the Sample Reads input data with the relevant Reference Sequences input data for each batch unit.



Configure batching

Define batch units
○ Use organization of input data
● Use metadata

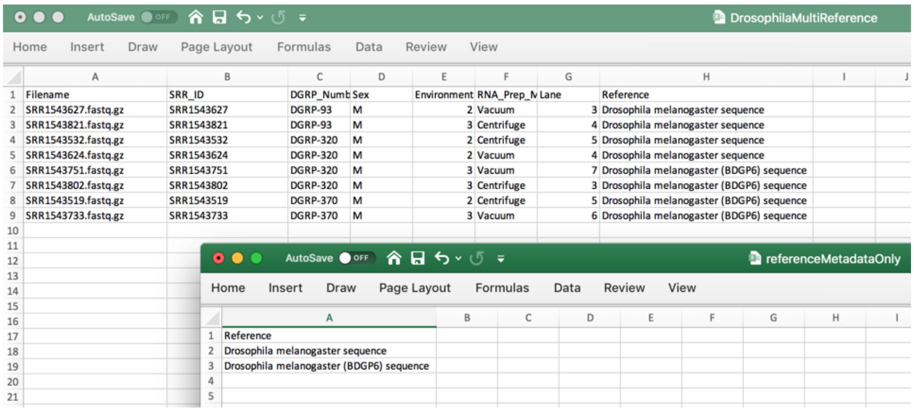
Select metadata
For Sample Reads osTutorial/InputData/DrosophilaMultiReference.xlsx
For Reference Sequences osTutorial/InputData/referenceMetadataOnly.xlsx

Workflow-level batching
Primary input Sample Reads
Define batch units using metadata column SRR_ID
Match Sample Reads and Reference Sequences using Reference

Figure 13.73: *A workflow with 2 inputs, where the Batch checkbox had been checked for both in the relevant launch steps. Metadata is used to define the batch units since the correct inputs must be matched together for each run.*

In the Workflow-level batching section of the launch wizard, the following are specified:

- The primary input. The input that determines the number of times the workflow should be run.
- The column in the metadata for the primary input that specifies the group the data belongs to. Each group makes up a single batch unit.
- The column in both metadata files that together will be used to ensure that the correct data from each workflow input are included together in a given batch run. For example, a given set of sample reads will be mapped to the correct reference sequence. A column with this name must be present in each metadata file or table.



	A	B	C	D	E	F	G	H
1	Filename	SRR_ID	DGRP_Numb	Sex	Environment	RNA_Prep_M	Lane	Reference
2	SRR1543627.fastq.gz	SRR1543627	DGRP-93	M	2	Vacuum	3	Drosophila melanogaster sequence
3	SRR1543821.fastq.gz	SRR1543821	DGRP-93	M	3	Centrifuge	4	Drosophila melanogaster sequence
4	SRR1543532.fastq.gz	SRR1543532	DGRP-320	M	2	Centrifuge	5	Drosophila melanogaster sequence
5	SRR1543624.fastq.gz	SRR1543624	DGRP-320	M	2	Vacuum	4	Drosophila melanogaster sequence
6	SRR1543751.fastq.gz	SRR1543751	DGRP-320	M	3	Vacuum	7	Drosophila melanogaster (BDGP6) sequence
7	SRR1543802.fastq.gz	SRR1543802	DGRP-320	M	3	Centrifuge	3	Drosophila melanogaster (BDGP6) sequence
8	SRR1543519.fastq.gz	SRR1543519	DGRP-370	M	2	Centrifuge	5	Drosophila melanogaster (BDGP6) sequence
9	SRR1543733.fastq.gz	SRR1543733	DGRP-370	M	3	Vacuum	6	Drosophila melanogaster (BDGP6) sequence

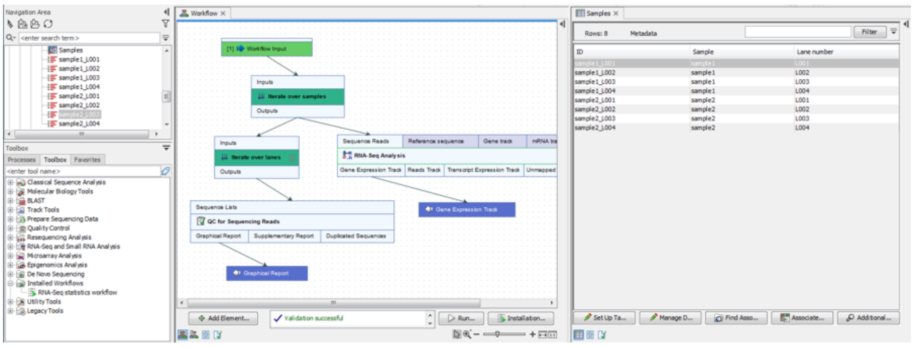
	A
1	Reference
2	Drosophila melanogaster sequence
3	Drosophila melanogaster (BDGP6) sequence

Figure 13.74: *Two Excel files containing information about the data for each batch unit for the workflow shown in figure 13.73. With the settings selected there, the number of batch runs will be based on the Sample Reads input, and will equal the number of unique SRR_ID entries in the DrosophilaMultiReference.xlsx file. The correct reference sequence to map to is determined by matching information in the Reference column of each Excel file.*

In figure 13.73, Sample Reads is the primary input: We wish to run the workflow once for each sample, which here, is once for each SRR_ID entry. The Reference sequence to use for each of these batch units is defined in a column called Reference, which is present in both the file containing information about the samples and the file containing information about the references.

13.4.2 Multiple levels of batching

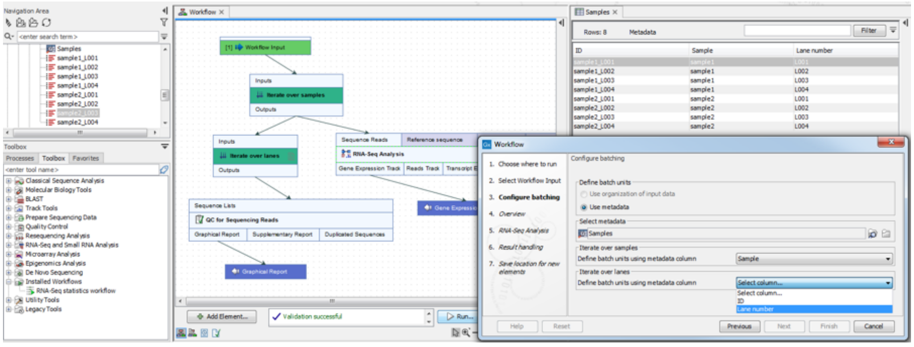
Sometimes it can be useful to batch or iterate over multiple levels. For example, suppose we have Illumina data from 4 lanes on the flow cell, such that each sample has 4 associated reads list. We may wish to run QC for Sequencing Reads per reads list, but the RNA-Seq Analysis tool per sample. A workflow like the one drawn in figure 13.75 allows us to do this, by connecting Iterate elements directly to each other. The top-level Iterate element results in a subdivision (grouping) of the data, and the innermost Iterate results in a further subdivision (grouping) of each of those groups. Note that it is not necessary for the workflow to include a Collect and Distribute element.



ID	Sample	Lane number
sample1_L001	sample1	L001
sample1_L002	sample1	L002
sample1_L003	sample1	L003
sample1_L004	sample1	L004
sample2_L001	sample2	L001
sample2_L002	sample2	L002
sample2_L003	sample2	L003
sample2_L004	sample2	L004

Figure 13.75: *The top-level Iterate element results in a subdivision (grouping) of the data, and the innermost Iterate results in a further subdivision (grouping) of each of those groups.*

When running the workflow, only metadata can be used to define the groups, because the workflow contains multiple levels of iterations (figure 13.76).



Rows: 8 Metadata

ID	Sample	Lane number
sample1_L001	sample1	L001
sample1_L002	sample1	L002
sample1_L003	sample1	L003
sample1_L004	sample1	L004
sample2_L001	sample2	L001
sample2_L002	sample2	L002
sample2_L003	sample2	L003
sample2_L004	sample2	L004

Workflow

1. Choose where to run
2. Select Workflow Input
3. **Configure batching**
4. *Overview*
5. *RNA-Seq Analysis*
6. *Result handling*
7. *Save location for new elements*

Configure batching

Define batch units

○ Use organization of input data

● Use metadata

Select metadata: Samples

Iterate over samples

Define batch units using metadata column: Sample

Iterate over lanes

Define batch units using metadata column: Select column...

Select column...
ID
Lane number

Figure 13.76: *When the workflow contains multiple levels of iterations, only metadata can be used to define the groups.*

It is always possible to execute a third level of batching by selecting the Batch checkbox when launching the workflow: this will run the whole workflow, including the inner batching processes, several times with different sets of data.

Control flow elements are described in more detail in section 13.2.5.

13.5 Template workflows

Template workflows are provided as example workflows (figure 13.77). They can be launched as they are from the under the Workflows menu, or copies can be opened, as described below, allowing you to optimize the workflow to fit your specific application. After making a copy and editing it, you can run the workflow from the Workflow Editor, or you can create an installer and install the workflow to your *CLC Workbench* or *CLC Server*. Links to documentation about these activities are provided later in this section.

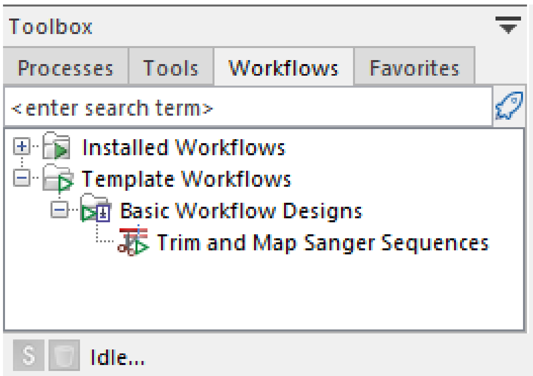


Figure 13.77: *The Template Workflows folder in the Workflows tab of the Toolbox*

Opening a template workflow for viewing or editing

You can open a copy of a template workflow in the Workflow Editor, where you can view or edit it:

- From under the Workflows tab in the Toolbox in the lower, left side of the Workbench: Right-click on the workflow name and select the option **Open Copy of Workflow** from the menu that appears.

  or

- From the Workflow Manager: Open the Workflow Manager by clicking on the **Manage Workflows** button ( ) in the toolbar, and choose the option **Manage Workflows**.

  Click on the **Template Workflows** tab and then select the workflow of interest. Then click on the **Open Copy of Workflow** button.

You can specify which settings can be adjusted when launching a workflow, and which cannot, by unlocking or locking parameters in workflow elements. Unlocked parameters can be adjusted when launching the workflow. For locked parameters, the value specified in the design is always used when the workflow is run.

Installed workflows cannot be edited directly, so by locking settings, and installing the workflow, you create a customized version of a template workflow, validated for your purposes, where you know exactly the settings that will be used for each workflow run.

Related documentation

The following manual pages contain information relevant to working with copies of template workflows:

- Configuring workflow elements, including locking and unlocking parameters: section 13.2.2
- Tips for configuring the view of workflows when editing them: section 13.1.9
- General information about editing workflows: section 13.1
- Installing a workflow: section 13.6.2

The template workflows distributed with the *CLC Main Workbench* are described after this section. Template workflows distributed with plugins are described in the relevant plugin manual.

13.5.1 Trim and Map Sanger Sequences

The **Trim and Map Sanger Sequences** template workflow adds trim annotations to sequences and then maps them to a reference. This workflow generates a stand-alone read mapping and a trimming report.

This workflow is aimed at working with data generated using Sanger sequencing. It is not intended for the analysis of high volumes of data. A maximum of 100,000 sequences can be supplied as input.

Importing Sanger sequences (with trace information) is described in section 21.1.

Launching the workflow

To launch the **Trim and Map Sanger Sequences** template workflow, go to:

Workflows | **Template Workflows** | **Basic Workflow Designs**| **Trim and Map Sanger Sequences** ( )

Tools in the workflow and outputs generated

The **Trim and Map Sanger Sequences** template workflow contains two tools (figur 13.78):

- **Trim Sequences**. Adds Trim annotations to sequences. Trimming options can be configured when launching the workflow. A trimming report is generated. See section 21.2 for more information.
- **Assemble Sequences to Reference**. Maps the trimmed sequences to the specified reference sequence(s). Mapping options can be configured when launching the workflow. A stand-alone read mapping is generated. See section 21.4) for more information.

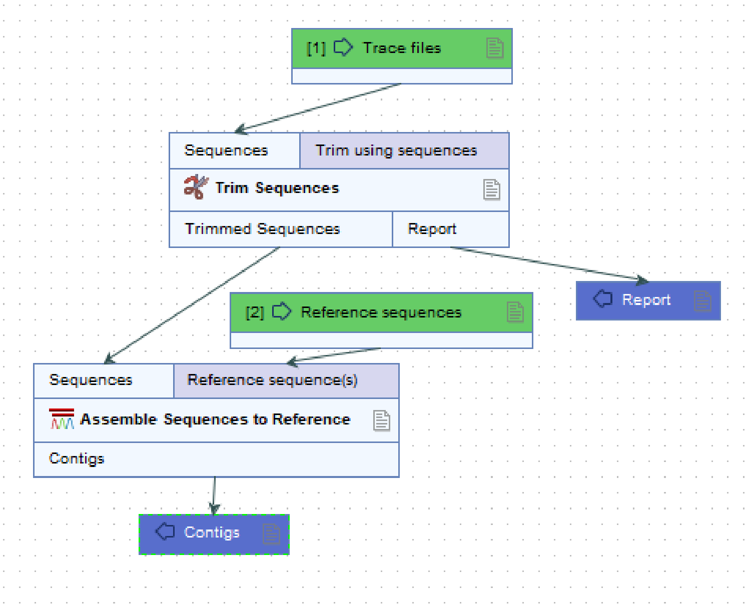


Figure 13.78: *The Trim and Map Sanger Sequences template workflow*

13.6 Managing workflows

Workflows can be managed from the Workflow Manager:

Utilities | **Manage Workflows** ( )

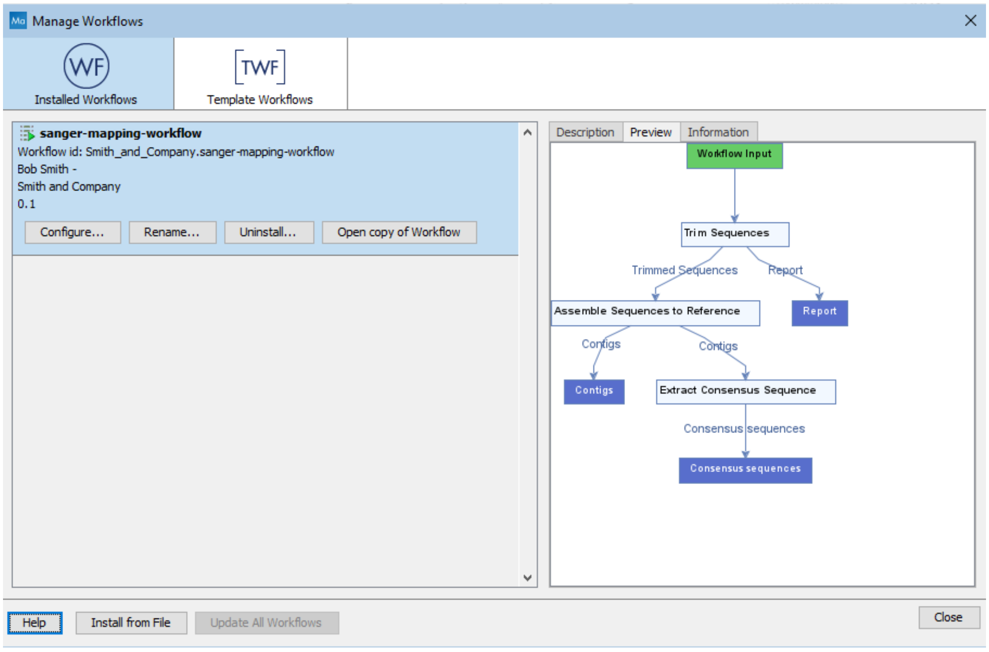
or using the "Workflows" button ( ) in the toolbar and then select "Manage Workflows..." ( ).

The Workflow Manager (figure 13.79) lists workflows installed on your system under the Installed Workflows tab. Workflows under the Installed Workflows tab can be configured, renamed and uninstalled, as described below.

Workflows provided by QIAGEN are listed under the Template Workflows tab.

Copies of workflows can be made by clicking on the "Open Copy of Workflow" button for the relevant workflow. This opens an editable copy of the workflow in the Workflow Editor. That workflow can then, if desired, be saved as an element in Navigation Area, and installed so it appears under the Installed Workflows folder of the Toolbox.

Note: Copies of installed and template workflows can also be opened from under the Workflows tab in the Toolbox at the bottom left side of the Workbench. Right-click on the workflow name and choose "Open Copy of Workflow" from the menu that appears.



sanger-mapping-workflow
Workflow id: Smith_and_Company.sanger-mapping-workflow
Bob Smith -
Smith and Company
0.1

Figure 13.79: *An installed workflow has been selected in the Workflow Manager. Some actions can be carried out on this workflow, and a preview pane showing the workflow design is open on the right hand side.*

Configure

Clicking on the **Configure** button for an installed workflow will open a dialog where configurable steps in the workflow are shown (figure 13.80). Settings can be configured, and unlocked settings can be locked if desired.

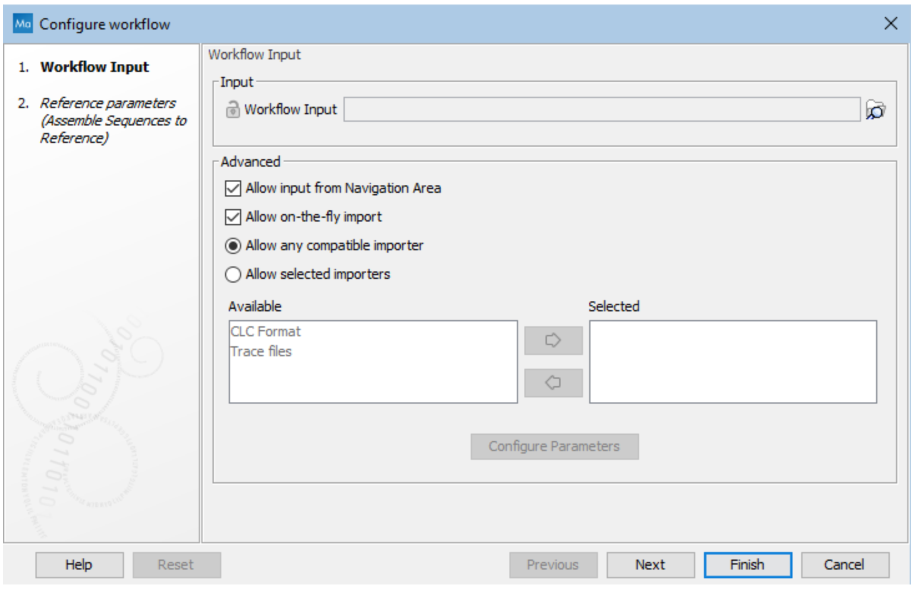
Note: Parameters locked in the original workflow design cannot be unlocked. Those locked using the Configure functionality of the Workbench Manager can be unlocked again later in the same way, if desired.

Parameter locking is described further in section 13.2.2.

Note that parameters requiring the selection of data should only be locked if the workflow will only be installed in a setting where there is access to the same data, in the same location, as the system where the workflow was created, or if the parameter is optional and no data should be selected. If the workflow is intended to be executed on a *CLC Server*, it is important to select data that is located on the *CLC Server*.

Rename

Clicking on the **Rename** button for an installed workflow allows you to change the name. The workflow will then be listed with that new name in the "Installed Workflows" folder in the Workflows menu.



Configure workflow

1. **Workflow Input**
2. *Reference parameters (Assemble Sequences to Reference)*

Workflow Input

Input

Workflow Input

Advanced

☑ Allow input from Navigation Area

☑ Allow on-the-fly import

● Allow any compatible importer

○ Allow selected importers

Available: CLC Format, Trace files

Selected

Figure 13.80: *Configuring parameters for a workflow.*

The workflow id will remain the same.

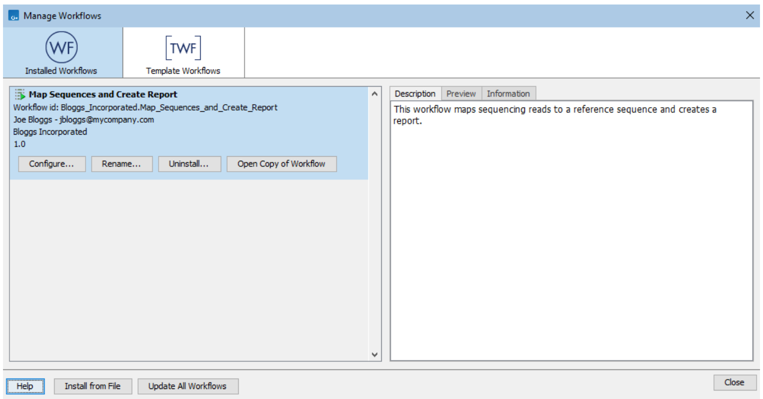
Uninstall Use this button to uninstall am installed workflow.

Description, Preview and Information In the right hand pane of the Workflow Manager, are three tabs.

- **Description** Contains the description that was entered when creating the workflow installer (figure 13.81). See section 13.6.2.
- **Preview** Contains a graphical representation of the workflow
- **Information** Contains general information about that workflow, such as the name, id, author, etc. (figure 13.82, and described in detail below).

The Information tab (figure 13.82) contains the following:

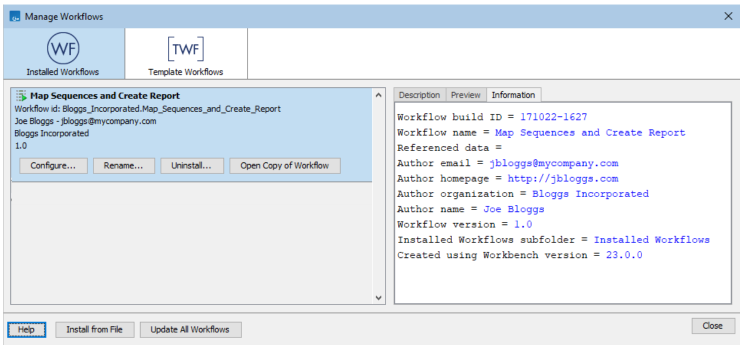
- **Workflow build id** The date (day month year) followed by the time (based on a 24 hour time) when the workflow installer was created. If the workflow was installed locally without an installation file being explicitly created, the build ID will reflect the time of installation.
- **Workflow name** The name of workflow
- **Referenced data** If reference data was referred to by the workflow and the option Bundled or Reference was selected when the installer was made, the reference data referred to is listed in this field. See section 13.6.2 for further details about these options.
- **Author email** The email address the workflow author entered when creating the workflow installer.



Map Sequences and Create Report
Workflow id: Bloggs_Incorporated.Map_Sequences_and_Create_Report
Joe Bloggs - jbloggs@mycompany.com
Bloggs Incorporated
1.0

This workflow maps sequencing reads to a reference sequence and creates a report.

Figure 13.81: *The description provided when creating the workflow installer is available in the Description tab in the Workflow Manager.*



Map Sequences and Create Report
Workflow id: Bloggs_Incorporated.Map_Sequences_and_Create_Report
Joe Bloggs - jbloggs@mycompany.com
Bloggs Incorporated
1.0

```
Workflow build ID = 171022-1627
Workflow name = Map Sequences and Create Report
Referenced data =
Author email = jbloggs@mycompany.com
Author homepage = http://jbloggs.com
Author organization = Bloggs Incorporated
Author name = Joe Bloggs
Workflow version = 1.0
Installed Workflows subfolder = Installed Workflows
Created using Workbench version = 23.0.0
```

Figure 13.82: *The Information tab contains the information provided when the workflow installer was created as well as the workflow build-id.*

- **Author homepage** The homepage the workflow author entered when creating the workflow installer.
- **Author organization** The organization the workflow author entered when creating the workflow installer.
- **Author name** The workflow author's name.
- **Workflow version** The version that the author assigned to the workflow when creating the installer.
- **Created using Workbench version** The version of the CLC Workbench used when the workflow installer was created.

13.6.1 Updating workflows

After installing a new version of a *CLC Workbench*, workflows may need to be updated before they can be used. Three situations are described in this section:

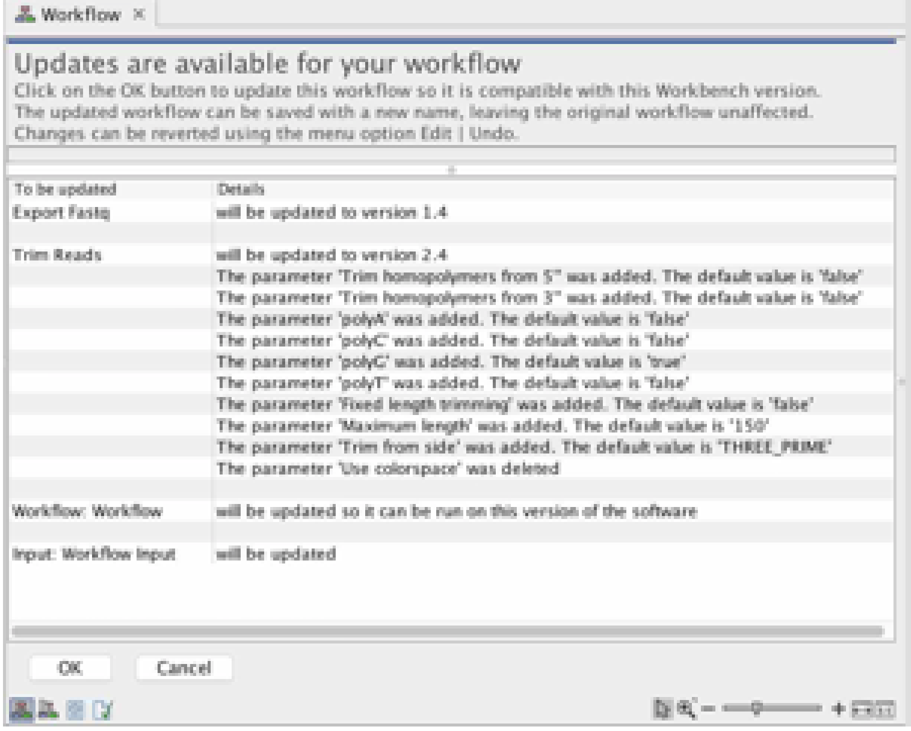
- Updating workflows stored in the Navigation area
- Updating installed and template workflows when using a upgraded Workbench in the *same* major version line

- Updating installed workflows when using software in a *higher* major version line

"Major version line" refers to the first digit in the version number. For example, versions 23.0.1 and 23.0.5 are part of the same major release line (23). Version 22.0 is part of a different major version line (22).

Updating workflows stored in the Navigation area

When you open a workflow stored in the Navigation Area that needs to be updated, an editor will open listing the tools that need to be updated, along with additional information about the changes to the tools (figure 13.83).



Updates are available for your workflow

Click on the OK button to update this workflow so it is compatible with this Workbench version.
The updated workflow can be saved with a new name, leaving the original workflow unaffected.
Changes can be reverted using the menu option Edit | Undo.

To be updated	Details
Export Fastq	will be updated to version 1.4
Trim Reads	will be updated to version 2.4 The parameter 'Trim homopolymers from 5'' was added. The default value is 'false' The parameter 'Trim homopolymers from 3'' was added. The default value is 'false' The parameter 'polyA' was added. The default value is 'false' The parameter 'polyC' was added. The default value is 'false' The parameter 'polyG' was added. The default value is 'true' The parameter 'polyT' was added. The default value is 'false' The parameter 'Fixed length trimming' was added. The default value is 'false' The parameter 'Maximum length' was added. The default value is '150' The parameter 'Trim from side' was added. The default value is 'THREE_PRIME' The parameter 'Use colorspace' was deleted
Workflow: Workflow	will be updated so it can be run on this version of the software
Input: Workflow Input	will be updated

Figure 13.83: *The workflow update editor lists tools and parameters that will be updated.*

To update the workflow, click on the **OK** button at the bottom of the editor.

The updated workflow can be saved under a new name, leaving the original workflow unaffected.

Updating installed and template workflows when using an upgraded Workbench in the *same* major version line

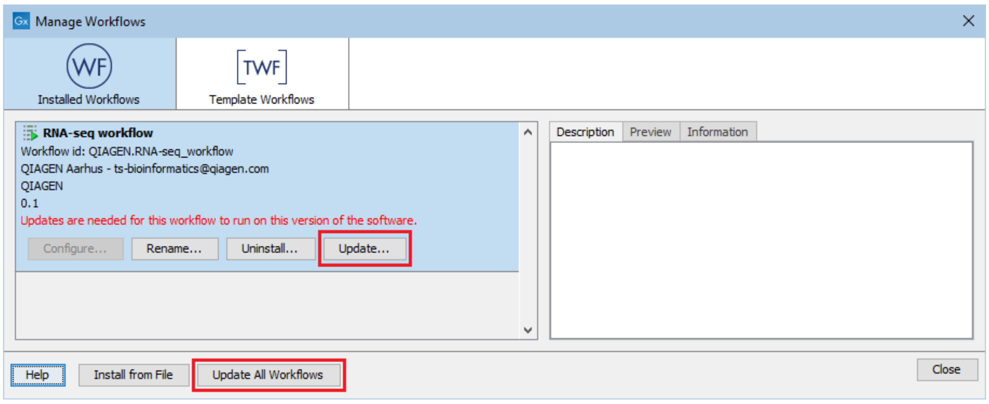
When working on an upgraded *CLC Workbench* in the same major version line, installed and template workflows are updated using the Workflow Manager.

To start the Workflow Manager, go to:

Utilities | Manage Workflows ( )

or click on the "Workflows" button ( ) in the toolbar, and select "Manage Workflow..." ( ) from the menu that appears.

A red message is displayed for each workflow that needs to be updated. An individual workflow can be updated by selecting it and then clicking on the **Update...** button. Alternatively, click on the **Update All Workflows** button to carry out all updates in a single action (figure 13.84).



RNA-seq workflow
Workflow id: QIAGEN.RNA-seq_workflow
QIAGEN Aarhus - ts-bioinformatics@qiagen.com
QIAGEN
0.1
Updates are needed for this workflow to run on this version of the software.

Figure 13.84: *A message in red text indicates a workflow needs to be updated. The Update button can be used to update an individual workflow. Alternatively, update all workflows that need updating by clicking on the Update All Workflows button.*

When you update a workflow through the Workflow Manager, the old version is overwritten.

To update a workflow you must have permission to write to the area the workflow is stored in. Usually, you will not need special permissions to do this for workflows you installed. However, to update template workflows, distributed via plugins, the *CLC Workbench* will usually need to be run as an administrative user.

When one or more installed workflows or template workflows needs to be updated, you are informed when you start up the *CLC Workbench*. A dialog listing these workflows is presented, prompting you to open the Workflow Manager (figure 13.85).

Updating installed workflows when using software in a *higher* major version line

To update an installed workflow after upgrading to software in a higher major version line, you need a copy of the older Workbench version, which the installed workflow can be run on, as well as the latest version of the Workbench.

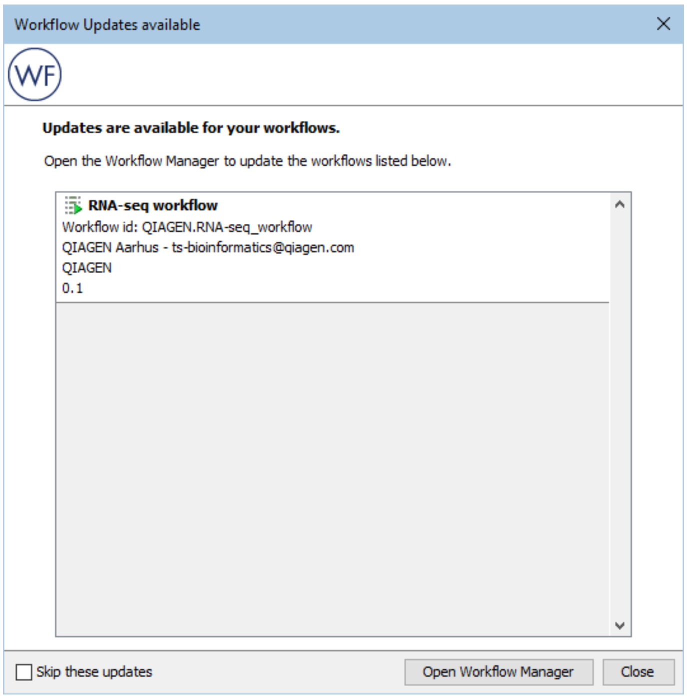
To start, open a copy of the installed workflow in a version of the Workbench it can be run on. To do this, right-click on the workflow's name in the **Installed Workflows**, folder under the **Workflows** tab in the Toolbox panel in the bottom left side of the Workbench, and choose the option "Open Copy of Workflow" from the menu that appears (figure 13.86).

Save the copy of the workflow. One way to do this is to drag and drop the tab to the location of your choice in the Navigation Area.

Close the older Workbench and open the new Workbench version. In the new version, open the workflow you just saved. Click on the **OK** button if you are prompted to update the workflow.

After checking that the workflow has been updated correctly, including that any reference data is configured as expected, save the updated workflow. Finally, click the **Installation** button to install the workflow, if desired.

If the above process does not work when upgrading directly from a much older Workbench version,



Updates are available for your workflows.

Open the Workflow Manager to update the workflows listed below.

RNA-seq workflow
Workflow id: QIAGEN.RNA-seq_workflow
QIAGEN Aarhus - ts-bioinformatics@qiagen.com
QIAGEN
0.1

☐ Skip these updates

Figure 13.85: *A dialog reporting that an installed workflow needs to be updated to be used on this version of the Workbench.*

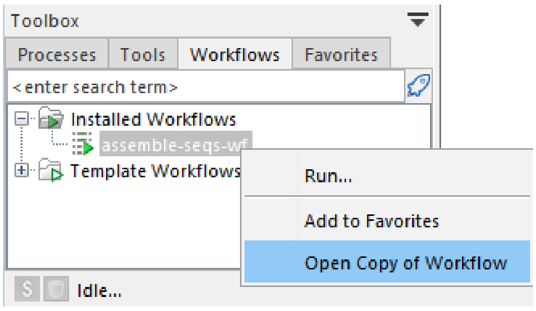


Figure 13.86: *Open a copy of an installed workflow by right-clicking on its name in the Workflows tab in the Toolbox and choosing the "Open Copy of Workflow" option from the menu.*

it may be necessary to upgrade step-wise by upgrading the workflow in sequentially higher major versions of the Workbench.

13.6.2 Workflow installation

Installing workflows allows you to provide and use a controlled version of the workflow. Only parameters left unlocked in such workflows can be configured when launching. Installed workflows are available for use from the Installed Workflows subfolder under the Workflows menu.

To create a workflow installer, or to directly install the workflow, click on the **Installation...** button at the bottom of the workflow editor (keyboard shortcut Shift + Alt + I).

At the end of this process, you will have the option to install the workflow directly to your Workbench or to create an installer file, which can be used to install the workflow on any compatible *CLC Workbench* or *CLC Server*. If you are logged into a *CLC Server* as a user with appropriate permissions, you will also have the option to install the workflow directly on the *CLC Server*.

Information about the workflow

In the Create Installer dialog, you are prompted for information about the workflow (figure 13.87). After the workflow is installed, this information will be visible in the Workflow Manager, as described in section 13.6.

The information requested when creating a workflow installer is described below. The workflow name and organization information are required- Other fields are optional.

Author name The name of the workflow author.

Author email The email address of workflow author.

Author homepage A URL.

Organization (Required) The organization name. This is used as part of the workflow id (section 13.6).

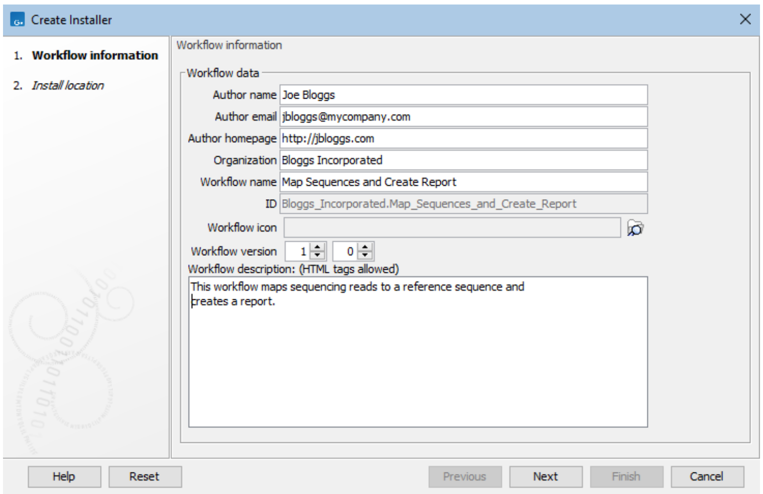
Workflow name (Required) The name of the workflow, as it should appear under the Workflows menu after installation. Changing this does not affect the name of the original workflow (as appears in your Navigation Area). This name is also used as part of the workflow id (section 13.6).

ID The workflow id. This is created using information provided in other fields. It cannot be directly edited.

Workflow icon An icon to use for this workflow in the Workflows menu once the workflow is installed. Icons use a 16 x 16 pixel gif or png file. If your icon file is larger, it will automatically be resized to fit 16 x 16 pixels.

Workflow version A major and minor version for this workflow. This version will be visible via the Workflow Manager after the workflow is installed, and will be included in the history of elements generated using this workflow. The version of the workflow open in the Workflow Editor, from which this installer is being created, will also be updated to the version specified here.

Workflow description A textual description of the workflow. After installation, this is shown in the Description tab of the Workflow Manager (section 13.6) and is also shown in a tooltip when the cursor is hovered over the installed workflow in the Workflows menu in the Toolbox panel at the bottom, left of the Workbench.



Author name: Joe Bloggs
Author email: jbloggs@mycompany.com
Author homepage: http://jbloggs.com
Organization: Bloggs Incorporated
Workflow name: Map Sequences and Create Report
ID: Bloggs_Incorporated.Map_Sequences_and_Create_Report
Workflow icon:
Workflow version: 1 0
Workflow description: (HTML tags allowed)
This workflow maps sequencing reads to a reference sequence and
creates a report.

Figure 13.87: *Provide information about the workflow that you are creating an installer for.*

Choosing how reference data inputs should be handled

If data elements were preconfigured as inputs to any workflow element, then the next step when creating the workflow installer is to specify how those elements and associated data should be handled (figure 13.88).

In workflows where no data elements were preconfigured as inputs, this step is not shown.

The data handling options are:

Ignore The data elements selected as inputs in the original workflow are not included in the workflow installer.

Input options where *Ignore* is selected should generally be kept unlocked. If locked, the data element referred to must present in the exact relative location used on your system when creating the installer. If the option is locked, and the selected data element is not present in the expected location, an error message is shown in the Workflow Manager when the workflow is installed. It will not be possible to run that workflow until the relevant data element is present in the expected location.

See section 13.2.2 for information on locking and unlocking parameters.

Bundle The data elements selected as inputs in the original workflow are included in the workflow installer. This is a good choice when sharing the workflow with others who may not have the relevant reference data on their system.

When installing a workflow with bundled data on a *CLC Workbench*, you are prompted where to save the bundled data elements. If the workflow is on a *CLC Server*, the data elements are saved automatically, as described in the *CLC Server* manual at:

`http://resources.qiagenbioinformatics.com/manuals/clcserver/current/admin/index.php?manual=Installing_configuring_workflows.html`

Bundling is intended for use with **small** reference data elements. With larger elements, the workflow installer can quickly become very big. In addition, each time such a workflow is installed, a copy of the bundled data is included, even if the relevant data element is already present on the system elsewhere.

When working with large data elements, leaving the input option unlocked and choosing the Ignore option is recommended. In this case, the relevant data elements should be shared using other means. For example, export the data and share this separately. The benefit with this, over bundling, is that the data can be shared once, rather than with every workflow installer that refers to it.

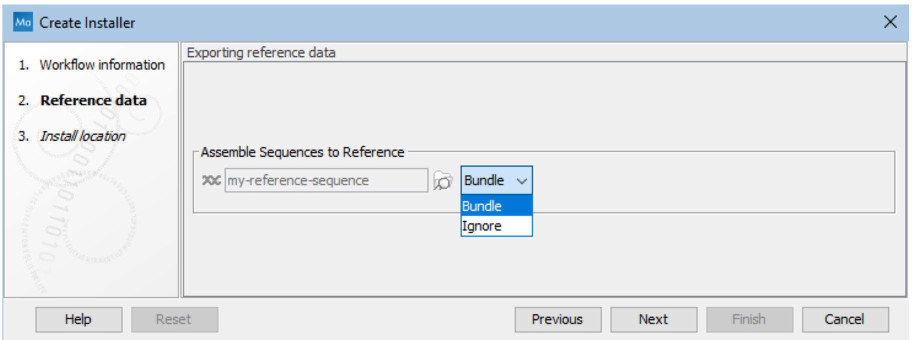


Figure 13.88: *Bundling data with the workflow installer.*

Installation options

The final step asks you to indicate whether to install the workflow directly to your Workbench or to create an installer file, which can be used to install the workflow on any compatible *CLC Main Workbench* or *CLC Server* (figure 13.89). If you are logged into a *CLC Server* as a user with appropriate permissions, you will also have the option to install the workflow directly on the *CLC Server*.

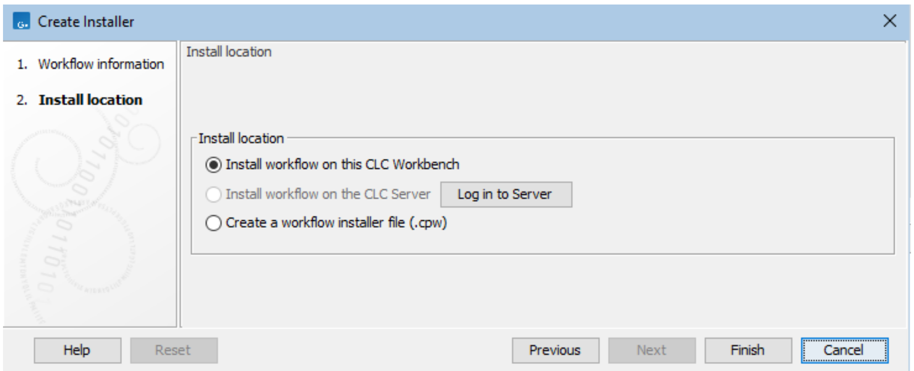
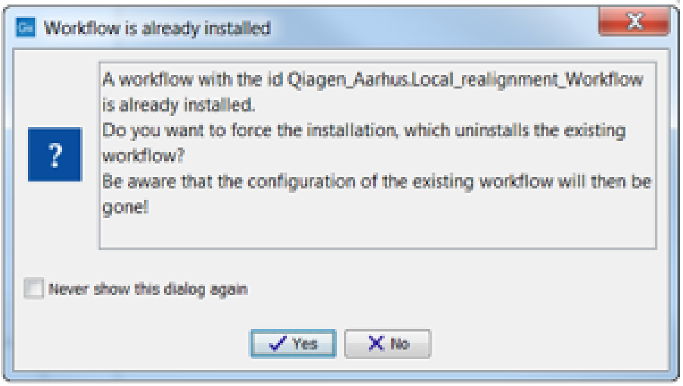


Figure 13.89: *Select whether the workflow should be installed to your CLC Workbench or an installer file (.cpw) should be created. Installation to the CLC Server is only available if you are logged in as a user with permission to administer workflows on the CLC Server.*

Workflows installed on a *CLC Workbench* cannot be updated in place. To update such a workflow, make a copy, modify it, and then create a new installer. We recommend that you increase the version number when creating the installer to help track your changes.

When you then install the updated copy of the workflow, a dialog will pop up with the message "Workflow is already installed" (figure 13.90). You have the option to force the installation. This will uninstall the existing workflow and install the modified version of the workflow. If you choose to do this, the configuration of the original workflow will be gone.


Workflow is already installed

A workflow with the id Qiagen_Aarhus.Local_realignment_Workflow is already installed.
Do you want to force the installation, which uninstalls the existing workflow?
Be aware that the configuration of the existing workflow will then be gone!

□ Never show this dialog again

Figure 13.90: *Select whether you wish to force the installation of the workflow or keep the original workflow.*

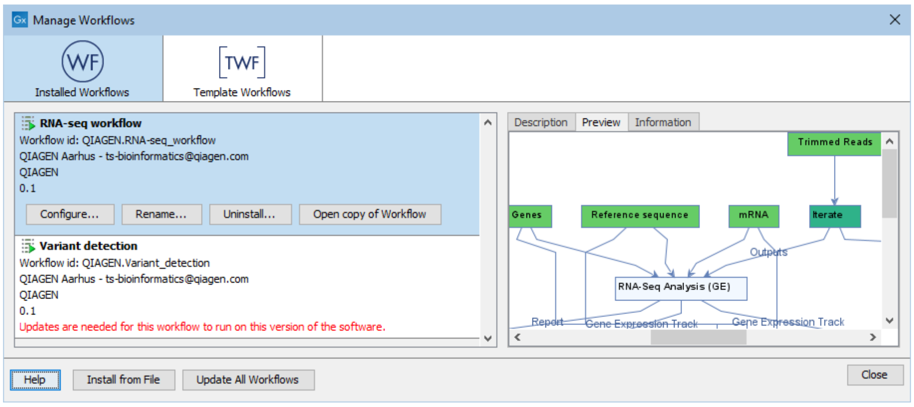
13.6.3 Using workflow installation files

Workflow installation files (.cpw files) can be installed on a *CLC Workbench* using the Workflow Manager:

Utilities | **Manage Workflows** ( )

or press the **Manage Workflows** button ( ) in the toolbar and then select "Manage Workflows..." ( ).

To install a workflow, click on Install from File and select a .cpw file. If the workflow has bundled data, you will be prompted for a location for that data. Once installed, the workflow will appear under the Installed Workflows tab (figure 13.91).


Manage Workflows

RNA-seq workflow
Workflow id: QIAGEN.RNA-seq_workflow
QIAGEN Aarhus - ts-bioinformatics@qiagen.com
QIAGEN
0.1

Variant detection
Workflow id: QIAGEN.Variant_detection
QIAGEN Aarhus - ts-bioinformatics@qiagen.com
QIAGEN
0.1
Updates are needed for this workflow to run on this version of the software.

Figure 13.91: *Workflows available in the workflow manager. The alert on the "Variant detection" workflow means that this workflow needs to be updated.*

See section 13.6.2 for information about options for handling reference data inputs.

Information about installing workflows on a *CLC Server* is provided in the *CLC Server* manual at:

http://resources.qiagenbioinformatics.com/manuals/clcserver/current/admin/index.php?manual=Installing_ configuring_workflows.html

Part III

Bioinformatics

Chapter 14

Viewing and editing sequences

Contents

Sequence information is stored in sequence elements or sequence lists. This chapter describes basic functionality for creating an working with these types of elements. Most functionality available for sequence elements is also available for sequence lists.

When you import multiple sequences, they are generally put into a sequence list, and this is the element type in use for most types of work. See chapter 7.

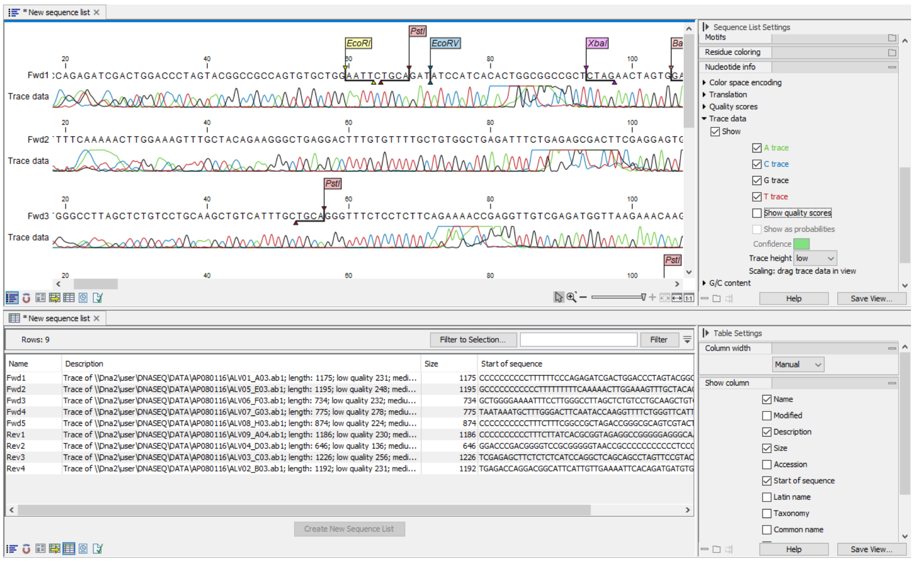
14.1 Sequence Lists

Sequence List elements contain one or more nucleotide sequences *or* one or more peptide sequences. They are used as input to many tools, and are generated as output by many tools. Sequence lists can contain single end sequences or paired end sequences, but not a mixture of both. Handling paired data is described at the end of this section.

When open in the viewing area, there are 5 icons at the bottom providing access to different views of the data. Side panel settings on views allow customization of what is shown. These settings can be saved, as described in section 4.6.

In figure 14.1, the graphical view and Table view of a sequence list are open in linked views (section 2.1). With linked views, clicking in one view can lead to updates in the other view. For example, selecting a row in the table view will cause the graphical view to update so the focus is on that sequence.

Much functionality is contained in right-click menus, with options differing depending on which view is open and where you click in it. Some actions are unique to a particular view, while others are available from more than one view. For example, sequence attributes can only be edited in the Table view but sequences can be added from the graphical view and Table view.



Sequence List Settings
- Motifs
- Residue coloring
- Nucleotide info
  - Color space encoding
  - Translation
  - Quality scores
  - Trace data
    - ☑ Show
    - ☑ A trace
    - ☑ C trace
    - ☑ G trace
    - ☑ T trace
    - ☐ Show quality scores
    - ☐ Show as probabilities
    - Confidence 🟩
    - Trace height: low
    - Scaling: drag trace data in view
  - G/C content

Rows: 9

Name	Description	Size	Start of sequence
Fwd1	Trace of \\Dna2\user\DNASEQ\DATA\AP080116\ALV01_A03.ab1; length: 1175; low quality 231; medi...	1175	CCCCCCCCCCTTTTTTCCCAGAGATCGACTGGACCCTAGTACGGC
Fwd2	Trace of \\Dna2\user\DNASEQ\DATA\AP080116\ALV05_E03.ab1; length: 1195; low quality 248; medi...	1195	GCCCCCCCCCCCTTTTTTTTTCAAAAACTTGGAAAGTTTGCTACAC
Fwd3	Trace of \\Dna2\user\DNASEQ\DATA\AP080116\ALV06_F03.ab1; length: 734; low quality 232; mediu...	734	GCTGGGGAAAATTTCCTTGGGCCTTAGCTCTGTCCTGCAAGCTGT
Fwd4	Trace of \\Dna2\user\DNASEQ\DATA\AP080116\ALV07_G03.ab1; length: 775; low quality 278; mediu...	775	TAATAAATGCTTTGGACTTCAATACCAAGGTTTTCTGGGTTCATT
Fwd5	Trace of \\Dna2\user\DNASEQ\DATA\AP080116\ALV08_H03.ab1; length: 874; low quality 224; mediu...	874	CCCCCCCCCCTTTCTTTCGGCCGCTAGACCGGGCGCAGTCGTACT
Rev1	Trace of \\Dna2\user\DNASEQ\DATA\AP080116\ALV09_A04.ab1; length: 1186; low quality 230; medi...	1186	CCCCCCCCCCTTTCTTATCACGCGGTAGAGGCCGGGGGAGGGCA
Rev2	Trace of \\Dna2\user\DNASEQ\DATA\AP080116\ALV04_D03.ab1; length: 646; low quality 136; mediu...	646	GGACCCGACGGGGTCCGCGGGGGTAACCGCCCCCCCCCCCTCCC
Rev3	Trace of \\Dna2\user\DNASEQ\DATA\AP080116\ALV03_C03.ab1; length: 1226; low quality 256; medi...	1226	TCGAGAGCTTCTCTCTCATCCAGGCTCAGCAGCCTAGTTCCGTAC
Rev4	Trace of \\Dna2\user\DNASEQ\DATA\AP080116\ALV02_B03.ab1; length: 1192; low quality 231; medi...	1192	TGAGACCAGGACGGCATTCATTGTTGAAAATTCACAGATGATGTG

Table Settings
- Column width: Manual
- Show column
  - ☑ Name
  - ☐ Modified
  - ☑ Description
  - ☑ Size
  - ☐ Accession
  - ☑ Start of sequence
  - ☐ Latin name
  - ☐ Taxonomy
  - ☐ Common name

Figure 14.1: *Two views of a sequence list are open in linked views, graphical at the top, and tabular at the bottom. Each view can be customized individually using settings in its side panel on the right.*

14.1.1 Creating sequence lists

Sequence lists are created in various ways, including:

- When sequences are imported. See chapter 7.
- As outputs of analysis tools.
- When sequences are downloaded, for example using tools under the Download menu.
- Using **Create Sequence List**, found at:

  Tools |Utility Tools ( ) | Sequence Lists ( ) | Create Sequence List ( )

or launched via a button at the bottom sequence lists in Table () view.

This tool can also be accessed at File | New | Sequence List.

14.1.2 Graphical view of sequence lists

In the graphical view of sequence lists, you can see sequences at the residue level, or zoomed right out to get an overview of the full sequence. Options in the Side Panel on the right allow detailed customization, including aspects like viewing trace data and quality scores, where available (figure 14.1).

Much of the functionality available when working with sequence lists is also available when working with individual sequence elements. Shared functionality is described in section 14.2. This section describes options relevant only to sequence lists.

Menus available when you right-click give access to much functionality for manipulating sequence lists.

Right-click on a blank area to access the following options (figure 14.2):

- **Extract Sequences** Extracts all sequences from the sequence list. If your aim is to extract a subset of the sequences, this can be done from the Table () view (see section 14.1.3) or using Split Sequence List () (see section 27.7).
- **Add Sequences** Add sequences from sequence elements or sequence lists to this sequence list.
- **Delete All Annotations from All Sequences** Deleting all annotations on all sequences can be done with this option for sequence lists with 1000 or fewer sequences. In other cases, or for more control over the annotations to delete, use the Annotation Table () view, described further below.

Right-click on a sequence or sequence name to access options (figure 14.3) to:

- **Rename, select, open,** or **delete** that sequence.
- **Sort the sequence list** alphabetically by sequence name, by length or by marked status. These options are only available for sequence lists with 1000 or fewer sequences.
- **Delete sequences that have been marked** This option is enabled when at least one sequence has been marked. Marking sequences is described below.

Tips for working with larger sequence lists are given later in this section.

Marking sequences:

Sequences in a list can be marked. Once marked, those sequences can be deleted, or the sequence list can be sorted based on whether sequences are marked or not. It is easy to adjust markings on many sequences using the options in the right-click menu on selection boxes (figure 14.4).

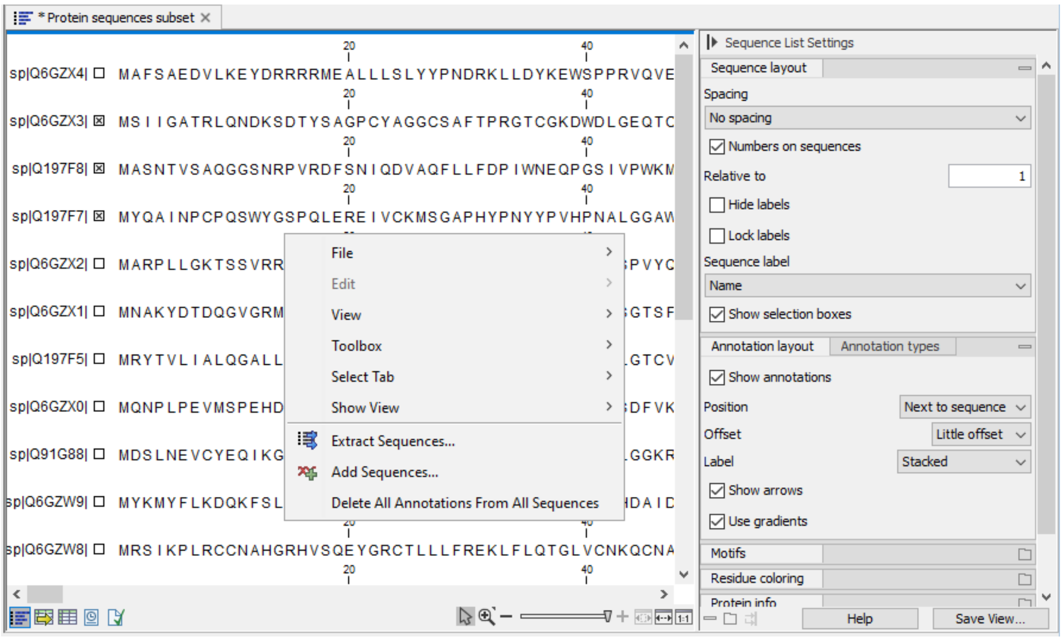
To mark sequences:

1. Check the **Show selection boxes** option in the "Sequence List Settings" section of the side panel settings on the right hand side.

   This makes checkboxes visible to the right of each sequence name.

2. Click in the checkbox beside a sequence name to mark the sequence.

   Clicking in the box for a marked sequence will remove the mark.



```
sp|Q6GZX4| □ MAFSAEDVLKEYDRRRRMEALLLSLYYPNDRKLLDYKEWSPPRVQVE
sp|Q6GZX3| ☒ MSIIGATRLQNDKSDTYSAGPCYAGGCSAFTPRGTCGKDWDLGEQTC
sp|Q197F8| ☒ MASNTVSAQGGSNRPVRDFSNIQDVAQFLLFDPIWNEQPGSIVPWKM
sp|Q197F7| ☒ MYQAINPCPQSWYGSPQLEREIVCKMSGAPHYPNYYPVHPNALGGAW
sp|Q6GZX2| □ MARPLLGKTSSVRR
sp|Q6GZX1| □ MNAKYDTDQGVGRM
sp|Q197F5| □ MRYTVLIALQGALL
sp|Q6GZX0| □ MQNPLPEVMSPEHD
sp|Q91G88| □ MDSLNEVCYEQIKG
sp|Q6GZW9| □ MYKMYFLKDQKFSL
sp|Q6GZW8| □ MRSIKPLRCCNAHGRHVSQEYGRCTLLLFREKLFLQTGLVCNKQCNA
```

Sequence List Settings

Sequence layout

Spacing: No spacing

☑ Numbers on sequences

Relative to: 1

□ Hide labels

□ Lock labels

Sequence label: Name

☑ Show selection boxes

Annotation layout | Annotation types

☑ Show annotations

Position: Next to sequence

Offset: Little offset

Label: Stacked

☑ Show arrows

☑ Use gradients

Motifs

Residue coloring

Protein info

Figure 14.2: *Options to extract the sequences in the list, add sequences to the list, and to delete all annotations on all sequences are available when you right-click on a blank area of the graphical view of a sequence list.*

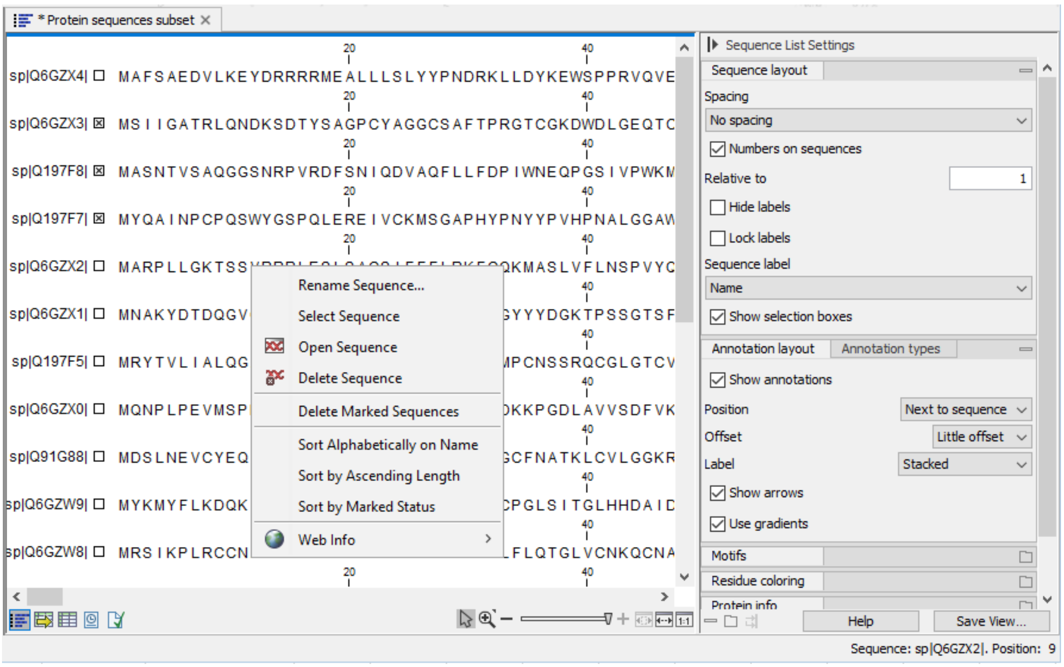
Tips when working with large sequence lists

- **Sorting long lists** can be done in Table (▦) view. For example, to sort on length, ensure the Size column is enabled in the side panel to the right of the table, and then click on the Size column header to sort the list. If a Table view is open as a linked view with the graphical view, clicking on a row in the table will highlight that sequence in the graphical view. See section 2.1 for information on linked views and section 9 for information about working with tables.

- **Deleting annotations on sequences** can be done in the Annotation Table (▦) view. Right click anywhere in this view to reveal a menu containing relevant options. To delete all annotations on the sequence list, ensure all annotation types are enabled in the side panel settings to the right.

- **To delete many sequences** from a list, you can mark the few you wish to retain, and then invert the marking by right-clicking on any selection checkbox and choosing the option **Invert All Marks** (figure 14.4).

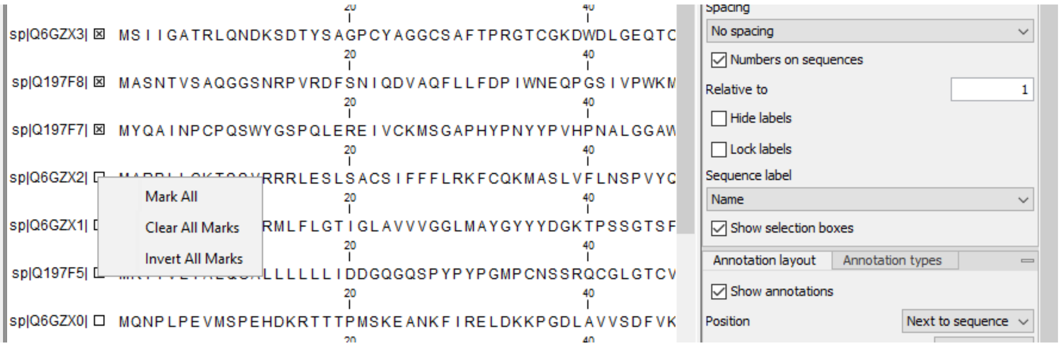
  Then right-click on any sequence or sequence name and choose the option to **Delete Marked Sequences** (figure 14.3). If the sequence list contains more than 1000 sequences, a warning will appear noting that, if you proceed, the deletion cannot be undone.



Spacing: No spacing
☑ Numbers on sequences
Relative to: 1
☐ Hide labels
☐ Lock labels
Sequence label: Name
☑ Show selection boxes
☑ Show annotations
Position: Next to sequence
Offset: Little offset
Label: Stacked
☑ Show arrows
☑ Use gradients

Sequence: sp|Q6GZX2|. Position: 9

Figure 14.3: *Options to rename, select, open, or delete a sequence are available when you right-click on the name or residues for a given sequence. Also in this menu are options for sorting the list and deleting marked sequences.*



Spacing: No spacing
☑ Numbers on sequences
Relative to: 1
☐ Hide labels
☐ Lock labels
Sequence label: Name
☑ Show selection boxes
☑ Show annotations
Position: Next to sequence

Figure 14.4: *Which sequences are marked can be quickly adjusted using the options in the right-click menu for any selection checkbox. The Show Selection boxes option in the side panel must be enabled to see these boxes.*

- **Renaming multiple sequences** in a list following the same renaming pattern can be done using the dedicated tool, **Rename Sequences in Lists**, described in section 27.9.

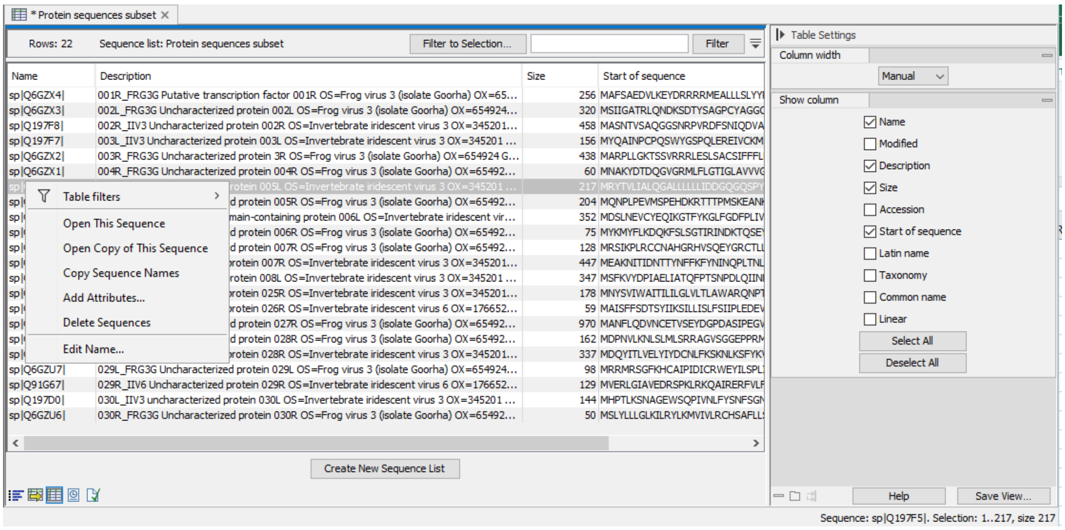
14.1.3 Table view of sequence lists

Sequence attributes are listed the Table (▦) view (figure 14.5). This includes information like the name, description, accession, length, etc. of each sequence, as well as any other attributes for that element, such as those added using Update Sequence Attributes in Lists (section 27.6), or custom attributes for that location (section 3.3).

The number of rows reported at the top is the number of sequences in the list (figure 14.5).

Standard functionality for working with tables applies (see section 9).

Actions on the sequence list can be taken directly, or via a right-click menu (figure 14.5):



Rows: 22 Sequence list: Protein sequences subset

Name	Description	Size	Start of sequence
sp|Q6GZX4|	001R_FRG3G Putative transcription factor 001R OS=Frog virus 3 (isolate Goorha) OX=65...	256	MAFSAEDVLKEYDRRRRMEALLLSLYYI
sp|Q6GZX3|	002L_FRG3G Uncharacterized protein 002L OS=Frog virus 3 (isolate Goorha) OX=654924...	320	MSIIGATRLQNDKSDTYSAGPCYAGGC
sp|Q197F8|	002R_IIV3 Uncharacterized protein 002R OS=Invertebrate iridescent virus 3 OX=345201...	458	MASNTVSAQGGSNRPVRDFSNIQDVA
sp|Q197F7|	003L_IIV3 Uncharacterized protein 003L OS=Invertebrate iridescent virus 3 OX=345201 ...	156	MYQAINPCPQSWYGSPQLEREIVCKM
sp|Q6GZX2|	003R_FRG3G Uncharacterized protein 3R OS=Frog virus 3 (isolate Goorha) OX=654924 G...	438	MARPLLGKTSSVRRRLESLSACSIFFFL
sp|Q6GZX1|	004R_FRG3G Uncharacterized protein 004R OS=Frog virus 3 (isolate Goorha) OX=65492...	60	MNAKYDTDQGVGRMLFLGTIGLAVVVG
sp|	rotein 005L OS=Invertebrate iridescent virus 3 OX=345201 ...	217	MRYTVLIALQGALLLLLLIDDGQGQSPY
sp|	d protein 005R OS=Frog virus 3 (isolate Goorha) OX=65492...	204	MQNPLPEVMSPEHDKRTTTPMSKEANK
sp|	main-containing protein 006L OS=Invertebrate iridescent vir...	352	MDSLNEVCYEQIKGTFYKGLFGDFPLIV
sp|	d protein 006R OS=Frog virus 3 (isolate Goorha) OX=65492...	75	MYKMYFLKDQKFSLSGTIRINDKTQSEY
sp|	d protein 007R OS=Frog virus 3 (isolate Goorha) OX=65492...	128	MRSIKPLRCCNAHGRHVSQEYGRCTLL
sp|	protein 007R OS=Invertebrate iridescent virus 3 OX=345201...	447	MEAKNITIDNTTYNFFKFYNINQPLTNL
sp|	rotein 008L OS=Invertebrate iridescent virus 3 OX=345201 ...	347	MSFKVYDPIAELIATQFPTSNPDLQIINI
sp|	protein 025R OS=Invertebrate iridescent virus 3 OX=345201...	178	MNYSVIWAITILILGLVLTLAWARQNPT
sp|	protein 026R OS=Invertebrate iridescent virus 6 OX=176652...	59	MAISFFSDTSYIIKSILLISLFSIIPLEDEV
sp|	d protein 027R OS=Frog virus 3 (isolate Goorha) OX=65492...	970	MANFLQDVNCETVSEYDGPDASIPEGV
sp|	d protein 028R OS=Frog virus 3 (isolate Goorha) OX=65492...	162	MDPNVLKNLSLMLSRRAGVSGGEPPRM
sp|	protein 028R OS=Invertebrate iridescent virus 3 OX=345201...	337	MDQYITLVELYIYDCNLFKSKNLKSFYKV
sp|Q6GZU7|	029L_FRG3G Uncharacterized protein 029L OS=Frog virus 3 (isolate Goorha) OX=654924...	98	MRRMRSGFKHCAIPIDICRWEYILSPLI
sp|Q91G67|	029R_IIV6 Uncharacterized protein 029R OS=Invertebrate iridescent virus 6 OX=176652...	129	MVERLGIAVEDRSPKLRKQAIRERFVLF
sp|Q197D0|	030L_IIV3 uncharacterized protein 030L OS=Invertebrate iridescent virus 3 OX=345201 ...	144	MHPTLKSNAGEWSQPIVNLFYSNFSGN
sp|Q6GZU6|	030R_FRG3G Uncharacterized protein 030R OS=Frog virus 3 (isolate Goorha) OX=65492...	50	MSLYLLLGLKILRYLKMVIVLRCHSAFLL

Column width: Manual

Show column:
- ☑ Name
- □ Modified
- ☑ Description
- ☑ Size
- □ Accession
- ☑ Start of sequence
- □ Latin name
- □ Taxonomy
- □ Common name
- □ Linear

Sequence: sp|Q197F5|. Selection: 1..217, size 217

Figure 14.5: *In Table view there is a row for each sequence in the sequence list. The number of rows equates to the number of sequences and is reported at the top left side. Right-click to display a menu with actions. This menu differs slightly depending on which column you click upon.*

- **Add sequences** Add sequences to this list by dragging and dropping sequence elements or sequence lists from the Navigation Area into the table. Sequences can also be added from the graphical view using a right-click option, as described earlier in this section.
- **Remove sequences** by selecting the relevant rows and either:
  - Clicking on the **Delete** ( ) icon in the top toolbar.
  - Right-clicking and choose the option **Delete Sequences** from the menu (figure 14.5).

  Sequences can also be deleted from other views.
- **Copy sequence names** Select the relevant rows, right-click and choose **Copy Sequence Names** from the menu. This list can be used within the Workbench, for example, in table filters with the action "is in list" or "is not in list" to find these names in other elements, or they can be pasted to other programs that accept text lists, such as Excel or text editors.
- **Edit attributes** Right-click in the the cell you wish to edit, and then update the contents of that cell. For example, if you right-click on a cell in the Name column, an option called "Edit Name..." will be in the menu presented (figure 14.5).

  If you select multiple rows, you will be able to edit the attribute, with the value you provide being applied to all the selected rows.

  Values calculated from the sequence itself cannot be edited directly. E.g. The Size column contains the length of each sequence, and the Start of sequence column contains the first 50 residues.
- **Extract selected sequences** Specified sequences in the list can be extracted:
  - **To a new sequence list** by selecting relevant rows and clicking on the **Create New Sequence List** button. This new list must be saved if you wish to keep it.
  - **To a individual sequence elements** by selecting relevant rows and dragging them into the Navigation Area.

Adding attributes

Attributes (columns in Table view) can be added using the right-click menu option **Add Attributes**. This is good for small lists and simple changes. You are prompted for an attribute name and a single value. A new column is added to the table with the name you provide, and the value you provided is added for all of the selected rows. This option can also be used to edit contents of an existing column, if desired.

The **Update Sequence Attributes in Lists** tool supports more detailed work, including importing from external sources, such as Excel and CSV format files. See (section 27.6) for more details.

14.1.4 Annotation Table view of sequence lists

Options in the Annotation Table ( ) view are the same as those in this view for sequences. They are described in the following sections:

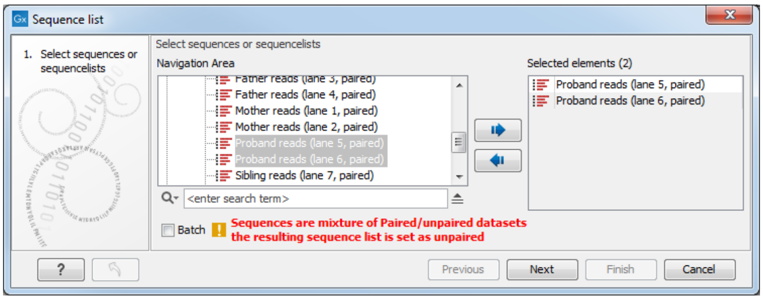
- Working with the Annotation Table ( ) view: section 14.3.1
- Adding annotations: section 14.3.2
- Editing annotations: section 14.3.3
- Exporting annotations to a gff3 format file: section 14.3.4
- Deleting annotations: section 14.3.5

14.1.5 Working with paired sequences in lists

When paired sequences are imported, the sequence list created is marked as containing paired data. This can be seen in the Element info view and can be edited within that view (section 14.4).

A sequence lists can contain single end reads or it can contain paired end reads. A given list cannot contain a mixture of these.

To create a paired sequence list from existing sequence lists, for example by merging lists, the input lists must be marked as paired and must have the same distance settings. If the input lists do not meet these criteria, a message is shown warning that the resulting sequence list will be unpaired (figure 14.6).



Father reads (lane 3, paired)
Father reads (lane 4, paired)
Mother reads (lane 1, paired)
Mother reads (lane 2, paired)
Proband reads (lane 5, paired)
Proband reads (lane 6, paired)
Sibling reads (lane 7, paired)

<enter search term>

□ Batch ! Sequences are mixture of Paired/unpaired datasets
the resulting sequence list is set as unpaired

Proband reads (lane 5, paired)
Proband reads (lane 6, paired)

Figure 14.6: *A warning appears when trying to create a new sequence list from a mixture of paired and unpaired sequence lists.*

14.2 View sequences

This section describes options available when viewing and editing sequences. Many of these options also apply to sequence lists (section 14.1) and alignments (section 16.2).

14.2.1 Sequence settings in Side Panel

Settings for a view are available in the **Side Panel** at the right hand side of the view (figure 14.7). The view is instantly updated when these settings are changed. The options available for sequence views are described in the sections that follow.

Note: Side Panel settings are not automatically saved when you save the sequence. See section 4.6 for information on saving and applying view settings.

For general information about Side Panels, see section 2.1.6.

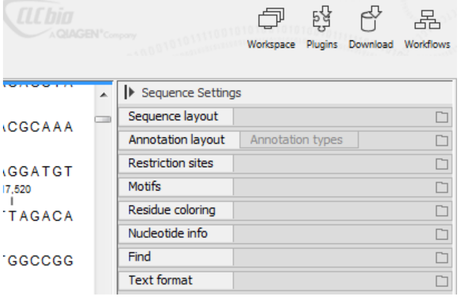


Figure 14.7: *Overview of the Side Panel for a sequence. Each tab can be expanded to reveal settings that can be configured.*

Sequence Layout

These preferences determine the overall layout of the sequence:

- **Spacing.** Inserts a space at a specified interval:
  - **No spacing.** The sequence is shown with no spaces.
  - **Every 10 residues.** There is a space every 10 residues, starting from the beginning of the sequence.
  - **Every 3 residues, frame 1.** There is a space every 3 residues, corresponding to the reading frame starting at the first residue.
  - **Every 3 residues, frame 2.** There is a space every 3 residues, corresponding to the reading frame starting at the second residue.
  - **Every 3 residues, frame 3.** There is a space every 3 residues, corresponding to the reading frame starting at the third residue.
- **Wrap sequences.** Shows the sequence on more than one line.
  - **No wrap.** The sequence is displayed on one line.
  - **Auto wrap.** Wraps the sequence to fit the width of the view, not matter if it is zoomed in our out (displays minimum 10 nucleotides on each line).
  - **Fixed wrap.** Makes it possible to specify when the sequence should be wrapped. In the text field below, you can choose the number of residues to display on each line.

- **Double stranded.** Shows both strands of a sequence (only applies to DNA sequences).
- **Numbers on sequences.** Shows residue positions along the sequence. The starting point can be changed by setting the number in the field below. If you set it to e.g. 101, the first residue will have the position of -100. This can also be done by right-clicking an annotation and choosing **Set Numbers Relative to This Annotation**.
- **Numbers on plus strand.** Whether to set the numbers relative to the positive or the negative strand in a nucleotide sequence (only applies to DNA sequences).
- **Lock numbers.** When you scroll vertically, the position numbers remain visible. (Only possible when the sequence is not wrapped.)
- **Lock labels.** When you scroll horizontally, the label of the sequence remains visible.
- **Sequence label.** Defines the label to the left of the sequence.
  - Name (this is the default information to be shown).
  - Accession (sequences downloaded from databases like GenBank have an accession number).
  - Latin name.
  - Latin name (accession).
  - Common name.
  - Common name (accession).
- **Matching residues as dots** Residues in aligned sequences identical to residues in the first (reference) sequence will be presented as dots. An option that is only available for "Alignments" and "Read mappings".

Annotation Layout and Annotation Types See section 14.3.1.

Restriction sites

Please see section 23.1.1.

Motifs

See section 18.9.1.

Residue coloring

These preferences make it possible to color both the residue letter and set a background color for the residue.

- **Non-standard residues.** For nucleotide sequences this will color the residues that are not C, G, A, T or U. For amino acids only B, Z, and X are colored as non-standard residues.
  - **Foreground color.** Sets the color of the letter. Click the color box to change the color.

  - **Background color.** Sets the background color of the residues. Click the color box to change the color.

- **Rasmol colors.** Colors the residues according to the Rasmol color scheme.
  See http://www.openrasmol.org/doc/rasmol.html
  - **Foreground color.** Sets the color of the letter. Click the color box to change the color.
  - **Background color.** Sets the background color of the residues. Click the color box to change the color.

- **Polarity colors (only protein).** Colors the residues according to the following categories:
  - **Green** neutral, polar
  - **Black** neutral, nonpolar
  - **Red** acidic, polar
  - **Blue** basic ,polar
  - As with other options, you can choose to set or change the coloring for either the residue letter or its background:
    - **Foreground color.** Sets the color of the letter. Click the color box to change the color.
    - **Background color.** Sets the background color of the residues. Click the color box to change the color.

- **Trace colors (only DNA).** Colors the residues according to the color conventions of chromatogram traces: A=green, C=blue, G=black, and T=red.
  - **Foreground color.** Sets the color of the letter.
  - **Background color.** Sets the background color of the residues.

Nucleotide info

These preferences apply only to nucleotide sequences.

The data points for graph representations can be exported (see section 8.3).

- **Translation.** Displays a translation into protein just below the nucleotide sequence. Depending on the zoom level, the amino acids are displayed with three letters or one letter. In cases where variants are present in the reads, synonymous variants are shown in orange in the translated sequence whereas non-synonymous are shown in red.
  - **Frame.** Determines where to start the translation.
    - **ORF/CDS**. If the sequence is annotated, the translation will follow the CDS or ORF annotations. If annotations overlap, only one translation will be shown. If only one annotation is visible, the Workbench will attempt to use this annotation to mark the start and stop for the translation. In cases where this is not possible, the first annotation will be used (i.e. the one closest to the 5' end of the sequence).

        - **Selection.** This option will only take effect when you make a selection on the sequence. The translation will start from the first nucleotide selected. Making a new selection will automatically display the corresponding translation. Read more about selecting in section 14.2.2.
        - **+1 to -1.** Select one of the six reading frames.
        - **All forward/All reverse.** Shows either all forward or all reverse reading frames.
        - **All.** Select all reading frames at once. The translations will be displayed on top of each other.
    - **Table.** The translation table to use in the translation. For more about translation tables, see section 19.4.
    - **Only AUG start codons.** For most genetic codes, a number of codons can be start codons (TTG, CTG, or ATG). These will be colored green, unless selecting the "Only AUG start codons" option, which will result in only the AUG codons colored in green.
    - **Single letter codes.** Choose to represent the amino acids with a single letter instead of three letters.

- **Quality scores.** For sequencing data containing quality scores, the quality score information can be displayed along the sequence.
    - **Show as probabilities.** Converts quality scores to error probabilities on a 0-1 scale, i.e. not log-transformed.
    - **Foreground color.** Colors the letter using a gradient, where the left side color is used for low quality and the right side color is used for high quality. The sliders just above the gradient color box can be dragged to highlight relevant levels. The colors can be changed by clicking the box. This will show a list of gradients to choose from.
    - **Background color.** Sets a background color of the residues using a gradient in the same way as described above.
    - **Graph.** The quality scores are displayed as a graph.
        - **Height.** Specifies the height of the graph.
        - **Type.** The graph can be displayed as Line plot, Bar plot or as a Color bar.
        - **Color box.** For Line and Bar plots, the color of the plot can be set by clicking the color box. For Colors, the color box is replaced by a gradient color box as described under Foreground color.

- **Trace data.** See section 21.1.

- **G/C content.** Calculates the G/C content of a part of the sequence and shows it as a gradient of colors or as a graph below the sequence.
    - **Window length.** Determines the length of the part of the sequence to calculate. A window length of 9 will calculate the G/C content for the nucleotide in question plus the 4 nucleotides to the left and the 4 nucleotides to the right. A narrow window will focus on small fluctuations in the G/C content level, whereas a wider window will show fluctuations between larger parts of the sequence.
    - **Foreground color.** Colors the letter using a gradient, where the left side color is used for low levels of G/C content and the right side color is used for high levels of G/C content. The sliders just above the gradient color box can be dragged to highlight relevant levels of G/C content. The colors can be changed by clicking the box. This will show a list of gradients to choose from.

  - **Background color.** Sets a background color of the residues using a gradient in the same way as described above.
  - **Graph.** The G/C content levels are displayed as a graph.
    - **Height.** Specifies the height of the graph.
    - **Type.** The graph can be displayed as Line plot, Bar plot or as a Color bar.
    - **Color box.** For Line and Bar plots, the color of the plot can be set by clicking the color box. For Colors, the color box is replaced by a gradient color box as described under Foreground color.

    When zoomed out, the graph displays G/C content only for a subset of evenly spaced positions. Because insertions shifts reference positions, zoomed-out graphs with and without insertions may not be directly comparable, as G/C content may be displayed for different positions.

- **Secondary structure.** Allows you to choose how to display a symbolic representation of the secondary structure along the sequence.

  See section 24.2.3 for a detailed description of the settings.

Protein info

These preferences only apply to proteins. The first nine items are different hydrophobicity scales. These are described in section 20.3.1.

- **Kyte-Doolittle.** The Kyte-Doolittle scale is widely used for detecting hydrophobic regions in proteins. Regions with a positive value are hydrophobic. This scale can be used for identifying both surface-exposed regions as well as transmembrane regions, depending on the window size used. Short window sizes of 5-7 generally work well for predicting putative surface-exposed regions. Large window sizes of 19-21 are well suited for finding transmembrane domains if the values calculated are above 1.6 [Kyte and Doolittle, 1982]. These values should be used as a rule of thumb and deviations from the rule may occur.

- **Cornette.** Cornette *et al.* computed an optimal hydrophobicity scale based on 28 published scales [Cornette et al., 1987]. This optimized scale is also suitable for prediction of alpha-helices in proteins.

- **Engelman.** The Engelman hydrophobicity scale, also known as the GES-scale, is another scale which can be used for prediction of protein hydrophobicity [Engelman et al., 1986]. As the Kyte-Doolittle scale, this scale is useful for predicting transmembrane regions in proteins.

- **Eisenberg.** The Eisenberg scale is a normalized consensus hydrophobicity scale which shares many features with the other hydrophobicity scales [Eisenberg et al., 1984].

- **Rose.** The hydrophobicity scale by Rose *et al.* is correlated to the average area of buried amino acids in globular proteins [Rose et al., 1985]. This results in a scale which is not showing the helices of a protein, but rather the surface accessibility.

- **Janin.** This scale also provides information about the accessible and buried amino acid residues of globular proteins [Janin, 1979].

- **Hopp-Woods.** Hopp and Woods developed their hydrophobicity scale for identification of potentially antigenic sites in proteins. This scale is basically a hydrophilic index where apolar residues have been assigned negative values. Antigenic sites are likely to be predicted when using a window size of 7 [Hopp and Woods, 1983].

- **Welling.** [Welling et al., 1985] Welling et al. used information on the relative occurrence of amino acids in antigenic regions to make a scale which is useful for prediction of antigenic regions. This method is better than the Hopp-Woods scale of hydrophobicity which is also used to identify antigenic regions.

- **Kolaskar-Tongaonkar.** A semi-empirical method for prediction of antigenic regions has been developed [Kolaskar and Tongaonkar, 1990]. This method also includes information of surface accessibility and flexibility and at the time of publication the method was able to predict antigenic determinants with an accuracy of 75%.

- **Surface Probability.** Display of surface probability based on the algorithm by [Emini et al., 1985]. This algorithm has been used to identify antigenic determinants on the surface of proteins.

- **Chain Flexibility.** Display of backbone chain flexibility based on the algorithm by [Karplus and Schulz, 1985]. It is known that chain flexibility is an indication of a putative antigenic determinant.

Find

The Find function can be used for searching the sequence and is invoked by pressing Ctrl + Shift + F (⌘ + Shift + F on Mac). Initially, specify the 'search term' to be found, select the type of search (see various options in the following) and finally click on the Find button. The first occurrence of the search term will then be highlighted. Clicking the find button again will find the next occurrence and so on. If the search string is found, the corresponding part of the sequence will be selected.

- **Search term.** Enter the text or number to search for. The search function does not discriminate between lower and upper case characters.

- **Sequence search.** Search the nucleotides or amino acids. For amino acids, the single letter abbreviations should be used for searching. The sequence search also has a set of advanced search parameters:

  - Include negative strand. This will search on the negative strand as well.
  - Treat ambiguous characters as wildcards in search term. If you search for e.g. ATN, you will find both ATG and ATC. If you wish to find literally exact matches for ATN (i.e. only find ATN - not ATG), this option should not be selected.
  - Treat ambiguous characters as wildcards in sequence. If you search for e.g. ATG, you will find both ATG and ATN. If you have large regions of Ns, this option should not be selected.

  Note that if you enter a position instead of a sequence, it will automatically switch to position search.

- **Annotation search.** Search the annotations on the sequence. The search is performed both on the labels of the annotations, but also on the text appearing in the tooltip that you see when you keep the mouse cursor fixed. If the search term is found, the part of the sequence corresponding to the matching annotation is selected. The option "Include translations" means that you can choose to search for translations *which are part of an annotation* (in some cases, CDS annotations contain the amino acid sequence in a "/translation" field). But it will not dynamically translate nucleotide sequences, nor will it search the translations that can enabled using the "Nucleotide info" side panel.

- **Position search.** Find a specific position on the sequence. In order to find an interval, e.g. from position 500 to 570, enter "500..570" in the search field. This will make a selection from position 500 to 570 (both included). Notice the two periods (..) between the start an end number. If you enter positions including thousands separators like 123,345, the comma will just be ignored and it would be equivalent to entering 123345.

- **Include negative strand.** When searching the sequence for nucleotides or amino acids, you can search on both strands.

- **Name search.** Search for sequence names. This is useful for searching sequence lists and mapping results for example.

This concludes the description of the **View Preferences**. Next, the options for selecting and editing sequences are described.

Text format

These preferences allow you to adjust the format of all the text in the view (both residue letters, sequence name and translations if they are shown).

- **Text size.** Specify a font size for the text in the view.

- **Font.** Specify a font for the text in the view.

- **Bold.** Make the text for the residues bold.

Restriction sites in the Side Panel

Please see section 23.1.1.

14.2.2 Selecting parts of the sequence

You can select parts of a sequence:

> **Click Selection (↖) in Toolbar | Press and hold down the mouse button on the sequence where you want the selection to start | move the mouse to the end of the selection while holding the button | release the mouse button**

Alternatively, you can search for a specific interval using the find function described above.

If you have made a selection and wish to adjust it:

> **drag the edge of the selection (you can see the mouse cursor change to a horizontal arrow**

or **press and hold the Shift key while using the right and left arrow keys to adjust the right side of the selection.**

If you wish to select the entire sequence:

double-click the sequence name to the left

Selecting non-contiguous parts of a sequence (multiselect) You can select non-contiguous parts of sequence by holding down the **Ctrl** button while making selections. Holding down the **Shift** button lets you extend or reduce an existing selection to the position you clicked.

To select a part of a sequence covered by an annotation:

right-click the annotation | Select annotation

or **double-click the annotation**

To select a fragment between two restriction sites that are shown on the sequence:

double-click the sequence between the two restriction sites

(Read more about restriction sites in section 14.2.1.)

Open a selection in a new view A selection can be opened in a new view and saved as a new sequence:

right-click the selection | Open selection in New View ( )

This opens the annotated part of the sequence in a new view. The new sequence can be saved by dragging the tab of the sequence view into the **Navigation Area**.

The process described above is also the way to manually translate coding parts of sequences (CDS) into protein. You simply translate the new sequence into protein. This is done by:

right-click the tab of the new sequence | Tools | Nucleotide Analysis ( )| Translate to Protein ( )

A selection can also be copied to the clipboard and pasted into another program:

make a selection | Ctrl + C (⌘ + C on Mac)

Note! The annotations covering the selection will not be copied.

A selection of a sequence can be edited as described in the following section.

14.2.3 Editing the sequence

When you make a selection, it can be edited by:

right-click the selection | Edit Selection ( )

A dialog appears displaying the sequence. You can add, remove or change the text and click **OK**. The original selected part of the sequence is now replaced by the sequence entered in the dialog. This dialog also allows you to paste text into the sequence using Ctrl + V (⌘ + V on Mac).

If you delete the text in the dialog and press **OK**, the selected text on the sequence will also be deleted. Another way to delete a part of the sequence is to:

right-click the selection | Delete Selection ( )

If you wish to correct only one residue, this is possible by simply making the selection cover only one residue and then type the new residue.

Another way to edit the sequence is by inserting a restriction site. See section 23.1.3.

Note When editing annotated nucleotide sequences, the annotation content is not updated automatically (but its position is). Please refer to section 14.3.3 for details on annotation editing.

Before exporting annotated nucleotide sequences in GenBank format, ensure that the annotations in the Annotations Table reflect the edits that have been made to the sequence.

14.2.4 Sequence region types

The various annotations on sequences cover parts of the sequence. Some cover an interval, some cover intervals with unknown endpoints, some cover more than one interval etc. In the following, all of these will be referred to as *regions*. Regions are generally illustrated by markings (often arrows) on the sequences. An arrow pointing to the right indicates that the corresponding region is located on the positive strand of the sequence. Figure 14.8 is an example of three regions with separate colors.

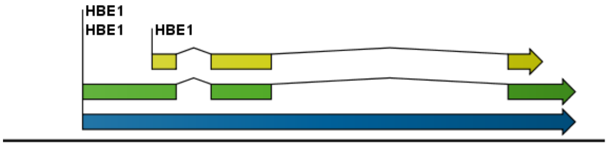


Figure 14.8: *Three regions on a human beta globin DNA sequence (HUMHBB).*

Figure 14.9 shows an artificial sequence with all the different kinds of regions.

14.2.5 Circular DNA

A sequence can be shown as a circular molecule:

Select a sequence in the Navigation Area and right-click on the file name | Hold the mouse over "Show" to enable a list of options | Select "Circular View" ( )

or **If the sequence is already open | Click "Show Circular View" ( ) at the lower left part of the view**

This will open a view of the molecule similar to the one in figure 14.10.

This view of the sequence shares some of the properties of the linear view of sequences as described in section 14.2, but there are some differences. The similarities and differences are listed below:

- **Similarities**:
  - The editing options.
  - Options for adding, editing and removing annotations.
  - **Restriction Sites**, **Annotation Types**, **Find** and **Text Format** preferences groups.

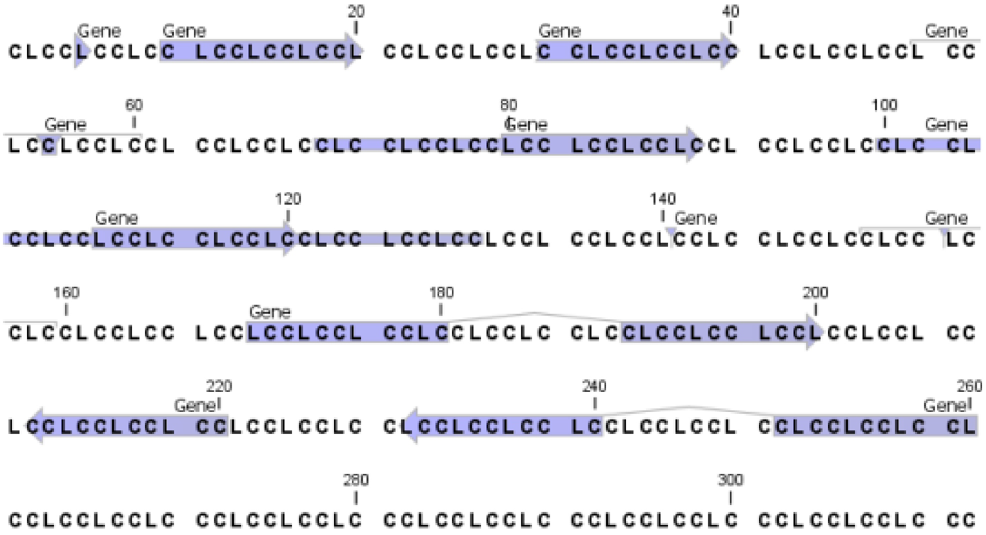

Gene
Gene
20
Gene
40
Gene
CLCCLCCLCC LCCLCCLCCL CCLCCLCCLC CLCCLCCLCC LCCLCCLCCL CC
Gene
60
80
Gene
100
Gene
LCCLCCLCCL CCLCCLCCLC CLCCLCCLCC LCCLCCLCCL CCLCCLCCLC CL
Gene
120
140
Gene
Gene
CCLCCLCCLC CLCCLCCLCC LCCLCCLCCL CCLCCLCCLC CLCCLCCLCC LC
160
Gene
180
200
CLCCLCCLCC LCCLCCLCCL CCLCCLCCLC CLCCLCCLCC LCCLCCLCCL CC
220
Gene
240
260
Gene
LCCLCCLCCL CCLCCLCCLC CLCCLCCLCC LCCLCCLCCL CCLCCLCCLC CL
280
300
CCLCCLCCLC CCLCCLCCLC CCLCCLCCLC CCLCCLCCLC CCLCCLCCLC CC

Figure 14.9: *Region #1: A single residue, Region #2: A range of residues including both endpoints, Region #3: A range of residues starting somewhere before 30 and continuing up to and including 40, Region #4: A single residue somewhere between 50 and 60 inclusive, Region #5: A range of residues beginning somewhere between 70 and 80 inclusive and ending at 90 inclusive, Region #6: A range of residues beginning somewhere between 100 and 110 inclusive and ending somewhere between 120 and 130 inclusive, Region #7: A site between residues 140 and 141, Region #8: A site between two residues somewhere between 150 and 160 inclusive, Region #9: A region that covers ranges from 170 to 180 inclusive and 190 to 200 inclusive, Region #10: A region on negative strand that covers ranges from 210 to 220 inclusive, Region #11: A region on negative strand that covers ranges from 230 to 240 inclusive and 250 to 260 inclusive.*

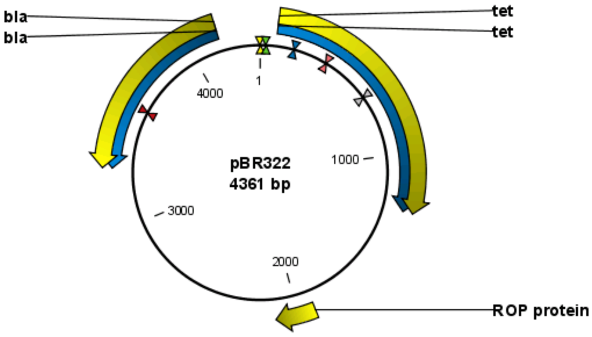


Figure 14.10: *A molecule shown in a circular view.*

- **Differences**:
  - In the **Sequence Layout** preferences, only the following options are available in the circular view: **Numbers on plus strand**, **Numbers on sequence** and **Sequence label**.
  - You cannot zoom in to see the residues in the circular molecule. If you wish to see these details, split the view with a linear view of the sequence
  - In the **Annotation Layout**, you also have the option of showing the labels as **Stacked**. This means that there are no overlapping labels and that all labels of both annotations and restriction sites are adjusted along the left and right edges of the view.

Using split views to see details of the circular molecule

To see the nucleotides of a circular molecule you can open a new view displaying a circular view of the molecule:

> **Press and hold the Ctrl button (⌘ on Mac) | click Show Sequence (ACT) at the bottom of the view**

This will open a linear view of the sequence below the circular view. When you zoom in on the linear view you can see the residues as shown in figure 14.11.

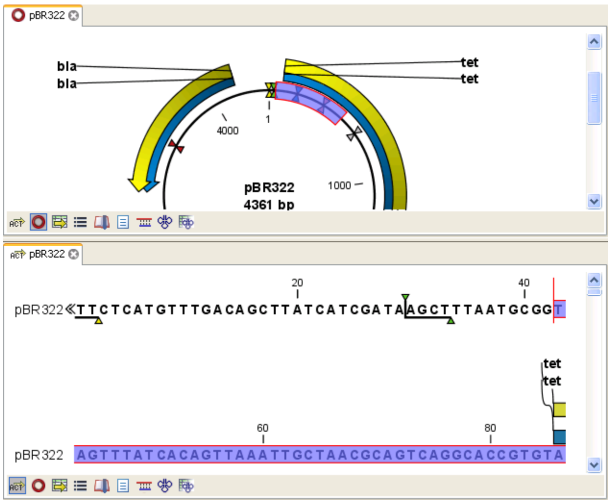

pBR322
bla
bla
tet
tet
4000
1
pBR322
4361 bp
1000
pBR322
20
40
pBR322《TTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGT
tet
tet
60
80
pBR322 AGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGTGTA

Figure 14.11: *Two views showing the same sequence. The bottom view is zoomed in.*

Note! If you make a selection in one of the views, the other view will also make the corresponding selection, providing an easy way for you to focus on the same region in both views.

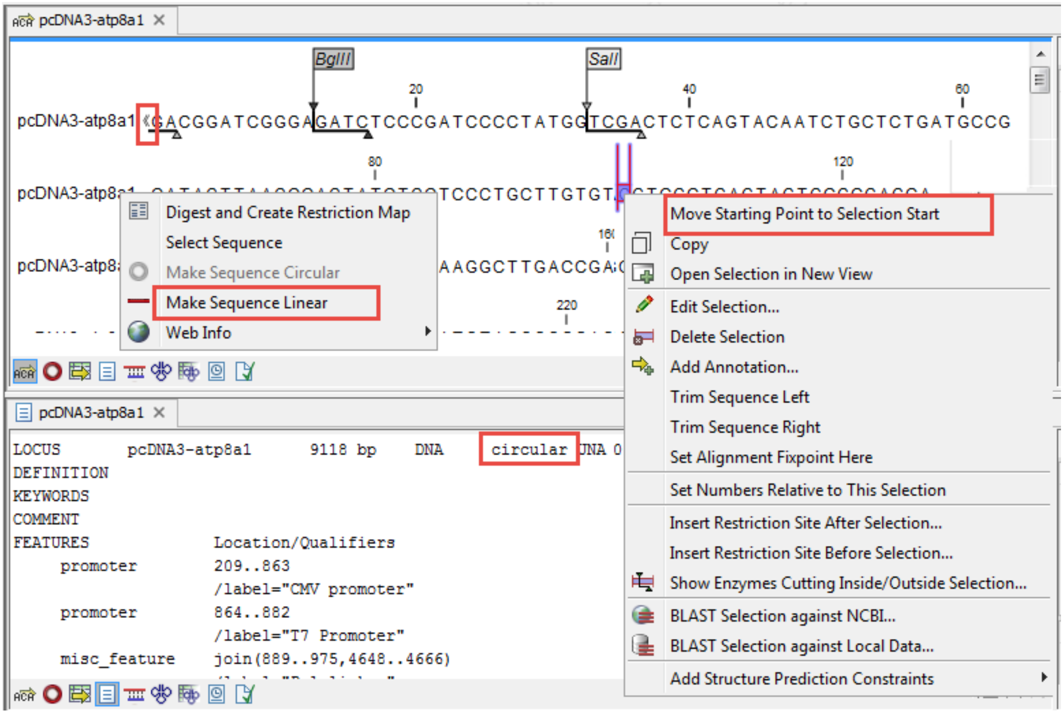
Mark molecule as circular and specify starting point

You can mark a DNA molecule as circular or linear by right-clicking on its name in either the Sequence view or the Circular view. If the sequence is linear, you will see the option to mark it as circular and vice versa (see figure 14.12).

In the Sequence view, a sequence marked as circular is indicated by the use of double angle brackets at the start and end of the sequence. The linear or circular status of a sequence can also be seen in the Locus line of the Text view for a Sequence, or in the Linear column of the Table view of a Sequence List.

The starting point of a circular sequence can be changed by selecting the position of the new starting point and right-clicking on that selection to choose the option **Move Starting Point to Selection Start** (figure 14.13).

.


pcDNA3-atp8a1
BglII
SalI
20
40
60
pcDNA3-atp8a1 «GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCG
80
120
Digest and Create Restriction Map
Select Sequence
Make Sequence Circular
Make Sequence Linear
Web Info
Move Starting Point to Selection Start
Copy
Open Selection in New View
Edit Selection...
Delete Selection
Add Annotation...
Trim Sequence Left
Trim Sequence Right
Set Alignment Fixpoint Here
Set Numbers Relative to This Selection
Insert Restriction Site After Selection...
Insert Restriction Site Before Selection...
Show Enzymes Cutting Inside/Outside Selection...
BLAST Selection against NCBI...
BLAST Selection against Local Data...
Add Structure Prediction Constraints
pcDNA3-atp8a1

```
LOCUS       pcDNA3-atp8a1      9118 bp    DNA     circular UNA 0
DEFINITION
KEYWORDS
COMMENT
FEATURES             Location/Qualifiers
     promoter        209..863
                     /label="CMV promoter"
     promoter        864..882
                     /label="T7 Promoter"
     misc_feature    join(889..975,4648..4666)
```

Figure 14.12: *Double angle brackets mark the start and end of a circular sequence in linear view (top). The first line in the text view (bottom) contains information that the sequence is circular.*

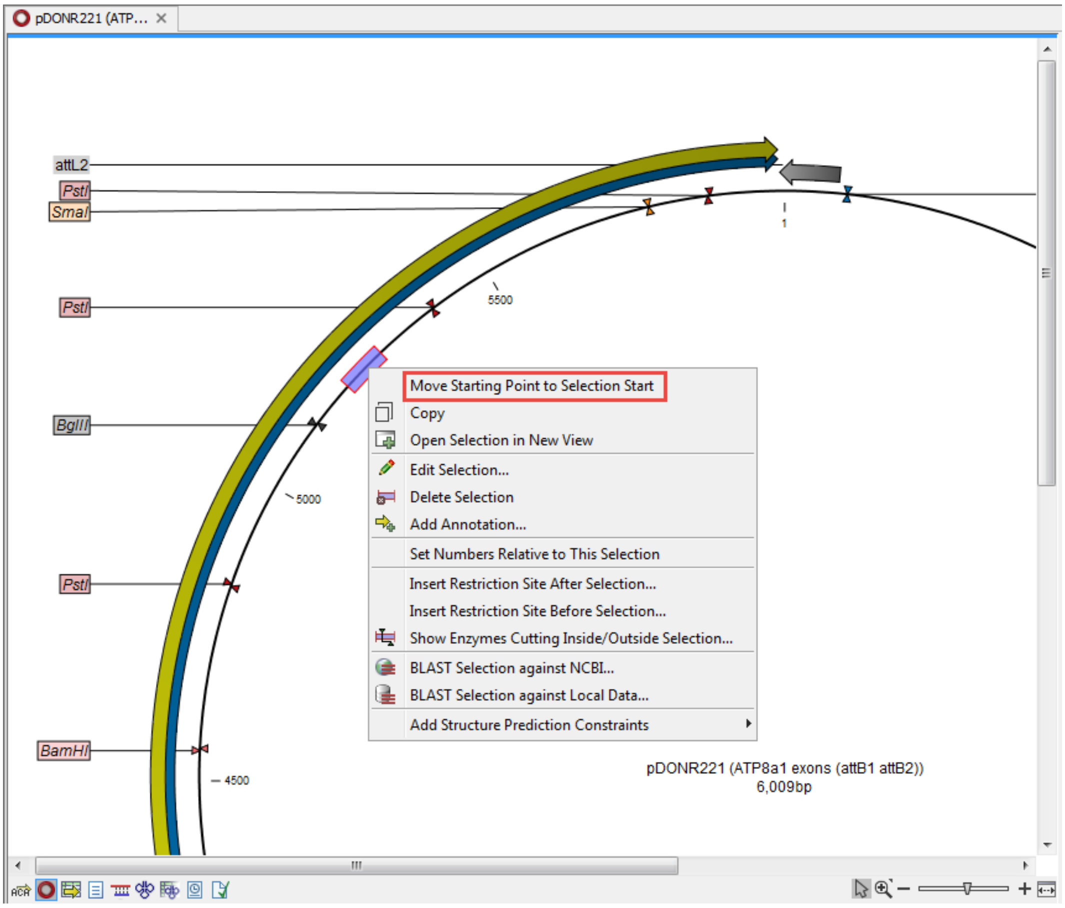

pDONR221 (ATP...
attL2
PstI
SmaI
PstI
BglII
PstI
BamHI
5500
5000
4500
1
Move Starting Point to Selection Start
Copy
Open Selection in New View
Edit Selection...
Delete Selection
Add Annotation...
Set Numbers Relative to This Selection
Insert Restriction Site After Selection...
Insert Restriction Site Before Selection...
Show Enzymes Cutting Inside/Outside Selection...
BLAST Selection against NCBI...
BLAST Selection against Local Data...
Add Structure Prediction Constraints
pDONR221 (ATP8a1 exons (attB1 attB2))
6,009bp

Figure 14.13: *Right-click on a circular sequence to move the starting point to the selected position.*

14.3 Working with annotations

Annotations provide information about specific regions of a sequence.

A typical example is the annotation of a gene on a genomic DNA sequence.

Annotations derive from different sources:

- Sequences downloaded from databases like GenBank are annotated.
- In some of the data formats that can be imported into *CLC Main Workbench*, sequences can have annotations (GenBank, EMBL and Swiss-Prot format).
- The result of a number of analyses in *CLC Main Workbench* are annotations on the sequence (e.g. finding open reading frames and restriction map analysis).
- A protein structure can be linked with a sequence (section 15.4.2), and atom groups defined on the structure transferred to sequence annotations or vica versa (section 15.4.3).
- You can manually add annotations to a sequence (described in the section 14.3.2).

If you would like to extract parts of a sequence (or several sequences) based on its annotations, you can find a description of how to do this in section 27.1.

Note! Annotations are included if you export the sequence in GenBank, Swiss-Prot, EMBL or CLC format. When exporting in other formats, annotations are not preserved in the exported file.

14.3.1 Viewing annotations

Annotations can be viewed in a number of different ways:

- As graphical arrows or boxes in all views displaying sequences (sequence lists, alignments etc)
- In a table in the Annotation () view
- In the text view of sequences ()

These views are described in more detail in the following sections.

View Annotations in sequence views

Figure 14.14 shows an annotation displayed on a sequence.

The various sequence views listed in section 14.3.1 have different default settings for showing annotations. However, they all have two groups in the **Side Panel** in common:

- **Annotation Layout**
- **Annotation Types**

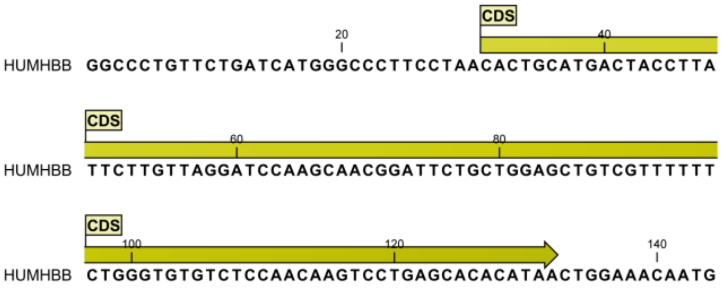
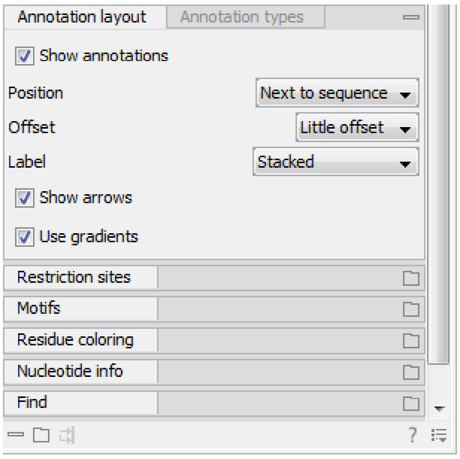

CDS
20
40
HUMHBB GGCCCTGTTCTGATCATGGGCCCTTCCTAACACTGCATGACTACCTTA
CDS
60
80
HUMHBB TTCTTGTTAGGATCCAAGCAACGGATTCTGCTGGAGCTGTCGTTTTTT
CDS
100
120
140
HUMHBB CTGGGTGTGTCTCCAACAAGTCCTGAGCACACATAACTGGAAACAATG

Figure 14.14: *An annotation showing a coding region on a genomic dna sequence.*



☑ Show annotations

Position: Next to sequence

Offset: Little offset

Label: Stacked

☑ Show arrows

☑ Use gradients

Figure 14.15: *The annotation layout in the Side Panel. The annotation types can be shown by clicking on the "Annotation types" tab.*

The two groups are shown in figure 14.15.

In the **Annotation layout** group, you can specify how the annotations should be displayed (notice that there are some minor differences between the different sequence views):

- **Show annotations.** Determines whether the annotations are shown.
- **Position.**
  - **On sequence.** The annotations are placed on the sequence. The residues are visible through the annotations (if you have zoomed in to 100%).
  - **Next to sequence.** The annotations are placed above the sequence.
  - **Separate layer.** The annotations are placed above the sequence and above restriction sites (only applicable for nucleotide sequences).
- **Offset.** If several annotations cover the same part of a sequence, they can be spread out.
  - **Piled.** The annotations are piled on top of each other. Only the one at front is visible.
  - **Little offset.** The annotations are piled on top of each other, but they have been offset a little.
  - **More offset.** Same as above, but with more spreading.

  - **Most offset.** The annotations are placed above each other with a little space between. This can take up a lot of space on the screen.

- **Label.** The name of the annotation can shown as a label. Additional information about the sequence is shown if you place the mouse cursor on the annotation and keep it still.
  - **No labels.** No labels are displayed.
  - **On annotation.** The labels are displayed in the annotation's box.
  - **Over annotation.** The labels are displayed above the annotations.
  - **Before annotation.** The labels are placed just to the left of the annotation.
  - **Flag.** The labels are displayed as flags at the beginning of the annotation.
  - **Stacked.** The labels are offset so that the text of all labels is visible. This means that there is varying distance between each sequence line to make room for the labels.

- **Show arrows.** Displays the end of the annotation as an arrow. This can be useful to see the orientation of the annotation (for DNA sequences). Annotations on the negative strand will have an arrow pointing to the left.

- **Use gradients.** Fills the boxes with gradient color.

In the **Annotation types** group, you can choose which kinds of annotations that should be displayed. This group lists all the types of annotations that are attached to the sequence(s) in the view. For sequences with many annotations, it can be easier to get an overview if you deselect the annotation types that are not relevant.

Unchecking the checkboxes in the **Annotation layout** will not remove this type of annotations them from the sequence - it will just hide them from the view.

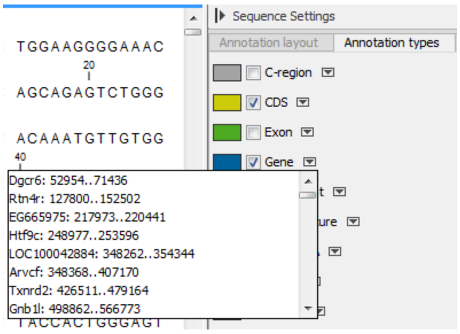
Besides selecting which types of annotations that should be displayed, the **Annotation types** group is also used to change the color of the annotations on the sequence. Click the colored square next to the relevant annotation type to change the color.

This will display a dialog with five tabs: Swatches, HSB, HSI, RGB, and CMYK. They represent five different ways of specifying colors. Apply your settings and click **OK**. When you click **OK**, the color settings cannot be reset. The **Reset** function only works for changes made before pressing **OK**.

Furthermore, the **Annotation types** can be used to easily browse the annotations by clicking the small button (▾) next to the type. This will display a list of the annotations of that type (see figure 14.16).

Clicking an annotation in the list will select this region on the sequence. In this way, you can quickly find a specific annotation on a long sequence.

Note: A waved end on an annotation (figure 14.17) means that the annotation is torn, i.e., it extends beyond the sequence displayed. An annotation can be torn when a new, smaller sequence has been created from a larger sequence. A common example of this situation is when you select a section of a stand-alone sequence and open it in a new view. If there are annotations present within this selected region that extend beyond the selection, then the selected sequence shown in the new view will exhibit these torn annotations.



Dgcr6: 52954..71436
Rtn4r: 127800..152502
EG665975: 217973..220441
Htf9c: 248977..253596
LOC100042884: 348262..354344
Arvcf: 348368..407170
Txnrd2: 426511..479164
Gnb1l: 498862..566773

Figure 14.16: *Browsing the gene annotations on a sequence.*

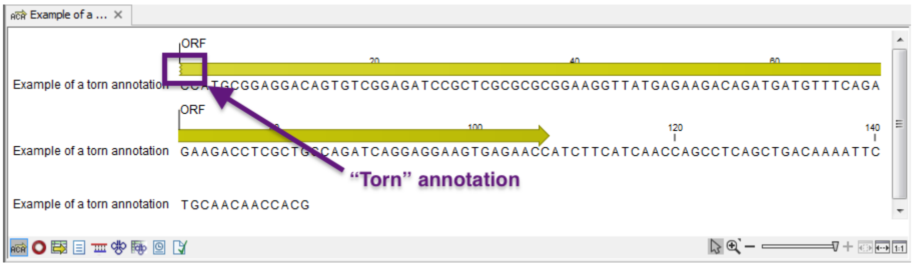

Example of a ...
ORF
20
40
60
Example of a torn annotation
CCATGCGGAGGACAGTGTCGGAGATCCGCTCGCGCGGAAGGTTATGAGAAGACAGATGATGTTTCAGA
ORF
100
120
140
Example of a torn annotation
GAAGACCTCGCTGCCAGATCAGGAGGAAGTGAGAACCATCTTCATCAACCAGCCTCAGCTGACAAAATTC
"Torn" annotation
Example of a torn annotation
TGCAACAACCACG

Figure 14.17: *Example of a torn annotation on a sequence.*

View Annotations in a table

The Annotation Table (⇨) view lists the annotations in a table. Standard functionality for working with tables applies, as described in section 9. Here we highlight functionality of particular interest when working with annotations.

This view is useful for getting a quick overview of annotations, and for filtering so that only the annotations of interest are listed. From this view, you can edit and add annotations, export selected annotations to a gff3 format file, and delete annotations. This functionality is described in more detail below.

To open the Annotation Table (⇨) view:

> **Select a sequence in the Navigation Area and right-click on the file name | Hold the mouse over "Show" to enable a list of options | Annotation Table (⇨)**

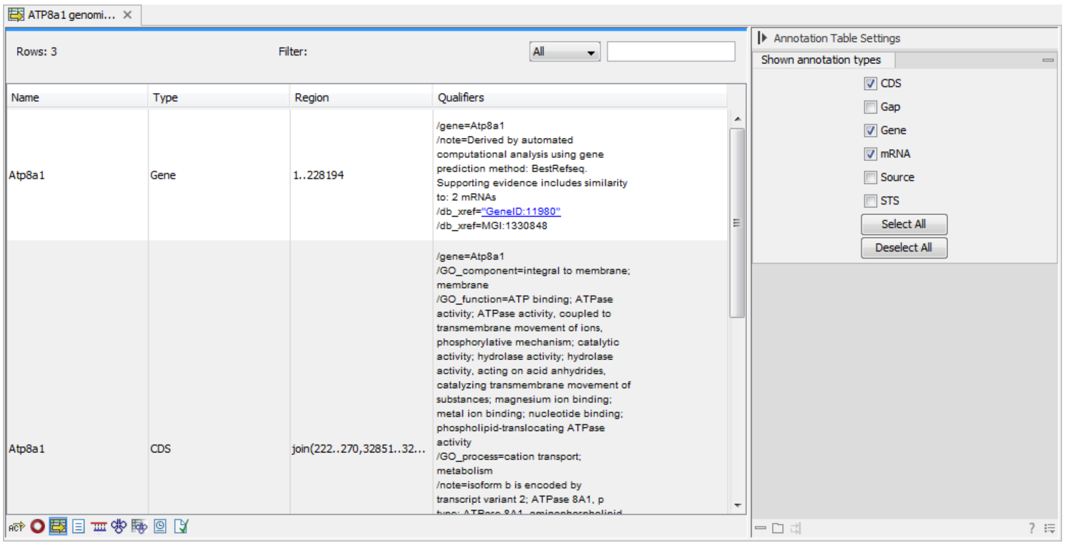
or **If the sequence is already open | Click Show Annotation Table (⇨) at the lower left part of the view**

This will open a view similar to the one in figure 14.18).

In the **Side Panel** you can show or hide individual annotation types in the table. E.g. if you only wish to see "gene" annotations, de-select the other annotation types so that only "gene" is selected.

Each row in the table is an annotation which is represented with the following information:

- **Name.**
- **Type.**
- **Region.**



Rows: 3 Filter: All

Name	Type	Region	Qualifiers
Atp8a1	Gene	1..228194	/gene=Atp8a1 /note=Derived by automated computational analysis using gene prediction method: BestRefseq. Supporting evidence includes similarity to: 2 mRNAs /db_xref="GeneID:11980" /db_xref=MGI:1330848
Atp8a1	CDS	join(222..270,32851..32...	/gene=Atp8a1 /GO_component=integral to membrane; membrane /GO_function=ATP binding; ATPase activity; ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism; catalytic activity; hydrolase activity; hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances; magnesium ion binding; metal ion binding; nucleotide binding; phospholipid-translocating ATPase activity /GO_process=cation transport; metabolism /note=isoform b is encoded by transcript variant 2; ATPase 8A1, p type: ATPase 8A1, aminophospholipid

Shown annotation types
☑ CDS
☐ Gap
☑ Gene
☑ mRNA
☐ Source
☐ STS

Figure 14.18: *A table showing annotations on the sequence.*

- **Qualifiers.**

This information corresponds to the information in the dialog when you edit and add annotations (see section 14.3.2).

The Name, Type and Region for each annotation can be edited simply by double-clicking, typing the change directly, and pressing **Enter**. See section 14.3.3 for further information about editing annotations.

14.3.2 Adding annotations

Annotations can be added to a sequence from the graphical view or the Annotation Table view.

Graphical view Select the region you want the annotation to cover, right-click on the selected region, and choose the option **Add Annotation (**➪**)** from the menu.

See section 14.2.2 on how to make selections that are not contiguous.

Annotation Table view Right-click anywhere in this view and select the option **Add Annotation (**➪**)** from the menu. (Note that this option is not enabled in sequence lists.)

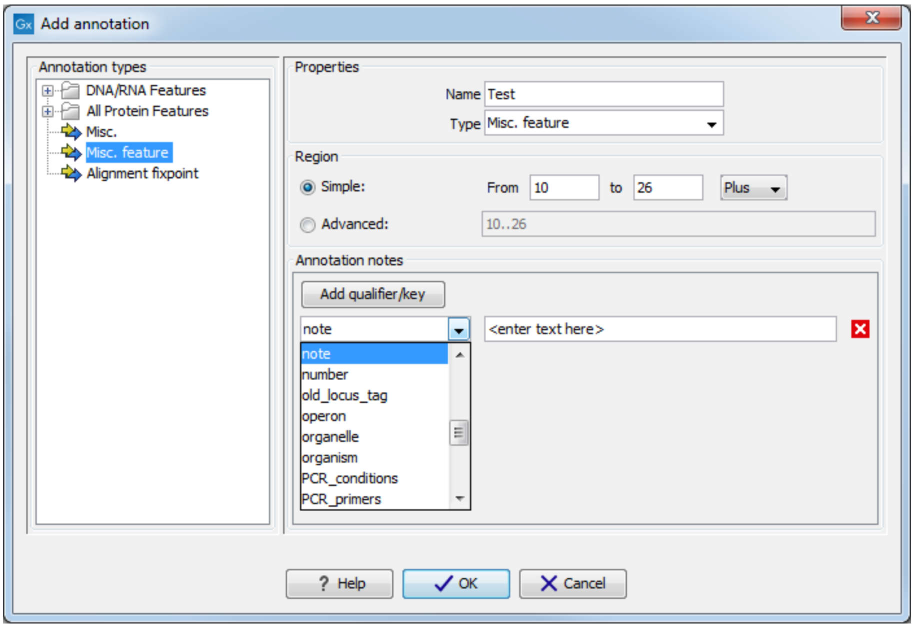
In both cases, a dialog opens where you can provide information about the annotation (figure 14.19).

The left-hand part of the dialog lists a number of **Annotation types**. When you have selected an annotation type, it appears in **Type** to the right. You can also select an annotation directly in this list. Choosing an annotation type is mandatory. If you wish to use an annotation type which is not present in the list, simply enter this type into the **Type** field [1].

The right-hand part of the dialog contains the following text fields:

- **Name.** The name of the annotation which can be shown on the label in the sequence views.

[1] Note that your own annotation types will be converted to "unsure" when exporting in GenBank format. As long as you use the sequence in CLC format, you own annotation type will be preserved



Name Test
Type Misc. feature
● Simple: From 10 to 26 Plus
○ Advanced: 10..26
note <enter text here>

Figure 14.19: *The Add annotation dialog.*

(Whether the name is actually shown depends on the **Annotation Layout** preferences, see section 14.3.1).

- **Type.** Reflects the left-hand part of the dialog as described above. You can also choose directly in this list or type your own annotation type.

- **Region.** If you have already made a selection, this field will show the positions of the selection. You can modify the region further using the conventions of DDBJ, EMBL and GenBank. The following are examples of how to use the syntax (based on `https://www.ncbi.nlm.nih.gov/collab/FT/`):

  - **467**. Points to a single residue in the presented sequence.
  - **340..565**. Points to a continuous range of residues bounded by and including the starting and ending residues.
  - **<345..500**. Indicates that the exact lower boundary point of a region is unknown. The location begins at some residue previous to the first residue specified (which is not necessarily contained in the presented sequence) and continues up to and including the ending residue.
  - **<1..888**. The region starts before the first sequenced residue and continues up to and including residue 888.
  - **1..>888**. The region starts at the first sequenced residue and continues beyond residue 888.
  - **(102.110)**. Indicates that the exact location is unknown, but that it is one of the residues between residues 102 and 110, inclusive.
  - **123ˆ124**. Points to a site between residues 123 and 124.
  - **join(12..78,134..202)**. Regions 12 to 78 and 134 to 202 should be joined to form one contiguous sequence.

  - **complement(34..126)** Start at the residue complementary to 126 and finish at the residue complementary to residue 34 (the region is on the strand complementary to the presented strand).
  - **complement(join(2691..4571,4918..5163))**. Joins regions 2691 to 4571 and 4918 to 5163, then complements the joined segments (the region is on the strand complementary to the presented strand).
  - **join(complement(4918..5163),complement(2691..4571))**. Complements regions 4918 to 5163 and 2691 to 4571, then joins the complemented segments (the region is on the strand complementary to the presented strand).

- **Annotations.** In this field, you can add more information about the annotation like comments and links. Click the **Add qualifier/key** button to enter information. Select a qualifier which describes the kind of information you wish to add. If an appropriate qualifier is not present in the list, you can type your own qualifier. The pre-defined qualifiers are derived from the GenBank format. You can add as many qualifier/key lines as you wish by clicking the button. Redundant lines can be removed by clicking the delete icon (☒). The information entered on these lines is shown in the annotation table (see section 14.3.1) and in the yellow box which appears when you place the mouse cursor on the annotation. If you write a hyperlink in the **Key** text field, like e.g. "digitalinsights.qiagen.com", it will be recognized as a hyperlink. Clicking the link in the annotation table will open a web browser.

Click **OK** to add the annotation.

Note! The annotation will be included if you export the sequence in GenBank, Swiss-Prot or CLC format. When exporting in other formats, annotations are not preserved in the exported file.

14.3.3 Editing annotations

Annotations can be edited from the graphical sequence view or from the Annotation Table () view.

For general information about the Annotation Table () view, see section 14.3.1.

Basic annotation editing in the sequence view

To edit an annotation from the sequence view:

Right-click on the annotation | Edit Annotation ()

A dialog like that in figure 14.19 will appear. Edit the fields as needed and click on **OK** to save your changes.

Editing annotations in the Annotation Table view

In the Annotation Table () view, each part of an annotation can be updated by double-clicking in a cell, editing the contents, and pressing **Enter**. In addition, editing options are among the options available in the right-click menu (figure 14.20).

Options related to editing annotations in this menu are:

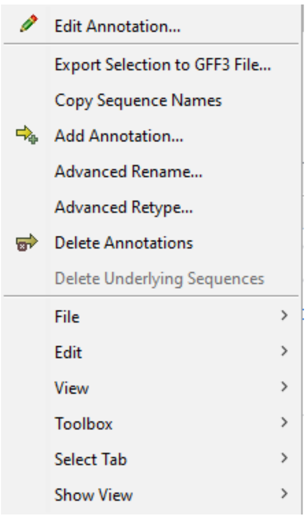
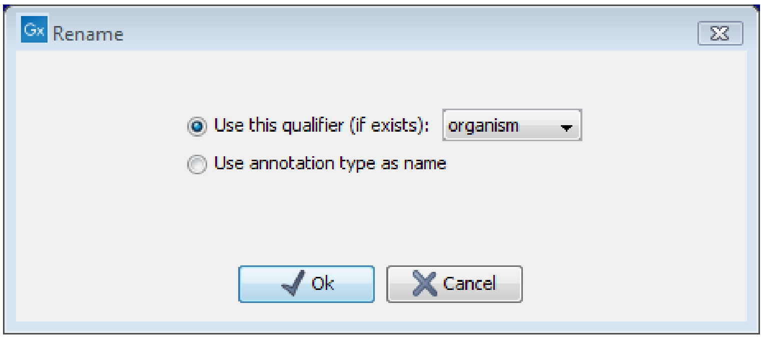


Figure 14.20: *The right-click menu in the Annotation Table view contains options for adding, editing, exporting and deleting annotations.*

- **Edit Annotation...** This option is only enabled if a single annotation is selected in the table. It will open the same dialog used to edit annotations from the sequence view (figure 14.19).
- **Advanced Rename...** Choose this to rename the selected annotations using qualifiers or annotation types. The options in the Rename dialog (figure 14.21) are:
  - **Use this qualifier** Choose the qualifier to use as that annotation name from a drop-down list of qualifiers available in the selected annotations. Selected annotations that do not include the selected qualifier will not be renamed. If an annotation has multiple qualifiers of the same type, the first is used for renaming.
  - **Use annotation type as name** The annotation's type will be used for the annotation name E.g. if you have an annotation of type "Promoter", it will get "Promoter" as its name by using this option.
- **Advanced Retype...** Choose this to edit the type of one or more annotations. The options in the Retype dialog (figure 14.22) are:
  - **Use this qualifier** Choose the qualifier to use as the annotation type from a drop-down list of qualifiers available in the selected annotations. Selected annotations that do not include the selected qualifier will not be retyped. If an annotation has multiple qualifiers of the same type, the first is used for the new type.
  - **New type** Enter an annotation type to apply or click on the arrows at the right of the field to see a drop-down list of pre-defined annotation types.
  - **Use annotation name as type** Use the annotation name as its type. E.g. if you have an annotation named "Promoter", it will get "Promoter" as its type by using this option.

14.3.4 Export annotations to a gff3 format file

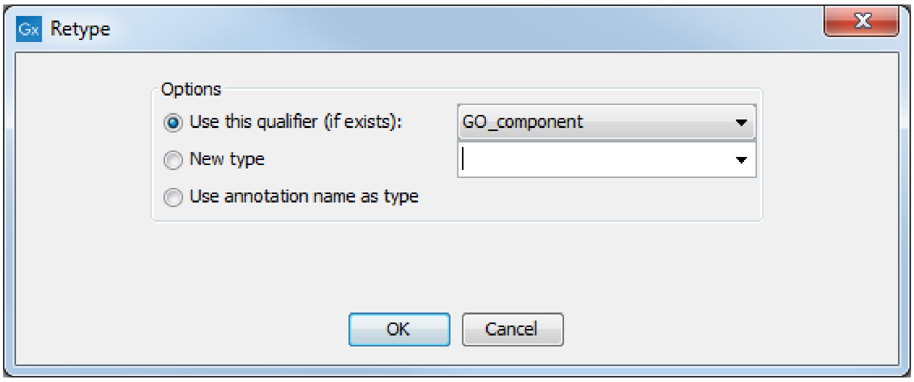
Annotations selected in the Annotation Table (▦) view can be exported to a gff3 format file by selecting the option **Export Selection to GFF3 File** in the right-click menu (figure 14.20).



● Use this qualifier (if exists): organism

○ Use annotation type as name

Figure 14.21: *The Advanced Rename dialog.*



● Use this qualifier (if exists): GO_component

○ New type

○ Use annotation name as type

Figure 14.22: *The Advanced Retype dialog.*

14.3.5 Removing annotations

Annotations can be hidden using the **Annotation Types** preferences in the **Side Panel** to the right of the view (see section 14.3.1). In order to completely remove the annotation:

> **right-click the annotation | Delete Annotation ()**

If you want to remove all annotations of one type:

> **right-click an annotation of the type you want to remove | Delete | Delete Annotations of Type "type"**

If you want to remove all annotations from a sequence:

> **right-click an annotation | Delete | Delete All Annotations**

The removal of annotations can be undone using Ctrl + Z or Undo () in the Toolbar.

If you have more sequences (e.g. in a sequence list, alignment or contig), you have two additional options:

> **right-click an annotation | Delete | Delete All Annotations from All Sequences**

> **right-click an annotation | Delete | Delete Annotations of Type "type" from All Sequences**

14.4 Element information

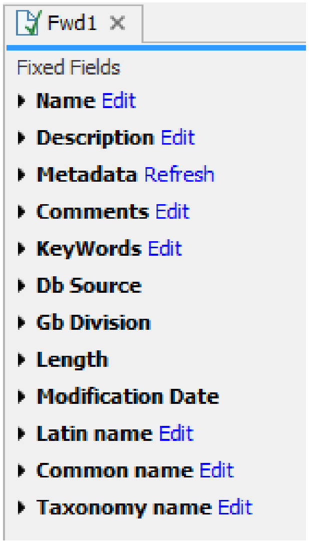
The normal view of a sequence (by double-clicking) shows the annotations as boxes along the sequence, but often there is more information available about sequences. This information is available through the **Element info** view.

To view the sequence information:

> **Select a sequence in the Navigation Area and right-click on the file name | Hold the mouse over "Show" to enable a list of options | Element Info (✓)**

Another way to show the text view is to open the sequence in the **View Area** and click on the "Show Element Info" icon (✓) found at the bottom of the window.

This will display a view similar to fig 14.23.



Fwd1 ✕

Fixed Fields

- **Name** Edit
- **Description** Edit
- **Metadata** Refresh
- **Comments** Edit
- **KeyWords** Edit
- **Db Source**
- **Gb Division**
- **Length**
- **Modification Date**
- **Latin name** Edit
- **Common name** Edit
- **Taxonomy name** Edit

Figure 14.23: *The initial display of sequence info for the HUMHBB DNA sequence from the Example data.*

All the lines in the view are headings, and the corresponding text can be shown by clicking the text. The information available depends on the origin of the sequence.

- **Name.** The name of the sequence which is also shown in sequence views and in the **Navigation Area**.
- **Description.** A description of the sequence.
- **Metadata.** The Metadata table and the detailed metadata values associated with the sequence.
- **Comments.** The author's comments about the sequence.
- **Keywords.** Keywords describing the sequence.
- **Db source.** Accession numbers in other databases concerning the same sequence.
- **Gb Division.** Abbreviation of GenBank divisions. See section 3.3 in the GenBank release notes for a full list of GenBank divisions.

- **Length.** The length of the sequence.
- **Modification date.** Modification date from the database. This means that this date does not reflect your own changes to the sequence. See the History view, described in section 2.5 for information about the latest changes to the sequence after it was downloaded from the database.
- **Latin name.** Latin name of the organism.
- **Common name.** Scientific name of the organism.
- **Taxonomy name.** Taxonomic classification levels.
- **Read group** Read group identifier "ID", technology used to produced the reads "Platform", and sample name "Sample".
- **Paired Status.** Unpaired or Paired sequences, with in this case the Minimum and Maximum distances as well as the Read orientation set during import.

Some of the information can be edited by clicking the blue **Edit** text. This means that you can add your own information to sequences that do not derive from databases.

14.5 View as text

A sequence can be viewed as text without any layout and text formatting. This displays all the information about the sequence in the GenBank file format. To view a sequence as text:

> **Select a sequence in the Navigation Area and right-click on the file name | Hold the mouse over "Show" to enable a list of options | Select "Text View" (☰)**

Another way to show the text view is to open the sequence in the **View Area** and click on the "Show Text View" icon (☰) found at the bottom of the window.

This makes it possible to see background information about e.g. the authors and the origin of DNA and protein sequences. Selections or the entire text of the **Sequence Text View** can be copied and pasted into other programs:

Much of the information is also displayed in the **Sequence info**, where it is easier to get an overview (see section 14.4.)

In the **Side Panel**, you find a search field for searching the text in the view.

Chapter 15

3D Molecule Viewer

Contents

Proteins are amino acid polymers that are involved in all aspects of cellular function. The structure of a protein is defined by its particular amino acid sequence, with the amino acid sequence being referred to as the primary protein structure. The amino acids fold up in local structural elements; helices and sheets, also called the secondary structure of the protein. These structural elements are then packed into globular folds, known as the tertiary structure or the three dimensional structure.

In order to understand protein function it is often valuable to see the three dimensional structure of the protein. This is possible when the structure of the protein has been resolved and published. Structure files are usually deposited in the Protein Data Bank (PDB) https://www.rcsb.org/, where the publicly available protein structure files can be searched and downloaded. The vast majority of the protein structures have been determined by X-ray crystallography (88%) while the rest of the structures predominantly have been obtained by Nuclear Magnetic Resonance techniques.

In addition to protein structures, the PDB entries also contain structural information about molecules that interact with the protein, such as nucleic acids, ligands, cofactors, and water. There are also entries, which contain nucleic acids and no protein structure. The **3D Molecule Viewer** in the *CLC Main Workbench* is an integrated viewer of such structure files.

> If you have problems viewing 3D structures, please check your system matches the requirements for 3D viewers. See section 1.3.

The **3D Molecule Viewer** offers a range of tools for inspection and visualization of molecular structures:

- Automatic sorting of molecules into categories: Proteins, Nucleic acids, Ligands, Cofactors, Water molecules
- Hide/unhide individual molecules from the view
- Four different atom-based molecule visualizations
- Backbone visualization for proteins and nucleic acids
- Molecular surface visualization
- Selection of different color schemes for each molecule visualization
- Customized visualization for user selected atoms
- Alignment of protein structures
- Browse amino acids and nucleic acids from sequence editors started from within the 3D Molecule Viewer
- Link a sequence or alignment to a protein structure
- Transfer annotations between the linked sequence and the structure

- Automatic sorting of molecules into categories: Proteins, Nucleic acids, Ligands, Cofactors, Water molecules
- Hide/unhide individual molecules from the view
- Four different atom-based molecule visualizations
- Backbone visualization for proteins and nucleic acids
- Molecular surface visualization
- Selection of different color schemes for each molecule visualization
- Customized visualization for user selected atoms
- Browse amino acids and nucleic acids from sequence editors started from within the 3D Molecule Viewer

15.1 Importing molecule structure files

The supported file format for three dimensional protein structures in the **3D Molecule Viewer** is the Protein Data Bank (PDB) format, which upon import is converted to a CLC Molecule Project. PDB files can be imported to a Molecule Project in three different ways:

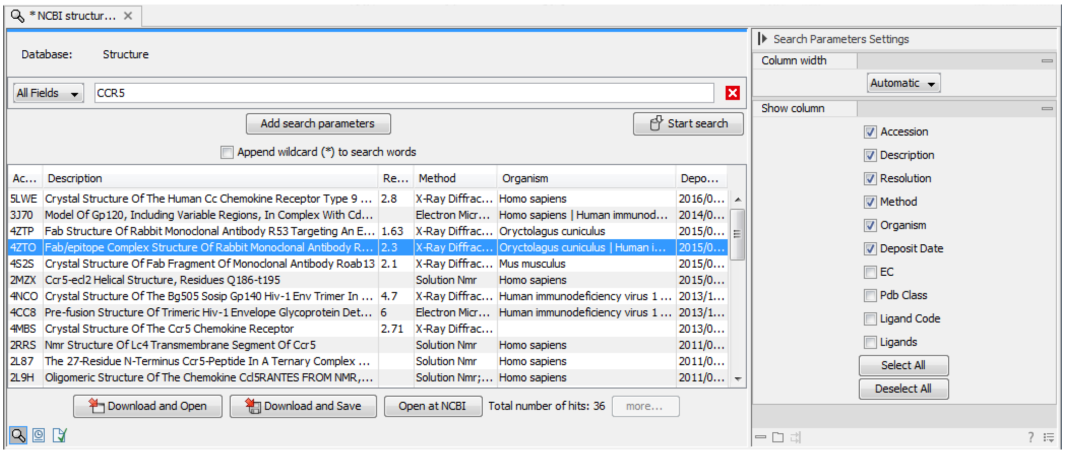
- from the Protein Data Bank
- from your own file system
- using BLAST search against the PDB database

15.1.1 From the Protein Data Bank

Molecule structures can be imported in the workbench from the Protein Data Bank using the "Download" function:

Toolbar | **Download** ( ) | **Search for PDB structures at NCBI** ( )

Type the molecule name or accession number into the search field and click on the "Start search" button (as shown in figure 15.1). The search hits will appear in the table below the search field.



Database: Structure

All Fields: CCR5

☐ Append wildcard (*) to search words

Ac...	Description	Re...	Method	Organism	Depo...
5LWE	Crystal Structure Of The Human Cc Chemokine Receptor Type 9 ...	2.8	X-Ray Diffrac...	Homo sapiens	2016/0...
3J70	Model Of Gp120, Including Variable Regions, In Complex With Cd...		Electron Micr...	Homo sapiens | Human immunod...	2014/0...
4ZTP	Fab Structure Of Rabbit Monoclonal Antibody R53 Targeting An E...	1.63	X-Ray Diffrac...	Oryctolagus cuniculus	2015/0...
4ZTO	Fab/epitope Complex Structure Of Rabbit Monoclonal Antibody R...	2.3	X-Ray Diffrac...	Oryctolagus cuniculus | Human i...	2015/0...
4S2S	Crystal Structure Of Fab Fragment Of Monoclonal Antibody Roab13	2.1	X-Ray Diffrac...	Mus musculus	2015/0...
2MZX	Ccr5-ecl2 Helical Structure, Residues Q186-t195		Solution Nmr	Homo sapiens	2015/0...
4NCO	Crystal Structure Of The Bg505 Sosip Gp140 Hiv-1 Env Trimer In ...	4.7	X-Ray Diffrac...	Human immunodeficiency virus 1 ...	2013/1...
4CC8	Pre-fusion Structure Of Trimeric Hiv-1 Envelope Glycoprotein Det...	6	Electron Micr...	Human immunodeficiency virus 1 ...	2013/1...
4MBS	Crystal Structure Of The Ccr5 Chemokine Receptor	2.71	X-Ray Diffrac...		2013/0...
2RRS	Nmr Structure Of Lc4 Transmembrane Segment Of Ccr5		Solution Nmr	Homo sapiens	2011/0...
2L87	The 27-Residue N-Terminus Ccr5-Peptide In A Ternary Complex ...		Solution Nmr	Homo sapiens	2011/0...
2L9H	Oligomeric Structure Of The Chemokine Ccl5RANTES FROM NMR,...		Solution Nmr;...	Homo sapiens	2011/0...

Total number of hits: 36

Column width: Automatic

Show column:
☑ Accession
☑ Description
☑ Resolution
☑ Method
☑ Organism
☑ Deposit Date
☐ EC
☐ Pdb Class
☐ Ligand Code
☐ Ligands

Figure 15.1: *Download protein structure from the Protein Data Bank. It is possible to open a structure file directly from the output of the search by clicking the "Download and Open" button or by double clicking directly on the relevant row.*

Select the molecule structure of interest and click on the button labeled "Download and Open" - or double click on the relevant row - in the table to open the protein structure.

Pressing the "Download and Save" button will save the molecule structure at a user defined destination in the Navigation Area.

The button "Open at NCBI" links directly to the structure summary page at NCBI: clicking this button will open individual NCBI pages describing each of the selected molecule structures.

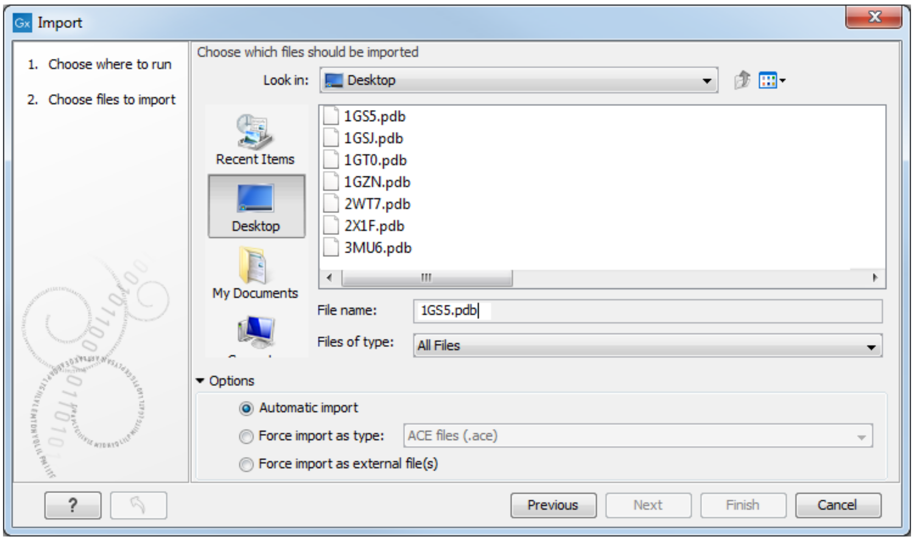
15.1.2 From your own file system

A PDB file can also be imported from your own file system using the standard import function:

Toolbar | **Import** ( ) | **Standard Import** ( )

In the Import dialog, select the structure(s) of interest from a data location and tick "Automatic import" (figure 15.2). Specify where to save the imported PDB file and click **Finish**.

Double clicking on the imported file in the **Navigation Area** will open the structure as a **Molecule Project** in the **View Area** of the *CLC Main Workbench*. Another option is to drag the PDB file from the **Navigation Area** to the **View Area**. This will automatically open the protein structure as a **Molecule Project**.



Import

1. Choose where to run
2. Choose files to import

Choose which files should be imported

Look in: Desktop

1GS5.pdb
1GSJ.pdb
1GT0.pdb
1GZN.pdb
2WT7.pdb
2X1F.pdb
3MU6.pdb

File name: 1GS5.pdb

Files of type: All Files

Options

● Automatic import
○ Force import as type: ACE files (.ace)
○ Force import as external file(s)

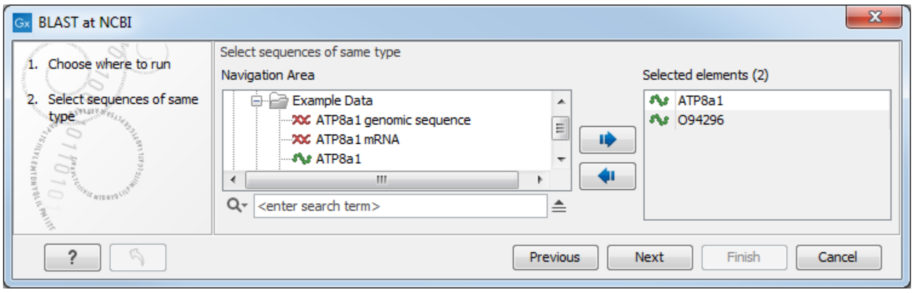
Figure 15.2: *A PDB file can be imported using the Standard Import tool.*

15.1.3 BLAST search against the PDB database

It is also possible to make a BLAST search against the PDB database, by going to:

Tools | **BLAST** ()| **BLAST at NCBI** ()

After selecting where to run the analysis, specify which input sequences to use for the BLAST search in the "BLAST at NCBI" dialog, within the box named "Select sequences of same type". More than one sequence can be selected at the same time, as long as the sequences are of the same type (figure 15.3).



BLAST at NCBI

1. Choose where to run
2. Select sequences of same type

Select sequences of same type

Navigation Area

Example Data
ATP8a1 genomic sequence
ATP8a1 mRNA
ATP8a1

<enter search term>

Selected elements (2)

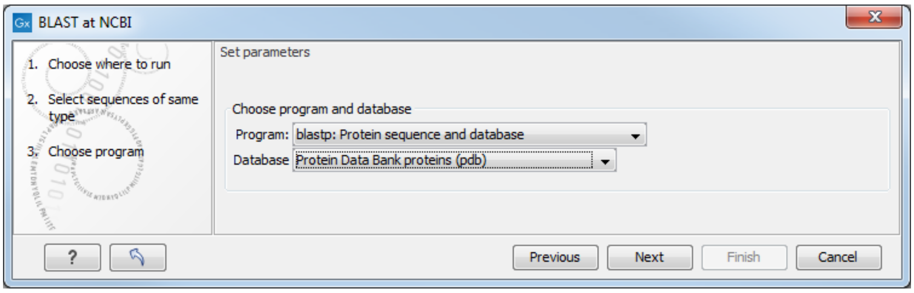
ATP8a1
O94296

Figure 15.3: *Select the input sequence of interest. In this example a protein sequence for ATPase class I type 8A member 1 and an ATPase ortholog from S. pombe have been selected.*

Click **Next** and choose program and database (figure 15.4). When a protein sequence has been used as input, select "Program: blastp: Protein sequence and database" and "Database: Protein

Data Bank proteins (pdb)".

It is also possible to use mRNA and genomic sequences as input. In such cases the program "blastx: Translated DNA sequence and protein database" should be used.



BLAST at NCBI

1. Choose where to run
2. Select sequences of same type
3. Choose program

Set parameters

Choose program and database

Program: blastp: Protein sequence and database

Database Protein Data Bank proteins (pdb)

Figure 15.4: *Select database and program.*

Please refer to section 26.1.1 for further description of the individual parameters in the wizard steps.

When you click on the button labeled **Finish**, a BLAST output is generated that shows local sequence alignments between your input sequence and a list of matching proteins with known structures available.

Note! The BLAST at NCBI search can take up to several minutes, especially when mRNA and genomic sequences are used as input.

Switch to the "BLAST Table" editor view to select the desired entry (figure 15.5). If you have performed a multi BLAST, to get access to the "BLAST Table" view, you must first double click on each row to open the entries individually.

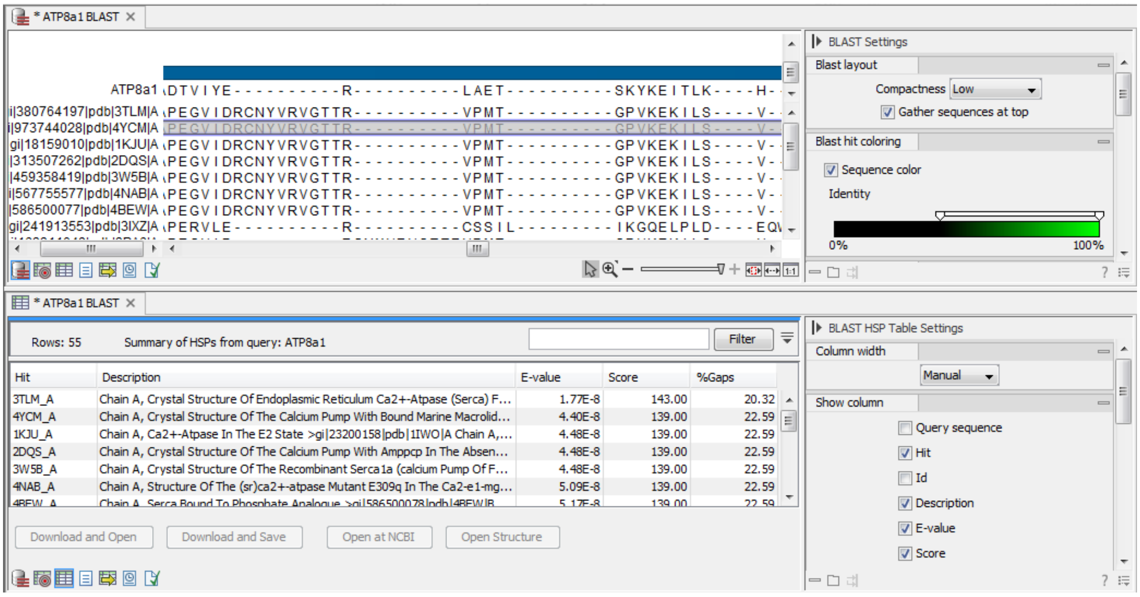
In this view four different options are available:

- **Download and Open** The sequence that has been selected in the table is downloaded and opened in the **View Area**.
- **Download and Save** The sequence that has been selected in the table is downloaded and saved in the **Navigation Area**.
- **Open at NCBI** The protein sequence that has been selected in the table is opened at NCBI.
- **Open Structure** Opens the selected structure in a **Molecule Project** in the **View Area**.

15.1.4 Import issues

When opening an imported molecule file for the first time, a notification is briefly shown in the lower left corner of the **Molecule Project** editor, with information of the number of issues encountered during import of the file. The issues are categorized and listed in a table view in the Issues view. The Issues list can be opened by selecting **Show | Issues** from the menu appearing when right-clicking in an empty space in the 3D view (figure 15.6).

Alternatively, the issues can be accessed from the lower left corner of the view, where buttons are shown for each available view. If you hold down the Ctrl key (Cmd on Mac) while clicking on the Issues icon (), the list will be shown in a split view together with the 3D view. The issues



* ATP8a1 BLAST

```
        ATP8a1 DTVIYE----------R----------LAET----------SKYKEITLK----H-
i|380764197|pdb|3TLM|A PEGVIDRCNYVRVGTTR----------VPMT----------GPVKEKILS----V-
i|973744028|pdb|4YCM|A PEGVIDRCNYVRVGTTR----------VPMT----------GPVKEKILS----V-
gi|18159010|pdb|1KJU|A PEGVIDRCNYVRVGTTR----------VPMT----------GPVKEKILS----V-
|313507262|pdb|2DQS|A PEGVIDRCNYVRVGTTR----------VPMT----------GPVKEKILS----V-
|459358419|pdb|3W5B|A PEGVIDRCNYVRVGTTR----------VPMT----------GPVKEKILS----V-
i|567755577|pdb|4NAB|A PEGVIDRCNYVRVGTTR----------VPMT----------GPVKEKILS----V-
|586500077|pdb|4BEW|A PEGVIDRCNYVRVGTTR----------VPMT----------GPVKEKILS----V-
gi|241913553|pdb|3IXZ|A PERVLE----------R----------CSSIL---------IKGQELPLD---EQ\
```

BLAST Settings

Blast layout

Compactness Low

☑ Gather sequences at top

Blast hit coloring

☑ Sequence color

Identity

0% 100%

* ATP8a1 BLAST

Rows: 55 Summary of HSPs from query: ATP8a1 Filter

Hit	Description	E-value	Score	%Gaps
3TLM_A	Chain A, Crystal Structure Of Endoplasmic Reticulum Ca2+-Atpase (Serca) F...	1.77E-8	143.00	20.32
4YCM_A	Chain A, Crystal Structure Of The Calcium Pump With Bound Marine Macrolid...	4.40E-8	139.00	22.59
1KJU_A	Chain A, Ca2+-Atpase In The E2 State >gi|23200158|pdb|1IWO|A Chain A,...	4.48E-8	139.00	22.59
2DQS_A	Chain A, Crystal Structure Of The Calcium Pump With Amppcp In The Absen...	4.48E-8	139.00	22.59
3W5B_A	Chain A, Crystal Structure Of The Recombinant Serca1a (calcium Pump Of F...	4.48E-8	139.00	22.59
4NAB_A	Chain A, Structure Of The (sr)ca2+-atpase Mutant E309q In The Ca2-e1-mg...	5.09E-8	139.00	22.59
4BEW_A	Chain A, Serca Bound To Phosphate Analogue >gi|586500078|pdb|4BEW|B	5.17E-8	139.00	22.59

Download and Open | Download and Save | Open at NCBI | Open Structure

BLAST HSP Table Settings

Column width

Manual

Show column

☐ Query sequence

☑ Hit

☐ Id

☑ Description

☑ E-value

☑ Score

Figure 15.5: *Top: The output from "BLAST at NCBI". Bottom: The "BLAST table". One of the protein sequences has been selected. This activates the four buttons under the table. Note that the table and the BLAST Graphics are linked, this means that when a sequence is selected in the table, the same sequence will be highlighted in the BLAST Graphics view.*

list is linked with the molecules in the 3D view, such that selecting an entry in the list will select the implicated atoms in the view, and zoom to put them into the center of the 3D view.

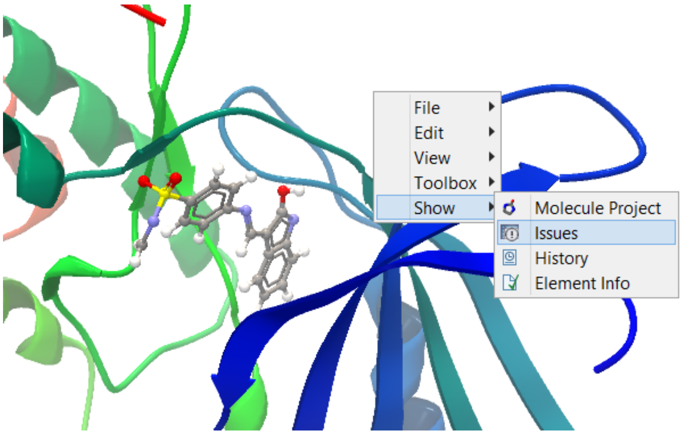


Figure 15.6: *At the bottom of the Molecule Project it is possible to switch to the "Show Issues" view by clicking on the "table-with-exclamation-mark" icon.*

15.2 Viewing molecular structures in 3D

An example of a 3D structure that has been opened as a **Molecule Project** is shown in figure 15.7.

> If you have problems viewing 3D structures, please check your system matches the requirements for 3D viewers. See section 1.3.

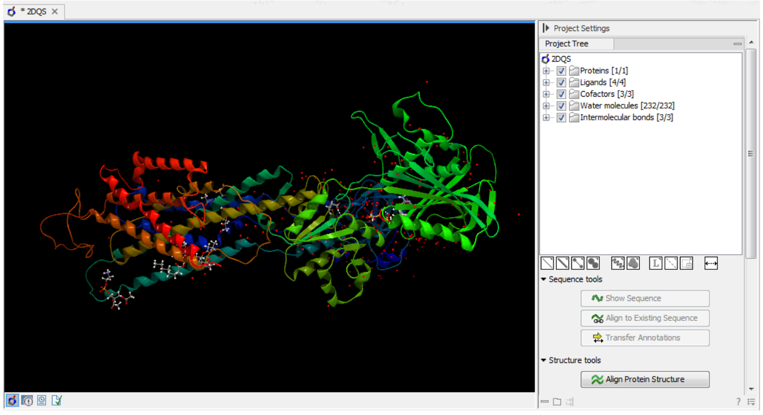


Figure 15.7: *3D view of a calcium ATPase. All molecules in the PDB file are shown in the Molecule Project. The Project Tree in the right side of the window lists the involved molecules.*

Moving and rotating The molecules can be rotated by holding down the left mouse button while moving the mouse. The right mouse button can be used to move the view.

Zooming can be done with the scroll-wheel or by holding down both left and right buttons while moving the mouse up and down.

All molecules in the **Molecule Project** are listed in categories in the **Project Tree**. The individual molecules or whole categories can be hidden from the view by un-cheking the boxes next to them.

It is possible to bring a particular molecule or a category of molecules into focus by selecting the molecule or category of interest in the **Project Tree** view and double-click on the molecule or category of interest. Another option is to use the zoom-to-fit button (↤↦) at the bottom of the **Project Tree** view.

Troubleshooting 3D graphics errors The 3D viewer uses OpenGL graphics hardware acceleration in order to provide the best possible experience. If you experience any graphics problems with the 3D view, please make sure that the drivers for your graphics card are up-to-date.

If the problems persist after upgrading the graphics card drivers, it is possible to change to a rendering mode, which is compatible with a wider range of graphic cards. To change the graphics mode go to Edit in the menu bar, select "Preferences", Click on "View", scroll down to the bottom and find "Molecule Project 3D Editor" and uncheck the box "Use modern OpenGL rendering".

Finally, it should be noted that certain types of visualization are more demanding than others. In particular, using multiple molecular surfaces may result in slower drawing, and even result in the graphics card running out of available memory. Consider creating a single combined surface (by using a selection) instead of creating surfaces for each single object. For molecules with a large number of atoms, changing to wireframe rendering and hiding hydrogen atoms can also greatly improve drawing speed.

15.3 Customizing the visualization

The molecular visualization of all molecules in the Molecule Project can be customized using different visualization styles. The styles can be applied to one molecule at a time, or to a whole category (or a mixture), by selecting the name of either the molecule or the category. Holding down the Ctrl (Cmd on Mac) or shift key while clicking the entry names in the **Project Tree** will select multiple molecules/categories.

The six leftmost quick-style buttons below the **Project Tree** view give access to the molecule visualization styles, while context menus on the buttons (accessible via right-click or left-click-hold) give access to the color schemes available for the visualization styles. Visualization styles and color schemes are also available from context menus directly on the selected entries in the **Project Tree**. Other quick-style buttons are available for displaying hydrogen bonds between Project Tree entries, for displaying labels in the 3D view and for creating custom atom groups. They are all described in detail below.

Note! Whenever you wish to change the visualization styles by right-clicking the entries in the **Project Tree**, please be aware that you must first click on the entry of interest, and ensure it is highlighted in blue, before right-clicking.

15.3.1 Visualization styles and colors

Wireframe, Stick, Ball and stick, Space-filling/CPK

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Four different ways of visualizing molecules by showing all atoms are provided: Wireframe, Stick, Ball and stick, and Space-filling/CPK.

The visualizations are mutually exclusive meaning that only one style can be applied at a time for each selected molecule or atom group.

Six color schemes are available and can be accessed via right-clicking on the quick-style buttons:

- Color by Element. Classic CPK coloring based on atom type (e.g. oxygen red, carbon gray, hydrogen white, nitrogen blue, sulfur yellow).
- Color by Temperature. For PDB files, this is based on the b-factors. For structure models created with tools in a CLC workbench, this is based on an estimate of the local model quality. The color scale goes from blue (0) over white (50) to red (100). The b-factors as well as the local model quality estimate are measures of uncertainty or disorder in the atom position; the higher the number, the higher the uncertainty.
- Color Carbons by Entry. Each entry (molecule or atom group) is assigned its own specific color. Only carbon atoms are colored by the specific color, other atoms are colored by element.
- Color by Entry. Each entry (molecule or atom group) is assigned its own specific color.
- Custom Color. The user selects a molecule color from a palette.
- Custom Carbon Color. The user selects a molecule color from a palette. Only carbon atoms are colored by the specific color, other atoms are colored by element.

Backbone

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For the molecules in the Proteins and Nucleic Acids categories, the backbone structure can be visualized in a schematic rendering, highlighting the secondary structure elements for proteins and matching base pairs for nucleic acids. The backbone visualization can be combined with any of the atom-level visualizations.

Five color schemes are available for backbone structures:

- Color by Residue Position. Rainbow color scale going from blue over green to yellow and red, following the residue number.
- Color by Type. For proteins, beta sheets are blue, helices red and loops/coil gray. For nucleic acids backbone ribbons are white while the individual nucleotides are indicated in green (T/U), red (A), yellow (G), and blue (C).
- Color by Backbone Temperature. For PDB files, this is based on the b-factors for the $C\alpha$ atoms (the central carbon atom in each amino acid). For structure models created with tools in the workbench, this is based on an estimate of the local model quality. The color scale goes from blue (0) over white (50) to red (100). The b-factors as well as the local model quality estimate are measures of uncertainty or disorder in the atom position; the higher the number, the higher the uncertainty.
- Color by Entry. Each chain/molecule is assigned its own specific color.
- Custom Color. The user selects a molecule color from a palette.

Surfaces

(●)

Molecular surfaces can be visualized.

Five color schemes are available for surfaces:

- Color by Charge. Charged amino acids close to the surface will show as red (negative) or blue (positive) areas on the surface, with a color gradient that depends on the distance of the charged atom to the surface.
- Color by Element. Smoothed out coloring based on the classic CPK coloring of the heteroatoms close to the surface.
- Color by Temperature. Smoothed out coloring based on the temperature values assigned to atoms close to the surface (See the "Wireframe, Stick, Ball and stick, Space-filling/CPK" section above).
- Color by Entry. Each surface is assigned its own specific color.
- Custom Color. The user selects a surface color from a palette.

A surface spanning multiple molecules can be visualized by creating a custom atom group that includes all atoms from the molecules (see section 15.3.1).

It is possible to adjust the opacity of a surface by adjusting the transparency slider at the bottom of the menu.

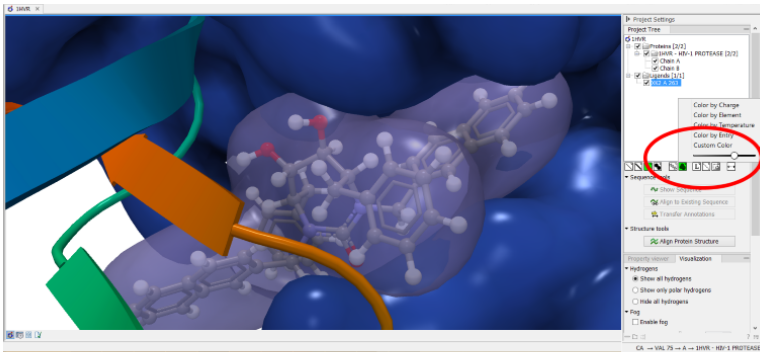

Figure 15.8: *Transparent surfaces*

Notice that visual artifacts may appear when rotating a transparent surface. These artifacts disappear as soon as the mouse is released.

Labels

(L)

Labels can be added to the molecules in the view by selecting an entry in the Project Tree and clicking the label button at the bottom of the Project Tree view. The color of the labels can be adjusted from the context menu by right clicking on the selected entry (which must be highlighted in blue first) or on the label button in the bottom of the Project Tree view (see figure 15.9).

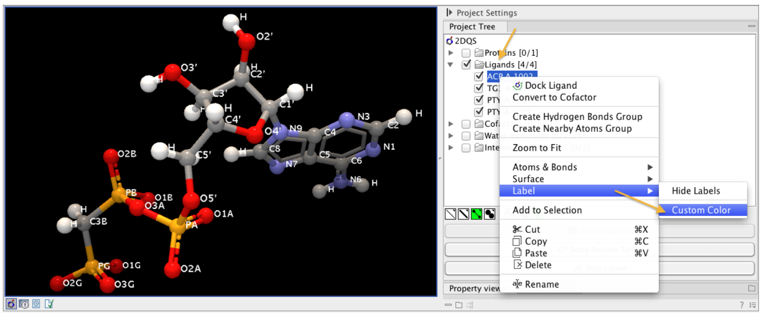

Figure 15.9: *The color of the labels can be adjusted in two different ways. Either directly using the label button by right clicking the button, or by right clicking on the molecule or category of interest in the Project Tree.*

- For proteins and nucleic acids, each residue is labeled with the PDB name and number.
- For ligands, each atom is labeled with the atom name as given in the input.
- For cofactors and water, one label is added with the name of the molecule.
- For atom groups including protein atoms, each protein residue is labeled with the PDB name and number.
- For atom groups not including protein atoms, each atom is labeled with the atom name as given in the input.

Labels can be removed again by clicking on the label button.

Hydrogen bonds

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The Show Hydrogen Bond visualization style may be applied to molecules and atom group entries in the project tree. If this style is enabled for a project tree entry, hydrogen bonds will be shown to all other currently visible objects. The hydrogen bonds are updated dynamically: if a molecule is toggled off, the hydrogen bonds to it will not be shown.

It is possible to customize the color of the hydrogen bonds using the context menu.

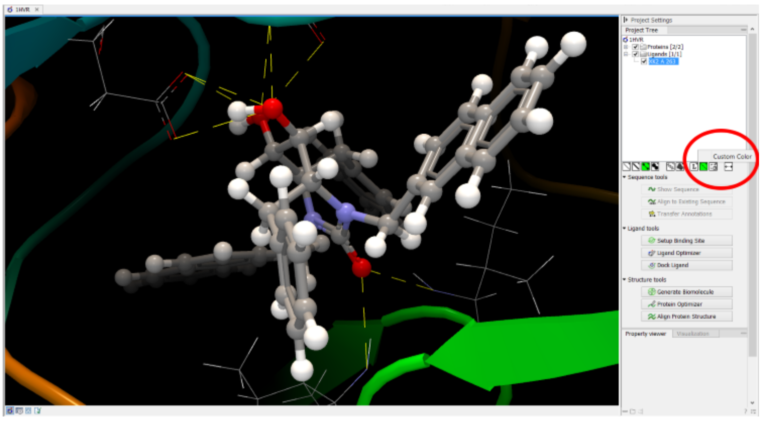

Figure 15.10: *The hydrogen bond visualization setting, with custom bond color.*

Create atom group

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Often it is convenient to use a unique visualization style or color to highlight a particular set of atoms, or to visualize only a subset of atoms from a molecule. This can be achieved by creating an atom group. Atom groups can be created based on atoms selected in the 3D view or entries selected in the Project Tree. When an atom group has been created, it appears as an entry in the Project Tree in the category "Atom groups". The atoms can then be hidden or shown, and the visualization changed, just as for the molecule entries in the Project Tree.

Note that an atom group entry can be renamed. Select the atom group in the Project Tree and invoke the right-click context menu. Here, the Rename option is found.

Create atom group based on atoms selected in 3D view

When atoms are selected in the 3D view, brown spheres indicate which atoms are included in the selection. The selection will appear as the entry "Current" in the Selections category in the Project Tree.

Once a selection has been made, press the "Create Atom Group" button and a context menu will show different options for creating a new atom group based on the selection:

- Selected Atoms. Creates an atom group containing exactly the selected atoms (those indicated by brown spheres). If an entire molecule or residue is selected, this option is not displayed.
- Selected Residue(s)/Molecules. Creates an atom group that includes all atoms in the

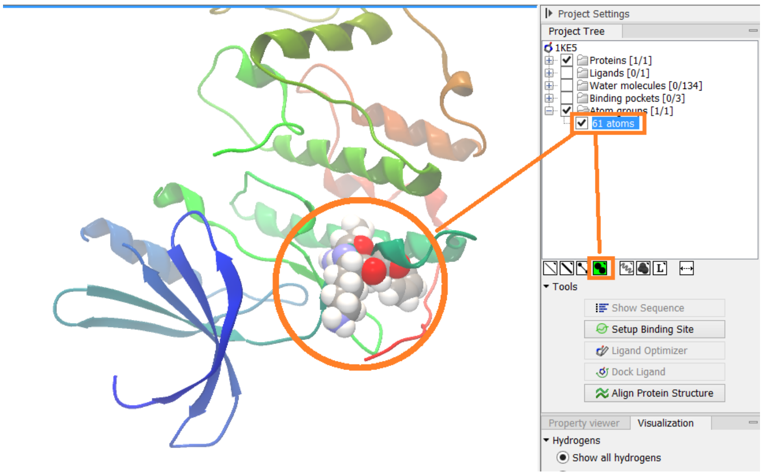


Figure 15.11: *An atom group that has been highlighted by adding a unique visualization style.*

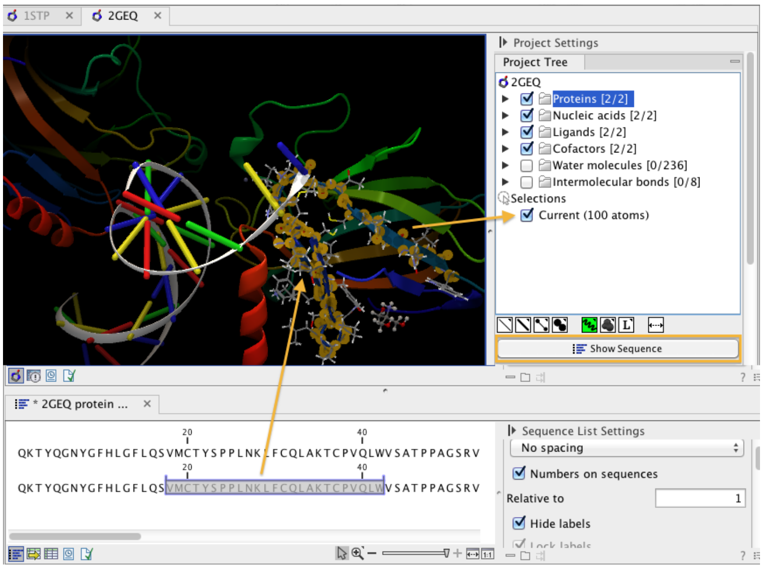
selected residues (for entries in the protein and nucleic acid categories) and molecules (for the other categories).

- Nearby Atoms. Creates an atom group that contains residues (for the protein and nucleic acid categories) and molecules (for the other categories) within 5 Å of the selected atoms. Only atoms from currently visible Project Tree entries are considered.
- Hydrogen Bonded Atoms. Creates an atom group that contains residues (for the protein and nucleic acid categories) and molecules (for the other categories) that have hydrogen bonds to the selected atoms. Only atoms from currently visible Project Tree entries are considered.

There are several ways to select atoms in the 3D view:

- Double click to select. Click on an atom to select it. When you double click on an atom that belongs to a residue in a protein or in a nucleic acid chain, the entire residue will be selected. For small molecules, the entire molecule will be selected.
- Adding atoms to a selection. Holding down Ctrl while picking atoms, will pile up the atoms in the selection. All atoms in a molecule or category from the Project Tree, can be added to the "Current" selection by choosing "Add to Current Selection" in the context menu. Similarly, entire molecules can be removed from the current selection via the context menu.
- Spherical selection. Hold down the shift-key, click on an atom and drag the mouse away from the atom. Then a sphere centered on the atom will appear, and all atoms inside the sphere, visualized with one of the all-atom representations will be selected. The status bar (lower right corner) will show the radius of the sphere.
- Show Sequence. Another option is to select protein or nucleic acid entries in the Project Tree, and click the "Show Sequence" button found below the Project Tree, see section 15.4.1. A split-view will appear with a sequence editor for each of the sequence data types (Protein, DNA, RNA) (figure 15.12). If you then select residues in the sequence view, the backbone atoms of the selected residues will show up as the "Current" selection in the 3D view and the Project Tree view. Notice that the link between the 3D view and the sequence editor is lost if either window is closed, or if the sequence is modified.

- Align to Existing Sequence. If a single protein chain is selected in the Project Tree, the "Align to Existing Sequence" button can be clicked, see section 15.4.2. This links the protein sequence with a sequence or sequence alignment found in the Navigation Area. A split-view appears with a sequence alignment where the sequence of the selected protein chain is linked to the 3D structure, and atoms can be selected in the 3D view, just as for the "Show Sequence" option.



☑ Proteins [2/2]
☑ Nucleic acids [2/2]
☑ Ligands [2/2]
☑ Cofactors [2/2]
□ Water molecules [0/236]
□ Intermolecular bonds [0/8]
☑ Current (100 atoms)

```
QKTYQGNYGFHLGFLQSVMCTYSPPLNKLFCQLAKTCPVQLWVSATPPAGSRV
QKTYQGNYGFHLGFLQSVMCTYSPPLNKLFCQLAKTCPVQLWVSATPPAGSRV
```

No spacing
☑ Numbers on sequences
Relative to 1
☑ Hide labels
☑ Lock labels

Figure 15.12: *The protein sequence in the split view is linked with the protein structure. This means that when a part of the protein sequence is selected, the same region in the protein structure will be selected.*

Create atom group based on entries selected in the Project Tree

Select one or more entries in the Project Tree, and press the "Create Atom Group" button, then a context menu will show different options for creating a new atom group based on the selected entries:

- Nearby Atoms. Creates an atom group that contains residues (for the protein and nucleic acid categories) and molecules (for the other categories) within 5 Å of the selected entries. Only atoms from currently visible Project Tree entries are considered.

- Hydrogen Bonded Atoms. Creates an atom group that contains residues (for the protein and nucleic acid categories) and molecules (for the other categories) that have hydrogen bonds to the selected entries. Only atoms from currently visible Project Tree entries are considered.

If a Binding Site Setup is present in the Project Tree (A Binding Site Setup could only be created using the now discontinued CLC Drug Discovery Workbench), and entries from the Ligands or Docking results categories are selected, two extra options are available under the header **Create Atom Group (Binding Site)**. For these options, atom groups are created considering all molecules included in the Binding Site Setup, and thus not taking into account which Project Tree entries are currently visible.

Zoom to fit

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The "Zoom to fit" button can be used to automatically move a region of interest into the center of the screen. This can be done by selecting a molecule or category of interest in the Project Tree view followed by a click on the "Zoom to fit" button (⇠⇢) at the bottom of the Project Tree view (figure 15.13). Double-clicking an entry in the Project Tree will have the same effect.

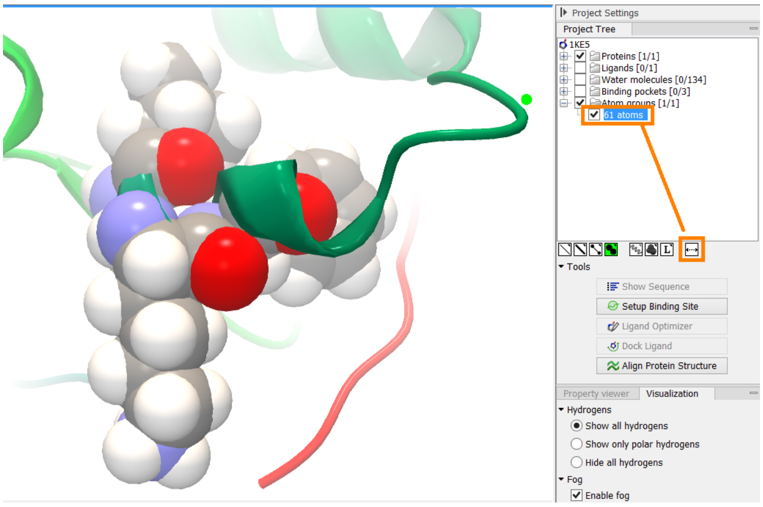


Figure 15.13: *The "Fit to screen" button can be used to bring a particular molecule or category of molecules in focus.*

15.3.2 Project settings

A number of general settings can be adjusted from the **Side Panel**. Personal settings as well as molecule visualizations can be saved by clicking in the lower right corner of the **Side Panel** (☰). This is described in detail in section 4.6.

Project Tree Tools

Just below the Project Tree, the following tools are available

- **Show Sequence** Select molecules which have sequences associated (Protein, DNA, RNA) in the Project Tree, and click this button. Then, a split-view will appear with a sequence editor for each of the sequence data types (Protein, DNA, RNA). This is described in section 15.4.1.
- **Align to Existing Sequence** Select a protein chain in the Project Tree, and click this button. Then protein sequences and sequence alignments found in the Navigation Area, can be linked with the protein structure. This is described in section 15.4.2.
- **Transfer Annotations** Select a protein chain in the Project Tree, that has been linked with a sequence using either the "Show Sequence" or "Align to Existing Sequence" options. Then it is possible to transfer annotations between the structure and the linked sequence. This is described in section 15.4.3.

- **Align Protein Structure** This will invoke the dialog for aligning protein structures based on global alignment of whole chains or local alignment of e.g. binding sites defined by atom groups. This is described in section 15.5.

Property viewer

The Property viewer, found in the Side Panel, lists detailed information about the atoms that the mouse hovers over. For all atoms the following information is listed:

- **Molecule** The name of the molecule the atom is part of.
- **Residue** For proteins and nucleic acids, the name and number of the residue the atom belongs to is listed, and the chain name is displayed in parentheses.
- **Name** The particular atom name, if given in input, with the element type (Carbon, Nitrogen, Oxygen...) displayed in parentheses.
- **Hybridization** The atom hybridization assigned to the atom.
- **Charge** The atomic charge as given in the input file. If charges are not given in the input file, some charged chemical groups are automatically recognized and a charge assigned.

For atoms in molecules imported from a PDB file, extra information is given:

- **Temperature** Here is listed the b-factor assigned to the atom in the PDB file. The b-factor is a measure of uncertainty or disorder in the atom position; the higher the number, the higher the disorder.
- **Occupancy** For each atom in a PDB file, the occupancy is given. It is typically 1, but if atoms are modeled in the PDB file, with no foundation in the raw data, the occupancy is 0. If a residue or molecule has been resolved in multiple positions, the occupancy is between 0 and 1.

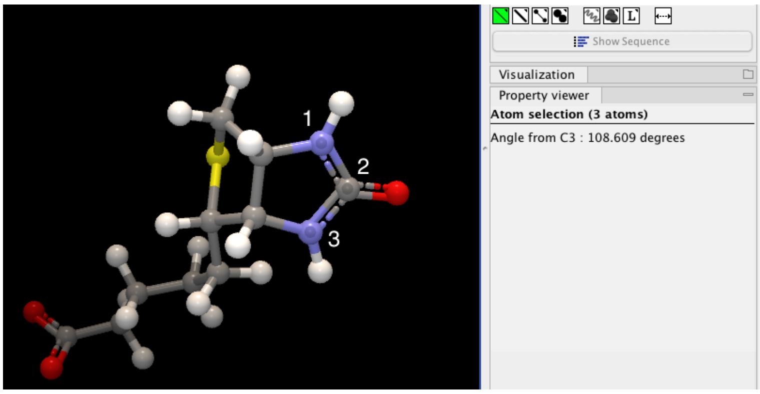
If an atom is selected, the Property view will be frozen with the details of the selected atom shown. If then a second atom is selected (by holding down Ctrl while clicking), the distance between the two selected atoms is shown. If a third atom is selected, the angle for the second atom selected is shown. If a fourth atom is selected, the dihedral angle measured as the angle between the planes formed by the three first and three last selected atoms is given.

If a molecule is selected in the Project Tree, the Property view shows information about this molecule. Two measures are always shown:

- **Atoms** Number of atoms in the molecule.
- **Weight** The weight of the molecule in Daltons.

Visualization settings

Under "Visualization" five options exist:



Atom selection (3 atoms)

Angle from C3 : 108.609 degrees

Figure 15.14: *Selecting two, three, or four atoms will display the distance, angle, or dihedral angle, respectively.*

- **Hydrogens** Hydrogen atoms can be shown (Show all hydrogens), hidden (Hide all hydrogens) or partially shown (Show only polar hydrogens).
- **Fog** "Fog" is added to give a sense of depth in the view. The strength of the fog can be adjusted or it can be disabled.
- **Clipping plane** This option makes it possible to add an imaginary plane at a specified distance along the camera's line of sight. Only objects behind this plane will be drawn. It is possible to clip only surfaces, or to clip surfaces together with proteins and nucleic acids. Small molecules, like ligands and water molecules, are never clipped.
- **3D projection** The view is opened up towards the viewer, with a "Perspective" 3D projection. The field of view of the perspective can be adjusted, or the perspective can be disabled by selecting an orthographic 3D projection.
- **Coloring** The background color can be selected from a color palette by clicking on the colored box.

Snapshots of the molecule visualization To save the current view as a picture, right-click in the **View Area** and select "File" and "Export Graphics". Another way to save an image is by pressing the "Graphics" button in the Workbench toolbar (). Next, select the location where you wish to save the image, select file format (PNG, JPEG, or TIFF), and provide a name, if you wish to use another name than the default name.

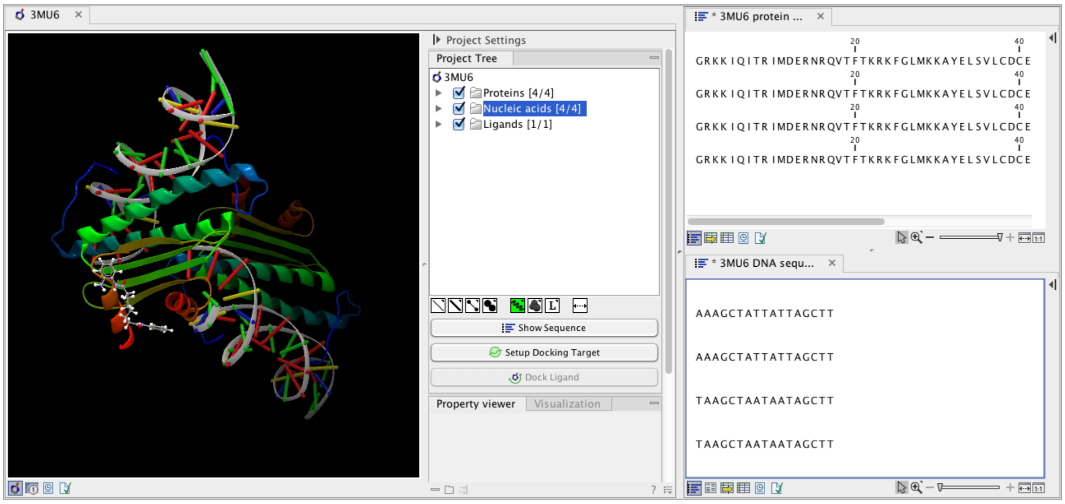
You can also save the current view directly on data with a custom name, so that it can later be applied (see section 4.6).

15.4 Tools for linking sequence and structure

The *CLC Main Workbench* has functionality that allows you to link a protein sequence to a protein structure. Selections made on the sequence will show up on the structure. This allows you to explore a protein sequence in a 3D structure context. Furthermore, sequence annotations can be transferred to annotations on the structure and annotations on the structure can be transferred to annotations on the sequence (see section 15.4.3).

15.4.1 Show sequence associated with molecule

From the Side Panel, sequences associated with the molecules in the Molecule Project can be opened as separate objects by selecting protein or nucleic acid entries in the Project Tree and clicking the button labeled "Show Sequence" (figure 15.15). This will generate a Sequence or Sequence List for each selected sequence type (protein, DNA, RNA). The sequences can be used to select atoms in the Molecular Project as described in section 15.3.1. The sequences can also be used as input for sequence analysis tools or be saved as independent objects. You can later re-link to the sequence using "Align to Existing Sequence" (see section 15.4.2).



```
                   20                  40
GRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCE
                   20                  40
GRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCE
                   20                  40
GRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCE
                   20                  40
GRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCE
```

```
AAAGCTATTATTAGCTT

AAAGCTATTATTAGCTT

TAAGCTAATAATAGCTT

TAAGCTAATAATAGCTT
```

Figure 15.15: *Protein chain sequences and DNA sequences are shown in separate views.*

15.4.2 Link sequence or sequence alignment to structure

The "Align to Existing Sequence" button can be used to map and link existing sequences or sequence alignments to a protein structure chain in a Molecule Project (3D view). It can also be used to reconnect a protein structure chain to a sequence or sequence alignment previously created by Show Sequence (section 15.4.1) or Align to Existing Sequence.

Select a single protein chain in the project tree (see figure 15.16). Pressing "Align to Existing Sequence" then opens a Navigation Area browser, where it is possible to select one or more Sequence, Sequence Lists, or Alignments, to link with the selected protein chain.

If the sequences or alignments already contain a sequence identical to the protein chain selected in the Molecule Project (i.e. same name and amino acid sequence), this sequence is linked to the protein structure. If no identical sequence is present, a sequence is extracted from the protein structure (as for Show Sequence, see section 15.4.1), and a sequence alignment is created between this sequence and the sequences or alignments selected from the Navigation Area. The new sequence alignment is created (see section 16.1) with the following settings:

- Gap open cost: 10.0
- Gap Extension cost: 1.0
- End gap cost: free
- Existing alignments are not redone

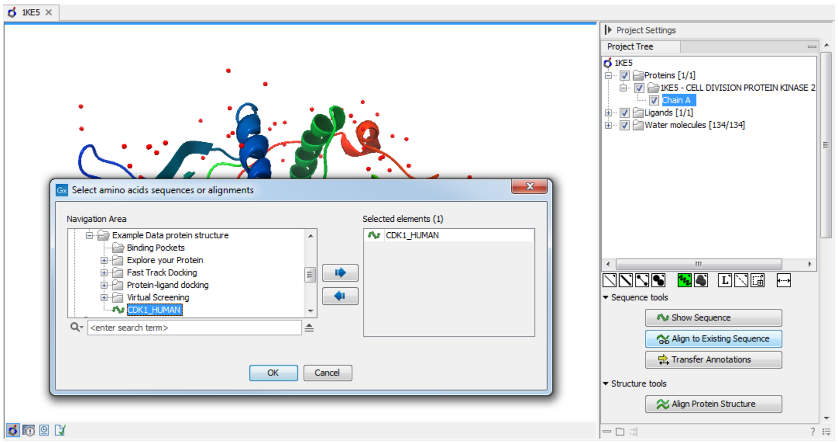


Figure 15.16: *Select a single protein chain in the Project Tree and invoke "Align to Existing Sequence".*

When the link is established, selections on the linked sequence in the sequence editor will create atom selections in the 3D view, and it is possible to transfer annotations between the linked sequence and the 3D protein chain (see section 15.4.3). Note that the link will be broken if either the sequence or the 3D protein chain is modified.

Two tips if the link is to a sequence in an alignment:

1. Read about how to change the layout of sequence alignments in section 16.2

2. It is only annotations present on the sequence linked to the 3D view that can be transferred to atom groups on the structure. To transfer sequence annotations from other sequences in the alignment, first copy the annotations to the sequence in the alignment that is linked to the structure (see figure 15.19 and section 16.3).

15.4.3 Transfer annotations between sequence and structure

The Transfer Annotations dialog makes it possible to create new atom groups (annotations on structure) based on protein sequence annotations and vice versa.

You can read more about sequence annotations in section 14.3 15.3.1 and more about atom groups in section 15.3.1.

Before it is possible to transfer annotations, a link between a protein sequence editor and a Molecule Project (a 3D view) must be established. This is done either by opening a sequence associated with a protein chain in the 3D view using the 'Show Sequence' button (see section 15.4.1 15.4.2) or by mapping to an existing sequence or sequence alignment using the 'Align to Existing Sequence' button (see section 15.4.2).

Invoke the Transfer Annotations dialog by selecting a linked protein chain in the Project Tree and press 'Transfer Annotations' (see figure 15.17).

The dialog contains two tables (see figure 15.18). The left table shows all atom groups in the Molecule Project, with at least one atom on the selected protein chain. The right table shows all annotations present on the linked sequence. While the Transfer Annotations dialog is open, it is not possible to make changes to neither the sequence nor the Molecule Project, however,

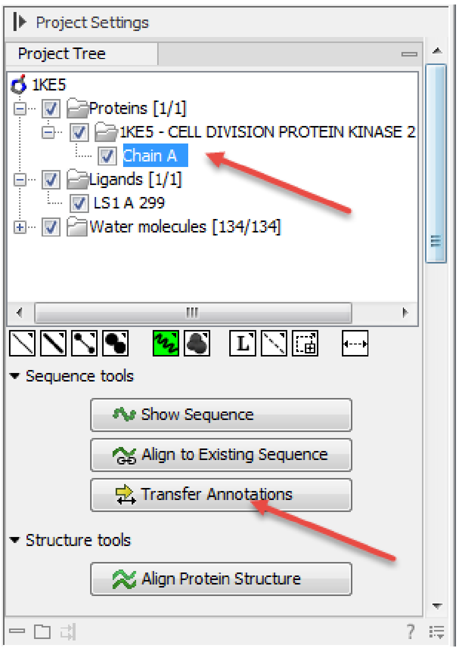


Figure 15.17: *Select a single protein chain in the Project Tree and invoke "Transfer Annotations".*

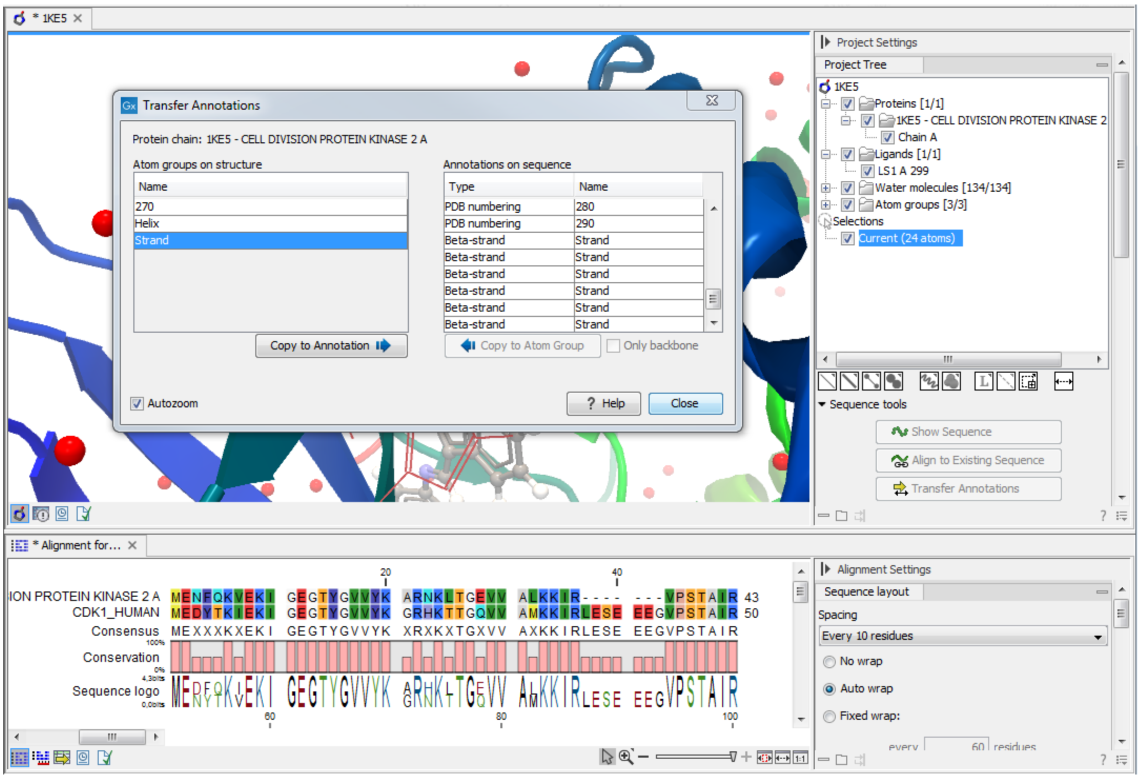
changes to the visualization styles are allowed.

How to undo annotation transfers

In order to undo operations made using the Transfer Annotations dialog, the dialog must first be closed. To undo atom groups added to the structure, activate the 3D view by clicking in it and press Undo in the Toolbar. To undo annotations added to the sequence, activate the sequence view by clicking in it and press Undo in the Toolbar.

Transfer sequence annotations from aligned sequences

It is only annotations present on the sequence linked to the 3D view that can be transferred to atom groups on the structure. If you wish to transfer annotations that are found on other sequences in a linked sequence alignment, you need first to copy the sequence annotations to the actual sequence linked to the 3D view (the sequence with the same name as the protein structure). This is done by invoking the context menu on the sequence annotation you wish to copy (see figure 15.19 and section 16.3).



Transfer Annotations

Protein chain: 1KE5 - CELL DIVISION PROTEIN KINASE 2 A

Atom groups on structure

Name
270
Helix
Strand

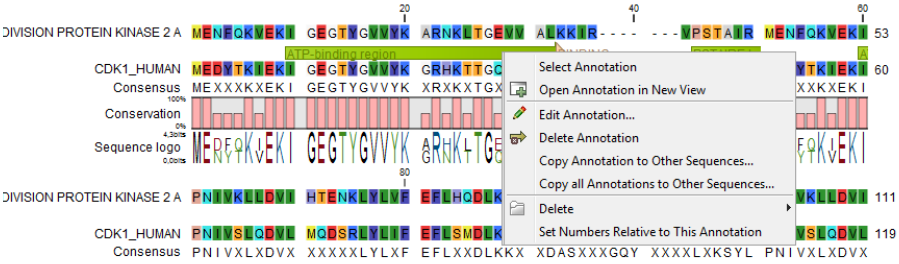
Annotations on sequence

Type	Name
PDB numbering	280
PDB numbering	290
Beta-strand	Strand
Beta-strand	Strand
Beta-strand	Strand
Beta-strand	Strand
Beta-strand	Strand
Beta-strand	Strand

☑ Autozoom

□ Only backbone

Figure 15.18: *The Transfer Annotations dialog allow you to select annotations listed in the two tables, and copy them from structure to sequence or vice versa.*



DIVISION PROTEIN KINASE 2 A
ATP-binding region
CDK1_HUMAN
Consensus
Conservation
Sequence logo
Select Annotation
Open Annotation in New View
Edit Annotation...
Delete Annotation
Copy Annotation to Other Sequences...
Copy all Annotations to Other Sequences...
Delete
Set Numbers Relative to This Annotation

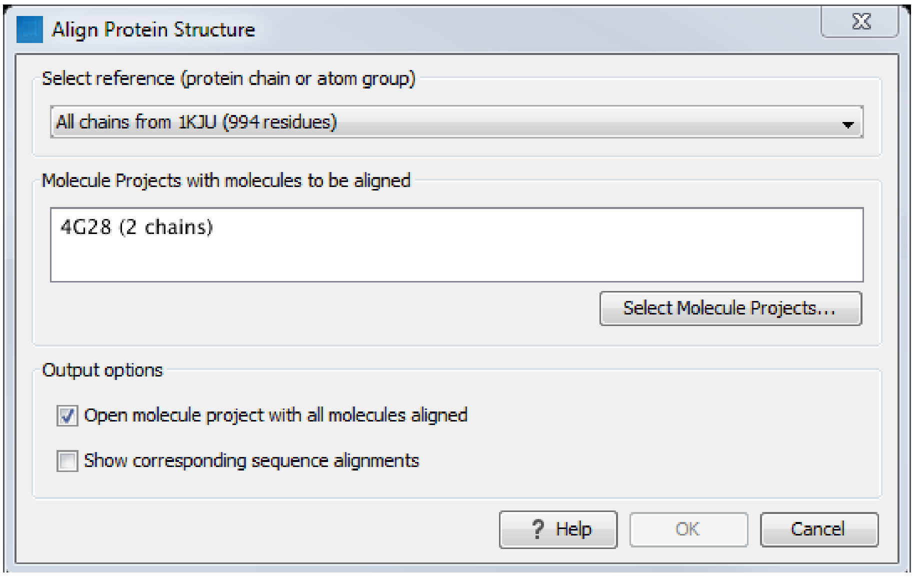
Figure 15.19: *Copy annotations from sequences in the alignment to the sequence linked to the 3D view.*

15.5 Align Protein Structure

The Align Protein Structure tool allows you to compare a protein or binding pocket in a **Molecule Project** with proteins from other **Molecule Projects**. The tool is invoked using the ( ) Align Protein Structure action from the **Molecule Project Side Panel**. This action will open an interactive dialog box (figure 15.20). By default, when the dialog box is closed with an "OK", a new **Molecule Project** will be opened containing all the input protein structures laid on top of one another. All molecules coming from the same input Molecule Project will have the same color in the initial visualization.

The dialog box contains three fields:

- **Select reference (protein chain or atom group)** This drop-down menu shows all the protein


Align Protein Structure

Select reference (protein chain or atom group)

All chains from 1KJU (994 residues)

Molecule Projects with molecules to be aligned

4G28 (2 chains)

Output options

☑ Open molecule project with all molecules aligned

□ Show corresponding sequence alignments

Figure 15.20: *The Align Protein Structure dialog box.*

chains and residue-containing atom groups in the current **Molecule Project**. If an atom group is selected, the structural alignment will be optimized in that area. The 'All chains from *Molecule Project* option will create a global alignment to all protein chains in the project, fitting e.g. a dimer to a dimer.

- **Molecule Projects with molecules to be aligned** One or more **Molecule Projects** containing protein chains may be selected.
- **Output options** The default output is a single **Molecule Project** containing all the input projects rotated onto the coordinate system of the reference. Several alignment statistics, including the RMSD, TM-score, and sequence identity, are added to the **History** of the output **Molecule Project**. Additionally, a sequence alignments of the aligned structures may be output, with the sequences linked to the 3D structure view.

15.5.1 Example: alignment of calmodulin

Calmodulin is a calcium binding protein. It is composed of two similar domains, each of which binds two calcium atoms. The protein is especially flexible, which can make structure alignment challenging. Here we will compare the calcium binding loops of two calmodulin crystal structures – PDB codes 1A29 and 4G28.

Initial global alignment The 1A29 project is opened and the Align Protein Structure dialog is filled out as in figure 15.20. Selecting "All chains from 1A29" tells the aligner to make the best possible global alignment, favoring no particular region. The output of the alignment is shown in figure 15.21. The blue chain is from 1A29, the brown chain is the corresponding calmodulin chain from 4G28 (a calmodulin-binding chain from the 4G28 file has been hidden from the view). Because calmodulin is so flexible, it is not possible to align both of its domains (enclosed in black boxes) at the same time. A good global alignment would require the brown protein to be translated in one direction to match the N-terminal domain, and in the other direction to match the C-terminal domain (see black arrows).

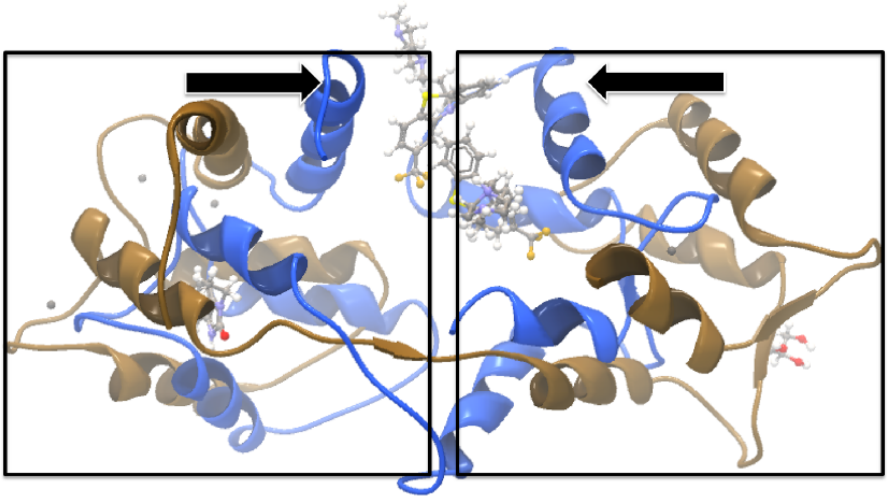


Figure 15.21: *Global alignment of two calmodulin structures (blue and brown). The two domains of calmodulin (shown within black boxes) can undergo large changes in relative orientation. In this case, the different orientation of the domains in the blue and brown structures makes a good global alignment impossible: the movement required to align the brown structure onto the blue is shown by arrows – as the arrows point in opposite directions, improving the alignment of one domain comes at the cost of worsening the alignment of the other.*

Focusing the alignment on the N-terminal domain To align only the N-terminal domain, we return to the 1A29 project and select the **Show Sequence** action from beneath the **Project Tree**. We highlight the first 62 residues, then convert them into an atom group by right-clicking on the "Current" selection in the **Project Tree** and choosing "Create Group from Selection" (figure 15.22). Using the new atom group as the reference in the alignment dialog leads to the alignment shown in figure 15.23. In addition to the original input proteins, the output now includes two Atom Groups, which contain the atoms on which the alignment was focused. The **History** of the output **Molecule Project** shows that the alignment has 0.9 Å RMSD over the 62 residues.

Aligning a binding site Two bound calcium atoms, one from each calmodulin structure, are shown in the black box of figure 15.23. We now wish to make an alignment that is as good as possible about these atoms so as to compare the binding modes. We return to the 1A29 project, right-click the calcium atom from the cofactors list in the **Project Tree** and select "Create Nearby Atoms Group". Using the new atom group as the reference in the alignment dialog leads to the alignment shown in figure 15.24.

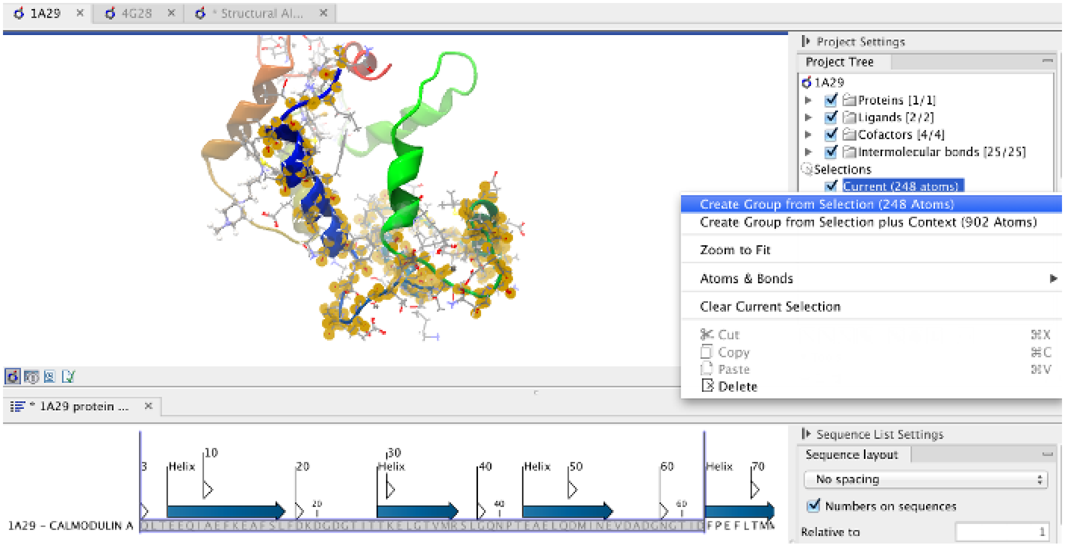
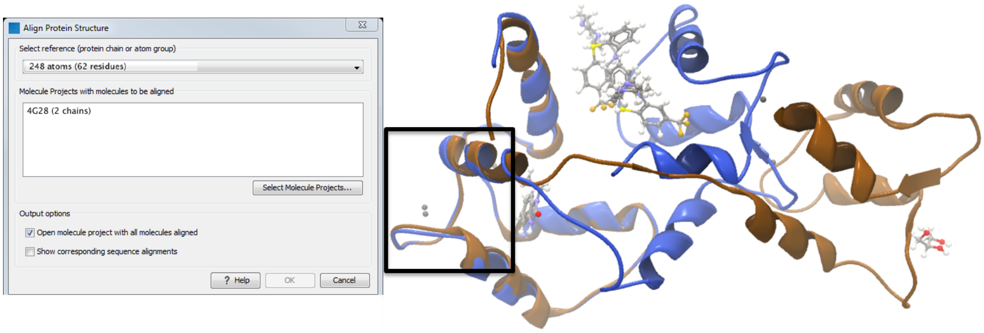


Figure 15.22: *Creation of an atom group containing the N-terminal domain of calmodulin.*



Align Protein Structure

Select reference (protein chain or atom group)

248 atoms (62 residues)

Molecule Projects with molecules to be aligned

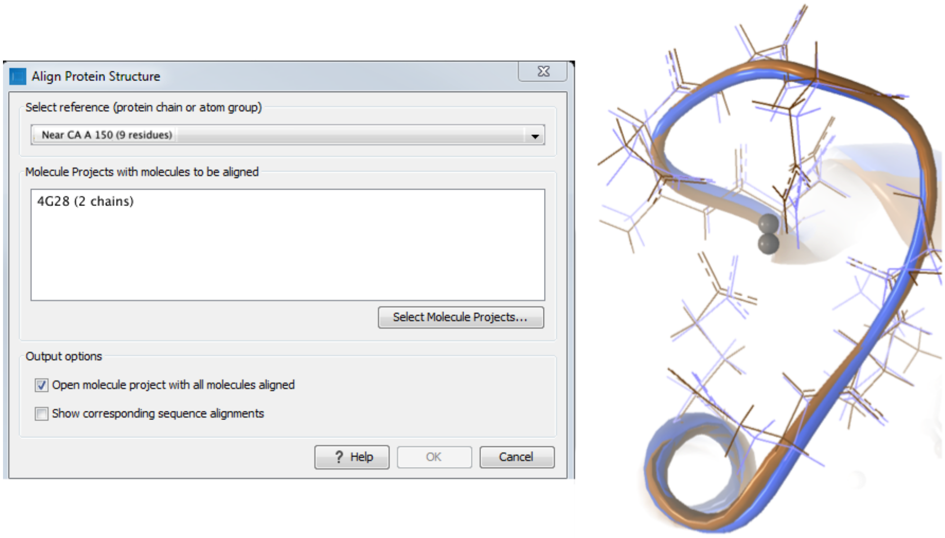
4G28 (2 chains)

Output options

☑ Open molecule project with all molecules aligned

□ Show corresponding sequence alignments

Figure 15.23: *Alignment of the same two calmodulin proteins as in figure* 15.21*, but this time with a focus on the N-terminal domain. The blue and brown structures are now well-superimposed in the N-terminal region. The black box encloses two calcium atoms that are bound to the structures.*



Align Protein Structure

Select reference (protein chain or atom group)

Near CA A 150 (9 residues)

Molecule Projects with molecules to be aligned

4G28 (2 chains)

Output options

☑ Open molecule project with all molecules aligned

□ Show corresponding sequence alignments

Figure 15.24: *Alignment of the same two calmodulin domains as in figure 15.21, but this time with a focus on the calcium atom within the black box of figure 15.23. The calcium atoms are less than 1 Å apart – compatible with thermal motion encoded in the atoms' temperature factors.*

15.5.2 The Align Protein Structure algorithm

Any approach to structure alignment must make a trade-off between alignment length and alignment accuracy. For example, is it better to align 200 amino acids at an RMSD of 3.0 Å or 150 amino acids at an RMSD of 2.5 Å? The Align Protein Structure algorithm determines the answer to this question by taking the alignment with the higher TM-score. For an alignment focused on a protein of length L, this is:

$$\text{TM-score} = \frac{1}{L}\sum_i \frac{1}{1+\frac{d_i}{d(L)}^2}$$

where i runs over the aligned pairs of residues, d_i is the distance between the i^{th} such pair, and $d(L)$ is a normalization term that approximates the average distance between two randomly chosen points in a globular protein of length L [Zhang and Skolnick, 2004]. A perfect alignment has a TM-score of 1.0, and two proteins with a TM-score $>$0.5 are often said to show structural homology [Xu and Zhang, 2010].

The Align Protein Structure Algorithm attempts to find the *structure alignment* with the highest TM-score. This problem reduces to finding a *sequence alignment* that pairs residues in a way that results in a high TM-score. Several sequence alignments are tried including an alignment with the BLOSUM62 matrix, an alignment of secondary structure elements, and iterative refinements of these alignments.

The Align Protein Structure Algorithm is also capable of aligning entire protein complexes. To do this, it must determine the correct pairing of each chain in one complex with a chain in the other. This set of chain pairings is determined by the following procedure:

1. Make structure alignments between every chain in one complex and every chain in the other. Discard pairs of chains that have a TM-score of < 0.4
2. Find all pairs of structure alignments that are consistent with each other i.e. are achieved by approximately the same rotation
3. Use a heuristic to combine consistent pairs of structure alignments into a single alignment

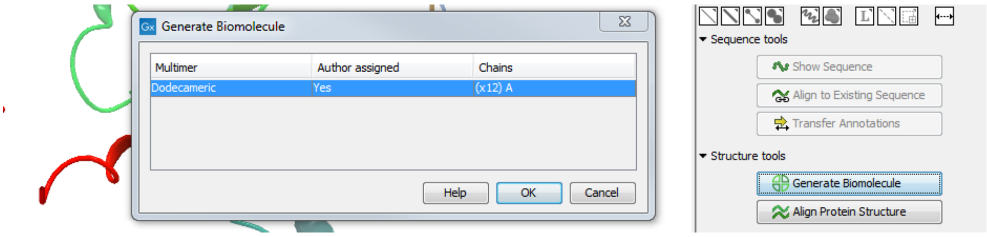
The heuristic used in the last step is similar to that of MM-align [Mukherjee and Zhang, 2009], whereas the first two steps lead to both a considerable speed up and increased accuracy. The alignment of two 30S ribosome subunits, each with 20 protein chains, can be achieved in less than a minute (PDB codes 2QBD and 1FJG).

15.6 Generate Biomolecule

Protein structures imported from a PBD file show the tertiary structure of proteins, but not necessarily the biologically relevant form (the quaternary structure). Oftentimes, several copies of a protein chain need to arrange in a multi-subunit complex to form a functioning biomolecule. In some PDB files several copies of a biomolecule are present and in others only one chain from a multi-subunit complex is present. In many cases, PDB files have information about how the molecule structures in the file can form biomolecules.

When a PDB file with biomolecule information available has been either downloaded directly to the workbench using the *Search for PDB Structures at NCBI* or imported using *Import Molecules with 3D Coordinates*, the information can be used to generate biomolecule structures in *CLC Main Workbench*.

The "Generate Biomolecule" dialog is invoked from the Side Panel of a Molecule Project (figure 15.25). The button (⊕) is found in the Structure tools section below the Project Tree.



Multimer	Author assigned	Chains
Dodecameric	Yes	(x12) A

Figure 15.25: *The Generate Biomolecule dialog lists all possibilities for biomolecules, as given in the PDB files imported to the Molecule Project. In this case, only one biomolecule option is available. The Generate Biomolecule button that invokes the dialog can be seen in the bottom right corner of the picture.*

There can be more than one biomolecule description available from the imported PDB files. The biomolecule definitions have either been assigned by the crystallographer solving the protein structure (Author assigned = "Yes") or suggested by a software prediction tool (Author assigned = "No"). The third column lists which protein chains are involved in the biomolecule, and how many copies will be made.

Select the preferred biomolecule definition and click OK.

A new Molecule Project will open containing the molecules involved in the selected biomolecule (example in figure 15.26). If required by the biomolecule definition, copies are made of protein chains and other molecules, and the copies are positioned according to the biomolecule information given in the PDB file. The copies will in that case have "s1", "s2", "s3" etc. at the end of the molecule names seen in the Project Tree.

If the proteins in the Molecule Project already are present in their biomolecule form, the message "The biological unit is already shown" is displayed, when the "Generate Biomolecule" button is clicked.

If the PDB files imported or downloaded to a Molecule Project did not hold biomolecule information, the message "No biological unit is associated with this Molecule Project" is shown, when the Generate Biomolecule button is clicked.

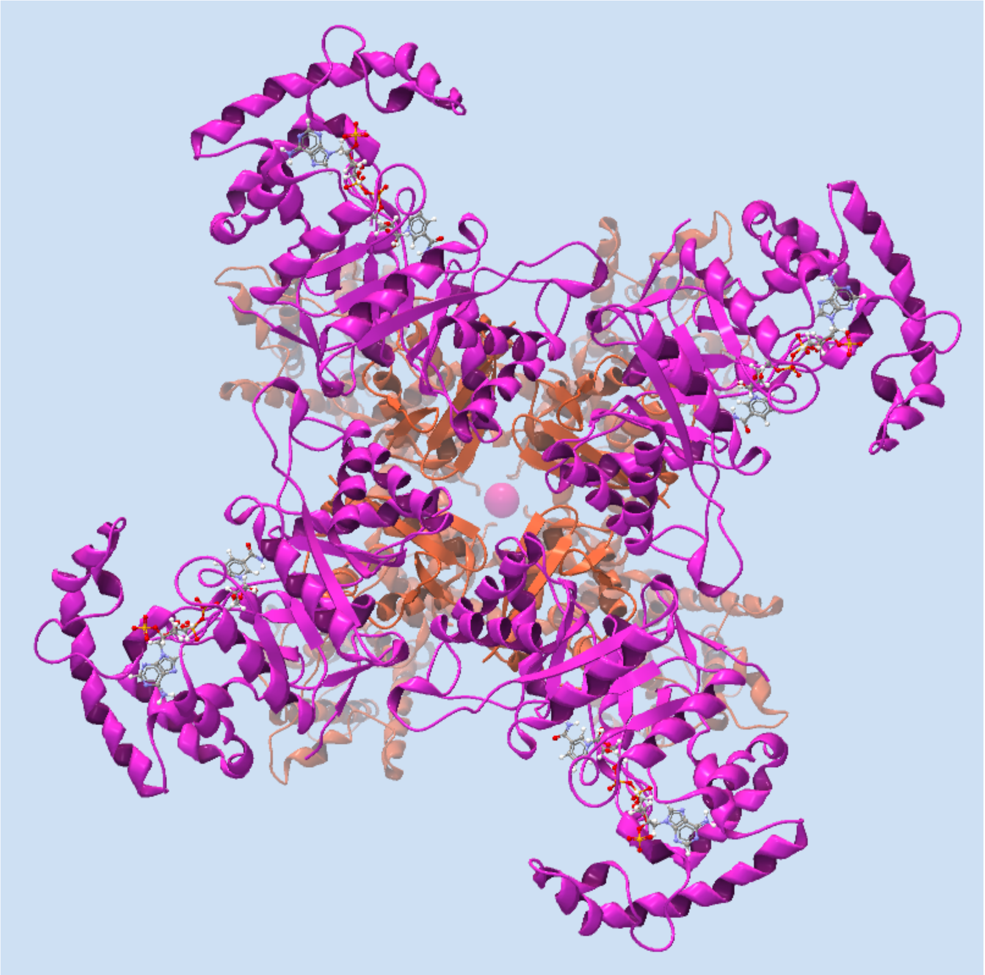


Figure 15.26: *One of the biomolecules that can be generated after downloading the PDB 2R9R to* **CLC Main Workbench**. *It is a voltage gated potassium channel.*

Chapter 16

Sequence alignment

Contents

CLC Main Workbench can align nucleotides and proteins using a *progressive alignment* algorithm (see section 16.5.2.

This chapter describes how to use the program to align sequences, and alignment algorithms in more general terms.

16.1 Create an alignment

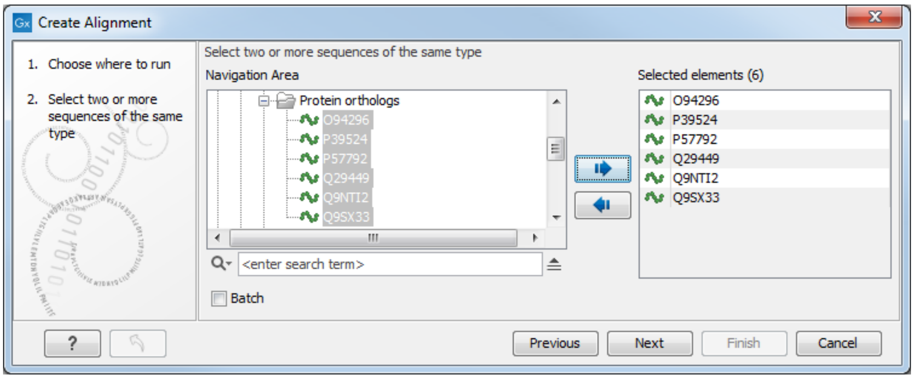
Alignments can be created from sequences, sequence lists (see section 14.1), existing alignments and from any combination of the three.

To create an alignment, run the **Create Alignment** tool, available at:

Tools | **Alignments and Trees** (▤)| **Create Alignment** (▦)

This opens the dialog shown in figure 16.1.

After selecting the elements to align, you are presented with options that can be configured (figure 16.2).


Create Alignment

1. Choose where to run
2. Select two or more sequences of the same type

Select two or more sequences of the same type

Navigation Area

- Protein orthologs
  - O94296
  - P39524
  - P57792
  - Q29449
  - Q9NTI2
  - Q9SX33

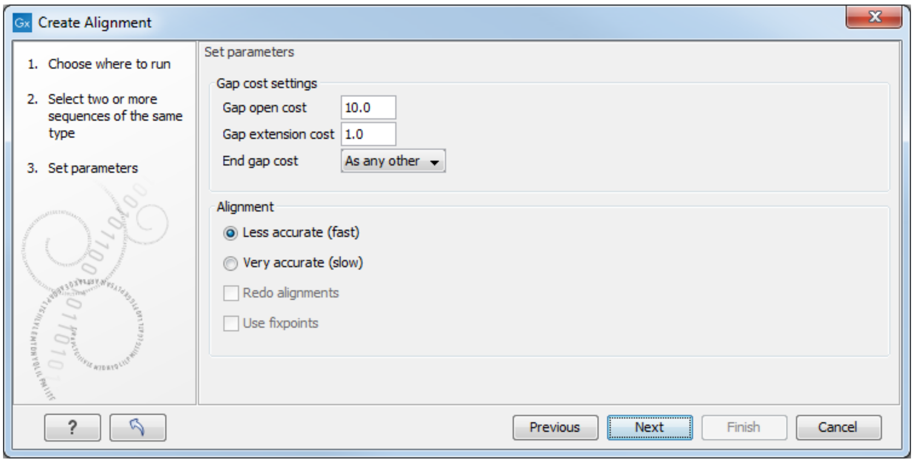
<enter search term>

☐ Batch

Selected elements (6)

- O94296
- P39524
- P57792
- Q29449
- Q9NTI2
- Q9SX33

Figure 16.1: *Creating an alignment.*


Create Alignment

1. Choose where to run
2. Select two or more sequences of the same type
3. Set parameters

Set parameters

Gap cost settings

Gap open cost 10.0

Gap extension cost 1.0

End gap cost As any other

Alignment

● Less accurate (fast)

○ Very accurate (slow)

☐ Redo alignments

☐ Use fixpoints

Figure 16.2: *Adjusting alignment algorithm parameters.*

16.1.1 Gap costs

The alignment algorithm has three parameters concerning gap costs: Gap open cost, Gap extension cost and End gap cost. The precision of these parameters is to one place of decimal.

- **Gap open cost**. The price for introducing gaps in an alignment.
- **Gap extension cost**. The price for every extension past the initial gap.

If you expect a lot of small gaps in your alignment, the Gap open cost should equal the Gap extension cost. On the other hand, if you expect few but large gaps, the Gap open cost should be set significantly higher than the Gap extension cost.

However, for most alignments it is a good idea to make the Gap open cost quite a bit higher than the Gap extension cost. The default values are 10.0 and 1.0 for the two parameters, respectively.

- **End gap cost**. The price of gaps at the beginning or the end of the alignment. One of the advantages of the *CLC Main Workbench* alignment method is that it provides flexibility in the treatment of gaps at the ends of the sequences. There are three possibilities:
  - **Free end gaps**. Any number of gaps can be inserted in the ends of the sequences without any cost.

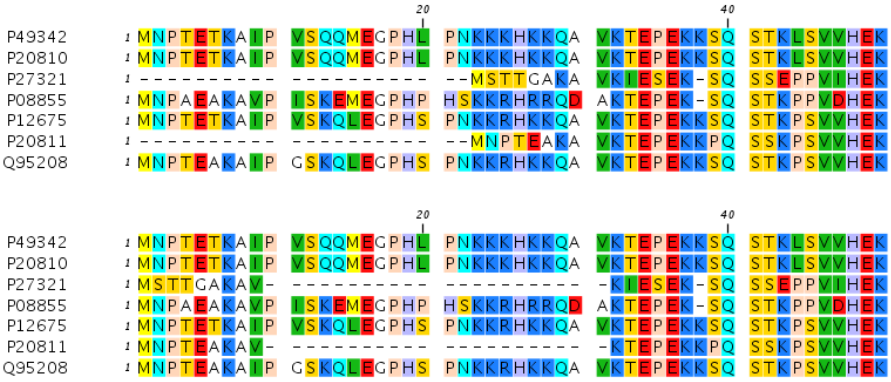
- **Cheap end gaps**. All end gaps are treated as gap extensions and any gaps past 10 are free.
- **End gaps as any other**. Gaps at the ends of sequences are treated like gaps in any other place in the sequences.

When aligning a long sequence with a short partial sequence, it is ideal to use free end gaps, since this will be the best approximation to the situation. The many gaps inserted at the ends are not due to evolutionary events, but rather to partial data.

Many homologous proteins have quite different ends, often with large insertions or deletions. This confuses alignment algorithms, but using the **Cheap end gaps** option, large gaps will generally be tolerated at the sequence ends, improving the overall alignment. This is the default setting of the algorithm.

Finally, treating end gaps like any other gaps is the best option when you know that there are no biologically distinct effects at the ends of the sequences.

Figures 16.3 and 16.4 illustrate the differences between the different gap scores at the sequence ends.



```
                                      20                      40
                                       |                       |
P49342   1 MNPTETKAIP VSQQMEGPHL PNKKKHKKQA VKTEPEKKSQ STKLSVVHEK
P20810   1 MNPTETKAIP VSQQMEGPHL PNKKKHKKQA VKTEPEKKSQ STKLSVVHEK
P27321   1 ---------- ---------- --MSTTGAKA VKIESEK-SQ SSEPPVIHEK
P08855   1 MNPAEAKAVP ISKEMEGPHP HSKKRHRRQD AKTEPEK-SQ STKPPVDHEK
P12675   1 MNPTETKAIP VSKQLEGPHS PNKKRHKKQA VKTEPEKKSQ STKPSVVHEK
P20811   1 ---------- ---------- --MNPTEAKA VKTEPEKKPQ SSKPSVVHEK
Q95208   1 MNPTEAKAIP GSKQLEGPHS PNKKRHKKQA VKTEPEKKSQ STKPSVVHEK

                                      20                      40
                                       |                       |
P49342   1 MNPTETKAIP VSQQMEGPHL PNKKKHKKQA VKTEPEKKSQ STKLSVVHEK
P20810   1 MNPTETKAIP VSQQMEGPHL PNKKKHKKQA VKTEPEKKSQ STKLSVVHEK
P27321   1 MSTTGAKAV- ---------- ---------- -KIESEK-SQ SSEPPVIHEK
P08855   1 MNPAEAKAVP ISKEMEGPHP HSKKRHRRQD AKTEPEK-SQ STKPPVDHEK
P12675   1 MNPTETKAIP VSKQLEGPHS PNKKRHKKQA VKTEPEKKSQ STKPSVVHEK
P20811   1 MNPTEAKAV- ---------- ---------- -KTEPEKKPQ SSKPSVVHEK
Q95208   1 MNPTEAKAIP GSKQLEGPHS PNKKRHKKQA VKTEPEKKSQ STKPSVVHEK
```

Figure 16.3: *The first 50 positions of two different alignments of seven calpastatin sequences. The top alignment is made with cheap end gaps, while the bottom alignment is made with end gaps having the same price as any other gaps. In this case it seems that the latter scoring scheme gives the best result.*

16.1.2 Fast or accurate alignment algorithm

CLC Main Workbench has two algorithms for calculating alignments:

- **Fast (less accurate).** Use an optimized alignment algorithm that is very fast. This is particularly useful for data sets with very long sequences.
- **Slow (very accurate).** The recommended choice unless the processing time is too long.

Both algorithms use progressive alignment. The faster algorithm builds the initial tree by doing more approximate pairwise alignments than the slower option.

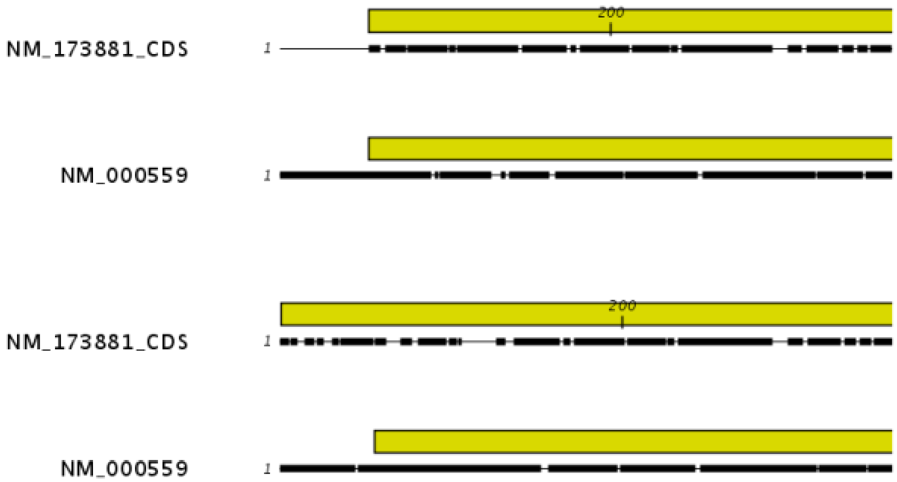


Figure 16.4: *The alignment of the coding sequence of bovine myoglobin with the full mRNA of human gamma globin. The top alignment is made with free end gaps, while the bottom alignment is made with end gaps treated as any other. The yellow annotation is the coding sequence in both sequences. It is evident that free end gaps are ideal in this situation as the start codons are aligned correctly in the top alignment. Treating end gaps as any other gaps in the case of aligning distant homologs where one sequence is partial leads to a spreading out of the short sequence as in the bottom alignment.*

16.1.3 Aligning alignments

The **Redo alignment** option becomes available when an alignment is provided as input to the tool (figure 16.1).

- **Leave this box unchecked** when aligning additional sequences to the original alignment. Equal sized gaps may be inserted in all sequences of the original alignment to accommodate the alignment of the new sequences (figure 16.5), but apart from this, positions in the original alignment are fixed.
- **Check this box** to realign the sequences in the alignment provided as input. This can be useful, for example, if you wish to realign using different gap costs than used originally.

A new alignment is output, whether or not **Redo alignment** option is checked.

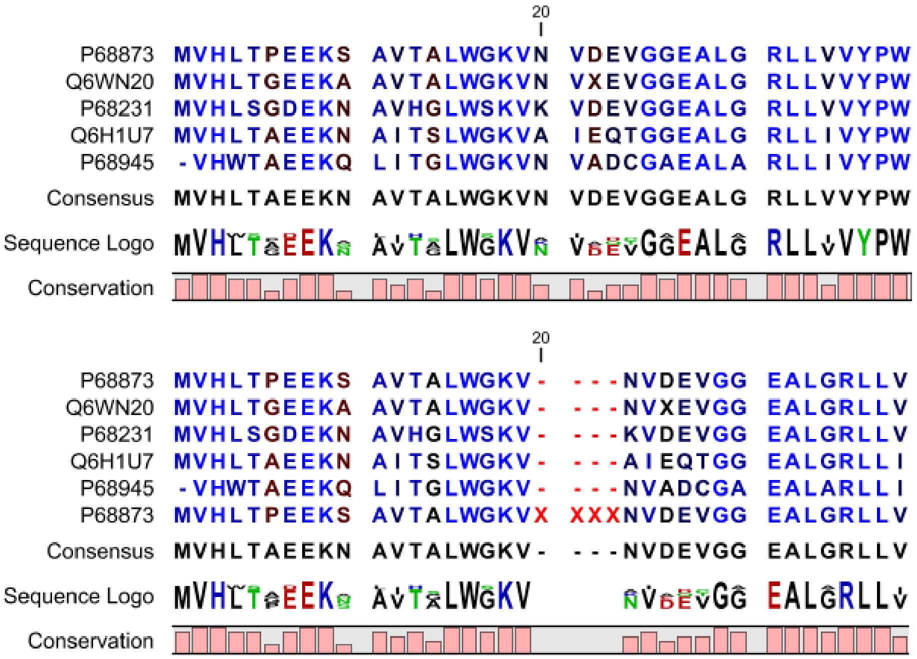
16.1.4 Fixpoints

To force particular regions of an alignment to be aligned to each other, there are two steps:

1. Add fixpoint annotations to these regions.
2. Check the "Use fixpoints" option when launching the Create Alignment tool.

To add a fixpoint, open the sequence or alignment and:

> **Select the region you want to use as a fixpoint | Right-click on the selection | Set Alignment Fixpoint Here**



```
                                        20
                                        |
P68873          MVHLTPEEKS AVTALWGKVN VDEVGGEALG RLLVVYPW
Q6WN20          MVHLTGEEKA AVTALWGKVN VXEVGGEALG RLLVVYPW
P68231          MVHLSGDEKN AVHGLWSKVK VDEVGGEALG RLLVVYPW
Q6H1U7          MVHLTAEEKN AITSLWGKVA IEQTGGEALG RLLIVYPW
P68945          -VHWTAEEKQ LITGLWGKVN VADCGAEALA RLLIVYPW
Consensus       MVHLTAEEKN AVTALWGKVN VDEVGGEALG RLLVVYPW
Sequence Logo
Conservation

                                        20
                                        |
P68873          MVHLTPEEKS AVTALWGKV- ---NVDEVGG EALGRLLV
Q6WN20          MVHLTGEEKA AVTALWGKV- ---NVXEVGG EALGRLLV
P68231          MVHLSGDEKN AVHGLWSKV- ---KVDEVGG EALGRLLV
Q6H1U7          MVHLTAEEKN AITSLWGKV- ---AIEQTGG EALGRLLI
P68945          -VHWTAEEKQ LITGLWGKV- ---NVADCGA EALARLLI
P68873          MVHLTPEEKS AVTALWGKVX XXXNVDEVGG EALGRLLV
Consensus       MVHLTAEEKN AVTALWGKV- ---NVDEVGG EALGRLLV
Sequence Logo
Conservation
```

Figure 16.5: *The original alignment is shown at the top. That alignment and a single additional sequence, with four Xs added for illustrative purposes, were used as input to Create Alignment. The "Redo alignment" option was left unchecked. The resulting alignment is shown at the bottom. Gaps have been added, compared to the original alignment, to accommodate the new sequence. All other positions are aligned as they were in the original alignment.*

This will add an annotation of type "Alignment fixpoint", with name "Fixpoint" to the sequence (figure 16.6).

Regions with fixpoint annotations with the *same name* are aligned to each other. Where there are multiple fixpoints of the same name on sequences, the first fixpoints on each sequence will be aligned to each other, the second on each sequence will be aligned to each other, and so on.

To adjust the name of a fixpoint annotation:

Right-click the Fixpoint annotation | Edit Annotation ( ) | Type the name in the 'Name' field

An example where assigning different names to fixpoints is useful: Given three sequences, A, B and C, where A and B each have one copy of a domain while sequence C has two copies of the domain, you can force sequence A to align to the first copy of the domain in sequence C and sequence B to align to the second copy of the domain in sequence C by naming the fixpoints accordingly. E.g. if the fixpoints in sequence C were named 'fp1' and 'fp2', the fixpoint in sequence A was named 'fp1' and the fixpoint in sequence B was named 'fp2', then when these sequences are aligned using fixpoints, the fixpoint in sequence A would be aligned to the first copy of the domain in sequence C, while the fixpoint in sequence B would be aligned to the second copy of the domain in sequence C.

The result of an alignment using fixpoints is shown in figure 16.7.

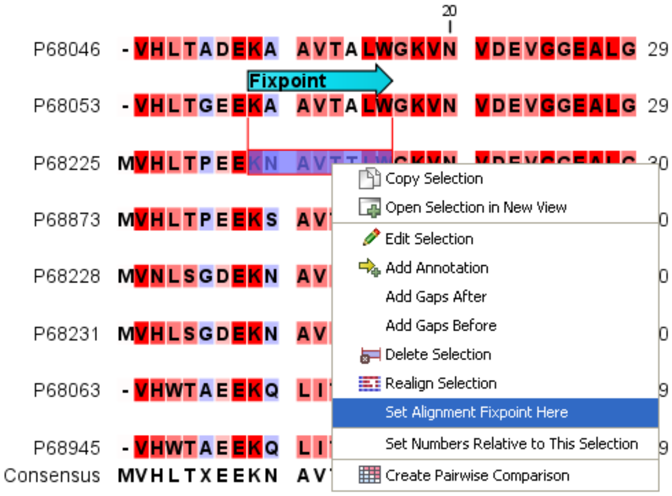


Figure 16.6: *Select a region and right-click on it to see the option to set a fixpoint. The second sequence in the list already has a Fixpoint annotation.*

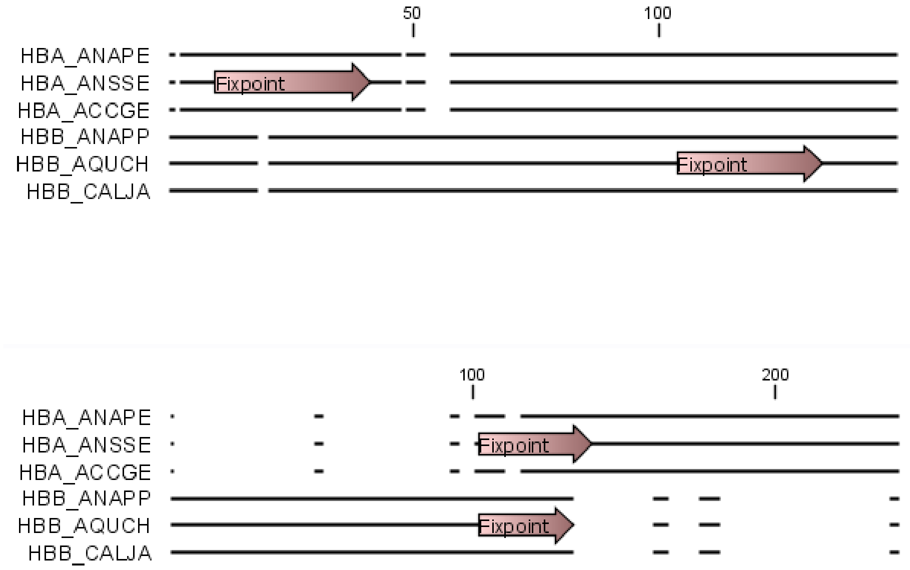


Figure 16.7: *Fixpoints have been added to 2 sequences in an alignment, where the first 3 sequences are very similar to each other and the last 3 sequences are very similar to each other (top). After realigning using just these 2 fixpoints (bottom), the alignment now shows clearly the 2 groups of sequences.*

16.2 View alignments

The basic options for viewing alignments are the same as for viewing sequences, described in section 14.2.

Alignment specific view options in the **Sequence layout**, **Nucleotide info**, **Alignment info**, and **Positional stats** side panel tabs, to the right of the view, are described here.

Sequence layout

In the side panel tab **Sequence layout** the option **Alignments on top** supports moving the aligned sequences relative to other elements shown in the alignment view. When checked, the alignment is shown at the top of the view, when unchecked the alignment is shown underneath other included summary information such as Consensus, Conservation and Sequence logo.

Nucleotide info

In the side panel tab **Nucleotide info** under **Translation**, there is an extra checkbox: **Relative to top sequence**. Checking this box will make the reading frames for the translation align with the top sequence so that you can compare the effect of nucleotide differences on the protein level.

Alignment info

The entire side panel tab **Alignment info** is specific to alignments. Each of the options in the **Alignment info** relate to each column in the alignment.

The data points for graph representations can be exported (see section 8.3).

Consensus Shows a consensus sequence at the bottom of the alignment. The consensus sequence is based on every single position in the alignment and reflects an artificial sequence which resembles the sequence information of the alignment, but only as one single sequence. If all sequences of the alignment is 100% identical the consensus sequence will be identical to all sequences found in the alignment. If the sequences of the alignment differ the consensus sequence will reflect the most common sequences in the alignment. Parameters for adjusting the consensus sequences are described below.

- **Limit** This option determines how conserved the sequences must be in order to agree on a consensus. Here you can also choose **IUPAC** which will display the ambiguity code when there are differences between the sequences. For example, an alignment with **A** and a **G** at the same position will display an **R** in the consensus line if the **IUPAC** option is selected. The IUPAC codes can be found in section G and F. Please note that the IUPAC codes are only available for nucleotide alignments.
- **No gaps** Checking this option will not show gaps in the consensus.
- **Ambiguous symbol** Select how ambiguities should be displayed in the consensus line (as **N**, **?**, *, . or **-**). This option has no effect if **IUPAC** is selected in the **Limit** list above.

The Consensus Sequence can be opened in a new view, simply by right-clicking the Consensus Sequence and click **Open Consensus in New View**.

Conservation Displays the level of conservation at each position in the alignment. The conservation shows the conservation of all sequence positions. The height of the bar, or the gradient of the color reflect how conserved that particular position is in the alignment. If one position is 100% conserved the bar will be shown in full height, and it is colored in the color specified at the right side of the gradient slider.

- **Foreground color** Colors the letters using a gradient, where the right side color is used for highly conserved positions and the left side color is used for positions that are less conserved.
- **Background color.** Sets a background color of the residues using a gradient in the same way as described above.
- **Graph** Displays the conservation level as a graph at the bottom of the alignment. The bar (default view) show the conservation of all sequence positions. The height of the graph reflects how conserved that particular position is in the alignment. If one position is 100% conserved the graph will be shown in full height.
  - **Height** Specifies the height of the graph.
  - **Type** The type of the graph: **Line plot**, **Bar plot**, or **Colors**, in which case the graph is seen as a color bar using a gradient like the foreground and background colors.
  - **Color box** Specifies the color of the graph for line and bar plots, and specifies a gradient for colors.

Gap fraction Which fraction of the sequences in the alignment that have gaps. The gap fraction is only relevant if there are gaps in the alignment.

- **Foreground color** Colors the letter using a gradient, where the left side color is used if there are relatively few gaps, and the right side color is used if there are relatively many gaps.
- **Background color** Sets a background color of the residues using a gradient in the same way as described above.
- **Graph** Displays the gap fraction as a graph at the bottom of the alignment.
  - **Height** Specifies the height of the graph.
  - **Type** The type of the graph: **Line plot**, **Bar plot**, or **Colors**, in which case the graph is seen as a color bar using a gradient like the foreground and background colors.
  - **Color box** Specifies the color of the graph for line and bar plots, and specifies a gradient for colors.

Color different residues Indicates differences in aligned residues.

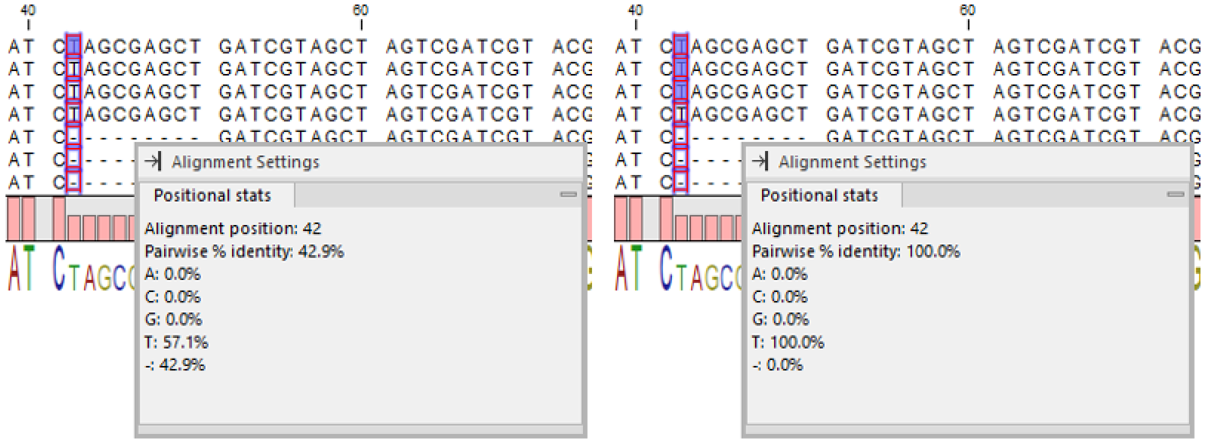
- **Foreground color** Colors the letter.
- **Background color.** Sets a background color of the residues.

Sequence logo A sequence logo displays the frequencies of residues at each position in an alignment. This is presented as the relative heights of letters, along with the degree of sequence conservation as the total height of a stack of letters, measured in bits of information. The vertical scale is in bits, with a maximum of 2 bits for nucleotides and approximately 4.32 bits for amino acid residues. See section 16.2.1 for more details.

- **Foreground color** Color the residues using a gradient according to the information content of the alignment column. Low values indicate columns with high variability whereas high values indicate columns with similar residues.
- **Background color** Sets a background color of the residues using a gradient in the same way as described above.
- **Logo** Displays sequence logo at the bottom of the alignment.
  - **Height** Specifies the height of the sequence logo graph.
  - **Color** The sequence logo can be displayed in black or Rasmol colors. For protein alignments, a polarity color scheme is also available, where hydrophobic residues are shown in black color, hydrophilic residues as green, acidic residues as red and basic residues as blue.

Positional stats

The side panel tab **Positional stats** provides site specific information about the alignment. Hover the mouse cursor over a position in the alignment or make a selection to populate the tab with information (figure 16.8).



Alignment position: 42
Pairwise % identity: 42.9%
A: 0.0%
C: 0.0%
G: 0.0%
T: 57.1%
-: 42.9%

Alignment position: 42
Pairwise % identity: 100.0%
A: 0.0%
C: 0.0%
G: 0.0%
T: 100.0%
-: 0.0%

Figure 16.8: *Contents of the Positional stats tab when a single sequence is selected (Left) and when three sequences are selected (Right). Note that the side panel can be dragged into the alignment view.*

When one position is selected, the information provided is calculated from all the sequences at the position. If more sequences at the same position are selected, the information is calculated for the selected sequences only.

The following information is provided:

- **Alignment position** The selected position in the alignment.
- **Pairwise % identity** Average percent identity. All pairs of bases at the same position are compared. The number of identical pairs is counted and divided by the total number

of pairs. The count of ambiguity characters is scaled to the number of bases they can represent, for example a G compared to an R (A or G) is given the value 0.5.

Example calculation for an alignment with the nucleotides A, A and G in the tested position: There are three pairwise comparisons, A to A = 1, A to G = 0, and A to G = 0. The pairwise % identity is then 1/3.

- **A, C, G, T and -** Percentage of A, C, G, T and "no nucleotide" at the selected position.

16.2.1 Bioinformatics explained: Sequence logo

In the search for homologous sequences, researchers are often interested in conserved sites/residues or positions in a sequence which tend to differ a lot. Most researches use alignments (see Bioinformatics explained*: multiple alignments*) for visualization of homology on a given set of either DNA or protein sequences. In proteins, active sites in a given protein family are often highly conserved. Thus, in an alignment these positions (which are not necessarily located in proximity) are fully or nearly fully conserved. On the other hand, antigen binding sites in the F$_{ab}$ unit of immunoglobulins tend to differ quite a lot, whereas the rest of the protein remains relatively unchanged.

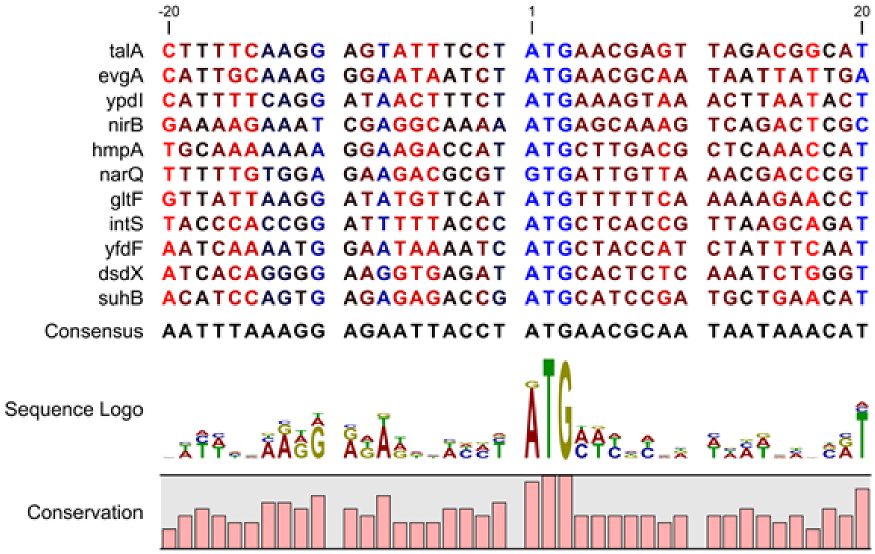
In DNA, promoter sites or other DNA binding sites are highly conserved (see figure 16.9). This is also the case for repressor sites as seen for the Cro repressor of bacteriophage λ.

When aligning such sequences, regardless of whether they are highly variable or highly conserved at specific sites, it is very difficult to generate a consensus sequence which covers the actual variability of a given position. In order to better understand the information content or significance of certain positions, a sequence logo can be used. The sequence logo displays the information content of all positions in an alignment as residues or nucleotides stacked on top of each other (see figure 16.9). The sequence logo provides a far more detailed view of the entire alignment than a simple consensus sequence. Sequence logos can aid to identify protein binding sites on DNA sequences and can also aid to identify conserved residues in aligned domains of protein sequences and a wide range of other applications.

Each position of the alignment and consequently the sequence logo shows the sequence information in a computed score based on Shannon entropy [Schneider and Stephens, 1990]. The height of the individual letters represent the sequence information content in that particular position of the alignment.

A sequence logo is a much better visualization tool than a simple consensus sequence. An example hereof is an alignment where in one position a particular residue is found in 70% of the sequences. If a consensus sequence is used, it typically only displays the single residue with 70% coverage. In figure 16.9 an un-gapped alignment of 11 *E. coli* start codons including flanking regions are shown. In this example, a consensus sequence would only display ATG as the start codon in position 1, but when looking at the sequence logo it is seen that a GTG is also allowed as a start codon.

Calculation of sequence logos A comprehensive walk-through of the calculation of the information content in sequence logos is beyond the scope of this document but can be found in the original paper by [Schneider and Stephens, 1990]. Nevertheless, the conservation of every position is defined as R_{seq} which is the difference between the maximal entropy (S_{max}) and the observed entropy for the residue distribution (S_{obs}),



```
          -20                                1                                20
talA      CTTTTCAAGG  AGTATTTCCT  ATGAACGAGT  TAGACGGCAT
evgA      CATTGCAAAG  GGAATAATCT  ATGAACGCAA  TAATTATTGA
ypdI      CATTTTCAGG  ATAACTTTCT  ATGAAAGTAA  ACTTAATACT
nirB      GAAAAGAAAT  CGAGGCAAAA  ATGAGCAAAG  TCAGACTCGC
hmpA      TGCAAAAAAA  GGAAGACCAT  ATGCTTGACG  CTCAAACCAT
narQ      TTTTTGTGGA  GAAGACGCGT  GTGATTGTTA  AACGACCCGT
gltF      GTTATTAAGG  ATATGTTCAT  ATGTTTTTCA  AAAAGAACCT
intS      TACCCACCGG  ATTTTTACCC  ATGCTCACCG  TTAAGCAGAT
yfdF      AATCAAAATG  GAATAAAATC  ATGCTACCAT  CTATTTCAAT
dsdX      ATCACAGGGG  AAGGTGAGAT  ATGCACTCTC  AAATCTGGGT
suhB      ACATCCAGTG  AGAGAGACCG  ATGCATCCGA  TGCTGAACAT

Consensus AATTTAAAGG  AGAATTACCT  ATGAACGCAA  TAATAAACAT
```

Figure 16.9: *Ungapped sequence alignment of eleven* E. coli *sequences defining a start codon. The start codons start at position 1. Below the alignment is shown the corresponding sequence logo. As seen, a GTG start codon and the usual ATG start codons are present in the alignment. This can also be visualized in the logo at position 1.*

$$R_{seq} = S_{max} - S_{obs} = \log_2 N - \left(- \sum_{n=1}^{N} p_n \log_2 p_n \right)$$

p_n is the observed frequency of a amino acid residue or nucleotide of symbol n at a particular position and N is the number of distinct symbols for the sequence alphabet, either 20 for proteins or four for DNA/RNA. This means that the maximal sequence information content per position is $\log_2 4 = 2\ bits$ for DNA/RNA and $\log_2 20 \approx 4.32\ bits$ for proteins.

The original implementation by Schneider does not handle sequence gaps.

We have slightly modified the algorithm so an estimated logo is presented in areas with sequence gaps.

If amino acid residues or nucleotides of one sequence are found in an area containing gaps, we have chosen to show the particular residue as the fraction of the sequences. Example; if one position in the alignment contain 9 gaps and only one alanine (A) the A represented in the logo has a hight of 0.1.

Other useful resources

The website of Tom Schneider
http://www-lmmb.ncifcrf.gov/~toms/

WebLogo
http://weblogo.berkeley.edu/

[Crooks et al., 2004]

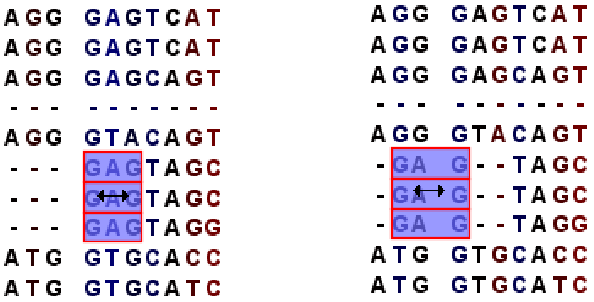
16.3 Edit alignments

Move residues and gaps The placement of gaps in the alignment can be changed by modifying the parameters when creating the alignment (see section 16.1). However, gaps and residues can also be moved after the alignment is created:

> **select one or more gaps or residues in the alignment | drag the selection to move**

This can be done both for single sequences, but also for multiple sequences by making a selection covering more than one sequence. When you have made the selection, the mouse pointer turns into a horizontal arrow indicating that the selection can be moved (see figure 16.10).

Note! Residues can only be moved when they are next to a gap.



```
AGG GAGTCAT
AGG GAGTCAT
AGG GAGCAGT
--- -------
AGG GTACAGT
--- GAGTAGC
--- GAGTAGC
--- GAGTAGG
ATG GTGCACC
ATG GTGCATC
```

```
AGG GAGTCAT
AGG GAGTCAT
AGG GAGCAGT
--- -------
AGG GTACAGT
-GA G--TAGC
-GA G--TAGC
-GA G--TAGG
ATG GTGCACC
ATG GTGCATC
```

Figure 16.10: *Moving a part of an alignment. Notice the change of mouse pointer to a horizontal arrow.*

Insert gaps The placement of gaps in the alignment can be changed by modifying the parameters when creating the alignment. However, gaps can also be added manually after the alignment is created.

To insert extra gaps:

> **select a part of the alignment | right-click the selection | Add gaps before/after**

If you have made a selection covering five residues for example, a gap of five will be inserted. In this way you can easily control the number of gaps to insert. Gaps will be inserted in the sequences that you selected. If you make a selection in two sequences in an alignment, gaps will be inserted into these two sequences. This means that these two sequences will be displaced compared to the other sequences in the alignment.

Delete residues and gaps Residues or gaps can be deleted for individual sequences or for the whole alignment. For individual sequences:

> **select the part of the sequence you want to delete | right-click the selection | Edit Selection (✎) | Delete the text in the dialog | Replace**

The selection shown in the dialog will be replaced by the text you enter. If you delete the text, the selection will be replaced by an empty text, i.e. deleted.

In order to delete entire columns:

> **manually select the columns to delete | right-click the selection | click 'Delete Selection'**

Copy annotations to other sequences Annotations on one sequence can be transferred to other sequences in the alignment:

right-click the annotation | Copy Annotation to other Sequences

This will display a dialog listing all the sequences in the alignment. Next to each sequence is a checkbox which is used for selecting which sequences the annotation should be copied to. Click **Copy** to copy the annotation.

If you wish to copy all annotations on the sequence, click the **Copy All Annotations to other Sequences**.

Copied/transferred annotations will contain the same qualifier text as the original, i.e., the text is not updated. As an example, if the annotation contains 'translation' as qualifier text, this translation will be copied to the new sequence and will thus reflect the translation of the original sequence, and not the new sequence which may differ.

Move sequences up and down Sequences can be moved up and down in the alignment:

drag the name of the sequence up or down

When you move the mouse pointer over the label, the pointer will turn into a vertical arrow indicating that the sequence can be moved.

The sequences can also be sorted automatically to let you save time moving the sequences around. To sort the sequences alphabetically:

Right-click the name of a sequence | Sort Sequences Alphabetically

If you change the Sequence name (in the **Sequence Layout** view preferences), you will have to ask the program to sort the sequences again.

If you have one particular sequence that you would like to use as a reference sequence, it can be useful to move this to the top. This can be done manually, but it can also be done automatically:

Right-click the name of a sequence | Move Sequence to Top

The sequences can also be sorted by similarity, grouping similar sequences together:

Right-click the name of a sequence | Sort Sequences by Similarity

Delete, rename and add sequences Sequences can be removed from the alignment by right-clicking the label of a sequence:

right-click label | Delete Sequence

If you wish to delete several sequences, you can check all the sequences, right-click and choose **Delete Marked Sequences**. To show the checkboxes, you first have to click the **Show Selection Boxes** in the **Side Panel**.

A sequence can also be renamed:

right-click label | Rename Sequence

This will show a dialog, letting you rename the sequence. This will not affect the sequence that the alignment is based on.

Extra sequences can be added to the alignment by creating a new alignment where you select the current alignment and the extra sequences (see section 16.1).

The same procedure can be used for joining two alignments.

16.3.1 Realignment

This section describes realigning parts of an existing alignment. To realign an entire alignment, consider using the "Redo alignment" option of the **Create Alignment** tool, described in section 16.1.3

Examples where realigning part of an alignment can be helpful include:

- **Adjusting the number of gaps** If a region has more gaps than is useful, select the region of interest and realign using a higher gap cost.
- **Combine with fixpoints** When you have an alignment where two residues are not aligned although they should have been, you can set an alignment fixpoint on each of those residues. and then realign the section of interest using those fixpoints, as described in section 16.1.4. This should result in the two residues being aligned, and everything in the selected region around them being adjusted to accommodate that change.

Realigning a subsection of an alignment

There are two steps to realigning a subsection of an alignment:

1. Select the region of interest.
2. Realign the selected region.

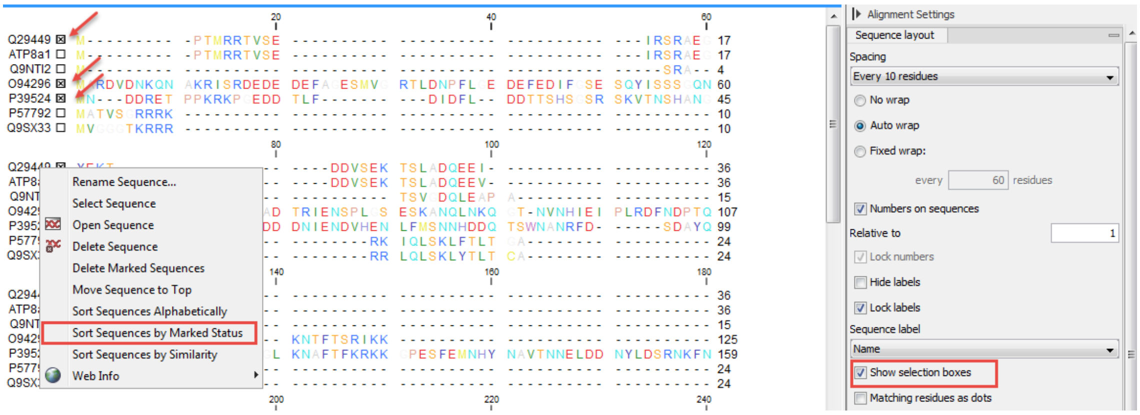
Selecting a region

Click and drag to select the regions of interest. For small regions in a small number of sequences, this may be easiest while zoomed in fully, such that each residue is visible. For realigning entire sequences, zooming out fully may be helpful.

As selection involves clicking and dragging the mouse, all regions of interest must be contiguous. That is, you must be able to drag over the relevant regions in a single motion. This may mean gathering particular sequences into a block. There are two ways to achieve this:

1. Click on the name of an individual sequence and drag it to the desired location in the alignment. Do this with each relevant sequence until all those of interest are placed as desired.
2. Check the option "Show selection boxes" in the Alignment settings section of the side panel settings (figure 16.11). Click in the checkbox next to the names of the sequences you wish to select. Then right-click on the name of one of the sequences and choose the option "Sort Sequences by Marked Status". This will bring all selected sequences to the top of the alignment.

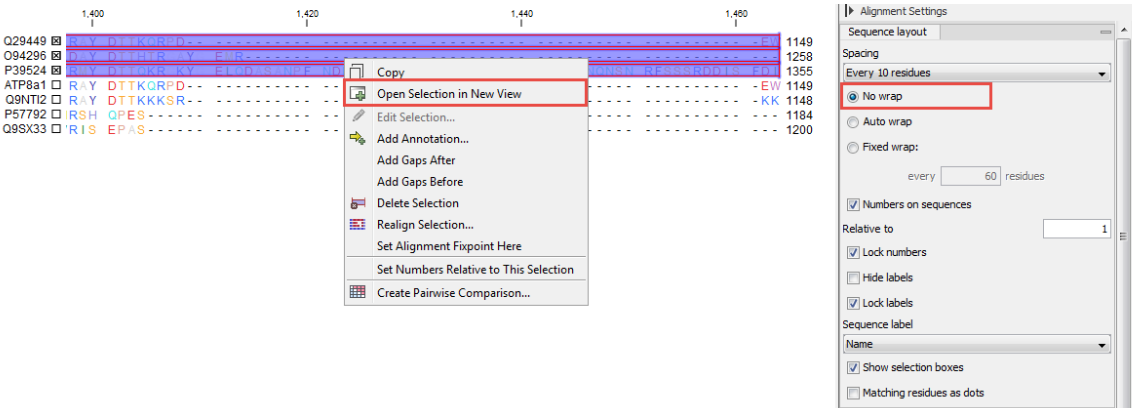
If you have many sequences to select, it can be easiest to select the few that are not of interest, and then invert the selection by right-clicking on any of the checkboxes and choosing the option "Invert All Marks".



Spacing: Every 10 residues
○ No wrap
● Auto wrap
○ Fixed wrap: every 60 residues
☑ Numbers on sequences
Relative to 1
☑ Lock numbers
☐ Hide labels
☑ Lock labels
Sequence label: Name
☑ Show selection boxes
☐ Matching residues as dots

Figure 16.11: *Marking and sorting sequences by marked status in an alignment*

You can then easily click-and-drag your selection of sequences (this is made easier if you select the "No wrap" setting in the right-hand side panel). By right-clicking on the selected sequences (not on their names, but on the sequences themselves as seen in figure 16.12), you can choose the option "Open selection in a new view", with the ability to run any relevant tool on that sub-alignment.



Spacing: Every 10 residues
● No wrap
○ Auto wrap
○ Fixed wrap: every 60 residues
☑ Numbers on sequences
Relative to 1
☑ Lock numbers
☐ Hide labels
☑ Lock labels
Sequence label: Name
☑ Show selection boxes
☐ Matching residues as dots

Figure 16.12: *Open the selected sequences in a new window to realign them.*

Realign the selected region

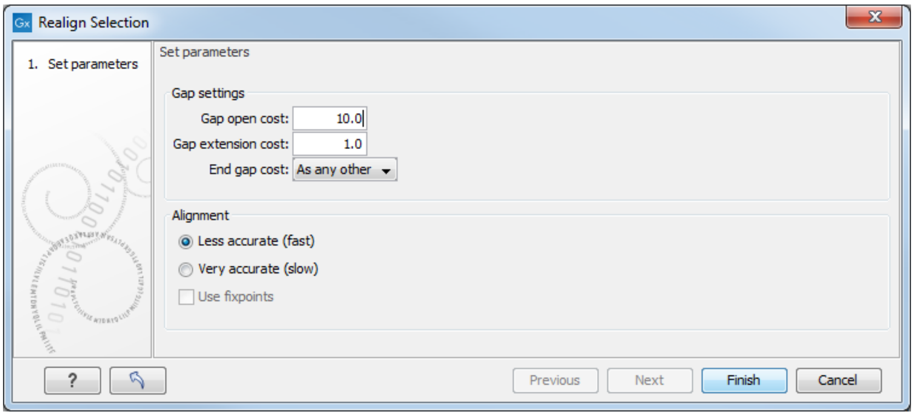
To realign the selected region:

Right-click the selection | Choose the option "Realign selection"

This will open a window allowing you to set the parameters for the realignment (see figure 16.13).

It is possible for an alignment to become shorter or longer as a result of the realignment of a region. This is because gaps may have to be inserted in, or deleted from, the sequences not selected for realignment. This will only occur for entire columns of gaps in these sequences, ensuring that their relative alignment is unchanged.

Learn more about the options available to you in the sections 16.1.1 and 16.1.2.



1. Set parameters

Set parameters

Gap settings

Gap open cost: 10.0

Gap extension cost: 1.0

End gap cost: As any other

Alignment

● Less accurate (fast)

○ Very accurate (slow)

□ Use fixpoints

Figure 16.13: *Options available when realigning a subset of an alignment.*

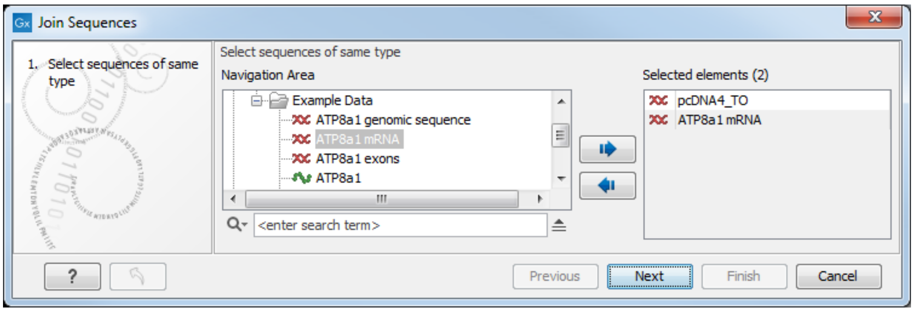
16.4 Join alignments

CLC Main Workbench can join several alignments into one. This feature can for example be used to construct "supergenes" for phylogenetic inference by joining alignments of several disjoint genes into one spliced alignment. Note, that when alignments are joined, all their annotations are carried over to the new spliced alignment.

Alignments can be joined using the **Join Alignments** tool, available at:

Tools | Alignments and Trees ( )| Join Alignments ( )

This opens the dialog shown in figure 16.14.



1. Select sequences of same type

Select sequences of same type

Navigation Area

Example Data

ATP8a1 genomic sequence

ATP8a1 mRNA

ATP8a1 exons

ATP8a1

<enter search term>

Selected elements (2)

pcDNA4_TO

ATP8a1 mRNA

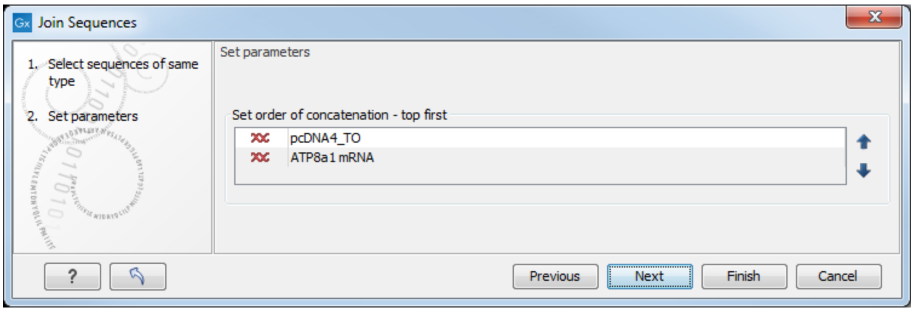
Figure 16.14: *Selecting two alignments to be joined.*

If you have selected some alignments before launching the tool, they will be pre-selected in the **Selected Elements** window of the dialog. Use the arrows to add or remove alignments from the selected elements. In this example seven alignments are selected. Each alignment represents one gene that have been sequenced from five different bacterial isolates from the genus Nisseria. Clicking **Next** opens the dialog shown in figure 16.15.

To adjust the order of concatenation, click the name of one of the alignments, and move it up or down using the arrow buttons.

The result is seen in the lower part of figure 16.16.

How alignments are joined Alignments are joined by considering the sequence names in the individual alignments. If two sequences from different alignments have identical names, they are considered to have the same origin and are thus joined. Consider the joining of the alignments



Join Sequences

1. Select sequences of same type
2. Set parameters

Set parameters

Set order of concatenation - top first

pcDNA4_TO
ATP8a1 mRNA

Figure 16.15: *Selecting order of concatenation.*

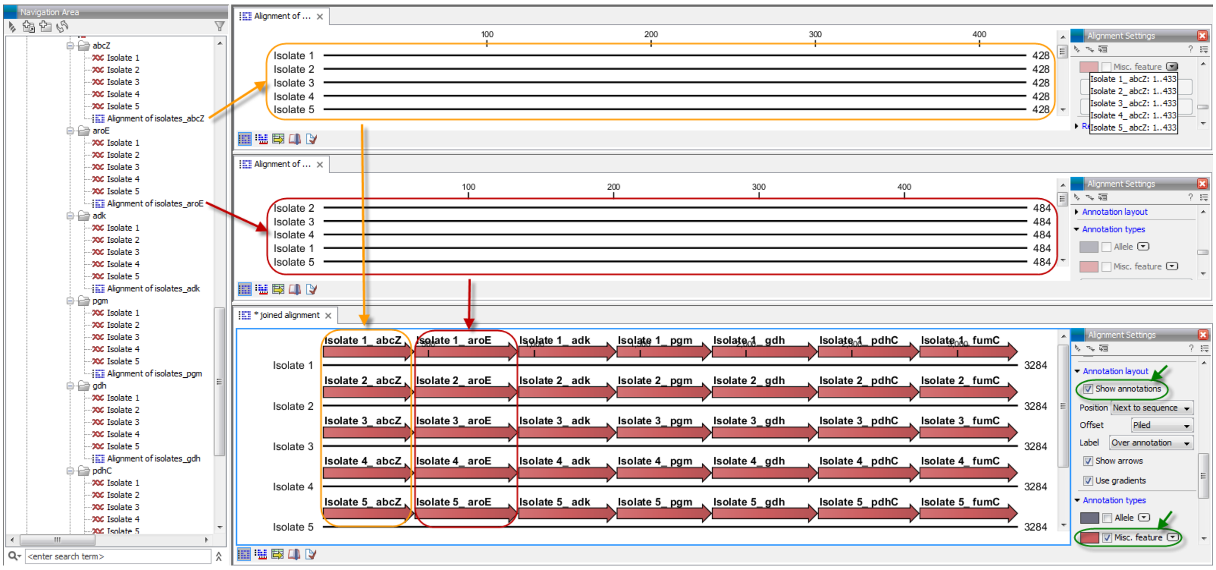


Figure 16.16: *The upper part of the figure shows two of the seven alignments for the genes "abcZ" and "aroE" respectively. Each alignment consists of sequences from one gene from five different isolates. The lower part of the figure shows the result of "Join Alignments". Seven genes have been joined to an artificial gene fusion, which can be useful for construction of phylogenetic trees in cases where only fractions of a genome is available. Joining of the alignments results in one row for each isolate consisting of seven fused genes. Each fused gene sequence corresponds to the number of uniquely named sequences in the joined alignments.*

shown in figure 16.16 "Alignment of isolates_abcZ", "Alignment of isolates_aroE", "Alignment of isolates_adk" etc. If a sequence with the same name is found in the different alignments (in this case the name of the isolates: Isolate 1, Isolate 2, Isolate 3, Isolate 4, and Isolate 5), a joined alignment will exist for each sequence name. In the joined alignment the selected alignments will be fused with each other in the order they were selected (in this case the seven different genes from the five bacterial isolates). Note that annotations have been added to each individual sequence before aligning the isolates for one gene at the time in order to make it clear which sequences were fused to each other.

16.5 Pairwise comparison

For a given set of aligned sequences it is possible to make a pairwise comparison in which each pair of sequences are compared to each other. This provides an overview of the diversity among the sequences in the alignment.

In *CLC Main Workbench* this is done by creating a comparison table:

Tools | Alignments and Trees ( )| Create Pairwise Comparison ( )

This opens the dialog displayed in figure 16.17:

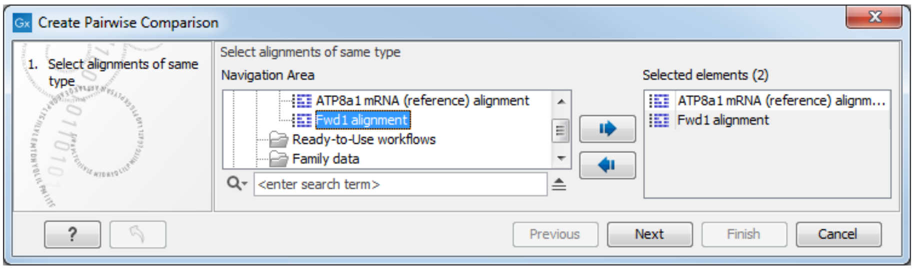
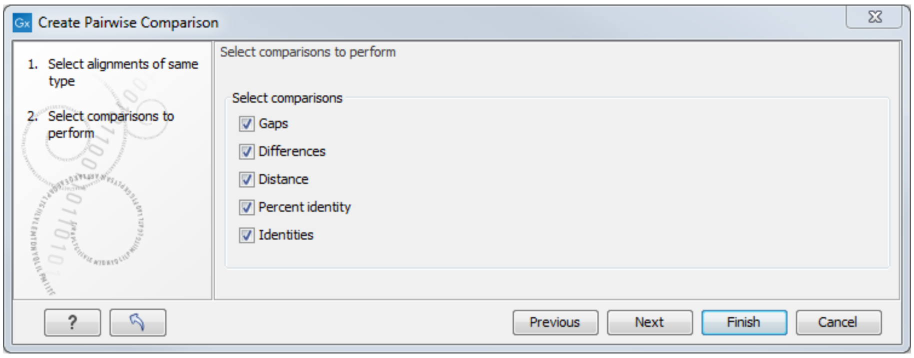

Create Pairwise Comparison
1. Select alignments of same type
Select alignments of same type
Navigation Area
ATP8a1 mRNA (reference) alignment
Fwd1 alignment
Ready-to-Use workflows
Family data
<enter search term>
Selected elements (2)
ATP8a1 mRNA (reference) alignm...
Fwd1 alignment
?
Previous
Next
Finish
Cancel

Figure 16.17: *Creating a pairwise comparison table.*

Select at least two alignments alignment to compare. A pairwise comparison can also be performed for a selected part of an alignment:

right-click on an alignment selection | Pairwise Comparison ( )

There are five kinds of comparison that can be made between the sequences in the alignment, as shown in figure 16.18.


Create Pairwise Comparison
1. Select alignments of same type
2. Select comparisons to perform
Select comparisons to perform
Select comparisons
?
Previous
Next
Finish
Cancel

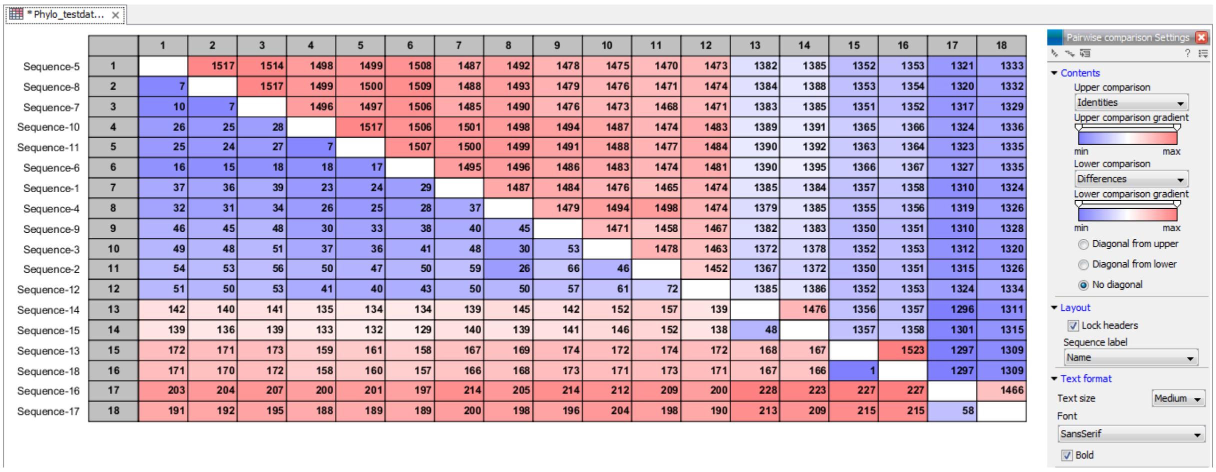
☑ Gaps
☑ Differences
☑ Distance
☑ Percent identity
☑ Identities

Figure 16.18: *Adjusting parameters for pairwise comparison.*

- **Gaps** Calculates the number of alignment positions where one sequence has a gap and the other does not.
- **Identities** Calculates the number of identical alignment positions to overlapping alignment positions between the two sequences. An overlapping alignment position is a position where at least one residue is present, rather than only gaps.
- **Differences** Calculates the number of alignment positions where one sequence is different from the other. This includes gap differences as in the Gaps comparison.
- **Distance** Calculates the Jukes-Cantor distance between the two sequences. This number is given as the Jukes-Cantor correction of the proportion between identical and overlapping alignment positions between the two sequences.
- **Percent identity** Calculates the percentage of identical residues in alignment positions to overlapping alignment positions between the two sequences.

16.5.1 The pairwise comparison table

The table shows the results of selected comparisons (see an example in figure 16.19). Since comparisons are often symmetric, the table can show the results of two comparisons at the same time, one in the upper-right and one in the lower-left triangle.



*Phylo_testdat...

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Sequence-5	1		1517	1514	1498	1499	1508	1487	1492	1478	1475	1470	1473	1382	1385	1352	1353	1321	1333
Sequence-8	2	7		1517	1499	1500	1509	1488	1493	1479	1476	1471	1474	1384	1388	1353	1354	1320	1332
Sequence-7	3	10	7		1496	1497	1506	1485	1490	1476	1473	1468	1471	1383	1385	1351	1352	1317	1329
Sequence-10	4	26	25	28		1517	1506	1501	1498	1494	1487	1474	1483	1389	1391	1365	1366	1324	1336
Sequence-11	5	25	24	27	7		1507	1500	1499	1491	1488	1477	1484	1390	1392	1363	1364	1323	1335
Sequence-6	6	16	15	18	18	17		1495	1496	1486	1483	1474	1481	1390	1395	1366	1367	1327	1335
Sequence-1	7	37	36	39	23	24	29		1487	1484	1476	1465	1474	1385	1384	1357	1358	1310	1324
Sequence-4	8	32	31	34	26	25	28	37		1479	1494	1498	1474	1379	1385	1355	1356	1319	1326
Sequence-9	9	46	45	48	30	33	38	40	45		1471	1458	1467	1382	1383	1350	1351	1310	1328
Sequence-3	10	49	48	51	37	36	41	48	30	53		1478	1463	1372	1378	1352	1353	1312	1320
Sequence-2	11	54	53	56	50	47	50	59	26	66	46		1452	1367	1372	1350	1351	1315	1326
Sequence-12	12	51	50	53	41	40	43	50	50	57	61	72		1385	1386	1352	1353	1324	1334
Sequence-14	13	142	140	141	135	134	134	139	145	142	152	157	139		1476	1356	1357	1296	1311
Sequence-15	14	139	136	139	133	132	129	140	139	141	146	152	138	48		1357	1358	1301	1315
Sequence-13	15	172	171	173	159	161	158	167	169	174	172	174	172	168	167		1523	1297	1309
Sequence-18	16	171	170	172	158	160	157	166	168	173	171	173	171	167	166	1		1297	1309
Sequence-16	17	203	204	207	200	201	197	214	205	214	212	209	200	228	223	227	227		1466
Sequence-17	18	191	192	195	188	189	189	200	198	196	204	198	190	213	209	215	215	58	

Pairwise comparison Settings

Contents

Upper comparison: Identities

Upper comparison gradient

Lower comparison: Differences

Lower comparison gradient

○ Diagonal from upper
○ Diagonal from lower
● No diagonal

Layout

☑ Lock headers

Sequence label: Name

Text format

Text size: Medium

Font: SansSerif

☑ Bold

Figure 16.19: *A pairwise comparison table.*

Note that you can change the minimum and maximum values of the gradient coloring by sliding the corresponding cursor along the gradient in the right side panel of the comparison table. The values that appears when you slide the cursor reflect the percentage of the range of values in the table, and not absolute values.

The following settings are present in the side panel:

- **Contents**
  - **Upper comparison** Selects the comparison to show in the upper triangle of the table.
  - **Upper comparison gradient** Selects the color gradient to use for the upper triangle.
  - **Lower comparison** Selects the comparison to show in the lower triangle. Choose the same comparison as in the upper triangle to show all the results of an asymmetric comparison.
  - **Lower comparison gradient** Selects the color gradient to use for the lower triangle.
  - **Diagonal from upper** Use this setting to show the diagonal results from the upper comparison.
  - **Diagonal from lower** Use this setting to show the diagonal results from the lower comparison.
  - **No Diagonal.** Leaves the diagonal table entries blank.
- **Layout**
  - **Lock headers** Locks the sequence labels and table headers when scrolling the table.
  - **Sequence label** Changes the sequence labels.
- **Text format**
  - **Text size** Changes the size of the table and the text within it.

- **Font** Changes the font in the table.
- **Bold** Toggles the use of boldface in the table.

16.5.2 Bioinformatics explained: Multiple alignments

Multiple alignments are at the core of bioinformatical analysis. Often the first step in a chain of bioinformatical analyses is to construct a multiple alignment of a number of homologs DNA or protein sequences. However, despite their frequent use, the development of multiple alignment algorithms remains one of the algorithmically most challenging areas in bioinformatical research.

Constructing a multiple alignment corresponds to developing a hypothesis of how a number of sequences have evolved through the processes of character substitution, insertion and deletion. The input to multiple alignment algorithms is a number of homologous sequences, i.e., sequences that share a common ancestor and most often also share molecular function. The generated alignment is a table (see figure 16.20) where each row corresponds to an input sequence and each column corresponds to a position in the alignment. An individual column in this table represents residues that have all diverged from a common ancestral residue. Gaps in the table (commonly represented by a '-') represent positions where residues have been inserted or deleted and thus do not have ancestral counterparts in all sequences.

Use of multiple alignments

Once a multiple alignment is constructed it can form the basis for a number of analyses:

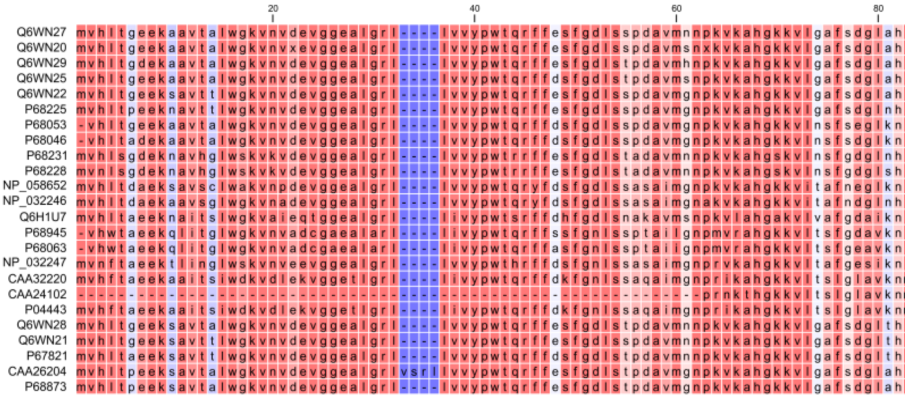
- The phylogenetic relationship of the sequences can be investigated by tree-building methods based on the alignment.
- Annotation of functional domains, which may only be known for a subset of the sequences, can be transferred to aligned positions in other un-annotated sequences.
- Conserved regions in the alignment can be found which are prime candidates for holding functionally important sites.
- Comparative bioinformatical analysis can be performed to identify functionally important regions.

Constructing multiple alignments

Whereas the optimal solution to the pairwise alignment problem can be found in reasonable time, the problem of constructing a multiple alignment is much harder.

The first major challenge in the multiple alignment procedure is how to rank different alignments, i.e., which *scoring function* to use. Since the sequences have a shared history they are correlated through their *phylogeny* and the scoring function should ideally take this into account. Doing so is, however, not straightforward as it increases the number of model parameters considerably. It is therefore commonplace to either ignore this complication and assume sequences to be unrelated, or to use heuristic corrections for shared ancestry.

The second challenge is to find the optimal alignment given a scoring function. For pairs of sequences this can be done by *dynamic programming* algorithms, but for more than three sequences this approach demands too much computer time and memory to be feasible.



```
                             20                  40                  60                  80
   Q6WN27 mvhltgeekaavtalwgkvnvdevggealgrl----lvvypwtqrffesfgdlsspdavmnnpkvkahgkkvlgafsdglah
   Q6WN20 mvhltgeekaavtalwgkvnvxevggealgrl----lvvypwtqrffesfgdlsspdavmsnxkvkahgkkvlgafsdglah
   Q6WN29 mvhltgdekaavtalwgkvnvdevggealgrl----lvvypwtqrffesfgdlstpdavmhnpkvkahgkkvlgafsdglah
   Q6WN25 mvhltgeekaavtalwgkvnvdevggealgrl----lvvypwtqrffdsfgdlstpdavmsnpkvkahgkkvlgafsdglah
   Q6WN22 mvhltgeeksavttlwgkvnvdevggealgrl----lvvypwtqrffesfgdlsspdavmgnpkvkahgkkvlgafsdglah
   P68225 mvhltpeeknavttlwgkvnvdevggealgrl----lvvypwtqrffesfgdlsspdavmgnpkvkahgkkvlgafsdglnh
   P68053 -vhltgeekaavtalwgkvnvdevggealgrl----lvvypwtqrffdsfgdlsspdavmgnpkvkahgkkvlnsfseglkn
   P68046 -vhltadekaavtalwgkvnvdevggealgrl----lvvypwtqrffdsfgdlsspdavmgnpkvkahgkkvlnsfsdglkn
   P68231 mvhlsgdeknavhglwskvkvdevggealgrl----lvvypwtrrffesfgdlstadavmnnpkvkahgskvlnsfgdglnh
   P68228 mvnlsgdeknavhglwskvkvdevggealgrl----lvvypwtrrffesfgdlstadavmnnpkvkahgskvlnsfgdglsh
NP_058652 mvhltdaeksavsclwakvnpdevggealgrl----lvvypwtqryfdsfgdlssasaimgnpkvkahgkkvitafneglkn
NP_032246 mvhltdaekaavsglwgkvnadevggealgrl----lvvypwtqryfdsfgdlssasaimgnakvkahgkkvitafndglnh
   Q6H1U7 mvhltaeeknaitslwgkvaieqtggealgrl----livypwtsrffdhfgdlsnakavmsnpkvlahgakvlvafgdaikn
   P68945 -vhwtaeekqlitglwgkvnvadcgaealarl----livypwtqrffssfgnlssptaillgnpmvrahgkkvltsfgdavkn
   P68063 -vhwtaeekqlitglwgkvnvadcgaealarl----livypwtqrffasfgnlssptaiignpmvrahgkkvltsfgeavkn
NP_032247 mvnftaeektlinglwskvnveevggealgrl----lvvypwthrffdsfgnlssasaimgnprvkahgkkvltafgesikn
 CAA32220 mvhftaeekaaitsiwdkvdlekvggetlgrl----livypwtqrffdkfgnlssaqaimgnprikahgkkvltslglavkn
 CAA24102 --------------------------------------------------------------prnkthgkkvltslglavkn
   P04443 mvhftaeekaaitsiwdkvdlekvggetlgrl----livypwtqrffdkfgnlssaqaimgnprikahgkkvltslglavkn
   Q6WN28 mvhltgeeksavtalwgkvnvdevggealgrl----lvvypwtqrffesfgdlstpdavmnnpkvkahgkkvlgafsdglth
   Q6WN21 mvhltgeeksavttlwgkvnvdevggealgrl----lvvypwtqrffesfgdlsspdavmnnpkvkahgkkvlgafsdglth
   P67821 mvhltaeeksavttlwgkvnvdevggealgrl----lvvypwtqrffdsfgdlstpdavmnnpkvkahgkkvlgafsdglth
 CAA26204 mvhltpeeksavtalwgkvnvdevggealgrlvsrllvvypwtqrffesfgdlstpdavmgnpkvkahgkkvlgafsdglah
   P68873 mvhltpeeksavtalwgkvnvdevggealgrl----lvvypwtqrffesfgdlstpdavmgnpkvkahgkkvlgafsdglah
```

Figure 16.20: *The tabular format of a multiple alignment of 24 Hemoglobin protein sequences. Sequence names appear at the beginning of each row and the residue position is indicated by the numbers at the top of the alignment columns. The level of sequence conservation is shown on a color scale with blue residues being the least conserved and red residues being the most conserved.*

A commonly used approach is therefore to do *progressive alignment* [Feng and Doolittle, 1987] where multiple alignments are built through the successive construction of pairwise alignments. These algorithms provide a good compromise between time spent and the quality of the resulting alignment

The method has the inherent drawback that once two sequences are aligned, there is no way of changing their relative alignment based on the information that additional sequences may contribute later in the process. It is therefore important to make the best possible alignments early in the procedure, to avoid accumulating errors. To accomplish this, a tree of the sequences is usually constructed to guide the progressive alignment algorithm. And to overcome the problem of a time consuming tree construction step, we are using word matching, a method that group sequences in a very efficient way, saving much time, without reducing the resulting alignment accuracy significantly.

Our algorithm (developed by QIAGEN Aarhus) has two speed settings: "standard" and "fast". The **standard method** makes a fairly standard progressive alignment using the fast method of generating a guide tree. When aligning two alignments to each other, two matching columns are scored as the average of all the pairwise scores of the residues in the columns. The gap cost is affine, allowing a different cost for the first gapped position and for the consecutive gaps. This ensures that gaps are not spread out too much.

The **fast method** of alignment uses the same overall method, except that it uses fixpoints in the alignment algorithm based on short subsequences that are identical in the sequences that are being aligned. This allows similar sequences to be aligned much more efficiently, without reducing accuracy very much.

Chapter 17

Phylogenetic trees

Contents

Phylogenetics describes the taxonomic classification of organisms based on their evolutionary history i.e. their phylogeny. Phylogenetics is therefore an integral part of the science of systematics that aims to establish the phylogeny of organisms based on their characteristics. Furthermore, phylogenetics is central to evolutionary biology as a whole as it is the condensation of the overall paradigm of how life arose and developed on earth. The focus of this module is the reconstruction and visualization of phylogenetic trees. Phylogenetic trees illustrate the inferred evolutionary history of a set of organisms, and makes it possible to e.g. identify groups of closely related organisms and observe clustering of organisms with common traits. See 17.4.1 for a more detailed introduction to phylogenetic trees.

The viewer for visualizing and working with phylogenetic trees allows the user to create high-quality, publication-ready figures of phylogenetic trees. Large trees can be explored in two alternative tree layouts; circular and radial. The viewer supports importing, editing and visualization of metadata associated with nodes in phylogenetic trees.

Below is an overview of the main features of the phylogenetic tree editor. Further details can be found in the subsequent sections.

Main features of the phylogenetic tree editor:

- Circular and radial layouts.
- Import of metadata in Excel and CSV format.
- Tabular view of metadata with support for editing.
- Options for collapsing nodes based on bootstrap values.
- Re-ordering of tree nodes.
- Legends describing metadata.
- Visualization of metadata though e.g. node color, node shape, branch color, etc.
- Minimap navigation.
- Coloring and labeling of subtrees.
- Curved edges.
- Editable node sizes and line width.
- Intelligent visualization of overlapping labels and nodes.

For a given set of aligned sequences (see section 16.1) it is possible to infer their evolutionary relationships. In *CLC Main Workbench* this may be done either by using a distance based method or by using maximum likelihood (ML) estimation, which is a statistical approach (see Bioinformatics explained in section 17.4.1). Both approaches generate a phylogenetic tree.

Three tools are available for generating phylogenetic trees:

- **K-mer Based Tree Construction** ( ) Is a distance-based method that can create trees based on multiple single sequences. K-mers are used to compute distance matrices for distance-based phylogenetic reconstruction tools such as neighbor joining and UPGMA (see section 17.4.1). This method is less precise than the Create Tree tool but it can cope with a very large number of long sequences as it does not require a multiple alignment. The k-mer based tree construction tool is especially useful for whole genome phylogenetic reconstruction where the genomes are closely releated, i.e. they differ mainly by SNPs and contain no or few structural variations.
- **Maximum Likelihood Phylogeny** ( ) The most advanced and time consuming method of the three mentioned. The maximum likelihood tree estimation is performed under the assumption of one of five substitution models: the Jukes-Cantor, the Kimura 80, the HKY and the GTR (also known as the REV model) models (see section 17.4 for further information

about the models). Prior to using the Maximum Likelihood Phylogeny tool for creating a phylogenetic tree it is recommended to run the Model Testing tool (see section 17.3) in order to identify the best suitable models for creating a tree.

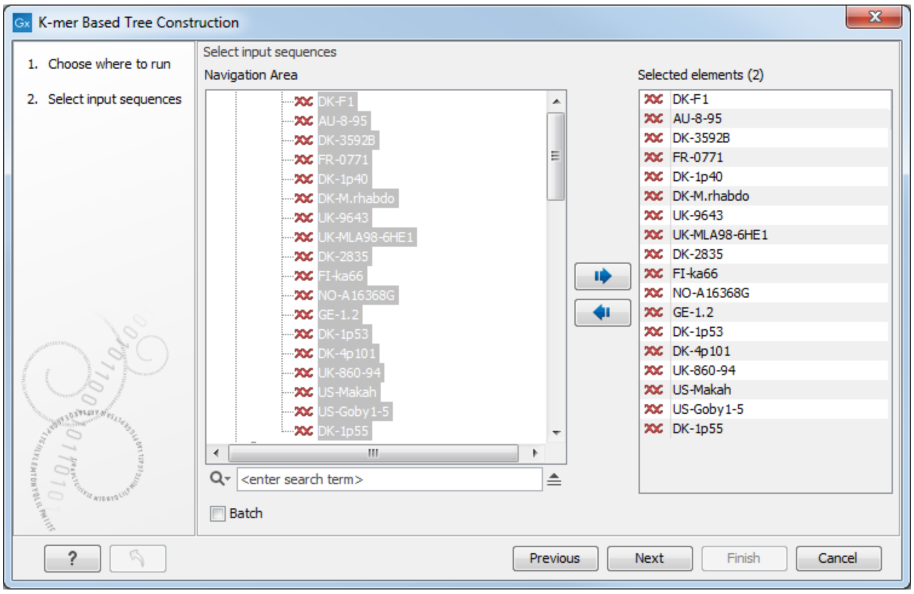
- **Create Tree** ( ) Is a tool that uses distance estimates computed from a multiple sequence alignment to create a tree. The user can select whether to use Jukes-Cantor distance correction or Kimura distance correction (Kimura 80 for nucleotides/Kimura protein for proteins) in combination with either the neighbor joining or UPGMA method (see section 17.4.1).

17.1 K-mer Based Tree Construction

The K-mer Based Tree Construction tool uses single sequences or sequence lists as input and is the simplest way of creating a distance-based phylogenetic tree. To run the K-mer Based Tree Construction tool:

Tools | Alignments and Trees ( )| K-mer Based Tree Construction ( )

Select sequences or a sequence list (figure 17.1):



K-mer Based Tree Construction

1. Choose where to run
2. Select input sequences

Select input sequences

Navigation Area

DK-F1
AU-8-95
DK-3592B
FR-0771
DK-1p40
DK-M.rhabdo
UK-9643
UK-MLA98-6HE1
DK-2835
FI-ka66
NO-A16368G
GE-1.2
DK-1p53
DK-4p101
UK-860-94
US-Makah
US-Goby1-5
DK-1p55

<enter search term>

☐ Batch

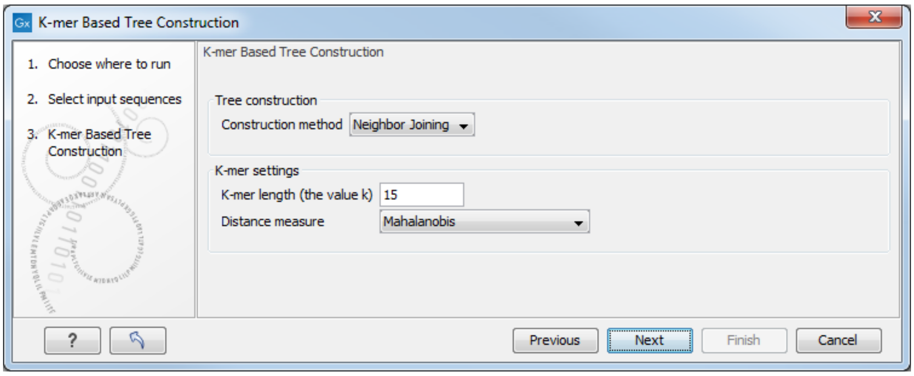
Selected elements (2)

DK-F1
AU-8-95
DK-3592B
FR-0771
DK-1p40
DK-M.rhabdo
UK-9643
UK-MLA98-6HE1
DK-2835
FI-ka66
NO-A16368G
GE-1.2
DK-1p53
DK-4p101
UK-860-94
US-Makah
US-Goby1-5
DK-1p55

Figure 17.1: *Select sequences needed for creating a tree with K-mer based tree construction.*

Next, select the construction method, specify the k-mer length and select a distance measure for tree construction (figure 17.2):

- **Tree construction**
  - **Tree construction method** The user is asked to specify which distance-based method to use for tree construction. There are two options (see section 17.4.1):
    - The **UPGMA** method. Assumes constant rate of evolution.
    - The **Neighbor Joining** method. Well suited for trees with varying rates of evolution.
- **K-mer settings**



K-mer Based Tree Construction

1. Choose where to run
2. Select input sequences
3. K-mer Based Tree Construction

K-mer Based Tree Construction

Tree construction

Construction method: Neighbor Joining

K-mer settings

K-mer length (the value k): 15

Distance measure: Mahalanobis

Figure 17.2: *Select the construction method, and specify the k-mer length and a distance measure.*

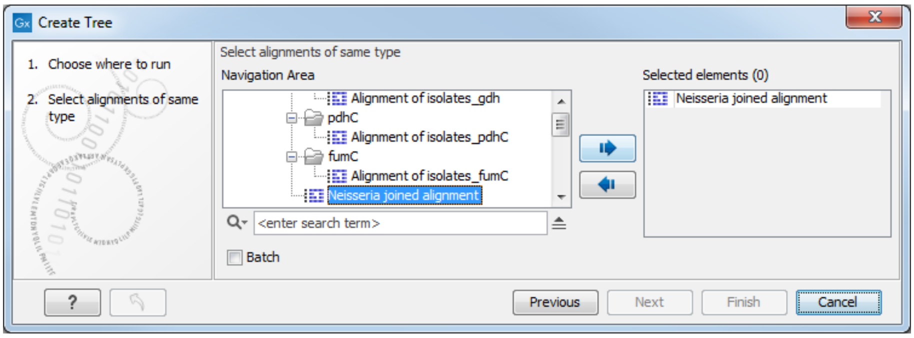
- **K-mer length (the value k)** Allows specification of the k-mer length, which can be a number between 3 and 50.
- **Distance measure** The distance measure is used to compute the distances between two counts of k-mers. Three options exist: Euclidian squared, Mahalanobis, and Fractional common K-mer count. See section 17.4.1 for further details.

17.2 Create tree

The Create tree tool can be used to generate a distance-based phylogenetic tree from an input alignment:

Tools | **Alignments and Trees** ()| **Create Tree** ()

This will open the dialog displayed in figure 17.3:



Create Tree

1. Choose where to run
2. Select alignments of same type

Select alignments of same type

Navigation Area

- Alignment of isolates_gdh
- pdhC
  - Alignment of isolates_pdhC
- fumC
  - Alignment of isolates_fumC
- Neisseria joined alignment

<enter search term>

□ Batch

Selected elements (0)

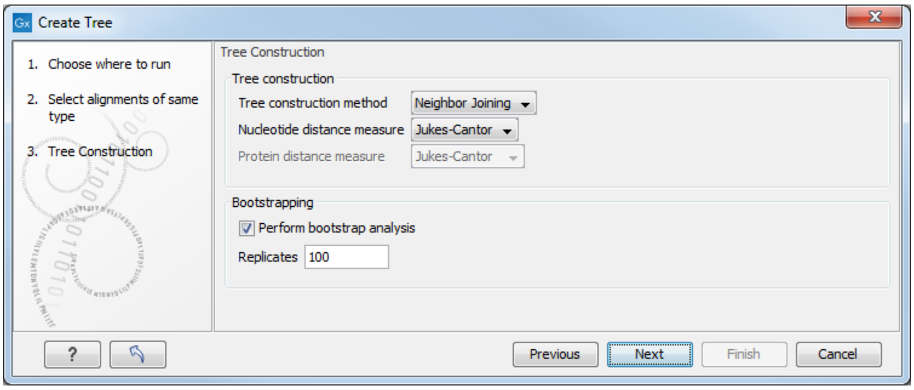
- Neisseria joined alignment

Figure 17.3: *Creating a tree.*

If an alignment was selected before the tool was launched, that alignment will be listed in the **Selected Elements** window of the dialog. Use the arrows to add or remove elements from the **Navigation Area**. Click **Next** to adjust parameters.

Figure 17.4 shows the parameters that can be set for this distance-based tree creation:

- Tree construction (see section 17.4.1)
  - Tree construction method
    - The **UPGMA** method. Assumes constant rate of evolution.
    - The **Neighbor Joining** method. Well suited for trees with varying rates of evolution.



1. Choose where to run
2. Select alignments of same type
3. Tree Construction

Tree Construction

Tree construction

Tree construction method: Neighbor Joining

Nucleotide distance measure: Jukes-Cantor

Protein distance measure: Jukes-Cantor

Bootstrapping

☑ Perform bootstrap analysis

Replicates: 100

Figure 17.4: *Adjusting parameters for distance-based methods.*

- - Nucleotide distance measure
    - **Jukes-Cantor**. Assumes equal base frequencies and equal substitution rates.
    - **Kimura 80**. Assumes equal base frequencies but distinguishes between transitions and transversions.
  - Protein distance measure
    - **Jukes-Cantor**. Assumes equal amino acid frequency and equal substitution rates.
    - **Kimura protein**. Assumes equal amino acid frequency and equal substitution rates. Includes a small correction term in the distance formula that is intended to give better distance estimates than Jukes-Cantor.
- Bootstrapping.
  - Perform bootstrap analysis. To evaluate the reliability of the inferred trees, *CLC Main Workbench* allows the option of doing a **bootstrap** analysis (see section 17.4.1). A bootstrap value will be attached to each node, and this value is a measure of the confidence in the subtree rooted at the node. The number of replicates used in the bootstrap analysis can be adjusted in the wizard. The default value is 100 replicates which is usually enough to distinguish between reliable and unreliable nodes in the tree. The bootstrap value assigned to each inner node in the output tree is the percentage (0-100) of replicates which contained the same subtree as the one rooted at the inner node.

For a more detailed explanation, see Bioinformatics explained in section 17.4.1.

17.3 Model Testing

As the Model Testing tool can help identify the best substitution model (17.4.1) to be used for Maximum Likelihood Phylogeny tree construction, it is recommended to run Model Testing before running the Maximum Likelihood Phylogeny tool.

The Model Testing tool uses four different statistical analyses:

- Hierarchical likelihood ratio test (hLRT)
- Bayesian information criterion (BIC)

- Minimum theoretical information criterion (AIC)
- Minimum corrected theoretical information criterion (AICc)

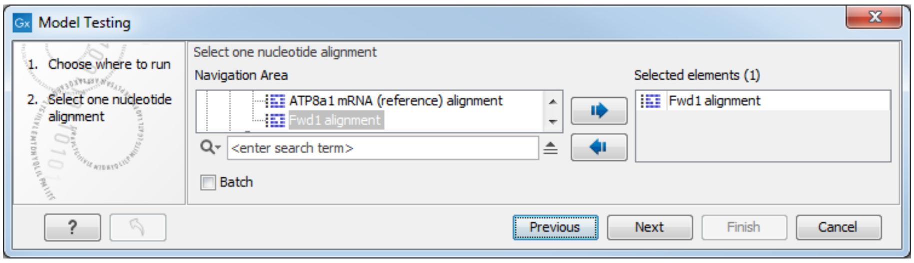
to test the substitution models:

- Jukes-Cantor [Jukes and Cantor, 1969]
- Felsenstein 81 [Felsenstein, 1981]
- Kimura 80 [Kimura, 1980]
- HKY [Hasegawa et al., 1985]
- GTR (also known as the REV model) [Yang, 1994a]

To do model testing:

Tools | **Alignments and Trees** (**)**| **Model Testing** (**)**

Select the alignment that you wish to use for the tree construction (figure 17.5):



1. Choose where to run
2. Select one nucleotide alignment

Select one nucleotide alignment

Navigation Area

ATP8a1 mRNA (reference) alignment
Fwd1 alignment

<enter search term>

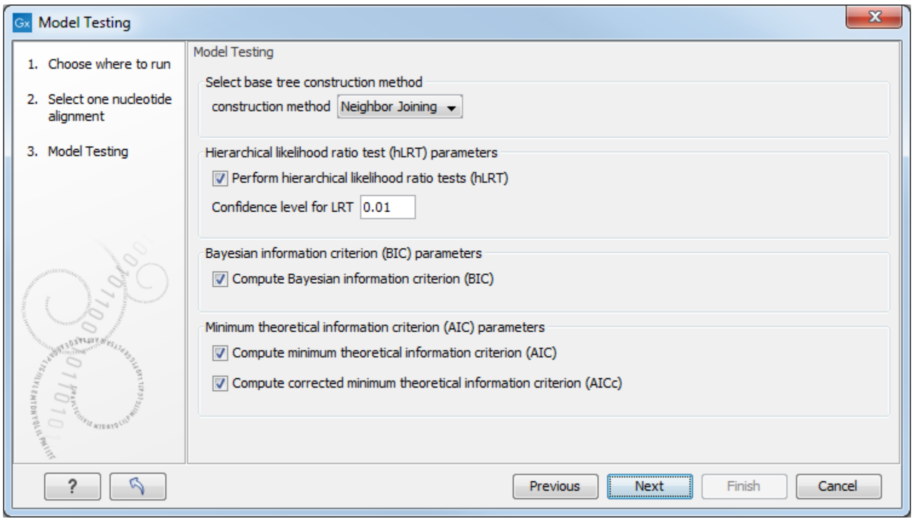
□ Batch

Selected elements (1)

Fwd1 alignment

Figure 17.5: *Select alignment for model testing.*

Specify the parameters to be used for model testing (figure 17.6):



1. Choose where to run
2. Select one nucleotide alignment
3. Model Testing

Model Testing

Select base tree construction method

construction method: Neighbor Joining

Hierarchical likelihood ratio test (hLRT) parameters

☑ Perform hierarchical likelihood ratio tests (hLRT)

Confidence level for LRT: 0.01

Bayesian information criterion (BIC) parameters

☑ Compute Bayesian information criterion (BIC)

Minimum theoretical information criterion (AIC) parameters

☑ Compute minimum theoretical information criterion (AIC)

☑ Compute corrected minimum theoretical information criterion (AICc)

Figure 17.6: *Specify parameters for model testing.*

- **Select base tree construction method**

A base tree (a guiding tree) is required in order to be able to determine which model(s) would be the most appropriate to use to make the best possible phylogenetic tree from a specific alignment. The topology of the base tree is used in the hierarchical likelihood ratio test (hLRT), and the base tree is used as starting point for topology exploration in Bayesian information criterion (BIC), Akaike information criterion (or minimum theoretical information criterion) (AIC), and AICc (AIC with a correction for the sample size) ranking.

  - **Construction method** A base tree is created automatically using one of two methods from the Create Tree tool:
    - The **UPGMA** method. Assumes constant rate of evolution.
    - The **Neighbor Joining** method. Well suited for trees with varying rates of evolution.

- **Hierarchical likelihood ratio test (hLRT) parameters** A statistical test of the goodness-of-fit between two models that compares a relatively more complex model to a simpler model to see if it fits a particular dataset significantly better.
  - **Perform hierarchical likelihood ratio test (hLRT)**
  - **Confidence level for LRT** The confidence level used in the likelihood ratio tests.
- **Bayesian information criterion (BIC) parameters**
  - **Compute Bayesian information criterion (BIC)** Rank substitution models based on Bayesian information criterion (BIC). Formula used is BIC = -2ln(L)+Kln(n), where ln(L) is the log-likelihood of the best tree, K is the number of parameters in the model, and ln(n) is the logarithm of the length of the alignment.
- **Minimum theoretical information criterion (AIC) parameters**
  - **Compute minimum theoretical information criterion (AIC)** Rank substitution models based on minimum theoretical information criterion (AIC). Formula used is AIC = -2ln(L)+2K, where ln(L) is the log-likelihood of the best tree, K is the number of parameters in the model.
  - **Compute corrected minimum theoretical information criterion (AIC)** Rank substitution models based on minimum corrected theoretical information criterion (AICc). Formula used is AICc = -2ln(L)+2K+2K(K+1)/(n-K-1), where ln(L) is the log-likelihood of the best tree, K is the number of parameters in the model, n is the length of the alignment. AICc is recommended over AIC roughly when n/K is less than 40.

The output from model testing is a report that lists all test results in table format. For each tested model the report indicate whether it is recommended to use rate variation or not. Topology variation is recommended in all cases.

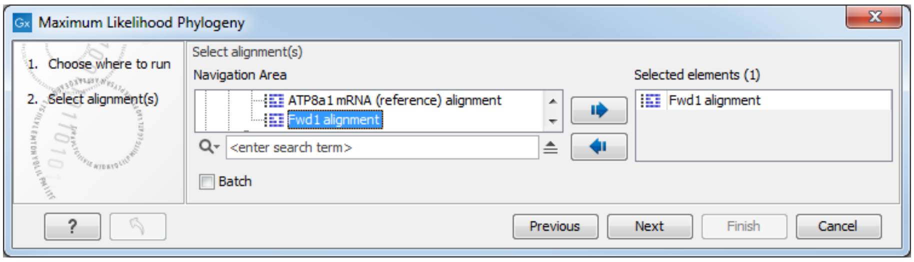
From the listed test results, it is up to the user to select the most appropriate model. The different statistical tests will usually agree on which models to recommend although variations may occur. Hence, in order to select the best possible model, it is recommended to select the model that has proven to be the best by most tests.

17.4 Maximum Likelihood Phylogeny

To generate a maximum likelihood based phylogenetic tree, go to:

Tools | Alignments and Trees ( )| Maximum Likelihood Phylogeny ( )

First, select the alignment to be used for the reconstruction (figure 17.7).



Maximum Likelihood Phylogeny

1. Choose where to run
2. Select alignment(s)

Select alignment(s)

Navigation Area

- ATP8a1 mRNA (reference) alignment
- Fwd1 alignment

<enter search term>

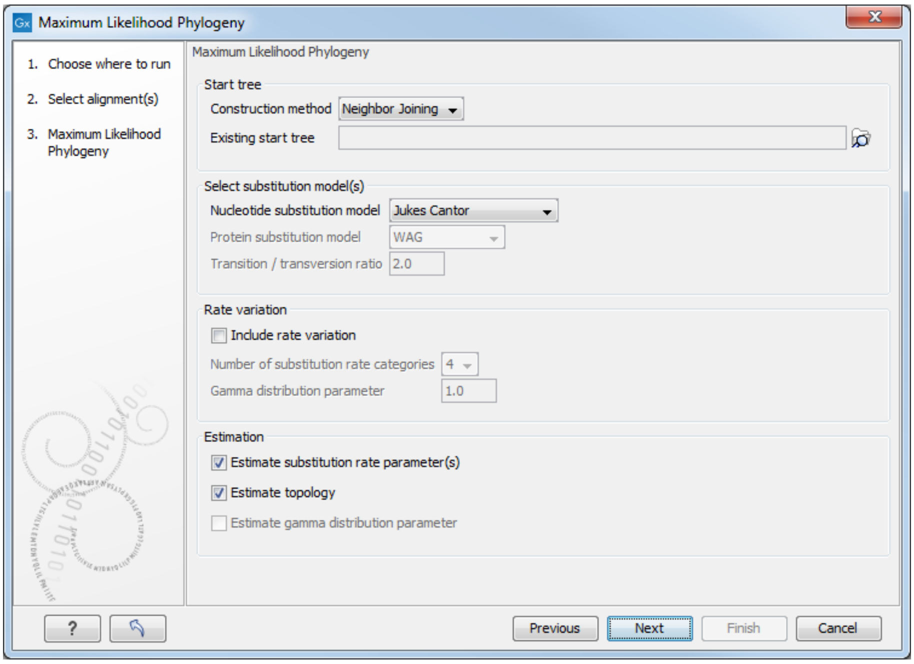
□ Batch

Selected elements (1)

- Fwd1 alignment

Figure 17.7: *Select the alignment for tree construction.*

You can then set up the following parameters (figure 17.8):



Maximum Likelihood Phylogeny

1. Choose where to run
2. Select alignment(s)
3. Maximum Likelihood Phylogeny

Maximum Likelihood Phylogeny

Start tree

Construction method: Neighbor Joining

Existing start tree:

Select substitution model(s)

Nucleotide substitution model: Jukes Cantor

Protein substitution model: WAG

Transition / transversion ratio: 2.0

Rate variation

□ Include rate variation

Number of substitution rate categories: 4

Gamma distribution parameter: 1.0

Estimation

☑ Estimate substitution rate parameter(s)

☑ Estimate topology

□ Estimate gamma distribution parameter

Figure 17.8: *Adjusting parameters for maximum likelihood phylogeny*

- **Start tree**
  - **Construction method** Specify the tree construction method which should be used to create the initial tree, Neighbor Joining or UPGMA
  - **Existing start tree** Alternatively, an existing tree can be used as starting tree for the tree reconstruction. Click on the folder icon to the right of the text field to specify the desired starting tree.
- **Select substitution model**
  - **Nucleotice substitution model** *CLC Main Workbench* allows maximum likelihood tree estimation to be performed under the assumption of one of five nucleotide substitution models:
    - ∗ Jukes-Cantor [Jukes and Cantor, 1969]

    - Felsenstein 81 [Felsenstein, 1981]
    - Kimura 80 [Kimura, 1980]
    - HKY [Hasegawa et al., 1985]
    - General Time Reversible (GTR) (also known as the REV model) [Yang, 1994a]

    All models are time-reversible. In the Kimura 80 and HKY models, the user may set a transtion/transversion ratio value, which will be used as starting value for optimization or as a fixed value, depending on the level of estimation chosen by the user. For further details, see 17.4.1.

  - **Protein substitution model** *CLC Main Workbench* allows maximum likelihood tree estimation to be performed under the assumption of one of four protein substitution models:

    - Bishop-Friday [Bishop and Friday, 1985]
    - Dayhoff (PAM) [Dayhoff et al., 1978]
    - JTT [Jones et al., 1992]
    - WAG [Whelan and Goldman, 2001]

    The Bishop-Friday substitution model is similar to the Jukes-Cantor model for nucleotide sequences, i.e. it assumes equal amino acid frequencies and substitution rates. This is an unrealistic assumption and we therefore recommend using one of the remaining three models. The Dayhoff, JTT and WAG substitution models are all based on large scale experiments where amino acid frequencies and substitution rates have been estimated by aligning thousands of protein sequences. For these models, the maximum likelihood tool does not estimate parameters, but simply uses those determined from these experiments.

- **Rate variation**

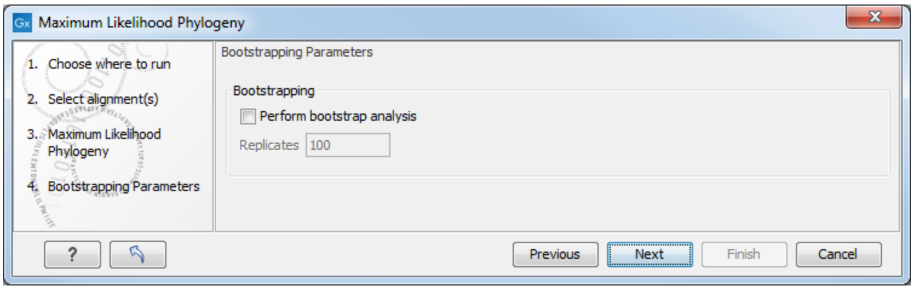
  To enable variable substitution rates among individual nucleotide sites in the alignment, select the **include rate variation** box. When selected, the discrete gamma model of Yang [Yang, 1994b] is used to model rate variation among sites. The number of categories used in the discretization of the gamma distribution as well as the gamma distribution parameter may be adjusted by the user (as the gamma distribution is restricted to have mean 1, there is only one parameter in the distribution).

- **Estimation**

  Estimation is done according to the maximum likelihood principle, that is, a search is performed for the values of the free parameters in the model assumed that results in the highest likelihood of the observed alignment [Felsenstein, 1981]. By ticking the **Estimate substitution rate parameters** box, maximum likelihood values of the free parameters in the rate matrix describing the assumed substitution model are found. If the **Estimate topology** box is selected, a search in the space of tree topologies for that which best explains the alignment is performed. If left un-ticked, the starting topology is kept fixed at that of the starting tree.

  The **Estimate Gamma distribution parameter** is active if rate variation has been included in the model and in this case allows estimation of the Gamma distribution parameter to be switched on or off. If the box is left un-ticked, the value is fixed at that given in the **Rate variation** part. In the absence of rate variation estimation of substitution parameters and branch lengths are carried out according to the expectation maximization algorithm [Dempster et al., 1977]. With rate variation the maximization algorithm is performed. The topology space is searched according to the PHYML method [Guindon and Gascuel, 2003], allowing efficient search and estimation of large phylogenies. **Branch lengths are given in terms of expected numbers of substitutions per nucleotide site**.

In the next step of the wizard it is possible to perform bootstrapping (figure 17.9).


Maximum Likelihood Phylogeny

1. Choose where to run
2. Select alignment(s)
3. Maximum Likelihood Phylogeny
4. Bootstrapping Parameters

Bootstrapping Parameters

Bootstrapping

□ Perform bootstrap analysis

Replicates 100

Figure 17.9: *Adjusting parameters for ML phylogeny*

To evaluate the reliability of the inferred trees, *CLC Main Workbench* allows the option of doing a **bootstrap** analysis (see section 17.4.1). A bootstrap value will be attached to each node, and this value is a measure of the confidence in the subtree rooted at the node. The number of replicates in the bootstrap analysis can be adjusted in the wizard by specifying the number of times to resample the data. The default value is 100 resamples. The bootstrap value assigned to a node in the output tree is the percentage (0-100) of the bootstrap resamples which resulted in a tree containing the same subtree as that rooted at the node.

17.4.1 Bioinformatics explained

The phylogenetic tree

The evolutionary hypothesis of a phylogeny can be graphically represented by a phylogenetic tree.

Figure 17.10 shows a proposed phylogeny for the great apes, *Hominidae*, taken in part from Purvis [Purvis, 1995]. The tree consists of a number of nodes (also termed vertices) and branches (also termed edges). These nodes can represent either an individual, a species, or a higher grouping and are thus broadly termed taxonomic units. In this case, the terminal nodes (also called leaves or tips of the tree) represent extant species of *Hominidae* and are the *operational taxonomic units* (OTUs). The internal nodes, which here represent extinct common ancestors of the great apes, are termed *hypothetical taxonomic units* since they are not directly observable.

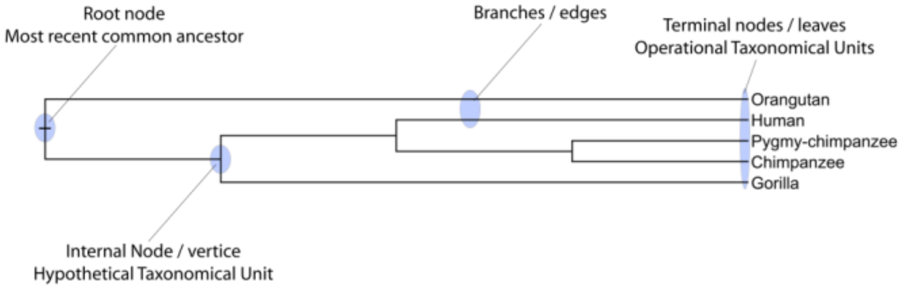


Figure 17.10: *A proposed phylogeny of the great apes (Hominidae). Different components of the tree are marked, see text for description.*

The ordering of the nodes determine the tree *topology* and describes how lineages have diverged over the course of evolution. The branches of the tree represent the amount of evolutionary divergence between two nodes in the tree and can be based on different measurements. A tree is completely specified by its topology and the set of all edge lengths.

The phylogenetic tree in figure 17.10 is rooted at the most recent common ancestor of all *Hominidae* species, and therefore represents a hypothesis of the direction of evolution e.g. that the common ancestor of gorilla, chimpanzee and man existed before the common ancestor of chimpanzee and man. In contrast, an unrooted tree would represent relationships without assumptions about ancestry.

Besides evolutionary biology and systematics the inference of phylogenies is central to other areas of research.

As more and more genetic diversity is being revealed through the completion of multiple genomes, an active area of research within bioinformatics is the development of comparative machine learning algorithms that can simultaneously process data from multiple species [Siepel and Haussler, 2004]. Through the comparative approach, valuable evolutionary information can be obtained about which amino acid substitutions are functionally tolerant to the organism and which are not. This information can be used to identify substitutions that affect protein function and stability, and is of major importance to the study of proteins [Knudsen and Miyamoto, 2001]. Knowledge of the underlying phylogeny is, however, paramount to comparative methods of inference as the phylogeny describes the underlying correlation from shared history that exists between data from different species.

In molecular epidemiology of infectious diseases, phylogenetic inference is also an important tool. The very fast substitution rate of microorganisms, especially the RNA viruses, means that these show substantial genetic divergence over the time-scale of months and years. Therefore, the phylogenetic relationship between the pathogens from individuals in an epidemic can be resolved and contribute valuable epidemiological information about transmission chains and epidemiologically significant events [Leitner and Albert, 1999], [Forsberg et al., 2001].

Substitution models and distance estimation

When estimating the evolutionary distance between organisms, one needs a model of how frequently different mutations occur in the DNA. Such models are known as substitution models. Our Model Testing and Maximum Likelihood Phylogeny tools currently support the five nucleotide substitution models listed here:

- Jukes-Cantor [Jukes and Cantor, 1969]
- Felsenstein 81 [Felsenstein, 1981]
- Kimura 80 [Kimura, 1980]
- HKY [Hasegawa et al., 1985]
- GTR (also known as the REV model) [Yang, 1994a]

Common to all these models is that they assume mutations at different sites in the genome occur independently and that the mutations at each site follow the same common probability distribution. Thus all five models provide relative frequencies for each of the 16 possible DNA substitutions (e.g. $C \rightarrow A$, $C \rightarrow C$, $C \rightarrow G$,...).

The Jukes-Cantor and Kimura 80 models assume equal base frequencies and the HKY and GTR models allow the frequencies of the four bases to differ (they will be estimated by the observed frequencies of the bases in the alignment). In the Jukes-Cantor model all substitutions are assumed to occur at equal rates, in the Kimura 80 and HKY models transition and transversion rates are allowed to differ (substitution between two purines $(A \leftrightarrow G)$ or two pyrimidines $(C \leftrightarrow T)$ are transitions and purine - pyrimidine substitutions are transversions). The GTR model is the general time reversible model that allows all substitutions to occur at different rates. For the substitution rate matrices describing the substitution models we use the parametrization of Yang [Yang, 1994a].

For protein sequences, our Maximum Likelihood Phylogeny tool supports four substitution models:

- Bishop-Friday [Bishop and Friday, 1985]
- Dayhoff (PAM) [Dayhoff et al., 1978]
- JTT [Jones et al., 1992]
- WAG [Whelan and Goldman, 2001]

As with nucleotide substitution models, it is assumed that mutations at different sites in the genome occur independently and according to the same probability distribution.

The Bishop-Friday model assumes all amino acids occur with same frequency and that all substitutions are equally likely. This is the simplest model, but also the most unrealistic. The remaining three models use amino acid frequencies and substitution rates which have been determined from large scale experiments where huge sets of protein sequences have been aligned and rates have been estimated. These three models reflect the outcome of three different experiments. We recommend using WAG as these rates where estimated from the largest experiment.

K-mer based distance estimation

K-mer based distance estimation is an alternative to estimating evolutionary distance based on multiple alignments. At a high level, the distance between two sequences is defined by first collecting the set of k-mers (subsequences of length k) occuring in the two sequences. From these two sets, the evolutionary distance between the two organisms is now defined by measuring how different the two sets are. The more the two sets look alike, the smaller is the evolutionary distance. The main motivation for estimating evolutionary distance based on k-mers, is that it is computationally much faster than first constructing a multiple alignment. Experiments show that phylogenetic tree reconstruction using k-mer based distances can produce results comparable to the slower multiple alignment based methods [Blaisdell, 1989].

All of the k-mer based distance measures completely ignores the ordering of the k-mers inside the input sequences. Hence, if the selected k value (the length of the sequences) is too small, very distantly related organisms may be assigned a small evolutionary distance (in the extreme case where k is 1, two organisms will be treated as being identical if the frequency of each nucleotide/amino-acid is the same in the two corresponding sequences). In the other extreme, the k-mers should have a length (k) that is somewhat below the average distance between mismatches if the input sequences were aligned (in the extreme case of k=the length of the sequences, two organisms have a maximum distance if they are not identical). Thus the selected k value should not be too large and not too small. A general rule of thumb is to only use k-mer based distance estimation for organisms that are not too distantly related.

Formal definition of distance. In the following, we give a more formal definition of the three supported distance measures: Euclidian-squared, Mahalanobis and Fractional common k-mer count. For all three, we first associate a point $p(s)$ to every input sequence s. Each point $p(s)$ has one coordinate for every possible length k sequence (e.g. if s represent nucleotide sequences, then $p(s)$ has 4^k coordinates). The coordinate corresponding to a length k sequence x has the value: "number of times x occurs as a subsequence in s". Now for two sequences s_1 and s_2, their evolutionary distance is defined as follows:

- **Euclidian squared**: For this measure, the distance is simply defined as the (squared Euclidian) distance between the two points $p(s_1)$ and $p(s_2)$, i.e.

$$\text{dist}(s_1, s_2) = \sum_i (p(s_1)_i - p(s_2)_i)^2.$$

- **Mahalanobis**: This measure is essentially a fine-tuned version of the Euclidian squared distance measure. Here all the counts $p(s_j)_i$ are "normalized" by dividing with the standard deviation σ_j of the count for the k-mer. The revised formula thus becomes:

$$\text{dist}(s_1, s_2) = \sum_i (p(s_1)_i/\sigma_i - p(s_2)_i/\sigma_i)^2.$$

  Here the standard deviations can be computed directly from a set of equilibrium frequencies for the different bases, see [Gentleman and Mullin, 1989].

- **Fractional common k-mer count**: For the last measure, the distance is computed based on the minimum count of every k-mer in the two sequences, thus if two sequences are very different, the minimums will all be small. The formula is as follows:

$$\text{dist}(s_1, s_2) = \log(0.1 + \sum_i (\min(p(s_1)_i, p(s_2)_i)/(\min(n, m) - k + 1))).$$

  Here n is the length of s_1 and m is the length of s_2. This method has been described in [Edgar, 2004].

In experiments performed in [Höhl et al., 2007], the Mahalanobis distance measure seemed to be the best performing of the three supported measures.

Distance based reconstruction methods

Distance based phylogenetic reconstruction methods use a pairwise distance estimate between the input organisms to reconstruct trees. The distances are an estimate of the evolutionary distance between each pair of organisms which are usually computed from DNA or amino acid sequences. Given two homologous sequences a distance estimate can be computed by aligning the sequences and then counting the number of positions where the sequences differ. The number of differences is called the observed number of substitutions and is usually an underestimate of the real distance as multiple mutations could have occurred at any position. To correct for these hidden substitutions a substitution model, such as Jukes-Cantor or Kimura 80, can be used to get a more precise distance estimate (see section 17.4.1).

To correct for these hidden substitutions a substitution model, such as Jukes-Cantor or Kimura 80, can be used to get a more precise distance estimate.

Alternatively, k-mer based methods or SNP based methods can be used to get a distance estimate without the use of substitution models.

After distance estimates have been computed, a phylogenetic tree can be reconstructed using a distance based reconstruction method. Most distance based methods perform a bottom up reconstruction using a greedy clustering algorithm. Initially, each input organism is put in its own cluster which corresponds to a leaf node in the resulting tree. Next, pairs of clusters are iteratively joined into higher level clusters, which correspond to connecting two nodes in the tree with a new parent node. When a single node remains, the tree is reconstructed.

The *CLC Main Workbench* provides two of the most widely used distance based reconstruction methods:

- The **UPGMA** method [Michener and Sokal, 1957] which assumes a constant rate of evolution (molecular clock hypothesis) in the different lineages. This method reconstruct trees by iteratively joining the two nearest clusters until there is only one cluster left. The result of the UPGMA method is a rooted bifurcating tree annotated with branch lengths.

- The **Neighbor Joining** method [Saitou and Nei, 1987] attempts to reconstruct a minimum evolution tree (a tree where the sum of all branch lengths is minimized). Opposite to the UPGMA method, the neighbor joining method is well suited for trees with varying rates of evolution in different lineages. A tree is reconstructed by iteratively joining clusters which are close to each other but at the same time far from all other clusters. The resulting tree is a bifurcating tree with branch lenghts. Since no particular biological hypothesis is made about the placement of the root in this method, the resulting tree is unrooted.

Maximum Likelihood reconstruction methods

Maximum Likelihood (ML) based reconstruction methods [Felsenstein, 1981] seek to identify the most probable tree given the data available, i.e. maximize $P(tree|data)$ where the $tree$ refers to a tree topology with branch lengths while $data$ is usually a set of sequences. However, it is not possible to compute $P(tree|data)$ so instead ML based methods have to compute the probability of the data given a tree, i.e. $P(data|tree)$. The ML tree is then the tree which makes the data most probable. In other words, ML methods search for the tree that gives the highest probability of producing the observed sequences. This is done by searching through the space of all possible trees while computing an ML estimate for each tree. Computing an ML estimate for a tree is time consuming and since the number of tree topologies grows exponentially with the number of leaves in a tree, it is infeasible to explore all possible topologies. Consequently, ML methods must employ search heuristics that quickly converges towards a tree with a likelihood close to the real ML tree.

The likelihood of trees are computed using an explicit model of evolution such as the Jukes-Cantor or Kimura 80 models. Choosing the right model is often important to get a good result. To help users choose the correct model for a data set, the Model Testing tool (see section 17.3) can be used to test a range of different models for input nucleotide sequences.

The search heuristics which are commonly used in ML methods requires an initial phylogenetic tree as a starting point for the search. An initial tree which is close to the optimal solution, can reduce the running time of ML methods and improve the chance of finding a tree with a large likelihood. A common way of reconstructing a good initial tree is to use a distance based method such as UPGMA or neighbor-joining to produce a tree based on a multiple alignment.

Bootstrap tests

Bootstrap tests [Felsenstein, 1985] is one of the most common ways to evaluate the reliability of the topology of a phylogenetic tree. In a bootstrap test, trees are evaluated using Efron's re-sampling technique [Efron, 1982], which samples nucleotides from the original set of sequences as follows:

Given an alignment of n sequences (rows) of length l (columns), we randomly choose l columns in the alignment with replacement and use them to create a new alignment. The new alignment has n rows and l columns just like the original alignment but it may contain duplicate columns and some columns in the original alignment may not be included in the new alignment. From this new alignment we reconstruct the corresponding tree and compare it to the original tree. For each subtree in the original tree we search for the same subtree in the new tree and add a score of one to the node at the root of the subtree if the subtree is present in the new tree. This procedure is repeated a number of times (usually around 100 times). The result is a counter for each interior node of the original tree, which indicate how likely it is to observe the exact same subtree when the input sequences are sampled. A bootstrap value is then computed for each interior node as the percentage of resampled trees that contained the same subtree as that rooted at the node.

Bootstrap values can be seen as a measure of how reliably we can reconstruct a tree, given the sequence data available. If all trees reconstructed from resampled sequence data have very different topologies, then most bootstrap values will be low, which is a strong indication that the topology of the original tree cannot be trusted.

Scale bar

The scale bar unit depends on the distance measure used and the tree construction algorithm used. The trees produced using the Maximum Likelihood Phylogeny tool has a very specific interpretation: A distance of x means that the expected number of substitutions/changes per nucleotide (amino acid for protein sequences) is x. i.e. if the distance between two taxa is 0.01, you expected a change in each nucleotide independently with probability 1 %. For the remaining algorithms, there is not as nice an interpretation. The distance depends on the weight given to different mutations as specified by the distance measure.

17.5 Tree Settings

The Tree Settings Side Panel found in the left side of the view area can be used to adjust the tree layout and to visualize metadata that is associated with the tree nodes. The following section describes the visualization options available from the Tree Settings side panel. Note however that editing legend boxes related to metadata can be done directly from editing the metadata table (see section 17.6).

The preferred tree layout settings (user defined tree settings) can be saved and applied via the top right **Save Tree Settings** (figure 17.11). Settings can either be saved **For This Tree Only** or for all saved phylogenetic trees (**For Tree View in General**). The first option will save the layout of the tree for that tree only and it ensures that the layout is preserved even if it is exported and opened by a different user. The second option stores the layout globally in the Workbench and makes it available to other trees through the **Apply Saved Settings** option.

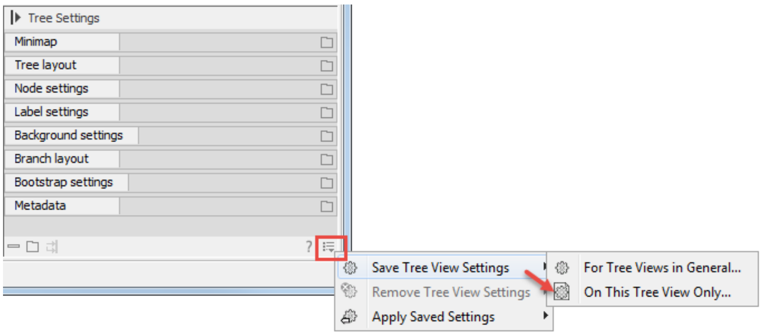


Figure 17.11: *Save, remove or apply preferred layout settings.*

17.5.1 Minimap

The Minimap is a navigation tool that shows a small version of the tree. A grey square indicates the specific part of the tree that is visible in the View Area (figure 17.12). To navigate the tree using the Minimap, click on the Minimap with the mouse and move the grey square around within the Minimap.

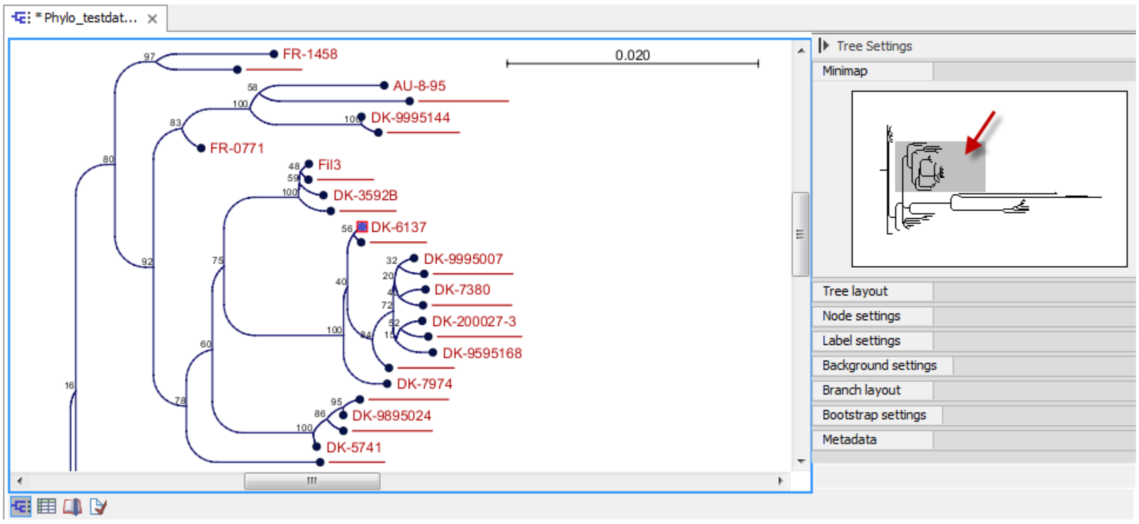


Figure 17.12: *Visualization of a phylogenetic tree. The grey square in the Minimap shows the part of the tree that is shown in the View Area.*

17.5.2 Tree layout

The **Tree Layout** can be adjusted in the Side Panel (figure 17.13).

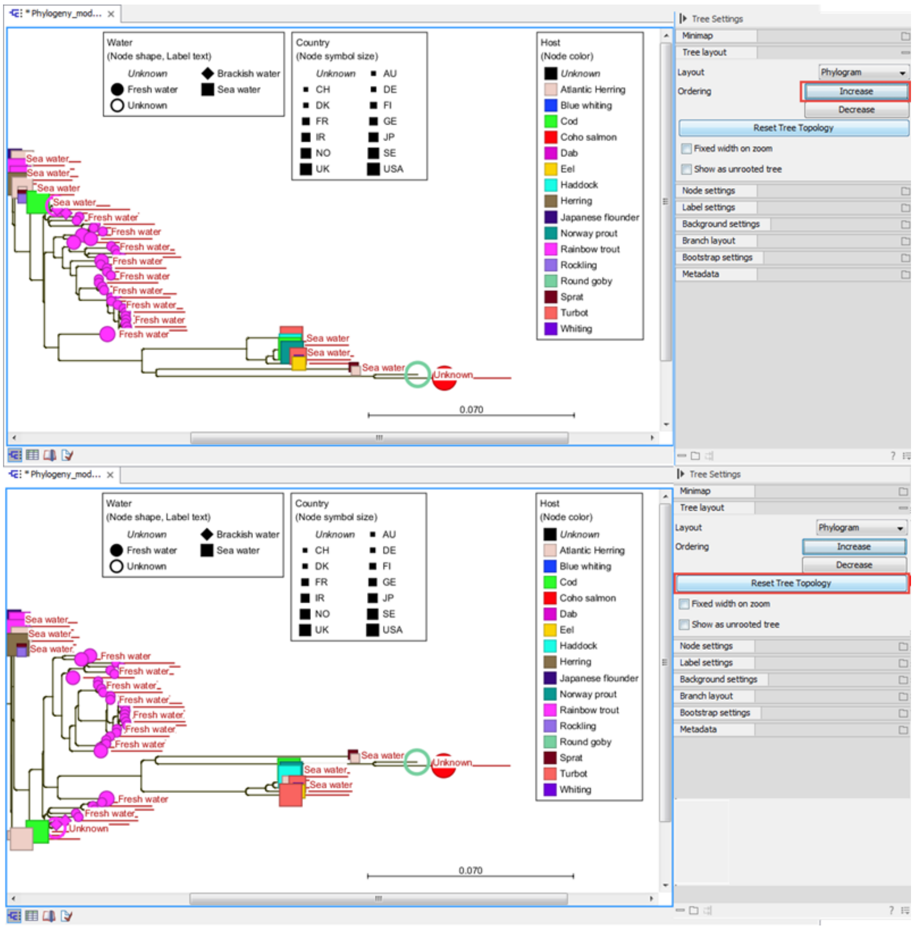


Figure 17.13: *The tree layout can be adjusted in the Side Panel. The top part of the figure shows a tree with increasing node order. In the bottom part of the figure the tree has been reverted to the original tree topology.*

- **Layout** Selects one of the five layout types: Phylogram, Cladogram, Circular Phylogram, Circular Cladogram or Radial. Note that only the Cladogram layouts are available if all branches in the tree have zero length.
  - **Phylogram** is a rooted tree where the edges have "lengths", usually proportional to the inferred amount of evolutionary change to have occurred along each branch.
  - **Cladogram** is a rooted tree without branch lengths which is useful for visualizing the topology of trees.
  - **Circular Phylogram** is also a phylogram but with the leaves in a circular layout.
  - **Circular Cladogram** is also a cladogram but with the leaves in a circular layout.
  - **Radial** is an unrooted tree that has the same topology and branch lengths as the rooted styles, but lacks any indication of evolutionary direction.
- **Ordering** The nodes can be ordered after the branch length; either **Increasing** (shown in figure 17.13) or **Decreasing**.
- **Reset Tree Topology** Resets to the default tree topology and node order (see figure 17.13). Any previously collapsed nodes will be uncollapsed.

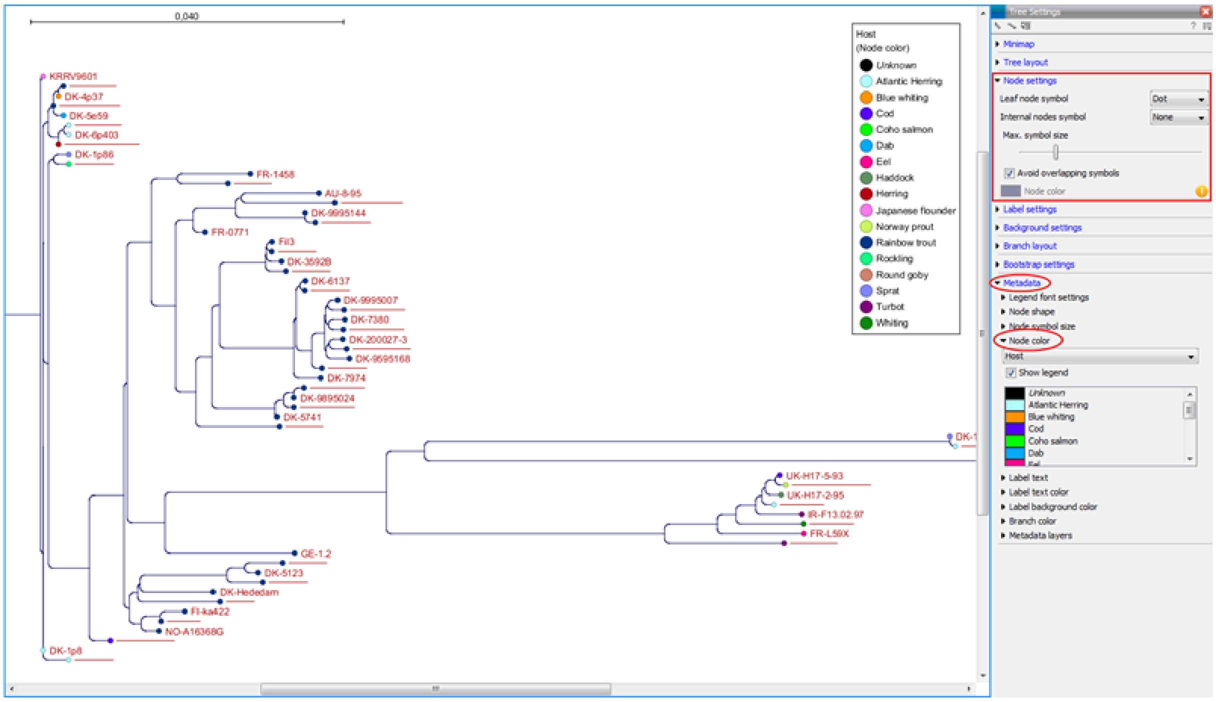
- **Fixed width on zoom** Locks the horizontal size of the tree to the size of the main window. Zoom is therefore only performed on the vertical axis when this option is enabled.
- **Show as unrooted tree** The tree can be shown with or without a root.

17.5.3 Node settings

The nodes can be manipulated in several ways.

- **Leaf node symbol** Leaf nodes can be shown as a range of different symbols (Dot, Box, Circle, etc.).
- **Internal node symbols** The internal nodes can also be shown with a range of different symbols (Dot, Box, Circle, etc.).
- **Max. symbol size** The size of leaf- and internal node symbols can be adjusted.
- **Avoid overlapping symbols** The symbol size will be automatically limited to avoid overlaps between symbols in the current view.
- **Node color** Specify a fixed color for all nodes in the tree.

The node layout settings in the Side Panel are shown in figure 17.14.



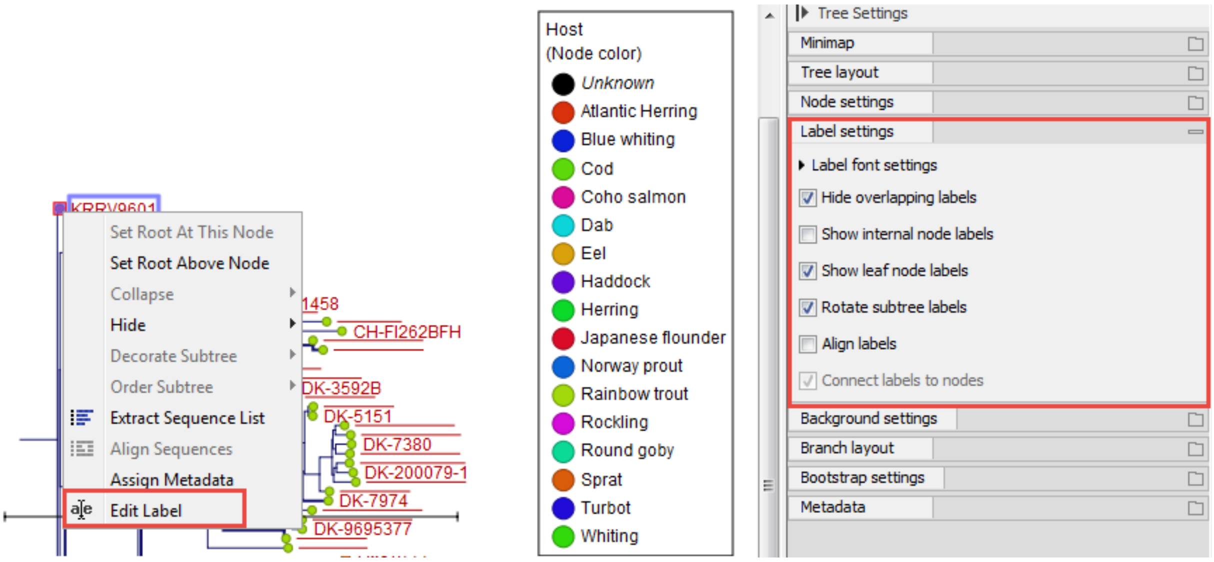
Leaf node symbol: Dot
Internal nodes symbol: None
Max. symbol size
☑ Avoid overlapping symbols
Node color
Node color: Host
☑ Show legend

Figure 17.14: *The Node Layout settings. Node color is specified by metadata and is therefore inactive in this example.*

17.5.4 Label settings

- **Label font settings** Can be used to specify/adjust font type, size and typography (Bold, Italic or normal).

- **Hide overlapping labels** Disable automatic hiding of overlapping labels and display all labels even if they overlap.
- **Show internal node labels** Labels for internal nodes of the tree (if any) can be displayed. Please note that subtrees and nodes can be labeled with a custom text. This is done by right clicking the node and selecting **Edit Label** (see figure 17.15).
- **Show leaf node labels** Leaf node labels can be shown or hidden.
- **Rotate Subtree labels** Subtree labels can be shown horizontally or vertically. Labels are shown vertically when "Rotate subtree labels" has been selected. Subtree labels can be added with the right click option "Set Subtree Label" that is enabled from "Decorate subtree" (see section 17.5.9).
- **Align labels** Align labels to the node furthest from the center of the tree so that all labels are positioned next to each other. The exact behavior depends on the selected tree layout.
- **Connect labels to nodes** Adds a thin line from the leaf node to the aligned label. Only possible when Align labels option is selected.



☑ Hide overlapping labels
□ Show internal node labels
☑ Show leaf node labels
☑ Rotate subtree labels
□ Align labels
☑ Connect labels to nodes

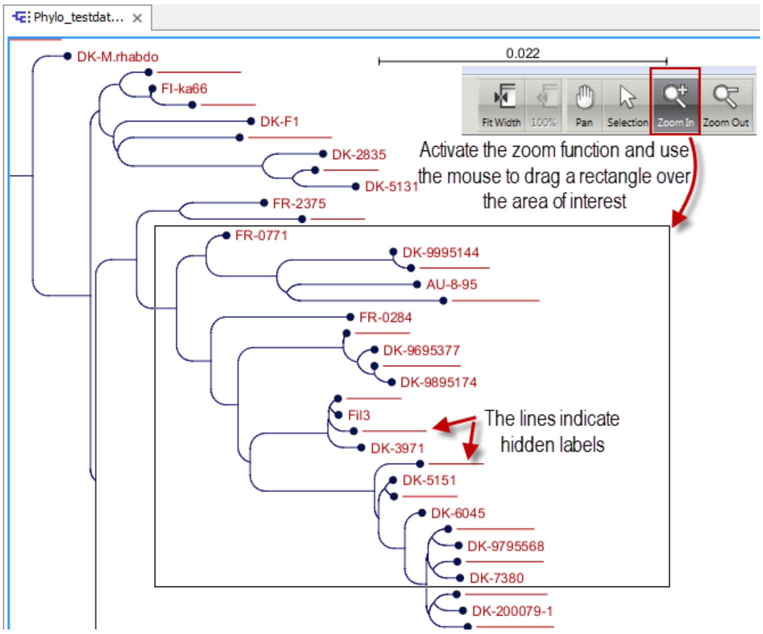
Figure 17.15: *"Edit label" in the right click menu can be used to customize the label text. The way node labels are displayed can be controlled through the labels settings in the right side panel.*

When working with big trees there is typically not enough space to show all labels. As illustrated in figure 17.15, only some of the labels are shown. The hidden labels are illustrated with thin horizontal lines (figure 17.16).

There are different ways of showing more labels. One way is to reduce the font size of the labels, which can be done under **Label font settings** in the Side Panel. Another option is to zoom in on specific areas of the tree (figure 17.16 and figure 17.17). The last option is to disable **Hide overlapping labels** under "Label settings" in the right side panel. When this option is unchecked all labels are shown even if the text overlaps. When allowing overlapping labels it is usually a good idea to disable **Show label background** under "Background settings" (see section 17.5.5).

Note! When working with a tree with hidden labels, it is possible to make the hidden label text appear by moving the mouse over the node with the hidden label.

Note! The text within labels can be edited by editing the metadata table values directly.



Activate the zoom function and use the mouse to drag a rectangle over the area of interest

The lines indicate hidden labels

Figure 17.16: *The zoom function in the upper right corner of the Workbench can be used to zoom in on a particular region of the tree. When the zoom function has been activated, use the mouse to drag a rectangle over the area that you wish to zoom in at.*

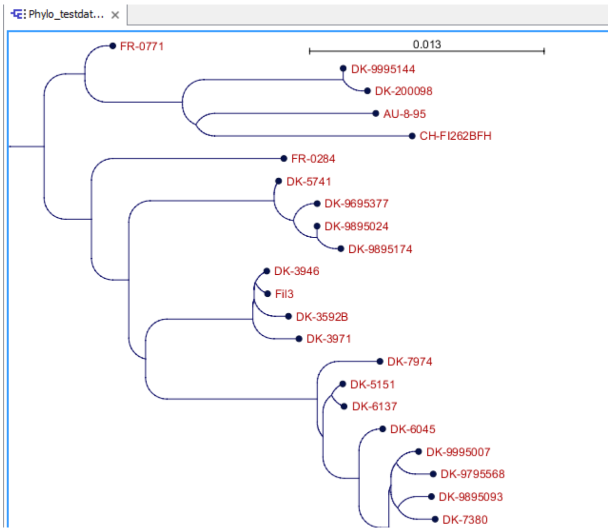


Figure 17.17: *After zooming in on a region of interest more labels become visible. In this example all labels are now visible.*

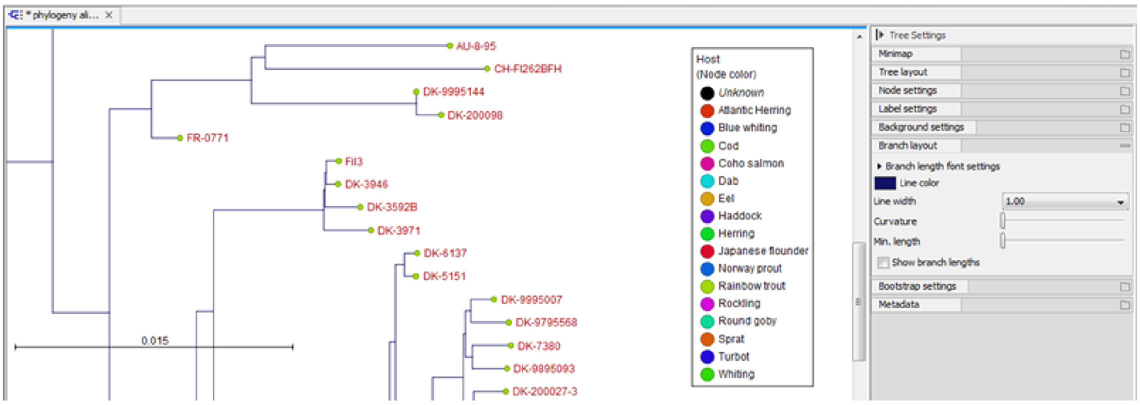
17.5.5 Background settings

- **Show label background** Show a background color for each label. Once ticked, it is possible to specify whether to use a fixed color or to use the color that is associated with the selected metadata category.

17.5.6 Branch layout

- **Branch length font settings** Specify/adjust font type, size and typography (Bold, Italic or normal).
- **Line color** Select the default line color.
- **Line width** Select the width of branches (1.0-3.0 pixels).
- **Curvature** Adjust the degree of branch curvature to get branches with round corners.
- **Min. length** Select a minimum branch length. This option can be used to prevent nodes connected with a short branch to cluster at the parent node.
- **Show branch lengths** Show or hide the branch lengths.

The branch layout settings in the Side Panel are shown in figure 17.18.



Line width 1.00

□ Show branch lengths

Figure 17.18: *Branch Layout settings.*

17.5.7 Bootstrap settings

Bootstrap values can be shown on the internal nodes. The bootstrap values are shown in percent and can be interpreted as confidence levels where a bootstrap value close to 100 indicate a clade, which is strongly supported by the data from which the tree was reconstructed. Bootstrap values are useful for identifying clades in the tree where the topology (and branch lengths) should not be trusted.

Some branches in rooted trees may not have bootstrap values. Trees constructed with neighbour joining are unrooted and to correctly visualize them, the "Radial" view is required. In all other tree views we need a root to visualize the tree. An "artificial node" and therefore an extra branch are created for such visualization to achieve this, which makes it look like a bootstrap value is missing

- **Bootstrap value font settings** Specify/adjust font type, size and typography (Bold, Italic or normal).
- **Show bootstrap values (%)** Show or hide bootstrap values. When selected, the bootstrap values (in percent) will be displayed on internal nodes if these have been computed during the reconstruction of the tree.

- **Bootstrap threshold (%)** When specifying a bootstrap threshold, the branch lengths can be controlled manually by collapsing internal nodes with bootstrap values under a certain threshold.
- **Highlight bootstrap $\geq$ (%)** Highlights branches where the bootstrap value is above the user defined threshold.

17.5.8 Visualizing metadata

Metadata associated with a phylogenetic tree (described in detail in section 17.6) can be visualized in a number of different ways:

- **Node shape** Different node shapes are available to visualize metadata.
- **Node symbol size** Change the node symbol size to visualize metadata.
- **Node color** Change the node color to visualize metadata.
- **Label text** The metadata can be shown directly as text labels as shown in figure 17.19.
- **Label text color** The label text can be colored and used to visualize metadata (see figure 17.19).
- **Label background color** The background color of node text labels can be used to visualize metadata.
- **Branch color** Branch colors can be changed according to metadata.
- **Metadata layers** Color coded layers shown next to leaf nodes.

Please note that when visualizing metadata through a tree property that can be adjusted in the right side panel (such as node color or node size), an exclamation mark will appear next to the control for that property to indicate that the setting is inactive because it is defined by metadata (see figure 17.14).

17.5.9 Node right click menu

Additional options for layout and extraction of subtree data are available when right clicking the nodes (figure 17.15):

- **Set Root At This Node** Re-root the tree using the selected node as root. Please note that re-rooting will change the tree topology. This option is only available for internal nodes, not leaf nodes.
- **Set Root Above Node** Re-root the tree by inserting a node between the selected node and its parent. Useful for rooting trees using an outgroup.
- **Collapse** Branches associated with a selected node can be collapsed with or without the associated labels. Collapsed branches can be uncollapsed using the *Uncollapse* option in the same menu.

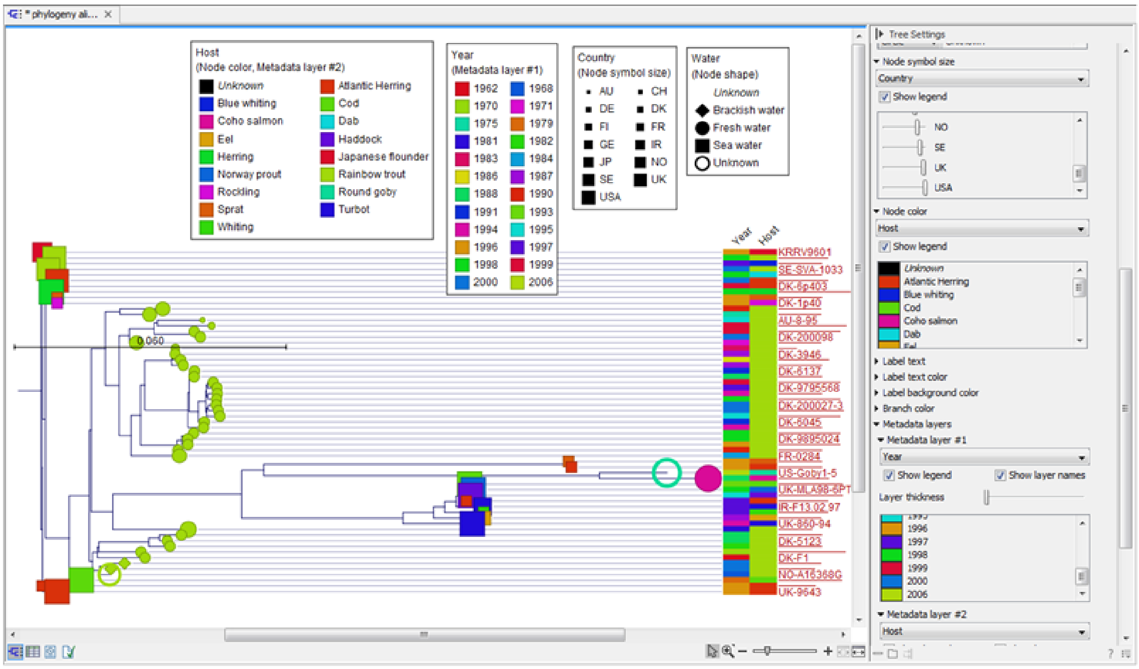


Figure 17.19: *Different types of metadata kan be visualized by adjusting node size, shape, and color. Two color-code metadata layers (Year and Host) are shown in the right side of the tree.*

- **Hide** Can be used to hide a node or a subtree. Hidden nodes or subtrees can be shown again using the *Show Hidden Subtree* function on a node which is root in a subtree containing hidden nodes (see figure 17.20). When hiding nodes, a new button appears labeled "Show X hidden nodes" in the Side Panel under "Tree Layout" (figure 17.21). When pressing this button, all hidden nodes are shown again.

- **Decorate Subtree** A subtree can be labeled with a customized name, and the subtree lines and/or background can be colored. To save the decoration, see figure 17.11 and use option: **Save/Restore Settings** | **Save Tree View Settings On This Tree View only**.

- **Order Subtree** Rearrange leaves and branches in a subtree by Increasing/Decreasing depth, respectively. Alternatively, change the order of a node's children by left clicking and dragging one of the node's children.

- **Extract Sequence List** Sequences associated with selected leaf nodes are extracted to a new sequence list.

- **Align Sequences** Sequences associated with selected leaf nodes are extracted and used as input to the *Create Alignment* tool.

- **Assign Metadata** Metadata can be added, deleted or modified. To add new metadata categories a new "Name" must be assigned. (This will be the column header in the metadata table). To add a new metadata category, enter a value in the "Value" field. To delete values, highlight the relevant nodes and right click on the selected nodes. In the dialog that appears, use the drop-down list to select the name of the desired metadata category and leave the value field empty. When pressing "Add" the values for the selected metadata category will be deleted from the selected nodes. Metadata can be modified in the same way, but instead of leaving the value field empty, the new value should be entered.

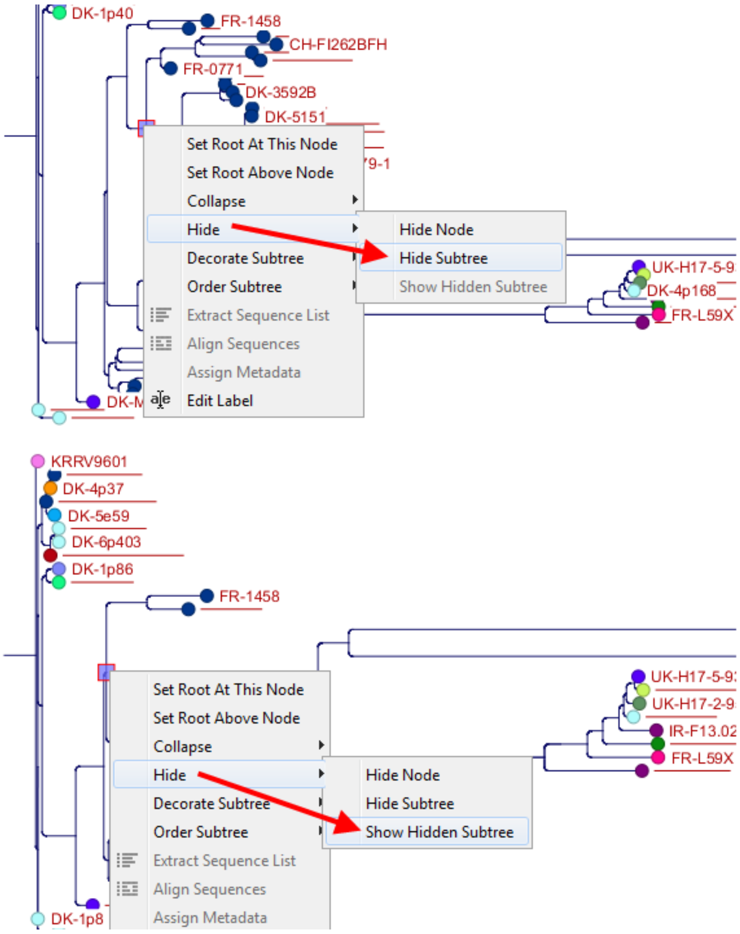
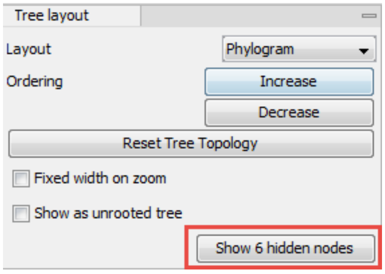


Figure 17.20: *A subtree can be hidden by selecting "Hide Subtree" and is shown again when selecting "Show Hidden Subtree" on a parent node.*



Tree layout

Layout: Phylogram

Ordering: Increase / Decrease

Reset Tree Topology

☐ Fixed width on zoom

☐ Show as unrooted tree

Show 6 hidden nodes

Figure 17.21: *When hiding nodes, a new button labeled "Show X hidden nodes" appears in the Side Panel under "Tree Layout". When pressing this button, all hidden nodes are brought back.*

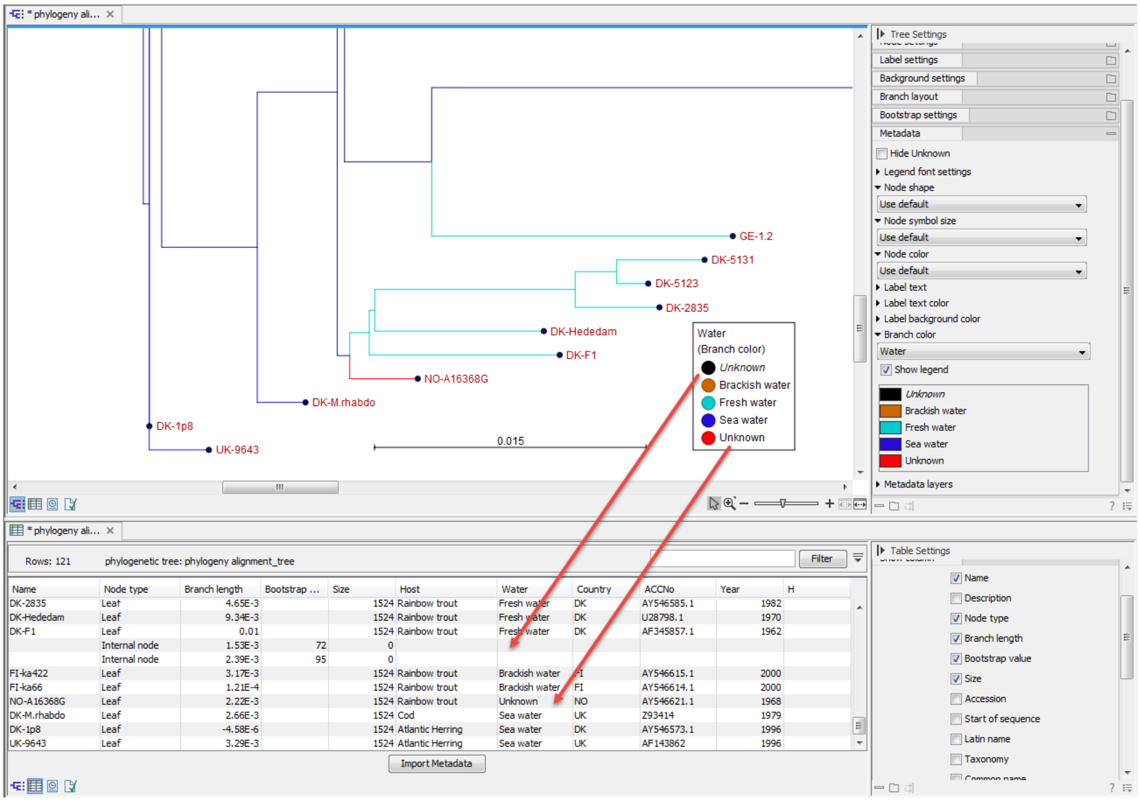
- **Edit label** Edit the text in the selected node label. Labels can be shown or hidden by using the Side Panel: **Label settings | Show internal node labels**

17.6 Metadata and phylogenetic trees

When a tree is reconstructed, some mandatory metadata will be added to nodes in the tree. These metadata are special in the sense that the tree viewer has specialized features for visualizing the data and some of them cannot be edited. The mandatory metadata include:

- **Node name** The node name.
- **Branch length** The length of the branch, which connects a node to the parent node.
- **Bootstrap value** The bootstrap value for internal nodes.
- **Size** The length of the sequence which corresponds to each leaf node. This only applies to leaf nodes.
- **Start of sequence** The first 50bp of the sequence corresponding to each leaf node.

To view metadata associated with a phylogenetic tree, click on the table icon (▦) at the bottom of the tree. If you hold down the Ctrl key (or ⌘ on Mac) while clicking on the table icon (▦), you will be able to see both the tree and the table in a split view (figure 17.22).



□ Hide Unknown
Legend font settings
Node shape: Use default
Node symbol size: Use default
Node color: Use default
Label text
Label text color
Label background color
Branch color: Water
☑ Show legend
■ *Unknown*
■ Brackish water
■ Fresh water
■ Sea water
■ Unknown
Metadata layers

Rows: 121 phylogenetic tree: phylogeny alignment_tree

Name	Node type	Branch length	Bootstrap ...	Size	Host	Water	Country	ACCNo	Year	H
DK-2835	Leaf	4.65E-3		1524	Rainbow trout	Fresh water	DK	AY546585.1	1982	
DK-Hededam	Leaf	9.34E-3		1524	Rainbow trout	Fresh water	DK	U28798.1	1970	
DK-F1	Leaf	0.01		1524	Rainbow trout	Fresh water	DK	AF345857.1	1962	
	Internal node	1.53E-3	72	0						
	Internal node	2.39E-3	95	0						
FI-ka422	Leaf	3.17E-3		1524	Rainbow trout	Brackish water	FI	AY546615.1	2000	
FI-ka66	Leaf	1.21E-4		1524	Rainbow trout	Brackish water	FI	AY546614.1	2000	
NO-A16368G	Leaf	2.22E-3		1524	Rainbow trout	Unknown	NO	AY546621.1	1968	
DK-M.rhabdo	Leaf	2.66E-3		1524	Cod	Sea water	UK	Z93414	1979	
DK-1p8	Leaf	-4.58E-6		1524	Atlantic Herring	Sea water	DK	AY546573.1	1996	
UK-9643	Leaf	3.29E-3		1524	Atlantic Herring	Sea water	UK	AF143862	1996	

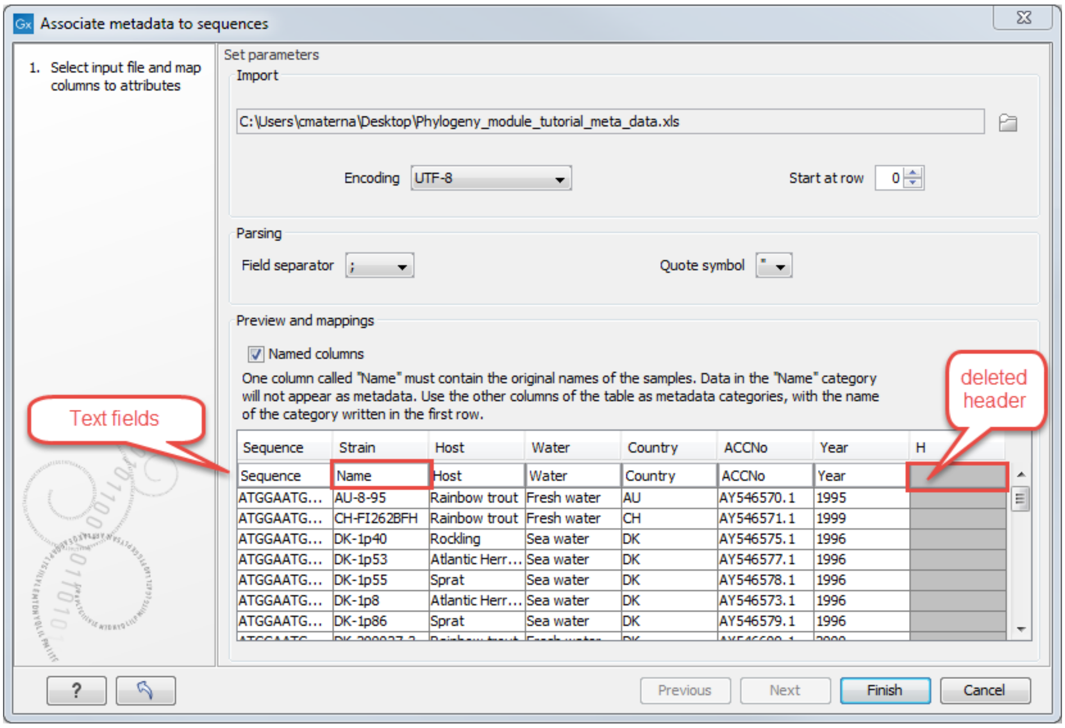
☑ Name
□ Description
☑ Node type
☑ Branch length
☑ Bootstrap value
☑ Size
□ Accession
□ Start of sequence
□ Latin name
□ Taxonomy

Figure 17.22: *Tabular metadata that is associated with an existing tree shown in a split view. Note that Unknown written in italics (black branches) refer to missing metadata, while Unknown in regular font refers to metadata labeled as "Unknown".*

Additional metadata can be associated with a tree by clicking the **Import Metadata** button. This will open up the dialog shown in figure 17.23.

To associate metadata with an existing tree a common denominator is required. This is achieved by mapping the node names in the "Name" column of the metadata table to the names that have been used in the metadata table to be imported. In this example the "Strain" column holds the names of the nodes and this column must be assigned "Name" to allow the importer to associate metadata with nodes in the tree.

It is possible to import a subset of the columns in a set of metadata. An example is given in figure 17.23. The column "H" is not relevant to import and can be excluded simply by leaving the text field at the top row of the column empty.



Associate metadata to sequences

1. Select input file and map columns to attributes

Set parameters

Import

C:\Users\cmaterna\Desktop\Phylogeny_module_tutorial_meta_data.xls

Encoding UTF-8

Start at row 0

Parsing

Field separator ;

Quote symbol "

Preview and mappings

☑ Named columns

One column called "Name" must contain the original names of the samples. Data in the "Name" category will not appear as metadata. Use the other columns of the table as metadata categories, with the name of the category written in the first row.

Sequence	Strain	Host	Water	Country	ACCNo	Year	H
Sequence	Name	Host	Water	Country	ACCNo	Year	
ATGGAATG...	AU-8-95	Rainbow trout	Fresh water	AU	AY546570.1	1995	
ATGGAATG...	CH-FI262BFH	Rainbow trout	Fresh water	CH	AY546571.1	1999	
ATGGAATG...	DK-1p40	Rockling	Sea water	DK	AY546575.1	1996	
ATGGAATG...	DK-1p53	Atlantic Herr...	Sea water	DK	AY546577.1	1996	
ATGGAATG...	DK-1p55	Sprat	Sea water	DK	AY546578.1	1996	
ATGGAATG...	DK-1p8	Atlantic Herr...	Sea water	DK	AY546573.1	1996	
ATGGAATG...	DK-1p86	Sprat	Sea water	DK	AY546579.1	1996	

Figure 17.23: *Import of metadata for a tree. The second column named "Strain" is choosen as the common denominator by entering "Name" in the text field of the column. The column labeled "H" is ignored by not assigning a column heading to this column.*

17.6.1 Table Settings and Filtering

How to use the metadata table (see figure 17.24):

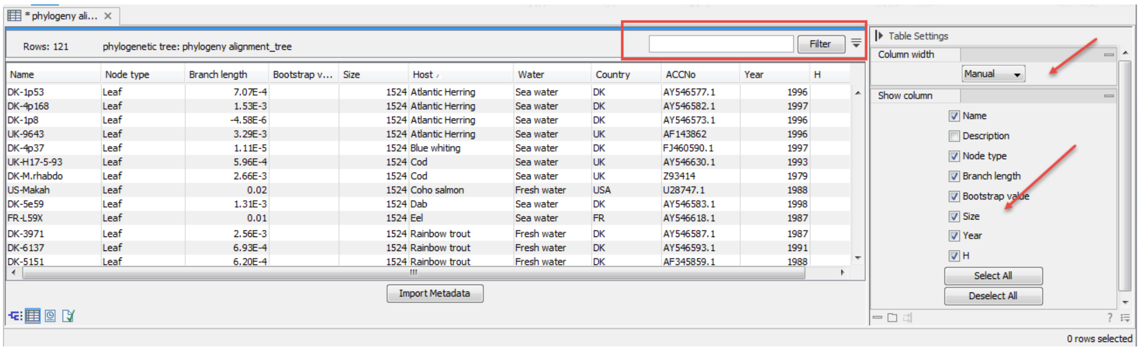
- **Column width** The column width can be adjusted in two ways; *Manually* or *Automatically*.
- **Show column** Selects which metadata categories that are shown in the table layout.
- **Filtering Metadata information** Metadata information in a table can be filtered by a simple- or advanced mode (this is described in section 9.2).

17.6.2 Add or modify metadata on a tree

It is possible to add and modify metadata from both the tree view and the table view.

Metadata can be added and edited in the metadata table by using the following right click options (see figure 17.25):

- **Assign Metadata** The right click option "Assign Metadata" can be used for four purposes.



Rows: 121   phylogenetic tree: phylogeny alignment_tree

Name	Node type	Branch length	Bootstrap v...	Size	Host	Water	Country	ACCNo	Year	H
DK-1p53	Leaf	7.07E-4		1524	Atlantic Herring	Sea water	DK	AY546577.1	1996	
DK-4p168	Leaf	1.53E-3		1524	Atlantic Herring	Sea water	DK	AY546582.1	1997	
DK-1p8	Leaf	-4.58E-6		1524	Atlantic Herring	Sea water	DK	AY546573.1	1996	
UK-9643	Leaf	3.29E-3		1524	Atlantic Herring	Sea water	UK	AF143862	1996	
DK-4p37	Leaf	1.11E-5		1524	Blue whiting	Sea water	DK	FJ460590.1	1997	
UK-H17-5-93	Leaf	5.96E-4		1524	Cod	Sea water	UK	AY546630.1	1993	
DK-M.rhabdo	Leaf	2.66E-3		1524	Cod	Sea water	UK	Z93414	1979	
US-Makah	Leaf	0.02		1524	Coho salmon	Fresh water	USA	U28747.1	1988	
DK-5e59	Leaf	1.31E-3		1524	Dab	Sea water	DK	AY546583.1	1998	
FR-L59X	Leaf	0.01		1524	Eel	Sea water	FR	AY546618.1	1987	
DK-3971	Leaf	2.56E-3		1524	Rainbow trout	Fresh water	DK	AY546587.1	1987	
DK-6137	Leaf	6.93E-4		1524	Rainbow trout	Fresh water	DK	AY546593.1	1991	
DK-5151	Leaf	6.20E-4		1524	Rainbow trout	Fresh water	DK	AF345859.1	1988	

Column width: Manual

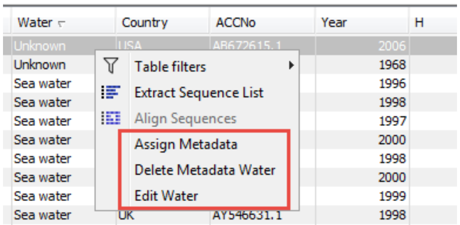
Show column

☑ Name
☐ Description
☑ Node type
☑ Branch length
☑ Bootstrap value
☑ Size
☑ Year
☑ H

Select All
Deselect All

0 rows selected

Figure 17.24: *Metadata table. The column width can be adjusted manually or automatically. Under "Show column" it is possible to select which columns should be shown in the table. Filtering using specific criteria can be performed.*



Water	Country	ACCNo	Year	H
Unknown	USA	AB672615.1	2006	
Unknown			1968	
Sea water			1996	
Sea water			1998	
Sea water			1997	
Sea water			2000	
Sea water			1998	
Sea water			2000	
Sea water			1999	
Sea water	UK	AY546631.1	1998	

Figure 17.25: *Right click options in the metadata table.*

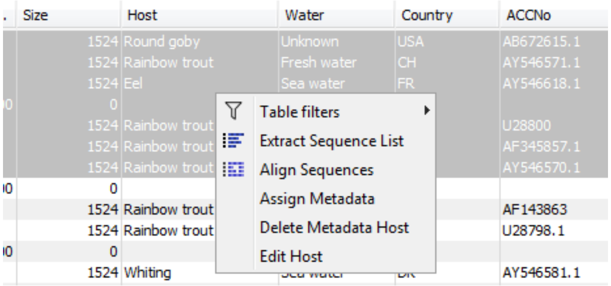
  - To add new metadata categories (columns). In this case, a new "Name" must be assigned, which will be the column header. To add a new column requires that a value is entered in the "Value" field. This can be done by right clicking anywhere in the table.
  - To add values to one or more rows in an existing column. In this case, highlight the relevant rows and right click on the selected rows. In the dialog that appears, use the drop-down list to select the name of the desired column and enter a value.
  - To delete values from an existing column. This is done in the same way as when adding a new value, with the only exception that the value field should be left empty.
  - To delete metadata columns. This is done by selecting all rows in the table followed by a right click anywhere in the table. Select the name of the column to delete from the drop down menu and leave the value field blank. When pressing "Add", the selected column will disappear.

- **Delete Metadata "column header"** This is the most simple way of deleting a metadata column. Click on one of the rows in the column to delete and select "Delete *column header*".

- **Edit "column header"** To modify existing metadata point, right click on a cell in the table and select the "Edit *column header*". To edit multiple entries at once, select multiple rows in the table, right click a selected cell in the column you want to edit and choose "Edit *column header*" (see an example in figure 17.26). This will change values in all selected rows in the column that was clicked.

17.6.3 Undefined metadata values on a tree

When visualizing a metadata category where one or more nodes in the tree have undefined values (empty fields in the table), these nodes will be visualized using a default value in **italics** in the



Size	Host	Water	Country	ACCNo
1524	Round goby	Unknown	USA	AB672615.1
1524	Rainbow trout	Fresh water	CH	AY546571.1
1524	Eel	Sea water	FR	AY546618.1
0				
1524	Rainbow trout			U28800
1524	Rainbow trout			AF345857.1
1524	Rainbow trout			AY546570.1
0				
1524	Rainbow trout			AF143863
1524	Rainbow trout			U28798.1
0				
1524	Whiting			AY546581.1

Figure 17.26: *To modify existing metadata, click on the specific field, select "Edit <column header>" and provide a new value.*

top of the legend (see the entry "*Unknown*" in figure 17.27). To remove this entry in the legend, all nodes must have a value assigned in the corresponding metadata category.

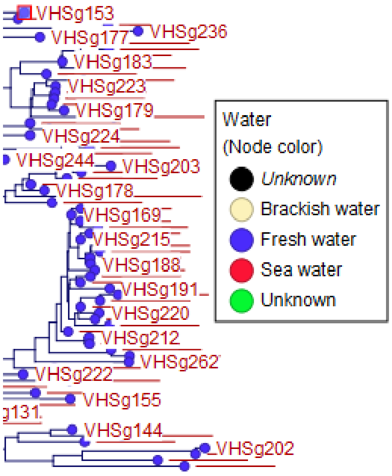


Figure 17.27: *A legend for a metadata category where one or more values are undefined. Fill your metadata table with a value of your choice to edit the mention of "(*"Unknown"* in the legend. Note that the "Unknown" that is not in italics is used for data that had a value written as "Unknown" in the metadata table.*

17.6.4 Selection of specific nodes

Selection of nodes in a tree is automatically synchronized to the metadata table and the other way around. Nodes in a tree can be selected in three ways:

- *Selection of a single node* Click once on a single node. Additional nodes can be added by holding down Ctrl (or ⌘ for Mac) and clicking on them (see figure 17.28).

- *Selecting all nodes in a subtree* Double clicking on a inner node results in the selection of all nodes in the subtree rooted at the node.

- *Selection via the Metadata table* Select one or more entries in the table. The corresponding nodes will now be selected in the tree.

It is possible to extract a subset of the underlying sequence data directly through either the tree viewer or the metadata table as follows. Select one or more nodes in the tree where at least one node has a sequence attached. Right click one of the selected nodes and choose **Extract Sequence List**. This will generate a new sequence list containing all sequences attached to the selected nodes. The same functionality is available in the metadata table where sequences can be extracted from selected rows using the right click menu. Please note that all extracted sequences are copies and any changes to these sequences will not be reflected in the tree.

When analyzing a phylogenetic tree it is often convenient to have a multiple alignment of sequences from e.g. a specific clade in the tree. A quick way to generate such an alignment is to first select one or more nodes in the tree (or the corresponding entries in the metadata table) and then select **Align Sequences** in the right click menu. This will extract the sequences corresponding to the selected elements and use a copy of them as input to the multiple alignment tool (see section 16.5.2). Next, change relevant option in the multiple alignment wizard that pops up and click **Finish**. The multiple alignment will now be generated.

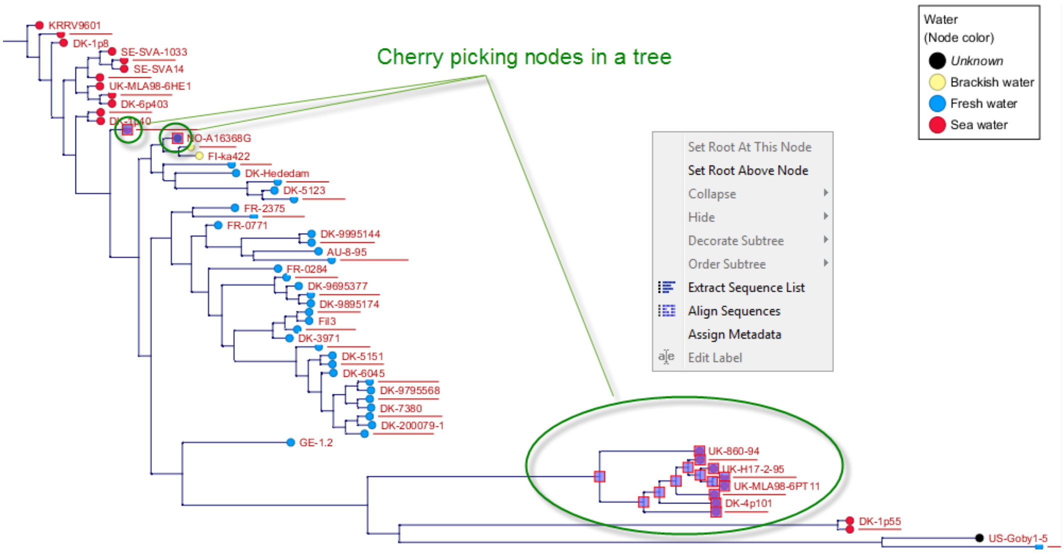


Figure 17.28: *Cherry picking nodes in a tree. The selected leaf sequences can be extracted by right clicking on one of the selected nodes and selecting "Extract Sequence List". It is also possible to Align Sequences directly by right clicking on the nodes or leaves.*

Chapter 18

General sequence analyses

Contents

CLC Main Workbench offers different kinds of sequence analyses that apply to both protein and DNA.

The analyses are described in this chapter.

18.1 Annotate with GFF/GTF/GVF file

Use **Annotate with GFF/GTF/GVF file** to add annotations from a GFF3, GTF or GVF file onto a sequence, or sequences in a sequence list. The names in the first column in the file must match the names of the sequences to be annotated. If this is not the case, either the names in the annotation file, or the names of the sequences, must be updated.

Tools are available for renaming sequences or sequences in sequence lists:

- Rename Elements, described in section 27.8
- Rename Sequences in Lists, described in section 27.9

See http://gmod.org/wiki/GFF3 for information about the GFF3 format and https://mblab.wustl.edu/GTF22.html for information on the GTF format.

How annotations are applied

Annotations from each line in the annotation file are placed on the sequence with the name given in the first column. Special treatment is given to annotations of the types CDS, exon, mRNA, transcript and gene. For these, the following applies:

- A gene annotation is generated for each gene_id. The region annotated extends from the leftmost to the rightmost positions of all annotations that have the gene_id (gtf-style).
- CDS annotations that have the same transcriptID are joined to one CDS annotation (gtf-style). Similarly, CDS annotations that have the same parent are joined to one CDS annotation (gff-style).
- If there is more than one exon annotation with the same transcriptID these are joined to one mRNA annotation. If there is only one exon annotation with a particular transcriptID, and no CDS with this transcriptID, a transcript annotation is added instead of the exon annotation (gtf-style).
- Exon annotations that have the same mRNA as parent are joined to one mRNA annotation. Similarly, exon annotations that have the same transcript as parent, are joined to one transcript annotation (gff-style).

Note that genes and transcripts are linked by name only (not by position, ID etc).

Running the tool

To run the **Annotate with GFF/GTF/GVF file** tool, go to:

Tools| General Sequence Analysis ( )| Annotate with GFF/GTF/GVF file ( )

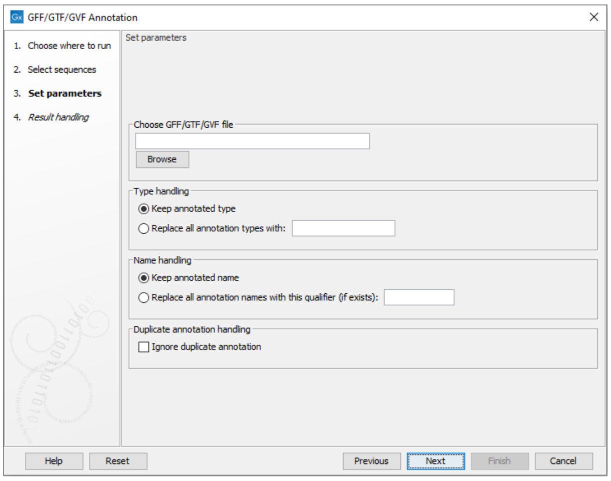
After selecting the sequence to annotate, the next step will look like that shown in figure 18.1.

Click on **Browse** to select a GFF, GTF or GVF file. After working through handling options, described below, your sequences will be annotated by the information from that file.

Name handling

Annotations are named in the following, prioritized way:

1. If one of the following qualifiers are present, it will be used for naming (prioritized):



GFF/GTF/GVF Annotation

1. Choose where to run
2. Select sequences
3. **Set parameters**
4. *Result handling*

Set parameters

Choose GFF/GTF/GVF file

Type handling
● Keep annotated type
○ Replace all annotation types with:

Name handling
● Keep annotated name
○ Replace all annotation names with this qualifier (if exists):

Duplicate annotation handling
□ Ignore duplicate annotation

Figure 18.1: *Select a GFF, GTF or GVR file by clicking on the Browse button.*

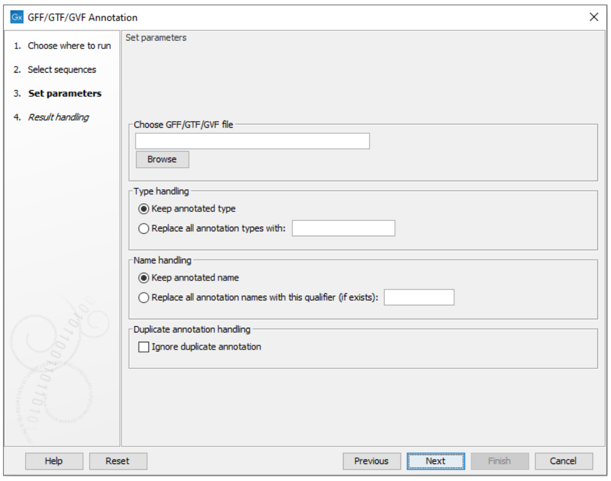
(a) Name
(b) Gene_name
(c) Gene_ID
(d) Locus_tag
(e) ID

2. If none of these are found, the annotation type will be used as name.

You can overrule this naming convention by choosing **Replace all annotation names with this qualifier** and specifying another qualifier (see figure 18.2).

If you provide a qualiifer, it must be written *identically* to the corresponding qualifier name in the annotation file.

Transcript annotations are handled separately, since they inherit the name from the gene annotation.



GFF/GTF/GVF Annotation

1. Choose where to run
2. Select sequences
3. **Set parameters**
4. *Result handling*

Set parameters

Choose GFF/GTF/GVF file

Type handling
● Keep annotated type
○ Replace all annotation types with:

Name handling
● Keep annotated name
○ Replace all annotation names with this qualifier (if exists):

Duplicate annotation handling
□ Ignore duplicate annotation

Figure 18.2: *You can choose Replace all annotation names with the specified qualifier.*

Type handling

You can overrule feature types in the annotation file by choosing **Replace all annotation types with** and specifying a type to use.

Ignore duplicate annotation

When the **Ignore duplicate annotation** option is checked, only one instance of duplicate annotations will be added to the sequence.

Create log

In the Result handling section of the wizard, check the **Create log** box results to create a log that includes information like the number of annotations found and if there are any that are could not be placed on the sequence. This information can help with troubleshooting when annotations are not added to a sequence when they were expected to be.

18.2 Extract sequences

Extract Sequences extracts all the sequences from any of the element types below into a sequence list or into individual sequence elements:

- Alignments ()
- BLAST result () For BLAST results, the sequence hits are extracted but not the original query sequence or the consensus sequence.
- BLAST overview tables ()
- Contigs and read mappings () For mappings, only the read sequences are extracted. Reference and consensus sequences are not extracted using this tool.
- Read mapping tables ()
- Read mapping tracks ()
- RNA-Seq mapping results ()
- Sequence lists () See further notes below about running this tool on sequence lists.

If only a **subset** of the sequences are of interest, create an element containing just this subset first, and then run Extract Sequences on this. See the documentation for the relevant element types for further details. For example, for extracting a subset of a mapping, see section 21.7.6.

Paired reads are extracted in accordance with the read group settings, which are specified during the original import of the reads. If the orientation has since been changed (for example using the Element Info tab for the sequence list), the read group information will be modified and reads will be extracted as specified by the modified read group. The default read group orientation is forward-reverse.

Extracting sequences from sequence lists: As all sequences will be extracted, the main reason to run this tool on a sequence list would be if you wished to create individual sequence elements from each sequence in the list. This is somewhat uncommon. If your aim is to create a list containing a subset of the sequences from another list, this can be done directly from the table view of sequence lists (see section 14.1.3), or using Split Sequence List (see section 27.7).

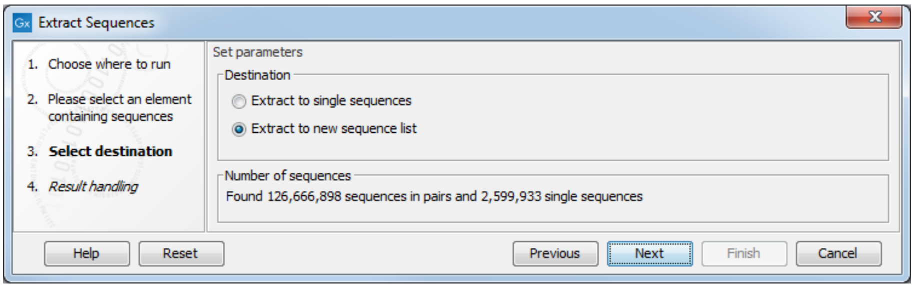
Running Extract Sequences

Launch **Extract Sequences** by going to:

Tools | **General Sequence Analysis** ()| **Extract Sequences** ()

After selecting the elements to extract sequences from, you are offered the choice of extracting them to individual sequence elements or to a sequence list (figure 18.3). For most data types, a sequence list will be the best choice.

Below these options, the number of sequences that will be extracted is reported.



Extract Sequences

1. Choose where to run
2. Please select an element containing sequences
3. **Select destination**
4. *Result handling*

Set parameters

Destination

○ Extract to single sequences

● Extract to new sequence list

Number of sequences

Found 126,666,898 sequences in pairs and 2,599,933 single sequences

Figure 18.3: *Extracted sequences can be put into a new sequence list or split into individual sequence elements.*

18.3 Shuffle sequence

In some cases, it is beneficial to shuffle a sequence, for example, when for statistical analyses when comparing an alignment score with the distribution of scores of shuffled sequences.

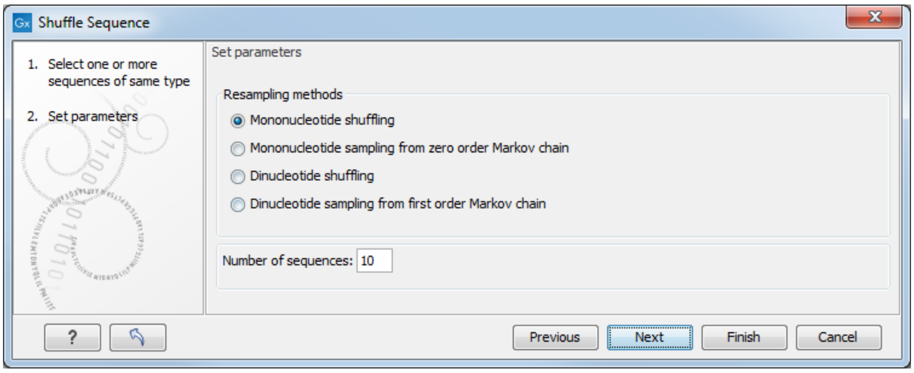
Shuffling a sequence removes all annotations that relate to the residues. To launch the **Shuffle Sequence** tool, go to:

Tools | **General Sequence Analysis** ()| **Shuffle Sequence** ()

Use the arrows to add or remove sequences or sequence lists from the selected elements list.

Click **Next** to determine how the shuffling should be performed.

In this step, shown in figure 18.4:



Shuffle Sequence

1. Select one or more sequences of same type
2. Set parameters

Set parameters

Resampling methods

● Mononucleotide shuffling

○ Mononucleotide sampling from zero order Markov chain

○ Dinucleotide shuffling

○ Dinucleotide sampling from first order Markov chain

Number of sequences: 10

Figure 18.4: *Parameters for shuffling.*

For nucleotides, the following parameters can be set:

- **Mononucleotide shuffling.** Shuffle method generating a sequence of the exact same mononucleotide frequency
- **Dinucleotide shuffling.** Shuffle method generating a sequence of the exact same dinucleotide frequency
- **Mononucleotide sampling from zero order Markov chain.** Resampling method generating a sequence of the same expected mononucleotide frequency.
- **Dinucleotide sampling from first order Markov chain.** Resampling method generating a sequence of the same expected dinucleotide frequency.

For proteins, the following parameters can be set:

- **Single amino acid shuffling.** Shuffle method generating a sequence of the exact same amino acid frequency.
- **Single amino acid sampling from zero order Markov chain.** Resampling method generating a sequence of the same expected single amino acid frequency.
- **Dipeptide shuffling.** Shuffle method generating a sequence of the exact same dipeptide frequency.
- **Dipeptide sampling from first order Markov chain.** Resampling method generating a sequence of the same expected dipeptide frequency.

For further details of these algorithms, see [Clote et al., 2005]. In addition to the shuffle method, you can specify the number of randomized sequences to output.

Click **Finish** to start the tool.

This will open a new view in the **View Area** displaying the shuffled sequence. The new sequence is not saved automatically. To save the sequence, drag it into the **Navigation Area** or press ctrl + S (⌘ + S on Mac) to activate a save dialog.

18.4 Dot plots

Dot plots provide a powerful visual comparison of two sequences. Dot plots can also be used to compare regions of similarity within a sequence.

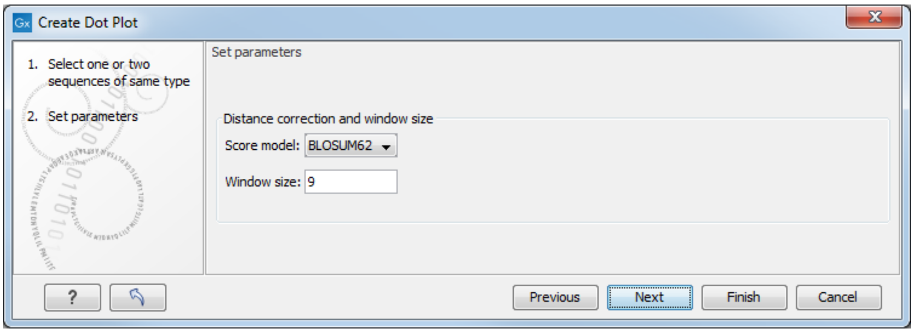
A dot plot is a simple, yet intuitive way of comparing two sequences, either DNA or protein, and is probably the oldest way of comparing two sequences [Maizel and Lenk, 1981]. A dot plot is a 2 dimensional matrix where each axis of the plot represents one sequence. By sliding a fixed size window over the sequences and making a sequence match by a dot in the matrix, a diagonal line will emerge if two identical (or very homologous) sequences are plotted against each other. Dot plots can also be used to visually inspect sequences for direct or inverted repeats or regions with low sequence complexity. Various smoothing algorithms can be applied to the dot plot calculation to avoid noisy background of the plot. Moreover, various substitution matrices can be applied in order to take the evolutionary distance of the two sequences into account.

18.4.1 Create dot plots

To create a dot plot, go to:

Tools | **General Sequence Analysis ()**| **Create Dot Plot ()**

In the dialog that opens, select a sequence and click **Next** to adjust dot plot parameters (figure 18.5).



Create Dot Plot

1. Select one or two sequences of same type
2. Set parameters

Set parameters

Distance correction and window size

Score model: BLOSUM62

Window size: 9

Figure 18.5: *Setting the dot plot parameters.*

There are two parameters for calculating the dot plot:

- **Distance correction (only valid for protein sequences)** In order to treat evolutionary transitions of amino acids, a distance correction measure can be used when calculating the dot plot. These distance correction matrices (substitution matrices) take into account the likeliness of one amino acid changing to another.

- **Window size** A residue by residue comparison (window size = 1) would undoubtedly result in a very noisy background due to a lot of similarities between the two sequences of interest. For DNA sequences the background noise will be even more dominant as a match between only four nucleotide is very likely to happen. Moreover, a residue by residue comparison (window size = 1) can be very time consuming and computationally demanding. Increasing the window size will make the dot plot more 'smooth'.

Note! Calculating dot plots takes up a considerable amount of memory in the computer. Therefore, you will see a warning message if the sum of the number of nucleotides/amino acids in the sequences is higher than 8000. If you insist on calculating a dot plot with more residues the Workbench may shut down, but still allowing you to save your work first. However, this depends on your computer's memory configuration.

Click **Finish** to start the tool.

18.4.2 View dot plots

A view of a dot plot can be seen in figure 18.6. You can select **Zoom in** () in the Toolbar and click the dot plot to zoom in to see the details of particular areas.

The **Side Panel** to the right let you specify the dot plot preferences. The gradient color box can be adjusted to get the appropriate result by dragging the small pointers at the top of the box. Moving the slider from the right to the left lowers the thresholds which can be directly seen in the dot plot, where more diagonal lines will emerge. You can also choose another color gradient by clicking on the gradient box and choose from the list.

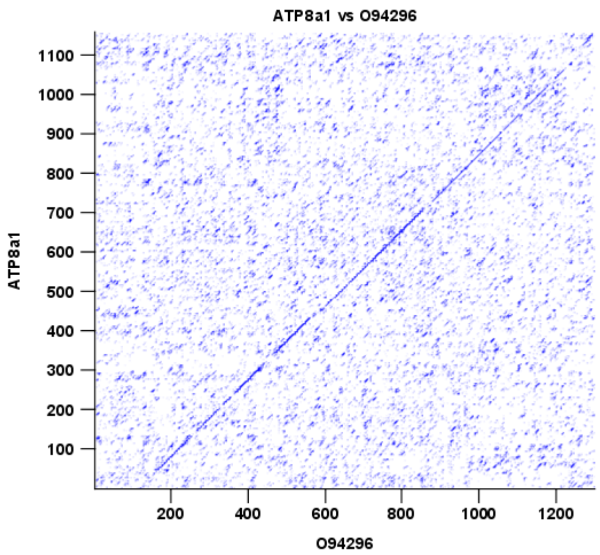


Figure 18.6: *A view is opened showing the dot plot.*

Adjusting the sliders above the gradient box is also practical, when producing an output for printing where too much background color might not be desirable. By crossing one slider over the other (the two sliders change side) the colors are inverted, allowing for a white background (figure 18.7).

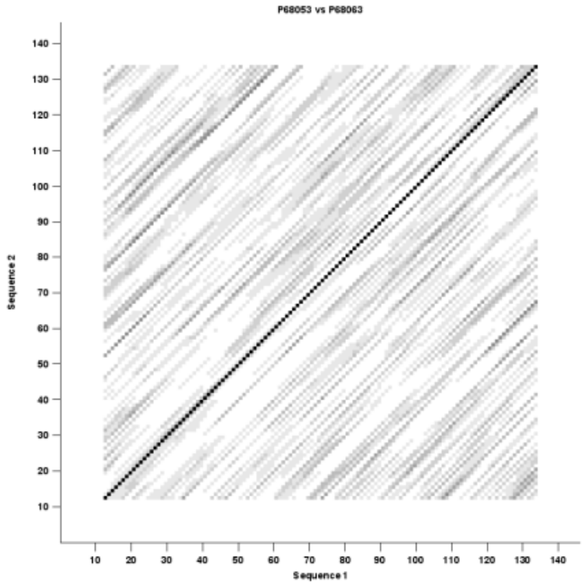


Figure 18.7: *Dot plot with inverted colors, practical for printing.*

18.4.3 Bioinformatics explained: Dot plots

Dot plots are two-dimensional plots where the x-axis and y-axis each represents a sequence and the plot itself shows a comparison of these two sequences by a calculated score for each position of the sequence. If a window of fixed size on one sequence (one axis) match to the other sequence a dot is drawn at the plot. Dot plots are one of the oldest methods for comparing two sequences [Maizel and Lenk, 1981].

The scores that are drawn on the plot are affected by several issues.

- Scoring matrix for distance correction.
  Scoring matrices (BLOSUM and PAM) contain substitution scores for every combination of two amino acids. Thus, these matrices can only be used for dot plots of protein sequences.
- Window size
  The single residue comparison (bit by bit comparison(window size = 1)) in dot plots will undoubtedly result in a noisy background of the plot. You can imagine that there are many successes in the comparison if you only have four possible residues like in nucleotide sequences. Therefore you can set a window size which is smoothing the dot plot. Instead of comparing single residues it compares subsequences of length set as window size. The score is now calculated with respect to aligning the subsequences.
- Threshold
  The dot plot shows the calculated scores with colored threshold. Hence you can better recognize the most important similarities.

Examples and interpretations of dot plots

Contrary to simple sequence alignments dot plots can be a very useful tool for spotting various evolutionary events which may have happened to the sequences of interest.

Below is shown some examples of dot plots where sequence insertions, low complexity regions, inverted repeats etc. can be identified visually.

Similar sequences The most simple example of a dot plot is obtained by plotting two homologous sequences of interest. If very similar or identical sequences are plotted against each other a diagonal line will occur.

The dot plot in figure 18.8 shows two related sequences of the Influenza A virus nucleoproteins infecting ducks and chickens. Accession numbers from the two sequences are: DQ232610 and DQ023146. Both sequences can be retrieved directly from http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi.

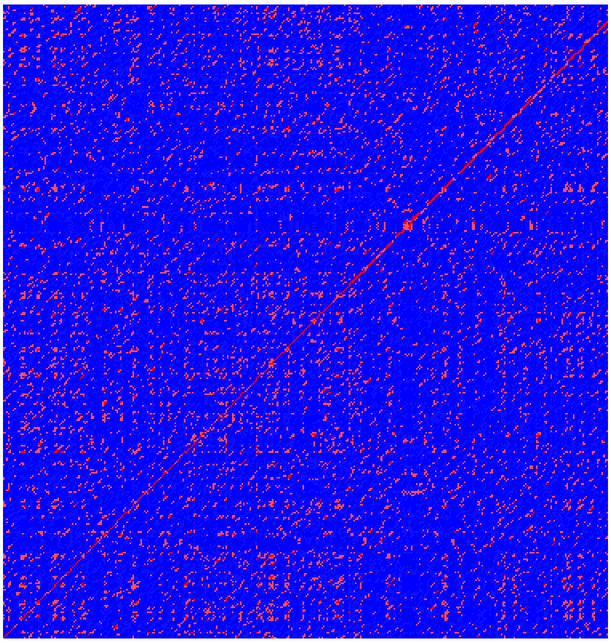


Figure 18.8: *Dot plot of DQ232610 vs. DQ023146 (Influenza A virus nucleoproteins) showing and overall similarity*

Repeated regions Sequence repeats can also be identified using dot plots. A repeat region will typically show up as lines parallel to the diagonal line.

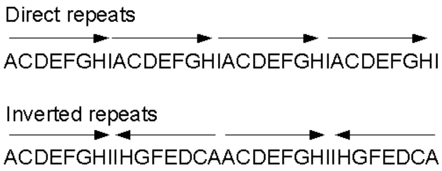

Direct repeats
ACDEFGHIACDEFGHIACDEFGHIACDEFGHI
Inverted repeats
ACDEFGHIIHGFEDCAACDEFGHIIHGFEDCA

Figure 18.9: *Direct and inverted repeats shown on an amino acid sequence generated for demonstration purposes.*

If the dot plot shows more than one diagonal in the same region of a sequence, the regions depending to the other sequence are repeated. In figure 18.10 you can see a sequence with repeats.

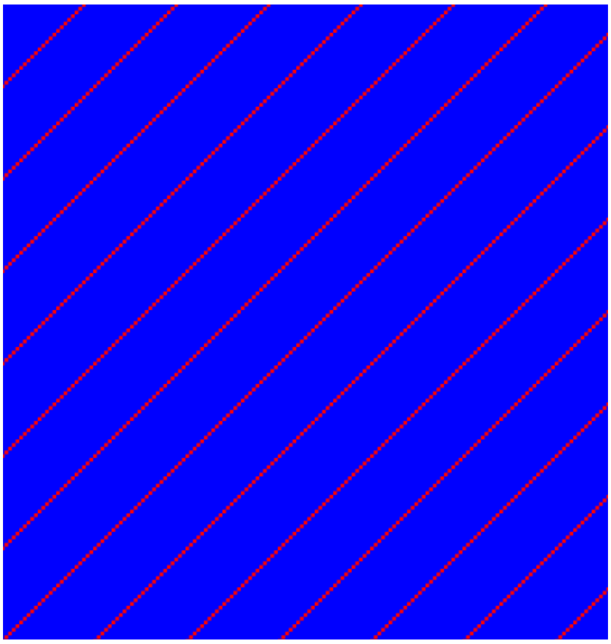


Figure 18.10: *The dot plot of a sequence showing repeated elements. See also figure 18.9.*

Frame shifts Frame shifts in a nucleotide sequence can occur due to insertions, deletions or mutations. Such frame shifts can be visualized in a dot plot as seen in figure 18.11. In this figure, three frame shifts for the sequence on the y-axis are found.

1. Deletion of nucleotides
2. Insertion of nucleotides
3. Mutation (out of frame)

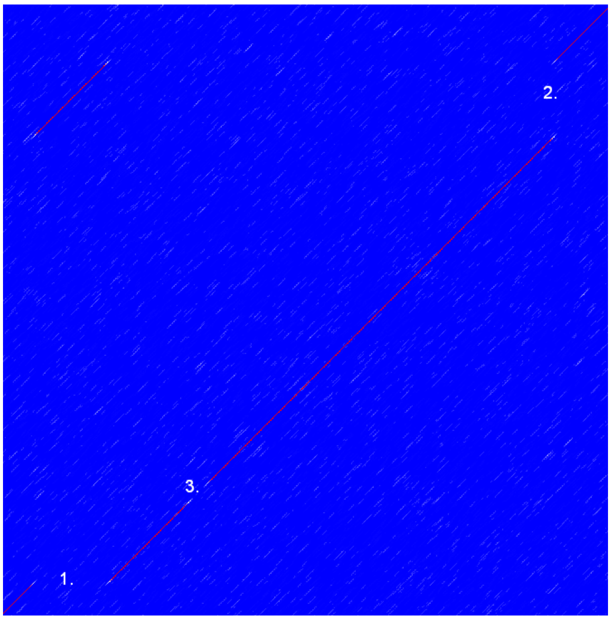


Figure 18.11: *This dot plot show various frame shifts in the sequence. See text for details.*

Sequence inversions In dot plots you can see an inversion of sequence as contrary diagonal to the diagonal showing similarity. In figure 18.12 you can see a dot plot (window length is 3) with an inversion.

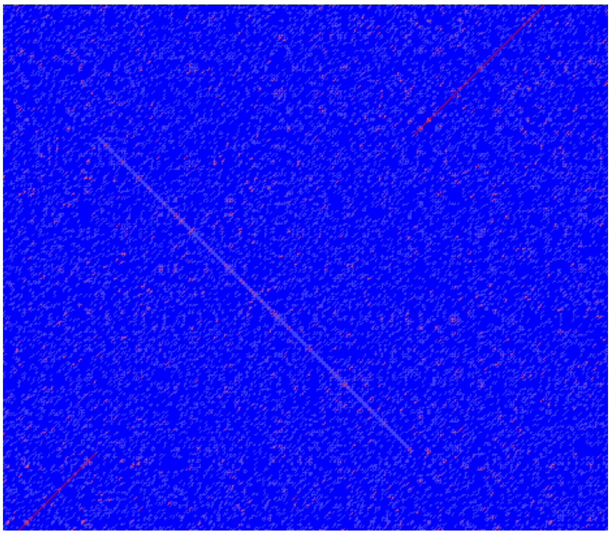


Figure 18.12: *The dot plot showing an inversion in a sequence. See also figure 18.9.*

Low-complexity regions Low-complexity regions in sequences can be found as regions around the diagonal all obtaining a high score. Low complexity regions are calculated from the redundancy of amino acids within a limited region [Wootton and Federhen, 1993]. These are most often seen as short regions of only a few different amino acids. In the middle of figure 18.13 is a square shows the low-complexity region of this sequence.

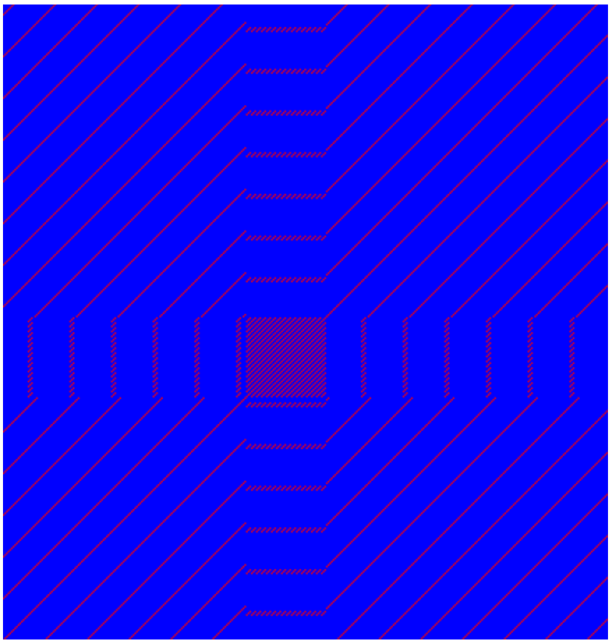


Figure 18.13: *The dot plot showing a low-complexity region in the sequence. The sequence is artificial and low complexity regions do not always show as a square.*

18.4.4 Bioinformatics explained: Scoring matrices

Biological sequences have evolved throughout time and evolution has shown that not all changes to a biological sequence is equally likely to happen. Certain amino acid substitutions (change of one amino acid to another) happen often, whereas other substitutions are very rare. For instance, tryptophan (W) which is a relatively rare amino acid, will only — on very rare occasions — mutate into a leucine (L).

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4	-1	-2	-2	0	-1	-1	0	-2	-1	-1	-1	-1	-2	-1	1	0	-3	-2	0
R	-1	5	0	-2	-3	1	0	-2	0	-3	-2	2	-1	-3	-2	-1	-1	-3	-2	-3
N	-2	0	6	1	-3	0	0	0	1	-3	-3	0	-2	-3	-2	1	0	-4	-2	-3
D	-2	-2	1	6	-3	0	2	-1	-1	-3	-4	-1	-3	-3	-1	0	-1	-4	-3	-3
C	0	-3	-3	-3	9	-3	-4	-3	-3	-1	-1	-3	-1	-2	-3	-1	-1	-2	-2	-1
Q	-1	1	0	0	-3	5	2	-2	0	-3	-2	1	0	-3	-1	0	-1	-2	-1	-2
E	-1	0	0	2	-4	2	5	-2	0	-3	-3	1	-2	-3	-1	0	-1	-3	-2	-2
G	0	-2	0	-1	-3	-2	-2	6	-2	-4	-4	-2	-3	-3	-2	0	-2	-2	-3	-3
H	-2	0	1	-1	-3	0	0	-2	8	-3	-3	-1	-2	-1	-2	-1	-2	-2	2	-3
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4	2	-3	1	0	-3	-2	-1	-3	-1	3
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4	-2	2	0	-3	-2	-1	-2	-1	1
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5	-1	-3	-1	0	-1	-3	-2	-2
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5	0	-2	-1	-1	-1	-1	1
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6	-4	-2	-2	1	3	-1
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7	-1	-1	-4	-3	-2
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4	1	-3	-2	-2
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5	-2	-2	0
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11	2	-3
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	-1
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Table 18.1: **The BLOSUM62 matrix.** A tabular view of the BLOSUM62 matrix containing all possible substitution scores [Henikoff and Henikoff, 1992].

Based on evolution of proteins it became apparent that these changes or substitutions of amino acids can be modeled by a scoring matrix also refereed to as a substitution matrix. See an example of a scoring matrix in table 18.1. This matrix lists the substitution scores of every single amino acid. A score for an aligned amino acid pair is found at the intersection of the corresponding column and row. For example, the substitution score from an arginine (R) to a lysine (K) is 2. The diagonal show scores for amino acids which have not changed. Most substitutions changes have a negative score. Only rounded numbers are found in this matrix.

The two most used matrices are the BLOSUM [Henikoff and Henikoff, 1992] and PAM [Dayhoff and Schwartz, 1978].

Different scoring matrices

- **PAM**

  The first PAM matrix (Point Accepted Mutation) was published in 1978 by Dayhoff et al. The PAM matrix was build through a global alignment of related sequences all having sequence similarity above 85% [Dayhoff and Schwartz, 1978]. A PAM matrix shows the probability that any given amino acid will mutate into another in a given time interval. As an example, PAM1 gives that one amino acid out of a 100 will mutate in a given time interval. In the other end of the scale, a PAM256 matrix, gives the probability of 256 mutations in a 100 amino acids (see figure 18.14).

There are some limitation to the PAM matrices which makes the BLOSUM matrices somewhat more attractive. The dataset on which the initial PAM matrices were build is very old by now, and the PAM matrices assume that all amino acids mutate at the same rate - this is not a correct assumption.

- **BLOSUM**

  In 1992, 14 years after the PAM matrices were published, the BLOSUM matrices (BLOcks SUbstitution Matrix) were developed and published [Henikoff and Henikoff, 1992].

  Henikoff et al. wanted to model more divergent proteins, thus they used locally aligned sequences where none of the aligned sequences share less than 62% identity. This resulted in a scoring matrix called BLOSUM62. In contrast to the PAM matrices the BLOSUM matrices are calculated from alignments without gaps emerging from the BLOCKS database `http://blocks.fhcrc.org/`.

  Sean Eddy recently wrote a paper reviewing the BLOSUM62 substitution matrix and how to calculate the scores [Eddy, 2004].

Use of scoring matrices Deciding which scoring matrix you should use in order of obtain the best alignment results is a difficult task. If you have no prior knowledge on the sequence the BLOSUM62 is probably the best choice. This matrix has become the *de facto* standard for scoring matrices and is also used as the default matrix in BLAST searches. The selection of a "wrong" scoring matrix will most probable strongly influence on the outcome of the analysis. In general a few rules apply to the selection of scoring matrices.

- For closely related sequences choose BLOSUM matrices created for highly similar alignments, like BLOSUM80. You can also select low PAM matrices such as PAM1.
- For distant related sequences, select low BLOSUM matrices (for example BLOSUM45) or high PAM matrices such as PAM250.

The BLOSUM matrices with low numbers correspond to PAM matrices with high numbers. (See figure 18.14) for correlations between the PAM and BLOSUM matrices. To summarize, if you want to find distant related proteins to a sequence of interest using BLAST, you could benefit of using BLOSUM45 or similar matrices.

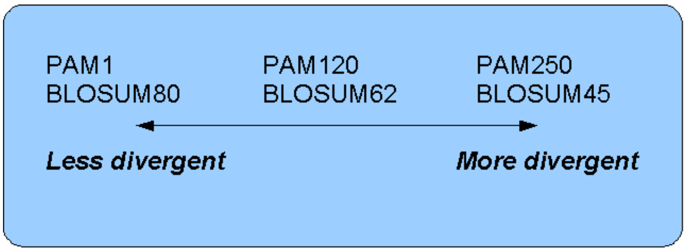


Figure 18.14: *Relationship between scoring matrices. The BLOSUM62 has become a* de facto *standard scoring matrix for a wide range of alignment programs. It is the default matrix in BLAST.*

Other useful resources BLOKS database
`http://blocks.fhcrc.org/`

NCBI help site
`http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs`

18.5 Local complexity plot

In *CLC Main Workbench* it is possible to calculate local complexity for both DNA and protein sequences. The local complexity is a measure of the diversity in the composition of amino acids within a given range (window) of the sequence. The K2 algorithm is used for calculating local complexity [Wootton and Federhen, 1993]. To conduct a complexity calculation do the following:

Tools | **General Sequence Analysis** ()| **Create Complexity Plot** ()

This opens a dialog. In **Step 1** you can use the arrows to change, remove and add DNA and protein sequences in the **Selected Elements** window.

When the relevant sequences are selected, clicking **Next** takes you to **Step 2**. This step allows you to adjust the window size from which the complexity plot is calculated. Default is set to 11 amino acids and the number should always be odd. The higher the number, the less volatile the graph.

Figure 18.15 shows an example of a local complexity plot.

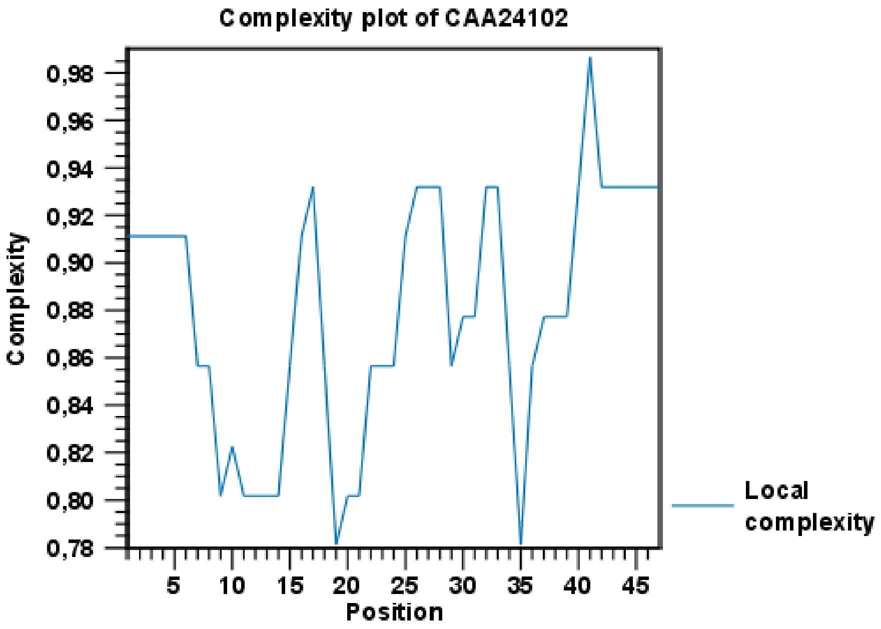


Figure 18.15: *An example of a local complexity plot.*

Click **Finish** to start the tool. The values of the complexity plot approaches 1.0 as the distribution of amino acids become more complex.

See section A in the appendix for information about the graph view.

18.6 Sequence statistics

CLC Main Workbench can produce an output with many relevant statistics for protein sequences. Some of the statistics are also relevant to produce for DNA sequences. Therefore, this section deals with both types of statistics. The required steps for producing the statistics are the same.

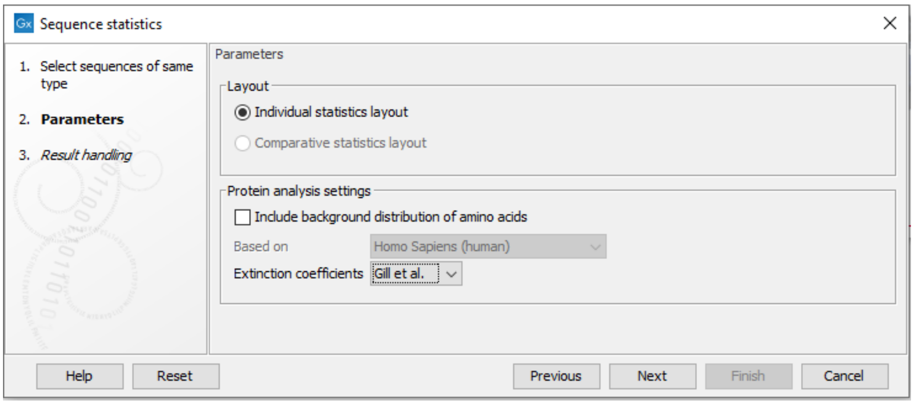
To create a statistic for the sequence, do the following:

Tools | General Sequence Analysis ( )| Create Sequence Statistics ( )

Select one or more sequence(s) or/and one or more sequence list(s). **Note!** You cannot create statistics for DNA and protein sequences at the same time, they must be run separately.

Next (figure 18.16), the dialog offers to adjust the following parameters:

- **Individual statistics layout.** If more sequences were selected in **Step 1**, this function generates separate statistics report for each sequence.
- **Comparative statistics layout.** If more sequences were selected in **Step 1**, this function generates statistics with comparisons between the sequences.



Sequence statistics

1. Select sequences of same type
2. **Parameters**
3. *Result handling*

Parameters

Layout

● Individual statistics layout

○ Comparative statistics layout

Protein analysis settings

□ Include background distribution of amino acids

Based on: Homo Sapiens (human)

Extinction coefficients: Gill et al.

Figure 18.16: *Setting parameters for the Sequence statistics tool.*

For protein seqences, you can choose to include Background distribution of amino acids. If this box is ticked, an extra column with amino acid distribution of the chosen species, is included in the table output. (The distributions are calculated from UniProt `www.uniprot.org` version 6.0, dated September 13 2005.)

You can also choose between two different sets of values for calculation of extinction coefficients:

- [Gill and von Hippel, 1989]: Ext(Cystine) = 120, Ext(Tyr) = 1280 and Ext(Trp) = 5690
- [Pace et al., 1995]: Ext(Cystine) = 125, Ext(Tyr) = 1490 and Ext(Trp) = 5500

Read more about calculation of extinction coefficients in section section 18.6.1.

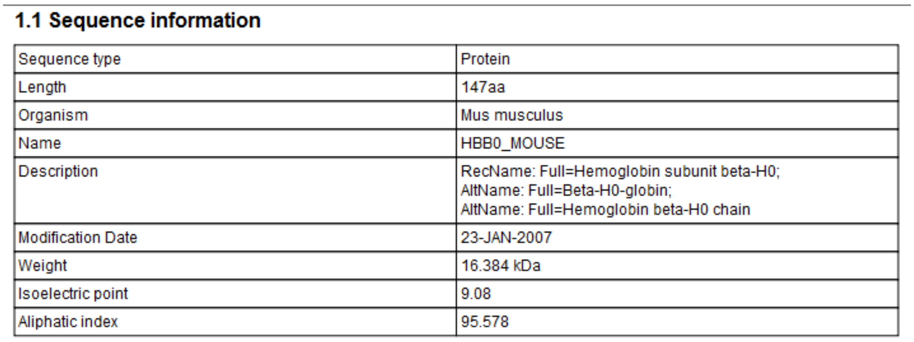
Click **Finish** to start the tool. An example of protein sequence statistics is shown in figure 18.17.

Nucleotide sequence statistics are generated using the same dialog as used for protein sequence statistics. However, the output of Nucleotide sequence statistics is less extensive than that of the protein sequence statistics.

Note! The headings of the tables change depending on whether you calculate individual or comparative sequence statistics.

The output of protein sequence statistics includes:

- **Sequence Information**:
  - Sequence type



1.1 Sequence information

Sequence type	Protein
Length	147aa
Organism	Mus musculus
Name	HBB0_MOUSE

Description	RecName: Full=Hemoglobin subunit beta-H0;
	AltName: Full=Beta-H0-globin;
	AltName: Full=Hemoglobin beta-H0 chain

Modification Date	23-JAN-2007
Weight	16.384 kDa
Isoelectric point	9.08
Aliphatic index	95.578

Figure 18.17: *Example of protein sequence statistics.*

  - Length
  - Organism
  - Name
  - Description
  - Modification Date
  - Weight. This is calculated like this: $sum_{unitsinsequence}(weight(unit)) - links * weight(H2O)$ where `links` is the sequence length minus one and `units` are amino acids. The atomic composition is defined the same way.
  - Isoelectric point
  - Aliphatic index

- Amino acid counts, frequencies

- Annotation counts

The output of nucleotide sequence statistics include:

- General statistics:
  - Sequence type
  - Length
  - Organism
  - Name
  - Description
  - Modification Date
  - Weight (calculated as single-stranded and double-stranded DNA)

- Annotation table

- Nucleotide distribution table

If nucleotide sequences are used as input, and these are annotated with CDS, a section on codon statistics for coding regions is included. This represents statistics for all codons; however, only codons that contribute with amino acids to the translated sequence will be counted.

A short description of the different areas of the statistical output is given in section 18.6.1.

18.6.1 Bioinformatics explained: Protein statistics

Every protein holds specific and individual features which are unique to that particular protein. Features such as isoelectric point or amino acid composition can reveal important information of a novel protein. Many of the features described below are calculated in a simple way.

- **Molecular weight** The molecular weight is the mass of a protein or molecule. The molecular weight is simply calculated as the sum of the atomic mass of all the atoms in the molecule.

  The weight of a protein is usually represented in Daltons (Da).

  A calculation of the molecular weight of a protein does not usually include additional post-translational modifications. For native and unknown proteins it tends to be difficult to assess whether posttranslational modifications such as glycosylations are present on the protein, making a calculation based solely on the amino acid sequence inaccurate. The molecular weight can be determined very accurately by mass-spectrometry in a laboratory.

- **Isoelectric point** The isoelectric point (pI) of a protein is the pH where the proteins has no net charge. The pI is calculated from the pKa values for 20 different amino acids. At a pH below the pI, the protein carries a positive charge, whereas if the pH is above pI the proteins carry a negative charge. In other words, pI is high for basic proteins and low for acidic proteins. This information can be used in the laboratory when running electrophoretic gels. Here the proteins can be separated, based on their isoelectric point.

- **Aliphatic index** The aliphatic index of a protein is a measure of the relative volume occupied by aliphatic side chain of the following amino acids: alanine, valine, leucine and isoleucine. An increase in the aliphatic index increases the thermostability of globular proteins. The index is calculated by the following formula.

  $Aliphaticindex = X(Ala) + a * X(Val) + b * X(Leu) + b * (X)Ile$

  X(Ala), *X(Val)*, *X(Ile)* and *X(Leu)* are the amino acid compositional fractions. The constants a and b are the relative volume of valine (a=2.9) and leucine/isoleucine (b=3.9) side chains compared to the side chain of alanine [Ikai, 1980].

- **Estimated half-life** The half life of a protein is the time it takes for the protein pool of that particular protein to be reduced to the half. The half life of proteins is highly dependent on the presence of the N-terminal amino acid, thus overall protein stability [Bachmair et al., 1986,Gonda et al., 1989,Tobias et al., 1991]. The importance of the N-terminal residues is generally known as the 'N-end rule'. The N-end rule and consequently the N-terminal amino acid, simply determines the half-life of proteins. The estimated half-life of proteins have been investigated in mammals, yeast and *E. coli* (see Table 18.2). If leucine is found N-terminally in mammalian proteins the estimated half-life is 5.5 hours.

- **Extinction coefficient** This measure indicates how much light is absorbed by a protein at a particular wavelength. The extinction coefficient is measured by UV spectrophotometry, but can also be calculated. The amino acid composition is important when calculating the extinction coefficient. The extinction coefficient is calculated from the absorbance of cysteine, tyrosine and tryptophan.

  Two values are reported. The first value, "Non-reduced cysteines", is computed assuming that all cysteine residues appear as half cystines, meaning they form di-sulfide bridges to other cysteines:

Amino acid	**Mammalian**	**Yeast**	**E. coli**
Ala (A)	4.4 hour	>20 hours	>10 hours
Cys (C)	1.2 hours	>20 hours	>10 hours
Asp (D)	1.1 hours	3 min	>10 hours
Glu (E)	1 hour	30 min	>10 hours
Phe (F)	1.1 hours	3 min	2 min
Gly (G)	30 hours	>20 hours	>10 hours
His (H)	3.5 hours	10 min	>10 hours
Ile (I)	20 hours	30 min	>10 hours
Lys (K)	1.3 hours	3 min	2 min
Leu (L)	5.5 hours	3 min	2 min
Met (M)	30 hours	>20 hours	>10 hours
Asn (N)	1.4 hours	3 min	>10 hours
Pro (P)	>20 hours	>20 hours	?
Gln (Q)	0.8 hour	10 min	>10 hours
Arg (R)	1 hour	2 min	2 min
Ser (S)	1.9 hours	>20 hours	>10 hours
Thr (T)	7.2 hours	>20 hours	>10 hours
Val (V)	100 hours	>20 hours	>10 hours
Trp (W)	2.8 hours	3 min	2 min
Tyr (Y)	2.8 hours	10 min	2 min

Table 18.2: **Estimated half life.** Half life of proteins where the N-terminal residue is listed in the first column and the half-life in the subsequent columns for mammals, yeast and *E. coli*.

$$\mathrm{Ext}(Protein) = \frac{\mathrm{count}(Cys)}{2} \cdot \mathrm{Ext}(Cys) + \mathrm{count}(Tyr) \cdot \mathrm{Ext}(Tyr) + \mathrm{count}(Trp) \cdot \mathrm{Ext}(Trp).$$

The second value, "Reduced cysteines", assumes that no di-sulfide bonds are formed:

$$\mathrm{Ext}(Protein) = \mathrm{count}(Tyr) \cdot \mathrm{Ext}(Tyr) + \mathrm{count}(Trp) \cdot \mathrm{Ext}(Trp).$$

The extinction coefficient values of the three important amino acids at different wavelengths are found in [Gill and von Hippel, 1989] or in [Pace et al., 1995]. At 280nm the extinction coefficients are

- [Gill and von Hippel, 1989]: Ext(Cystine) = 120, Ext(Tyr) = 1280 and Ext(Trp) = 5690
- [Pace et al., 1995]: Ext(Cystine) = 125, Ext(Tyr) = 1490 and Ext(Trp) = 5500

This equation is only valid under the following conditions:

- pH 6.5
- 6.0 M guanidium hydrochloride
- 0.02 M phosphate buffer

Knowing the extinction coefficient, the absorbance (optical density) can be calculated using the following formula: $Absorbance(Protein) = \frac{Ext(Protein)}{Molecular\ weight}$

- **Atomic composition** Amino acids are indeed very simple compounds. All 20 amino acids consist of combinations of only five different atoms. The atoms which can be found in these simple structures are: Carbon, Nitrogen, Hydrogen, Sulfur, Oxygen. The atomic composition of a protein can for example be used to calculate the precise molecular weight of the entire protein.

- **Total number of negatively charged residues (Asp + Glu)** At neutral pH, the fraction of negatively charged residues provides information about the location of the protein. Intracellular proteins tend to have a higher fraction of negatively charged residues than extracellular proteins.

- **Total number of positively charged residues (Arg + Lys)** At neutral pH, nuclear proteins have a high relative percentage of positively charged amino acids. Nuclear proteins often bind to the negatively charged DNA, which may regulate gene expression or help to fold the DNA. Nuclear proteins often have a low percentage of aromatic residues [Andrade et al., 1998].

- **Amino acid distribution** Amino acids are the basic components of proteins. The amino acid distribution in a protein is simply the percentage of the different amino acids represented in a particular protein of interest. Amino acid composition is generally conserved through family-classes in different organisms which can be useful when studying a particular protein or enzymes across species borders. Another interesting observation is that amino acid composition variate slightly between proteins from different subcellular localizations. This fact has been used in several computational methods, used for prediction of subcellular localization.

- **Annotation table** This table provides an overview of all the different annotations associated with the sequence and their incidence.

- **Dipeptide distribution** This measure is simply a count, or frequency, of all the observed adjacent pairs of amino acids (dipeptides) found in the protein. It is only possible to report neighboring amino acids. Knowledge on dipeptide composition have previously been used for prediction of subcellular localization.

18.7 Join Sequences

CLC Main Workbench can join several nucleotide or protein sequences into one sequence. This feature can for example be used to construct "supergenes" for phylogenetic inference by joining several disjoint genes into one. Note, that when sequences are joined, all their annotations are carried over to the new spliced sequence.

Two (or more) sequences can be joined using the **Join Sequences** tool, available at:

> **Tools** | **Classical Sequence Analysis** ( ) | **General Sequence Analyses** ( ) | **Join Sequences** ( )

This opens the dialog shown in figure 18.18.

Use the arrows to add or remove sequences from the selected elements list.

In the next wizard step, you can define the order in which the sequences should be joined (figure 18.19).

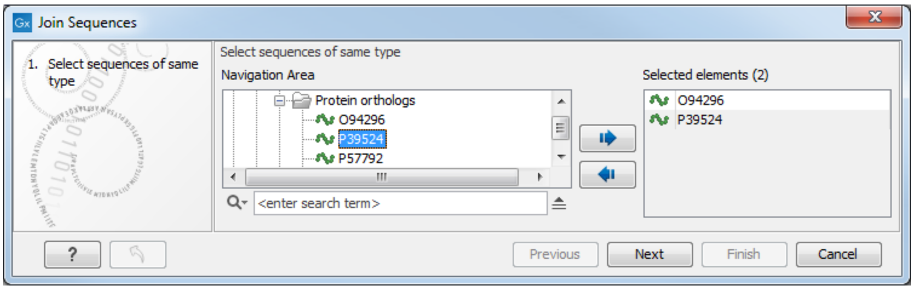


Figure 18.18: *Selecting two sequences to be joined.*

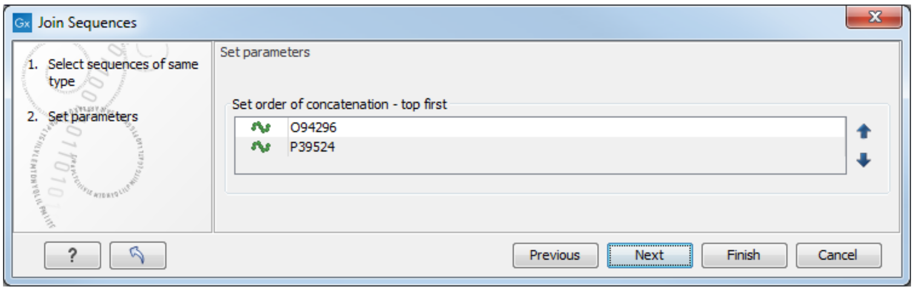


Figure 18.19: *Setting the order in which sequences are joined.*

In step 2 you can change the order in which the sequences will be joined. Select a sequence and use the arrows to move the selected sequence up or down.

Click **Finish** to start the tool.

The result is shown in figure 18.20.

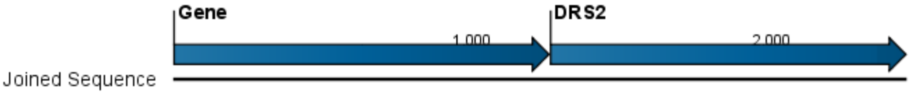


Figure 18.20: *The result of joining sequences is a new sequence containing the annotations of the joined sequences (they each had a HBB annotation).*

18.8 Pattern discovery

With *CLC Main Workbench* you can perform pattern discovery on both DNA and protein sequences. Advanced hidden Markov models can help to identify unknown sequence patterns across single or even multiple sequences.

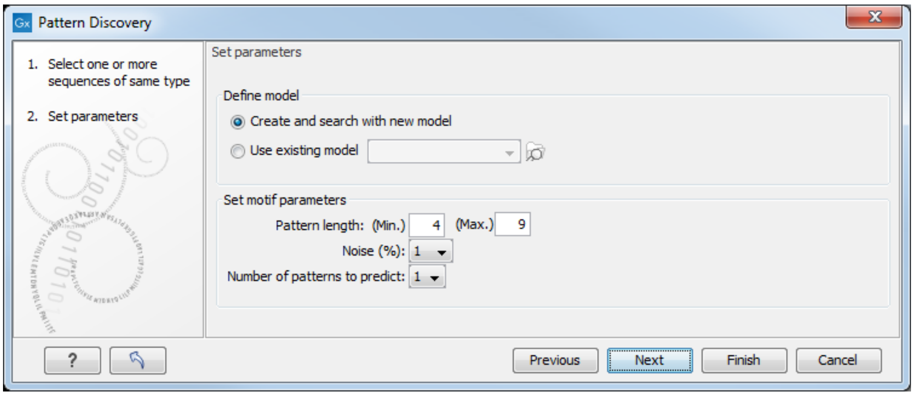
In order to search for unknown patterns:

Tools | **General Sequence Analysis** ()| **Pattern Discovery** ()

Choose one or more sequence(s) or sequence list(s). You can perform the analysis on several DNA or several protein sequences at a time. If the analysis is performed on several sequences at a time the method will search for patterns which is common between all the sequences. Annotations will be added to all the sequences and a view is opened for each sequence.

Click **Next** to adjust parameters (see figure 18.21).

In order to search unknown sequences with an already existing model:



Define model

● Create and search with new model

○ Use existing model

Set motif parameters

Pattern length: (Min.) 4 (Max.) 9

Noise (%): 1

Number of patterns to predict: 1

Figure 18.21: *Setting parameters for the pattern discovery. See text for details.*

Select to use an already existing model which is seen in figure 18.21. Models are represented with the following icon in the **Navigation Area** ( ).

18.8.1 Pattern discovery search parameters

Various parameters can be set prior to the pattern discovery. The parameters are listed below and a screenshot of the parameter settings can be seen in figure 18.21.

- **Create and search with new model.** This will create a new HMM model based on the selected sequences. The found model will be opened after the run and presented in a table view. It can be saved and used later if desired.
- **Use existing model.** It is possible to use already created models to search for the same pattern in new sequences.
- **Minimum pattern length.** Here, the minimum length of patterns to search for, can be specified.
- **Maximum pattern length.** Here, the maximum length of patterns to search for, can be specified.
- **Noise (%).** Specify noise-level of the model. This parameter has influence on the level of degeneracy of patterns in the sequence(s). The noise parameter can be 1,2,5 or 10 percent.
- **Number of different kinds of patterns to predict.** Number of iterations the algorithm goes through. After the first iteration, we force predicted pattern-positions in the first run to be member of the background: In that way, the algorithm finds new patterns in the second iteration. Patterns marked 'Pattern1' have the highest confidence. The maximal iterations to go through is 3.
- **Include background distribution.** For protein sequences it is possible to include information on the background distribution of amino acids from a range of organisms.

Click **Finish** to start the tool. This will open a view showing the patterns found as annotations on the original sequence (see figure 18.22). If you have selected several sequences, a corresponding number of views will be opened.

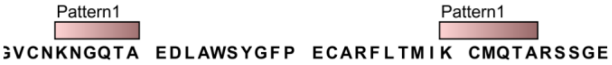


Figure 18.22: *Sequence view displaying two discovered patterns.*

18.8.2 Pattern search output

If the analysis is performed on several sequences at a time the method will search for patterns in the sequences and open a new view for each of the sequences, in which a pattern was discovered. Each novel pattern will be represented as an annotation of the type **Region**. More information on each found pattern is available through the tool-tip, including detailed information on the position of the pattern and quality scores.

It is also possible to get a tabular view of all found patterns in one combined table. Then each found pattern will be represented with various information on obtained scores, quality of the pattern and position in the sequence.

A table view of emission values of the actual used HMM model is presented in a table view. This model can be saved and used to search for a similar pattern in new or unknown sequences.

18.9 Motif Search

Known motifs in nucleotide or peptide sequences can be searched for using a specific sequence or, more flexibly, by using a regular expression.

There are two ways to search for known motifs:

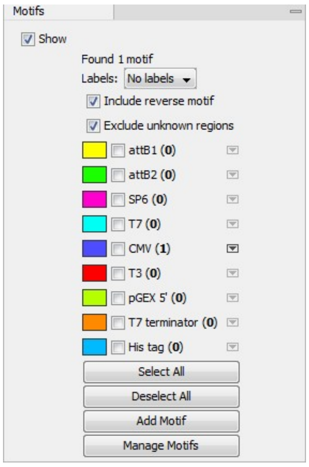
- **Search in an open sequence** Common motifs and custom motifs can be quickly scanned for and visualized on a sequence while working with that sequence interactively. See section 18.9.1 for details.
- **Use the Motif Search tool** A more refined and systematic search for motifs can be performed using the **Motif Search** tool. This generates a table and can optionally add annotations to the sequences. See section section 18.9.2 for details.

18.9.1 Dynamic motifs

In the Side Panel settings of a sequence that is open for viewing, there is a **Motifs** palette (figure 18.23). Using these options, motifs can be added to or removed from the list, as well as moved up and down in the list. All listed motifs are searched for, and the number of instances found is reported in brackets by the motif name.

If the box by a particular motif is checked, that motif will be highlighted on the sequence itself (figure 18.24). By default, a motif is shown as a faded arrow, where the direction of the arrow indicates the strand of the motif. Hovering the mouse cursor over a motif on the sequence reveals information about the motif (figure 18.25).

To add **Labels** to the motif, select the **Flag** or **Stacked** option. They will put the name of the motif as a flag above the sequence. The stacked option will stack the labels when there is more than one motif so that all labels are shown.



Motifs

☑ Show

Found 1 motif

Labels: No labels

☑ Include reverse motif

☑ Exclude unknown regions

🟨 □ attB1 (0)

🟩 □ attB2 (0)

🟪 □ SP6 (0)

🟦 □ T7 (0)

🟦 □ CMV (1)

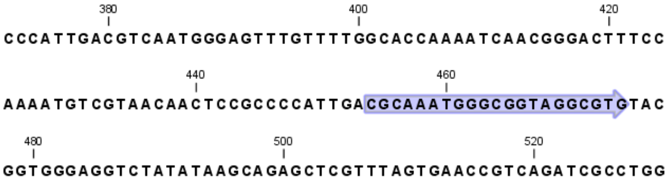
🟥 □ T3 (0)

🟩 □ pGEX 5' (0)

🟧 □ T7 terminator (0)

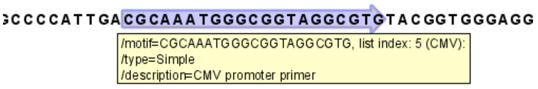
🟦 □ His tag (0)

Figure 18.23: *The Motifs palette of the Side Panel of an open sequence. A single instance of the CMV motif has been detected.*



380
400
420
CCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCC
440
460
AAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTAC
480
500
520
GGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGG

Figure 18.24: *When the box next to a motif type is checked, any instances of that motif in a sequence will be highlighted in the view.*



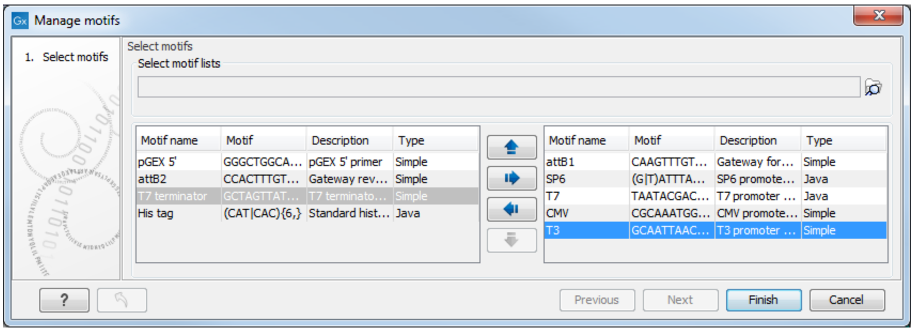
/motif=CGCAAATGGGCGGTAGGCGTG, list index: 5 (CMV):
/type=Simple
/description=CMV promoter primer

Figure 18.25: *Hover the mouse cursor over a motif region on the sequence to reveal a tool tip with information about the motif.*

Below the labels option there are two options for controlling the way the sequence should be searched for motifs:

- **Include reverse motifs**. This will also find motifs on the negative strand (only available for nucleotide sequences)
- **Exclude matches in N-regions for simple motifs.** The motif search handles ambiguous characters in the way that two residues are different if they do not have any residues in common. For example: For nucleotides, *N* matches any character and *R* matches *A,G*. For proteins, *X* matches any character and *Z* matches *E,Q*. Genome sequence often have large regions with unknown sequence. These regions are very often padded with N's. Ticking this checkbox will not display hits found in N-regions and if a one residue in a motif matches to an N, it will be treated as a mismatch.

The list of motifs shown in figure 18.23 is a pre-defined list that is included with the workbench, but you can define your own set of motifs to use instead. In order to do this, you can either launch the Create Motif List tool from the Navigation Area or using the **Add Motif** button in the Side Panel (see section 18.10). Once your list of custom motif(s) is saved, you can click the **Manage Motifs** button in the Side Panel, which will bring up the dialog shown in figure 18.26.



Motif name	Motif	Description	Type
pGEX 5'	GGGCTGGCA...	pGEX 5' primer	Simple
attB2	CCACTTTGT...	Gateway rev...	Simple
T7 terminator	GCTAGTTAT...	T7 terminato...	Simple
His tag	(CAT|CAC){6,}	Standard hist...	Java

Motif name	Motif	Description	Type
attB1	CAAGTTTGT...	Gateway for...	Simple
SP6	(G|T)ATTTA...	SP6 promote...	Java
T7	TAATACGAC...	T7 promoter ...	Java
CMV	CGCAAATGG...	CMV promote...	Simple
T3	GCAATTAAC...	T3 promoter ...	Simple

Figure 18.26: *Managing the motifs to display*

At the top, select a motif list by clicking the **Browse** ( ) button. When the motif list is selected, its motifs are listed in the panel in the left-hand side of the dialog. The right-hand side panel contains the motifs that will be listed in the **Side Panel** when you click **Finish**.

See section section 18.9.2 for a non-interactive option for detecting motifs.

18.9.2 The Motif Search tool

The **Motif Search** tool supports searching for motifs in nucleotide or peptide sequences, including in alignments. Motifs to search for can be provided as simple text, as regular expressions or in lists, and a match accuracy for a successful match can be defined. Searches on the negative strand of nucleotide sequences is optional. Results can be added as annotation to the sequences, and a table of results can also be output.

To start **Motif Search** tool, go to:

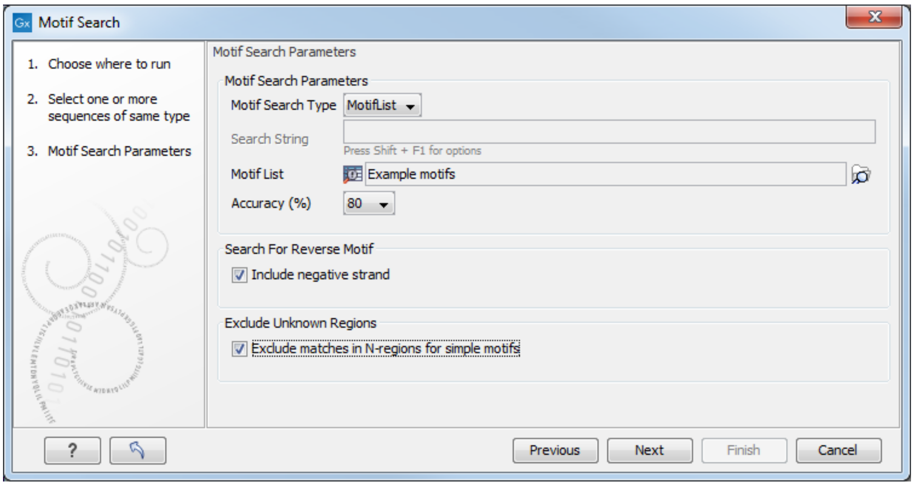
Tools | General Sequence Analysis ( )| Motif Search ( )

In the launch wizard, select the sequences, sequence lists, or alignments from the Navigation Area.

Search options are provided in the "Motif Search Parameters" wizard step (see figure 18.27).

The Motif Search options are:

- **Motif types.** Choose what kind of motif to be used:
  - Simple motif. Choosing this option means that you enter a simple motif, e.g. ATGATGNNATG.
  - Java regular expression. See section 18.9.3.
  - Prosite regular expression. For proteins, you can enter different protein patterns from the PROSITE database (protein patterns using regular expressions and describing specific amino acid sequences). The PROSITE database contains a great number of patterns and have been used to identify related proteins (see https://prosite.expasy.org/cgi-bin/prosite/prosite-list.pl).


Motif Search

1. Choose where to run
2. Select one or more sequences of same type
3. Motif Search Parameters

Motif Search Parameters

Motif Search Parameters

Motif Search Type: MotifList

Search String:

Press Shift + F1 for options

Motif List: Example motifs

Accuracy (%): 80

Search For Reverse Motif

☑ Include negative strand

Exclude Unknown Regions

☑ Exclude matches in N-regions for simple motifs

Figure 18.27: *Specifying the options for the Motif Search tool.*

- Use motif list. Clicking the small button ( ) will allow you to select a saved motif list (see section 18.10).

- **Motif.** If you choose to search with a simple motif, you should enter a literal string as your motif. Ambiguous amino acids and nucleotides are allowed. Example; ATGATGNNATG. If your motif type is Java regular expression, you should enter a regular expression according to the syntax rules described in section 18.9.3. Press **Shift + F1** key for options. For proteins, you can search with a Prosite regular expression and you should enter a protein pattern from the PROSITE database.

- **Accuracy.** If you search with a simple motif, you can adjust the accuracy of the motif to the match on the sequence. If you type in a simple motif and let the accuracy be 80%, the motif search algorithm runs through the input sequence and finds all subsequences of the same length as the simple motif such that the fraction of identity between the subsequence and the simple motif is at least 80%. A motif match is added to the sequence as an annotation with the exact fraction of identity between the subsequence and the simple motif. If you use a list of motifs, the accuracy applies only to the simple motifs in the list.

- **Search for reverse motif.** This enables searching on the negative strand on nucleotide sequences.

- **Exclude unknown regions.** Genome sequence often have large regions with unknown sequence. These regions are very often padded with N's. Ticking this checkbox will not display hits found in N-regions.Motif search handles ambiguous characters in the way that two residues are different if they do not have any residues in common. For example: For nucleotides, *N* matches any character and *R* matches *A,G*. For proteins, *X* matches any character and *Z* matches *E,Q*.

Click **Next** to adjust how to handle the results and then click **Finish**. There are multiple types of results that can be produced:

- **Create report**. This will create a report with summary information about motifs found.

- **Create table**. This will create an overview table of all the motifs found for all the input sequences.

- **Add annotations to sequences**. This will add an annotation to the sequence when a motif is found (an example is shown in figure 18.28). For details on viewing annotations see section 14.3.1.

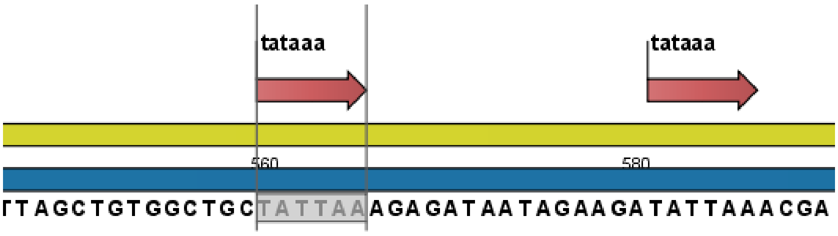


Figure 18.28: *Sequence view displaying the pattern found. The search string was 'tataaa'.*

18.9.3 Java regular expressions

A regular expressions is a string that describes or matches a set of strings, according to certain syntax rules. They are usually used to give a concise description of a set, without having to list all elements. The simplest form of a regular expression is a literal string. The syntax used for the regular expressions is the Java regular expression syntax (see `https://java.sun.com/docs/books/tutorial/essential/regex/index.html`). Below is listed some of the most important syntax rules which are also shown in the help pop-up when you press Shift + F1:

[A-Z] will match the characters *A* through *Z* (Range). You can also put single characters between the brackets: The expression *[AGT]* matches the characters *A*, *G* or *T*.

[A-D[M-P]] will match the characters *A* through *D* and *M* through *P* (Union). You can also put single characters between the brackets: The expression *[AG[M-P]]* matches the characters *A*, *G* and *M* through *P*.

[A-M&&[H-P]] will match the characters between *A* and *M* lying between *H* and *P* (Intersection). You can also put single characters between the brackets. The expression *[A-M&&[HGTDA]]* matches the characters *A* through *M* which is *H*, *G*, *T*, *D* or *A*.

[ˆA-M] will match any character except those between *A* and *M* (Excluding). You can also put single characters between the brackets: The expression *[ˆAG]* matches any character except *A* and *G*.

[A-Z&&[ˆM-P]] will match any character *A* through *Z* except those between *M* and *P* (Subtraction). You can also put single characters between the brackets: The expression *[A-P&&[ˆCG]]* matches any character between *A* and *P* except *C* and *G*.

The symbol . matches any character.

X{n} will match a repetition of an element indicated by following that element with a numerical value or a numerical range between the curly brackets. For example, *ACG{2}* matches the string *ACGG* and *(ACG){2}* matches *ACGACG*.

X{n,m} will match a certain number of repetitions of an element indicated by following that element with two numerical values between the curly brackets. The first number is a lower limit on the number of repetitions and the second number is an upper limit on the number of repetitions. For example, *ACT{1,3}* matches *ACT, ACTT* and *ACTTT*.

X{n,} represents a repetition of an element at least *n* times. For example, *(AC){2,}* matches all strings *ACAC*, *ACACAC*, *ACACACAC*,...

The symbol ^ restricts the search to the beginning of your sequence. For example, if you search through a sequence with the regular expression *^AC*, the algorithm will find a match if *AC* occurs in the beginning of the sequence.

The symbol *$* restricts the search to the end of your sequence. For example, if you search through a sequence with the regular expression *GT$*, the algorithm will find a match if *GT* occurs in the end of the sequence.

Examples
The expression *[ACG][^AC]G{2}* matches all strings of length *4*, where the first character is *A,C* or *G* and the second is any character except *A,C* and the third and fourth character is *G*. The expression *G.[^A]$* matches all strings of length *3* in the end of your sequence, where the first character is *C*, the second any character and the third any character except *A*.

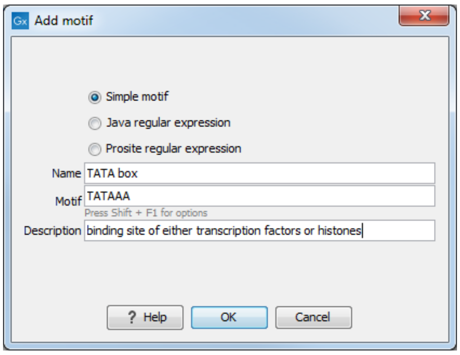
18.10 Create motif list

CLC Main Workbench offers advanced and versatile options to create lists of sequence patterns or known motifs, represented either by a literal string or a regular expression.

A motif list can be created using:

Tools | **General Sequence Analysis** ()| **Create Motif List** ()

Click on the **Add** () button at the bottom of the view. This will open a dialog shown in figure 18.29.



Add motif

● Simple motif
○ Java regular expression
○ Prosite regular expression

Name: TATA box
Motif: TATAAA
Press Shift + F1 for options
Description: binding site of either transcription factors or histones

Figure 18.29: *Entering a new motif in the list.*

In this dialog, you can enter the following information:

- **Name**. The name of the motif. In the result of a motif search, this name will appear as the name of the annotation and in the result table.
- **Motif**. The actual motif. See section 18.9.2 for more information about the syntax of motifs.

- **Description**. You can enter a description of the motif. In the result of a motif search, the description will appear in the result table and will be added as a note to the annotation on the sequence (visible in the **Annotation table** () or by placing the mouse cursor on the annotation).
- **Type**. You can enter three different types of motifs: Simple motifs, java regular expressions or PROSITE regular expression. Read more in section 18.9.2.

The motif list can contain a mix of different types of motifs. This is practical because some motifs can be described with the simple syntax, whereas others need the more advanced regular expression syntax.

Instead of manually adding motifs, you can **Import From Fasta File** (). This will show a dialog where you can select a fasta file on your computer and use this to create motifs. This will automatically take the name, description and sequence information from the fasta file, and put it into the motif list. The motif type will be "simple". Note that reformatting Prosite file into FASTA format for import will fail, as only simple motifs can be imported this way and regular expressions are not supported.

Besides adding new motifs, you can also edit and delete existing motifs in the list. To edit a motif, either double-click the motif in the list, or select and click the **Edit** () button at the bottom of the view.

To delete a motif, select it and press the Delete key on the keyboard. Alternatively, click **Delete** () in the **Tool bar**.

Save the motif list in the **Navigation Area**, and you will be able to use for Motif Search () (see section 18.9).

Chapter 19

Nucleotide analyses

Contents

CLC Main Workbench offers different kinds of sequence analyses, which only apply to DNA and RNA.

19.1 Convert DNA to RNA

Use the **Convert DNA to RNA** tool to convert a DNA sequence into an RNA sequence, substituting the T residues (Thymine) for U residues (Uracil). It is available at:

Tools | **Nucleotide Analysis** ()| **Convert DNA to RNA** ()

This opens the dialog displayed in figure 19.1:

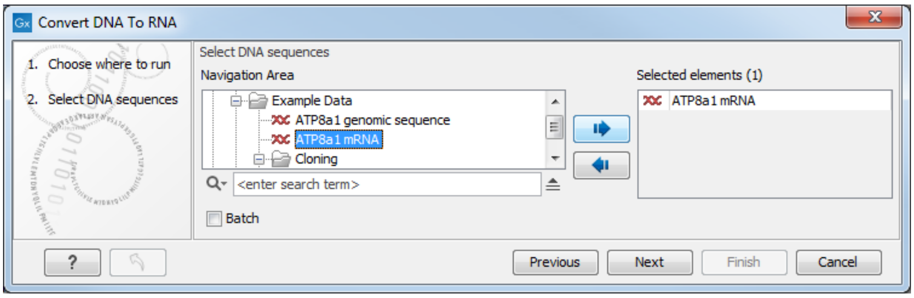


Figure 19.1: *Translating DNA to RNA.*

Use the arrows to add or remove sequences or sequence lists from the selected elements list.

You can select multiple DNA sequences and sequence lists for conversion. If a sequence list contains RNA sequences, those sequences will not be converted.

Click **Finish** to start the tool.

19.2 Convert RNA to DNA

Use the **Convert RNA to DNA** tool to convert an RNA sequence into DNA, substituting the U residues (Uracil) for T residues (Thymine). It is available at:

Tools | Nucleotide Analysis ( )| Convert RNA to DNA ( )

This opens the dialog displayed in figure 19.2:

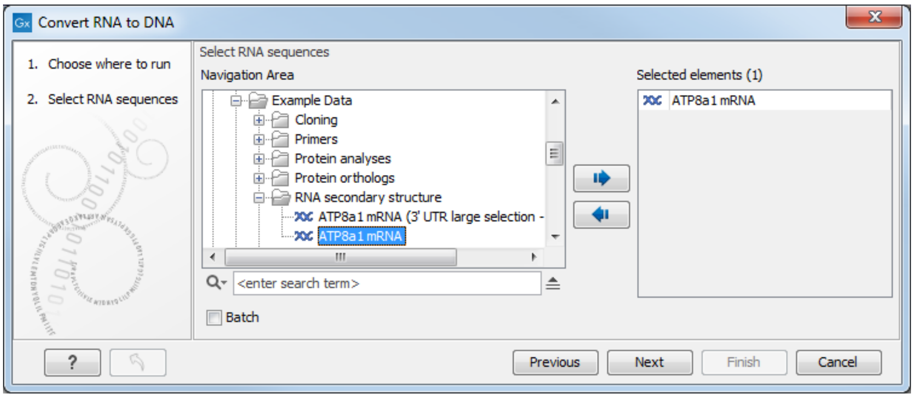


Figure 19.2: *Translating RNA to DNA.*

Use the arrows to add or remove sequences or sequence lists from the selected elements list.

You can select multiple RNA sequences and sequence lists for conversion. If a selected sequence list contains DNA sequences, those sequences will not be converted.

Click **Finish** to start the tool.

This will open a new view in the **View Area** displaying the new DNA sequence. The new sequence is not saved automatically. To save the sequence, drag it into the **Navigation Area** or press Ctrl + S (⌘ + S on Mac) to activate a save dialog.

19.3 Reverse complements of sequences

CLC Main Workbench is able to create the reverse complement of a nucleotide sequence. By doing that, a new sequence is created which also has all the annotations reversed since they now occupy the opposite strand of their previous location.

To quickly obtain the reverse complement of a sequence or part of a sequence, you may select a region on the negative strand and open it in a new view:

right-click a selection on the negative strand | Open selection in New View ( )

By doing that, the sequence will be reversed. This is only possible when the double stranded view option is enabled. It is possible to copy the selection and paste it in a word processing program or an e-mail. To obtain a reverse complement of an entire sequence:

Tools | Nucleotide Analysis ( )| Reverse Complement Sequence ( )

This opens the dialog displayed in figure 19.3:

Use the arrows to add or remove sequences or sequence lists from the selected elements list.

Click **Finish** to start the tool.

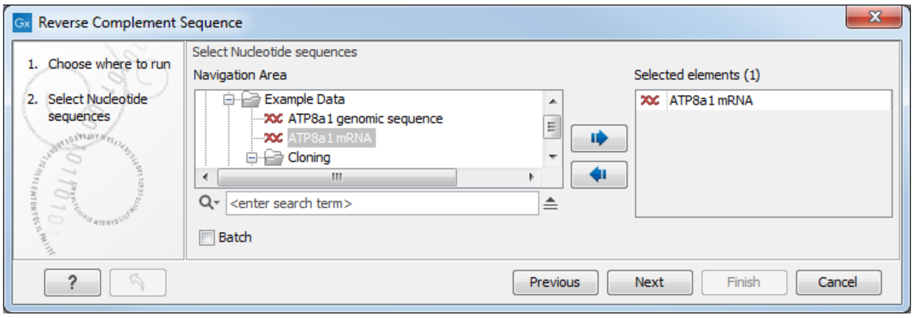


Figure 19.3: *Creating a reverse complement sequence.*

This will open a new view in the **View Area** displaying the reverse complement of the selected sequence. The new sequence is not saved automatically. To save the sequence, drag it into the **Navigation Area** or press Ctrl + S (⌘ + S on Mac) to activate a save dialog.

19.4 Translation of DNA or RNA to protein

To translate a nucleotide sequence into a protein sequence use the **Translate to Protein** tool, available at:

Tools | **Nucleotide Analysis** ()| **Translate to Protein** ()

This opens the dialog displayed in figure 19.4:

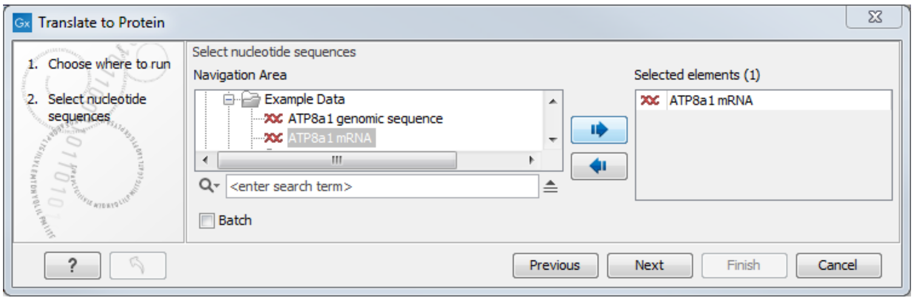


Figure 19.4: *Choosing sequences for translation.*

Use the arrows to add or remove sequences or sequence lists from the selected elements list.

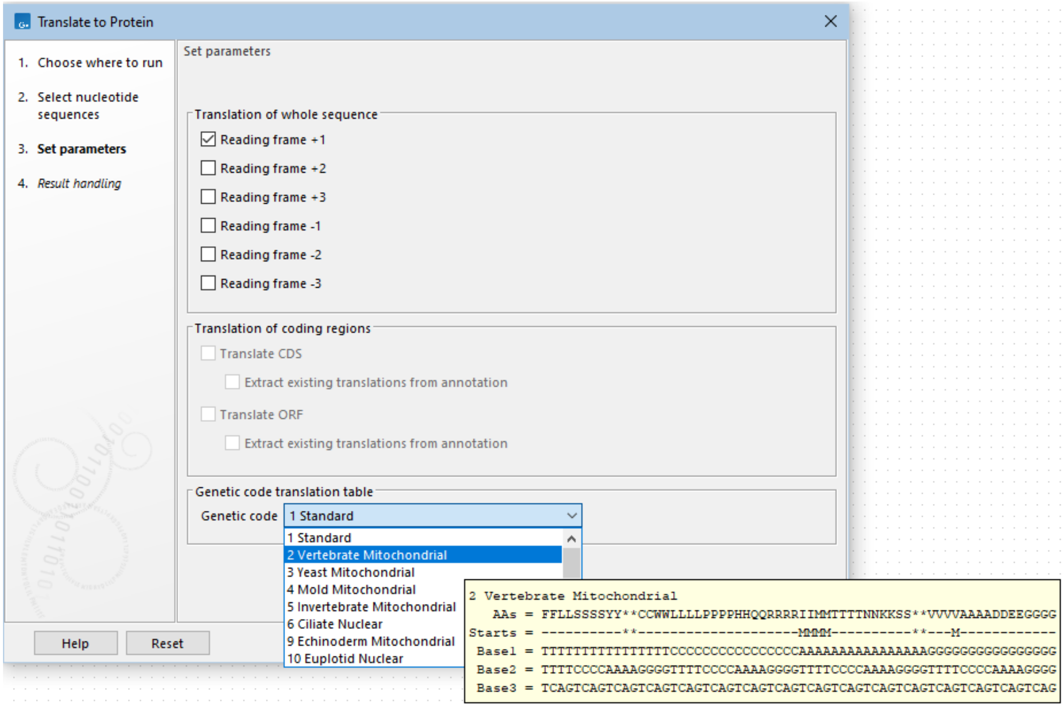
Clicking **Next** generates the dialog seen in figure 19.5:

Here you have the following options:

Reading frames If you wish to translate the whole sequence, you must specify the reading frame for the translation. If you select e.g. two reading frames, two protein sequences are generated.

Translate CDS You can choose to translate regions marked by and CDS or ORF annotation. This will generate a protein sequence for each CDS or ORF annotation on the sequence. The "Extract existing translations from annotation" allows to list the amino acid CDS sequence shown in the tool tip annotation (e.g. interstate from NCBI download) and does therefore not represent a translation of the actual nt sequence.

Genetic code Specify the genetic code to use. Hover the mouse cursor over an item in this list to reveal a tooltip containing the relevant translation table (figure 19.5). The translation tables



Set parameters

Translation of whole sequence

☑ Reading frame +1
☐ Reading frame +2
☐ Reading frame +3
☐ Reading frame -1
☐ Reading frame -2
☐ Reading frame -3

Translation of coding regions

☐ Translate CDS
☐ Extract existing translations from annotation
☐ Translate ORF
☐ Extract existing translations from annotation

Genetic code translation table

Genetic code: 1 Standard

1 Standard
2 Vertebrate Mitochondrial
3 Yeast Mitochondrial
4 Mold Mitochondrial
5 Invertebrate Mitochondrial
6 Ciliate Nuclear
9 Echinoderm Mitochondrial
10 Euplotid Nuclear

```
2 Vertebrate Mitochondrial
   AAs = FFLLSSSSYY**CCWWLLLLPPPPHHQQRRRRIIMMTTTTNNKKSS**VVVVAAAADDEEGGGG
Starts = ----------**--------------------MMMM----------**---M------------
 Base1 = TTTTTTTTTTTTTTTTCCCCCCCCCCCCCCCCAAAAAAAAAAAAAAAAGGGGGGGGGGGGGGGG
 Base2 = TTTTCCCCAAAAGGGGTTTTCCCCAAAAGGGGTTTTCCCCAAAAGGGGTTTTCCCCAAAAGGGG
 Base3 = TCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAG
```

Figure 19.5: *Configure the translation options. Hover the mouse cursor over a genetic code option to reveal a tooltip containing the relevant translation table.*

are sourced from the NCBI (https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi).

Stop codons result in an asterisk being inserted in the protein sequence at the corresponding position.

Click **Finish** to start the tool. The newly created protein is shown, but is not saved automatically.

To save a protein sequence, drag it into the **Navigation Area** or press Ctrl + S (⌘ + S on Mac) to activate a save dialog.

The name for a coding region translation consists of the name of the input sequence followed by the annotation type and finally the annotation name.

Translate part of a nucleotide sequence If you want to make separate translations of *all* the coding regions of a nucleotide sequence, you can check the option: "Translate CDS/ORF..." in the translation dialog (see figure 19.5).

If you want to translate a *specific* coding region, which is annotated on the sequence, use the following procedure:

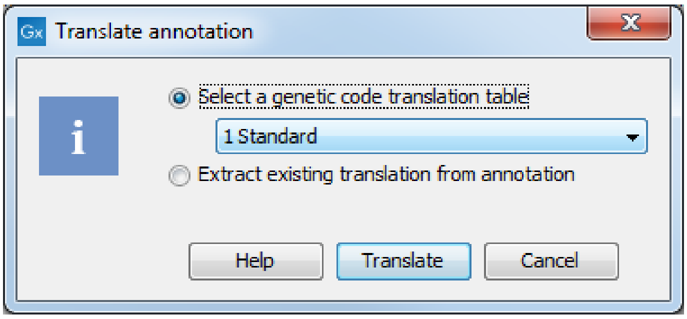
> **Open the nucleotide sequence | right-click the ORF or CDS annotation | Translate CDS/ORF... ( )**

A dialog opens to offer you the following choices (figure 19.6):

- **Select a genetic code translation table** Translates the ORF/CDS to protein using the selected translation table. Hover the mouse cursor over an item in this list to reveal a tooltip containing the relevant translation table (figure 19.5). The translation tables are sourced from the NCBI (`https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi`).

- **Extract existing translation from annotation** Translates the ORF/CDS to protein using existing translation information available in the annotation.

Choose the option needed and click **Translate**.



Translate annotation

● Select a genetic code translation table

1 Standard

○ Extract existing translation from annotation

Figure 19.6: *Choosing how to translate CDS or ORF annotations.*

The CDS and ORF annotations are colored yellow as default.

19.5 Find open reading frames

Find Open Reading Frames identifies open reading frames (ORFs) in sequences, and can be used as a rudimentary gene finder.

During translation of a transcript, protein is generated from the first start codon to the stop codon, internal start codons are translated to their respective amino acids. **Find Open Reading Frames** correspondingly always reports ORFs using the first possible start codon and ignores internal start codons.

Identified ORFs are shown as annotations on the sequence. Different genetic codes are available, but it is also possible to manually specify start codons.

In one analysis, **Find Open Reading Frames** can process a maximum of 100,000 sequences or 50 million base pairs. Sequences may be provided to the tool as individual sequences or as sequence lists.

To run **Find Open Reading Frames**, go to:

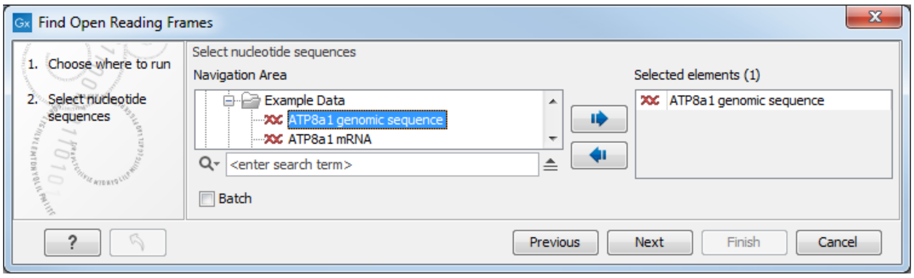
Tools | **Nucleotide Analysis** ()| **Find Open Reading Frames** ()

This opens the dialog displayed in figure 19.7

Use the arrows to add or remove sequences or sequence lists from the selected elements list.

Next, specify which parameters should be used (figure 19.8)

- **Start codon**
  - **AUG** Most commonly used start codon. When selected, only AUG (or ATG) codons are used as start codons.



Find Open Reading Frames

1. Choose where to run
2. Select nucleotide sequences

Select nucleotide sequences

Navigation Area

- Example Data
  - ATP8a1 genomic sequence
  - ATP8a1 mRNA

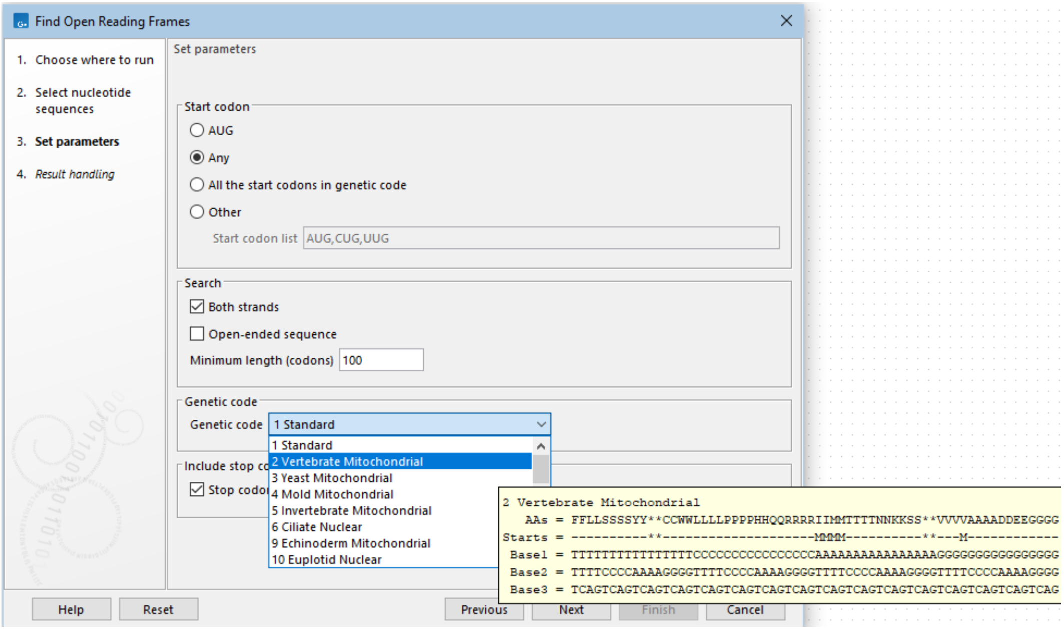
<enter search term>

□ Batch

Selected elements (1)

ATP8a1 genomic sequence

Figure 19.7: *Select a sequence or a sequence list as input.*



Find Open Reading Frames

1. Choose where to run
2. Select nucleotide sequences
3. **Set parameters**
4. *Result handling*

Set parameters

Start codon

○ AUG

● Any

○ All the start codons in genetic code

○ Other

Start codon list AUG,CUG,UUG

Search

☑ Both strands

□ Open-ended sequence

Minimum length (codons) 100

Genetic code

Genetic code 1 Standard

- 1 Standard
- 2 Vertebrate Mitochondrial
- 3 Yeast Mitochondrial
- 4 Mold Mitochondrial
- 5 Invertebrate Mitochondrial
- 6 Ciliate Nuclear
- 9 Echinoderm Mitochondrial
- 10 Euplotid Nuclear

Include stop co

☑ Stop codo

```
2 Vertebrate Mitochondrial
   AAs = FFLLSSSSYY**CCWWLLLLPPPPHHQQRRRRIIMMTTTTNNKKSS**VVVVAAAADDEEGGGG
Starts = ----------**--------------------MMMM----------**---M------------
 Base1 = TTTTTTTTTTTTTTTTCCCCCCCCCCCCCCCCAAAAAAAAAAAAAAAAGGGGGGGGGGGGGGGG
 Base2 = TTTTCCCCAAAAGGGGTTTTCCCCAAAAGGGGTTTTCCCCAAAAGGGGTTTTCCCCAAAAGGGG
 Base3 = TCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAG
```

Figure 19.8: *Configure the options for finding open reading frames. Hover the mouse cursor over a genetic code option to reveal a tooltip containing the relevant translation table.*

- **Any** Any codon can be used as the start codon. For identification of the open reading frames, the first possible codon in the same reading frame as the stop codon is used as the start codon.
- **All the start codons in genetic code** Select to use the start codons that are specific to the genetic code specified under **Genetic code**.
- **Other** Identifies open reading frames that start with one of the codons provided in the start codon list.

- **Both strands** Find reading frames on both strands.
- **Open-ended sequence** Allow ORFs to extend up to the sequence start or end not considering the sequence context. This can be relevant when only a fragment of a sequence is analyzed, and there may be up- or downstream start and stop codons that are not included in the sequence. When predicting the open reading frames, stop codons are always used, but a given start codon is only used if it is the first one after the last stop codon. Start codons that are not preceded by a stop codon are ignored, because there may be another start codon upstream that is not included in the sequence.
- **Minimum length (codons)** The minimum number of codons that must be present for an open reading frame to be reported.

- **Genetic code** Specify the genetic code to use. Hover the mouse cursor over an item in this list to reveal a tooltip containing the relevant translation table (figure 19.8). The translation tables are sourced from the NCBI (`https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi`).
- **Stop codon included in annotation** Include the stop codon in the open reading frame annotations on the sequences.

Using open reading frames to find genes is a fairly simple approach which is likely to predict genes which are not real. Setting a relatively high minimum length of the ORFs will reduce the number of false positive predictions, but at the same time short genes may be missed (see figure 19.9).

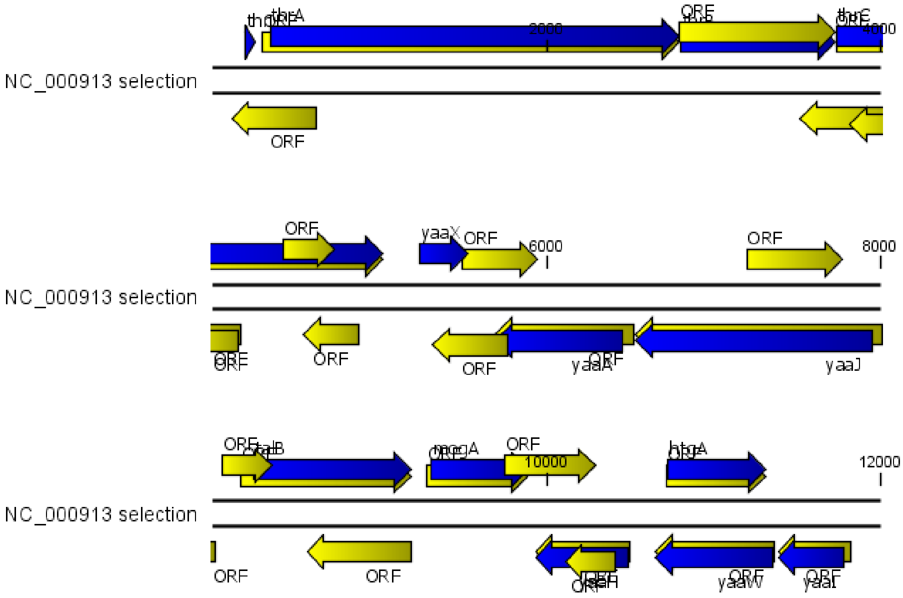


Figure 19.9: *The first 12,000 positions of the* E. coli *sequence NC_000913 downloaded from GenBank. The blue (dark) annotations are the genes while the yellow (brighter) annotations are the ORFs with a length of at least 100 amino acids. On the positive strand around position 11,000, a gene starts before the ORF. This is due to the use of the standard genetic code rather than the bacterial code. This particular gene starts with CTG, which is a start codon in bacteria. Two short genes are entirely missing, while a handful of open reading frames do not correspond to any of the annotated genes.*

Click **Finish** to start the tool.

Finding open reading frames is often a good first step in annotating sequences such as cloning vectors or bacterial genomes. For eukaryotic genes, ORF determination may not always be very helpful since the intron/exon structure is not part of the algorithm.

Chapter 20

Protein analyses

Contents

CLC Main Workbench offers a number of analyses of proteins as described in this chapter.

Note that the SignalP and TMHMM plugin allows you to predict signal peptides. For more information, please read the plugin manual at https://resources.qiagenbioinformatics.com/manuals/signalpandtmhmm/current/User_Manual.pdf.

The TMHMM plugin allows you to predict transmembrane helix. For more information, please read the plugin manual at http://resources.qiagenbioinformatics.com/manuals/tmhmm/current/Tmhmm_User_Manual.pdf.

20.1 Protein charge

In *CLC Main Workbench* you can create a graph in the electric charge of a protein as a function of pH. This is particularly useful for finding the net charge of the protein at a given pH. This knowledge can be used e.g. in relation to isoelectric focusing on the first dimension of 2D-gel electrophoresis. The isoelectric point (pI) is found where the net charge of the protein is zero. The calculation of the protein charge does not include knowledge about any potential post-translational modifications the protein may have.

The pKa values reported in the literature may differ slightly, thus resulting in different looking graphs of the protein charge plot compared to other programs.

In order to calculate the protein charge:

Tools | **Protein Analysis** ( )| **Create Protein Charge Plot** ( )

This opens the dialog displayed in figure 20.1:

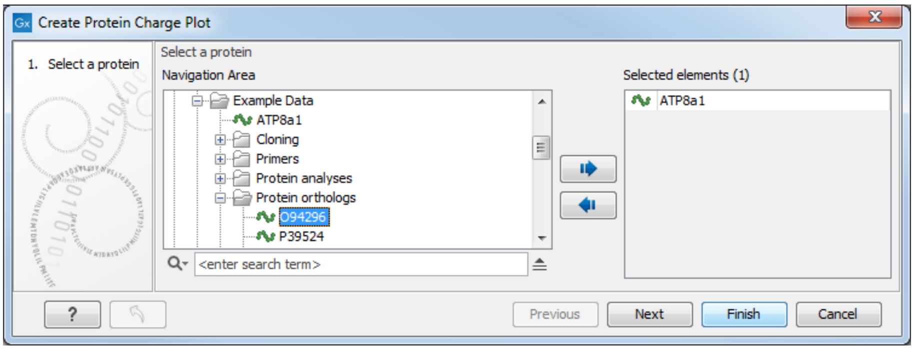


Figure 20.1: *Choosing protein sequences to calculate protein charge.*

If a sequence was selected before running the tool, the sequence will be listed in the **Selected Elements** pane of the dialog. Use the arrows to add or remove sequences or sequence lists from the selected elements.

You can perform the analysis on several protein sequences at a time. This will result in one output graph showing protein charge graphs for the individual proteins.

Click **Finish** to start the tool.

Figure 20.2 shows the electrical charges for three proteins. In the **Side Panel** to the right, you can modify the layout of the graph.

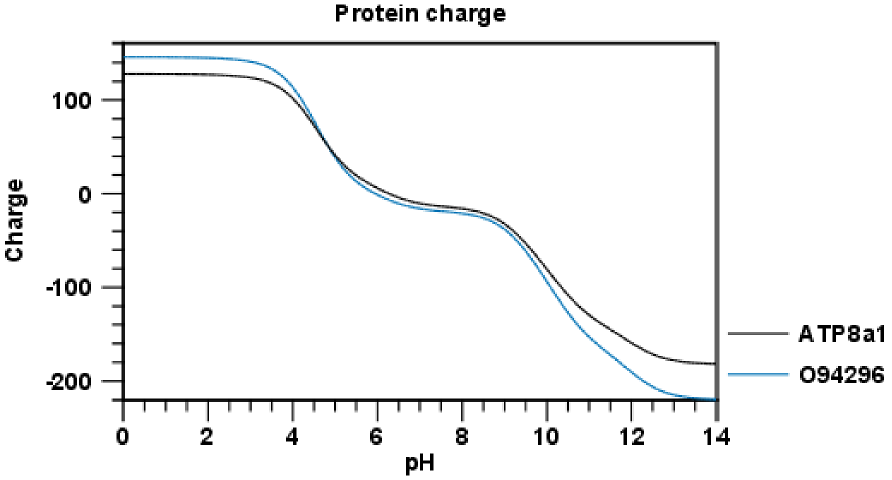


Figure 20.2: *View of the protein charge.*

See section A in the appendix for information about the graph view.

20.2 Antigenicity

CLC Main Workbench can help to identify antigenic regions in protein sequences in different ways, using different algorithms. The algorithms provided in the Workbench, merely plot an index of antigenicity over the sequence.

Two different methods are available:

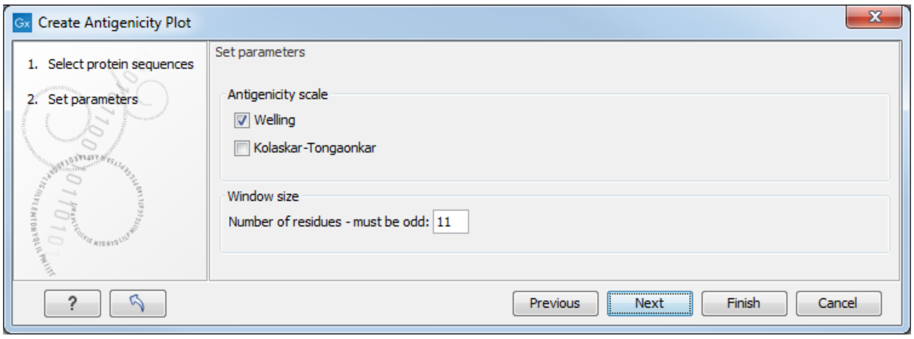
- [Welling et al., 1985] Welling et al. used information on the relative occurrence of amino acids in antigenic regions to make a scale which is useful for prediction of antigenic regions. This method is better than the Hopp-Woods scale of hydrophobicity which is also used to identify antigenic regions.
- A semi-empirical method for prediction of antigenic regions has been developed [Kolaskar and Tongaonkar, 1990]. This method also includes information of surface accessibility and flexibility and at the time of publication the method was able to predict antigenic determinants with an accuracy of 75%.

Note! Similar results from the two methods can not always be expected as the two methods are based on different training sets.

Displaying the antigenicity for a protein sequence in a plot is done in the following way:

Tools | Protein Analysis ( )| Create Antigenicity Plot ( )

This opens a dialog. The first step allows you to add or remove sequences. If you had already selected sequences in the Navigation Area before running the tool, these will be listed in the **Selected Elements** pane. Clicking **Next** takes you through to **Step 2**, which is displayed in figure 20.3.



Set parameters

Antigenicity scale

☑ Welling

□ Kolaskar-Tongaonkar

Window size

Number of residues - must be odd: 11

Figure 20.3: *Step two in the Antigenicity Plot allows you to choose different antigenicity scales and the window size.*

The **Window size** is the width of the window where, the antigenicity is calculated. The wider the window, the less volatile the graph. You can chose from a number of antigenicity scales. Click **Finish** to start the tool. The result can be seen in figure 20.4.

See section A in the appendix for information about the graph view.

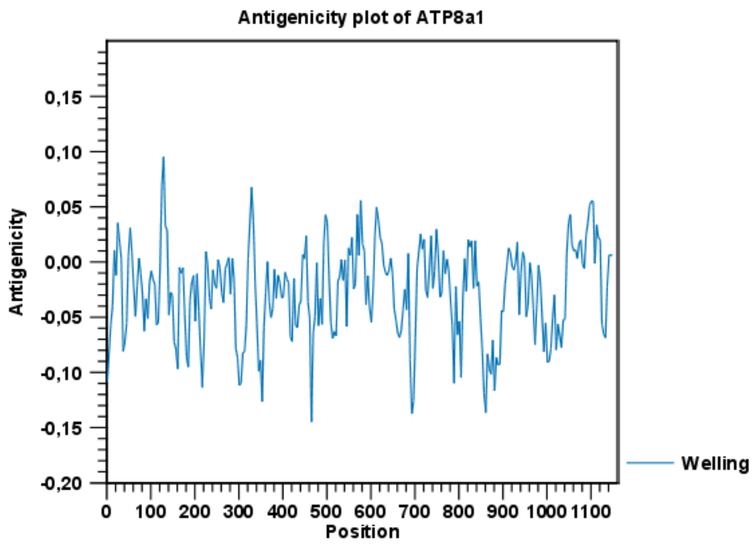


Figure 20.4: *The result of the antigenicity plot calculation and the associated Side Panel.*

The level of antigenicity is calculated on the basis of the different scales. The different scales add different values to each type of amino acid. The antigenicity score is then calculated as the sum of the values in a 'window', which is a particular range of the sequence. The window length can be set from 5 to 25 residues. The wider the window, the less fluctuations in the antigenicity scores.

Antigenicity graphs along the sequence can be displayed using the **Side Panel**. The functionality is similar to hydrophobicity (see section 20.3.1).

20.3 Hydrophobicity

CLC Main Workbench can calculate the hydrophobicity of protein sequences in different ways, using different algorithms (see section 20.3.2). Furthermore, hydrophobicity of sequences can be displayed as hydrophobicity plots and as graphs along sequences. In addition, *CLC Main Workbench* can calculate hydrophobicity for several sequences at the same time, and for alignments.

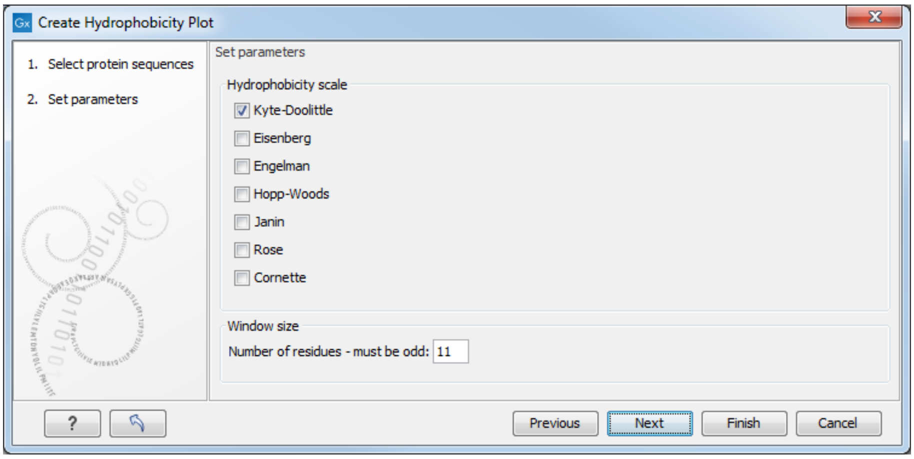
Displaying the hydrophobicity for a protein sequence in a plot is done in the following way:

Tools | **Protein Analysis ( )**| **Create Hydrophobicity Plot ( )**

This opens a dialog. The first step allows you to add or remove sequences. If you had already selected a sequence in the Navigation Area, this will be shown in the **Selected Elements**.Clicking **Next** takes you through to **Step 2**, which is displayed in figure 20.5.

The **Window size** is the width of the window where the hydrophobicity is calculated. The wider the window, the less volatile the graph. You can chose from a number of hydrophobicity scales which are further explained in section 20.3.2 Click **Finish** to start the tool. The result can be seen in figure 20.6.

See section A in the appendix for information about the graph view.



Set parameters

Hydrophobicity scale

☑ Kyte-Doolittle

☐ Eisenberg

☐ Engelman

☐ Hopp-Woods

☐ Janin

☐ Rose

☐ Cornette

Window size

Number of residues - must be odd: 11

Figure 20.5: *Step two in the Hydrophobicity Plot allows you to choose hydrophobicity scale and the window size.*

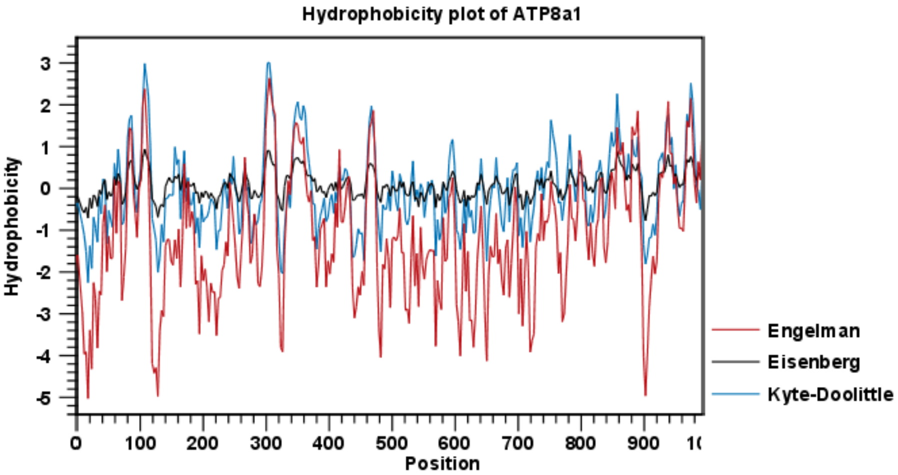


Figure 20.6: *The result of the hydrophobicity plot calculation and the associated Side Panel.*

20.3.1 Hydrophobicity graphs along sequence

Hydrophobicity graphs along sequence can be displayed easily by activating the calculations from the **Side Panel** for a sequence. Simply right-click or double click on a protein sequence in the Navigation Area, and choose

Show | **Sequence** | **open Protein info in Side Panel**

These actions result in the view displayed in figure 20.7.

The level of hydrophobicity is calculated on the basis of the different scales. The different scales add different values to each type of amino acid. The hydrophobicity score is then calculated as the sum of the values in a 'window', which is a particular range of the sequence. The window length can be set from 5 to 25 residues. The wider the window, the less fluctuations in the hydrophobicity scores. (For more about the theory behind hydrophobicity, see 20.3.2).

In the following we will focus on the different ways that the Workbench offers to display the hydrophobicity scores. We use Kyte-Doolittle to explain the display of the scores, but the different options are the same for all the scales. Initially there are three options for displaying the

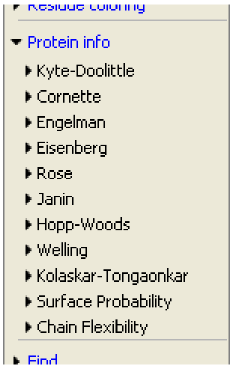
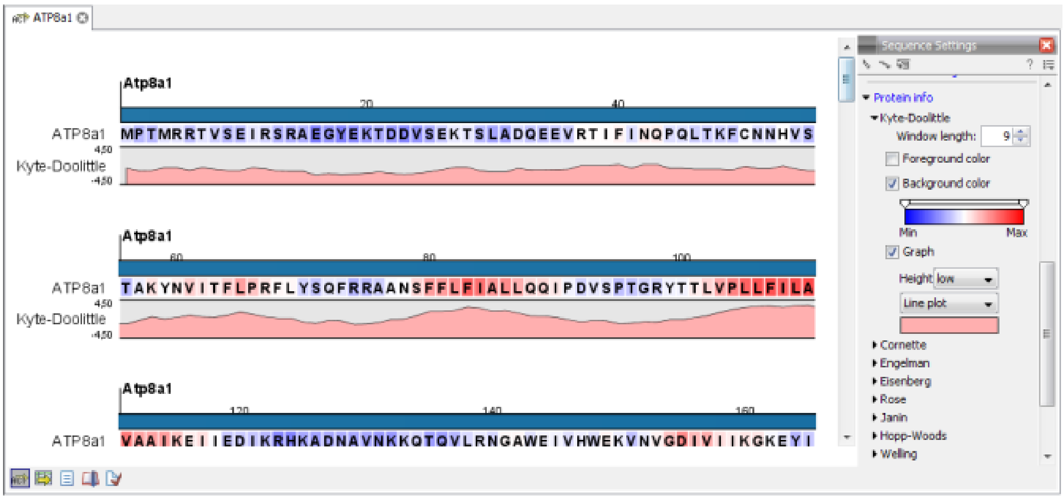


Figure 20.7: *The different available scales in Protein info.*

hydrophobicity scores. You can choose one, two or all three options by selecting the boxes (figure 20.8).



Window length: 9

☐ Foreground color

☑ Background color

☑ Graph

Height: low

Line plot

Figure 20.8: *The different ways of displaying the hydrophobicity scores, using the Kyte-Doolittle scale.*

Coloring the letters and their background. When choosing coloring of letters or coloring of their background, the color red is used to indicate high scores of hydrophobicity. A 'color-slider' allows you to amplify the scores, thereby emphasizing areas with high (or low, blue) levels of hydrophobicity. The color settings mentioned are default settings. By clicking the color bar just below the color slider you get the option of changing color settings.

Graphs along sequences. When selecting graphs, you choose to display the hydrophobicity scores underneath the sequence. This can be done either by a line-plot or bar-plot, or by coloring. The latter option offers you the same possibilities of amplifying the scores as applies for coloring of letters. The different ways to display the scores when choosing 'graphs' are displayed in figure 20.8. Notice that you can choose the height of the graphs underneath the sequence.

20.3.2 Bioinformatics explained: Protein hydrophobicity

Calculation of hydrophobicity is important to the identification of various protein features. This can be membrane spanning regions, antigenic sites, exposed loops or buried residues. Usually, these calculations are shown as a plot along the protein sequence, making it easy to identify the location of potential protein features.

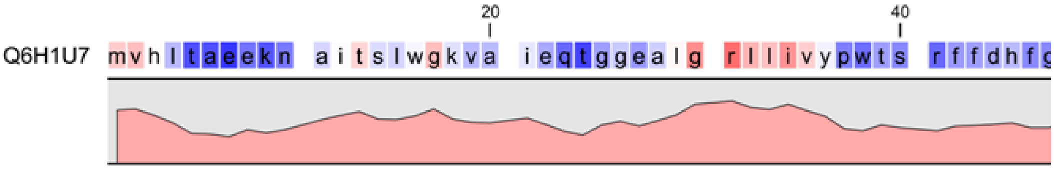

20
40
Q6H1U7
m v h l t a e e k n a i t s l w g k v a i e q t g g e a l g r l l i v y p w t s r f f d h f g

Figure 20.9: *Plot of hydrophobicity along the amino acid sequence. Hydrophobic regions on the sequence have higher numbers according to the graph below the sequence, furthermore hydrophobic regions are colored on the sequence. Red indicates regions with high hydrophobicity and blue indicates regions with low hydrophobicity.*

The hydrophobicity is calculated by sliding a fixed size window (of an odd number) over the protein sequence. At the central position of the window, the average hydrophobicity of the entire window is plotted (see figure 20.9).

Hydrophobicity scales Several hydrophobicity scales have been published for various uses. Many of the commonly used hydrophobicity scales are described below.

- **Kyte-Doolittle scale.** The Kyte-Doolittle scale is widely used for detecting hydrophobic regions in proteins. Regions with a positive value are hydrophobic. This scale can be used for identifying both surface-exposed regions as well as transmembrane regions, depending on the window size used. Short window sizes of 5-7 generally work well for predicting putative surface-exposed regions. Large window sizes of 19-21 are well suited for finding transmembrane domains if the values calculated are above 1.6 [Kyte and Doolittle, 1982]. These values should be used as a rule of thumb and deviations from the rule may occur.
- **Engelman scale.** The Engelman hydrophobicity scale, also known as the GES-scale, is another scale which can be used for prediction of protein hydrophobicity [Engelman et al., 1986]. As the Kyte-Doolittle scale, this scale is useful for predicting transmembrane regions in proteins.
- **Eisenberg scale.** The Eisenberg scale is a normalized consensus hydrophobicity scale which shares many features with the other hydrophobicity scales [Eisenberg et al., 1984].
- **Hopp-Woods scale.** Hopp and Woods developed their hydrophobicity scale for identification of potentially antigenic sites in proteins. This scale is basically a hydrophilic index where apolar residues have been assigned negative values. Antigenic sites are likely to be predicted when using a window size of 7 [Hopp and Woods, 1983].
- **Cornette scale.** Cornette *et al.* computed an optimal hydrophobicity scale based on 28 published scales [Cornette et al., 1987]. This optimized scale is also suitable for prediction of alpha-helices in proteins.
- **Rose scale.** The hydrophobicity scale by Rose *et al.* is correlated to the average area of buried amino acids in globular proteins [Rose et al., 1985]. This results in a scale which is not showing the helices of a protein, but rather the surface accessibility.
- **Janin scale.** This scale also provides information about the accessible and buried amino acid residues of globular proteins [Janin, 1979].

aa	aa	Kyte-	Hopp-	Cornette	Eisenberg	Rose	Janin	Engelman
		Doolittle	Woods					(GES)
A	Alanine	1.80	-0.50	0.20	0.62	0.74	0.30	1.60
C	Cysteine	2.50	-1.00	4.10	0.29	0.91	0.90	2.00
D	Aspartic acid	-3.50	3.00	-3.10	-0.90	0.62	-0.60	-9.20
E	Glutamic acid	-3.50	3.00	-1.80	-0.74	0.62	-0.70	-8.20
F	Phenylalanine	2.80	-2.50	4.40	1.19	0.88	0.50	3.70
G	Glycine	-0.40	0.00	0.00	0.48	0.72	0.30	1.00
H	Histidine	-3.20	-0.50	0.50	-0.40	0.78	-0.10	-3.00
I	Isoleucine	4.50	-1.80	4.80	1.38	0.88	0.70	3.10
K	Lysine	-3.90	3.00	-3.10	-1.50	0.52	-1.80	-8.80
L	Leucine	3.80	-1.80	5.70	1.06	0.85	0.50	2.80
M	Methionine	1.90	-1.30	4.20	0.64	0.85	0.40	3.40
N	Asparagine	-3.50	0.20	-0.50	-0.78	0.63	-0.50	-4.80
P	Proline	-1.60	0.00	-2.20	0.12	0.64	-0.30	-0.20
Q	Glutamine	-3.50	0.20	-2.80	-0.85	0.62	-0.70	-4.10
R	Arginine	-4.50	3.00	1.40	-2.53	0.64	-1.40	-12.3
S	Serine	-0.80	0.30	-0.50	-0.18	0.66	-0.10	0.60
T	Threonine	-0.70	-0.40	-1.90	-0.05	0.70	-0.20	1.20
V	Valine	4.20	-1.50	4.70	1.08	0.86	0.60	2.60
W	Tryptophan	-0.90	-3.40	1.00	0.81	0.85	0.30	1.90
Y	Tyrosine	-1.30	-2.30	3.20	0.26	0.76	-0.40	-0.70

Table 20.1: *Hydrophobicity scales. This table shows seven different hydrophobicity scales which are generally used for prediction of e.g. transmembrane regions and antigenicity.*

- **Welling scale.** Welling et al. used information on the relative occurrence of amino acids in antigenic regions to make a scale which is useful for prediction of antigenic regions. This method is better than the Hopp-Woods scale of hydrophobicity which is also used to identify antigenic regions.

- **Kolaskar-Tongaonkar.** A semi-empirical method for prediction of antigenic regions has been developed [Kolaskar and Tongaonkar, 1990]. This method also includes information of surface accessibility and flexibility and at the time of publication the method was able to predict antigenic determinants with an accuracy of 75%.

- **Surface Probability.** Display of surface probability based on the algorithm by [Emini et al., 1985]. This algorithm has been used to identify antigenic determinants on the surface of proteins.

- **Chain Flexibility.** Display of backbone chain flexibility based on the algorithm by [Karplus and Schulz, 1985]. It is known that chain flexibility is an indication of a putative antigenic determinant.

Many more scales have been published throughout the last three decades. Even though more advanced methods have been developed for prediction of membrane spanning regions, the simple and very fast calculations are still highly used.

Other useful resources

AAindex: Amino acid index database
`http://www.genome.ad.jp/dbget/aaindex.html`

20.4 Download Pfam Database

To run **Pfam Domain Search** you must first download the Pfam database. The Pfam database can be downloaded using:

Tools | Protein Analysis ()| Download Pfam Database ()

This element is designed for use with Pfam Domain Search. If you open it directly from the Navigation Area, only the element history is accessible.

20.5 Pfam domain search

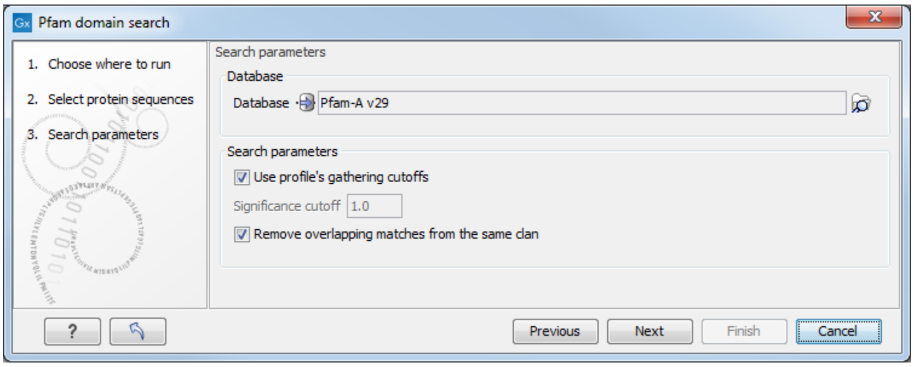
Pfam Domain Search searches for domains in protein sequences using the Pfam database [Bateman et al., 2004], a large collection of multiple sequence alignments and hidden Markov models (HMMs) covering many common protein domains. It can add **Region** annotations on the input sequences where domains were found (figure 20.11) and it can output a table listing the domains found.

Why search against Pfam? Many proteins have a unique combination of domains, which can be responsible for the catalytic activities of enzymes. Annotating sequences based on pairwise alignment methods by simply transferring annotation from a known protein to the unknown partner does not take domain organization into account [Galperin and Koonin, 1998]. For example, a protein may be annotated incorrectly as an enzyme if the pairwise alignment only finds a regulatory domain.

After the Pfam database has been downloaded (see section 20.4), start **Pfam Domain Search** by going to:

Tools | Protein Analysis ()| Pfam Domain Search ()

By selecting several input sequences, you can perform the analysis on all these at once. Options can be configured (figure 20.10).



Pfam domain search

1. Choose where to run
2. Select protein sequences
3. Search parameters

Search parameters

Database

Database Pfam-A v29

Search parameters

☑ Use profile's gathering cutoffs

Significance cutoff 1.0

☑ Remove overlapping matches from the same clan

Figure 20.10: *Setting parameters for Pfam Domain Search.*

Pfam Domain Search options

- **Database** Choose the database to use when searching for Pfam domains.
- Significance cutoff:

  - **Use profile's gathering cutoffs** Use cutoffs specifically assigned to each family by the curator instead of manually assigning the **Significance cutoff**.
  - **Significance cutoff** The E-value (expectation value) describes the number of hits one would expect to see by chance when searching a database of a particular size. Essentially, a hit with a low E-value is more significant than a hit with a high E-value. By lowering the significance threshold the domain search will become more specific and less sensitive, i.e. fewer hits will be reported but the reported hits will be more significant on average.

- **Remove overlapping matches from the same clan** Perform post-processing of the results where overlaps between hits are resolved by keeping the hit with the smallest E-value.

If annotations were added but are not initially visible on your sequences, check under the "Annotation types" tab of the side panel settings to ensure the **Region** annotation type has been checked.

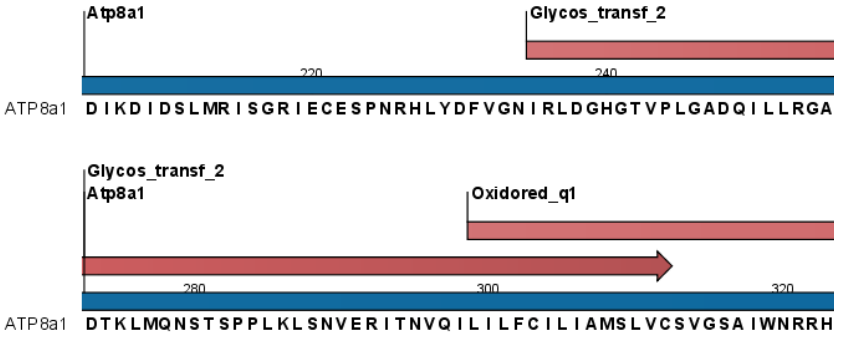

Atp8a1
Glycos_transf_2
220
240
ATP8a1
DIKDIDSLMRISGRIECESPNRHLYDFVGNIRLDGHGTVPLGADQILLRGA
Glycos_transf_2
Atp8a1
Oxidored_q1
280
300
320
ATP8a1
DTKLMQNSTSPPLKLSNVERITNVQILILFCILIAMSLVCSVGSAIWNRRH

Figure 20.11: *Annotations (in red) that were added by the Pfam search tool.*

Detailed information for each domain annotation is available in the annotation tool tip as well as in the Annotation Table view of the sequence list.

The domain search is performed using the hmmsearch tool from the HMMER3 package version 3.4 `http://hmmer.org/`. Detailed information about the scores in the Region annotations added can be found in the HMMER User Guide `http://eddylab.org/software/hmmer/Userguide.pdf`.

Individual domain annotations can be removed manually, if desired. See section 14.3.5.

20.6 Download 3D Protein Structure Database

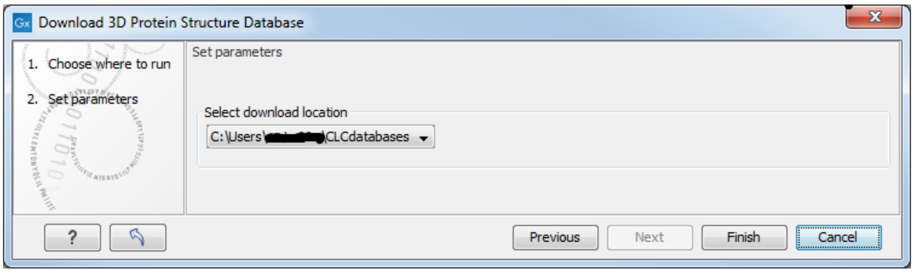
This tool downloads the 3D Protein Structure Database from a public accessible HTTP location hosted by QIAGEN Aarhus.

The database contains a curated set of sequences with known 3D structures, which are obtained from the Protein Data Bank (`https://www.wwpdb.org`) [Berman et al., 2003]. The information stored in the database (e.g. protein sequence, X-ray resolution) is used to identify suitable structural templates when using the **Link Variants to 3D Protein Structure** tool.

To download the database, go to:

Tools | **Protein Analysis** ( )| **Download 3D Protein Structure Database** ( )

If you are connected to a server, you will first be asked about whether you want to download the data locally or on a server. In the next wizard step you are asked to select the download location (see figure 20.12).



Download 3D Protein Structure Database

1. Choose where to run
2. Set parameters

Set parameters

Select download location: C:\Users\[redacted]CLCdatabases

Figure 20.12: *Select the download location.*

The downloaded database will be installed in the same location as local BLAST databases (e.g. <username>/CLCdatabases) or at a server location if the tool was executed on a CLC Server. From the wizard it is possible to select alternative locations if more than one location is available.

When new databases are released, a new version of the database can be downloaded by invoking the tool again (the existing database will be replaced).

If needed, the **Manage BLAST Databases** tool can be used to inspect or delete the database (the database is listed with the name 'ProteinStructureSequences'). You can find the tool here:

BLAST ( )| Manage BLAST Databases ( )

20.7 Find and Model Structure

This tool is used to find suitable protein structures for representing a given protein sequence. From the resulting table, a structure model (homology model) of the sequence can be created by one click, using one of the found protein structures as template.

To run the *Find and Model Structure* tool, go to:

Tools | **Sequence Analysis** ( ) | **Find and Model Structure** ( )

Note: Before running the tool, a protein structure sequence database must be downloaded and installed using the 'Download Find Structure Database' tool (see section 20.6).

In the tool wizard step 1, select the amino acid sequence to use as query from the Navigation Area.

In step 2, specify if the output table should be opened or saved.

The Find and Model Structure tool carries out the following steps, to find and rank available structures representing the query sequence:

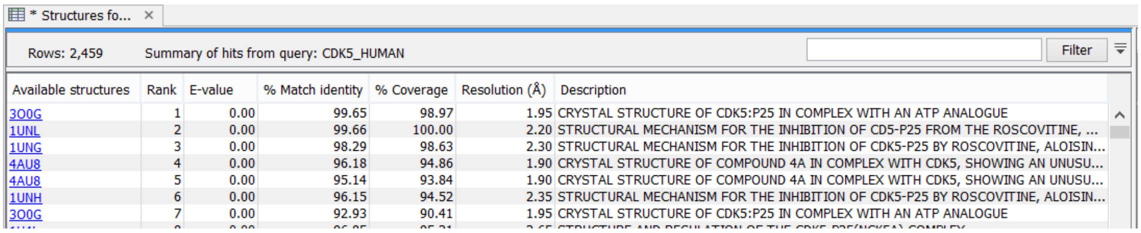
Input: Query protein sequence

1. BLAST against protein structure sequence database
2. Filter away low quality hits

3. Rank the available structures

Output: Table listing available structures

In the output table (figure 20.13), the column named "Available Structures" contains links that will invoke a menu with the options to either create a structure model of the query sequence or just download the structure. This is further described in section 20.7.1. The remaining columns contain additional information originating from the PDB file or from the BLAST search.



Rows: 2,459 Summary of hits from query: CDK5_HUMAN

Available structures	Rank	E-value	% Match identity	% Coverage	Resolution (Å)	Description
3O0G	1	0.00	99.65	98.97	1.95	CRYSTAL STRUCTURE OF CDK5:P25 IN COMPLEX WITH AN ATP ANALOGUE
1UNL	2	0.00	99.66	100.00	2.20	STRUCTURAL MECHANISM FOR THE INHIBITION OF CD5-P25 FROM THE ROSCOVITINE, ...
1UNG	3	0.00	98.29	98.63	2.30	STRUCTURAL MECHANISM FOR THE INHIBITION OF CDK5-P25 BY ROSCOVITINE, ALOISIN...
4AU8	4	0.00	96.18	94.86	1.90	CRYSTAL STRUCTURE OF COMPOUND 4A IN COMPLEX WITH CDK5, SHOWING AN UNUSU...
4AU8	5	0.00	95.14	93.84	1.90	CRYSTAL STRUCTURE OF COMPOUND 4A IN COMPLEX WITH CDK5, SHOWING AN UNUSU...
1UNH	6	0.00	96.15	94.52	2.35	STRUCTURAL MECHANISM FOR THE INHIBITION OF CDK5-P25 BY ROSCOVITINE, ALOISIN...
3O0G	7	0.00	92.93	90.41	1.95	CRYSTAL STRUCTURE OF CDK5:P25 IN COMPLEX WITH AN ATP ANALOGUE

Figure 20.13: *Table output from Find and Model Structure.*

The three steps carried out by the *Find and Model Structure* tool are described in short below.

BLAST against protein structure sequence database A local BLAST search is carried out for the query sequence against the protein structure sequence database (see section 20.6).

BLAST hits with E-value > 0.0001 are rejected and a maximum of 2500 BLAST hits are retrieved. Read more about BLAST in section 26.5.

Filter away low quality hits From the list of BLAST hits, entries are rejected based on the following rules:

- PDB structures with a resolution lower than 4 Å are removed since they cannot be expected to represent a trustworthy atomistic model.
- BLAST hits with an identity to the query sequence lower than 20 % are removed since they most likely would result in inaccurate models.

Rank the available structures For the resulting list of available structures, each structure is scored based on its homology to the query sequence, and the quality of the structure itself. The *Template quality score* is used to rank the structures in the table, and the rank of each structure is shown in the "Rank" column (see figure 20.13). Read more about the *Template quality score* in section 20.7.2.

20.7.1 Create structure model

Clicking on a link in the "Available structures" column will show a menu with three options:

- Download and Open
- Download and Create Model

- Help

The "Download and Open" option will do the following:

1. **Download and import** the PDB file containing the structure.
2. **Create an alignment** between the query and structure sequences.
3. **Open a 3D view** (Molecule Project) with the molecules from the PDB file and open the created sequence alignment. The sequence originating from the structure will be linked to the structure in the 3D view, so that selections on the sequence will show up on the structure (see section 15.4).

The "Download and Create Model" option will do the following:

1. **Download and import** the PDB file containing the structure.
2. **Generate a biomolecule** involving the protein chain to be modeled. Biomolecule information available in the template PDB file is used (see section 15.6). If several biomolecules involving the chain are available, the first one is applied.
3. **Create an alignment** between the query and structure sequences.
4. **Create a model structure** by mapping the query sequence onto the structure based on the sequence alignment (see section 20.7.2). If multiple copies of the template protein chain have been made to generate a biomolecule, all copies are modeled at the same time.
5. **Open a 3D view** (a Molecule Project) with the structure model shown in both backbone and wireframe representation. The model is colored by temperature (see figure 20.14), to indicate local model uncertainty (see section 20.7.2). Other molecules from the template PDB file are shown in orange or yellow coloring. The created sequence alignment is also opened and linked with the 3D views so that selections on the model sequence will show up on the model structure (see section 15.4).

The template structure is also available from the Proteins category in the Project Tree, but hidden in the initial view. The initial view settings are saved on the Molecule Project as "Initial visualization", and can always be reapplied from the View Settings menu (☰) found in the bottom right corner of the Molecule Project (see section 4.6).

> If you have problems viewing 3D structures, please check your system matches the requirements for 3D Viewers. See section 1.3.

20.7.2 Model structure

Protein coloring to visualize local structural uncertainties

The default coloring scheme for modeled structures in *CLC Main Workbench* is "Color by Temperature". This coloring indicates the uncertainty or disorder of each atom position in the structure.

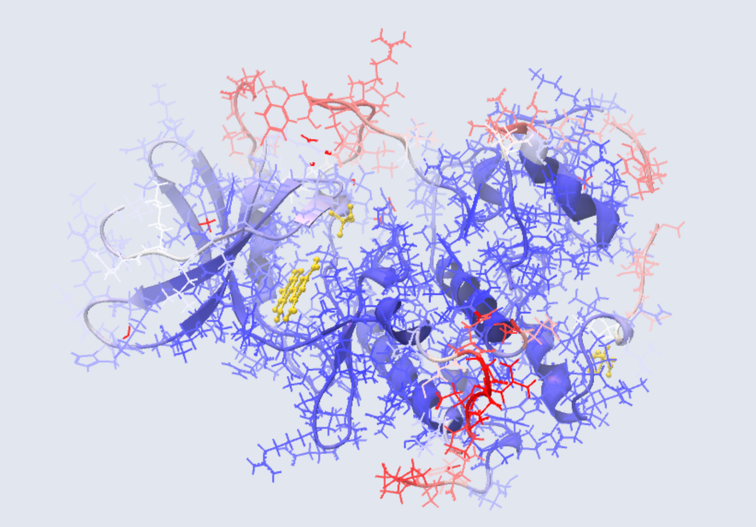


Figure 20.14: *Structure Model of CDK5_HUMAN. The atoms and backbone are colored by temperature, showing uncertain structure in red and well defined structure in blue.*

For crystal structures, the temperature factor (also called the B-factor) is given in the PDB file as a measure of the uncertainty or disorder of each atom position. The temperature factor has the unit Å^2, and is typically in the range [0, 100].

The temperature color scale ranges from blue (0) over white (50) to red (100) (see section 15.3.1).

For structure models created in *CLC Main Workbench*, the temperature factor assigned to each atom combines three sources of positional uncertainty:

- **PDB Temp.** The atom position uncertainty for the template structure, represented by the temperature factor of the backbone atoms in the template structure.
- **P(alignment)** The probability that the alignment of a residue in the query sequence to a particular position on the structure is correct.
- **Clash?** It is evaluated if atoms in the structure model seem to clash, thereby indicating a problem with the model.

The three aspects are combined to give a temperature value between zero and 100, as illustrated in figure 20.15 and 20.16.

When holding the mouse over an atom, the Property Viewer in the Side Panel will show various information about the atom. For atoms in structure models, the contributions to the assigned temperature are listed as seen in figure 20.17.

Note: For NMR structures, the temperature factor is set to zero in the PDB file, and the "Color by Temperature" will therefore suggest that the structure is more well determined than is actually the case.

P(alignment) Alignment error is one of the largest causes of model inaccuracy, particularly when the model is built from a template sharing low sequence identity (e.g. lower than 60%).

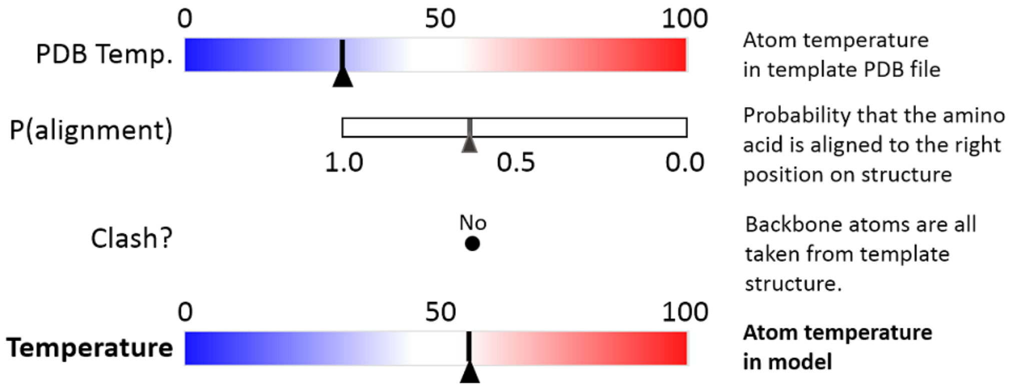

0
50
100
PDB Temp.
Atom temperature in template PDB file
P(alignment)
1.0
0.5
0.0
Probability that the amino acid is aligned to the right position on structure
Clash?
No
Backbone atoms are all taken from template structure.
0
50
100
Temperature
Atom temperature in model

Figure 20.15: *Evaluation of temperature color for backbone atoms in structure models.*

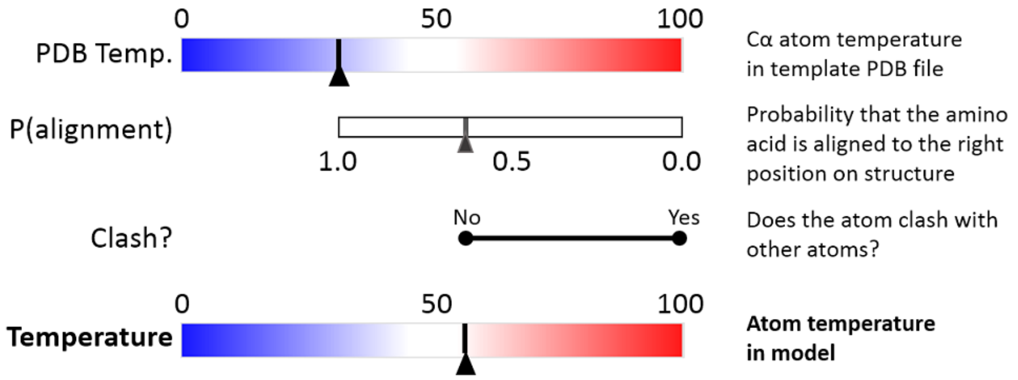
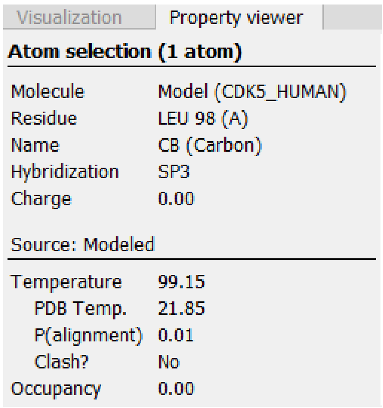

0
50
100
PDB Temp.
Cα atom temperature in template PDB file
P(alignment)
1.0
0.5
0.0
Probability that the amino acid is aligned to the right position on structure
Clash?
No
Yes
Does the atom clash with other atoms?
0
50
100
Temperature
Atom temperature in model

Figure 20.16: *Evaluation of temperature color for side chain atoms in structure models.*



Atom selection (1 atom)

Molecule	Model (CDK5_HUMAN)
Residue	LEU 98 (A)
Name	CB (Carbon)
Hybridization	SP3
Charge	0.00

Source: Modeled

Temperature	99.15
PDB Temp.	21.85
P(alignment)	0.01
Clash?	No
Occupancy	0.00

Figure 20.17: *Information displayed in the Side Panel Property viewer for a modeled atom.*

Misaligning a single amino acid by one position will cause a ca. 3.5 Å shift of its atoms from their true positions.

The estimate of the probability that two amino acids are correctly aligned, P(alignment), is obtained by averaging over all the possible alignments between two sequences, similar to [Knudsen and Miyamoto, 2003].

This allows local alignment uncertainty to be detected even in similar sequences. For example the position of the D in this alignment:

```
Template   GGACDAEDRSTRSTACE---GG
Target     GGACD---RSTRSTACEKLMGG
```

is uncertain, because an alternative alignment is as likely:

```
Template   GGACDAEDRSTRSTACE---GG
Target     GGAC---DRSTRSTACEKLMGG
```

Clash? Clashes are evaluated separately for each atom in a side chain. If the atom is considered to clash, it will be assigned a temperature of 100.

Note: Clashes within the modeled protein chain as well as with all other molecules in the downloaded PDB file (except water) are considered.

Ranking structures

The protein sequence of the gene affected by the variant (the query sequence) is BLASTed against the protein structure sequence database (section 20.6).

A *template quality score* is calculated for the available structures found for the query sequence. The purpose of the score is to rank structures considering both their quality and their homology to the query sequence.

The five descriptors contributing to the score are:

- E-value
- % Match identity
- % Coverage
- Resolution (of crystal structure)
- Free R-value (R_{free} of crystal structure)

Each of the five descriptors are scaled to [0,1], based on the linear functions seen in figure 20.18. The five scaled descriptors are combined into the *template quality score*, weighting them to emphasize homology over structure qualities.

Template quality score $= 3 \cdot S_{\text{E-value}} + 3 \cdot S_{\text{Identity}} + 1.5 \cdot S_{\text{Coverage}} + S_{\text{Resolution}} + 0.5 \cdot S_{\text{Rfree}}$

E-value is a measure of the quality of the match returned from the BLAST search. You can read more about BLAST and E-values in section 26.5.

% Match identity is the identity between the query sequence and the BLAST hit in the matched region. It is evaluated as

$$\text{\% Match identity} = 100\% \cdot (\text{Identity in BLAST alignment})/L_{\text{B}}$$

where L_{B} is the length of the BLAST alignment of the matched region, as indicated in figure 20.19, and "Identity in BLAST alignment" is the number of identical positions in the matched region.

% Coverage indicates how much of the query sequence has been covered by a given BLAST hit (see figure 20.19). It is evaluated as

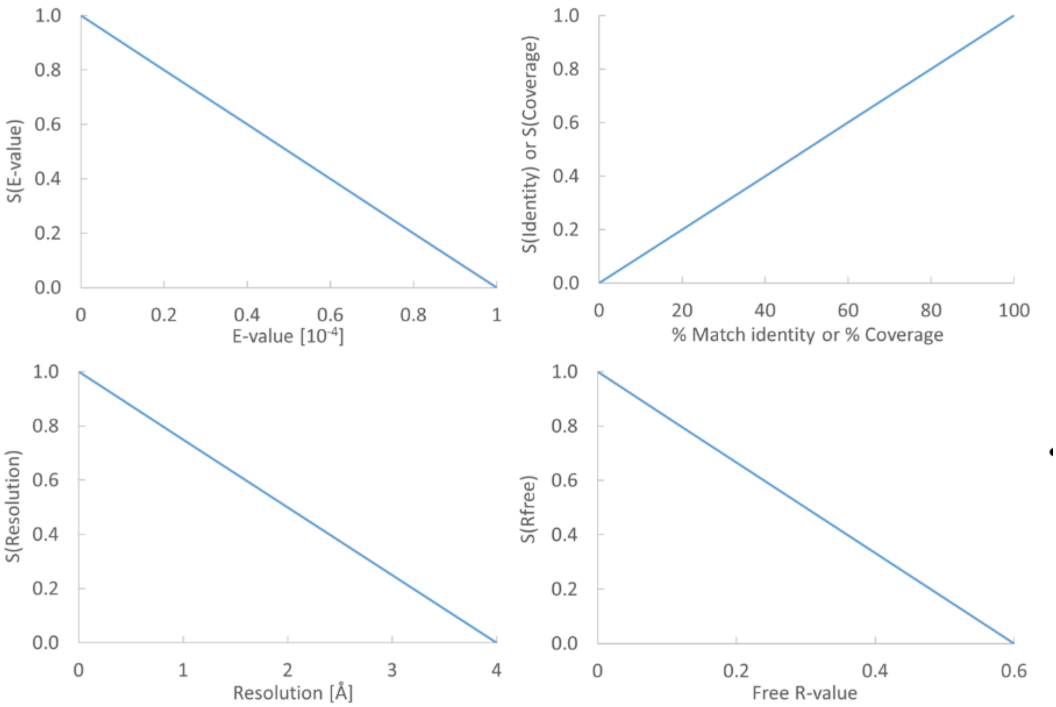


Figure 20.18: *From the E-value, % Match identity, % Coverage, Resolution, and Free R-value, the contributions to the "Template quality score" are determined from the linear functions shown in the graphs.*

$$\text{\% Coverage} = 100\% \cdot (L_\text{B} - L_\text{G})/L_\text{Q}$$

where L_G is the total length of gaps in the BLAST alignment and L_Q is the length of the query sequence.

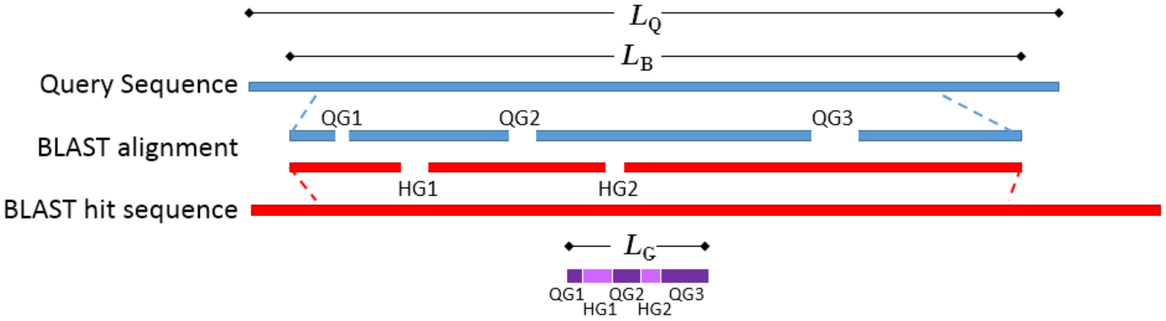


Figure 20.19: *Schematic of a query sequence matched to a BLAST hit. L_Q is the length of the query sequence, L_B is the length of the BLAST alignment of the matched region, QG1-3 are gaps in the matched region of the query sequence, HG1-2 are gaps in the matched region of the BLAST hit sequence, L_G is the total length of gaps in the BLAST alignment.*

The **resolution** of a crystal structure is related to the size of structural features that can be resolved from the raw experimental data.

R$_\text{free}$ is used to assess possible overmodeling of the experimental data.

Resolution and R$_\text{free}$ are only given for crystal structures. NMR structures will therefore usually be ranked lower than crystal structures. Likewise, structures where R_{free} has not been given will tend to receive a lower rank. This often coincides with structures of older date.

How a model structure is created

A structure model is created by mapping the query sequence onto the template structure based on a sequence alignment (see figure 20.20):

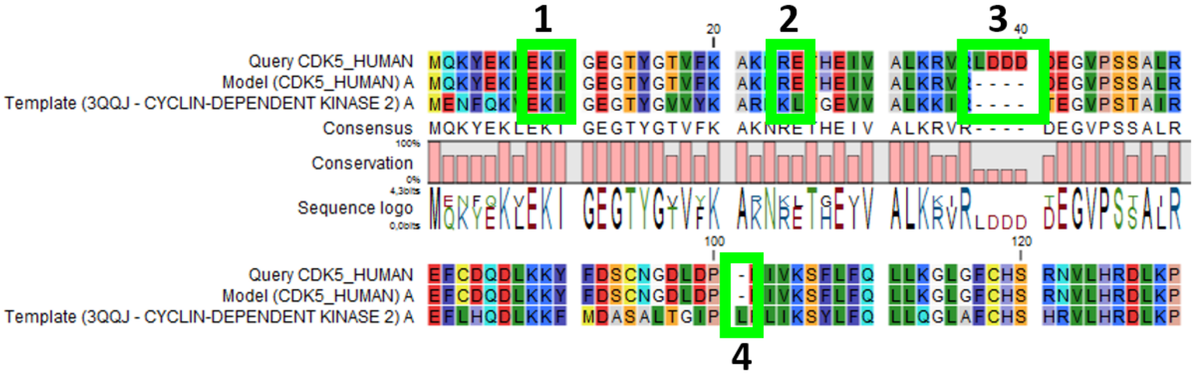

1
2
3
20
40
Query CDK5_HUMAN MQKYEKLEKI GEGTYGTVFK AKNRETHEIV ALKRVRLDDD DEGVPSSALR
Model (CDK5_HUMAN) A MQKYEKLEKI GEGTYGTVFK AKNRETHEIV ALKRVR---- DEGVPSSALR
Template (3QQJ - CYCLIN-DEPENDENT KINASE 2) A MENFQKVEKI GEGTYGVVYK ARNKLTGEVV ALKKIR---- TEGVPSTAIR
Consensus MQKYEKLEKI GEGTYGTVFK AKNRETHEIV ALKRVR---- DEGVPSSALR
Conservation
100%
0%
Sequence logo
4,3bits
0,0bits
100
120
Query CDK5_HUMAN EFCDQDLKKY FDSCNGDLDP -EIVKSFLFQ LLKGLGFCHS RNVLHRDLKP
Model (CDK5_HUMAN) A EFCDQDLKKY FDSCNGDLDP -EIVKSFLFQ LLKGLGFCHS RNVLHRDLKP
Template (3QQJ - CYCLIN-DEPENDENT KINASE 2) A EFLHQDLKKF MDASALTGIP LPLIKSYLFQ LLQGLAFCHS HRVLHRDLKP
4

Figure 20.20: *Sequence alignment mapping query sequence (Query CDK5_HUMAN) to the structure with sequence "Template(3QQJ - CYCLIN-DEPENDENT KINASE 2)", producing a structure with sequence "Model(CDK5_HUMAN)". Examples are highlighted: 1. Identical amino acids, 2. Amino acid changes, 3. Amino acids in query sequence not aligned to a position on the template structure, and 4. Amino acids on the template structure, not aligned to query sequence.*

- For identical amino acids (example 1 in figure 20.20) => Copy atom positions from the PDB file. If the side chain is missing atoms in the PDB file, the side chain is rebuilt (section 20.7.2).
- For amino acid changes (example 2 in figure 20.20) => Copy backbone atom positions from the PDB file. Model side chain atom positions to match the query sequence (section 20.7.2).
- For amino acids in the query sequence not aligned to a position on the template structure (example 3 in figure 20.20) => No atoms are modeled. The model backbone will have a gap at this position and a "Structure modeling" issue is raised (see section 15.1.4).
- For amino acids on the template structure, not aligned to the query sequence (example 4 in figure 20.20) => The residues are deleted from the structure and a "Structure modeling" issue is raised (see section 15.1.4).

How side chains are modeled

Amino acid side chains tend to assume one of a discrete number of "rotamer" conformations. The rotamers used in *CLC Main Workbench* have been calculated from a non-redundant set of high-resolution crystal structures.

Side chains are modeled using a heat bath Monte Carlo simulated annealing algorithm, similar to the OPUS-Rota method [Lu et al., 2008]. The algorithm consists of approximately 100 cycles of simulation. In a single cycle, rotamers are selected for each side chain with a probability according to their energy. As the simulation proceeds, the selection increasingly favors the rotamers with the lowest energy, and the algorithm converges.

A local minimization of the modeled side chains is then carried out, to reduce unfavorable interactions with the surroundings.

Calculating the energy of a side chain rotamer

The total energy is composed of several terms:

- Statistical potential: This score accounts for interactions between the given side chain and the local backbone, and is estimated from a database of high-resolution crystal structures. It depends only on the rotamer and the local backbone dihedral angles ϕ and ψ.
- Atom interaction potential: This score is used to evaluate the interaction between a given side chain atom and its surroundings.
- Disulfide potential: Only applies to cysteines. It follows the form used in the RASP program [Miao et al., 2011] and serves to allow disulfide bridges between cysteine residues. It penalizes deviations from ideal disulfide geometry. A distance filter is applied to determine if the disulfide potential should be used, and when it is applied the atom interaction potential between the two sulfur atoms is turned off. Note that disulfide bridges are not formed between separate chains.

Note: The atom interaction potential considers interactions within the modeled protein chain as well as with all other molecules in the downloaded PDB file (except water).

Local minimization of side chain

After applying a side chain rotamer from the library to the backbone, a local minimization may be carried out for rotations around single bonds in the side chain.

The potential to minimize with respect to bond rotation is composed of the following terms:

- Atom interaction potential: Same as for calculating the energy of a rotamer.
- Disulfide potential: Same as for calculating the energy of a rotamer.
- Harmonic potential: This penalizes small deviations from ideal rotamers according to a harmonic potential. This is motivated by the concept of a rotamer representing a minimum energy state for a residue without external interactions.

20.8 Secondary structure prediction

An important issue when trying to understand protein function is to know the actual structure of the protein. Many questions that are raised by molecular biologists are directly targeted at protein structure. The alpha-helix forms a coiled rod like structure whereas a beta-sheet show an extended sheet-like structure. Some proteins are almost devoid of alpha-helices such as chymotrypsin (PDB_ID: 1AB9) whereas others like myoglobin (PDB_ID: 101M) have a very high content of alpha-helices.

With *CLC Main Workbench* one can predict the secondary structure of proteins very fast. Predicted elements are alpha-helix, beta-sheet (same as beta-strand) and other regions.

Based on extracted protein sequences from the Protein Data Bank (`https://www.rcsb.org/`) a hidden Markov model (HMM) was trained and evaluated for performance. Machine learning methods have shown superior when it comes to prediction of secondary structure of proteins [Rost, 2001]. By far the most common structures are Alpha-helices and beta-sheets which can be predicted, and predicted structures are automatically added to the query as annotation which later can be edited.

In order to predict the secondary structure of proteins:

Tools | **Protein Analysis** (▨)| **Predict secondary structure** (▨)

This opens the dialog displayed in figure 20.21:

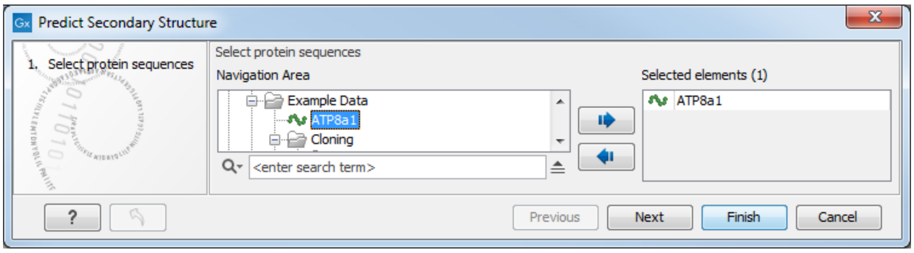


Figure 20.21: *Choosing one or more protein sequences for secondary structure prediction.*

If a sequence was selected before running the tool, that sequence will be listed in the **Selected Elements** pane of the dialog. Use the arrows to add or remove sequences or sequence lists from the selected elements.

You can perform the analysis on several protein sequences at a time. This will add annotations to all the sequences and open a view for each sequence.

Click **Finish** to start the tool.

After running the prediction as described above, the protein sequence will show predicted alpha-helices and beta-sheets as annotations on the original sequence (see figure 20.22).

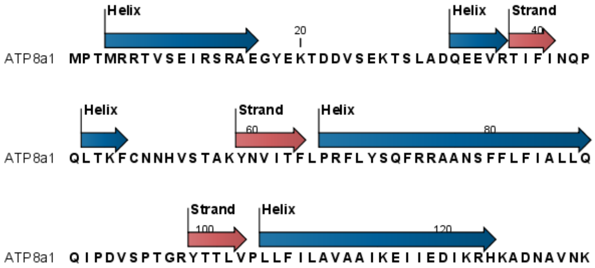

Helix
20
Helix
Strand
40
ATP8a1 MPTMRRTVSEIRSRAEGYEKTDDVSEKTSLADQEEVRTIFINQP
Helix
Strand
60
Helix
80
ATP8a1 QLTKFCNNHVSTAKYNVITFLPRFLYSQFRRAANSFFLFIALLQ
Strand
100
Helix
120
ATP8a1 QIPDVSPTGRYTTLVPLLFILAVAAIKEIIEDIKRHKADNAVNK

Figure 20.22: *Alpha-helices and beta-strands shown as annotations on the sequence.*

Each annotation will carry a tooltip note saying that the corresponding annotation is predicted with *CLC Main Workbench*. Additional notes can be added through the **Edit Annotation** (▨) right-click mouse menu. See section 14.3.2.

Undesired alpha-helices or beta-sheets can be removed through the **Delete Annotation** (▨) right-click mouse menu. See section 14.3.5.

20.9 Protein report

CLC Main Workbench is able to produce protein reports, a collection of some of the protein analyses described elsewhere in this manual.

To create a protein report do the following:

Tools | Protein Analysis ( )| Create Protein Report ( )

This opens a dialog where you can choose which proteins to create a report for. If you had already selected a sequence in the Navigation Area before running the tool, that sequence will be listed in the **Selected Elements** pane. Use the arrows to add and remove elements from the list of selected elements.

In the next dialog, you can choose which analyses you want to include in the report. The following list shows which analyses are available and explains where to find more details.

- **Sequence statistics.** Will produce a section called Protein statistics, as described in section 18.6.1.
- **Protein charge plot.** Plot of charge as function of pH, see section 20.1.
- **Hydrophobicity plot.** See section 20.3.
- **Complexity plot.** See section 18.5.
- **Dot plot.** See section 18.4.
- **Secondary structure prediction.** See section 20.8.
- **Pfam domain search.** See section 20.5.
- **BLAST against NCBI databases.** See section 26.1.1.

When you have selected the relevant analyses, click **Next**. In the following dialogs, adjust the parameters for the different analyses you selected. The parameters are explained in more details in the relevant chapters or sections (mentioned in the list above).

For sequence statistics:

- **Individual Statistics Layout. Comparative** is disabled because reports are generated for one protein at a time.
- **Include Background Distribution of Amino Acids.** Includes distributions from different organisms. Background distributions are calculated from UniProt `www.uniprot.org` version 6.0, dated September 13 2005.

For hydrophobicity plots:

- **Hydrophobicity scales.** Lets you choose between different scales.
- **Window size.** Width of window on sequence (it must be an odd number).

For complexity plots:

- **Window size.** Width of window on sequence (must be odd).

For dot plots:

- **Score model.** Different scoring matrices.
- **Window size.** Width of window on sequence.

For Pfam domain search:

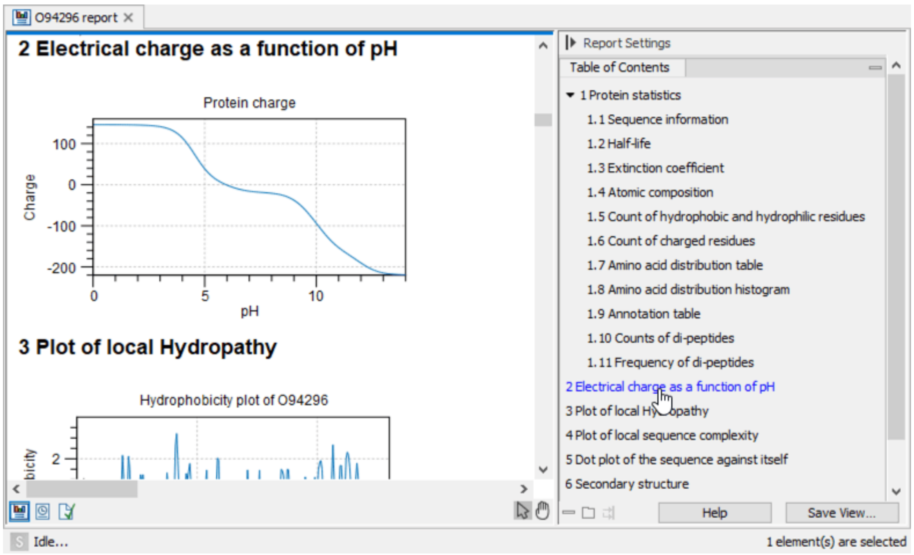
- **Database and search type** lets you choose different databases and specify the search for full domains or fragments.
- **Significance cutoff** lets you set your E-value.

For BLAST against NCBI databases:

- **Program** lets you choose between different BLAST programs.
- **Database** lets you limit your search to a particular database.
- **Genetic code** lets you choose a genetic code for the sequence or the database.

Also set the BLAST parameters as explained in section 26.1.1.

An example of Protein report can be seen in figure 20.23.



O94296 report

2 Electrical charge as a function of pH

3 Plot of local Hydropathy

Report Settings

Table of Contents

- 1 Protein statistics
  - 1.1 Sequence information
  - 1.2 Half-life
  - 1.3 Extinction coefficient
  - 1.4 Atomic composition
  - 1.5 Count of hydrophobic and hydrophilic residues
  - 1.6 Count of charged residues
  - 1.7 Amino acid distribution table
  - 1.8 Amino acid distribution histogram
  - 1.9 Annotation table
  - 1.10 Counts of di-peptides
  - 1.11 Frequency of di-peptides
- 2 Electrical charge as a function of pH
- 3 Plot of local Hydropathy
- 4 Plot of local sequence complexity
- 5 Dot plot of the sequence against itself
- 6 Secondary structure

Idle...

1 element(s) are selected

Figure 20.23: *A protein report. There is a Table of Contents in the Side Panel that makes it easy to browse the report.*

By double clicking a graph in the output, this graph is shown in a different view (*CLC Main Workbench* generates another tab). The report output and the new graph views can be saved by dragging the tab into the **Navigation Area**.

The content of the tables in the report can be copy/pasted out of the program and e.g. into Microsoft Excel. You can also **Export** (📤) the report in Excel format.

20.10 Reverse translation from protein into DNA

A protein sequence can be back-translated into DNA using *CLC Main Workbench*. Due to degeneracy of the genetic code every amino acid could translate into several different codons (only 20 amino acids but 64 different codons). Thus, the program offers a number of choices for determining which codons should be used. These choices are explained in this section. For background information see section 20.10.1.

In order to make a reverse translation:

Tools | **Protein Analysis** ( )| **Reverse Translate** ( )

This opens the dialog displayed in figure 20.24:

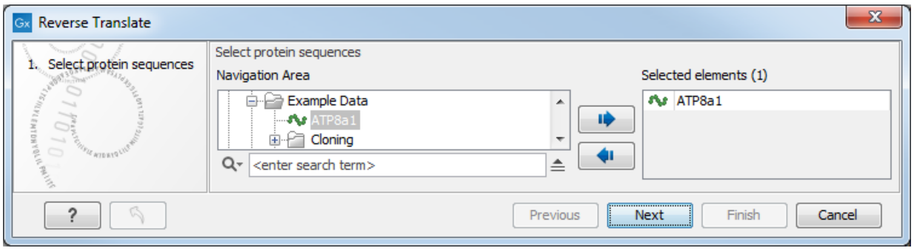
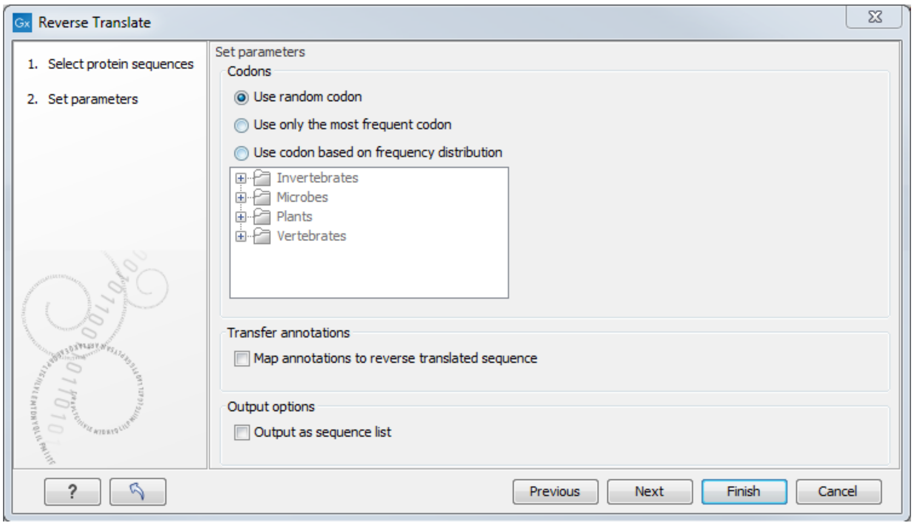


Figure 20.24: *Choosing a protein sequence for reverse translation.*

If a sequence was selected before running the tool, that sequence will be listed in the **Selected Elements** pane of the dialog. Use the arrows to add or remove sequences or sequence lists from the selected elements. You can translate several protein sequences at a time.

Adjust the parameters for the translation in the dialog shown in figure 20.25.



● Use random codon
○ Use only the most frequent codon
○ Use codon based on frequency distribution

□ Map annotations to reverse translated sequence

□ Output as sequence list

Figure 20.25: *Choosing parameters for the reverse translation.*

- **Use random codon.** This will randomly back-translate an amino acid to a codon assuming the genetic code to be 1, but without using the codon frequency tables. Every time you perform the analysis you will get a different result.
- **Use only the most frequent codon.** On the basis of the selected translation table, this parameter/option will assign the codon that occurs most often. When choosing this option,

the results of performing several reverse translations will always be the same, contrary to the other two options.

- **Use codon based on frequency distribution.** This option is a mix of the other two options. The selected translation table is used to attach weights to each codon based on its frequency. The codons are assigned randomly with a probability given by the weights. A more frequent codon has a higher probability of being selected. Every time you perform the analysis, you will get a different result. This option yields a result that is closer to the translation behavior of the organism (assuming you choose an appropriate codon frequency table).

- **Map annotations to reverse translated sequence.** If this checkbox is checked, then all annotations on the protein sequence will be mapped to the resulting DNA sequence. In the tooltip on the transferred annotations, there is a note saying that the annotation derives from the original sequence.

The **Codon Frequency Table** is used to determine the frequencies of the codons. Select a frequency table from the list that fits the organism you are working with. A translation table of an organism is created on the basis of counting all the codons in the coding sequences. Every codon in a **Codon Frequency Table** has its own count, frequency (per thousand) and fraction which are calculated in accordance with the occurrences of the codon in the organism. The tables provided were made using Codon Usage database https://www.kazusa.or.jp/codon/ that was built on The NCBI-GenBank Flat File Release 160.0 [June 15 2007]. You can customize the list of codon frequency tables for your installation, see Appendix J.

Click **Finish** to start the tool. The newly created nucleotide sequence is shown, and if the analysis was performed on several protein sequences, there will be a corresponding number of views of nucleotide sequences.

20.10.1 Bioinformatics explained: Reverse translation

In all living cells containing hereditary material such as DNA, a transcription to mRNA and subsequent a translation to proteins occur. This is of course simplified but is in general what is happening in order to have a steady production of proteins needed for the survival of the cell. In bioinformatics analysis of proteins it is sometimes useful to know the ancestral DNA sequence in order to find the genomic localization of the gene. Thus, the translation of proteins back to DNA/RNA is of particular interest, and is called reverse translation or back-translation.

The Genetic Code In 1968 the Nobel Prize in Medicine was awarded to Robert W. Holley, Har Gobind Khorana and Marshall W. Nirenberg for their interpretation of the Genetic Code (http://nobelprize.org/medicine/laureates/1968/). The Genetic Code represents translations of all 64 different codons into 20 different amino acids. Therefore it is no problem to translate a DNA/RNA sequence into a specific protein. But due to the degeneracy of the genetic code, several codons may code for only one specific amino acid. This can be seen in the table below. After the discovery of the genetic code it has been concluded that different organism (and organelles) have genetic codes which are different from the "standard genetic code". Moreover, the amino acid alphabet is no longer limited to 20 amino acids. The 21'st amino acid, selenocysteine, is encoded by an 'UGA' codon which is normally a stop codon. The discrimination of a selenocysteine over a stop codon is carried out by the translation machinery. Selenocysteines are very rare amino acids.

The table below shows the Standard Genetic Code which is the default translation table.

TTT F Phe	TCT S Ser	TAT Y Tyr	TGT C Cys
TTC F Phe	TCC S Ser	TAC Y Tyr	TGC C Cys
TTA L Leu	TCA S Ser	TAA * Ter	TGA * Ter
TTG L Leu i	TCG S Ser	TAG * Ter	TGG W Trp
CTT L Leu	CCT P Pro	CAT H His	CGT R Arg
CTC L Leu	CCC P Pro	CAC H His	CGC R Arg
CTA L Leu	CCA P Pro	CAA Q Gln	CGA R Arg
CTG L Leu i	CCG P Pro	CAG Q Gln	CGG R Arg
ATT I Ile	ACT T Thr	AAT N Asn	AGT S Ser
ATC I Ile	ACC T Thr	AAC N Asn	AGC S Ser
ATA I Ile	ACA T Thr	AAA K Lys	AGA R Arg
ATG M Met i	ACG T Thr	AAG K Lys	AGG R Arg
GTT V Val	GCT A Ala	GAT D Asp	GGT G Gly
GTC V Val	GCC A Ala	GAC D Asp	GGC G Gly
GTA V Val	GCA A Ala	GAA E Glu	GGA G Gly
GTG V Val	GCG A Ala	GAG E Glu	GGG G Gly

Solving the ambiguities of reverse translation A particular protein follows from the translation of a DNA sequence whereas the reverse translation need not have a specific solution according to the Genetic Code. The Genetic Code is degenerate which means that a particular amino acid can be translated into more than one codon. Hence there are ambiguities of the reverse translation.

In order to solve these ambiguities of reverse translation you can define how to prioritize the codon selection, e.g:

- Choose a codon randomly.
- Select the most frequent codon in a given organism.
- Randomize a codon, but with respect to its frequency in the organism.

As an example we want to translate an alanine to the corresponding codon. Four different codons can be used for this reverse translation; GCU, GCC, GCA or GCG. By picking either one by random choice we will get an alanine.

The most frequent codon, coding for an alanine in *E. coli* is GCG, encoding 33.7% of all alanines. Then comes GCC (25.5%), GCA (20.3%) and finally GCU (15.3%). The data are retrieved from the Codon usage database, see below. Always picking the most frequent codon does not necessarily give the best answer.

By selecting codons from a distribution of calculated codon frequencies, the DNA sequence obtained after the reverse translation, holds the correct (or nearly correct) codon distribution. It should be kept in mind that the obtained DNA sequence is not necessarily identical to the original one encoding the protein in the first place, due to the degeneracy of the genetic code.

In order to obtain the best possible result of the reverse translation, one should use the codon frequency table from the correct organism or a closely related species. The codon usage of the mitochondrial chromosome are often different from the native chromosome(s), thus mitochondrial codon frequency tables should only be used when working specifically with mitochondria.

Other useful resources

The Genetic Code at NCBI:
http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi?mode=c

Codon usage database:
http://www.kazusa.or.jp/codon/

Wikipedia on the genetic code
http://en.wikipedia.org/wiki/Genetic_code

20.11 Proteolytic cleavage detection

Given a protein sequence, *CLC Main Workbench* detects proteolytic cleavage sites in accordance with detection parameters and shows the detected sites as annotations on the sequence as well as in a table below the sequence view.

Detection of proteolytic cleavage sites is initiated by:

Tools | **Protein Analysis** ( )| **Proteolytic Cleavage** ( )

If you are connected to a server, the first wizard step will ask you where you would like to run the analysis. Next, you must provide one or more sequences figure 20.26.

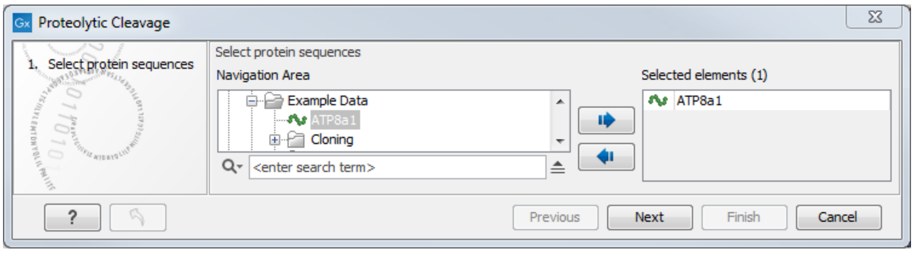
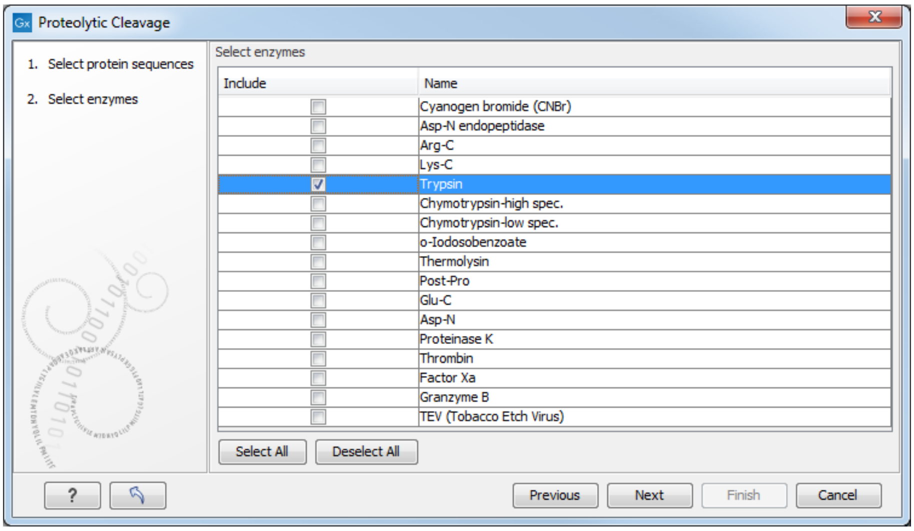


Figure 20.26: *Choosing a protein sequence for proteolytic cleavage.*

In the second dialog, you can select proteolytic cleavage enzymes. Presently, the list contains the enzymes shown in figure 20.27. The full list of enzymes and their cleavage patterns can be seen in Appendix, section C.

You can then set parameters for the detection. This limits the number of detected cleavages (figure 20.28).

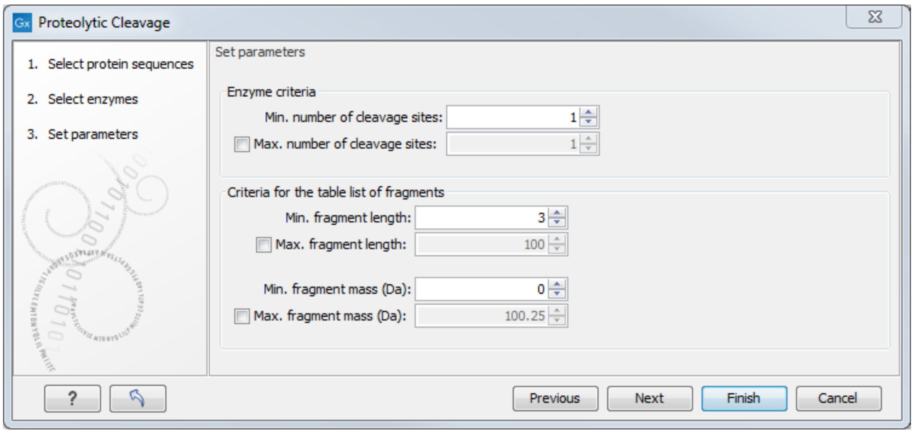
- **Min. and max. number of cleavage sites.** Certain proteolytic enzymes cleave at many positions in the amino acid sequence. For instance proteinase K cleaves at nine different amino acids, regardless of the surrounding residues. Thus, it can be very useful to limit the number of actual cleavage sites before running the analysis.



Select enzymes

Include	Name
□	Cyanogen bromide (CNBr)
□	Asp-N endopeptidase
□	Arg-C
□	Lys-C
☑	Trypsin
□	Chymotrypsin-high spec.
□	Chymotrypsin-low spec.
□	o-Iodosobenzoate
□	Thermolysin
□	Post-Pro
□	Glu-C
□	Asp-N
□	Proteinase K
□	Thrombin
□	Factor Xa
□	Granzyme B
□	TEV (Tobacco Etch Virus)

Figure 20.27: *Setting parameters for proteolytic cleavage detection.*



Set parameters

Enzyme criteria

Min. number of cleavage sites: 1

□ Max. number of cleavage sites: 1

Criteria for the table list of fragments

Min. fragment length: 3

□ Max. fragment length: 100

Min. fragment mass (Da): 0

□ Max. fragment mass (Da): 100.25

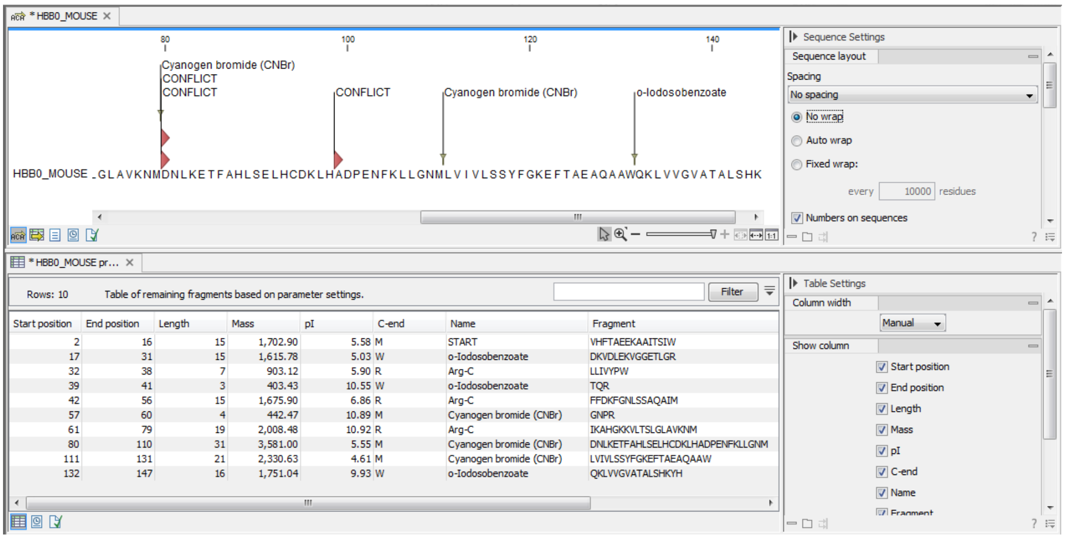
Figure 20.28: *Setting parameters for proteolytic cleavage detection.*

- **Min. and max. fragment length** Likewise, it is possible to limit the output to only display sequence fragments between a chosen length. Both a lower and upper limit can be chosen.
- **Min. and max. fragment mass** The molecular weight is not necessarily directly correlated to the fragment length as amino acids have different molecular masses. For that reason it is also possible to limit the search for proteolytic cleavage sites to mass-range.

For example, if you have one protein sequence but you only want to show which enzymes cut between two and four times. Then you should select "The enzymes has more cleavage sites than 2" and select "The enzyme has less cleavage sites than 4". In the next step you should simply select all enzymes. This will result in a view where only enzymes which cut 2,3 or 4 times are presented.

Click **Finish** to start the tool. The result of the detection is displayed in figure 20.29.

Depending on the settings in the program, the output of the proteolytic cleavage site detection will display two views on the screen. The top view shows the actual protein sequence with the predicted cleavage sites indicated by small arrows. If no labels are found on the arrows they can be enabled by setting the labels in the "annotation layout" in the preference panel. The bottom view shows a text output of the detection, listing the individual fragments and information on



Rows: 10 Table of remaining fragments based on parameter settings.

Start position	End position	Length	Mass	pI	C-end	Name	Fragment
2	16	15	1,702.90	5.58	M	START	VHFTAEEKAAITSIW
17	31	15	1,615.78	5.03	W	o-Iodosobenzoate	DKVDLEKVGGETLGR
32	38	7	903.12	5.90	R	Arg-C	LLIVYPW
39	41	3	403.43	10.55	W	o-Iodosobenzoate	TQR
42	56	15	1,675.90	6.86	R	Arg-C	FFDKFGNLSSAQAIM
57	60	4	442.47	10.89	M	Cyanogen bromide (CNBr)	GNPR
61	79	19	2,008.48	10.92	R	Arg-C	IKAHGKKVLTSLGLAVKNM
80	110	31	3,581.00	5.55	M	Cyanogen bromide (CNBr)	DNLKETFAHLSELHCDKLHADPENFKLLGNM
111	131	21	2,330.63	4.61	M	Cyanogen bromide (CNBr)	LVIVLSSYFGKEFTAEAQAAW
132	147	16	1,751.04	9.93	W	o-Iodosobenzoate	QKLVVGVATALSHKYH

Figure 20.29: *The result of the proteolytic cleavage detection.*

these.

20.11.1 Bioinformatics explained: Proteolytic cleavage

Proteolytic cleavage is basically the process of breaking the peptide bonds between amino acids in proteins. This process is carried out by enzymes called peptidases, proteases or proteolytic cleavage enzymes.

Proteins often undergo proteolytic processing by specific proteolytic enzymes (proteases/peptidases) before final maturation of the protein. Proteins can also be cleaved as a result of intracellular processing of, for example, misfolded proteins. Another example of proteolytic processing of proteins is secretory proteins or proteins targeted to organelles, which have their signal peptide removed by specific signal peptidases before release to the extracellular environment or specific organelle.

Below a few processes are listed where proteolytic enzymes act on a protein substrate.

- N-terminal methionine residues are often removed after translation.
- Signal peptides or targeting sequences are removed during translocation through a membrane.
- Viral proteins that were translated from a monocistronic mRNA are cleaved.
- Proteins or peptides can be cleaved and used as nutrients.
- Precursor proteins are often processed to yield the mature protein.

Proteolytic cleavage of proteins has shown its importance in laboratory experiments where it is often useful to work with specific peptide fragments instead of entire proteins.

Proteases also have commercial applications. As an example proteases can be used as detergents for cleavage of proteinaceous stains in clothing.

The general nomenclature of cleavage site positions of the substrate were formulated by Schechter and Berger, 1967-68 [Schechter and Berger, 1967], [Schechter and Berger, 1968]. They designate the cleavage site between P1-P1', incrementing the numbering in the N-terminal direction of the cleaved peptide bond (P2, P3, P4, etc..). On the carboxyl side of the cleavage site the numbering is incremented in the same way (P1', P2', P3' etc.). This is visualized in figure 20.30.

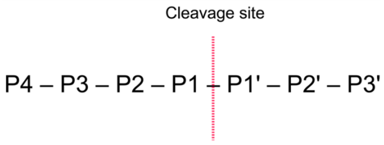


Figure 20.30: *Nomenclature of the peptide substrate. The substrate is cleaved between position P1-P1'.*

Proteases often have a specific recognition site where the peptide bond is cleaved. As an example trypsin only cleaves at lysine or arginine residues, but it does not matter (with a few exceptions) which amino acid is located at position P1'(carboxyterminal of the cleavage site). Another example is trombin which cleaves if an arginine is found in position P1, but not if a D or E is found in position P1' at the same time. (See figure 20.31).

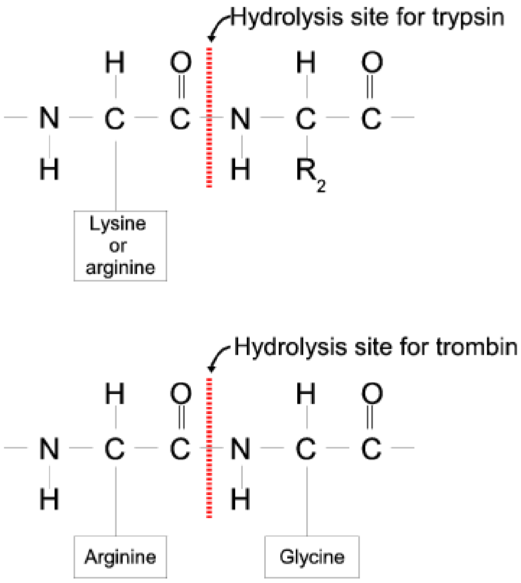


Figure 20.31: *Hydrolysis of the peptide bond between two amino acids. Trypsin cleaves unspecifically at lysine or arginine residues whereas trombin cleaves at arginines if asparate or glutamate is absent.*

Bioinformatics approaches are used to identify potential peptidase cleavage sites. Fragments can be found by scanning the amino acid sequence for patterns which match the corresponding cleavage site for the protease. When identifying cleaved fragments it is relatively important to know the calculated molecular weight and the isoelectric point.

Other useful resources

The Peptidase Database: http://merops.sanger.ac.uk/

Chapter 21

Sequencing data analyses and Assembly

Contents

CLC Main Workbench lets you import, trim and assemble DNA sequence reads from automated sequencing machines. A number of different formats are supported (see section 7.1).

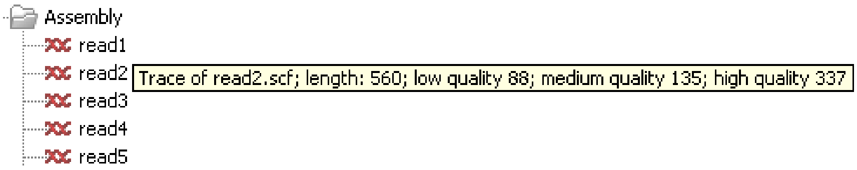
This chapter first explains how to trim sequence reads. Next follows a description of how to assemble reads into contigs both with and without a reference sequence. In the final section, the options for viewing and editing contigs are explained.

21.1 Importing and viewing trace data

A number of different binary trace data formats can be imported into the program, including *Standard Chromatogram Format (.SCF)*, *ABI sequencer data files (.ABI and .AB1)*, *PHRED output files (.PHD)* and *PHRAP output files (.ACE)* (see section 7.1).

After import, the sequence reads and their trace data are saved as DNA sequences. This means that all analyses that apply to DNA sequences can be performed on the sequence reads.

You can see additional information about the quality of the traces by holding the mouse cursor on the imported sequence. This will display a tool tip as shown in figure 21.1.



Assembly
read1
read2 Trace of read2.scf; length: 560; low quality 88; medium quality 135; high quality 337
read3
read4
read5

Figure 21.1: *A tooltip displaying information about the quality of the chromatogram.*

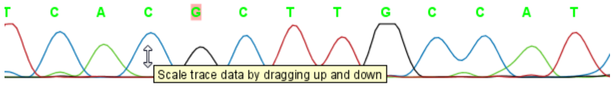
The qualities are based on the phred scoring system, with scores below 19 counted as low quality, scores between 20 and 39 counted as medium quality, and those 40 and above counted as high quality.

If the trace file does not contain information about quality, only the sequence length will be shown.

To view the trace data, open the sequence read in a standard sequence view ().

The traces can be scaled by dragging the trace vertically as shown in figure figure 21.2. The Workbench automatically adjust the height of the traces to be readable, but if the trace height varies a lot, this manual scaling is very useful.

The height of the area available for showing traces can be adjusted in the **Side Panel** as described insection 21.1.1.



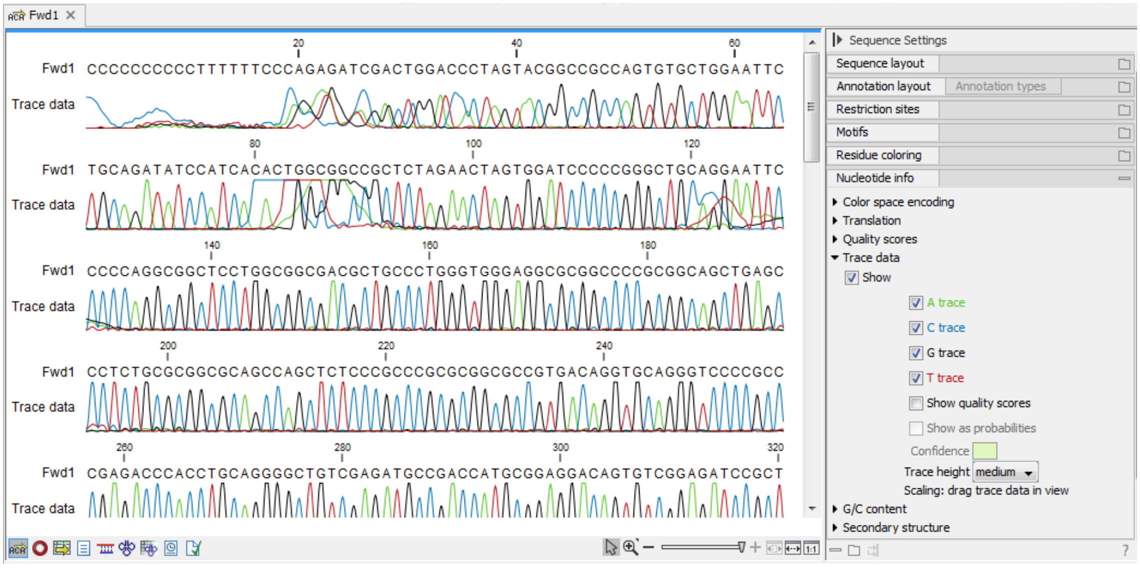
Scale trace data by dragging up and down

Figure 21.2: *Grab the traces to scale.*

21.1.1 Trace settings in the Side Panel

In the Nucleotide info preference group the display of trace data can be selected and unselected. When selected, the trace data information is shown as a plot beneath the sequence. The appearance of the plot can be adjusted using the following options (see figure 21.3):

- **Nucleotide trace.** For each of the four nucleotides the trace data can be selected and unselected.
- **Scale traces.** A slider which allows the user to scale the height of the trace area. Scaling the traces individually is described in section 21.1.



```
                    20                    40                    60
Fwd1        CCCCCCCCCCTTTTTTCCCAGAGATCGACTGGACCCTAGTACGGCCGCCAGTGTGCTGGAATTC
Trace data
                    80                    100                   120
Fwd1        TGCAGATATCCATCACACTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTC
Trace data
                    140                   160                   180
Fwd1        CCCCAGGCGGCTCCTGGCGGCGACGCTGCCCTGGGTGGGAGGCGCGGCCCCGCGGCAGCTGAGC
Trace data
                    200                   220                   240
Fwd1        CCTCTGCGCGGCGCAGCCAGCTCTCCCGCCCGCGCGGCGCCGTGACAGGTGCAGGGTCCCCGCC
Trace data
                    260                   280                   300                   320
Fwd1        CGAGACCCACCTGCAGGGGCTGTCGAGATGCCGACCATGCGGAGGACAGTGTCGGAGATCCGCT
Trace data
```

Sequence Settings
- Sequence layout
- Annotation layout | Annotation types
- Restriction sites
- Motifs
- Residue coloring
- Nucleotide info
  - Color space encoding
  - Translation
  - Quality scores
  - Trace data
    - ☑ Show
      - ☑ A trace
      - ☑ C trace
      - ☑ G trace
      - ☑ T trace
      - ☐ Show quality scores
      - ☐ Show as probabilities
      - Confidence
      - Trace height: medium
      - Scaling: drag trace data in view
  - G/C content
  - Secondary structure

Figure 21.3: *A sequence with trace data. The preferences for viewing the trace are shown in the Side Panel.*

When working with stand-alone mappings containing reads with trace data, you can view the traces by turning on the trace setting options as described here **and** choosing **Not compact** in the Read layout setting for the mapping.

21.2 Trim sequences

Trimming as described in this section involves marking of low quality and/or vector sequence with a Trim annotation as shown in figure 21.4). Such annotated regions are then ignored when using downstream analysis tools located in the same area in the Tools menu, for example Assembly (see section 21.3). The trimming described here annotates, but does not remove data, allowing you to explore the output of different trimming schemes easily.

Trimming as a separate task can be done manually or using a tool designed specifically for this task.

To remove existing trimming information from a sequence, simply remove its trim annotation (see section 14.3.2). When exporting to fasta format, there is an option to remove sequence ends covered by Trim annotations.

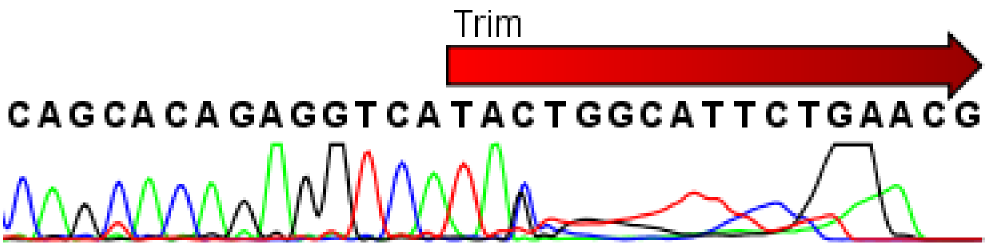


Figure 21.4: *Trimming creates annotations on the regions that will be ignored in the assembly process.*

21.2.1 Trimming using the Trim Sequences tool

Sequence reads can be trimmed based on a number of different criteria. Using a trimming tool for this is particularly useful if:

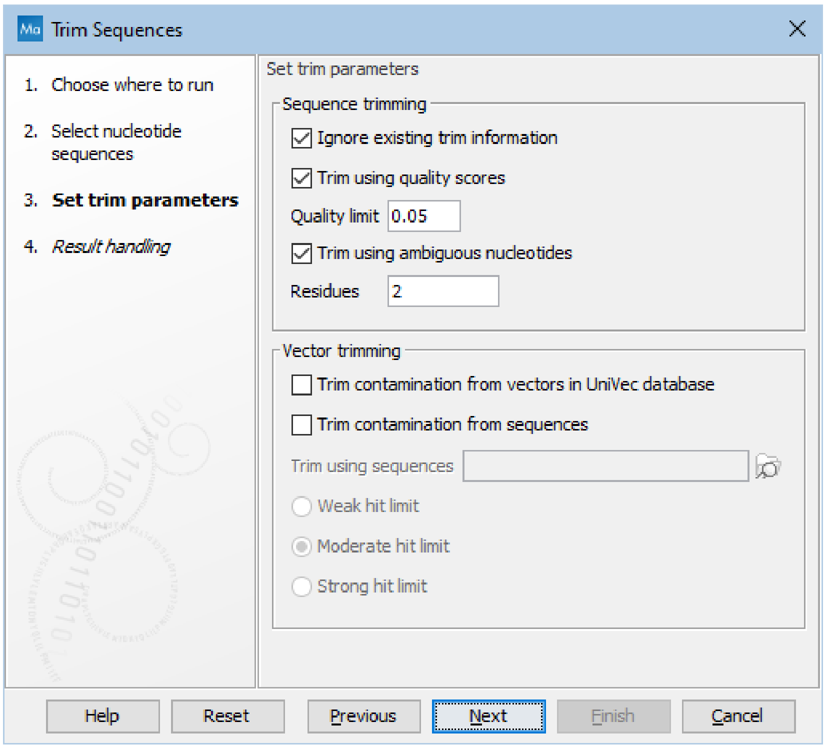
- You have many sequences to trim.
- You wish to trim vector contamination from sequencing reads.
- You wish to ensure that consistency when trimming. That is, you wish to ensure the same criteria are used for all the sequences in a set.

To start up the Trim Sequences tool in the Workbench, go to the menu option:

Tools | **Sanger Sequencing Analysis** (**)**| **Trim Sequences** (**)**

This opens a dialog where you can choose the sequences to trim, by using the arrows to move them between the Navigation Area and the 'Selected Elements' box.

You can then specify the trim parameters as displayed in figure 21.5.



1. Choose where to run
2. Select nucleotide sequences
3. **Set trim parameters**
4. *Result handling*

Set trim parameters

Sequence trimming

☑ Ignore existing trim information

☑ Trim using quality scores

Quality limit 0.05

☑ Trim using ambiguous nucleotides

Residues 2

Vector trimming

☐ Trim contamination from vectors in UniVec database

☐ Trim contamination from sequences

Trim using sequences

○ Weak hit limit

● Moderate hit limit

○ Strong hit limit

Figure 21.5: *Setting parameters for trimming.*

The following parameters can be adjusted in the dialog:

- **Ignore existing trim information.** If you have previously trimmed the sequences, you can check this to remove existing trimming annotation prior to analysis.
- **Trim using quality scores.** If the sequence files contain quality scores from a base caller algorithm this information can be used for trimming sequence ends. The program uses the modified-Mott trimming algorithm for this purpose (Richard Mott, personal communication):

Quality scores in the Workbench are on a Phred scale, and formats using other scales will be converted during import. The Phred quality scores (Q), defined as: $Q = -10log10(P)$, where P is the base-calling error probability, can then be used to calculate the error probabilities, which in turn can be used to set the limit for, which bases should be trimmed.

Hence, the first step in the trim process is to convert the quality score (Q) to an error probability: $p_{error} = 10^{\frac{Q}{-10}}$. (This now means that low values are high quality bases.)

Next, for every base a new value is calculated: $Limit - p_{error}$. This value will be negative for low quality bases, where the error probability is high.

For every base, the Workbench calculates the running sum of this value. If the sum drops below zero, it is set to zero. The part of the sequence not trimmed will be the region ending at the highest value of the running sum and starting at the last zero value before this highest score. Everything before and after this region will be trimmed. A read will be completely removed if the score never makes it above zero.

At https://resources.qiagenbioinformatics.com/testdata/trim.zip you find an example sequence and an Excel sheet showing the calculations done for this particular sequence to illustrate the procedure described above.

- **Trim ambiguous nucleotides.** This option trims the sequence ends based on the presence of ambiguous nucleotides (typically N). Note that the automated sequencer generating the data must be set to output ambiguous nucleotides in order for this option to apply. The algorithm takes as input the *maximal number of ambiguous nucleotides allowed in the sequence after trimming*. If this maximum is set to e.g. 3, the algorithm finds the maximum length region containing 3 or fewer ambiguities and then trims away the ends not included in this region. The "Trim ambiguous nucleotides" option trims all types of ambiguous nucleotides (see Appendix G).

- **Trim contamination from vectors in UniVec database.** If selected, the program will match the sequence reads against all vectors in the UniVec database and mark sequence ends with significant matches with a 'Trim' annotation.

  The UniVec database build 10.1 is included when you install the *CLC Main Workbench*. A list of all the vectors in the database can be found at https://www.ncbi.nlm.nih.gov/VecScreen/replist.html.

- **Trim contamination from sequences.** This option lets you use your own vector sequences that you have imported into the *CLC Main Workbench*. If selected, **Trim using sequences** will be enabled and you can choose one or more sequences.

- **Hit limit for vector trimming.** When at least one vector trimming parameter is selected, the strictness for vector contamination trimming can be specified. Since vector contamination usually occurs at the beginning or end of a sequence, different criteria are applied for terminal and internal matches. A match is considered terminal if it is located within the first 25 bases at either sequence end. Three match categories are defined according to the expected frequency of an alignment with the same score occurring between random sequences. The *CLC Main Workbench* uses the same settings as VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/):

  - **Weak hit limit** Expect 1 random match in 40 queries of length 350 kb.
    - Terminal match with Score 16 to 18.

  - * Internal match with Score 23 to 24.
  - **Moderate hit limit** Expect 1 random match in 1,000 queries of length 350 kb.
    - * Terminal match with Score 19 to 23.
    - * Internal match with Score 25 to 29.
  - **Strong hit limit** Expect 1 random match in 1,000,000 queries of length 350 kb.
    - * Terminal match with Score at least 24.
    - * Internal match with Score at least 30.

In the last step of the wizard, you can choose to create a report, summarizing how each sequence has been trimmed. Click **Finish** to start the tool. This will start the trimming process. Views of each trimmed sequence will be shown, and you can inspect the result by looking at the "Trim" annotations (they are colored red as default). Note that the trim annotations are used to signal that this part of the sequence is to be ignored during further analyses, hence the trimmed sequences are not deleted. If there are no trim annotations, the sequence has not been trimmed.

21.2.2 Manual trimming

Sequence reads can be trimmed manually while inspecting their trace and quality data.

Trimming sequences manually involves adding an annotation of type Trim, with the special condition that this annotation can only be applied to the ends of a sequence:

> **double-click the sequence to trim in the Navigation Area | select the region you want to trim | right-click the selection | Trim sequence left/right to determine the direction of the trimming**

This will add a trimming annotation to the end of the sequence in the selected direction. No sequence is being deleted here. Rather, the regions covered by trim annotations are noted by downstream analyses (in the same section of the Tools menu as the Trim Sequences tool) as regions to be ignored.

21.3 Assemble sequences

This section describes how to assemble a number of sequence reads into a contig without the use of a reference sequence (a known sequence that can be used for comparison with the other sequences, see section 21.4).

Note! You can assemble a maximum of 10,000 sequences at a time.

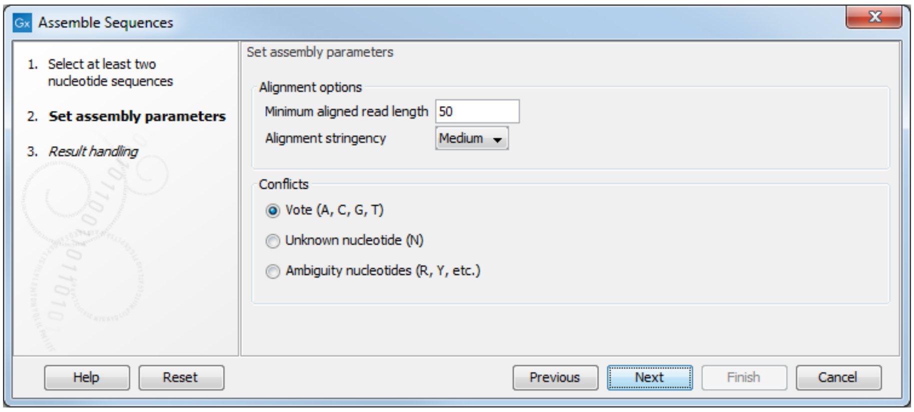
Tools to assemble a larger number of sequences are available in the *CLC Genomics Workbench*: https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-clc-genomics-workbench/

To perform the assembly:

> **Tools | Sanger Sequencing Analysis ( )| Assemble Sequences ( )**

This will open a dialog where you can select sequences to assemble. If you already selected sequences in the Navigation Area, these will be shown in 'Selected Elements'. You can alter your choice of sequences to assemble, or add others, by using the arrows to move sequences between the Navigation Area and the 'Selected Elements' box. You can also add sequence lists.

When the sequences are selected, click **Next**. This will show the dialog in figure 21.6



Assemble Sequences

1. Select at least two nucleotide sequences
2. **Set assembly parameters**
3. *Result handling*

Set assembly parameters

Alignment options

Minimum aligned read length: 50

Alignment stringency: Medium

Conflicts

● Vote (A, C, G, T)

○ Unknown nucleotide (N)

○ Ambiguity nucleotides (R, Y, etc.)

Figure 21.6: *Setting assembly parameters.*

This dialog gives you the following options for assembly:

- **Minimum aligned read length.** The minimum number of nucleotides in a read which must be successfully aligned to the contig. If this criteria is not met by a read, the read is excluded from the assembly.
- **Alignment stringency.** Specifies the stringency (Low, Medium or High) of the scoring function used by the alignment step in the contig assembly algorithm. A higher stringency level will tend to produce contigs with fewer ambiguities but will also tend to omit more sequencing reads and to generate more and shorter contigs.
- **Conflicts.** If there is a conflict, i.e. a position where there is disagreement about the residue (A, C, T or G), you can specify how the contig sequence should reflect the conflict:
  - **Vote (A, C, G, T).** The conflict will be solved by counting instances of each nucleotide and then letting the majority decide the nucleotide in the contig. In case of equality, ACGT are given priority over one another in the stated order.
  - **Unknown nucleotide (N).** The contig will be assigned an 'N' character in all positions with conflicts (conflicts are registered already when two nucleotides differ).
  - **Ambiguity nucleotides (R, Y, etc.).** The contig will display an ambiguity nucleotide reflecting the different nucleotides found in the reads (nucleotide ambiguity is registered already when two nucleotides differ). For an overview of ambiguity codes, see Appendix G.

  Note, that conflicts will always be highlighted no matter which of the options you choose. Furthermore, each conflict will be marked as annotation on the contig sequence and will be present if the contig sequence is extracted for further analysis. As a result, the details of any experimental heterogeneity can be maintained and used when the result of single-sequence analyzes is interpreted. Read more about conflicts in section 21.7.4.
- **Create full contigs, including trace data.** This will create a contig where all the aligned reads are displayed below the contig sequence. (You can always extract the contig sequence without the reads later on.) For more information on how to use the contigs that are created, see section 21.7.

- **Show tabular view of contigs.** A contig can be shown both in a graphical as well as a tabular view. If you select this option, a tabular view of the contig will also be opened (Even if you do not select this option, you can show the tabular view of the contig later on by clicking **Table** () at the bottom of the view.) For more information about the tabular view of contigs, see section 21.7.7.
- **Create only consensus sequences.** This will not display a contig but will only output the assembled contig sequences as single nucleotide sequences. If you choose this option it is not possible to validate the assembly process and edit the contig based on the traces.

When the assembly process has ended, a number of views will be shown, each containing a contig of two or more sequences that have been matched. If the number of contigs seem too high or low, try again with another **Alignment stringency** setting. Depending on your choices of output options above, the views will include trace files or only contig sequences. However, the calculation of the contig is carried out the same way, no matter how the contig is displayed.

See section 21.7 on how to use the resulting contigs.

21.4 Assemble sequences to reference

This section describes how to assemble a number of sequence reads into a contig using a reference sequence, a process called read mapping. A reference sequence can be particularly helpful when the objective is to characterize SNP variation in the data.

Note! You can assemble a maximum of 10,000 sequences at a time.

Tools to assemble a larger number of sequences are available in the *CLC Genomics Workbench*: https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-clc-genomics-workbench/

To run the **Assemble Sequences to Reference** tool, go to:

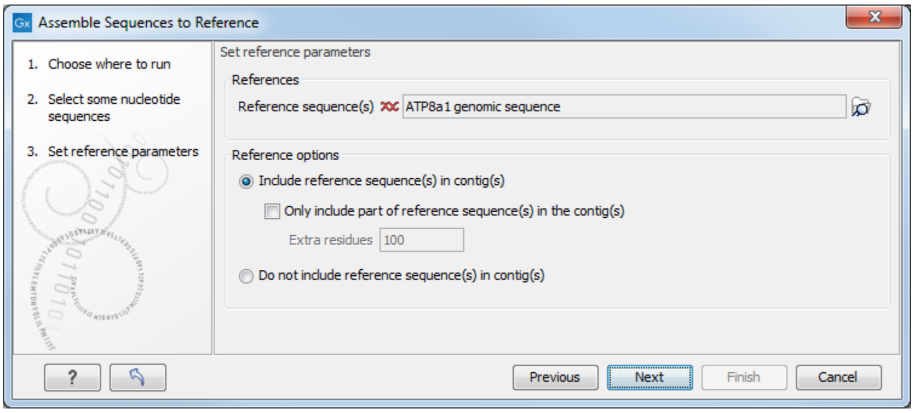
Tools | **Sanger Sequencing Analysis** ()| **Assemble Sequences to Reference** ()

This opens a dialog where you can alter your choice of sequences to assemble. If you have already selected sequences in the Navigation Area, these will be shown in Selected Elements, however you can remove these or add others, by using the arrows to move sequences between the Navigation Area and Selected Elements boxes. You can also add sequence lists.

When the sequences are selected, click **Next**, and you will see the dialog shown in figure 21.7

This dialog gives you the following options for assembling:

- **Reference sequence.** Click the **Browse and select element** icon () in order to select one or more sequences to use as reference(s).
- **Include reference sequence(s) in contig(s).** This will create a contig for each reference with the corresponding reference sequence at the top and the aligned sequences below. This option is useful when comparing sequence reads to a closely related reference sequence e.g. when sequencing for SNP characterization.
  - **Only include part of reference sequence(s) in the contig(s).** If the aligned sequences only cover a small part of a reference sequence, it may not be desirable to include the



Assemble Sequences to Reference

1. Choose where to run
2. Select some nucleotide sequences
3. Set reference parameters

Set reference parameters

References

Reference sequence(s) ATP8a1 genomic sequence

Reference options

● Include reference sequence(s) in contig(s)

□ Only include part of reference sequence(s) in the contig(s)

Extra residues 100

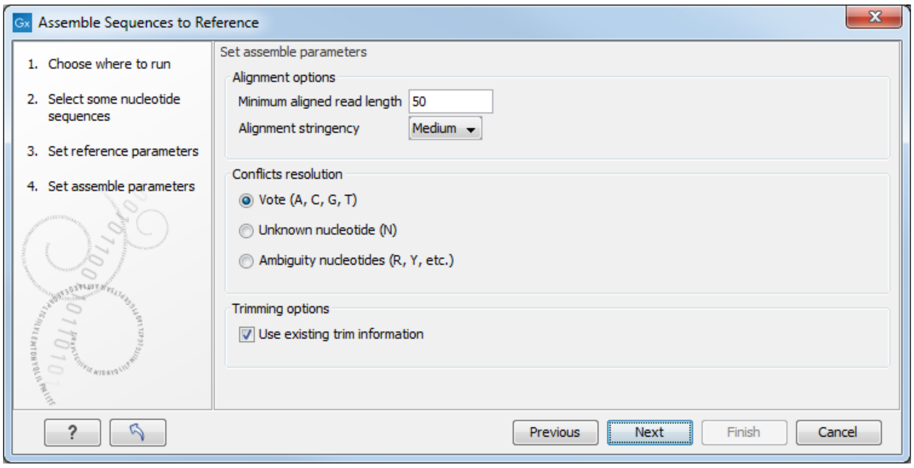
○ Do not include reference sequence(s) in contig(s)

Figure 21.7: *Parameters for how the reference should be handled when assembling sequences to a reference sequence.*

whole reference sequence in a contig. When this option is selected, you can specify the number of residues from reference sequences that should be included on each side of regions spanned by aligned sequences using the **Extra residues** field.

- **Do not include reference sequence(s) in contig(s).** This will produce contigs without any reference sequence where the input sequences have been assembled using reference sequences as a scaffold. The input sequences are first aligned to the reference sequence(s). Next, the consensus sequence for regions spanned by aligned sequences are extracted and output as contigs. This option is useful when performing assembling sequences where the reference sequences that are not closely related to the input sequencing.

When the reference sequence has been selected, click **Next**, to see the dialog shown in figure 21.8



Assemble Sequences to Reference

1. Choose where to run
2. Select some nucleotide sequences
3. Set reference parameters
4. Set assemble parameters

Set assemble parameters

Alignment options

Minimum aligned read length 50

Alignment stringency Medium

Conflicts resolution

● Vote (A, C, G, T)

○ Unknown nucleotide (N)

○ Ambiguity nucleotides (R, Y, etc.)

Trimming options

☑ Use existing trim information

Figure 21.8: *Options for how the input sequences should be aligned and how nucleotide conflicts should be handled.*

In this dialog, you can specify the following options:

- **Minimum aligned read length.** The minimum number of nucleotides in a read which must match a reference sequence. If an input sequence does not meet this criteria, the sequence is excluded from the assembly.

- **Alignment stringency.** Specifies the stringency (Low, Medium or High) of the scoring function used for aligning the input sequences to the reference sequence(s). A higher stringency level often produce contigs with lower levels of ambiguity but also reduces the ability to align distant homologs or sequences with a high error rate to reference sequences. The result of a higher stringency level is often that the number of contigs increases and the average length of contigs decreases while the quality of each contig increases.

  The stringency settings Low, Medium and High are based on the following score values (mt=match, ti=transition, tv=transversion, un=unknown):

Score values			
	Low	Medium	High
Match (mt)	2	2	2
Transversion (tv)	-6	-10	-20
Transition (ti)	-2	-6	-16
Unknown (un)	-2	-6	-16
Gap	-8	-16	-36

Score Matrix					
	A	C	G	T	N
A	mt	tv	ti	tv	un
C	tv	mt	tv	ti	un
G	ti	tv	mt	tv	un
T	tv	ti	tv	mt	un
N	un	un	un	un	un

- **Conflicts resolution.** If there is a conflict, i.e. a position where aligned sequences disagreement about the residue (A, C, T or G), you can specify how the contig sequence should reflect this conflict:

  - **Unknown nucleotide (N).** The contig will be assigned an 'N' character in all positions with conflicts (conflicts are registered already when two nucleotides differ).
  - **Ambiguity nucleotides (R, Y, etc.).** The contig will display an ambiguity nucleotide reflecting the different nucleotides found in the aligned sequences (nucleotide ambiguity is registered when two nucleotides differ). For an overview of ambiguity codes, see Appendix G.
  - **Vote (A, C, G, T).** The conflict will be solved by counting instances of each nucleotide and then letting the majority decide the nucleotide in the contig. In case of equality, ACGT are given priority over one another in the stated order.

  Note, that conflicts will be highlighted for all options. Furthermore, conflicts will be marked with an annotation on each contig sequence which are preserved if the contig sequence is extracted for further analysis. As a result, the details of any experimental heterogeneity can be maintained and used when the result of single-sequence analyzes is interpreted.

- **Trimming options.** When aligning sequences to a reference sequence, trimming is generally not necessary, but if you wish to use trimming you can check this box. It requires that the sequence reads have been trimmed beforehand (see section 21.2 for more information about trimming).

Click **Finish** to start the tool. This will start the assembly process. See section 21.7 on how to use the resulting contigs.

21.5 Sort sequences by name

With this functionality you will be able to group sequencing reads based on their file name. A typical example would be that you have a list of files named like this:

```
...
A02__Asp_F_016_2007-01-10
A02__Asp_R_016_2007-01-10
A02__Gln_F_016_2007-01-11
A02__Gln_R_016_2007-01-11
A03__Asp_F_031_2007-01-10
A03__Asp_R_031_2007-01-10
A03__Gln_F_031_2007-01-11
A03__Gln_R_031_2007-01-11
...
```

In this example, the names have five distinct parts (we take the first name as an example):

- **A02** which is the position on the 96-well plate
- **Asp** which is the name of the gene being sequenced
- **F** which describes the orientation of the read (forward/reverse)
- **016** which is an ID identifying the sample
- **2007-01-10** which is the date of the sequencing run

To start mapping these data, you probably want to have them divided into groups instead of having all reads in one folder. If, for example, you wish to map each sample separately, or if you wish to map each gene separately, you cannot simply run the mapping on all the sequences in one step.

That is where **Sort Sequences by Name** comes into play. It will allow you to specify which part of the name should be used to divide the sequences into groups. We will use the example described above to show how it works:

> **Tools | Molecular Biology Tools () | Sanger Sequencing Analysis () | Sort Sequences by Name ()**

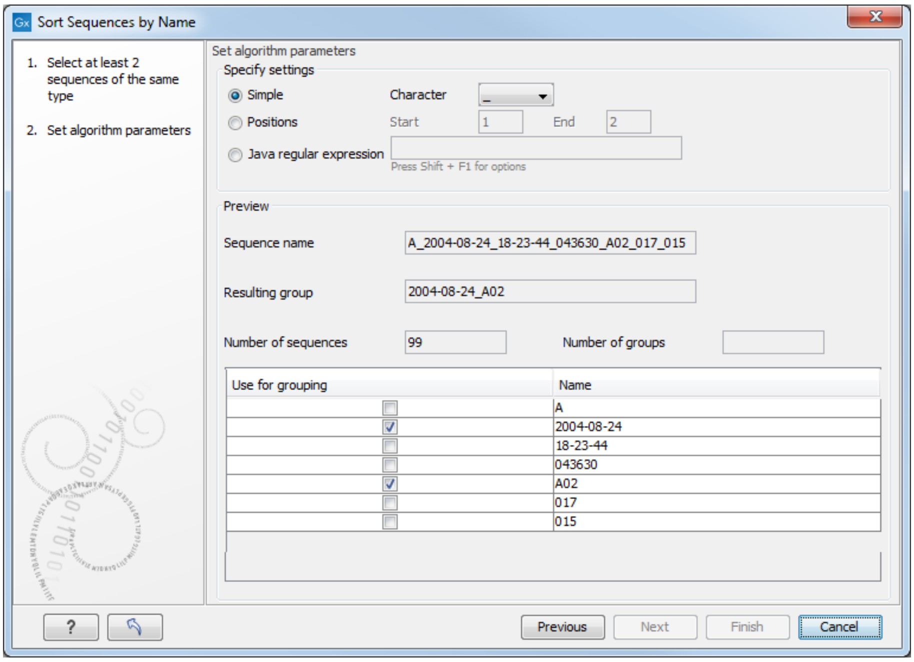
This opens a dialog where you can add the sequences you wish to sort, by using the arrows to move them between the Navigation Area and 'Selected Elements'. You can also add sequence lists or the contents of an entire folder by right-clicking the folder and choose: **Add folder contents**.

When you click **Next**, you will be able to specify the details of how the grouping should be performed. First, you have to choose how each part of the name should be identified. There are three options:

- **Simple**. This will simply use a designated character to split up the name. You can choose a character from the list:
  - Underscore _
  - Dash -
  - Hash (number sign / pound sign) #
  - Pipe |
  - Tilde ~
  - Dot .
- **Positions**. You can define a part of the name by entering the start and end positions, e.g. from character number 6 to 14. For this to work, the names have to be of equal lengths.
- **Java regular expression**. This is an option for advanced users where you can use a special syntax to have total control over the splitting. See more below.

In the example above, it would be sufficient to use a simple split with the underscore _ character, since this is how the different parts of the name are divided.

When you have chosen a way to divide the name, the parts of the name will be listed in the table at the bottom of the dialog. There is a checkbox next to each part of the name. This checkbox is used to specify which of the name parts should be used for grouping. In the example above, if we want to group the reads according to date and analysis position, these two parts should be checked as shown in figure 21.9.


Sort Sequences by Name
1. Select at least 2 sequences of the same type
2. Set algorithm parameters
Set algorithm parameters
Specify settings
Preview
Press Shift + F1 for options
?
Previous
Next
Finish
Cancel

● Simple — Character: _

○ Positions — Start: 1 End: 2

○ Java regular expression

Sequence name: A_2004-08-24_18-23-44_043630_A02_017_015

Resulting group: 2004-08-24_A02

Number of sequences: 99

Number of groups:

Use for grouping	Name
□	A
☑	2004-08-24
□	18-23-44
□	043630
☑	A02
□	017
□	015

Figure 21.9: *Splitting up the name at every underscore (_) and using the date and analysis position for grouping.*

At the middle of the dialog there is a preview panel listing:

- **Sequence name**. This is the name of the first sequence that has been chosen. It is shown here in the dialog in order to give you a sample of what the names in the list look like.

- **Resulting group**. The name of the group that this sequence would belong to if you proceed with the current settings.
- **Number of sequences**. The number of sequences chosen in the first step.
- **Number of groups**. The number of groups that would be produced when you proceed with the current settings.

This preview cannot be changed. It is shown to guide you when finding the appropriate settings.

Click **Finish** to start the tool. A new sequence list will be generated for each group. It will be named according to the group, e.g. *2004-08-24_A02* will be the name of one of the groups in the example shown in figure 21.9.

Advanced splitting using regular expressions

You can see a more detail explanation of the regular expressions syntax in section 18.9.3.

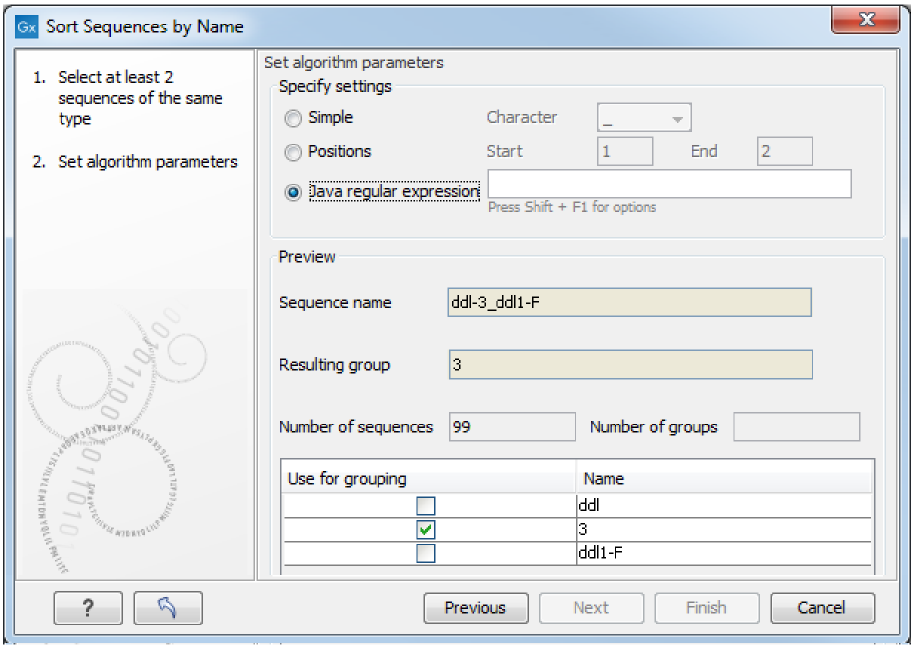
In this section you will see a practical example showing how to create a regular expression. Consider a list of files as shown below:

```
...
adk-29_adk1n-F
adk-29_adk2n-R
adk-3_adk1n-F
adk-3_adk2n-R
adk-66_adk1n-F
adk-66_adk2n-R
atp-29_atpA1n-F
atp-29_atpA2n-R
atp-3_atpA1n-F
atp-3_atpA2n-R
atp-66_atpA1n-F
atp-66_atpA2n-R
...
```

In this example, we wish to group the sequences into three groups based on the number after the "-" and before the "_" (i.e. 29, 3 and 66). The simple splitting as shown in figure 21.9 requires the same character before and after the text used for grouping, and since we now have both a "-" and a "_", we need to use the regular expressions instead (note that dividing by position would not work because we have both single and double digit numbers (3, 29 and 66)).

The regular expression for doing this would be `(.*)-(.*)_(.*)` as shown in figure 21.10.

The round brackets () denote the part of the name that will be listed in the groups table at the bottom of the dialog. In this example we actually did not need the first and last set of brackets, so the expression could also have been `.*-(.*)_.*` in which case only one group would be listed in the table at the bottom of the dialog.



Sort Sequences by Name

1. Select at least 2 sequences of the same type
2. Set algorithm parameters

Set algorithm parameters

Specify settings

○ Simple — Character: _

○ Positions — Start: 1 End: 2

● Java regular expression:

Press Shift + F1 for options

Preview

Sequence name: ddl-3_ddl1-F

Resulting group: 3

Number of sequences: 99 Number of groups:

Use for grouping	Name
□	ddl
☑	3
□	ddl1-F

Figure 21.10: *Dividing the sequence into three groups based on the number in the middle of the name.*

21.6 Add sequences to an existing contig

This section describes how to assemble sequences to an existing contig. This feature can be used for example to provide a steady work-flow when a number of exons from the same gene are sequenced one at a time and assembled to a reference sequence.

Note that the new sequences will be added to the existing contig, which will not be extended. If the new sequences extend beyond the existing contig, they will be cut off.

To run the **Add Sequences to Contig** tool, go to:

Tools | **Sanger Sequencing Analysis ( )**| **Add Sequences to Contig ( )**

or

or **Right-click in the empty white area of the contig** | **Add Sequences to Contig ( )**

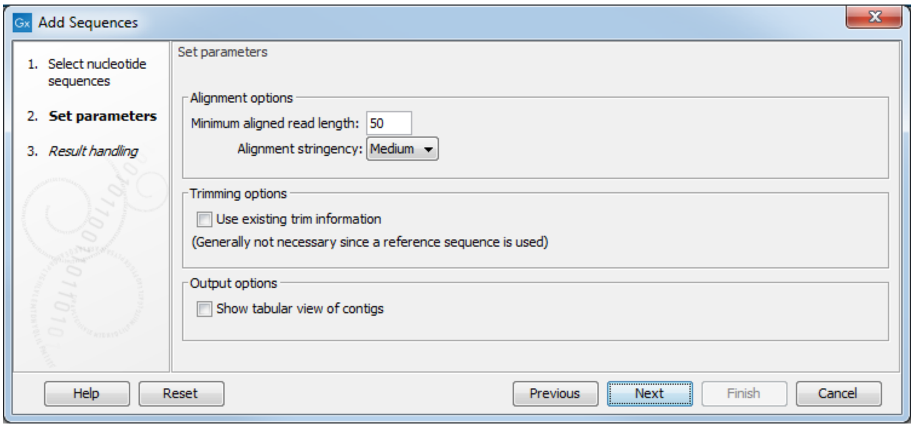
This opens a dialog where you can select one contig and a number of sequences to assemble. If you have already selected sequences in the Navigation Area, these will be shown in the 'Selected Elements' box. However, you can remove these, or add others, by using the arrows to move sequences between the Navigation Area and Selected Elements boxes. You can also add sequence lists.

Often, the results of the assembly will be better if the sequences are trimmed first (see section 21.2.1).

When the elements are selected, click **Next**, and you will see the dialog shown in figure 21.11

The options in this dialog are similar to the options that are available when assembling to a reference sequence (see section 21.4).

Click **Finish** to start the tool. This will start the assembly process. See section 21.7 on how to use the resulting contig.



Add Sequences

1. Select nucleotide sequences
2. **Set parameters**
3. *Result handling*

Set parameters

Alignment options

Minimum aligned read length: 50

Alignment stringency: Medium

Trimming options

□ Use existing trim information

(Generally not necessary since a reference sequence is used)

Output options

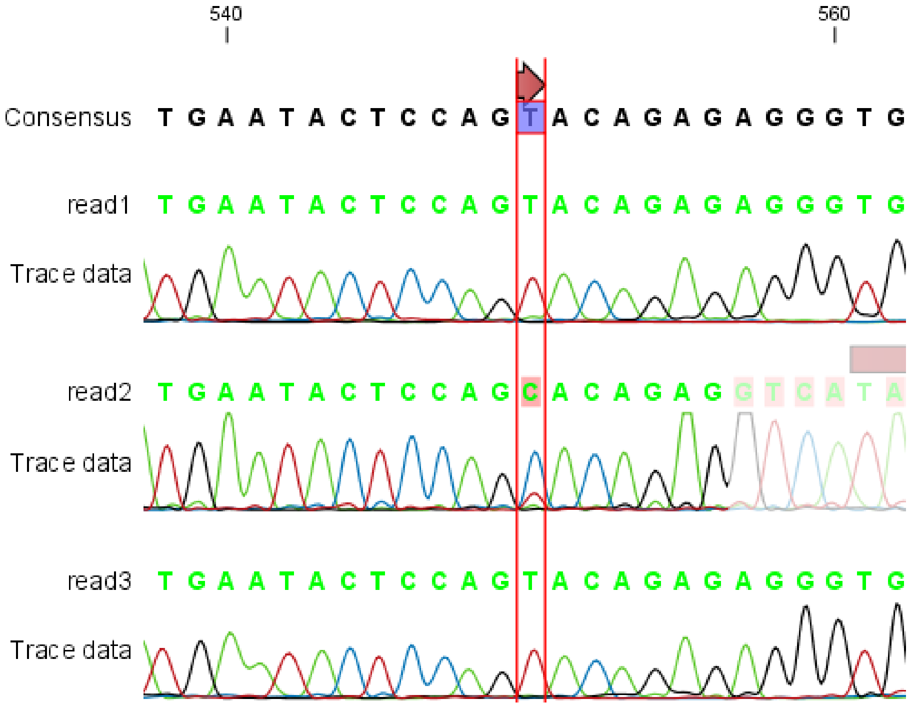
□ Show tabular view of contigs

Figure 21.11: *Setting assembly parameters when assembling to an existing contig.*

Note that the new sequences will be added to the existing contig which will not be extended. If the new sequences extend beyond the existing contig, they will be cut off.

21.7 View and edit contigs and read mappings

The results of Assemble Sequences or Assemble Sequences to Reference are contigs or read mappings, respectively (figure 21.12). A mapping is generated for each reference supplied as input to Assemble Sequences to Reference. Individual read mappings can be opened by double-clicking on a row in the table that lists the mappings.



540
560
Consensus T G A A T A C T C C A G T A C A G A G A G G G T G
read1 T G A A T A C T C C A G T A C A G A G A G G G T G
Trace data
read2 T G A A T A C T C C A G C A C A G A G G T C A T A
Trace data
read3 T G A A T A C T C C A G T A C A G A G A G G G T G
Trace data

Figure 21.12: *The view of a contig. Controls at the bottom allow you to zoom in and out, and settings to the right control how the mapping is displayed.*

Customizing how a contig or mapping looks is covered in section 21.7.1.

The residue colors and traces at the ends of reads are faded in regions that do not contribute to the final contig or mapping results. This may be due to trimming before or during the assembly or to misalignment with other reads (assembly) or the reference sequence (mapping).

Simply drag the edge of the faded section to adjust the trimmed area to include more of the read in the contig or mapping (figure 21.13).

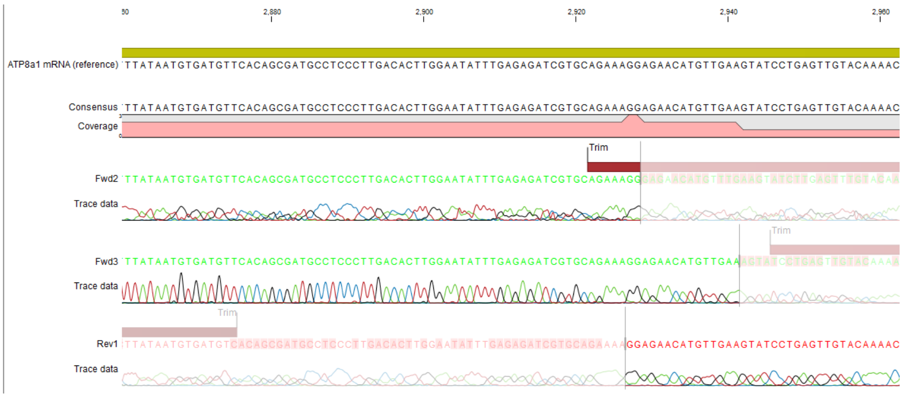

50
2,880
2,900
2,920
2,940
2,960
ATP8a1 mRNA (reference) TTATAATGTGATGTTCACAGCGATGCCTCCCTTGACACTTGGAATATTTGAGAGATCGTGCAGAAAGGAGAACATGTTGAAGTATCCTGAGTTGTACAAAAC
Consensus TTATAATGTGATGTTCACAGCGATGCCTCCCTTGACACTTGGAATATTTGAGAGATCGTGCAGAAAGGAGAACATGTTGAAGTATCCTGAGTTGTACAAAAC
Coverage
Trim
Fwd2
Trace data
Trim
Fwd3
Trace data
Trim
Rev1
Trace data

Figure 21.13: *Drag the edge of the faded area to customize how much of a read should be considered in the mapping.*

Note: Handles for dragging are only available when individual residues can be seen. For this, zoom fully in and chose a **Compactness** level of "Not compact", "Low" or "Packed".

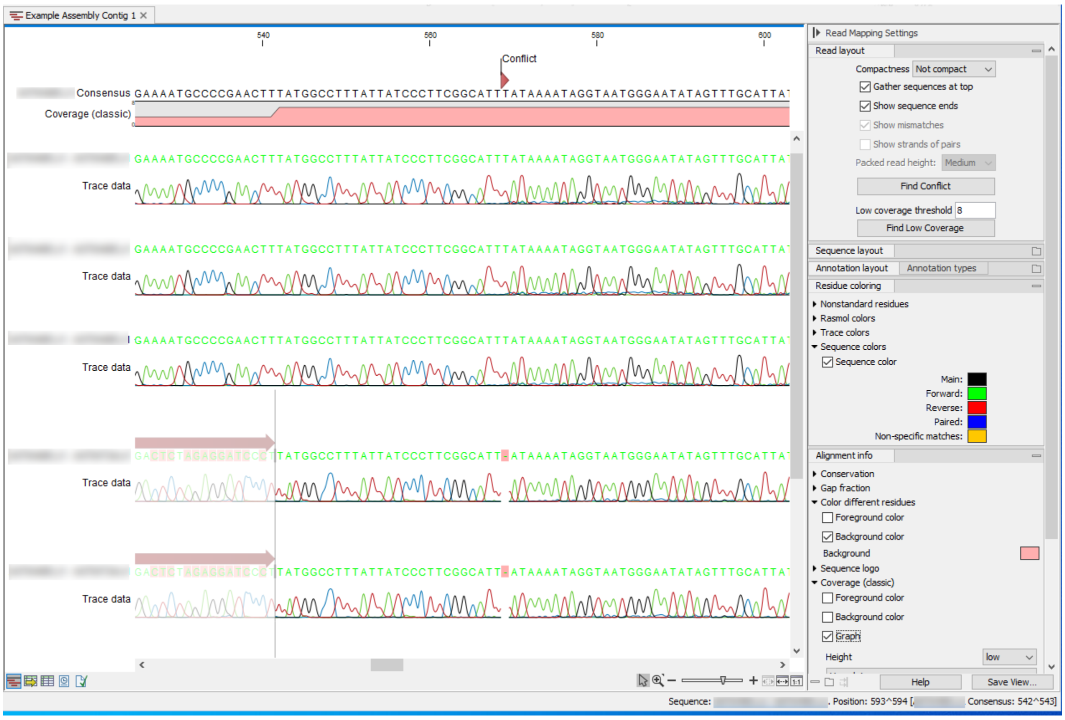
To reverse complement an entire contig or mapping, right-click in the empty white area of the contig or mapping and choose to **Reverse Complement Sequence**.

21.7.1 View settings in the Side Panel

Here we cover a few of the key settings available in the side panel for customizing how a contig or read mapping looks. We step through them according to the tab names in the side panel.

Read layout.

- **Compactness**. Set the level of detail to be displayed. The level of compactness affects other view settings as well as the overall view. For example: if **Compact** is selected, quality scores and annotations on the reads will not be visible, even if these options are turned on under the "Nucleotide info" palette. Compactness can also be changed by pressing and holding the Alt key while scrolling with the mouse wheel or touchpad.
  - **Not compact**. This allows the mapping to be viewed in full detail, including quality scores and trace data for the reads, where present. To view such information, additional viewing options under the **Nucleotide info** view settings must also selected. For further details on these, see section 21.1.1 and section 14.2.1.
  - **Low**. Hides trace data, quality scores and puts the reads' annotations on the sequence. The editing functions available when right-clicking on a nucleotide with compactness set to Low is shown in figure 21.15.
  - **Medium**. The labels of the reads and their annotations are hidden, and reads are shown as lines. The residues of the reads cannot be seen, even when zoomed in 100%.
  - **Compact**. Like Medium but with less space between the reads.



Read layout

Compactness: Not compact

☑ Gather sequences at top

☑ Show sequence ends

☑ Show mismatches

□ Show strands of pairs

Packed read height: Medium

Low coverage threshold: 8

☑ Sequence color

Main: ■ Forward: 🟩 Reverse: 🟥 Paired: 🟦 Non-specific matches: 🟨

□ Foreground color

☑ Background color

Background

□ Foreground color

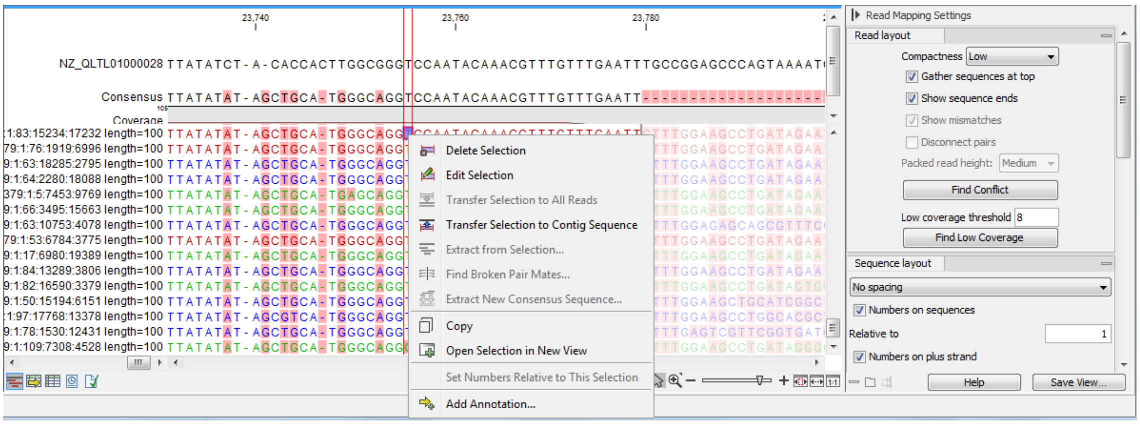
□ Background color

☑ Graph

Height: low

Sequence: . Position: 593^594 [Consensus: 542^543]

Figure 21.14: *Settings in the side panel allow customization of the view of read mappings and contigs from assemblies.*


23,740
23,760
23,780
NZ_QLTL01000028
Consensus
Coverage
Delete Selection
Edit Selection
Transfer Selection to All Reads
Transfer Selection to Contig Sequence
Extract from Selection...
Find Broken Pair Mates...
Extract New Consensus Sequence...
Copy
Open Selection in New View
Set Numbers Relative to This Selection
Add Annotation...
Read Mapping Settings
Find Conflict
Find Low Coverage
Sequence layout
Help
Save View...

Read layout

Compactness: Low

☑ Gather sequences at top

☑ Show sequence ends

☑ Show mismatches

□ Disconnect pairs

Packed read height: Medium

Low coverage threshold: 8

No spacing

☑ Numbers on sequences

Relative to: 1

☑ Numbers on plus strand

Figure 21.15: *An example showing the low compactness setting.*

- **Packed.** This uses all the horizontal space available for displaying the reads (figure 21.16). This differs from the other settings, which stack all reads vertically. When zoomed in to 100%, the individual residues are visible. When zoomed out, reads are represented as lines. Packed mode is useful when viewing large amounts of data, but some functionality is not available. For example, the read mapping cannot be edited, portions cannot be selected, and color coding changes are not possible.

- **Gather sequences at top**. When selected, the sequence reads contributing to the mapping at that position are placed right below the reference. This setting has no effect when the compactness level is Packed.
- **Show sequence ends**. When selected, trimmed regions are shown (faded traces and

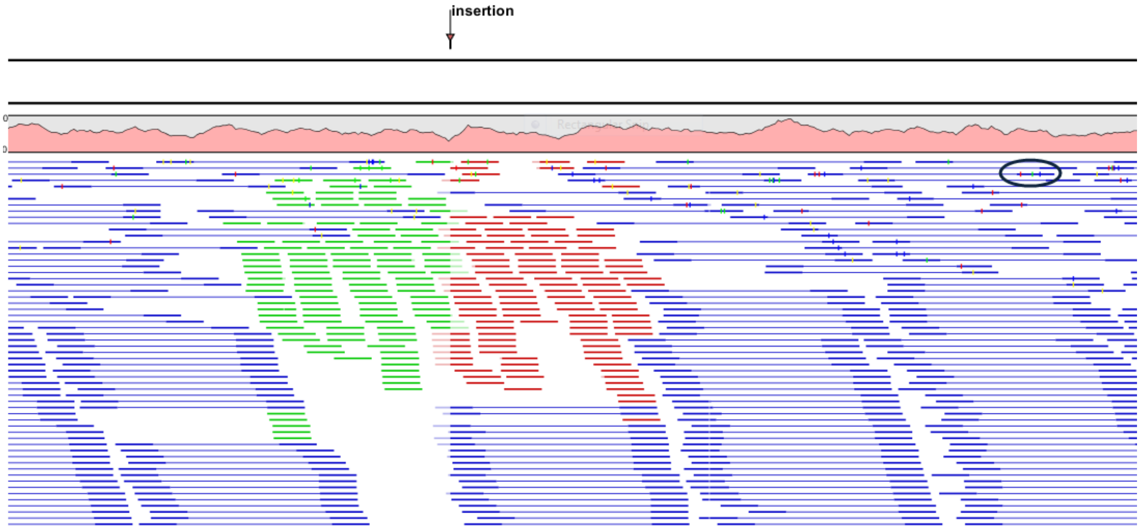


Figure 21.16: *An example of the Packed compactness setting. Highlighted in black is an example of 3 narrow vertical lines representing mismatching residues.*

residues). Trimmed regions do not contribute to the mapping or contig.

- **Show mismatches**. When selected and when the compactness is set to Packed, based that do not match the reference at that position are highlighted by coloring them according to the Rasmol color scheme. Reads with mismatches are floated to the top of the view.
- **Show strands of paired reads.** When the compactness is set to Packed, display each member of a read pair in full and color them according to direction. This is particularly useful for reviewing overlap regions in overlapping read pairs.
- **Packed read height**. When the compactness is set to "Packed", select a height for the visible reads.

  When there are more reads than the height specified, an overflow graph is displayed that uses the same colors as the sequences. Mismatches in reads are shown as narrow vertical lines, using colors representing the mismatching residue. Horizontal line colors correspond to those used for highlighting mismatches in the sequences (red = A, blue = C, yellow = G, and green = T). For example, a red line with half the height of the blue part of the overflow graph represents a mismatching "A" in half of the paired reads at that particular position.
- **Find Conflict**. Clicking this button selects the next position where there is an conflict. Mismatching residues are colored using the default color settings. You can also click on the Space bar of your keyboard to find the next conflict.
- **Low coverage threshold**. All regions with coverage up to and including this value are considered low coverage. Clicking the 'Find low coverage' button selects the next region in the read mapping with low coverage.

Sequence layout. There is one parameter in this section in addition to those described in section 14.2.1

- **Matching residues as dots**. When selected, matching residues are presented as dots instead of as letters.

Annotation Layout and Annotation Types See section 14.3.1.

Residue coloring. There is one parameter in this section in addition to those described in section 14.2.1.

- **Sequence colors**. This setting controls the coloring of sequences when working in most compactness modes. The exception is Packed mode, where colors are controlled with settings under the "Match coloring" tab, described below.
  - **Main**. The color of the consensus and reference sequence. Black by default.
  - **Forward**. The color of forward reads. Green by default.
  - **Reverse**. The color of reverse reads. Red by default.
  - **Paired**. The color of read pairs. Blue by default. Reads from **broken pairs** are colored according to their orientation (forward or reverse) or as a non-specific match, but with a darker hue than the color of ordinary reads.
  - **Non-specific matches**. When a read would have matched equally well another place in the mapping, it is considered a non-specific match and is colored yellow by default. Coloring to indicate a non-specific match overrules other coloring. For mappings with several reference sequences, a read is considered a non-specific match if it matches more than once *across all the contigs/references*.

  Colors can be adjusted by clicking on an individual color and selecting from the palette presented.

Alignment info. There are several parameters in this section in addition to the ones described in section 16.2.

- **Coverage**: Shows how many reads are contributing information to a given position in the read mapping. The level of coverage is relative to the overall number of reads.
- **Paired distance**: Plots the distance between the members of paired reads.
- **Single paired reads**: Plots the percentage of reads marked as single paired reads (when only one of the reads in a pair matches).
- **Non-specific matches**: Plots the percentage of reads that also match other places.
- **Non-perfect matches**: Plots the percentage of reads that do not match perfectly.
- **Spliced matches**: Plots the percentage of reads that are spliced.

Options for these parameters are:

- **Foreground color**. Colors the residues using a gradient, where the left side color is used for low coverage and the right side is used for maximum coverage.
- **Background color**. Colors the background of the residues using a gradient, where the left side color is used for low coverage and the right side is used for maximum coverage.
- **Graph**. Read coverage is displayed as a graph. The data points for the graph can be exported (see section 8.3).
  - **Height**. Specifies the height of the graph.
  - **Type**. The graph can be displayed as Line plot, Bar plot or as a Color bar.
  - **Color box**. For Line and Bar plots, the color of the plot can be set by clicking the color box. If a Color bar is chosen, the color box is replaced by a gradient color box as described under Foreground color.

Nucleotide info See section 14.2.1

Match coloring Coloring of the mapped reads when the Packed compactness option is selected. Colors can be adjusted by clicking on an individual color and selecting from the palette presented. Coloring of bases when other compactness settings are selected is controlled under the "Residue coloring" tab.

Find See section 14.2.1

Text format See section 14.2.1

21.7.2 Editing a contig or read mapping

When editing contigs and read mappings, you are typically interested in confirming or changing single bases.

To do this:

Select the base to edit | Type the desired base

If you want to replace a residue with a gap, use the **Delete** key.

If you wish to edit a selection of more than one residue:

right-click the selection | Edit Selection ( )

There are three shortcut keys for easily finding the positions where there are conflicts:

- Space bar: Finds the *next* conflict.
- "." (punctuation mark key): Finds the *next* conflict.
- "," (comma key): Finds the *previous* conflict.

In the contig or mapping view, you can use **Zoom in** ( ) to zoom to a greater level of detail than in other views (see figure 21.12).

Note: For contigs or mappings with more than 1,000 reads, you can only do single-residue replacements. When the compactness is **Packed**, you cannot edit any of the reads.

All changes are recorded in the history of the element (see section 2.5).

21.7.3 Sorting reads

To change the order of the sequence reads, simply drag the label of the sequence up and down. Note that this is not possible if you have chosen **Gather sequences at top** or set the compactness to **Packed** in the **Side Panel**.

You can also sort the reads by right-clicking a sequence label and choose from the following options:

- **Sort Reads by Alignment Start Position.** This will list the first read in the alignment at the top etc.
- **Sort Reads by Name.** Sort the reads alphabetically.

- **Sort Reads by Length.** The shortest reads will be listed at the top.

21.7.4 Read conflicts

After assembly or mapping, conflicts between the reads are annotated on the consensus sequence. The definition of a conflict is *a position where at least one of the reads has a different residue compared to the reference*.

A conflict can be in two states:

- **Conflict**. Both the annotation and the corresponding row in the Table (▦) are colored **red**.
- **Resolved**. Both the annotation and the corresponding row in the Table (▦) are colored **green**.

The conflict can be resolved by correcting the deviating residues in the reads as described above.

A fast way of making all the reads reflect the consensus sequence is to select the position in the consensus, right-click the selection, and choose **Transfer Selection to All Reads**.

The opposite is also possible: make a selection on one of the reads, right click, and **Transfer Selection to Contig Sequence**.

21.7.5 Using the mapping

Due to the integrated nature of *CLC Main Workbench* it is easy to use the consensus sequences as input for additional analyses. If you wish to extract the consensus sequence for further use, use the **Extract Consensus Sequence** tool (see section 21.10).

You can also right-click the consensus sequence and select **Open Sequence**. This will not create a new sequence but simply let you see the sequence in a sequence view. This means that the sequence still "belong" to the mapping and will be saved together with the mapping. It also means that if you add annotations to the sequence, they will be shown in the mapping view as well. This can be very convenient for Primer design (▤) for example.

If you wish to BLAST the consensus sequence, simply select the whole contig for your BLAST search. It will automatically extract the consensus sequence and perform the BLAST search.

In order to preserve the history of the changes you have made to the contig, the contig itself should be saved from the contig view, using either the save button (⇤) or by dragging it to the **Navigation Area**.

21.7.6 Extracting reads from mappings

Reads can be extracted from stand-alone read mappings in multiple ways:

- **Extract from Selection**. Available from the right-click menu of the reference sequence or consensus sequence (figure 21.17). A new stand-alone read mapping consisting of just the reads that are completely covered by the selected region will be created. Options are available to specify the nature of the extracted reads in the 'Specify reads to be included' wizard step, see below.

- **Extract Sequences**. Available from the right-click menu of the coverage graph or a read (figure 21.18), or from the Tools menu. It extracts all reads to a sequence list or individual sequences. See section 18.2.

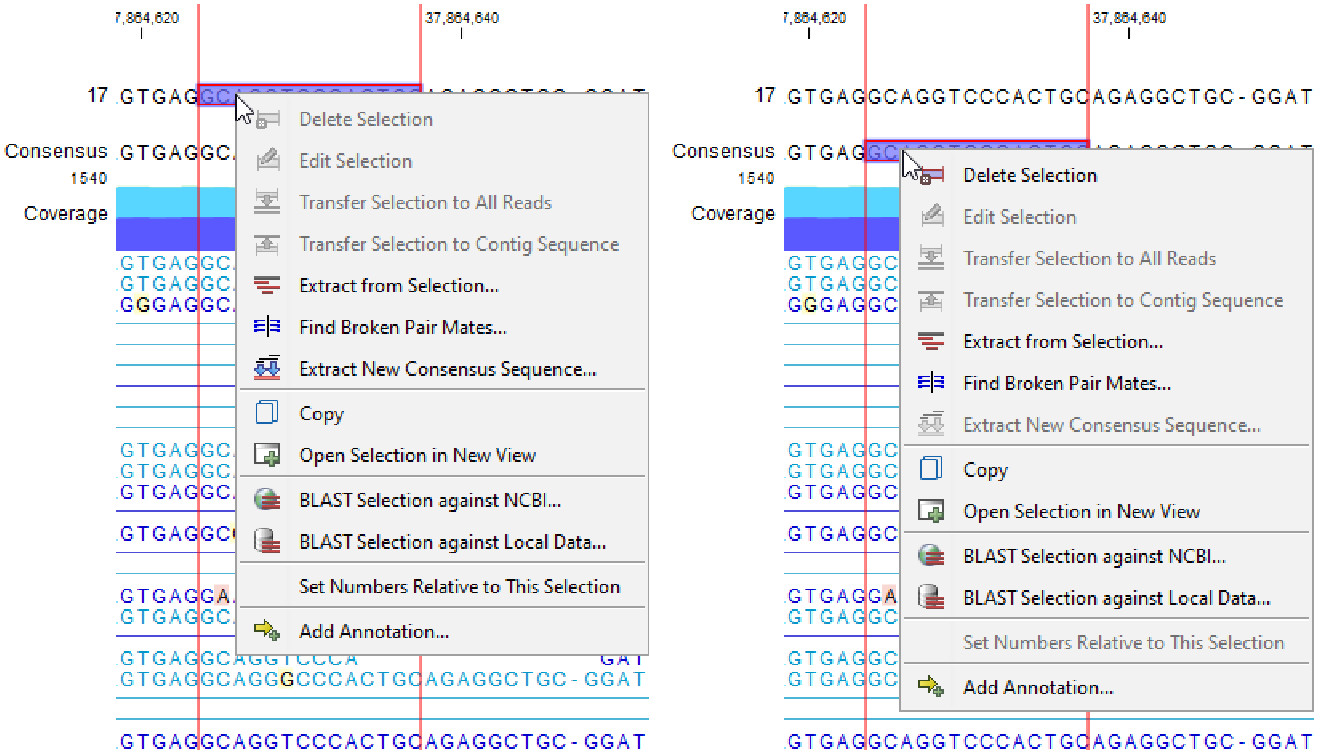

7,864,620
37,864,640
17
Consensus
1540
Coverage
Delete Selection
Edit Selection
Transfer Selection to All Reads
Transfer Selection to Contig Sequence
Extract from Selection...
Find Broken Pair Mates...
Extract New Consensus Sequence...
Copy
Open Selection in New View
BLAST Selection against NCBI...
BLAST Selection against Local Data...
Set Numbers Relative to This Selection
Add Annotation...
GTGAGGCAGGTCCCACTGCAGAGGCTGC-GGAT

Figure 21.17: *Right-click on the selected region on the reference sequence (left) or consensus sequence (right) in a stand-alone read mapping for revealing the available options.*

The 'Specify reads to be included' wizard step of **Extract from Selection** offers the following options (figure 21.19):

Match specificity

- **Include specific matches** Reads that mapped best to just a single position of the reference genome.
- **Include non-specific matches** Reads that have multiple, equally good alignments to the reference genome. These reads are colored yellow by default in read mappings.

Alignment quality

- **Include perfectly aligned reads** Reads where *the full read* is perfectly aligned to the reference genome. Reads that extend beyond the end of the reference are not considered perfectly aligned, because part of the read does not match the reference.
- **Include reads with less than perfect alignment** Reads with mismatches, insertions or deletions, or with unaligned ends.

Spliced status

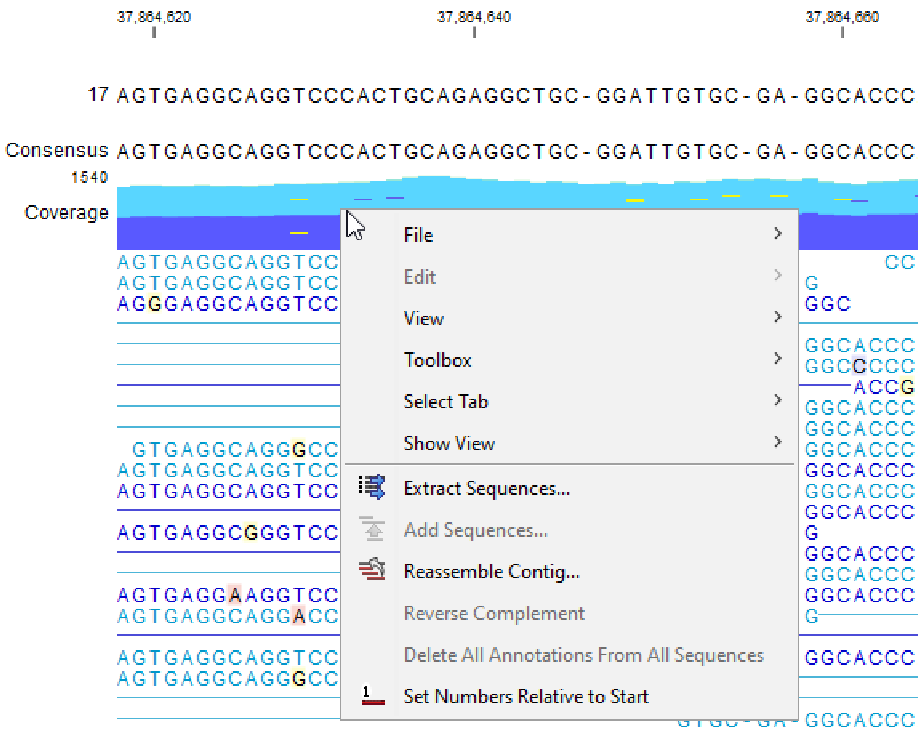

37,864,620
37,864,640
37,864,660
17 AGTGAGGCAGGTCCCACTGCAGAGGCTGC-GGATTGTGC-GA-GGCACCC
Consensus AGTGAGGCAGGTCCCACTGCAGAGGCTGC-GGATTGTGC-GA-GGCACCC
1540
Coverage
File
Edit
View
Toolbox
Select Tab
Show View
Extract Sequences...
Add Sequences...
Reassemble Contig...
Reverse Complement
Delete All Annotations From All Sequences
Set Numbers Relative to Start

Figure 21.18: *Right-click on the coverage graph or reads for revealing the available options.*

- **Include spliced reads** Reads mapped across an intron.
- **Include non spliced reads** Reads not mapped across an intron.

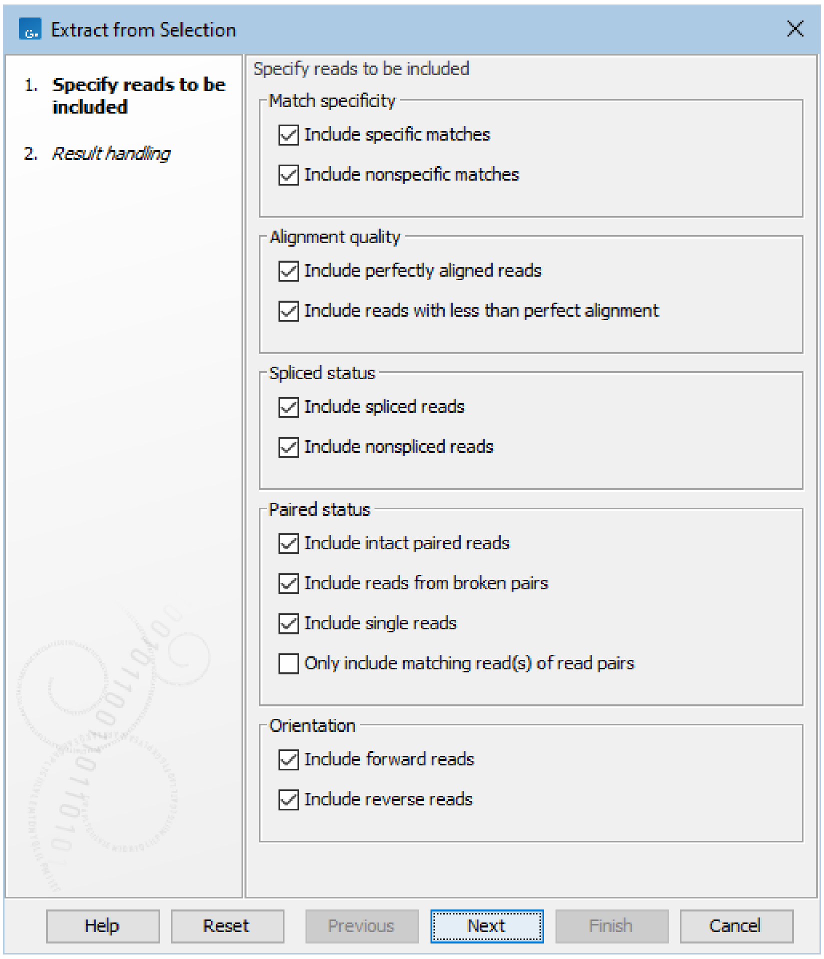
Paired status

- **Include intact paired reads** Paired reads mapped within the specified paired distance.
- **Include reads from broken pairs** Paired reads where only one of the reads mapped, either because only one read in the pair matched the reference, or because the distance or relative orientation of its mate was wrong.
- **Include single reads** Reads marked as single reads (as opposed to paired reads). Reads from broken pairs are not included. Reads marked as single reads after trimming paired sequence lists are included.
- **Only include matching read(s) of read pairs** If only one read of a read pair matches the criteria, then only include the matching read as a broken pair. For example if only one of the reads from the pair is inside the overlap region, then this option only includes the read found within the overlap region as a broken read. When both reads are inside the overlap region, the full paired read is included. Note that some tools ignore broken reads by default.

Orientation

- **Include forward reads** Reads mapped in the forward direction.
- **Include reverse reads** Reads mapped in the reverse direction.

  Note that excluding forward or reverse reads will generate broken pairs if reads in pairs are mapped in opposite directions, regardless of the **Only include matching read(s) of read pairs** option.



Extract from Selection

1. **Specify reads to be included**
2. *Result handling*

Specify reads to be included

Match specificity

☑ Include specific matches

☑ Include nonspecific matches

Alignment quality

☑ Include perfectly aligned reads

☑ Include reads with less than perfect alignment

Spliced status

☑ Include spliced reads

☑ Include nonspliced reads

Paired status

☑ Include intact paired reads

☑ Include reads from broken pairs

☑ Include single reads

☐ Only include matching read(s) of read pairs

Orientation

☑ Include forward reads

☑ Include reverse reads

Figure 21.19: *Options to include or exclude specific types of reads.*

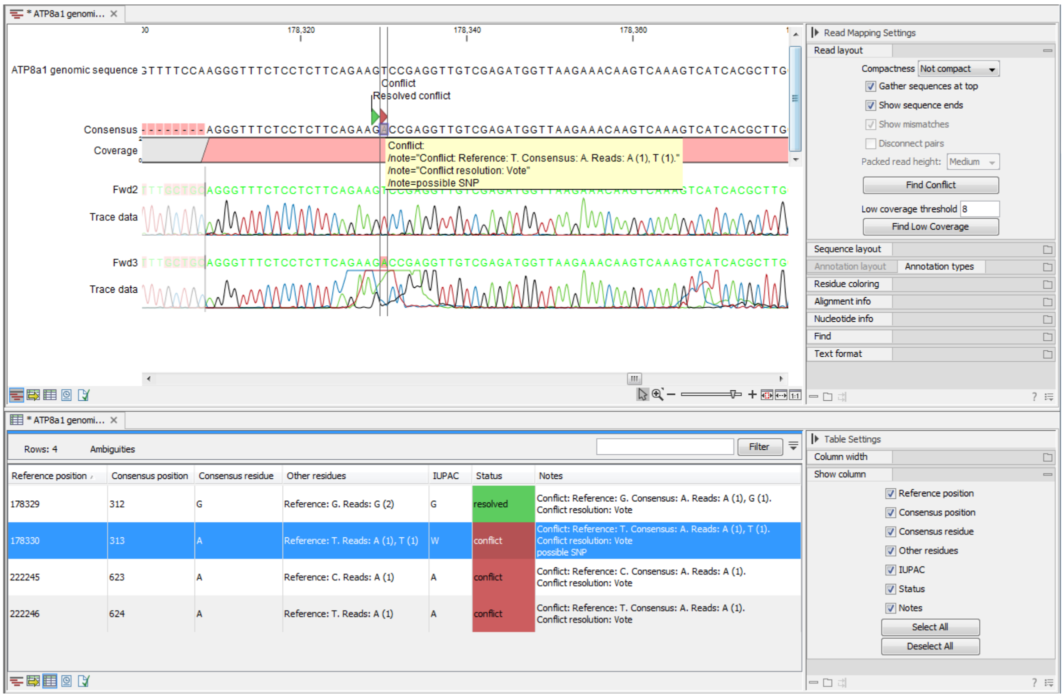
21.7.7 Variance table

In addition to the standard graphical display of a contig or mapping as described above, you can also see a tabular overview of the conflicts between the reads by clicking the **Table (▦)** icon at the bottom of the view.

This will display a new view of the conflicts as shown in figure 21.20.

The table has the following columns:

- **Reference position.** The position of the conflict measured from the starting point of the reference sequence.
- **Consensus position.** The position of the conflict measured from the starting point of the consensus sequence.
- **Consensus residue.** The consensus's residue at this position. The residue can be edited in the graphical view, as described above.
- **Other residues.** Lists the residues of the reads. Inside the brackets, you can see the number of reads having this residue at this position. In the example in figure 21.20, you



Conflict:
/note="Conflict: Reference: T. Consensus: A. Reads: A (1), T (1)."
/note="Conflict resolution: Vote"
/note=possible SNP

Read Mapping Settings

Read layout

Compactness: Not compact

☑ Gather sequences at top

☑ Show sequence ends

☑ Show mismatches

□ Disconnect pairs

Packed read height: Medium

Find Conflict

Low coverage threshold: 8

Find Low Coverage

Sequence layout · Annotation layout · Annotation types · Residue coloring · Alignment info · Nucleotide info · Find · Text format

Rows: 4 Ambiguities

Reference position	Consensus position	Consensus residue	Other residues	IUPAC	Status	Notes
178329	312	G	Reference: G. Reads: G (2)	G	resolved	Conflict: Reference: G. Consensus: A. Reads: A (1), G (1). Conflict resolution: Vote
178330	313	A	Reference: T. Reads: A (1), T (1)	W	conflict	Conflict: Reference: T. Consensus: A. Reads: A (1), T (1). Conflict resolution: Vote possible SNP
222245	623	A	Reference: C. Reads: A (1)	A	conflict	Conflict: Reference: C. Consensus: A. Reads: A (1). Conflict resolution: Vote
222246	624	A	Reference: T. Reads: A (1)	A	conflict	Conflict: Reference: T. Consensus: A. Reads: A (1). Conflict resolution: Vote

Table Settings

Column width

Show column

☑ Reference position

☑ Consensus position

☑ Consensus residue

☑ Other residues

☑ IUPAC

☑ Status

☑ Notes

Select All

Deselect All

Figure 21.20: *The graphical view is displayed at the top, and underneath the conflicts are shown in a table. At the conflict at position 313, the user has entered a comment in the table (to see it, make sure the Notes column is wide enough to display all text lines). This comment is now also added to the tooltip of the conflict annotation in the graphical view above.*

can see that at position 637 there is a 'C' in the top read in the graphical view. The other two reads have a 'T'. Therefore, the table displays the following text: 'C (1), T (2)'.

- **IUPAC.** The ambiguity code for this position. The ambiguity code reflects the residues in the reads - not in the consensus sequence. (The IUPAC codes can be found in section G.)
- **Status.** The status can either be conflict or resolved:
  - **Conflict.** Initially, all the rows in the table have this status. This means that there is one or more differences between the sequences at this position.
  - **Resolved.** If you edit the sequences, e.g. if there was an error in one of the sequences, and they now all have the same residue at this position, the status is set to *Resolved*.
- **Note.** Can be used for your own comments on this conflict. Right-click in this cell of the table to add or edit the comments. The comments in the table are associated with the conflict annotation in the graphical view. Therefore, the comments you enter in the table will also be attached to the annotation on the consensus sequence (the comments can be displayed by placing the mouse cursor on the annotation for one second - see figure 21.20). The comments are saved when you **Save** ( ).

By clicking a row in the table, the corresponding position is highlighted in the graphical view. Clicking the rows of the table is another way of navigating the contig or the mapping, as are using the **Find Conflict** button or using the **Space bar**. You can use the up and down arrow keys to navigate the rows of the table.

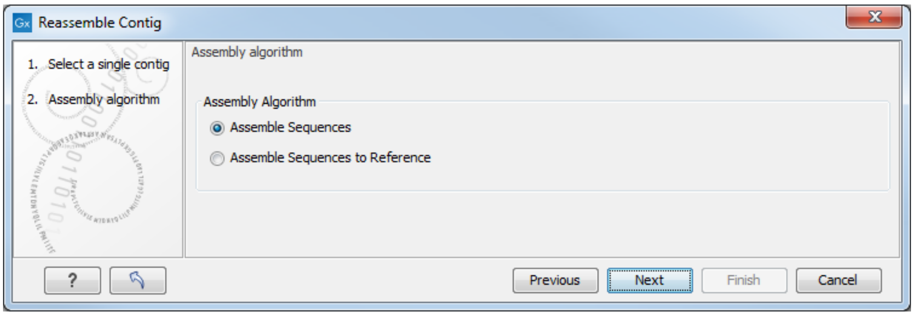
21.8 Reassemble contig

If you have edited a contig, changed trimmed regions, or added or removed reads, you may wish to reassemble the contig. This can be done in two ways:

Tools | Sanger Sequencing Analysis ()| Reassemble Contig ()

Select the contig from Navigation Area, move to 'Selected Elements' and click Next. You can also right-click in the empty white area of the contig and choose to **Reassemble contig** ().

This opens a dialog as shown in figure 21.21



Reassemble Contig

1. Select a single contig
2. Assembly algorithm

Assembly algorithm

Assembly Algorithm

● Assemble Sequences

○ Assemble Sequences to Reference

Figure 21.21: *Re-assembling a contig.*

In this dialog, you can choose:

- **De novo assembly**. This will perform a normal assembly in the same way as if you had selected the reads as individual sequences. When you click **Next**, you will follow the same steps as described in section 21.3. The consensus sequence of the contig will be ignored.
- **Reference assembly**. This will use the consensus sequence of the contig as reference. When you click **Next**, you will follow the same steps as described in section 21.4.

When you click **Finish**, a new contig is created, so you do not lose the information in the old contig.

21.9 Secondary peak calling

CLC Main Workbench is able to detect secondary peaks - a peak within a peak - to help discover heterozygous mutations. Looking at the height of the peak below the top peak, the *CLC Main Workbench* considers all positions in a sequence, and if a peak is higher than the threshold set by the user, it will be "called".

The peak detection investigates any secondary high peaks in the same interval as the already called peaks. The peaks must have a peak shape in order to be considered (i.e. a fading signal from the previous peak will be ignored). **Note!** The secondary peak caller does not call and annotate secondary peaks that have already been called by the Sanger sequencing machine and denoted with an ambiguity code.

Regions that are trimmed (i.e. covered by Trim annotations) are ignored in the analysis (section 21.2).

When a secondary peak is called, the residue is changed to an ambiguity character to reflect that two bases are possible at this position, and optionally an annotation is added at this position.

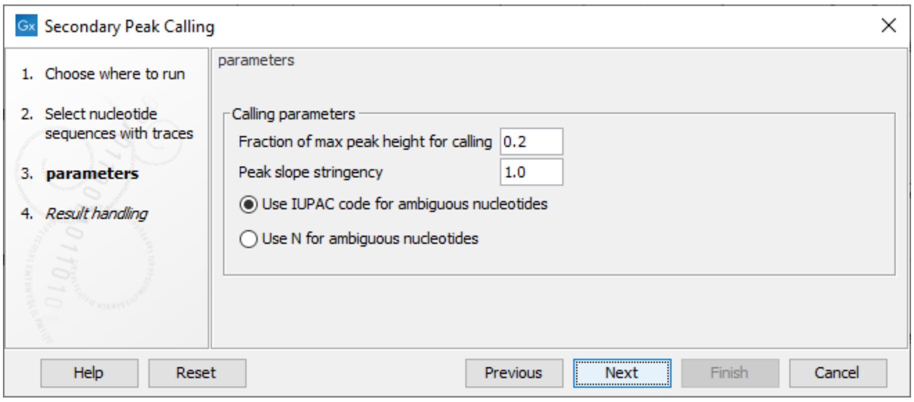
To call secondary peaks, go to:

Tools | **Sanger Sequencing Analysis (** **)**| **Call Secondary Peaks (** **)**

This opens a dialog where you can add the sequences to be analyzed. If you had already selected sequence in the Navigation Area, these will be shown in the 'Selected Elements' box. However you can remove these, or add others, by using the arrows to move sequences between the Navigation Area and Selected Elements boxes.

When the sequences are selected, click **Next**.

This opens the dialog displayed in figure 21.22.



Secondary Peak Calling

1. Choose where to run
2. Select nucleotide sequences with traces
3. **parameters**
4. *Result handling*

parameters

Calling parameters

Fraction of max peak height for calling: 0.2

Peak slope stringency: 1.0

● Use IUPAC code for ambiguous nucleotides

○ Use N for ambiguous nucleotides

Figure 21.22: *Setting parameters secondary peak calling.*

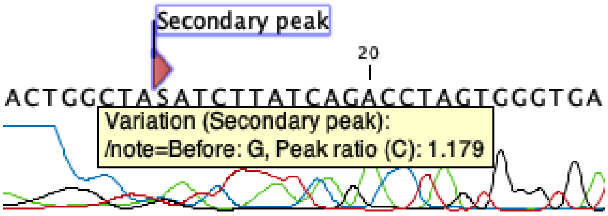
The following parameters can be adjusted in the dialog:

- **Fraction of max peak height for calling.** Adjust this value to specify how high the secondary peak must be to be called.
- **Peak slope stringency.** Control how pronounced each nucleotide peak must be. Decreasing this will detect more peaks. Increasing it will detect fewer.
- **Use IUPAC code / N for ambiguous nucleotides.** When a secondary peak is called, the residue at this position can either be replaced by an N or by a ambiguity character based on the IUPAC codes (see section G).

Clicking **Next** allows you to add annotations. In addition to changing the actual sequence, annotations can be added for each base that has been called. The annotations hold information about the fraction of the max peak height.

Click **Finish** to start the tool. This will start the secondary peak calling. A detailed history entry will be added to the history specifying all the changes made to the sequence.

Secondary peaks are marked in the output sequence as can be seen in figure 21.23. When the mouse is hovered over a secondary peak, **Before** and **Peak ratio** values are shown. The **Before** value refers to the original residue that was present in the sequence, while the **Peak ratio** shows the ratio between the original peak and the secondary peak signal strength values (the base associated with the secondary peak is shown in parentheses next to the peak ratio). In the case of figure 21.23, it can be seen that the original residue is G while the residue C yields a secondary peak. This then results in the ambiguity character S shown in the sequence.



Variation (Secondary peak):
/note=Before: G, Peak ratio (C): 1.179

Figure 21.23: *Secondary peaks are marked in the output sequence.*

21.10 Extract Consensus Sequence

Extract Consensus Sequence takes as input stand-alone read mappings or nucleotide BLAST results, and outputs a consensus sequence.

Consensus sequences can also be extracted directly from elements opened in the View Area by selecting **Extract New Consensus Sequence ()** from the right-click menu on:

- For stand-alone read mappings: the reference sequence or a selection within it, or the consensus sequence.
- For nucleotide BLAST results: a selection within the query sequence.

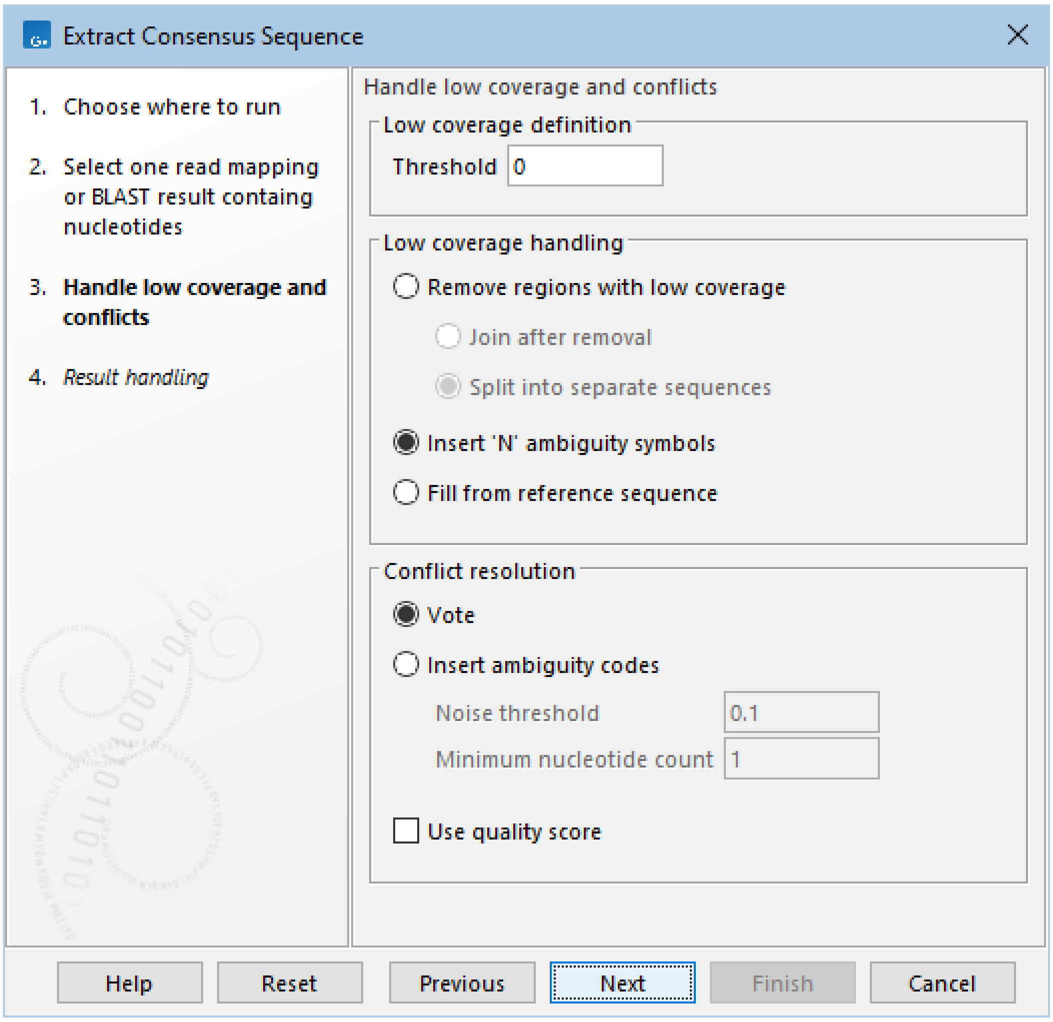
To run the tool, go to:

Tools | **Sanger Sequencing Analysis ()**| **Extract Consensus Sequence ()**

The following options for extracting the consensus sequence can be configured (figure 21.24):

- **Threshold** Coverage above this value is considered high, while coverage at or below it is considered low. Overlapping paired-end reads count as two when calculating the coverage.

  A higher threshold yields a more reliable consensus but reduces completeness.
- **Low coverage handling** Options for handling regions with low coverage.
  - **Remove regions with low coverage** No consensus is generated in regions of low coverage.
    - **Join after removal** High coverage regions are joined into a single consensus sequence.
    - **Split into separate sequences** Each high coverage region produces an individual consensus sequence.
  - **Insert 'N' ambiguity symbols** The consensus is set to 'N' at each position with low coverage.
  - **Fill from reference sequence** The consensus is set to the reference symbol at each position with low coverage.
- **Conflict resolution** Options for resolving read disagreements at individual positions within high coverage regions.
  - **Vote** The consensus is set to the most supported symbol (see **Use quality score**), excluding ambiguous symbols.



Extract Consensus Sequence

1. Choose where to run
2. Select one read mapping or BLAST result containg nucleotides
3. **Handle low coverage and conflicts**
4. *Result handling*

Handle low coverage and conflicts

Low coverage definition

Threshold 0

Low coverage handling

○ Remove regions with low coverage

○ Join after removal

● Split into separate sequences

● Insert 'N' ambiguity symbols

○ Fill from reference sequence

Conflict resolution

● Vote

○ Insert ambiguity codes

Noise threshold 0.1

Minimum nucleotide count 1

□ Use quality score

Figure 21.24: *Options for consensus sequence extraction.*

In case of a tie, symbols are chosen in the order: A > C > G > T.

To preserve biological heterozygous variation, see **Insert ambiguity codes**.

- **Insert ambiguity codes** The consensus is set to the IUPAC ambiguity code (section G) that best reflects the variation observed in the reads.

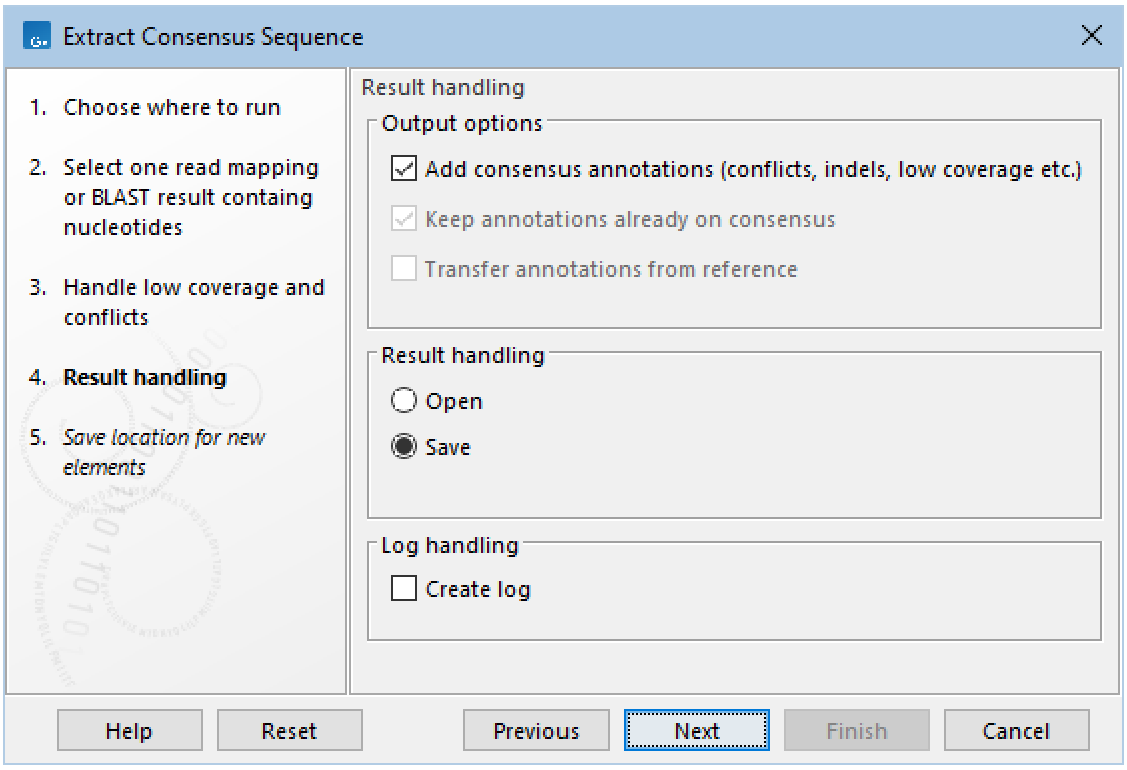
  The following options determine which symbols contribute to the ambiguity codes:

  * **Noise threshold** Only symbols with support greater than this value (see **Use quality score**) contribute to the ambiguity code.
  * **Minimum nucleotide count** Only symbols present in at least this number of reads contribute to the ambiguity code.

  Positions where no symbol qualifies to contribute to the ambiguity code are not included in the consensus.

- **Use quality score** The support of a symbol is determined by:
  - When unchecked: the percentage of reads containing the symbol.
  - When checked: the sum of the symbol's quality scores, expressed as a percentage of the total quality score at that position.

The following options for annotations the consensus sequence can be configured (figure 21.25):



1. Choose where to run
2. Select one read mapping or BLAST result containg nucleotides
3. Handle low coverage and conflicts
4. **Result handling**
5. *Save location for new elements*

Result handling

Output options

☑ Add consensus annotations (conflicts, indels, low coverage etc.)

☑ Keep annotations already on consensus

□ Transfer annotations from reference

Result handling

○ Open

● Save

Log handling

□ Create log

Figure 21.25: *Options for adding annotations to the extracted consensus sequence.*

- **Add consensus annotations (conflicts, indels, low coverage etc.)** When checked, annotations are added to the consensus sequence to indicate resolved conflicts, deletions relative to the reference, and low coverage regions, provided the **Split into separate sequences** option is not selected.

  For inputs containing many reads or long references, many such annotations may be generated.

- **Keep annotations already on consensus** and **Transfer annotations from reference** When checked, annotations present on the consensus or on the reference in the input stand-alone read mapping are copied to the extracted consensus sequence. The copied annotations are placed in regions corresponding to their original location in the input data, although actual coordinates may differ. Annotations may be split if the **Split into separate sequences** option is selected.

  These options are not enabled for types of input other than stand-alone read mapping.

Quality scores on the consensus sequence

When quality scores are present in the input, they are propagated to the extracted consensus as follows.

Consider a consensus symbol X, and let Y and Z be quality scores sums at its position, defined as:

- Y: The sum of quality scores from all reads.
- Z: The sum of quality scores from reads supporting X, defined as:
  - Reads containing the symbol X, if the **Vote** option is selected.
  - Reads containing symbols that contribute to the ambiguity code, if the **Insert ambiguity codes** option is selected.

The consensus symbol X is assigned a quality score $Q = Z - (Y - Z)$, bounded to a minimum of 0 and a maximum of 64.

Chapter 22

Primers and probes

Contents

CLC Main Workbench offers graphically and algorithmically advanced design of primers and probes for various purposes. This chapter begins with a brief introduction to the general concepts of the primer designing process. Then follows instructions on how to adjust parameters for primers, how to inspect and interpret primer properties graphically and how to interpret, save and analyze the output of the primer design analysis. After a description of the different reaction types for which primers can be designed, the chapter closes with sections on how to match primers with other sequences and how to create a primer order.

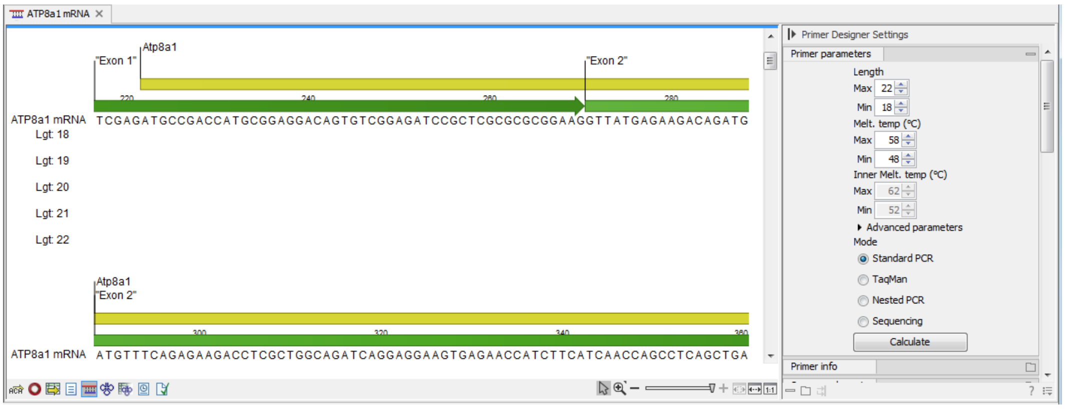
22.1 Primer design - an introduction

Primer design can be accessed in two ways:

Tools | **Primers and Probes (** **)**| **Design Primers (** **)** | **OK**

or **right-click sequence in Navigation Area** | **Show** | **Primer Designer (** **)**

In the primer view (see figure 22.1), the basic options for viewing the template sequence are the same as for the standard sequence view (see section 14.2 for an explanation of these options). This means that annotations such as known SNPs or exons can be displayed on the template sequence to guide the choice of primer regions. In addition, traces in sequencing reads can be shown along with the structure to guide the re-sequencing of poorly resolved regions.



```
ATP8a1 mRNA  TCGAGATGCCGACCATGCGGAGGACAGTGTCGGAGATCCGCTCGCGCGCGGAAGGTTATGAGAAGACAGATG
ATP8a1 mRNA  ATGTTTCAGAGAAGACCTCGCTGGCAGATCAGGAGGAAGTGAGAACCATCTTCATCAACCAGCCTCAGCTGA
```

Primer Designer Settings

Primer parameters

Length
Max 22
Min 18
Melt. temp (°C)
Max 58
Min 48
Inner Melt. temp (°C)
Max 62
Min 52

Mode
● Standard PCR
○ TaqMan
○ Nested PCR
○ Sequencing

Figure 22.1: *The initial view of the sequence used for primer design.*

22.1.1 General concept

The concept of the primer view is that the user first chooses the desired reaction type for the session in the Primer Parameters preference group, e.g. *Standard PCR*. Reflecting the choice of reaction type, it is now possibly to select one or more regions on the sequence and to use the right-click mouse menu to designate these as primer or probe regions (see figure 22.2).

When a region is chosen, graphical information about the properties of all possible primers in this region will appear in lines beneath it. By default, information is showed using a compact mode but the user can change to a more detailed mode in the Primer information preference group.

The number of information lines reflects the chosen length interval for primers and probes. In the compact information mode one line is shown for every possible primer-length and each of these lines contain information regarding all possible primers of the given length. At each potential primer starting position, a circular information point is shown which indicates whether the primer fulfills the requirements set in the primer parameters preference group. A green circle indicates a primer which fulfils all criteria and a red circle indicates a primer which fails to meet one or more of the set criteria. For more detailed information, place the mouse cursor over the circle

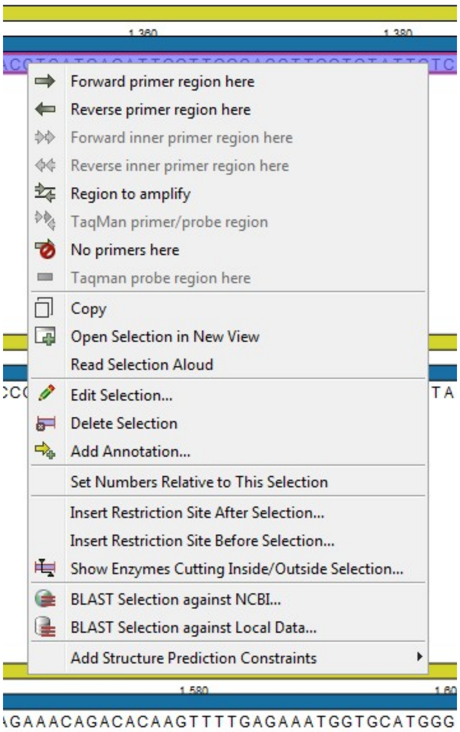

Forward primer region here
Reverse primer region here
Forward inner primer region here
Reverse inner primer region here
Region to amplify
TaqMan primer/probe region
No primers here
Taqman probe region here
Copy
Open Selection in New View
Read Selection Aloud
Edit Selection...
Delete Selection
Add Annotation...
Set Numbers Relative to This Selection
Insert Restriction Site After Selection...
Insert Restriction Site Before Selection...
Show Enzymes Cutting Inside/Outside Selection...
BLAST Selection against NCBI...
BLAST Selection against Local Data...
Add Structure Prediction Constraints
GAAACAGACACAAGTTTTGAGAAATGGTGCATGGG

Figure 22.2: *Right-click menu allowing you to specify regions for the primer design*

representing the primer of interest. A tool-tip will then appear on screen, displaying detailed information about the primer in relation to the set criteria. To locate the primer on the sequence, simply left-click the circle using the mouse.

The various primer parameters can now be varied to explore their effect and the view area will dynamically update to reflect this allowing for a high degree of interactivity in the primer design process.

After having explored the potential primers the user may have found a satisfactory primer and choose to export this directly from the view area using a mouse right-click on the primers information point. This does not allow for any design information to enter concerning the properties of primer/probe pairs or sets e.g. primer pair annealing and T_m difference between primers. If the latter is desired the user can use the **Calculate** button at the bottom of the Primer parameter preference group. This will activate a dialog, the contents of which depends on the chosen mode. Here, the user can set primer-pair specific setting such as allowed or desired T_m difference and view the single-primer parameters which were chosen in the Primer parameters preference group.

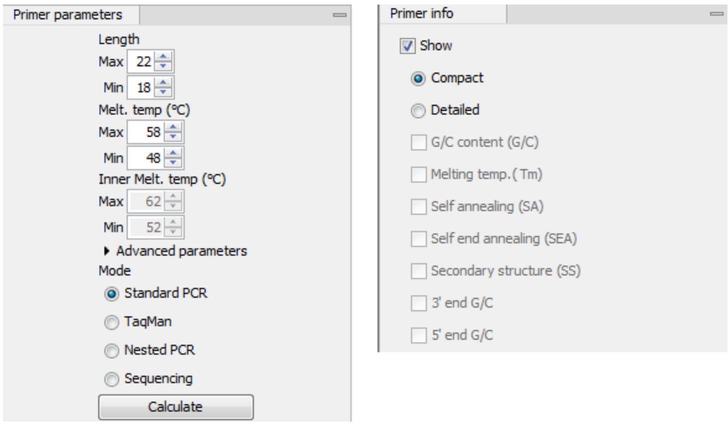
Upon pressing finish, an algorithm will generate all possible primer sets and rank these based on their characteristics and the chosen parameters. A list will appear displaying the 100 most high scoring sets and information pertaining to these. The search result can be saved to the navigator. From the result table, suggested primers or primer/probe sets can be explored since clicking an entry in the table will highlight the associated primers and probes on the sequence. It is also possible to save individual primers or sets from the table through the mouse right-click menu. For a given primer pair, the amplified PCR fragment can also be opened or saved using the mouse right-click menu.

22.1.2 Scoring primers

CLC Main Workbench employs a proprietary algorithm to rank primer and probe solutions. The algorithm considers both the parameters pertaining to single oligos, such as e.g. the secondary structure score and parameters pertaining to oligo-pairs such as e.g. the oligo pair-annealing score. The ideal score for a solution is 100 and solutions are thus ranked in descending order. Each parameter is assigned an ideal value and a tolerance. Consider for example oligo self-annealing, here the ideal value of the annealing score is 0 and the tolerance corresponds to the maximum value specified in the side panel. The contribution to the final score is determined by how much the parameter deviates from the ideal value and is scaled by the specified tolerance. Hence, a large deviation from the ideal and a small tolerance will give a large deduction in the final score and a small deviation from the ideal and a high tolerance will give a small deduction in the final score.

22.2 Setting parameters for primers and probes

The primer-specific view options and settings are found in the **Primer parameters** preference group in the **Side Panel** to the right of the view (see figure 22.3).



Primer parameters

Length

Max 22

Min 18

Melt. temp (°C)

Max 58

Min 48

Inner Melt. temp (°C)

Max 62

Min 52

Advanced parameters

Mode

● Standard PCR

○ TaqMan

○ Nested PCR

○ Sequencing

Calculate

Primer info

☑ Show

● Compact

○ Detailed

□ G/C content (G/C)

□ Melting temp.(Tm)

□ Self annealing (SA)

□ Self end annealing (SEA)

□ Secondary structure (SS)

□ 3' end G/C

□ 5' end G/C

Figure 22.3: *The two groups of primer parameters (in the program, the Primer information group is listed below the other group).*

22.2.1 Primer Parameters

In this preference group a number of criteria can be set, which the selected primers must meet. All the criteria concern *single primers*, as primer pairs are not generated until the **Calculate** button is pressed. Parameters regarding primer and probe sets are described in detail for each reaction mode (see below).

- **Length.** Determines the length interval within which primers can be designed by setting a maximum and a minimum length. The upper and lower lengths allowed by the program are 50 and 10 nucleotides respectively.

- **Melting temperature.** Determines the temperature interval within which primers must lie. When the *Nested PCR* or *TaqMan* reaction type is chosen, the first pair of melting temperature interval settings relate to the outer primer pair i.e. not the probe. Melting temperatures are calculated by a nearest-neighbor model which considers stacking interactions between neighboring bases in the primer-template complex. The model uses state-of-the-art thermodynamic parameters [SantaLucia, 1998] and considers the important contribution from the dangling ends that are present when a short primer anneals to a template sequence [Bommarito et al., 2000]. A number of parameters can be adjusted concerning the reaction mixture and which influence melting temperatures (see below). Melting temperatures are corrected for the presence of monovalent cations using the model of [SantaLucia, 1998] and temperatures are further corrected for the presence of magnesium, deoxynucleotide triphosphates (dNTP) and dimethyl sulfoxide (DMSO) using the model of [von Ahsen et al., 2001].

- **Inner melting temperature.** This option is only activated when the *Nested PCR* or *TaqMan* mode is selected. In *Nested PCR* mode, it determines the allowed melting temperature interval for the inner/nested pair of primers, and in *TaqMan* mode it determines the allowed temperature interval for the TaqMan probe.

- **Advanced parameters.** A number of less commonly used options

  - **Buffer properties.** A number of parameters concerning the reaction mixture which influence melting temperatures.

    - **Primer concentration.** Specifies the concentration of primers and probes in units of nanomoles (nM). Note that in the case of a mix of primers, the concentration here refers to the individual primer and not the combined primers concentration.
    - **Salt concentration.** Specifies the concentration of monovalent cations ($[NA^+]$, $[K^+]$ and equivalents) in units of millimoles (mM)
    - **Magnesium concentration.** Specifies the concentration of magnesium cations ($[Mg^{++}]$) in units of millimoles (mM)
    - **dNTP concentration.** Specifies the combined concentration of all deoxynucleotide triphosphates in units of millimoles (mM)
    - **DMSO concentration.** Specifies the concentration of dimethyl sulfoxide in units of volume percent ($vol.\%$)

  - **GC content.** Determines the interval of CG content (% C and G nucleotides in the primer) within which primers must lie by setting a maximum and a minimum GC content.

  - **Self annealing.** Determines the maximum self annealing value of all primers and probes. This determines the amount of base-pairing allowed between two copies of the same molecule. The self annealing score is measured in number of hydrogen bonds between two copies of primer molecules, with A-T base pairs contributing 2 hydrogen bonds and G-C base pairs contributing 3 hydrogen bonds.

  - **Self end annealing.** Determines the maximum self end annealing value of all primers and probes. This determines the number of consecutive base pairs allowed between the 3' end of one primer and another copy of that primer. This score is calculated in number of hydrogen bonds (the example below has a score of 4 - derived from 2 A-T base pairs each with 2 hydrogen bonds).

```
AATTCCCTACAATCCCCAAA
                  ||
  AAACCCCTAACATCCCTTAA
```

.

  - **Secondary structure.** Determines the maximum score of the optimal secondary DNA structure found for a primer or probe. Secondary structures are scored by the number of hydrogen bonds in the structure, and 2 extra hydrogen bonds are added for each stacking base-pair in the structure.

- **3' end G/C restrictions.** When this checkbox is selected it is possible to specify restrictions concerning the number of G and C molecules in the 3' end of primers and probes. A low G/C content of the primer/probe 3' end increases the specificity of the reaction. A high G/C content facilitates a tight binding of the oligo to the template but also increases the possibility of mispriming. Unfolding the preference groups yields the following options:
  - **End length.** The number of consecutive terminal nucleotides for which to consider the C/G content
  - **Max no. of G/C.** The maximum number of G and C nucleotides allowed within the specified length interval
  - **Min no. of G/C.** The minimum number of G and C nucleotides required within the specified length interval

- **5' end G/C restrictions.** When this checkbox is selected it is possible to specify restrictions concerning the number of G and C molecules in the 5' end of primers and probes. A high G/C content facilitates a tight binding of the oligo to the template but also increases the possibility of mis-priming. Unfolding the preference groups yields the same options as described above for the 3' end.

- **Mode.** Specifies the reaction type for which primers are designed:
  - **Standard PCR.** Used when the objective is to design primers, or primer pairs, for PCR amplification of a single DNA fragment.
  - **Nested PCR.** Used when the objective is to design two primer pairs for nested PCR amplification of a single DNA fragment.
  - **Sequencing.** Used when the objective is to design primers for DNA sequencing.
  - **TaqMan.** Used when the objective is to design a primer pair and a probe for TaqMan quantitative PCR.

  Each mode is described further below.

- **Calculate.** Pushing this button will activate the algorithm for designing primers

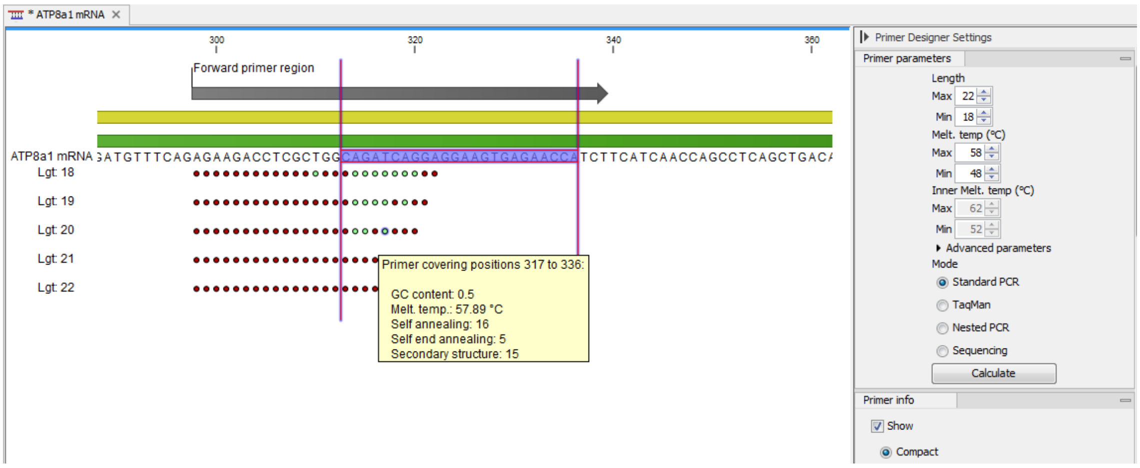
22.3 Graphical display of primer information

The primer information settings are found in the **Primer information** preference group in the **Side Panel** to the right of the view (see figure 22.3).

There are two different ways to display the information relating to a single primer, the detailed and the compact view. Both are shown below the primer regions selected on the sequence.

22.3.1 Compact information mode

This mode offers a condensed overview of all the primers that are available in the selected region. When a region is chosen primer information will appear in lines beneath it (see figure 22.4).



Primer covering positions 317 to 336:

GC content: 0.5
Melt. temp.: 57.89 °C
Self annealing: 16
Self end annealing: 5
Secondary structure: 15

Length
Max 22
Min 18
Melt. temp (°C)
Max 58
Min 48
Inner Melt. temp (°C)
Max 62
Min 52

Mode
● Standard PCR
○ TaqMan
○ Nested PCR
○ Sequencing

☑ Show
● Compact

Figure 22.4: *Compact information mode.*

The number of information lines reflects the chosen length interval for primers and probes. One line is shown for every possible primer-length, if the length interval is widened more lines will appear. At each potential primer starting position a circle is shown which indicates whether the primer fulfills the requirements set in the primer parameters preference group. A green primer indicates a primer which fulfils all criteria and a red primer indicates a primer which fails to meet one or more of the set criteria. For more detailed information, place the mouse cursor over the circle representing the primer of interest. A tool-tip will then appear on screen displaying detailed information about the primer in relation to the set criteria. To locate the primer on the sequence, simply left-click the circle using the mouse.

The various primer parameters can now be varied to explore their effect and the view area will dynamically update to reflect this. If e.g. the allowed melting temperature interval is widened more green circles will appear indicating that more primers now fulfill the set requirements and if e.g. a requirement for 3' G/C content is selected, rec circles will appear at the starting points of the primers which fail to meet this requirement.

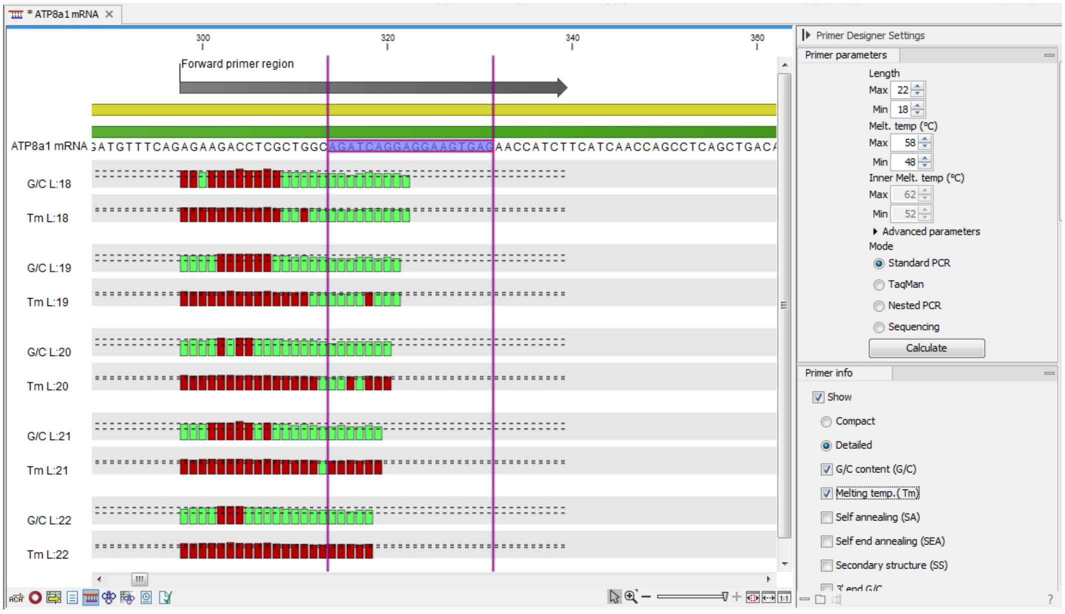
22.3.2 Detailed information mode

In this mode a very detailed account is given of the properties of all the available primers. When a region is chosen primer information will appear in groups of lines beneath it (see figure 22.5).

The number of information-line-groups reflects the chosen length interval for primers and probes. One group is shown for every possible primer length. Within each group, a line is shown for every primer property that is selected from the checkboxes in the primer information preference group. Primer properties are shown at each potential primer starting position and are of two types:

Properties with numerical values are represented by bar plots. A green bar represents the starting point of a primer that meets the set requirement and a red bar represents the starting point of a primer that fails to meet the set requirement:

- G/C content
- Melting temperature
- Self annealing score



Primer Designer Settings

Primer parameters

Length
Max 22
Min 18
Melt. temp (°C)
Max 58
Min 48
Inner Melt. temp (°C)
Max 62
Min 52
Advanced parameters
Mode
● Standard PCR
○ TaqMan
○ Nested PCR
○ Sequencing
Calculate

Primer info

☑ Show
○ Compact
● Detailed
☑ G/C content (G/C)
☑ Melting temp. (Tm)
☐ Self annealing (SA)
☐ Self end annealing (SEA)
☐ Secondary structure (SS)
☐ 3' end G/C

Figure 22.5: *Detailed information mode.*

- Self end annealing score
- Secondary structure score

Properties with Yes - No values. If a primer meets the set requirement a green circle will be shown at its starting position and if it fails to meet the requirement a red dot is shown at its starting position:

- C/G at 3' end
- C/G at 5' end

Common to both sorts of properties is that mouse clicking an information point (filled circle or bar) will cause the region covered by the associated primer to be selected on the sequence.

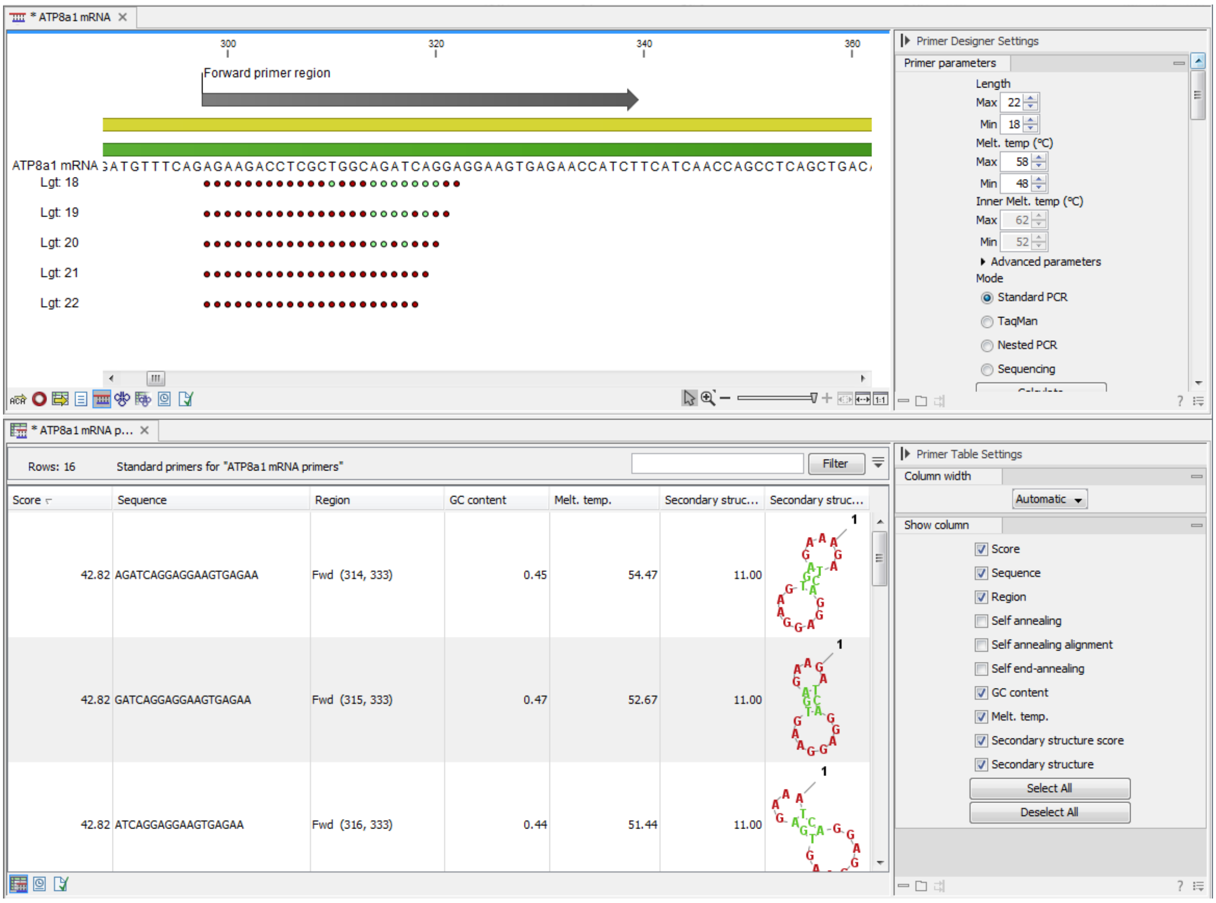
22.4 Output from primer design

The output generated by the primer design algorithm is a table of proposed primers or primer pairs with the accompanying information (see figure 22.6).

In the preference panel of the table, it is possible to customize which columns are shown in the table. See the sections below on the different reaction types for a description of the available information.

The columns in the output table can be sorted by the present information. For example the user can choose to sort the available primers by their score (default) or by their self annealing score, simply by right-clicking the column header.

The output table interacts with the accompanying primer editor such that when a proposed combination of primers and probes is selected in the table the primers and probes in this solution are highlighted on the sequence.



Primer Designer Settings

Primer parameters

Length
Max 22
Min 18
Melt. temp (°C)
Max 58
Min 48
Inner Melt. temp (°C)
Max 62
Min 52
Advanced parameters
Mode
● Standard PCR
○ TaqMan
○ Nested PCR
○ Sequencing

Rows: 16   Standard primers for "ATP8a1 mRNA primers"

Score	Sequence	Region	GC content	Melt. temp.	Secondary struc...	Secondary struc...
42.82	AGATCAGGAGGAAGTGAGAA	Fwd (314, 333)	0.45	54.47	11.00	
42.82	GATCAGGAGGAAGTGAGAA	Fwd (315, 333)	0.47	52.67	11.00	
42.82	ATCAGGAGGAAGTGAGAA	Fwd (316, 333)	0.44	51.44	11.00	

Primer Table Settings

Column width
Automatic

Show column
☑ Score
☑ Sequence
☑ Region
☐ Self annealing
☐ Self annealing alignment
☐ Self end-annealing
☑ GC content
☑ Melt. temp.
☑ Secondary structure score
☑ Secondary structure
Select All
Deselect All

Figure 22.6: *Proposed primers.*

Saving primers Primer solutions in a table row can be saved by selecting the row and using the right-click mouse menu. This opens a dialog that allows the user to save the primers to the desired location. Primers and probes are saved as DNA sequences in the program. This means that all available DNA analyzes can be performed on the saved primers. Furthermore, the primers can be edited using the standard sequence view to introduce e.g. mutations and restriction sites.

Saving PCR fragments The PCR fragment generated from the primer pair in a given table row can also be saved by selecting the row and using the right-click mouse menu. This opens a dialog that allows the user to save the fragment to the desired location. The fragment is saved as a DNA sequence and the position of the primers is added as annotation on the sequence. The fragment can then be used for further analysis and included in e.g. an in-silico cloning experiment using the cloning editor.

Adding primer binding annotation You can add an annotation to the template sequence specifying the binding site of the primer: Right-click the primer in the table and select **Mark primer annotation on sequence**.

22.5 Standard PCR

This mode is used to design primers for a PCR amplification of a single DNA fragment.

In this mode the user must define either a *Forward primer region*, a *Reverse primer region*, or both. These are defined by making a selection on the sequence and right-clicking the selection.

It is also possible to define a *Region to amplify* in which case a forward- and a reverse primer region are automatically placed so as to ensure that the designated region will be included in the PCR fragment. If areas are known where primers must not bind (e.g. repeat rich areas), one or more *No primers here* regions can be defined.

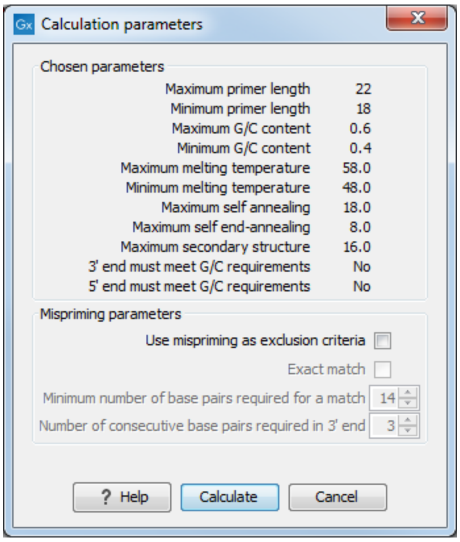
If two regions are defined, it is required that at least a part of the *Forward primer region* is located upstream of the *Reverse primer region*.

After exploring the available primers (see section 22.3) and setting the desired parameter values in the Primer Parameters preference group, the **Calculate** button will activate the primer design algorithm.

22.5.1 When a single primer region is defined

If only a single region is defined, only *single primers* will be suggested by the algorithm.

After pressing the **Calculate** button a dialog will appear (see figure 22.7).



Chosen parameters

Maximum primer length	22
Minimum primer length	18
Maximum G/C content	0.6
Minimum G/C content	0.4
Maximum melting temperature	58.0
Minimum melting temperature	48.0
Maximum self annealing	18.0
Maximum self end-annealing	8.0
Maximum secondary structure	16.0
3' end must meet G/C requirements	No
5' end must meet G/C requirements	No

Mispriming parameters

Use mispriming as exclusion criteria	□
Exact match	□
Minimum number of base pairs required for a match	14
Number of consecutive base pairs required in 3' end	3

Figure 22.7: *Calculation dialog for PCR primers when only a single primer region has been defined.*

The top part of this dialog shows the parameter settings chosen in the Primer parameters preference group which will be used by the design algorithm.

Mispriming: The lower part contains a menu where the user can choose to include mispriming as an exclusion criteria in the design process. If this option is selected the algorithm will search for competing binding sites of the primer within the rest of the sequence, to see if the primer would match to multiple locations. If a competing site is found (according to the parameters set), the primer will be excluded.

The adjustable parameters for the search are:

- **Exact match**. Choose only to consider exact matches of the primer, i.e. all positions must base pair with the template for mispriming to occur.
- **Minimum number of base pairs required for a match**. How many nucleotides of the primer that must base pair to the sequence in order to cause mispriming.

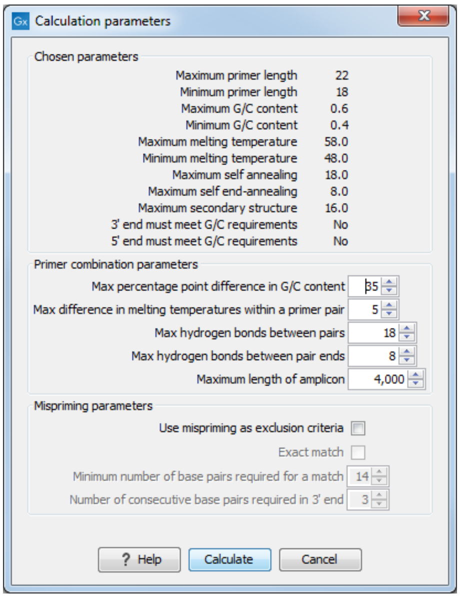
- **Number of consecutive base pairs required in 3' end**. How many consecutive 3' end base pairs in the primer that MUST be present for mispriming to occur. This option is included since 3' terminal base pairs are known to be essential for priming to occur.

Note! Including a search for potential mispriming sites will prolong the search time substantially if long sequences are used as template and if the minimum number of base pairs required for a match is low. If the region to be amplified is part of a very long molecule and mispriming is a concern, consider extracting part of the sequence prior to designing primers.

22.5.2 When both forward and reverse regions are defined

If both a forward and a reverse region are defined, *primer pairs* will be suggested by the algorithm.

After pressing the **Calculate** button a dialog will appear (see figure 22.8).



Calculation parameters

Chosen parameters

Maximum primer length	22
Minimum primer length	18
Maximum G/C content	0.6
Minimum G/C content	0.4
Maximum melting temperature	58.0
Minimum melting temperature	48.0
Maximum self annealing	18.0
Maximum self end-annealing	8.0
Maximum secondary structure	16.0
3' end must meet G/C requirements	No
5' end must meet G/C requirements	No

Primer combination parameters

Max percentage point difference in G/C content	35
Max difference in melting temperatures within a primer pair	5
Max hydrogen bonds between pairs	18
Max hydrogen bonds between pair ends	8
Maximum length of amplicon	4,000

Mispriming parameters

Use mispriming as exclusion criteria	□
Exact match	□
Minimum number of base pairs required for a match	14
Number of consecutive base pairs required in 3' end	3

Figure 22.8: *Calculation dialog for PCR primers when two primer regions have been defined.*

Again, the top part of this dialog shows the parameter settings chosen in the Primer parameters preference group which will be used by the design algorithm. The lower part again contains a menu where the user can choose to include mispriming of both primers as a criteria in the design process (see section 22.5.1). The central part of the dialog contains parameters pertaining to primer pairs. Here three parameters can be set:

- Maximum percentage point difference in G/C content - if this is set at e.g. 5 points a pair of primers with 45% and 49% G/C nucleotides, respectively, will be allowed, whereas a pair of primers with 45% and 51% G/C nucleotides, respectively will not be included.
- Maximal difference in melting temperature of primers in a pair - the number of degrees Celsius that primers in a pair are all allowed to differ.
- Max hydrogen bonds between pairs - the maximum number of hydrogen bonds allowed between the forward and the reverse primer in a primer pair.

- Max hydrogen bonds between pair ends - the maximum number of hydrogen bonds allowed in the consecutive ends of the forward and the reverse primer in a primer pair.
- Maximum length of amplicon - determines the maximum length of the PCR fragment.

22.5.3 Standard PCR output table

If only a single region is selected the following columns of information are available:

- Sequence - the primer's sequence.
- Score - measures how much the properties of the primer (or primer pair) deviates from the optimal solution in terms of the chosen parameters and tolerances. The higher the score, the better the solution. The scale is from 0 to 100.
- Region - the interval of the template sequence covered by the primer
- Self annealing - the maximum self annealing score of the primer in units of hydrogen bonds
- Self annealing alignment - a visualization of the highest maximum scoring self annealing alignment
- Self end annealing - the maximum score of consecutive end base-pairings allowed between the ends of two copies of the same molecule in units of hydrogen bonds
- GC content - the fraction of G and C nucleotides in the primer
- Melting temperature of the primer-template complex
- Secondary structure score - the score of the optimal secondary DNA structure found for the primer. Secondary structures are scored by adding the number of hydrogen bonds in the structure, and 2 extra hydrogen bonds are added for each stacking base-pair in the structure
- Secondary structure - a visualization of the optimal DNA structure found for the primer

If both a forward and a reverse region are selected a table of primer pairs is shown, where the above columns (excluding the score) are represented twice, once for the forward primer (designated by the letter F) and once for the reverse primer (designated by the letter R).

Before these, and following the score of the primer pair, are the following columns pertaining to primer pair-information available:

- Pair annealing - the number of hydrogen bonds found in the optimal alignment of the forward and the reverse primer in a primer pair
- Pair annealing alignment - a visualization of the optimal alignment of the forward and the reverse primer in a primer pair.
- Pair end annealing - the maximum score of consecutive end base-pairings found between the ends of the two primers in the primer pair, in units of hydrogen bonds
- Fragment length - the length (number of nucleotides) of the PCR fragment generated by the primer pair

22.6 Nested PCR

Nested PCR is a modification of Standard PCR, aimed at reducing product contamination due to the amplification of unintended primer binding sites (mispriming). If the intended fragment can not be amplified without interference from competing binding sites, the idea is to seek out a larger outer fragment which can be unambiguously amplified and which contains the smaller intended fragment. Having amplified the outer fragment to large numbers, the PCR amplification of the inner fragment can proceed and will yield amplification of this with minimal contamination.

Primer design for nested PCR thus involves designing two primer pairs, one for the outer fragment and one for the inner fragment.

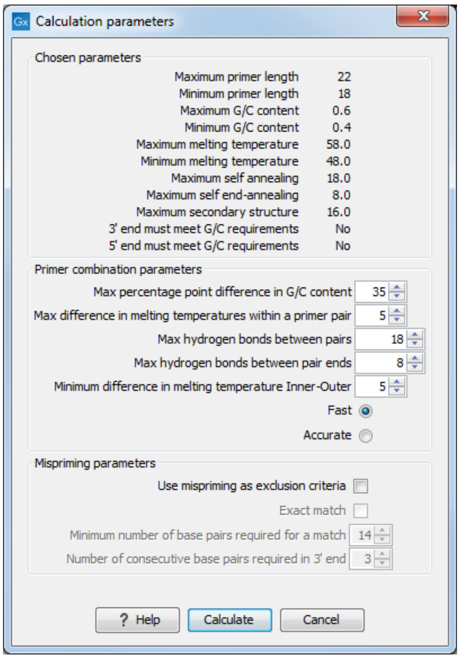
In *Nested PCR* mode the user must thus define four regions a *Forward primer region* (the outer forward primer), a *Reverse primer region* (the outer reverse primer), a *Forward inner primer region*, and a *Reverse inner primer region*. These are defined by making a selection on the sequence and right-clicking the selection. If areas are known where primers must not bind (e.g. repeat rich areas), one or more *No primers here* regions can be defined.

It is required that the *Forward primer region*, is located upstream of the *Forward inner primer region*, that the *Forward inner primer region*, is located upstream of the *Reverse inner primer region*, and that the *Reverse inner primer region*, is located upstream of the *Reverse primer region*.

In *Nested PCR* mode the *Inner melting temperature* menu in the Primer parameters panel is activated, allowing the user to set a separate melting temperature interval for the inner and outer primer pairs.

After exploring the available primers (see section 22.3) and setting the desired parameter values in the Primer parameters preference group, the **Calculate** button will activate the primer design algorithm.

After pressing the **Calculate** button a dialog will appear (see figure 22.9).



Chosen parameters

Maximum primer length	22
Minimum primer length	18
Maximum G/C content	0.6
Minimum G/C content	0.4
Maximum melting temperature	58.0
Minimum melting temperature	48.0
Maximum self annealing	18.0
Maximum self end-annealing	8.0
Maximum secondary structure	16.0
3' end must meet G/C requirements	No
5' end must meet G/C requirements	No

Primer combination parameters

Max percentage point difference in G/C content	35
Max difference in melting temperatures within a primer pair	5
Max hydrogen bonds between pairs	18
Max hydrogen bonds between pair ends	8
Minimum difference in melting temperature Inner-Outer	5

Fast ●
Accurate ○

Mispriming parameters

Use mispriming as exclusion criteria □
Exact match □
Minimum number of base pairs required for a match 14
Number of consecutive base pairs required in 3' end 3

Figure 22.9: *Calculation dialog for nested primers.*

The top and bottom parts of this dialog are identical to the *Standard PCR* dialog for designing primer pairs described above.

The central part of the dialog contains parameters pertaining to primer pairs and the comparison between the outer and the inner pair. Here five options can be set:

- Maximum percentage point difference in G/C content (described above under Standard PCR) - this criteria is applied to both primer pairs independently.
- Maximal difference in melting temperature of primers in a pair - the number of degrees Celsius that primers in a pair are all allowed to differ. This criteria is applied to both primer pairs independently.
- Maximum pair annealing score - the maximum number of hydrogen bonds allowed between the forward and the reverse primer in a primer pair. This criteria is applied to all possible combinations of primers.
- Minimum difference in the melting temperature of primers in the inner and outer primer pair - all comparisons between the melting temperature of primers from the two pairs must be at least this different, otherwise the primer set is excluded. This option is applied to ensure that the inner and outer PCR reactions can be initiated at different annealing temperatures. Please note that to ensure flexibility there is no directionality indicated when setting parameters for melting temperature differences between inner and outer primer pair, i.e. it is not specified whether the inner pair should have a lower or higher T_m. Instead this is determined by the allowed temperature intervals for inner and outer primers that are set in the primer parameters preference group in the side panel. If a higher T_m of inner primers is desired, choose a T_m interval for inner primers which has higher values than the interval for outer primers.
- Two radio buttons allowing the user to choose between a fast and an accurate algorithm for primer prediction.

Nested PCR output table In nested PCR there are four primers in a solution, forward outer primer (FO), forward inner primer (FI), reverse inner primer (RI) and a reverse outer primer (RO).

The output table can show primer-pair combination parameters for all four combinations of primers and single primer parameters for all four primers in a solution (see section on Standard PCR for an explanation of the available primer-pair and single primer information).

The fragment length in this mode refers to the length of the PCR fragment generated by the inner primer pair, and this is also the PCR fragment which can be exported.

22.7 TaqMan

CLC Main Workbench allows the user to design primers and probes for TaqMan PCR applications.

TaqMan probes are oligonucleotides that contain a fluorescent reporter dye at the 5' end and a quenching dye at the 3' end. Fluorescent molecules become excited when they are irradiated and usually emit light. However, in a TaqMan probe the energy from the fluorescent dye is transferred to the quencher dye by fluorescence resonance energy transfer as long as the quencher and the dye are located in close proximity i.e. when the probe is intact. TaqMan probes are designed to anneal within a PCR product amplified by a standard PCR primer pair. If a TaqMan probe is bound to a product template, the replication of this will cause the Taq polymerase to encounter the probe. Upon doing so, the 5'exonuclease activity of the polymerase will cleave the probe. This cleavage separates the quencher and the dye, and as a result the reporter dye starts to emit fluorescence.

The TaqMan technology is used in Real-Time quantitative PCR. Since the accumulation of fluorescence mirrors the accumulation of PCR products it can can be monitored in real-time and used to quantify the amount of template initially present in the buffer.

The technology is also used to detect genetic variation such as SNP's. By designing a TaqMan probe which will specifically bind to one of two or more genetic variants it is possible to detect genetic variants by the presence or absence of fluorescence in the reaction.

A specific requirement of TaqMan probes is that a G nucleotide can not be present at the 5' end since this will quench the fluorescence of the reporter dye. It is recommended that the melting temperature of the TaqMan probe is about 10 degrees celsius higher than that of the primer pair.

Primer design for TaqMan technology involves designing a primer pair and a TaqMan probe.

In *TaqMan* the user must thus define three regions: a *Forward primer region*, a *Reverse primer region*, and a *TaqMan probe region*. The easiest way to do this is to designate a *TaqMan primer/probe region* spanning the sequence region where TaqMan amplification is desired. This will automatically add all three regions to the sequence. If more control is desired about the placing of primers and probes the *Forward primer region*, *Reverse primer region* and *TaqMan probe region* can all be defined manually. If areas are known where primers or probes must not bind (e.g. repeat rich areas), one or more *No primers here* regions can be defined. The regions are defined by making a selection on the sequence and right-clicking the selection.

It is required that at least a part of the *Forward primer region* is located upstream of the *TaqMan Probe region*, and that the *TaqMan Probe region*, is located upstream of a part of the *Reverse primer region*.

In *TaqMan* mode the *Inner melting temperature* menu in the primer parameters panel is activated allowing the user to set a separate melting temperature interval for the TaqMan probe.

After exploring the available primers (see section 22.3) and setting the desired parameter values in the Primer Parameters preference group, the **Calculate** button will activate the primer design algorithm.

After pressing the **Calculate** button a dialog will appear (see figure 22.10) which is similar to the *Nested PCR* dialog described above (see section 22.6).

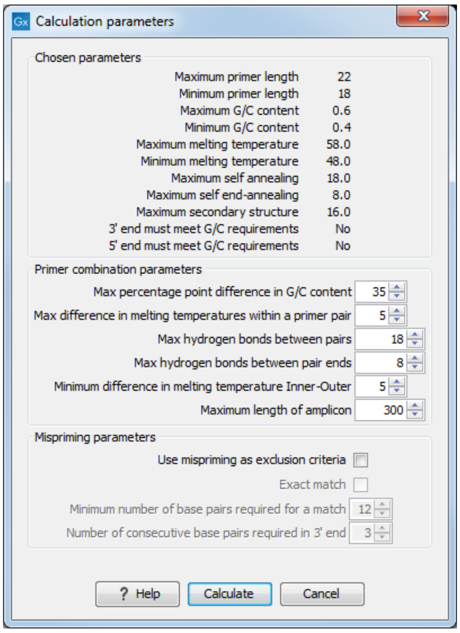
In this dialog the options to set a minimum and a desired melting temperature difference between outer and inner refers to primer pair and probe respectively.

Furthermore, the central part of the dialog contains an additional parameter

- Maximum length of amplicon - determines the maximum length of the PCR fragment generated in the TaqMan analysis.

TaqMan output table In TaqMan mode there are two primers and a probe in a given solution, forward primer (F), reverse primer (R) and a TaqMan probe (TP).

The output table can show primer/probe-pair combination parameters for all three combinations



Calculation parameters

Chosen parameters

Maximum primer length	22
Minimum primer length	18
Maximum G/C content	0.6
Minimum G/C content	0.4
Maximum melting temperature	58.0
Minimum melting temperature	48.0
Maximum self annealing	18.0
Maximum self end-annealing	8.0
Maximum secondary structure	16.0
3' end must meet G/C requirements	No
5' end must meet G/C requirements	No

Primer combination parameters

Max percentage point difference in G/C content	35
Max difference in melting temperatures within a primer pair	5
Max hydrogen bonds between pairs	18
Max hydrogen bonds between pair ends	8
Minimum difference in melting temperature Inner-Outer	5
Maximum length of amplicon	300

Mispriming parameters

Use mispriming as exclusion criteria ☐

Exact match ☐

Minimum number of base pairs required for a match 12

Number of consecutive base pairs required in 3' end 3

Figure 22.10: *Calculation dialog for taqman primers.*

of primers and single primer parameters for both primers and the TaqMan probe (see section on Standard PCR for an explanation of the available primer-pair and single primer information).

The fragment length in this mode refers to the length of the PCR fragment generated by the primer pair, and this is also the PCR fragment which can be exported.

22.8 Sequencing primers

This mode is used to design primers for DNA sequencing.

In this mode the user can define a number of *Forward primer regions* and *Reverse primer regions* where a sequencing primer can start. These are defined by making a selection on the sequence and right-clicking the selection. If areas are known where primers must not bind (e.g. repeat rich areas), one or more *No primers here* regions can be defined.

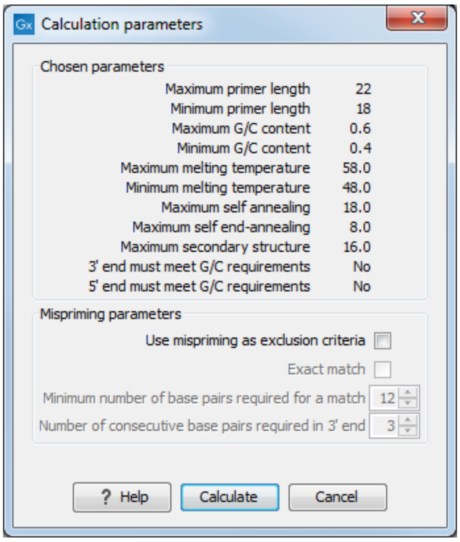
No requirements are instated on the relative position of the regions defined.

After exploring the available primers (see section 22.3) and setting the desired parameter values in the Primer Parameters preference group, the **Calculate** button will activate the primer design algorithm.

After pressing the **Calculate** button a dialog will appear (see figure 22.11).

Since design of sequencing primers does not require the consideration of interactions between primer pairs, this dialog is identical to the dialog shown in *Standard PCR* mode when only a single primer region is chosen (see section 22.5 for a description).

Sequencing primers output table In this mode primers are predicted independently for each region, but the optimal solutions are all presented in one table. The solutions are numbered consecutively according to their position on the sequence such that the forward primer region closest to the 5' end of the molecule is designated F1, the next one F2 etc.



Calculation parameters

Chosen parameters

Maximum primer length	22
Minimum primer length	18
Maximum G/C content	0.6
Minimum G/C content	0.4
Maximum melting temperature	58.0
Minimum melting temperature	48.0
Maximum self annealing	18.0
Maximum self end-annealing	8.0
Maximum secondary structure	16.0
3' end must meet G/C requirements	No
5' end must meet G/C requirements	No

Mispriming parameters

Use mispriming as exclusion criteria □

Exact match □

Minimum number of base pairs required for a match 12

Number of consecutive base pairs required in 3' end 3

Figure 22.11: *Calculation dialog for sequencing primers.*

For each solution, the single primer information described under Standard PCR is available in the table.

22.9 Alignment-based primer and probe design

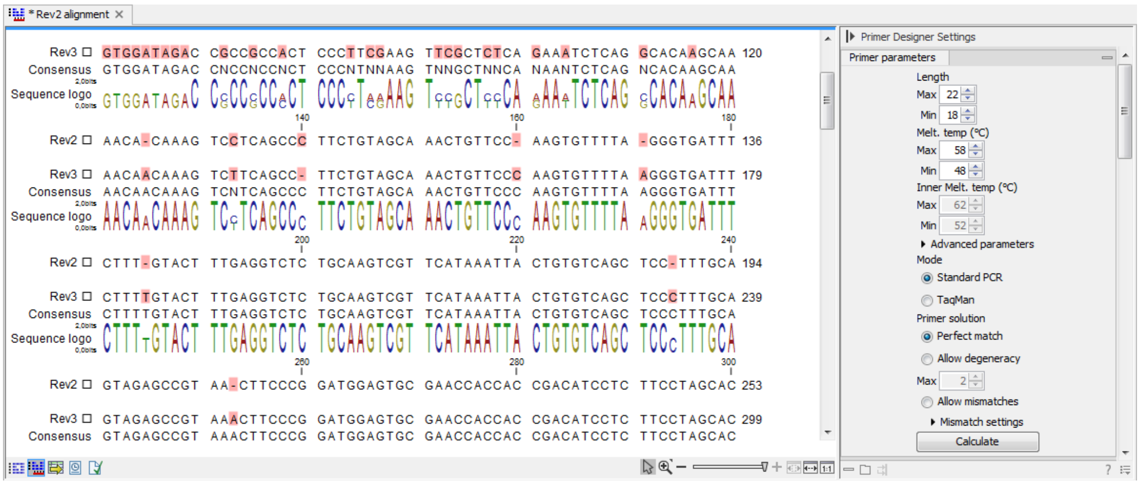
CLC Main Workbench allows the user to design PCR primers and TaqMan probes based on an alignment of multiple sequences.

The primer designer for alignments can be accessed with:

Tools | **Primers and Probes** ( )| **Design Primers** ( )

Or if the alignment is already open, click Primer Designer ( ) in the lower left part of the view.

In the alignment primer view (see figure 22.12), the basic options for viewing the template alignment are the same as for the standard view of alignments (see section 16 for an explanation of these options). This means that annotations such as known SNPs or exons can be displayed on the template sequence to guide the choice of primer regions.



```
      Rev3 □ GTGGATAGAC CGCCGCCACT CCCTTCGAAG TTCGCTCTCA GAAATCTCAG GCACAAGCAA 120
  Consensus  GTGGATAGAC CNCCNCCNCT CCCNTNNAAG TNNGCTNNCA NAANTCTCAG NCACAAGCAA
                                 140                  160                  180
      Rev2 □ AACA-CAAAG TCCTCAGCCC TTCTGTAGCA AACTGTTCC- AAGTGTTTTA -GGGTGATTT 136

      Rev3 □ AACAACAAAG TCTTCAGCC- TTCTGTAGCA AACTGTTCCC AAGTGTTTTA AGGGTGATTT 179
  Consensus  AACAACAAAG TCNTCAGCCC TTCTGTAGCA AACTGTTCCC AAGTGTTTTA AGGGTGATTT
                                 200                  220                  240
      Rev2 □ CTTT-GTACT TTGAGGTCTC TGCAAGTCGT TCATAAATTA CTGTGTCAGC TCC-TTTGCA 194

      Rev3 □ CTTTTGTACT TTGAGGTCTC TGCAAGTCGT TCATAAATTA CTGTGTCAGC TCCCTTTGCA 239
  Consensus  CTTTTGTACT TTGAGGTCTC TGCAAGTCGT TCATAAATTA CTGTGTCAGC TCCCTTTGCA
                                 260                  280                  300
      Rev2 □ GTAGAGCCGT AA-CTTCCCG GATGGAGTGC GAACCACCAC CGACATCCTC TTCCTAGCAC 253

      Rev3 □ GTAGAGCCGT AAACTTCCCG GATGGAGTGC GAACCACCAC CGACATCCTC TTCCTAGCAC 299
  Consensus  GTAGAGCCGT AAACTTCCCG GATGGAGTGC GAACCACCAC CGACATCCTC TTCCTAGCAC
```

Primer Designer Settings

Primer parameters

Length
Max 22
Min 18

Melt. temp (°C)
Max 58
Min 48

Inner Melt. temp (°C)
Max 62
Min 52

Advanced parameters

Mode
● Standard PCR
○ TaqMan

Primer solution
● Perfect match
○ Allow degeneracy
Max 2
○ Allow mismatches

Mismatch settings

Figure 22.12: *The initial view of an alignment used for primer design.*

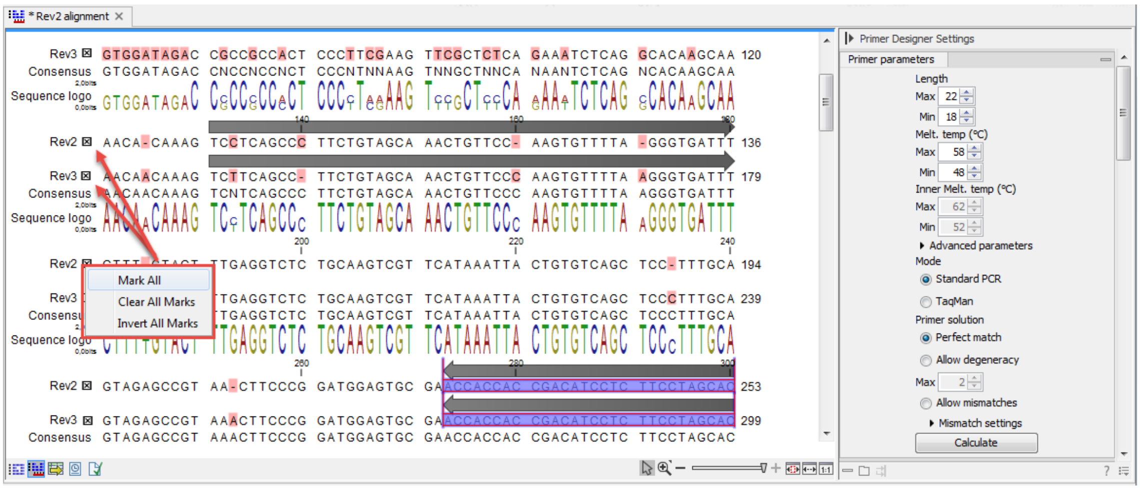
22.9.1 Specific options for alignment-based primer and probe design

Compared to the primer view of a single sequence, the most notable difference is that the alignment primer view has no available graphical information. Furthermore, the selection boxes found to the left of the names in the alignment play an important role in specifying the oligo design process.

The **Primer Parameters** group in the **Side Panel** has the same options as the ones defined for primers design based on single sequences, but differs by the following submenus(see figure 22.12):

- In the **Mode** submenu, specify either:
  - **Standard PCR.** Used when the objective is to design primers, or primer pairs, for PCR amplification of a single DNA fragment.
  - **TaqMan.** Used when the objective is to design a primer pair and a probe set for TaqMan quantitative PCR.
- In the **Primer solution** submenu, specify requirements for the match of a PCR primer against the template sequences. These options are described further below. It contains the following options:
  - **Perfect match**
  - **Allow degeneracy**
  - **Allow mismatches**

The workflow when designing alignment based primers and probes is as follows (see figure 22.13):



Length
Max 22
Min 18
Melt. temp (°C)
Max 58
Min 48
Inner Melt. temp (°C)
Max 62
Min 52
Advanced parameters
Mode
● Standard PCR
○ TaqMan
Primer solution
● Perfect match
○ Allow degeneracy
Max 2
○ Allow mismatches
Mismatch settings
Calculate

Figure 22.13: *The initial view of an alignment used for primer design.*

- Use selection boxes to specify groups of included and excluded sequences. To select all the sequences in the alignment, right-click one of the selection boxes and choose **Mark All**.
- Mark either a single forward primer region, a single reverse primer region or both on the sequence (and perhaps also a TaqMan region). Selections must cover all sequences in the included group. You can also specify that there should be no primers in a region (No Primers Here) or that a whole region should be amplified (Region to Amplify).

- Adjust parameters regarding single primers in the preference panel.
- Click the **Calculate** button.

22.9.2 Alignment based design of PCR primers

In this mode, a single or a pair of PCR primers are designed. *CLC Main Workbench* allows the user to design primers which will specifically amplify a group of *included* sequences but **not** amplify the remainder of the sequences, the *excluded* sequences. The selection boxes are used to indicate the status of a sequence, if the box is checked the sequence belongs to the included sequences, if not, it belongs to the excluded sequences. To design primers that are general for all primers in an alignment, simply add them all to the set of included sequences by checking all selection boxes. Specificity of priming is determined by criteria set by the user in the dialog box which is shown when the **Calculate** button is pressed (see below).

Different options can be chosen concerning the match of the primer to the template sequences in the included group:

- **Perfect match.** Specifies that the designed primers must have a perfect match to all relevant sequences in the alignment. When selected, primers will thus only be located in regions that are completely conserved within the sequences belonging to the included group.
- **Allow degeneracy.** Designs primers that may include ambiguity characters where heterogeneities occur in the included template sequences. The allowed fold of degeneracy is user defined and corresponds to the number of possible primer combinations formed by a degenerate primer. Thus, if a primer covers two 4-fold degenerate site and one 2-fold degenerate site the total fold of degeneracy is $4 * 4 * 2 = 32$ and the primer will, when supplied from the manufacturer, consist of a mixture of 32 different oligonucleotides. When scoring the available primers, degenerate primers are given a score which decreases with the fold of degeneracy.
- **Allow mismatches.** Designs primers which are allowed a specified number of mismatches to the included template sequences. The melting temperature algorithm employed includes the latest thermodynamic parameters for calculating T_m when single-base mismatches occur.

When in Standard PCR mode, clicking the **Calculate** button will prompt the dialog shown in figure 22.14.

The top part of this dialog shows the single-primer parameter settings chosen in the Primer parameters preference group which will be used by the design algorithm.

The central part of the dialog contains parameters pertaining to primer specificity (this is omitted if all sequences belong to the included group). Here, three parameters can be set:

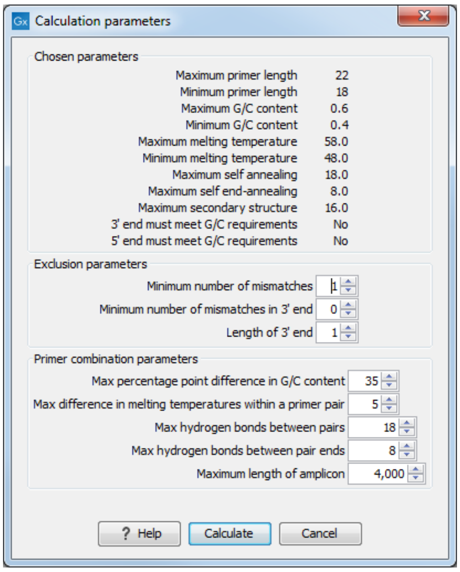
- Minimum number of mismatches - the minimum number of mismatches that a primer must have against all sequences in the excluded group to ensure that it does not prime these.
- Minimum number of mismatches in 3' end - the minimum number of mismatches that a primer must have in its 3' end against all sequences in the excluded group to ensure that it does not prime these.

- Length of 3' end - the number of consecutive nucleotides to consider for mismatches in the 3' end of the primer.

The lower part of the dialog contains parameters pertaining to primer pairs (this is omitted when only designing a single primer). Here, three parameters can be set:

- Maximum percentage point difference in G/C content - if this is set at e.g. 5 points a pair of primers with 45% and 49% G/C nucleotides, respectively, will be allowed, whereas a pair of primers with 45% and 51% G/C nucleotides, respectively will not be included.
- Maximal difference in melting temperature of primers in a pair - the number of degrees Celsius that primers in a pair are all allowed to differ.
- Max hydrogen bonds between pairs - the maximum number of hydrogen bonds allowed between the forward and the reverse primer in a primer pair.
- Maximum length of amplicon - determines the maximum length of the PCR fragment.

The output of the design process is a table of single primers or primer pairs as described for primer design based on single sequences. These primers are specific to the included sequences in the alignment according to the criteria defined for specificity. The only novelty in the table, is that melting temperatures are displayed with both a maximum, a minimum and an average value to reflect that degenerate primers or primers with mismatches may have heterogeneous behavior on the different templates in the group of included sequences.



Chosen parameters

Maximum primer length	22
Minimum primer length	18
Maximum G/C content	0.6
Minimum G/C content	0.4
Maximum melting temperature	58.0
Minimum melting temperature	48.0
Maximum self annealing	18.0
Maximum self end-annealing	8.0
Maximum secondary structure	16.0
3' end must meet G/C requirements	No
5' end must meet G/C requirements	No

Exclusion parameters

Minimum number of mismatches: 1
Minimum number of mismatches in 3' end: 0
Length of 3' end: 1

Primer combination parameters

Max percentage point difference in G/C content: 35
Max difference in melting temperatures within a primer pair: 5
Max hydrogen bonds between pairs: 18
Max hydrogen bonds between pair ends: 8
Maximum length of amplicon: 4,000

Figure 22.14: *Calculation dialog shown when designing alignment based PCR primers.*

22.9.3 Alignment-based TaqMan probe design

CLC Main Workbench allows the user to design solutions for TaqMan quantitative PCR which consist of four oligos: a general primer pair which will amplify all sequences in the alignment, a specific TaqMan probe which will match the group of *included* sequences but **not** match the *excluded* sequences and a specific TaqMan probe which will match the group of *excluded* sequences but **not** match the *included* sequences. As above, the selection boxes are used to indicate the status of a sequence, if the box is checked the sequence belongs to the included sequences, if not, it belongs to the excluded sequences. We use the terms included and excluded here to be consistent with the section above although a probe solution is presented for both groups. In TaqMan mode, primers are not allowed degeneracy or mismatches to any template sequence in the alignment, variation is only allowed/required in the TaqMan probes.

Pushing the **Calculate** button will cause the dialog shown in figure 22.15 to appear.

The top part of this dialog is identical to the *Standard PCR* dialog for designing primer pairs described above.

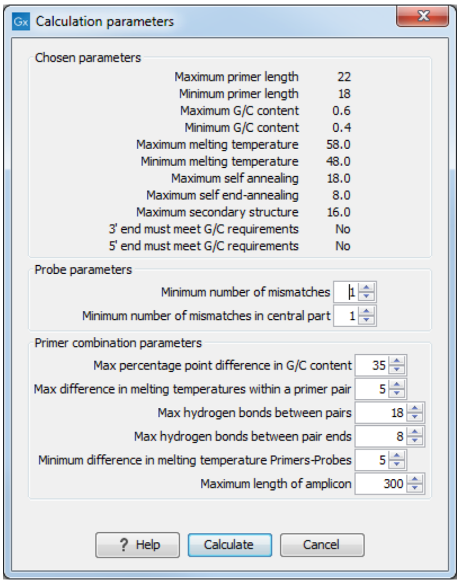
The central part of the dialog contains parameters to define the specificity of TaqMan probes. Two parameters can be set:

- Minimum number of mismatches - the minimum total number of mismatches that must exist between a specific TaqMan probe and all sequences which belong to the group not recognized by the probe.
- Minimum number of mismatches in central part - the minimum number of mismatches in the central part of the oligo that must exist between a specific TaqMan probe and all sequences which belong to the group not recognized by the probe.

The lower part of the dialog contains parameters pertaining to primer pairs and the comparison between the outer oligos(primers) and the inner oligos (TaqMan probes). Here, five options can be set:

- Maximum percentage point difference in G/C content (described above under Standard PCR).
- Maximal difference in melting temperature of primers in a pair - the number of degrees Celsius that primers in the primer pair are all allowed to differ.
- Maximum pair annealing score - the maximum number of hydrogen bonds allowed between the forward and the reverse primer in an oligo pair. This criteria is applied to all possible combinations of primers and probes.
- Minimum difference in the melting temperature of primer (outer) and TaqMan probe (inner) oligos - all comparisons between the melting temperature of primers and probes must be at least this different, otherwise the solution set is excluded.
- Desired temperature difference in melting temperature between outer (primers) and inner (TaqMan) oligos - the scoring function discounts solution sets which deviate greatly from this value. Regarding this, and the minimum difference option mentioned above, please note that to ensure flexibility there is no directionality indicated when setting parameters for melting temperature differences between probes and primers, i.e. it is not specified whether the probes should have a lower or higher T_m. Instead this is determined by the allowed temperature intervals for inner and outer oligos that are set in the primer parameters preference group in the side panel. If a higher T_m of probes is required, choose a T_m interval for probes which has higher values than the interval for outer primers.

The output of the design process is a table of solution sets. Each solution set contains the following: a set of primers which are general to all sequences in the alignment, a TaqMan probe which is specific to the set of included sequences (sequences where selection boxes are checked) and a TaqMan probe which is specific to the set of excluded sequences (marked by *). Otherwise, the table is similar to that described above for TaqMan probe prediction on single sequences.



Chosen parameters

Maximum primer length	22
Minimum primer length	18
Maximum G/C content	0.6
Minimum G/C content	0.4
Maximum melting temperature	58.0
Minimum melting temperature	48.0
Maximum self annealing	18.0
Maximum self end-annealing	8.0
Maximum secondary structure	16.0
3' end must meet G/C requirements	No
5' end must meet G/C requirements	No

Probe parameters

Minimum number of mismatches 1

Minimum number of mismatches in central part 1

Primer combination parameters

Max percentage point difference in G/C content 35

Max difference in melting temperatures within a primer pair 5

Max hydrogen bonds between pairs 18

Max hydrogen bonds between pair ends 8

Minimum difference in melting temperature Primers-Probes 5

Maximum length of amplicon 300

Figure 22.15: *Calculation dialog shown when designing alignment based TaqMan probes.*

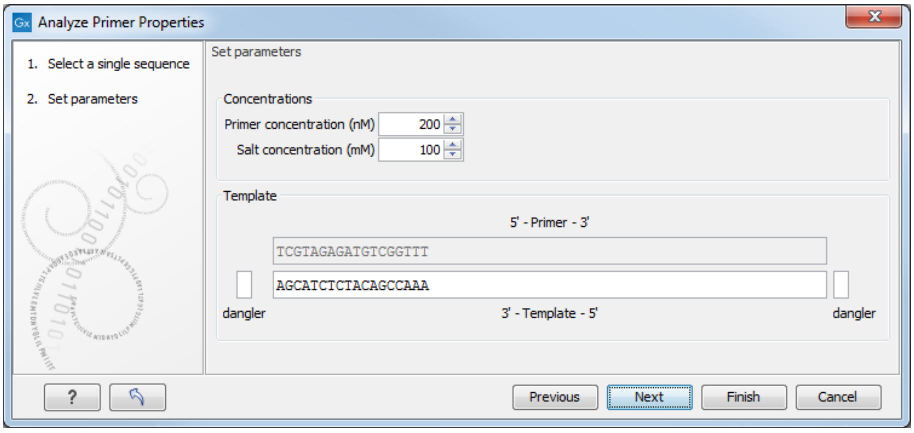
22.10 Analyze primer properties

CLC Main Workbench can calculate and display the properties of predefined primers and probes:

> **Tools | Primers and Probes ( )| Analyze Primer Properties ( )**

If a sequence was selected before launching the tool, this sequence will be listed in the **Selected Elements** window of the dialog. Use the arrows to add or remove a sequence from the selected elements. (Primers are represented as DNA sequences in the Navigation Area).

Clicking **Next** generates the dialog seen in figure 22.16:



Set parameters

Concentrations

Primer concentration (nM) 200

Salt concentration (mM) 100

Template

5' - Primer - 3'

TCGTAGAGATGTCGGTTT

□ AGCATCTCTACAGCCAAA □

dangler 3' - Template - 5' dangler

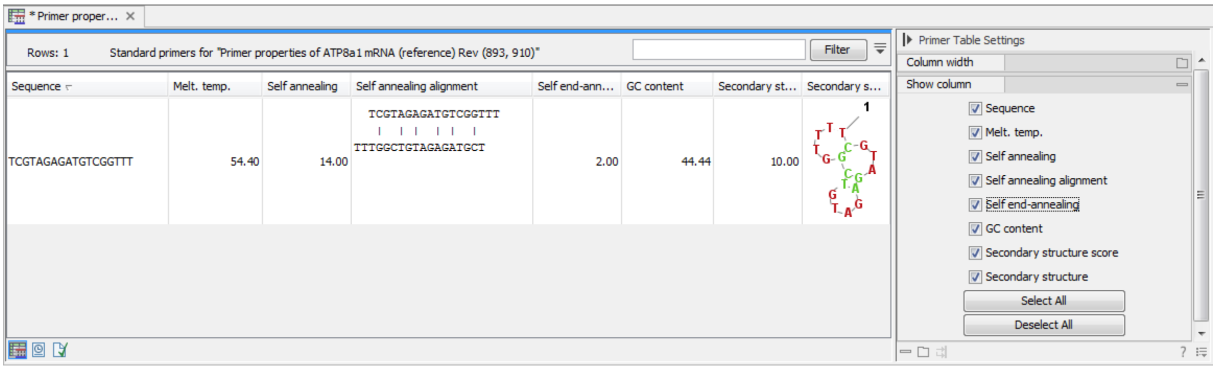
Figure 22.16: *The parameters for analyzing primer properties.*

In the *Concentrations* panel a number of parameters can be specified concerning the reaction mixture and which influence melting temperatures

- **Primer concentration.** Specifies the concentration of primers and probes in units of nanomoles (nM)
- **Salt concentration.** Specifies the concentration of monovalent cations $([NA^{+}]$, $[K^{+}]$ and equivalents) in units of millimoles (mM)

In the *Template panel* the sequences of the chosen primer and the template sequence are shown. The template sequence is as default set to the reverse complement of the primer sequence i.e. as perfectly base-pairing. However, it is possible to edit the template to introduce mismatches which may affect the melting temperature. At each side of the template sequence a text field is shown. Here, the dangling ends of the template sequence can be specified. These may have an important affect on the melting temperature [Bommarito et al., 2000]

Click **Finish** to start the tool. The result is shown in figure 22.17:



Rows: 1 Standard primers for "Primer properties of ATP8a1 mRNA (reference) Rev (893, 910)"

Sequence	Melt. temp.	Self annealing	Self annealing alignment	Self end-ann...	GC content	Secondary st...	Secondary s...
TCGTAGAGATGTCGGTTT	54.40	14.00	TCGTAGAGATGTCGGTTT TTTGGCTGTAGAGATGCT	2.00	44.44	10.00	

Primer Table Settings

Column width

Show column

- ☑ Sequence
- ☑ Melt. temp.
- ☑ Self annealing
- ☑ Self annealing alignment
- ☑ Self end-annealing
- ☑ GC content
- ☑ Secondary structure score
- ☑ Secondary structure

Figure 22.17: *Properties of a primer.*

In the **Side Panel** you can specify the information to display about the primer. The information parameters of the primer properties table are explained in section 22.5.3.

22.11 Find binding sites and create fragments

In *CLC Main Workbench* you have the possibility of matching known primers against one or more DNA sequences or a list of DNA sequences. This can be applied to test whether a primer used in a previous experiment is applicable to amplify a homologous region in another species, or to test for potential mispriming. This functionality can also be used to extract the resulting PCR product when two primers are matched. This is particularly useful if your primers have extensions in the 5' end. Note that this tool is not meant to analyze rapidly high-throughput data. The maximum amount of sequences the tool will handle in a reasonable amount of time depends on your computer processing capabilities.

To search for primer binding sites:

Tools | Primers and Probes ( )| Find Binding Sites and Create Fragments ( )

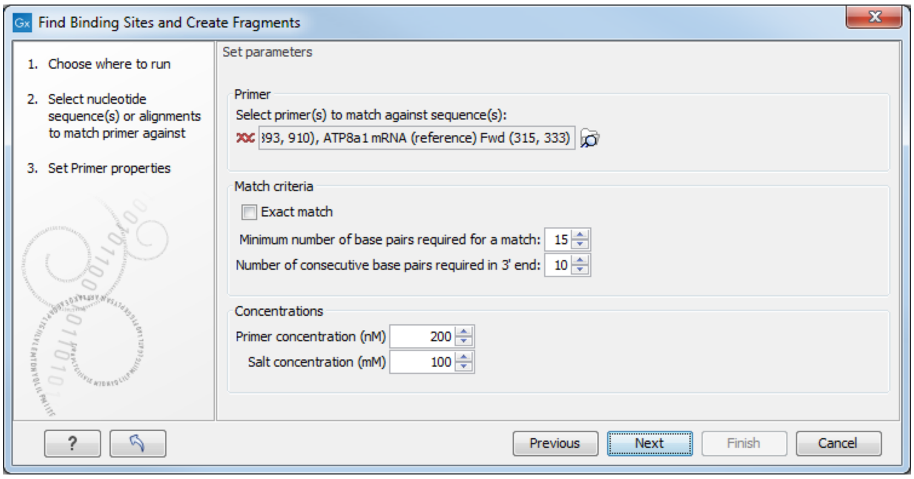
If a sequence was already selected in the Navigation Area, this sequence is now listed in the **Selected Elements** window of the dialog. Use the arrows to add or remove sequences or sequence lists from the selected elements.

Click **Next** when all the sequence have been added.

Note! You should not add the primer sequences at this step.

22.11.1 Binding parameters

This opens the dialog displayed in figure 22.18:



Find Binding Sites and Create Fragments

1. Choose where to run
2. Select nucleotide sequence(s) or alignments to match primer against
3. Set Primer properties

Set parameters

Primer

Select primer(s) to match against sequence(s):

393, 910), ATP8a1 mRNA (reference) Fwd (315, 333)

Match criteria

□ Exact match

Minimum number of base pairs required for a match: 15

Number of consecutive base pairs required in 3' end: 10

Concentrations

Primer concentration (nM) 200

Salt concentration (mM) 100

Figure 22.18: *Search parameters for finding primer binding sites.*

At the top, select one or more primers by clicking the browse ( ) button. In *CLC Main Workbench*, primers are just DNA sequences like any other, but there is a filter on the length of the sequence. Only sequences up to 400 bp can be added.

The **Match criteria** for matching a primer to a sequence are:

- **Exact match**. Choose only to consider exact matches of the primer, i.e. all positions must base pair with the template.
- **Minimum number of base pairs required for a match**. How many nucleotides of the primer that must base pair to the sequence in order to cause priming/mispriming.
- **Number of consecutive base pairs required in 3' end**. How many consecutive 3' end base pairs in the primer that MUST be present for priming/mispriming to occur. This option is included since 3' terminal base pairs are known to be essential for priming to occur.

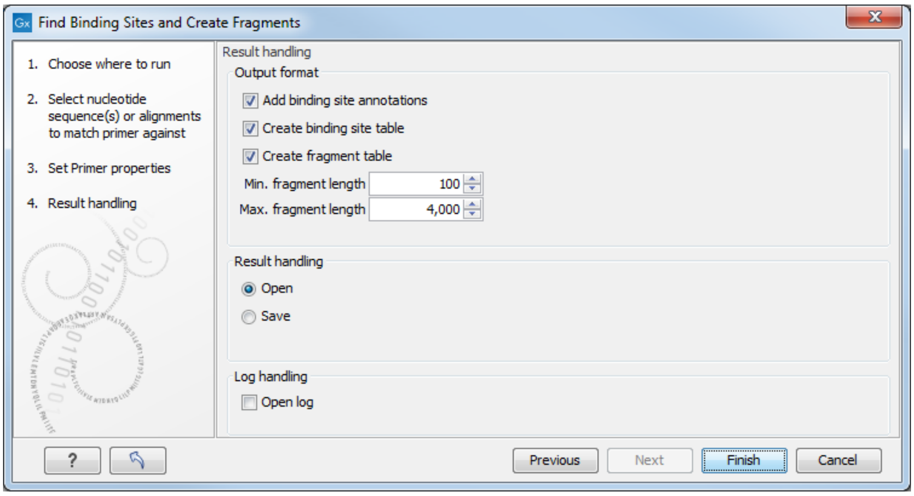
Note that the number of mismatches is reported in the output, so you will be able to filter on this afterwards (see below).

Below the match settings, you can adjust **Concentrations** concerning the reaction mixture. This is used when reporting melting temperatures for the primers.

- **Primer concentration.** Specifies the concentration of primers and probes in units of nanomoles (nM)
- **Salt concentration.** Specifies the concentration of monovalent cations ($[NA^+]$, $[K^+]$ and equivalents) in units of millimoles (mM)

22.11.2 Results - binding sites and fragments

Specify the output options as shown in figure 22.19:



Find Binding Sites and Create Fragments

1. Choose where to run
2. Select nucleotide sequence(s) or alignments to match primer against
3. Set Primer properties
4. Result handling

Result handling

Output format

☑ Add binding site annotations

☑ Create binding site table

☑ Create fragment table

Min. fragment length 100

Max. fragment length 4,000

Result handling

● Open

○ Save

Log handling

□ Open log

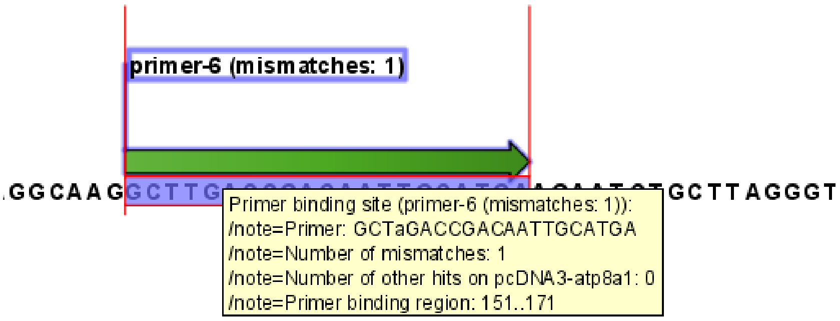
Figure 22.19: *Output options include reporting of binding sites and fragments.*

The output options are:

- **Add binding site annotations**. This will add annotations to the input sequences (see details below).
- **Create binding site table**. Creates a table of all binding sites. Described in details below.
- **Create fragment table**. Showing a table of all fragments that could result from using the primers. Note that you can set the minimum and maximum sizes of the fragments to be shown. The table is described in detail below.

Click **Finish** to start the tool.

An example of a **binding site annotation** is shown in figure 22.20.



Primer binding site (primer-6 (mismatches: 1)):
/note=Primer: GCTaGACCGACAATTGCATGA
/note=Number of mismatches: 1
/note=Number of other hits on pcDNA3-atp8a1: 0
/note=Primer binding region: 151..171

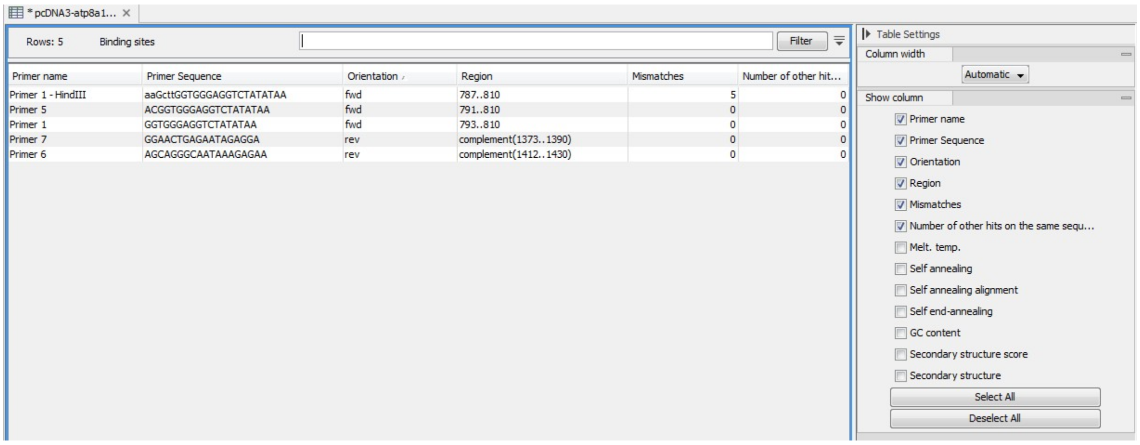
Figure 22.20: *Annotation showing a primer match.*

The annotation has the following information:

- **Sequence of the primer**. Positions with mismatches will be in lower-case (see the fourth position in figure 22.20 where the primer has an `a` and the template sequence has a `T`).

- **Number of mismatches**.
- **Number of other hits on the same sequence**. This number can be useful to check specificity of the primer.
- **Binding region**. This region ends with the 3' exact match and is simply the primer length upstream. This means that if you have 5' extensions to the primer, part of the binding region covers sequence that will actually not be annealed to the primer.

An example of the **primer binding site table** is shown in figure 22.21.



Rows: 5 Binding sites

Primer name	Primer Sequence	Orientation	Region	Mismatches	Number of other hit...
Primer 1 - HindIII	aaGcttGGTGGGAGGTCTATATAA	fwd	787..810	5	0
Primer 5	ACGGTGGGAGGTCTATATAA	fwd	791..810	0	0
Primer 1	GGTGGGAGGTCTATATAA	fwd	793..810	0	0
Primer 7	GGAACTGAGAATAGAGGA	rev	complement(1373..1390)	0	0
Primer 6	AGCAGGGCAATAAAGAGAA	rev	complement(1412..1430)	0	0

Column width: Automatic

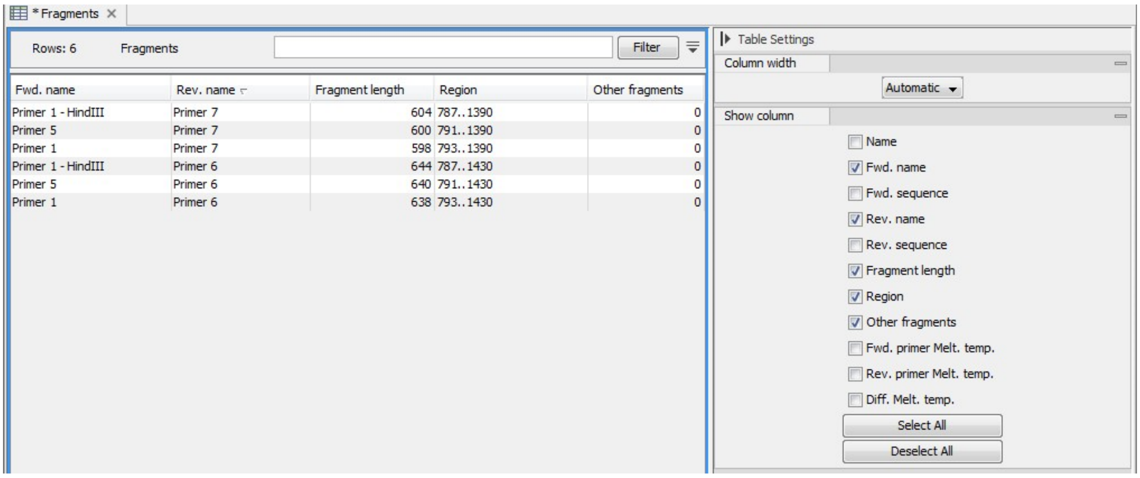
Show column

☑ Primer name
☑ Primer Sequence
☑ Orientation
☑ Region
☑ Mismatches
☑ Number of other hits on the same sequ...
□ Melt. temp.
□ Self annealing
□ Self annealing alignment
□ Self end-annealing
□ GC content
□ Secondary structure score
□ Secondary structure

Figure 22.21: *A table showing all binding sites.*

The information here is the same as in the primer annotation and furthermore you can see additional information about melting temperature etc. by selecting the options in the **Side Panel**. See a more detailed description of this information in section 22.5.3. You can use this table to browse the binding sites. If you make a split view of the table and the sequence (see section 2.1.4), you can browse through the binding positions by clicking in the table. This will cause the sequence view to jump to the position of the binding site.

An example of a **fragment table** is shown in figure 22.22.



Rows: 6 Fragments

Fwd. name	Rev. name	Fragment length	Region	Other fragments
Primer 1 - HindIII	Primer 7	604	787..1390	0
Primer 5	Primer 7	600	791..1390	0
Primer 1	Primer 7	598	793..1390	0
Primer 1 - HindIII	Primer 6	644	787..1430	0
Primer 5	Primer 6	640	791..1430	0
Primer 1	Primer 6	638	793..1430	0

Column width: Automatic

Show column

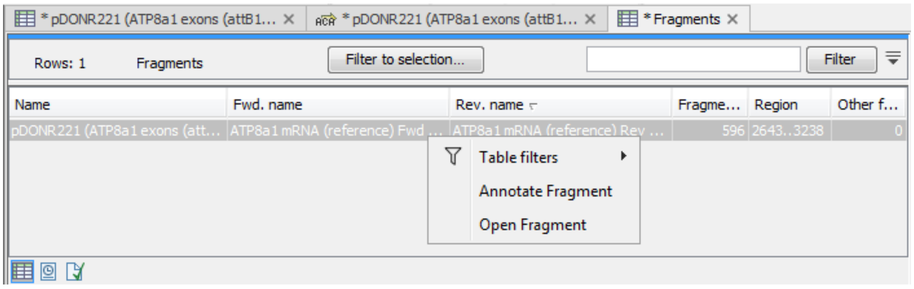
□ Name
☑ Fwd. name
□ Fwd. sequence
☑ Rev. name
□ Rev. sequence
☑ Fragment length
☑ Region
☑ Other fragments
□ Fwd. primer Melt. temp.
□ Rev. primer Melt. temp.
□ Diff. Melt. temp.

Figure 22.22: *A table showing all possible fragments of the specified size.*

The table first lists the names of the forward and reverse primers, then the length of the fragment and the region. The last column tells if there are other possible fragments fulfilling the length criteria on this sequence. This information can be used to check for competing products in the PCR. In the **Side Panel** you can show information about melting temperature for the primers as well as the difference between melting temperatures.

You can use this table to browse the fragment regions. If you make a split view of the table and the sequence (see section 2.1.4), you can browse through the fragment regions by clicking in the table. This will cause the sequence view to jump to the start position of the fragment.

There are some additional options in the fragment table. First, you can annotate the fragment on the original sequence. This is done by right-clicking (Ctrl-click on Mac) the fragment and choose **Annotate Fragment** as shown in figure 22.23.



Name	Fwd. name	Rev. name	Fragme...	Region	Other f...
pDONR221 (ATP8a1 exons (att...	ATP8a1 mRNA (reference) Fwd ...	ATP8a1 mRNA (reference) Rev ...	596	2643..3238	0

Figure 22.23: *Right-clicking a fragment allows you to annotate the region on the input sequence or open the fragment as a new sequence.*

This will put a *PCR fragment* annotations on the input sequence covering the region specified in the table. As you can see from figure 22.23, you can also choose to **Open Fragment**. This will create a new sequence representing the PCR product that would be the result of using these two primers. Note that if you have extensions on the primers, they will be used to construct the new sequence.

If you are doing restriction cloning using primers with restriction site extensions, you can use this functionality to retrieve the PCR fragment for us in the cloning editor (see section 23.3).

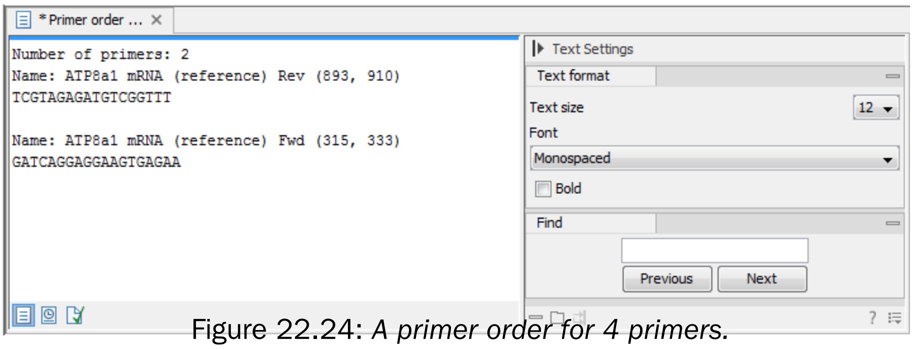
22.12 Order primers

To facilitate the ordering of primers and probes, *CLC Main Workbench* offers an easy way of displaying and saving a textual representation of one or more primers:

Tools | Primers and Probes ( )| Order Primers ( )

This opens a dialog where you can choose primers to generate a textual representation of the primers (see figure 22.24).

The first line states the number of primers being ordered and after this follows the names and nucleotide sequences of the primers in 5'-3' orientation. From the editor, the primer information can be copied and pasted to web forms or e-mails. This file can also be saved and exported as a text file.



```
Number of primers: 2
Name: ATP8a1 mRNA (reference) Rev (893, 910)
TCGTAGAGATGTCGGTTT

Name: ATP8a1 mRNA (reference) Fwd (315, 333)
GATCAGGAGGAAGTGAGAA
```

Figure 22.24: *A primer order for 4 primers.*

Chapter 23

Cloning and restriction sites

Contents

CLC Main Workbench offers graphically advanced *in silico* cloning and design of vectors, together with restriction enzyme analysis and functionalities for managing lists of restriction enzymes.

23.1 Restriction site analyses

There are two ways of finding and showing restriction sites:

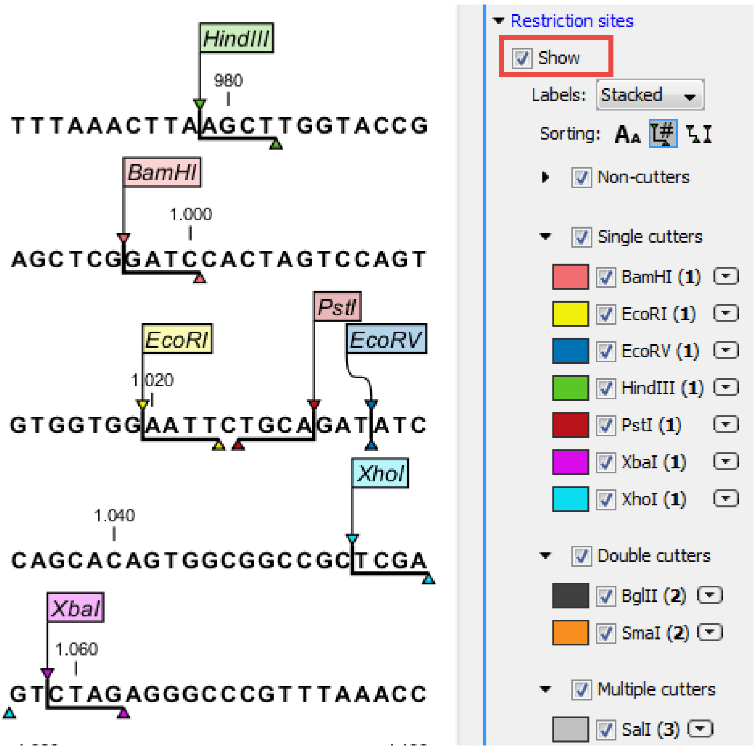
- In many cases, the dynamic restriction sites found in the **Side Panel** of sequence views is the fastest and easiest way of showing restriction sites.

- Under the **Tools** menu you will find the **Restriction Sites Analysis** tool, which provides more control over the analysis and more output options, such as a table of restriction sites. It also allows you to perform the same restriction map analysis on several sequences in one step.

23.1.1 Dynamic restriction sites

If you open a sequence or a sequence list, you will find a **Restriction sites** palette available in the Side Panel.

Restriction sites can be shown on the sequence as colored triangles and lines (figure 23.1): check the "Show" option on top of the Restriction sites section, then specify the enzymes that should be displayed.



Restriction sites

☑ Show

Labels: Stacked

Sorting:

☑ Non-cutters

☑ Single cutters

☑ BamHI (1)

☑ EcoRI (1)

☑ EcoRV (1)

☑ HindIII (1)

☑ PstI (1)

☑ XbaI (1)

☑ XhoI (1)

☑ Double cutters

☑ BglII (2)

☑ SmaI (2)

☑ Multiple cutters

☑ SalI (3)

Figure 23.1: *Showing restriction sites of ten restriction enzymes.*

The color of the restriction enzyme can be changed by clicking the colored box next to the enzyme's name. The name of the enzyme can also be shown next to the restriction site by selecting **Show** above the list of restriction enzymes.

There is also an option to specify how the **Labels** should be shown:

- **No labels**. This will just display the cut site with no information about the name of the enzyme. Placing the mouse button on the cut site will reveal this information as a tool tip.

- **Flag**. This will place a flag just above the sequence with the enzyme name (see an example in figure 23.2). Note that this option will make it hard to see when several cut sites are located close to each other. In the circular view, this option is replaced by the Radial option.

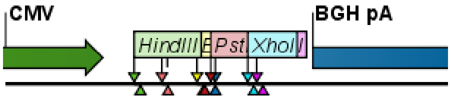


Figure 23.2: *Restriction site labels shown as flags.*

- **Radial**. This option is only available in the circular view. It will place the restriction site labels as close to the cut site as possible (see an example in figure 23.3).

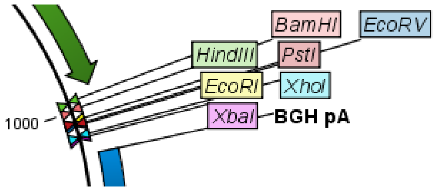


Figure 23.3: *Restriction site labels in radial layout.*

- **Stacked**. This is similar to the flag option for linear sequence views, but it will stack the labels so that all enzymes are shown. For circular views, it will align all the labels on each side of the circle. This can be useful for clearly seeing the order of the cut sites when they are located closely together (see an example in figure 23.4).

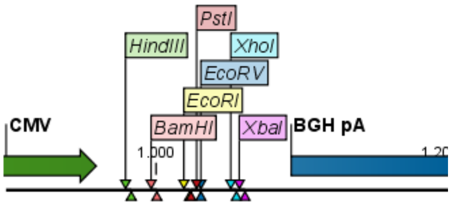


Figure 23.4: *Restriction site labels stacked.*

Note that in a circular view, the **Stacked** and **Radial** options also affect the layout of annotations.

Just above the list of enzymes, three buttons can be used for sorting the list (see figure 23.5).

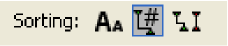


Figure 23.5: *Buttons to sort restriction enzymes.*

- **Sort enzymes alphabetically** (AA). Clicking this button will sort the list of enzymes alphabetically.
- **Sort enzymes by number of restriction sites** (#). This will divide the enzymes into four groups:
  - Non-cutters.
  - Single cutters.
  - Double cutters.

  - Multiple cutters.

  There is a checkbox for each group which can be used to hide / show all the enzymes in a group.

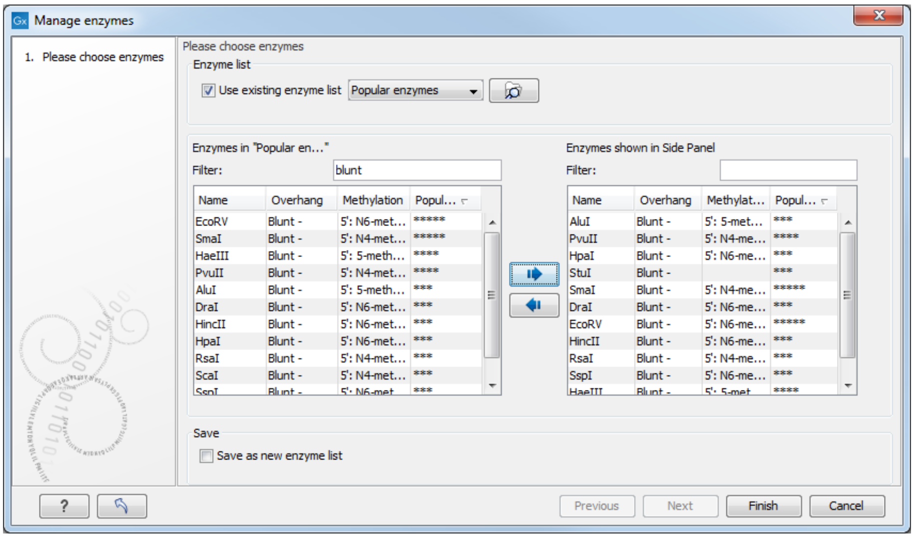
- **Sort enzymes by overhang** (). This will divide the enzymes into three groups:
  - Blunt. Enzymes cutting both strands at the same position.
  - 3'. Enzymes producing an overhang at the 3' end.
  - 5'. Enzymes producing an overhang at the 5' end.

  There is a checkbox for each group which can be used to hide / show all the enzymes in a group.

Manage enzymes

The list of restriction enzymes contains per default some of the most popular enzymes, but you can easily modify this list and add more enzymes by clicking the **Manage enzymes button** found at the bottom of the "Restriction sites" palette of the Side Panel.

This will open the dialog shown in figure 23.6.



Manage enzymes

1. Please choose enzymes

Please choose enzymes

Enzyme list

☑ Use existing enzyme list: Popular enzymes

Enzymes in "Popular en..."

Filter: blunt

Name	Overhang	Methylation	Popul...
EcoRV	Blunt -	5': N6-met...	*****
SmaI	Blunt -	5': N4-met...	*****
HaeIII	Blunt -	5': 5-meth...	****
PvuII	Blunt -	5': N4-met...	****
AluI	Blunt -	5': 5-meth...	***
DraI	Blunt -	5': N6-met...	***
HincII	Blunt -	5': N6-met...	***
HpaI	Blunt -	5': N6-met...	***
RsaI	Blunt -	5': N4-met...	***
ScaI	Blunt -	5': N4-met...	***
SspI	Blunt -	5': N6-met	***

Enzymes shown in Side Panel

Filter:

Name	Overhang	Methylat...	Popul...
AluI	Blunt -	5': 5-met...	***
PvuII	Blunt -	5': N4-me...	****
HpaI	Blunt -	5': N6-me...	***
StuI	Blunt -		***
SmaI	Blunt -	5': N4-me...	*****
DraI	Blunt -	5': N6-me...	***
EcoRV	Blunt -	5': N6-me...	*****
HincII	Blunt -	5': N6-me...	***
RsaI	Blunt -	5': N4-me...	***
SspI	Blunt -	5': N6-me...	***
HaeIII	Blunt -	5': 5-met	****

Save

□ Save as new enzyme list

Figure 23.6: *Adding or removing enzymes from the Side Panel.*

At the top, you can choose to **Use existing enzyme list**. Clicking this option lets you select an enzyme list which is stored in the **Navigation Area**. A list of popular enzymes is available in the Example Data folder you can download from the Help menu.

Below there are two panels:

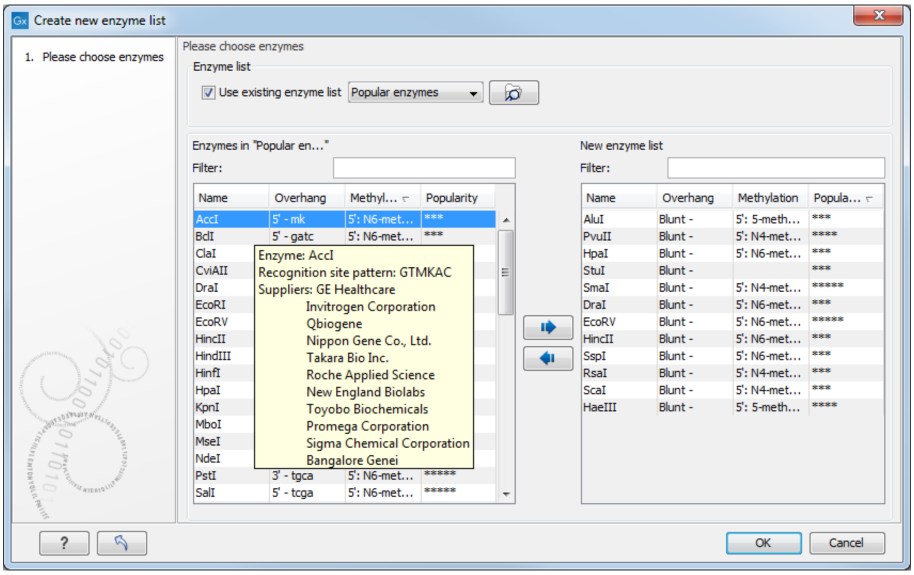
- To the **left**, you can see all the enzymes that are in the list selected above. If you have not chosen to use a specific enzyme list, this panel shows all the enzymes available.
- To the **right**, you can see the list of the enzymes that will be used.

Select enzymes in the left side panel and add them to the right panel by double-clicking or clicking the **Add** button (➡).

The enzymes can be sorted by clicking the column headings, i.e., Name, Overhang, Methylation or Popularity. This is particularly useful if you wish to use enzymes which produce a 3' overhang for example.

When looking for a specific enzyme, it is easier to use the Filter. You can type HindIII or blunt into the filter, and the list of enzymes will shrink automatically to only include respectively only the HindIII enzyme, or all enzymes producing a blunt cut.

If you need more detailed information and filtering of the enzymes, you can hover your mouse on an enzyme (see figure 23.7). You can also open a view of an enzyme list saved in the Navigation Area.



1. Please choose enzymes

Please choose enzymes

Enzyme list

☑ Use existing enzyme list: Popular enzymes

Enzymes in "Popular en..."

Filter:

Name	Overhang	Methyl...	Popularity
AccI	5' - mk	5': N6-met...	***
BclI	5' - gatc	5': N6-met...	***
ClaI			
CviAII			
DraI			
EcoRI			
EcoRV			
HincII			
HindIII			
HinfI			
HpaI			
KpnI			
MboI			
MseI			
NdeI			
PstI	3' - tgca	5': N6-met...	*****
SalI	5' - tcga	5': N6-met...	*****

Enzyme: AccI
Recognition site pattern: GTMKAC
Suppliers: GE Healthcare
Invitrogen Corporation
Qbiogene
Nippon Gene Co., Ltd.
Takara Bio Inc.
Roche Applied Science
New England Biolabs
Toyobo Biochemicals
Promega Corporation
Sigma Chemical Corporation
Bangalore Genei

New enzyme list

Filter:

Name	Overhang	Methylation	Popula...
AluI	Blunt -	5': 5-meth...	***
PvuII	Blunt -	5': N4-met...	****
HpaI	Blunt -	5': N6-met...	***
StuI	Blunt -		***
SmaI	Blunt -	5': N4-met...	*****
DraI	Blunt -	5': N6-met...	***
EcoRV	Blunt -	5': N6-met...	*****
HincII	Blunt -	5': N6-met...	***
SspI	Blunt -	5': N6-met...	***
RsaI	Blunt -	5': N4-met...	***
ScaI	Blunt -	5': N4-met...	***
HaeIII	Blunt -	5': 5-meth...	****

Figure 23.7: *Showing additional information about an enzyme like recognition sequence or a list of commercial vendors.*

At the bottom of the dialog, you can select to save the updated list of enzymes as a new file. When you click on **Finish**, the enzymes are added to the Side Panel and the cut sites are shown on the sequence. You can save the settings in the Side Panel, including the enzymes just added, as described in section 4.6).

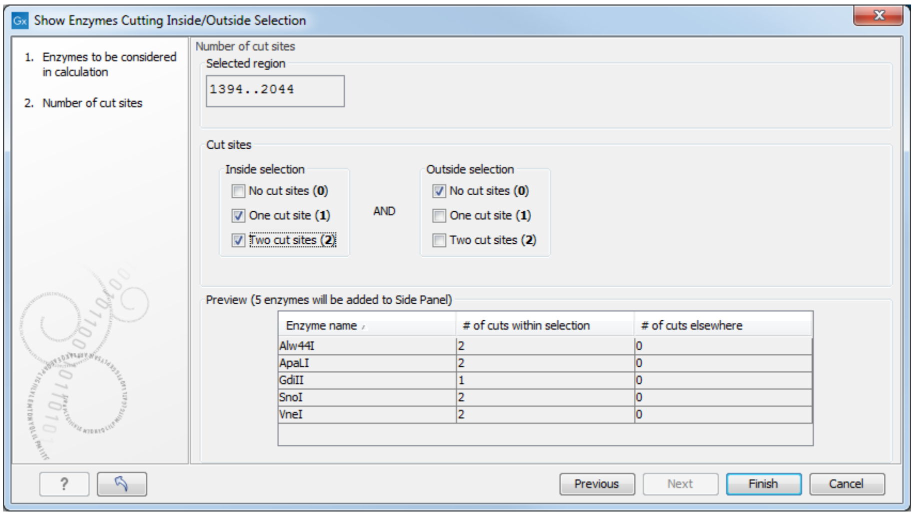
Show enzymes cutting inside/outside selection

In cases where you have a selection on a sequence, and you wish to find enzymes cutting within the selection but not outside, right-click the selection and choose the option **Show Enzymes Cutting Inside/Outside Selection** (**⊞**).

This will open a wizard where you can specify which enzymes should initially be considered (see section 23.1.1). You can for example select all the enzymes from a custom made list that correspond to all the enzymes that are already available in your lab.

In the following step (figure 23.8), you can define the terms of your search.

At the top of the dialog, you see the selected region, and below are two panels:


Show Enzymes Cutting Inside/Outside Selection
1. Enzymes to be considered in calculation
2. Number of cut sites
?
Previous
Next
Finish
Cancel

Number of cut sites

Selected region: 1394..2044

Cut sites

Inside selection
- ☐ No cut sites (0)
- ☑ One cut site (1)
- ☑ Two cut sites (2)

AND

Outside selection
- ☑ No cut sites (0)
- ☐ One cut site (1)
- ☐ Two cut sites (2)

Preview (5 enzymes will be added to Side Panel)

Enzyme name	# of cuts within selection	# of cuts elsewhere
Alw44I	2	0
ApaLI	2	0
GdiII	1	0
SnoI	2	0
VneI	2	0

Figure 23.8: *Deciding number of cut sites inside and outside the selection.*

- **Inside selection**. Specify how many times you wish the enzyme to cut inside the selection.
- **Outside selection**. Specify how many times you wish the enzyme to cut outside the selection (i.e. the rest of the sequence).

These panels offer a lot of flexibility for combining number of cut sites inside and outside the selection, respectively. To give a hint of how many enzymes will be added based on the combination of cut sites, the preview panel at the bottom lists the enzymes which will be added when you click **Finish**. Note that this list is dynamically updated when you change the number of cut sites. The enzymes shown in brackets [] are enzymes which are already present in the Side Panel.

If you have selected more than one region on the sequence (using Ctrl or ⌘), they will be treated as individual regions. This means that the criteria for cut sites apply to each region.

Show enzymes with compatible ends

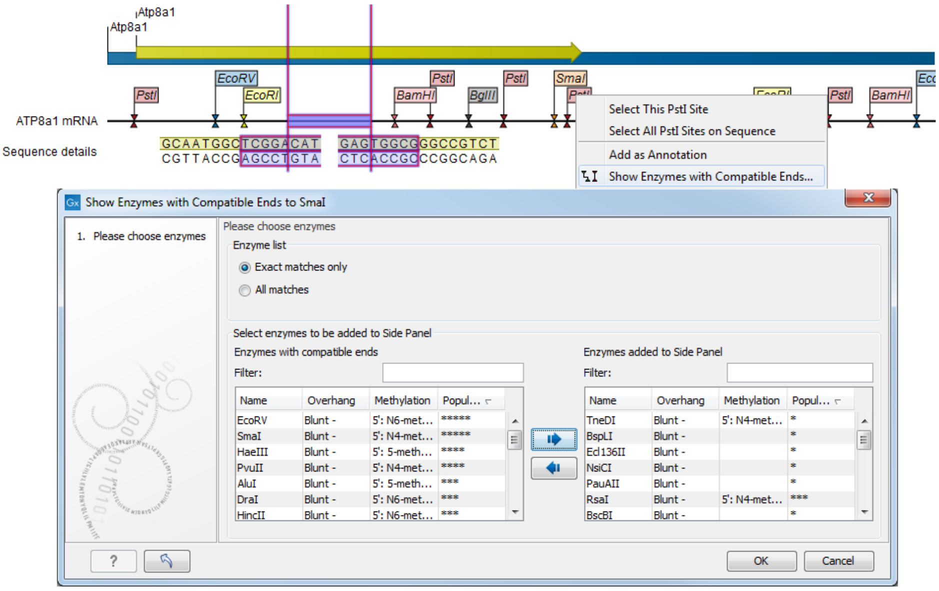
A third way of adding enzymes to the Side Panel and thereby displaying them on the sequence is based on the overhang produced by cutting with an enzyme. Right-click on a restriction site and choose to **Show Enzymes with Compatible Ends ( )** to find enzymes producing a compatible overhang (figure 23.9).

At the top you can choose whether the enzymes considered should have an exact match or not. We recommend trying **Exact match** first, and use **All matches** as an alternative if a satisfactory result cannot be achieved. Indeed, since a number of restriction enzymes have ambiguous cut patterns, there will be variations in the resulting overhangs. Choosing **All matches**, you cannot be 100% sure that the overhang will match, and you will need to inspect the sequence further afterwards.

Use the arrows between the two panels to select enzymes which will be displayed on the sequence and added to the Side Panel.

At the bottom of the dialog, the list of enzymes producing compatible overhangs is shown.

When you have added the relevant enzymes, click **Finish**, and the enzymes will be added to the



Show Enzymes with Compatible Ends to SmaI

1. Please choose enzymes

Please choose enzymes

Enzyme list

● Exact matches only

○ All matches

Select enzymes to be added to Side Panel

Enzymes with compatible ends

Filter:

Name	Overhang	Methylation	Popul...
EcoRV	Blunt -	5': N6-met...	*****
SmaI	Blunt -	5': N4-met...	*****
HaeIII	Blunt -	5': 5-meth...	****
PvuII	Blunt -	5': N4-met...	****
AluI	Blunt -	5': 5-meth...	***
DraI	Blunt -	5': N6-met...	***
HincII	Blunt -	5': N6-met...	***

Enzymes added to Side Panel

Filter:

Name	Overhang	Methylation	Popul...
TneDI	Blunt -	5': N4-met...	*
BspLI	Blunt -		*
Ecl136II	Blunt -		*
NsiCI	Blunt -		*
PauAII	Blunt -		*
RsaI	Blunt -	5': N4-met...	***
BscBI	Blunt -		*

Figure 23.9: *Enzymes with compatible ends.*

Side Panel and their cut sites displayed on the sequence.

This functionality does not work for enzymes where the cut site is located outside the recognition site.

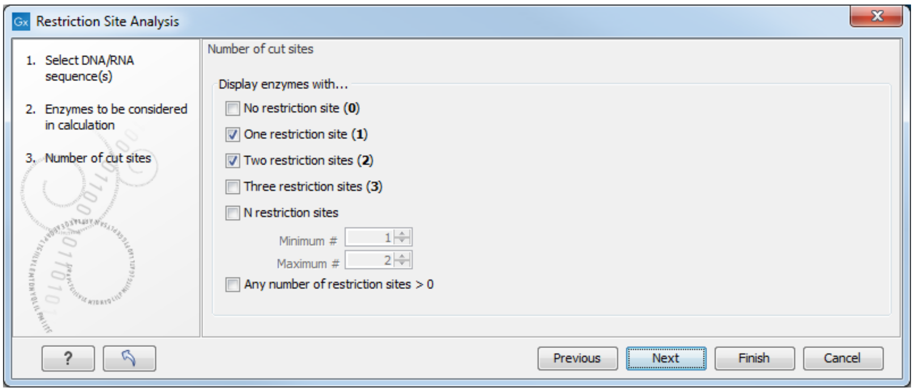
23.1.2 Restriction Site Analysis

To run the **Restriction Site Analysis** tool, go to:

Tools | **Cloning** ( )| **Restriction Site Analysis** ( )

You first specify the sequences to analyze, and in the next step, which enzymes to use. See section 23.1.1 for information about managing the restriction enzymes available.

In the next wizard step, you can limit the list of sites reported, depending on how many times they cut a sequence (figure 23.10). The default is to report enzymes that cut the sequence one or two times.



Restriction Site Analysis

1. Select DNA/RNA sequence(s)
2. Enzymes to be considered in calculation
3. Number of cut sites

Number of cut sites

Display enzymes with...

□ No restriction site (**0**)

☑ One restriction site (**1**)

☑ Two restriction sites (**2**)

□ Three restriction sites (**3**)

□ N restriction sites

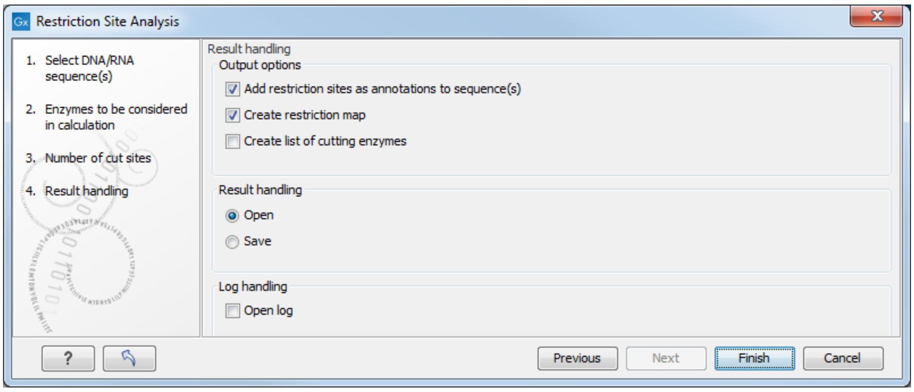
Minimum # 1

Maximum # 2

□ Any number of restriction sites > 0

Figure 23.10: *Selecting number of cut sites.*

The Result handling wizard step (figure 23.11) lets you specify how the result of the restriction map analysis should be presented.



Restriction Site Analysis

1. Select DNA/RNA sequence(s)
2. Enzymes to be considered in calculation
3. Number of cut sites
4. Result handling

Result handling

Output options

☑ Add restriction sites as annotations to sequence(s)

☑ Create restriction map

□ Create list of cutting enzymes

Result handling

● Open

○ Save

Log handling

□ Open log

Figure 23.11: *Choosing to add restriction sites as annotations or creating a restriction map.*

Add restriction sites as annotations to sequence(s) . This option makes it possible to see the restriction sites on the sequence (see figure 23.12) and save the annotations for later use.

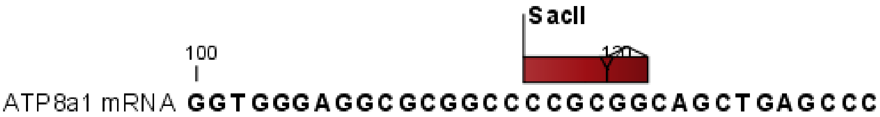
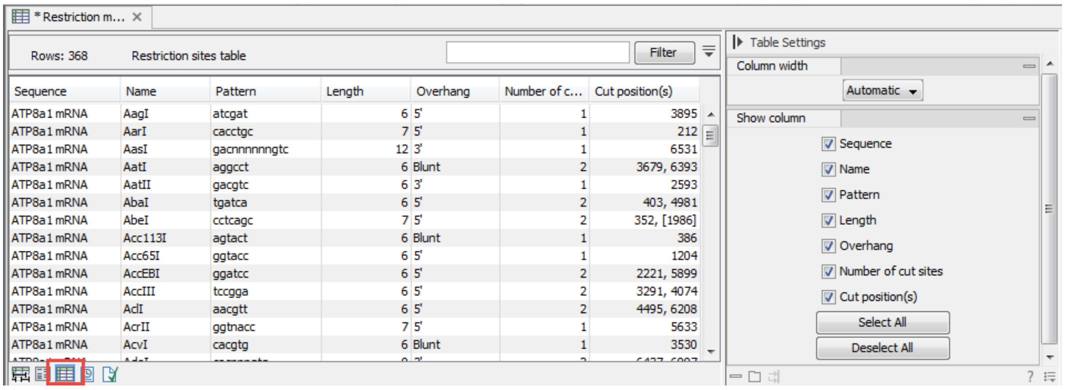


Figure 23.12: *The result of the restriction analysis shown as annotations.*

Create restriction map . When a restriction map is created, it can be shown in three different ways:

- As a **table of restriction sites** as shown in figure 23.13. If more than one sequence were selected, the table will include the restriction sites of all the sequences. This makes it easy to compare the result of the restriction map analysis for two sequences.



*Restriction m...

Rows: 368 Restriction sites table

Sequence	Name	Pattern	Length	Overhang	Number of c...	Cut position(s)
ATP8a1 mRNA	AagI	atcgat	6	5'	1	3895
ATP8a1 mRNA	AarI	cacctgc	7	5'	1	212
ATP8a1 mRNA	AasI	gacnnnnnngtc	12	3'	1	6531
ATP8a1 mRNA	AatI	aggcct	6	Blunt	2	3679, 6393
ATP8a1 mRNA	AatII	gacgtc	6	3'	1	2593
ATP8a1 mRNA	AbaI	tgatca	6	5'	2	403, 4981
ATP8a1 mRNA	AbeI	cctcagc	7	5'	2	352, [1986]
ATP8a1 mRNA	Acc113I	agtact	6	Blunt	1	386
ATP8a1 mRNA	Acc65I	ggtacc	6	5'	1	1204
ATP8a1 mRNA	AccEBI	ggatcc	6	5'	2	2221, 5899
ATP8a1 mRNA	AccIII	tccgga	6	5'	2	3291, 4074
ATP8a1 mRNA	AclI	aacgtt	6	5'	2	4495, 6208
ATP8a1 mRNA	AcrII	ggtnacc	7	5'	1	5633
ATP8a1 mRNA	AcvI	cacgtg	6	Blunt	1	3530

Table Settings

Column width: Automatic

Show column

☑ Sequence

☑ Name

☑ Pattern

☑ Length

☑ Overhang

☑ Number of cut sites

☑ Cut position(s)

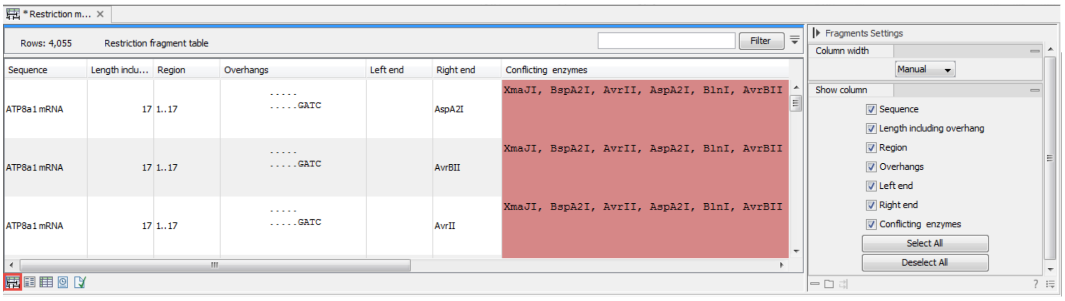
Figure 23.13: *The result of the restriction analysis shown as a table of restriction sites.*

Each row in the table represents a restriction enzyme. The following information is available for each enzyme:

- **Sequence**. The name of the sequence which is relevant if you have performed restriction map analysis on more than one sequence.

- **Name**. The name of the enzyme.
- **Pattern**. The recognition sequence of the enzyme.
- **Length**. the restriction site length.
- **Overhang**. The overhang produced by cutting with the enzyme (3', 5' or Blunt).
- **Number of cut sites**.
- **Cut position(s)**. The position of each cut.
    - [] If the enzyme's recognition sequence is on the negative strand, the cut position is put in brackets.
    - () Some enzymes cut the sequence twice for each recognition site, and in this case the two cut positions are surrounded by parentheses.

- As a **table of fragments** which shows the sequence fragments that would be the result of cutting the sequence with the selected enzymes (see figure23.14). Click the Fragments button (▦) at the bottom of the view.



Rows: 4,055 Restriction fragment table

Sequence	Length inclu...	Region	Overhangs	Left end	Right end	Conflicting enzymes
ATP8a1 mRNA	17	1..17	....GATC		AspA2I	XmaJI, BspA2I, AvrII, AspA2I, BlnI, AvrBII
ATP8a1 mRNA	17	1..17	....GATC		AvrBII	XmaJI, BspA2I, AvrII, AspA2I, BlnI, AvrBII
ATP8a1 mRNA	17	1..17	....GATC		AvrII	XmaJI, BspA2I, AvrII, AspA2I, BlnI, AvrBII

Figure 23.14: *The result of the restriction analysis shown as table of fragments.*

Each row in the table represents a fragment. If more than one enzyme cuts in the same region, or if an enzyme's recognition site is cut by another enzyme, there will be a fragment for each of the possible cut combinations. Furthermore, if this is the case, you will see the names of the other enzymes in the **Conflicting Enzymes** column.

The following information is available for each fragment.

- **Sequence**. The name of the sequence which is relevant if you have performed restriction map analysis on more than one sequence.
- **Length including overhang**. The length of the fragment. If there are overhangs of the fragment, these are included in the length (both 3' and 5' overhangs).
- **Region**. The fragment's region on the original sequence.
- **Overhangs**. If there is an overhang, this is displayed with an abbreviated version of the fragment and its overhangs. The two rows of dots (.) represent the two strands of the fragment and the overhang is visualized on each side of the dots with the residue(s) that make up the overhang. If there are only the two rows of dots, it means that there is no overhang.
- **Left end**. The enzyme that cuts the fragment to the left (5' end).
- **Right end**. The enzyme that cuts the fragment to the right (3' end).
- **Conflicting enzymes**. If more than one enzyme cuts at the same position, or if an enzyme's recognition site is cut by another enzyme, a fragment is displayed for each possible combination of cuts. At the same time, this column will display the enzymes

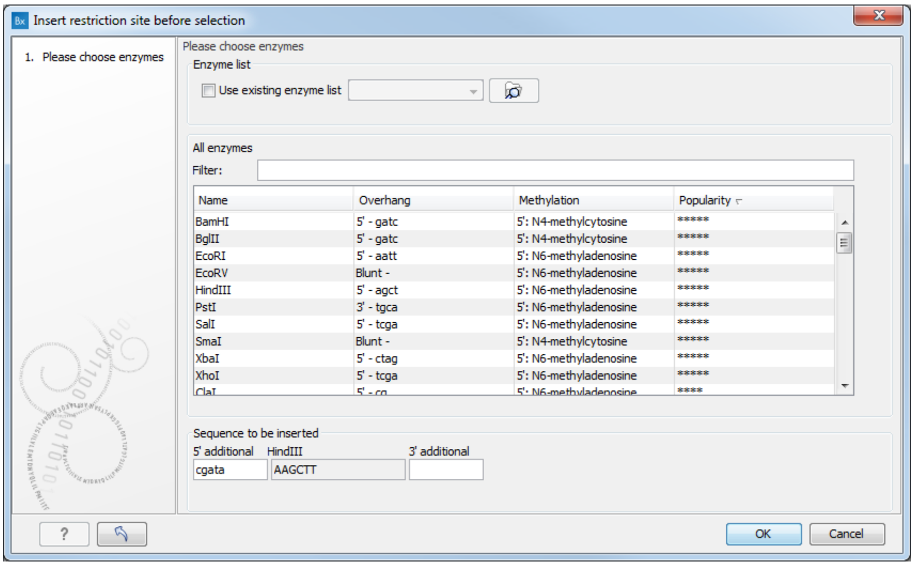
that are in conflict. If there are conflicting enzymes, they will be colored red to alert the user. If the same experiment were performed in the lab, conflicting enzymes could lead to wrong results. For this reason, this functionality is useful to simulate digestions with complex combinations of restriction enzymes.

If views of both the fragment table and the sequence are open, clicking in the fragment table will select the corresponding region on the sequence.

- As a **virtual gel** simulation which shows the fragments as bands on a gel (see figure 23.48). For more information about gel electrophoresis, see section 23.6.

23.1.3 Insert restriction site

If you right-click on a selected region of a sequence, you find this option for inserting the recognition sequence of a restriction enzyme before or after the region you selected. This will display a dialog as shown in figure 23.15.



Name	Overhang	Methylation	Popularity
BamHI	5' - gatc	5': N4-methylcytosine	*****
BglII	5' - gatc	5': N4-methylcytosine	*****
EcoRI	5' - aatt	5': N6-methyladenosine	*****
EcoRV	Blunt -	5': N6-methyladenosine	*****
HindIII	5' - agct	5': N6-methyladenosine	*****
PstI	3' - tgca	5': N6-methyladenosine	*****
SalI	5' - tcga	5': N6-methyladenosine	*****
SmaI	Blunt -	5': N4-methylcytosine	*****
XbaI	5' - ctag	5': N6-methyladenosine	*****
XhoI	5' - tcga	5': N6-methyladenosine	*****
ClaI	5' - cg	5': N6-methyladenosine	****

5' additional	HindIII	3' additional
cgata	AAGCTT	

Figure 23.15: *Inserting a restriction site and potentially a recognition sequence.*

At the top, you can select an existing enzyme list or you can use the full list of enzymes (default). Select an enzyme, and you will see its recognition sequence in the text field below the list (AAGCTT). If you wish to insert additional residues such as tags, this can be typed into the text fields adjacent to the recognition sequence.

Click **OK** will insert the restriction site and the tag(s) before or after the selection. If the enzyme selected was not already present in the list in the **Side Panel**, it will now be added and selected.

23.2 Restriction enzyme lists

CLC Main Workbench includes restriction enzymes available in the **REBASE** database, with methylation shown as performed by the cognate methylase rather than by Dam/Dcm. If you want to customize the enzyme database for your installation, see section D. However, when performing restriction site analyses, it is often an advantage to use a customized list of enzymes. In this case, the user can create special lists containing for example all enzymes available in the laboratory freezer, or all enzymes used to create a given restriction map or all enzymes that are available form the preferred vendor.

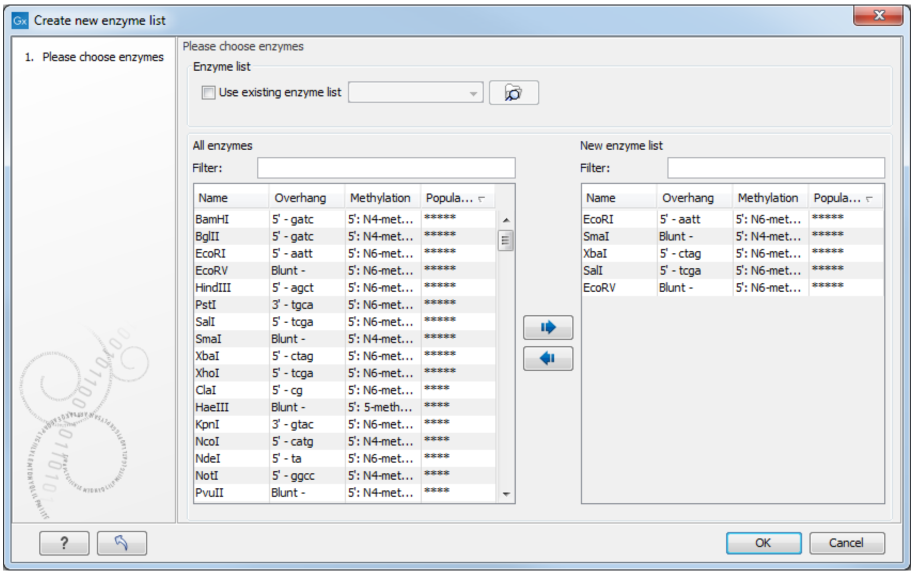
In the Example data (import in your Navigation Area using the Help menu), under Nucleotide->Restriction analysis, there are two enzyme lists: one with the 50 most popular enzymes, and another with all enzymes that are included in the *CLC Main Workbench*.

Create enzyme list *CLC Main Workbench* uses enzymes from the **REBASE** restriction enzyme database at `http://rebase.neb.com`. If you want to customize the enzyme database for your installation, see section D.

To create an enzyme list of a subset of these enzymes:

File | **New** | **Enzyme list** (▦)

This opens the dialog shown in figure 23.16



□ Use existing enzyme list

All enzymes

Filter:

Name	Overhang	Methylation	Popula...
BamHI	5' - gatc	5': N4-met...	*****
BglII	5' - gatc	5': N4-met...	*****
EcoRI	5' - aatt	5': N6-met...	*****
EcoRV	Blunt -	5': N6-met...	*****
HindIII	5' - agct	5': N6-met...	*****
PstI	3' - tgca	5': N6-met...	*****
SalI	5' - tcga	5': N6-met...	*****
SmaI	Blunt -	5': N4-met...	*****
XbaI	5' - ctag	5': N6-met...	*****
XhoI	5' - tcga	5': N6-met...	*****
ClaI	5' - cg	5': N6-met...	****
HaeIII	Blunt -	5': 5-meth...	****
KpnI	3' - gtac	5': N6-met...	****
NcoI	5' - catg	5': N4-met...	****
NdeI	5' - ta	5': N6-met...	****
NotI	5' - ggcc	5': N4-met...	****
PvuII	Blunt -	5': N4-met...	****

New enzyme list

Filter:

Name	Overhang	Methylation	Popula...
EcoRI	5' - aatt	5': N6-met...	*****
SmaI	Blunt -	5': N4-met...	*****
XbaI	5' - ctag	5': N6-met...	*****
SalI	5' - tcga	5': N6-met...	*****
EcoRV	Blunt -	5': N6-met...	*****

Figure 23.16: *Choosing enzymes for the new enzyme list.*

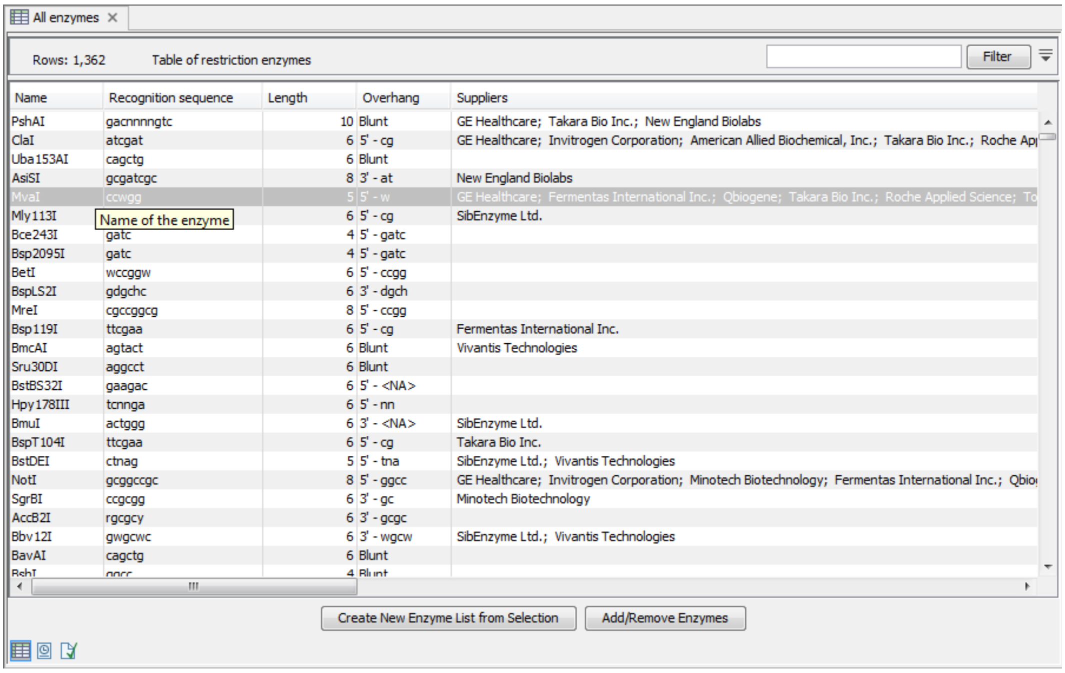
Choose which enzyme you want to include in the new enzyme list (see section 23.1.1), and click **Finish** to open the enzyme list.

View and modify enzyme list An enzyme list is shown in figure 23.17. It can be sorted by clicking the columns, and you can use the filter at the top right corner to search for specific enzymes, recognition sequences etc.

If you wish to remove or add enzymes, click the **Add/Remove Enzymes** button at the bottom of the view. This will present the same dialog as shown in figure 23.16 with the enzyme list shown to the right.

If you wish to extract a subset of an enzyme list, open the list, select the relevant enzymes, right-click on the selection and choose to **Create New Enzyme List from Selection** (▦).

If you combined this method with the filter located at the top of the view, you can extract a very specific set of enzymes. for example, if you wish to create a list of enzymes sold by a particular


All enzymes
Rows: 1,362
Table of restriction enzymes
Filter
Name of the enzyme
Create New Enzyme List from Selection
Add/Remove Enzymes

Name	Recognition sequence	Length	Overhang	Suppliers
PshAI	gacnnnngtc	10	Blunt	GE Healthcare; Takara Bio Inc.; New England Biolabs
ClaI	atcgat	6	5' - cg	GE Healthcare; Invitrogen Corporation; American Allied Biochemical, Inc.; Takara Bio Inc.; Roche Ap
Uba153AI	cagctg	6	Blunt	
AsiSI	gcgatcgc	8	3' - at	New England Biolabs
MvaI	ccwgg	5	5' - w	GE Healthcare; Fermentas International Inc.; Qbiogene; Takara Bio Inc.; Roche Applied Science; To
Mly113I		6	5' - cg	SibEnzyme Ltd.
Bce243I	gatc	4	5' - gatc	
Bsp2095I	gatc	4	5' - gatc	
BetI	wccggw	6	5' - ccgg	
BspLS2I	gdgchc	6	3' - dgch	
MreI	cgccggcg	8	5' - ccgg	
Bsp119I	ttcgaa	6	5' - cg	Fermentas International Inc.
BmcAI	agtact	6	Blunt	Vivantis Technologies
Sru30DI	aggcct	6	Blunt	
BstBS32I	gaagac	6	5' - <NA>	
Hpy178III	tcnnga	6	5' - nn	
BmuI	actggg	6	3' - <NA>	SibEnzyme Ltd.
BspT104I	ttcgaa	6	5' - cg	Takara Bio Inc.
BstDEI	ctnag	5	5' - tna	SibEnzyme Ltd.; Vivantis Technologies
NotI	gcggccgc	8	5' - ggcc	GE Healthcare; Invitrogen Corporation; Minotech Biotechnology; Fermentas International Inc.; Qbio
SgrBI	ccgcgg	6	3' - gc	Minotech Biotechnology
AccB2I	rgcgcy	6	3' - gcgc	
Bbv12I	gwgcwc	6	3' - wgcw	SibEnzyme Ltd.; Vivantis Technologies
BavAI	cagctg	6	Blunt	
BshI	ggcc	4	Blunt	

Figure 23.17: *An enzyme list.*

distributor, type the name of the distributor into the filter and select and create a new enzyme list from the selection.

23.3 Restriction Based Cloning

The *in silico* cloning process in *CLC Main Workbench* begins with the Restriction Based Cloning tool:

Cloning (🖼)| **Restriction Based Cloning** (🖼)

This will open a dialog where you can select both the sequences containing the fragments you want to clone, as well as the one to be used as vector (figure 23.18).

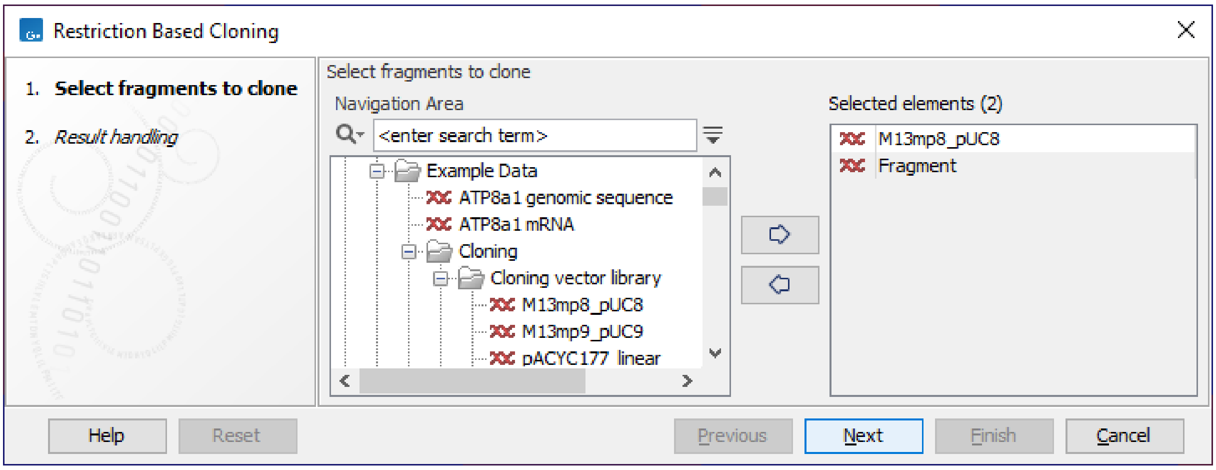
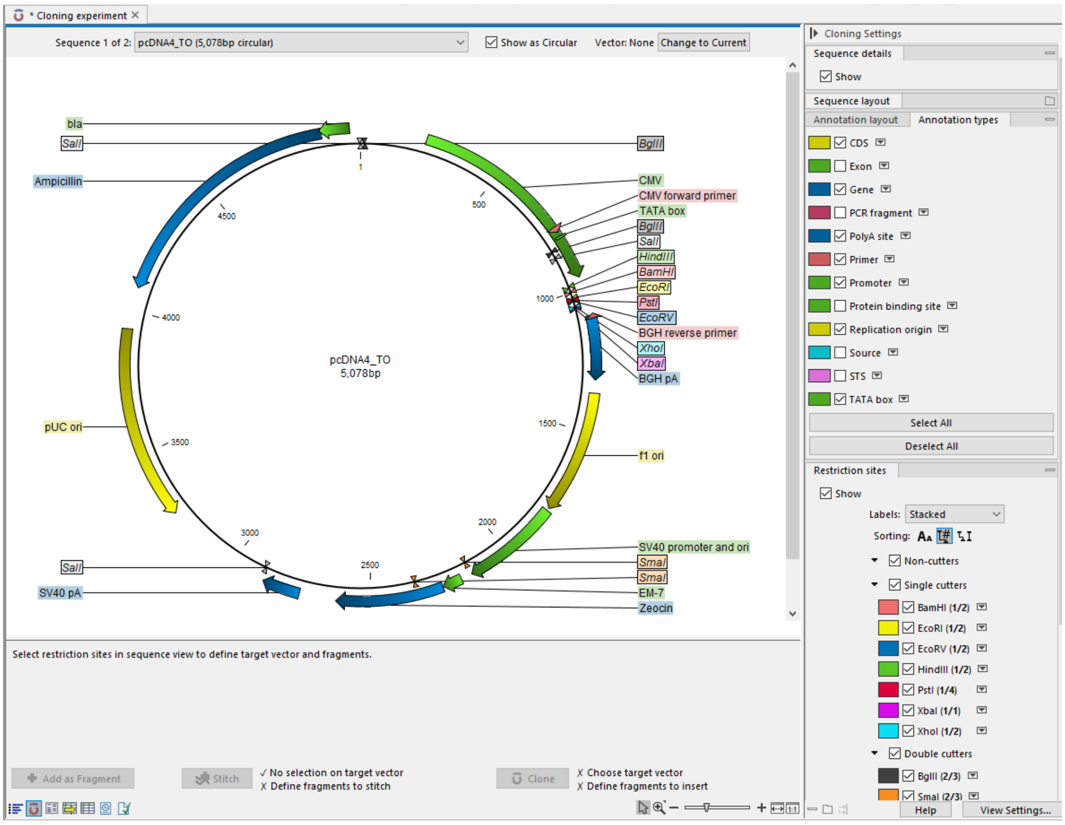


Figure 23.18: *Selecting the sequences containing the fragments you want to clone and the vector.*

CLC Main Workbench will now create a sequence list of the selected fragments and vector sequences. For cloning work, open the sequence list and switch to the **Cloning Editor** ( ) at the bottom of the view (figure 23.19).



Sequence 1 of 2: pcDNA4_TO (5,078bp circular) ☑ Show as Circular Vector: None

Select restriction sites in sequence view to define target vector and fragments.

✓ No selection on target vector
X Define fragments to stitch

X Choose target vector
X Define fragments to insert

Sequence details: ☑ Show

Annotation types: ☑ CDS, □ Exon, ☑ Gene, □ PCR fragment, ☑ PolyA site, ☑ Primer, ☑ Promoter, □ Protein binding site, ☑ Replication origin, □ Source, □ STS, ☑ TATA box

Restriction sites: ☑ Show

Labels: Stacked

Sorting:

☑ Non-cutters

☑ Single cutters: ☑ BamHI (1/2), ☑ EcoRI (1/2), ☑ EcoRV (1/2), ☑ HindIII (1/2), ☑ PstI (1/4), ☑ XbaI (1/1), ☑ XhoI (1/2)

☑ Double cutters: ☑ BglII (2/3), ☑ SmaI (2/3)

Figure 23.19: *Cloning editor view of the sequence list. Choose which sequence to display from the drop down menu.*

If you later in the process need additional sequences, right-click anywhere on the empty white area of the view and choose to "Add Sequences".

23.3.1 Introduction to the Cloning Editor

In the Cloning Editor, most of the basic options for viewing, selecting and zooming the sequences are the same as for the standard sequence view (section 14.2). In particular, this means that annotations can be displayed on the sequences to guide the choice of regions to clone.

However, the Cloning Editor has a special layout with three distinct areas (in addition to the **Side Panel** found in other sequence views as well):

- At the top, there is a panel to switch between the sequences selected as input for the cloning. You can also specify whether the sequence should be visualized **as circular** or as a fragment. On the right-hand side, you can select a vector: the button is by default set to **Change to Current**. Click on it to select the currently shown sequence as **vector**.
- In the middle, the selected sequence is shown. This is the central area for defining how the cloning should be performed.
- At the bottom, there is a panel where the selection of fragments and target vector is performed.

The Cloning Editor can be opened in the following ways:

- Click on the **Cloning Editor** icon ( ) in the view area when a sequence list has been opened in the sequence list editor.
- Create a new cloning experiment using the **Restriction Based Cloning** ( ) action from the toolbox. This tool collects a set of existing sequences and creates a new sequence list.

The Cloning Editor can be used in two ways:

- **Cloning mode** Opened when one of the sequences has been selected as 'Vector'. In this mode, you can apply one or more cuts to the vector, thereby creating an opening for insertion of other sequence fragments. From the remaining sequences in the cloning experiment/sequence list, either complete sequences or fragments created by cutting can be inserted into the vector. In the cloning adapter dialog, the order and direction of the inserted fragments can be adjusted prior to adjusting the overhangs to match the cloning conditions.
- **Stitch mode** If no sequence has ben selected as 'Vector', a number of fragments (either full sequences or cuttings) can be selected from the cloning experiment. These can then be stitched together into a single new sequence. In the stitching adapter dialog, the order and direction of the fragments can be adjusted prior to adjusting the overhangs to match the stitch conditions.

23.3.2 The restriction cloning workflow

The restriction cloning workflow consists of the following steps:

1. **Define one or more fragments**

   First, select the sequence containing the cloning fragment in the list at the top of the view. Next, make sure the restriction enzyme you wish to use is listed in the **Side Panel** (see section 23.1.1). To specify which part of the sequence should be treated as the fragment, first click one of the cut sites you wish to use. Then press and hold the Ctrl key (⌘ on Mac) while you click the second cut site. You can also right-click the cut sites and use the **Select This ... Site** to select a site. If you just wish to remove the selection of one of the sites, right-click the site on the sequence and choose **De-select This ... Site**.

   When this is done, the panel is updated to reflect the selections (see figure 23.20).

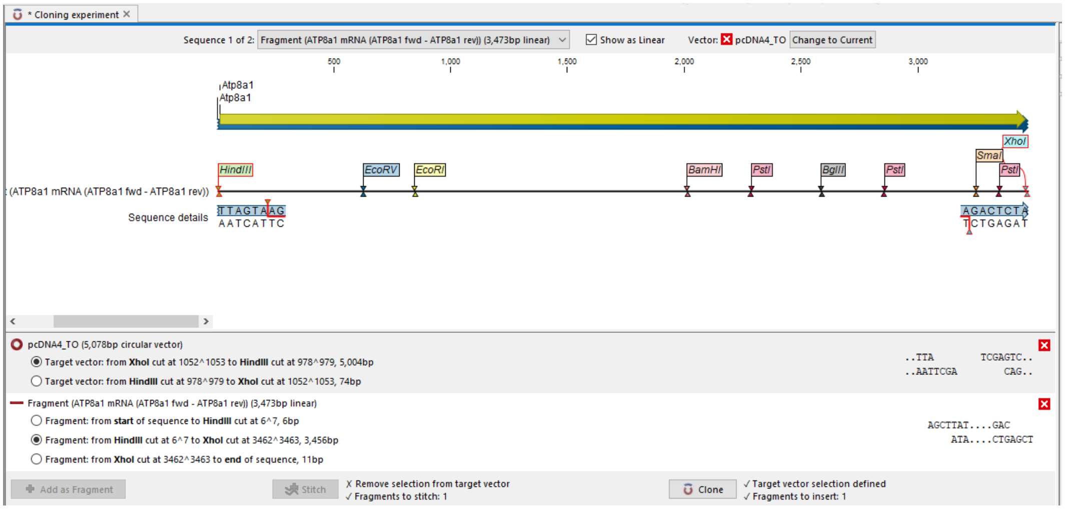
   In this example you can see that there are now three fragments that can be used for cloning listed in the panel below the view. The fragment selected per default is the one that is in between the cut sites selected.

   If the entire sequence should be selected as fragment, click **Add as Fragment** (✚).

   At any time, the selection of cut sites can be cleared by clicking the **Remove** (☒) icon to the right of the target vector selections.

2. **Defining target vector**

   The next step is to define where the vector should be cut. If the vector sequence should just be opened, click the restriction site you want to use for opening (figure 23.21).



Sequence 1 of 2: Fragment (ATP8a1 mRNA (ATP8a1 fwd - ATP8a1 rev)) (3,473bp linear) ☑ Show as Linear Vector: ☒ pcDNA4_TO Change to Current

pcDNA4_TO (5,078bp circular vector)

● Target vector: from **XhoI** cut at 1052^1053 to **HindIII** cut at 978^979, 5,004bp

○ Target vector: from **HindIII** cut at 978^979 to **XhoI** cut at 1052^1053, 74bp

```
..TTA        TCGAGTC..
..AATTCGA        CAG..
```

Fragment (ATP8a1 mRNA (ATP8a1 fwd - ATP8a1 rev)) (3,473bp linear)

○ Fragment: from **start** of sequence to **HindIII** cut at 6^7, 6bp

● Fragment: from **HindIII** cut at 6^7 to **XhoI** cut at 3462^3463, 3,456bp

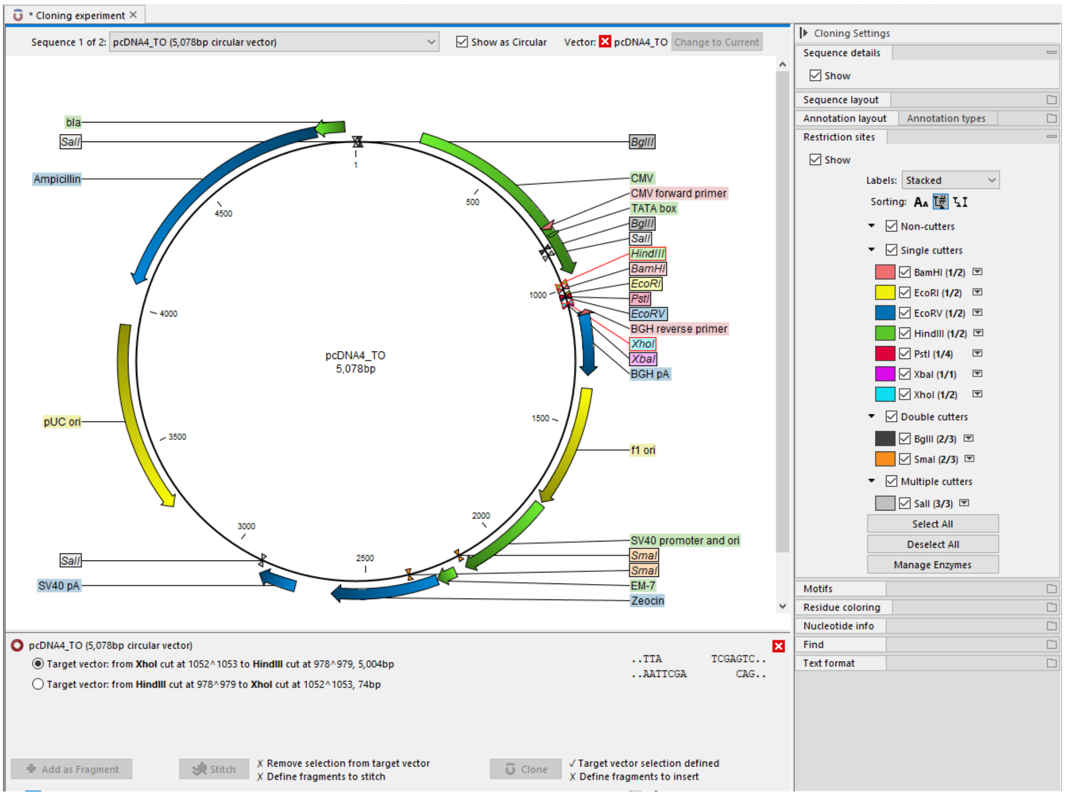
○ Fragment: from **XhoI** cut at 3462^3463 to **end** of sequence, 11bp

```
AGCTTAT....GAC
    ATA....CTGAGCT
```

X Remove selection from target vector
✓ Fragments to stitch: 1

✓ Target vector selection defined
✓ Fragments to insert: 1

Figure 23.20: *EcoRI cut sites selected to cut out fragment.*



Sequence 1 of 2: pcDNA4_TO (5,078bp circular vector) ☑ Show as Circular Vector: ☒ pcDNA4_TO Change to Current

Sequence details: ☑ Show

Restriction sites: ☑ Show

Labels: Stacked

Sorting: Aa # ↕I

☑ Non-cutters

☑ Single cutters

- 🟥 ☑ BamHI (1/2)
- 🟨 ☑ EcoRI (1/2)
- 🟦 ☑ EcoRV (1/2)
- 🟩 ☑ HindIII (1/2)
- 🟥 ☑ PstI (1/4)
- 🟪 ☑ XbaI (1/1)
- 🟦 ☑ XhoI (1/2)

☑ Double cutters

- ⬛ ☑ BglII (2/3)
- 🟧 ☑ SmaI (2/3)

☑ Multiple cutters

- ⬜ ☑ SalI (3/3)

pcDNA4_TO (5,078bp circular vector)

● Target vector: from **XhoI** cut at 1052^1053 to **HindIII** cut at 978^979, 5,004bp

○ Target vector: from **HindIII** cut at 978^979 to **XhoI** cut at 1052^1053, 74bp

```
..TTA        TCGAGTC..
..AATTCGA        CAG..
```

X Remove selection from target vector
X Define fragments to stitch

✓ Target vector selection defined
X Define fragments to insert

Figure 23.21: *EcoRI site used to open the vector. Note that the "Cloning" button has now been enabled as both criteria ("Target vector selection defined" and "Fragments to insert:...") have been defined.*

If you want to cut off part of the vector, click two restriction sites while pressing the Ctrl key (⌘ on Mac). You can also right-click the cut sites and use the **Select This ... Site** to select a site. This will display two options for what the target vector should be (for linear vectors there would have been three option). At any time, the selection of cut sites can be cleared by clicking the **Remove** (☒) icon to the right of the target vector selections.

3. **Perform cloning**

Once both fragments and vector are selected, click **Clone** ( ). This will display a dialog to adapt overhangs and change orientation as shown in figure 23.22.

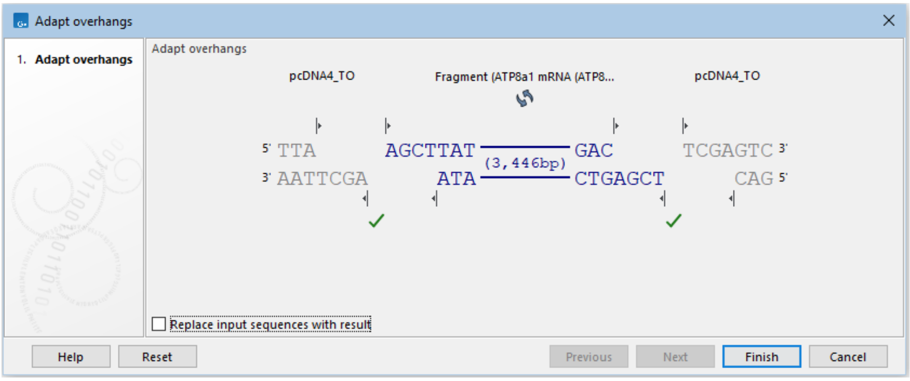


Figure 23.22: *Showing the insertion point of the vector.*

This dialog visualizes the details of the insertion. The vector sequence is on each side shown in a faded gray color. In the middle the fragment is displayed. If the overhangs of the sequence and the vector do not match (⊘), you will not be able to click **Finish**. But you can blunt end or fill in the overhangs using the **drag handles** ( ) until the overhangs match (✓).

The fragment can be reverse complemented by clicking the **Reverse complement fragment** ( ).

When several fragments are used, the order of the fragments can be changed by clicking the move buttons (➡)/ (⬅).

Per default, the construct will be opened in a new view and can be saved separately. But selecting the option **Replace input sequences with result** will add the construct to the input sequence list and delete the original fragment and vector sequences.

Note that the cloning experiment used to design the construct can be saved as well. If you check the **History** ( ) of the construct, you can see the details about restriction sites and fragments used for the cloning.

23.3.3 Manual cloning

Cloning steps can be done manually. For this, create a sequence list using **Restriction Based Cloning** and use the cloning actions provided via right-click menus. The general workflow is the same as that described in section 23.3.2, but carried out manually.

Manipulate the whole sequence

Right-click the sequence label to the left to see the menu shown in figure 23.23.

- **Duplicate sequence**. Adds a duplicate of the selected sequence to the sequence list accessible from the drop down menu on top of the Cloning view.
- **Insert sequence after this sequence** ( ). The sequence to be inserted can be selected from the sequence list via the drop down menu on top of the Cloning view. The inserted

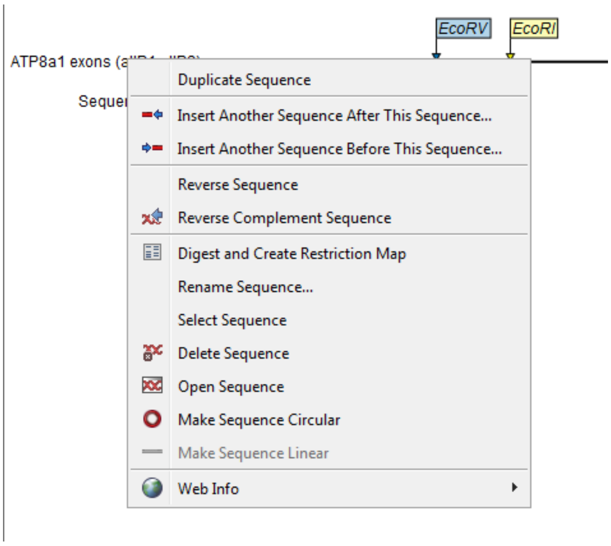


Figure 23.23: *Right click on the sequence in the cloning view.*

sequence remains on the list of sequences. If the two sequences do not have blunt ends, the ends' overhangs have to match each other.

- **Insert sequence before this sequence ()**. The sequence to be inserted can be selected from the sequence list via the drop down menu on top of the Cloning view. The inserted sequence remains on the list of sequences. If the two sequences do not have blunt ends, the ends' overhangs have to match each other.
- **Reverse sequence**. Reverses the sequence and replaces the original sequence in the list. This is sometimes useful when working with single stranded sequences. Note that this is *not* the same as creating the reverse *complement* of a sequence.
- **Reverse complement sequence ()**. Creates the reverse complement of a sequence and replaces the original sequence in the list. This is useful if the vector and the insert sequences are not oriented the same way.
- **Digest and Create Restriction Map ()**. See section 23.6
- **Rename sequence**. Renames the sequence.
- **Select sequence**. Selects the entire sequence.
- **Delete sequence ()**. Deletes the given sequence from the Cloning Editor.
- **Open sequence ()**. Opens the selected sequence in a normal sequence view.
- **Make sequence circular** (○). Converts a sequence from a linear to a circular form. If the sequence have matching overhangs at the ends, they will be merged together. If the sequence have incompatible overhangs, a dialog is displayed, and the sequence cannot be made circular. The circular form is represented by >> and << at the ends of the sequence.
- **Make sequence linear (—)**. Converts a sequence from a circular to a linear form, removing the << and >> at the ends.

Manipulate parts of the sequence

Right-click on a selected region of the sequence to see the menu shown in figure 23.24.

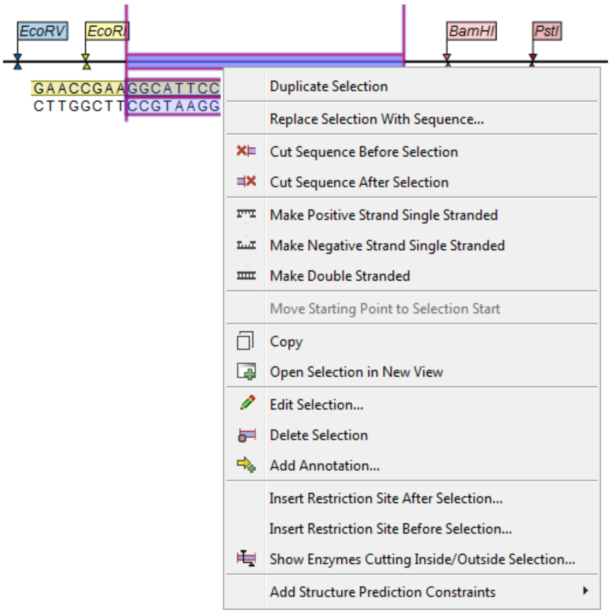

EcoRV
EcoRI
BamHI
PstI
GAACCGAAGGCATTCC
CTTGGCTTCCGTAAGG
Duplicate Selection
Replace Selection With Sequence...
Cut Sequence Before Selection
Cut Sequence After Selection
Make Positive Strand Single Stranded
Make Negative Strand Single Stranded
Make Double Stranded
Move Starting Point to Selection Start
Copy
Open Selection in New View
Edit Selection...
Delete Selection
Add Annotation...
Insert Restriction Site After Selection...
Insert Restriction Site Before Selection...
Show Enzymes Cutting Inside/Outside Selection...
Add Structure Prediction Constraints

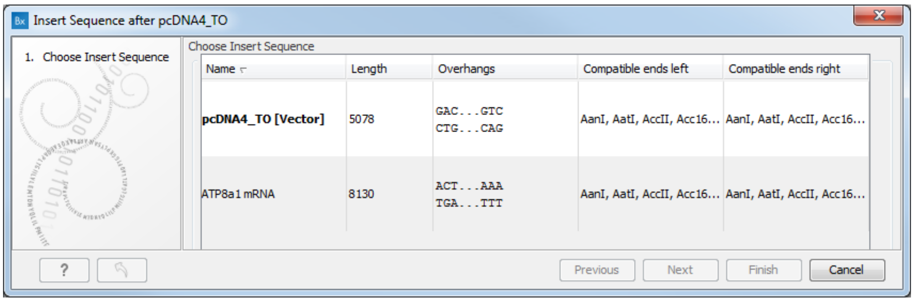
Figure 23.24: *Right click on a sequence selection in the cloning view.*

- **Duplicate Selection.** If a selection on the sequence is duplicated, the selected region will be added as a new sequence to the Cloning Editor. The new sequence name representing the length of the fragment. When double-clicking on a sequence, the region between the two closest restriction sites is automatically selected.
- **Replace Selection with sequence.** Replaces the selected region with a sequence selected from the drop down menu listing all sequences in the Cloning Editor.
- **Cut Sequence Before Selection ().** Cleaves the sequence before the selection and will result in two smaller fragments.
- **Cut Sequence After Selection ().** Cleaves the sequence after the selection and will result in two smaller fragments.
- **Make Positive Strand Single Stranded ().** Makes the positive strand of the selected region single stranded.
- **Make Negative Strand Single Stranded ().** Makes the negative strand of the selected region single stranded.
- **Make Double Stranded ().** This will make the selected region double stranded.
- **Move Starting Point to Selection Start.** This is only active for circular sequences. It will move the starting point of the sequence to the beginning of the selection.
- **Copy ().** Copies the selected region to the clipboard, which will enable it for use in other programs.
- **Open Selection in New View ().** Opens the selected region in the normal sequence view.

- **Edit Selection ( ).** Opens a dialog box in which is it possible to edit the selected residues.
- **Delete Selection ( ).** Deletes the selected region of the sequence.
- **Add Annotation ( ).** Opens the **Add annotation** dialog box.
- **Insert Restriction Sites After/Before Selection.** Shows a dialog where you can choose from a list restriction enzymes (see section 23.1.3).
- **Show Enzymes Cutting Inside/Outside Selection ( ).** Adds enzymes cutting this selection to the Side Panel.
- **Add Structure Prediction Constraints.** This is relevant for RNA secondary structure prediction:
  - **Force Stem Here** is activated after choosing 2 regions of equal length on the sequence. It will add an annotation labeled "Forced Stem" and will force the algorithm to compute minimum free energy and structure with a stem in the selected region.
  - **Prohibit Stem Here** is activated after choosing 2 regions of equal length on the sequence. It will add an annotation labeled "Prohibited Stem" to the sequence and will force the algorithm to compute minimum free energy and structure without a stem in the selected region.
  - **Prohibit From Forming Base Pairs** will add an annotation labeled "No base pairs" to the sequence, and will force the algorithm to compute minimum free energy and structure without a base pair containing any residues in the selected region.

Insert one sequence into another

Sequences can be inserted into each other in various ways as described in the lists above. When you choose to insert one sequence into another, you will be presented with a dialog where all sequences in the sequence list are present (see figure 23.25).

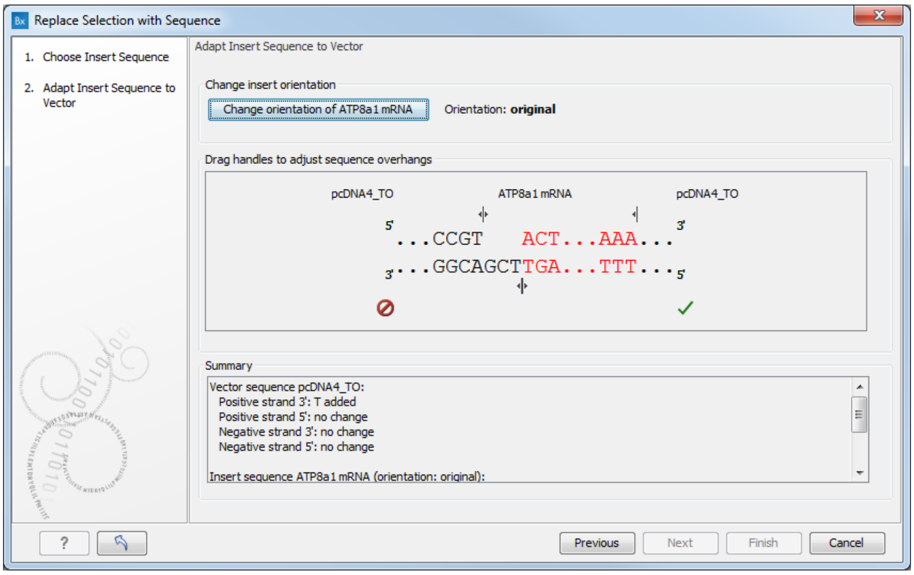


Name	Length	Overhangs	Compatible ends left	Compatible ends right
pcDNA4_TO [Vector]	5078	GAC...GTC CTG...CAG	AanI, AatI, AccII, Acc16...	AanI, AatI, AccII, Acc16...
ATP8a1 mRNA	8130	ACT...AAA TGA...TTT	AanI, AatI, AccII, Acc16...	AanI, AatI, AccII, Acc16...

Figure 23.25: *Select a sequence for insertion.*

The sequence that you have chosen to insert into will be marked with **bold** and the text **[vector]** is appended to the sequence name. Note that this is completely unrelated to the vector concept in the cloning workflow described in section 23.3.2.

Furthermore, the list includes the length of the fragment, an indication of the overhangs, and a list of enzymes that are compatible with this overhang (for the left and right ends, respectively). If not all the enzymes can be shown, place your mouse cursor on the enzymes, and a full list will be shown in the tool tip.



Replace Selection with Sequence

1. Choose Insert Sequence
2. Adapt Insert Sequence to Vector

Adapt Insert Sequence to Vector

Change insert orientation

Change orientation of ATP8a1 mRNA   Orientation: **original**

Drag handles to adjust sequence overhangs

Summary

Vector sequence pcDNA4_TO:
Positive strand 3': T added
Positive strand 5': no change
Negative strand 3': no change
Negative strand 5': no change

Insert sequence ATP8a1 mRNA (orientation: original):

Figure 23.26: *Drag the handles to adjust overhangs.*

Select the sequence you wish to insert and click **Next** to adapt insert sequence to vector dialog (figure 23.26).

At the top is a button to reverse complement the inserted sequence.

Below is a visualization of the insertion details. The inserted sequence is at the middle shown in red, and the vector has been split at the insertion point and the ends are shown at each side of the inserted sequence.

If the overhangs of the sequence and the vector do not match (⊘), you can blunt end or fill in the overhangs using the **drag handles** (⍿) until it does (✓).

At the bottom of the dialog is a summary field which records all the changes made to the overhangs. This contents of the summary will also be written in the history (🕓) of the cloning experiment.

When you click **Finish**, the sequence is inserted and highlighted by being selected.

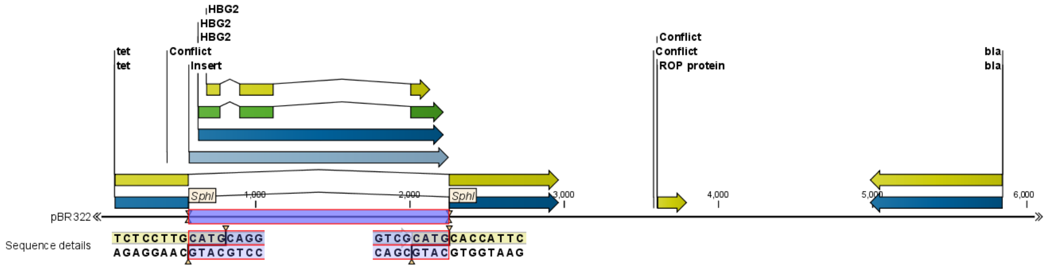


Figure 23.27: *One sequence is now inserted into the cloning vector. The sequence inserted is automatically selected.*

23.4 Homology Based Cloning

Using **Homology Based Cloning**, cloning experiments can be designed for methods such as Takara In-Fusion® and other ligation independent cloning techniques and Gibson Assembly®, where sequences with homologous ends need to be assembled.

The tool generates primers with overhangs so that appropriate homologous sequence is added to fragments that should be assembled. Subsequently, primers and overhangs can be inspected and adjusted.

Running the Homology Based Cloning tool

To run the tool, go to:

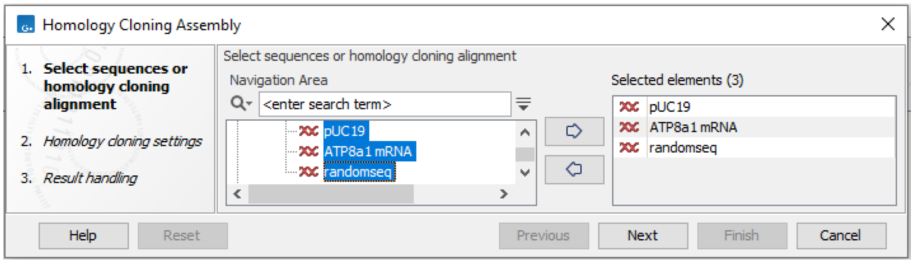
Cloning ()| Homology Based Cloning ()

In the first dialog, select the vector and all the DNA fragments that should be assembled in the cloning reaction (figure 23.28).

The first sequence used as input, will be considered the vector, but the order of sequences can be adjusted in the following wizard step.

You can select 1-50 individual sequences with a maximal combined length of 100,000 base pairs as input to the tool. However, the number of sequences selected should be in accordance with the relevant laboratory protocol.

Note that it is also possible to use an assembled vector that was previously generated by **Homology Based Cloning** as input. In that case, previously designed primers and overhangs will be shown in the following wizard step.



Homology Cloning Assembly

1. **Select sequences or homology cloning alignment**
2. *Homology cloning settings*
3. *Result handling*

Select sequences or homology cloning alignment

Navigation Area

<enter search term>

- pUC19
- ATP8a1 mRNA
- randomseq

Selected elements (3)

- pUC19
- ATP8a1 mRNA
- randomseq

Figure 23.28: *Select the vector and fragments that should be assembled in the homology based cloning reaction.*

Press **Next** to open the wizard allowing you to inspect and adjust primers and overhangs.

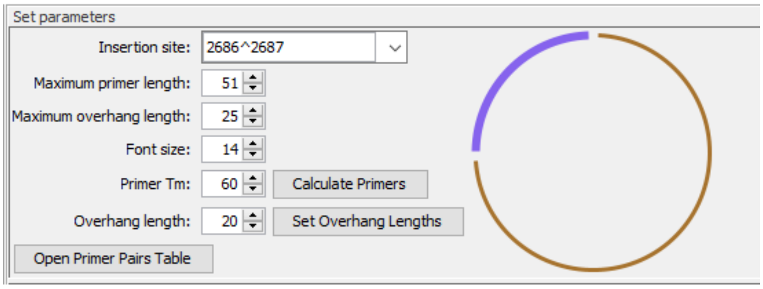
23.4.1 Working with homology based cloning

This section gives a quick overview of the Homology Based Cloning wizard. For a full description, see section 23.4.4.

General options

General options are at the top of the wizard. These include the position of the insertion site in the vector, the maximum primer and overhang lengths as well as option to set the Tm and overhang length for all primers at once. There is also a diagram of the vector including the inserts, where each sequence has a different colour (figure 23.29).

Sequences



Insertion site: 2686^2687

Maximum primer length: 51

Maximum overhang length: 25

Font size: 14

Primer Tm: 60

Overhang length: 20

Figure 23.29: *The top section of the wizard contains general options.*

Each sequence is displayed individually, with a coloured bar to the left and a vertical scroll bar at the bottom. The top sequence is the vector, with the insert sequences displayed further down.

The order of the sequences reflects how they will be assembled into the vector, and the overhangs on the primers support this assembly order.

Vector, inserts, primers and overhangs are color coded (figure 23.30):

- **Black** Vector or insert sequence
- **Grey** Vector or insert sequence not included in the PCR product
- **Brown** Primer sequence
- **Pink** Overhang sequence
- **Blue** Added bases that are inserted between primer and overhang

For each sequence, you can adjust primer and overhang lengths and add bases between primers and overhangs.

The vector sequence is considered circular and primers are depicted as pointing away from each other in order to amplify the circular sequence. Inserts are considered linear, and primers are placed at the ends of the insert sequence pointing towards each other in order to amplify the linear sequence (figure 23.30).

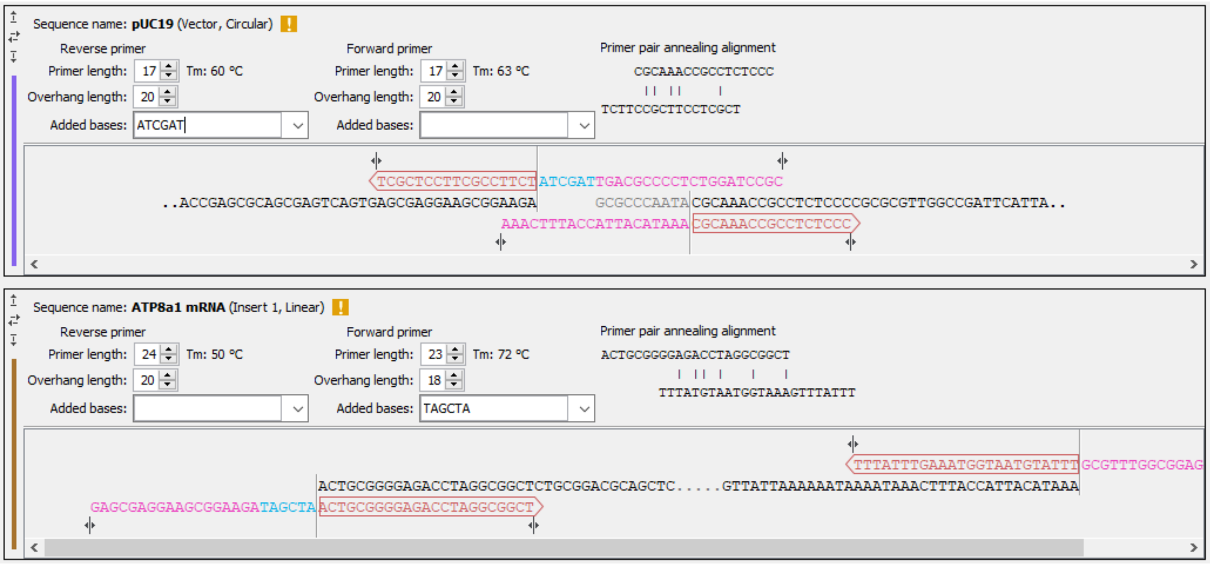
23.4.2 Adjust the homology based cloning design

This section describes how primers and overhangs can be adjusted and inspected.

The overhangs added by **Homology Based Cloning** are 20 base pairs long and are added to vector or insert primers:

- If one insert is assembled into a vector < 8 kb in length, overhangs are added to the vector primers.
- If one insert is assembled into a vector > 8 kb in length, overhangs are added to the insert primers.
- If more inserts are assembled into a vector, the overhangs are added to insert primers.

Primer and overhang lengths should be adjusted, according to the cloning kit used.



Sequence name: **pUC19** (Vector, Circular)

Reverse primer
Primer length: 17 Tm: 60 °C
Overhang length: 20
Added bases: ATCGAT

Forward primer
Primer length: 17 Tm: 63 °C
Overhang length: 20
Added bases:

Primer pair annealing alignment

```
CGCAAACCGCCTCTCCC
 || ||     |
TCTTCCGCTTCCTCGCT
```

```
                    TCGCTCCTTCGCCTTCTATCGATTGACGCCCCTCTGGATCCGC
..ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGA    GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTA..
                                     AAACTTTACCATTACATAAACGCAAACCGCCTCTCCC
```

Sequence name: **ATP8a1 mRNA** (Insert 1, Linear)

Reverse primer
Primer length: 24 Tm: 50 °C
Overhang length: 20
Added bases:

Forward primer
Primer length: 23 Tm: 72 °C
Overhang length: 18
Added bases: TAGCTA

Primer pair annealing alignment

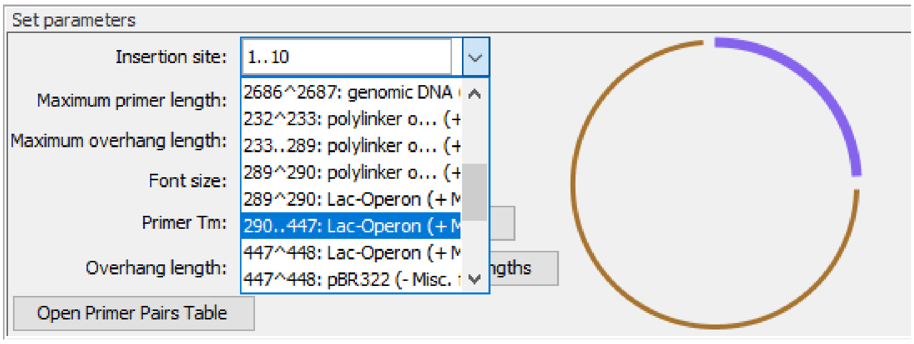
```
ACTGCGGGGAGACCTAGGCGGCT
     | || |   |   |
   TTTATGTAATGGTAAAGTTTATTT
```

```
                                                                               TTTATTTGAAATGGTAATGTATTTGCGTTTGGCGGAG
                        ACTGCGGGGAGACCTAGGCGGCTCTGCGGACGCAGCTC.....GTTATTAAAAAATAAAATAAACTTTACCATTACATAAA
GAGCGAGGAAGCGGAAGATAGCTAACTGCGGGGAGACCTAGGCGGCT
```

Figure 23.30: *Top: The vector sequence and primers with overhangs. The grey sequence between the primers is not included in the PCR product. Bottom: An insert sequence and primers with overhangs.*

Change the insertion site

Change the insertion site in the vector by typing a position or a range of positions directly into **Insertion site** text field. Alternatively, choose the start, end or complete range of an annotation from the drop down menu (figure 23.31). If you specify a range of bases, primers will be placed so that bases are replaced by the insert(s).



Set parameters

Insertion site: 1..10
Maximum primer length:
Maximum overhang length:
Font size:
Primer Tm:
Overhang length:

Figure 23.31: *Choose an insertion site from the drop down menu or type position(s) directly in the Insertion site text field.*

Change the assembly order

Use the arrows to the left of the sequence names to move a sequence up or down in the order. The sequence of primer overhangs is automatically updated to reflect the new order. The length of overhangs is not changed.

Adjust the length of primers and overhangs

You can adjust primers and overhangs using the following options:

- To adjust all primers at once, change the **Primer Tm** in the top section and press **Calculate primers** (figure 23.29). This will update primers on all sequences.
- To adjust all overhangs at once, change the **Overhang length** in the top section and press **Set Overhang Lengths** (figure 23.29). This will update overhangs on all sequences.
- To adjust the length of individual primers and overhangs use the **Primer length** and **Overhang length** options available for the forward and reverse primer on each sequence. You can also extend or shorten the the primer and overhang sequences by dragging the **arrow symbols** at the ends of the primers and overhangs (figure 23.30).

Insert additional bases

Insert additional bases between the primer and the overhang by typing directly in to the **Added bases** text fields. You can also choose the sequence of a restriction site from the drop down menu.

Inspect primer pairs

The designed primer pairs can be closely inspected in the table that opens when pressing **Open Primer Pairs Table**. The table contains information about secondary structure for each primer pair, both with and without overhang. For a description of each of the columns in the Primer Pairs table see section 22.5.3. Close the table before returning to the wizard to make further adjustments or complete the design.

The outputs created by **Homology Based Cloning** are described in section 23.4.3.

23.4.3 Homology Based Cloning outputs

Homology Based Cloning can create the following outputs:

- **Report** A report containing the following sections:
  - **Summary** Contains the number of fragments and primers used in the cloning reaction as well as their lengths and any warnings.
  - **Fragments** Lists the vector and fragments used in the cloning reaction.
  - **Warnings** Lists the warnings given for primer pairs.
  - **Primer pairs** Lists fragments for which primers were designed together with pair annealing and pair end annealign values for the primer pairs. See section 22.5.3 for information about annealing values.
  - **Primers** Lists individual primers and their sequence. Primer sequence is written with capital letters, whereas added bases and overhangs are in lowercase.
  - **Primer parts** Lists full and subparts of designed primers with characteristics such as length and G/C content. The following terms are used:
    - **Full** The full primer including overhang and added bases.
    - **Anneal** The part of the primer annealing to the original fragment (primer without overhang or any added bases).

  - **Overhang** The overhang added to primers to generate homologous sequence between fragments.
  - **Added** The bases inserted between primer and overhang.

- **Assembled vector** The vector as it will appear after all fragments have been assembled. The assembled vector will be annotated with the positions of primers, added bases and overhangs as well as with inserts and vector sequence. When the vector is opened, you can select which annotations should be shown on the sequence in the side panel under Annotation types.

  Note: the assembled vector can be used as input to **Homology Based Cloning** if you wish to adjust a previous design.

- **Primers sequence list** A sequence list containing the designed primers. The primers are annotated with primer, added bases and overhang, where primer is the part of the sequence that originally aligned to the insert or vector that was amplified.

- **PCR fragments sequence list** The PCR fragments generated from input sequences and designed primers including additional bases and overhangs.

- **Primer pairs table** A table providing information about melting temperatures, secondary structure, etc., for primer pairs with and without overhangs. For a description of each of the columns in the Primer Pairs table, see section 22.5.3.

23.4.4 Detailed description of the Homology Based Cloning wizard

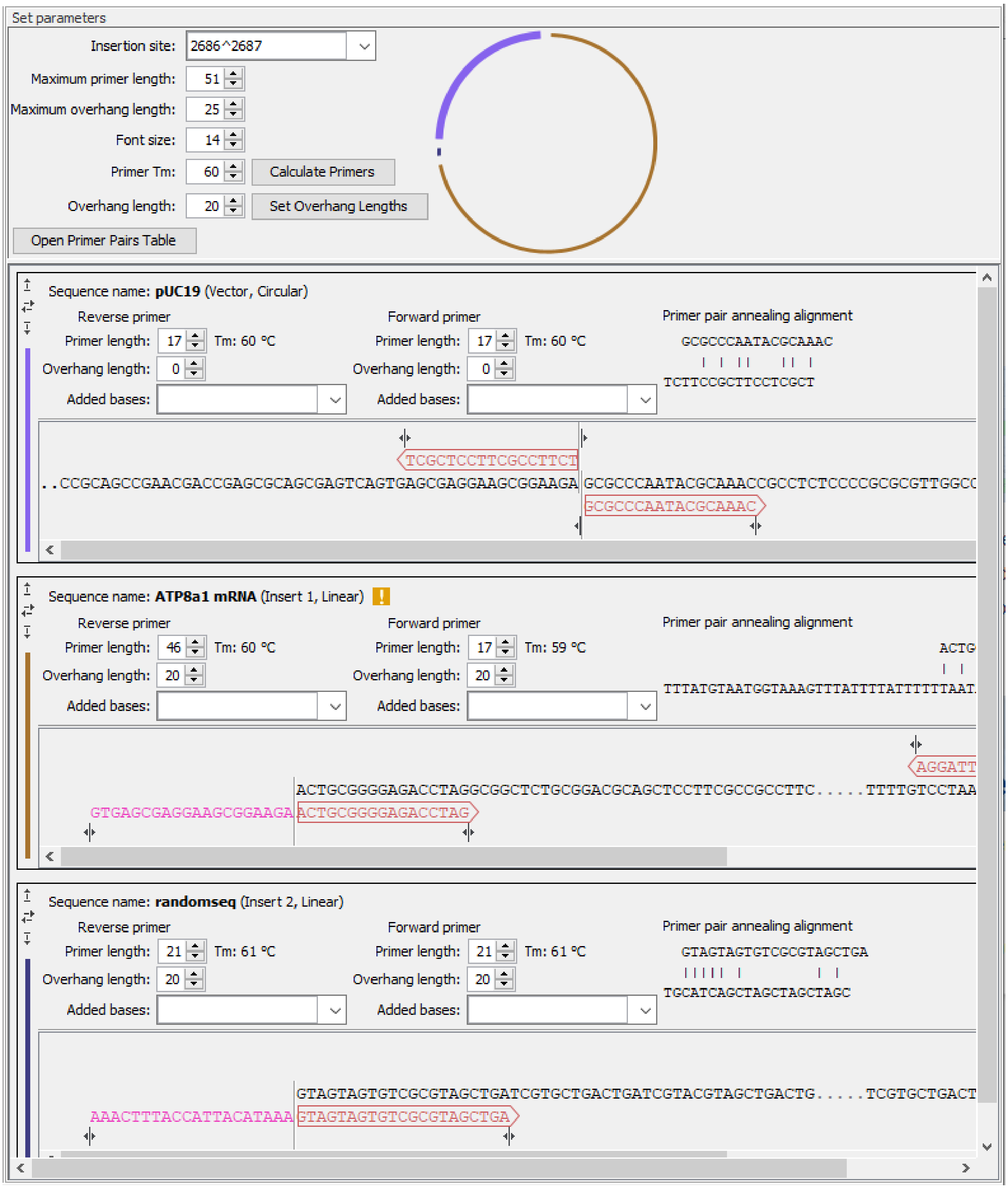
This section contains a detailed description of the **Homology Based Cloning** options (figure 23.32).

- **Insertion site** The position where fragments will be inserted in the vector. You can type in a specific position or a range of positions. You can also choose the start, the end, or the entire span of an annotation on the vector using the drop down menu (figure 23.33).

  The primers designed to amplify the vector will be placed so that their 5' ends are adjacent to the insertion site. If a range of positions are selected, the primers will be placed so that the selected positions are not included in the PCR product. When the insertion site is changed, the vector primers in the view below are updated accordingly.

  Insertion site examples:

  - 0 or **0^1** Assembles inserts into the vector between the last and the first base.
  - **1** or **1^2** Assembles inserts into the vector between the first and second base.
  - **1..10** Inserts replace bases 1-10 in the vector.
  - **Start of an annotation** Assembles inserts into the vector before the first base in the annotated region.
  - **Span of an annotation** Inserts replace all bases in the annotated region.
  - **End of an annotation** Assembles inserts into the vector after the last base in the annotated region.



Set parameters

Insertion site: 2686^2687

Maximum primer length: 51

Maximum overhang length: 25

Font size: 14

Primer Tm: 60

Overhang length: 20

Sequence name: **pUC19** (Vector, Circular)

Reverse primer: Primer length: 17, Tm: 60 °C, Overhang length: 0, Added bases:

Forward primer: Primer length: 17, Tm: 60 °C, Overhang length: 0, Added bases:

Primer pair annealing alignment

```
GCGCCCAATACGCAAAC
 | |  ||   || |
TCTTCCGCTTCCTCGCT
```

```
                                     TCGCTCCTTCGCCTTCT
..CCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGA GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCC
                                                      GCGCCCAATACGCAAAC
```

Sequence name: **ATP8a1 mRNA** (Insert 1, Linear) !

Reverse primer: Primer length: 46, Tm: 60 °C, Overhang length: 20, Added bases:

Forward primer: Primer length: 17, Tm: 59 °C, Overhang length: 20, Added bases:

Primer pair annealing alignment

```
                                      ACTG
                                      | |
TTTATGTAATGGTAAAGTTTATTTTATTTTTTAAT
```

```
                                                                                  AGGATT
                   ACTGCGGGGAGACCTAGGCGGCTCTGCGGACGCAGCTCCTTCGCCGCCTTC.....TTTTGTCCTAA
GTGAGCGAGGAAGCGGAAGA ACTGCGGGGAGACCTAG
```

Sequence name: **randomseq** (Insert 2, Linear)

Reverse primer: Primer length: 21, Tm: 61 °C, Overhang length: 20, Added bases:

Forward primer: Primer length: 21, Tm: 61 °C, Overhang length: 20, Added bases:

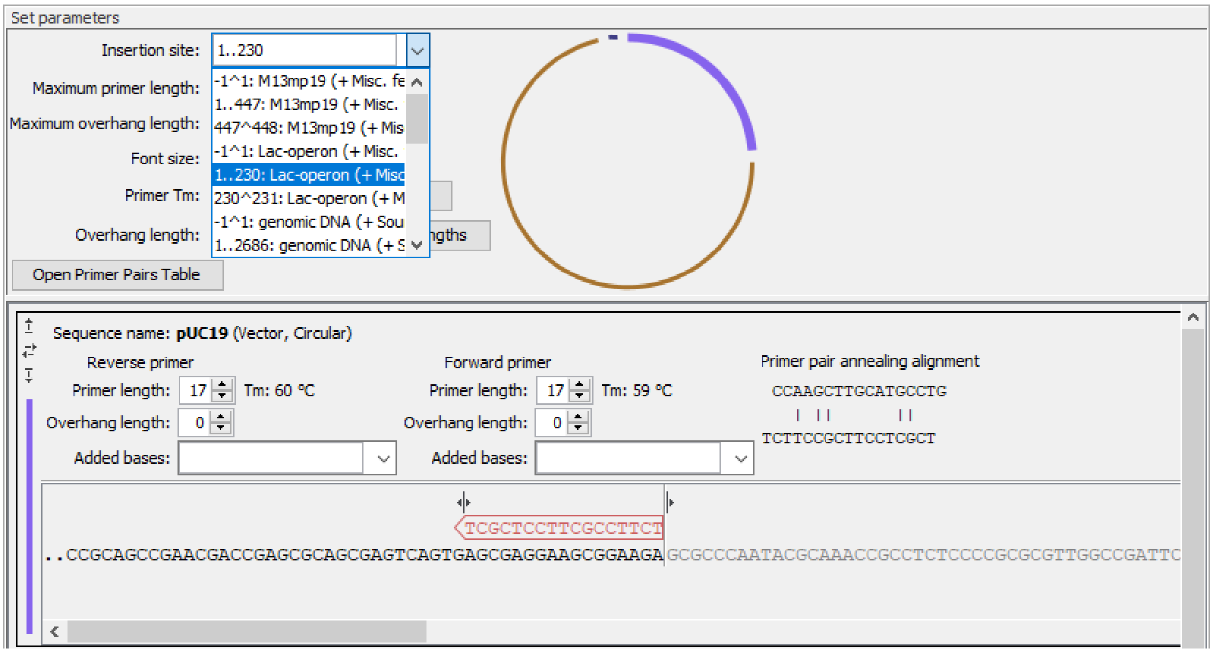
Primer pair annealing alignment

```
 GTAGTAGTGTCGCGTAGCTGA
 |||||  |        |  |
TGCATCAGCTAGCTAGCTAGC
```

```
                   GTAGTAGTGTCGCGTAGCTGATCGTGCTGACTGATCGTACGTAGCTGACTG.....TCGTGCTGACT
AAACTTTACCATTACATAAA GTAGTAGTGTCGCGTAGCTGA
```

Figure 23.32: *General options for the cloning experiment are provided at the top of the wizard, followed by sections for the vector and insert sequences, where many options relevant to the cloning experiment can be adjusted.*

- **Maximum primer length** The maximum length that primers for vectors and inserts can be. This is reflected in the number of nucleotides visible for each sequence in the views below.
- **Maximum overhang length** The maximum length that overhangs for vectors and inserts can be.



Set parameters

Insertion site: 1..230

Maximum primer length:

Maximum overhang length:

Font size:

Primer Tm:

Overhang length:

Sequence name: **pUC19** (Vector, Circular)

Reverse primer

Primer length: 17 Tm: 60 °C

Overhang length: 0

Added bases:

Forward primer

Primer length: 17 Tm: 59 °C

Overhang length: 0

Added bases:

Primer pair annealing alignment

```
CCAAGCTTGCATGCCTG
  |  ||       ||
TCTTCCGCTTCCTCGCT
```

```
                                                TCGCTCCTTCGCCTTCT
..CCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTC
```

Figure 23.33: *Specify the insertion site in the vector. Here the entire Lac-operon has been selected from the drop down menu. Notice that when a span of bases are chosen as insertion site, the vector sequence between the primers is grey and not included in the PCR product.*

- **Font size** The font size to use for vector and insert sequences, and for primers and overhangs.
- **Primer Tm** The primer melting temperature. This value does not take into account any added bases or overhangs. Click on **Calculate primers** to update all primers after changing this value.
- **Overhang length** The length of the overhang added to primers not including added bases. Click on **Set Overhang Lengths** to update overhangs after changing this value.
- **Open Primer Pairs Table** Opens a table listing each of the primer pairs shown on the sequences below. The primer pairs table contains primer pairs, both with and without overhangs and added bases. It also provides information about melting temperatures, secondary structure, etc. For a description of each of the columns in the Primer Pairs table, see section 22.5.3.
- **Vector map** A vector map showing the assembled vector. Each original fragment has its own color that matches the side bars of the sequences in the views below. If you hover over the sequence of the vector or an insert, it will become bold in the vector map. If you hover over a primer, it will appear on the vector map. The fragments, but not the primers are drawn to scale.
- **Sequence Name: n (vector, circular)** and **Sequence Name: n (insert y, linear)**. The sequences identified as the vector and inserts.
- **Arrows to the left of Sequence Name** Change the order of the sequences in the list using the up and down arrows. Reverse complement the sequence using the horizontal arrows.
- **Primer length** and **Overhang length** The primer and overhang lengths for the forward and reverse primer, respectively. These lengths can be adjusted by typing new values into the

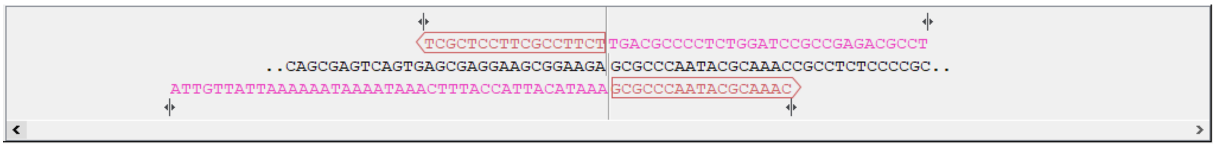
dialogs, or by using the up and down arrows to the right of the dialogs. Changes to the lengths are immediately updated in the sequence view below. The Tm and primer pair annealing alignment are also updated.

- **Tm** The primer melting temperature. This value does not include overhangs or any added bases.
- **Primer pair annealing alignment** Predicted primer-primer annealing of the forward and reverse primers. Overhangs and added bases are not included. The same plot is also available in the primer pairs table.
- **Added bases** Insert additional bases between the primer and overhang. You can either type the bases directly into the dialog, or you can choose the sequence of a specific restriction enzyme from the drop down menu.
- **Sequence and primer views** For each sequence included in the homology cloning reaction, you can see the part of the sequence that primers are designed to, as well as the primers and their overhangs. For the vector, the fragment is considered circular and the primers are placed pointing in opposite directions from the insertion site (figure 23.34). Inserts are considered linear and primers are placed at the ends (figure 23.35).

  Vector, inserts, primers and overhangs are color coded (figure 23.30):

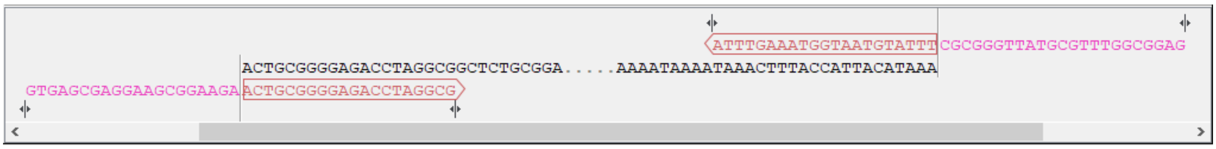
  - **Black** Vector or insert sequence
  - **Grey** Vector or insert sequence not included in the PCR product
  - **Brown** Primer sequence
  - **Pink** Overhang sequence
  - **Blue** Added bases that are inserted between primer and overhang

  The overhang of a primer for a given sequence is identical to the sequence that it will be adjacent to in the assembled vector. Figures 23.34 and 23.35 show an example where the linear sequence can be inserted into the circular sequence. Pink overhang bases on the primers for the circular fragment are either the same sequence or complementary to the black sequence of the linear DNA fragment. Overhangs are designed to assemble the fragments in the order they appear in the wizard. In this example, two sequences are assembled, but more than two can be used for homology based cloning.



```
                        TCGCTCCTTCGCCTTCTTGACGCCCCTCTGGATCCGCCGAGACGCCT
          ..CAGCGAGTCAGTGAGCGAGGAAGCGGAAGA GCGCCCAATACGCAAACCGCCTCTCCCCGC..
ATTGTTATTAAAAAATAAAATAAACTTTACCATTACATAAAGCGCCCAATACGCAAAC
```

Figure 23.34: *Vector sequence and corresponding primers.*

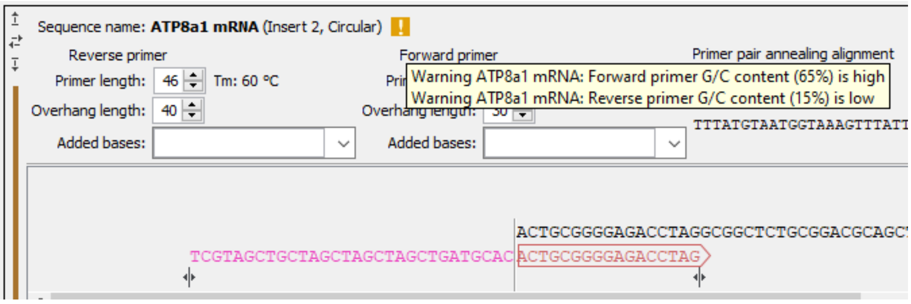


```
                                                     ATTTGAAATGGTAATGTATTTCGCGGGTTATGCGTTTGGCGGAG
                 ACTGCGGGGAGACCTAGGCGGCTCTGCGGA.....AAAATAAAATAAACTTTACCATTACATAAA
GTGAGCGAGGAAGCGGAAGAACTGCGGGGAGACCTAGGCG
```

Figure 23.35: *Insert sequence and corresponding primers.*

- **Warnings in sequence views** A yellow or red exclamation mark next to the sequence name warns of any problems 23.36. Hover over the primer to get more information from the tooltip or click on the warning to open a dialog showing the warning message. Examples of when warnings appear include:

- A primer does not have G/C content between 40 and 60 %.
- Only one primer is designed for a given fragment.
- An insert with a length that is not a multiple of 3 is placed inside a vector region annotated as a CDS and so may disrupt the reading frame.



Sequence name: **ATP8a1 mRNA** (Insert 2, Circular) !

Reverse primer
Primer length: 46 Tm: 60 °C
Overhang length: 40
Added bases:

Forward primer
Overhang length: 30
Added bases:

Primer pair annealing alignment

Warning ATP8a1 mRNA: Forward primer G/C content (65%) is high
Warning ATP8a1 mRNA: Reverse primer G/C content (15%) is low

```
TTTATGTAATGGTAAAGTTTATT
ACTGCGGGGAGACCTAGGCGGCTCTGCGGACGCAGC
TCGTAGCTGCTAGCTAGCTAGCTGATGCACACTGCGGGGAGACCTAG
```

Figure 23.36: *Hover over the yellow exclamation mark to see the warnings in the tooltip.*

23.4.5 Working with mutations

Mutations can be introduced using PCR amplification with primers containing the mutations. This can be mimicked in the software:

- Introduce mutations manually in the sequences before running Homology Based Cloning. Place primers over mutated sites to ensure the mutations are included in the primer sequence.
- Run Homology Based Cloning using the original sequences, and then introduce mutations into the assembled vector. Re-run Homology Based Cloning using the vector containing the mutations.

23.5 Gateway cloning

CLC Main Workbench offers tools to perform *in silico* Gateway cloning (Thermo Fisher Scientific), including Multi-site Gateway cloning.

The three tools for doing Gateway cloning in the *CLC Main Workbench* mimic the procedure followed in the lab:

- First, attB sites are added to a sequence fragment
- Second, the attB-flanked fragment is recombined into a donor vector (the BP reaction) to construct an entry clone
- Finally, the target fragment from the entry clone is recombined into an expression vector (the LR reaction) to construct an expression clone. For Multi-site gateway cloning, multiple entry clones can be created that can recombine in the LR reaction.

During this process, both the attB-flanked fragment and the entry clone can be saved.

For more information about the Gateway technology, please visit https://www.thermofisher.com/us/en/home/life-science/cloning/gateway-cloning/gateway-technology.html. To perform these analyses in *CLC Main Workbench*, you need to import donor and expression vectors. These can be found on the Thermo Fisher Scientific's website: find the relevant vector sequences, copy them, and paste them in the field that opens when you choose **New | Sequence** in the workbench. Fill in additional information appropriately (enter a "Name", check the "Circular" option) and save the sequences in the Navigation Area.

23.5.1 Add attB sites

The first step in the Gateway cloning process is to amplify the target sequence with primers including so-called attB sites:

Tools | **Cloning** ( )| **Gateway Cloning** ( ) | **Add attB Sites** ( )

This will open a dialog where you can select the input sequence, sequences, or sequence lists containing fewer than 10,000 sequences. Note that if a fragment is part of a longer sequence, you will need to extract it prior to starting the tool: select the relevant region (or an annotation) of the original sequence, right-click the selection and choose to **Open Annotation in New View**. **Save** ( ) the new sequence in the Navigation Area.

When you have selected your fragment(s), click **Next**.

This will allow you to choose which attB sites you wish to add to each end of the fragment as shown in figure 23.37.

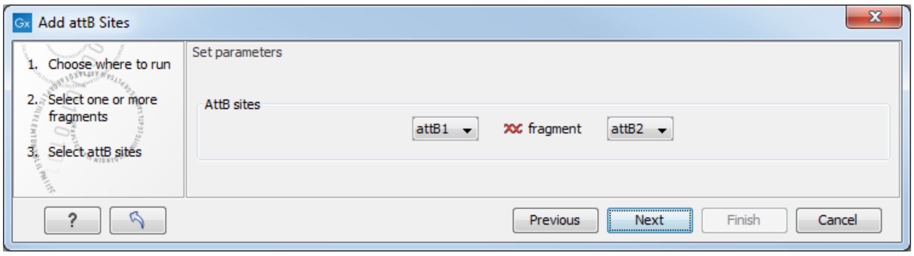


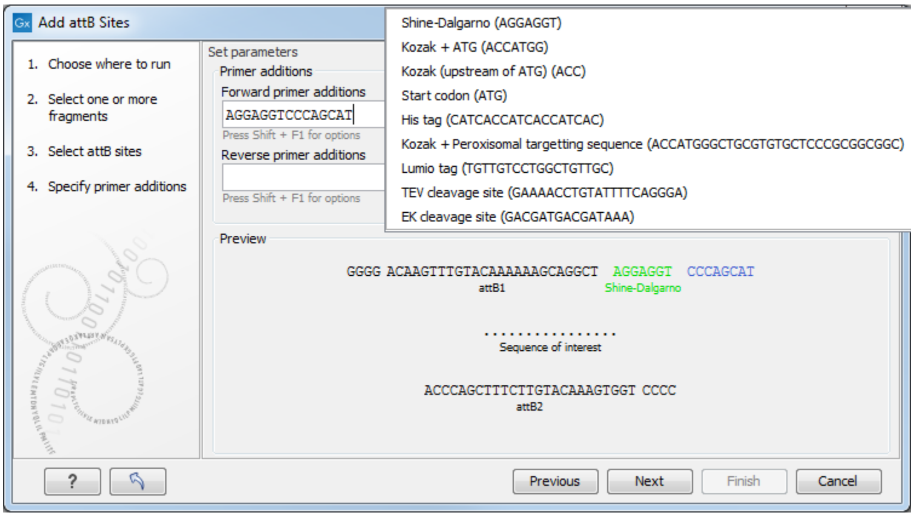
Figure 23.37: *Selecting which attB sites to add.*

The default option is to use the attB1 and attB2 sites. If you have selected several fragments and wish to add different combinations of sites, you will have to run this tool once for each combination.

Next, you are given the option to extend the fragment with additional sequences by extending the primers 5' of the template-specific part of the primer, i.e., between the template specific part and the attB sites.

You can manually type or paste in a sequence of your choice, but it is also possible to click in the text field and press **Shift + F1 (Shift + Fn + F1 on Mac)** to show some of the most common additions (see figure 23.38). Use the up and down arrow keys to select a tag and press **Enter**. To learn how to modify the default list of primer additions, see section 23.5.1.

At the bottom of the dialog, you can see a preview of what the final PCR product will look like. In the middle there is the sequence of interest. In the beginning is the attB1 site, and at the end is the attB2 site. The primer additions that you have inserted are shown in colors.



Forward primer additions: AGGAGGTCCCAGCAT

Reverse primer additions:

Shine-Dalgarno (AGGAGGT)
Kozak + ATG (ACCATGG)
Kozak (upstream of ATG) (ACC)
Start codon (ATG)
His tag (CATCACCATCACCATCAC)
Kozak + Peroxisomal targetting sequence (ACCATGGGCTGCGTGTGCTCCCGCGGCGGC)
Lumio tag (TGTTGTCCTGGCTGTTGC)
TEV cleavage site (GAAAACCTGTATTTTCAGGGA)
EK cleavage site (GACGATGACGATAAA)

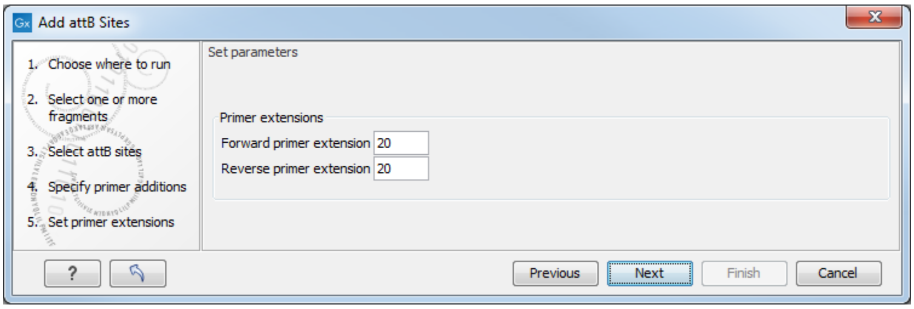
```
GGGG ACAAGTTTGTACAAAAAAGCAGGCT  AGGAGGT  CCCAGCAT
             attB1               Shine-Dalgarno

                  .................
                 Sequence of interest

        ACCCAGCTTTCTTGTACAAAGTGGT CCCC
                    attB2
```

Figure 23.38: *Primer additions 5' of the template-specific part of the primer where a Shine-Dalgarno site has been added between the attB site and the gene of interest.*

In the next step, specify the length of the template-specific part of the primers as shown in figure 23.39.



Forward primer extension 20

Reverse primer extension 20

Figure 23.39: *Specifying the length of the template-specific part of the primers.*

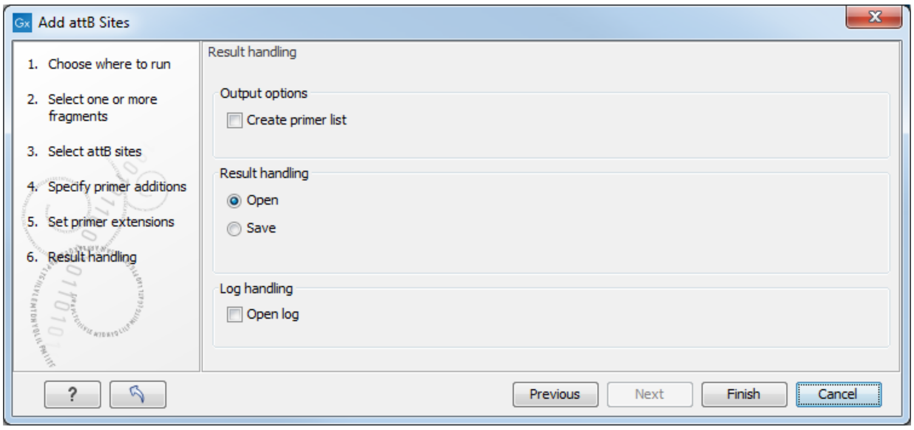
The Workbench is not doing any kind of primer design when adding the attB sites. As a user, you simply specify the length of the template-specific part of the primer, and together with the attB sites and optional primer additions, this will be the primer. The primer region will be annotated in the resulting attB-flanked sequence. You can also choose to get a list of primers in the Result handling dialog (see figure 23.40).

The attB sites, the primer additions and the primer regions are annotated in the final result as shown in figure 23.41 (you may need to switch on the relevant annotation types to show the sites and primer additions).

There will be one output sequence for each sequence you have selected for adding attB sites. **Save** (⇤) the resulting sequence as it will be the input to the next part of the Gateway cloning workflow (see section 23.5.2).

Extending the pre-defined list of primer additions

The list of primer additions shown when pressing **Shift+F1** (on Mac: Shift + fn + F1) in the dialog shown in figure 23.38 can be configured and extended. If there is a tag that you use a lot, you can add it to the list for convenient and easy access later on. This is done in the **Preferences**:



Result handling

Output options

□ Create primer list

Result handling

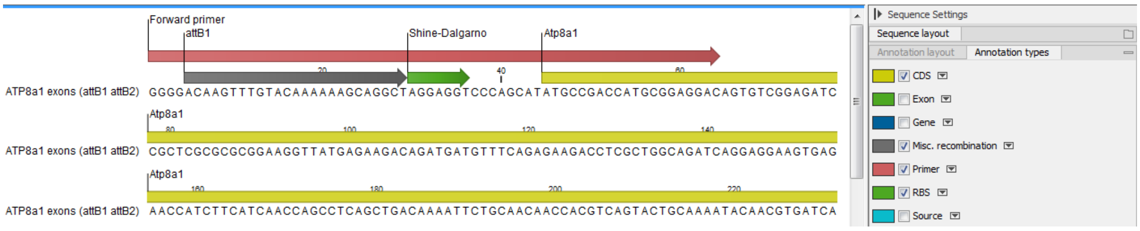
● Open

○ Save

Log handling

□ Open log

Figure 23.40: *Besides the main output which is a copy of the input sequence(s) now including attB sites and primer additions, you can get a list of primers as output.*



```
ATP8a1 exons (attB1 attB2)  GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGAGGTCCCAGCATATGCCGACCATGCGGAGGACAGTGTCGGAGATC
ATP8a1 exons (attB1 attB2)  CGCTCGCGCGCGGAAGGTTATGAGAAGACAGATGATGTTTCAGAGAAGACCTCGCTGGCAGATCAGGAGGAAGTGAG
ATP8a1 exons (attB1 attB2)  AACCATCTTCATCAACCAGCCTCAGCTGACAAAATTCTGCAACAACCACGTCAGTACTGCAAAATACAACGTGATCA
```

☑ CDS

□ Exon

□ Gene

☑ Misc. recombination

☑ Primer

☑ RBS

□ Source

Figure 23.41: *the attB site plus the Shine-Dalgarno primer addition is annotated.*

Edit | **Preferences** | **Data**

In the table **Multisite Gateway Cloning primer additions** (see figure 23.42), select which primer addition options you want to add to forward or reverse primers. You can edit the existing elements in the table by double-clicking any of the cells, or you can use the buttons below to **Add Row** or **Delete Row**. If you by accident have deleted or modified some of the default primer additions, you can press **Add Default Rows**. Note that this will not reset the table but only add all the default rows to the existing rows.

Each element in the list has the following information:

Name When the sequence fragment is extended with a primer addition, an annotation will be added displaying this name.

Sequence The actual sequence to be inserted, defined on the sense strand (although the reverse primer would be reverse complement).

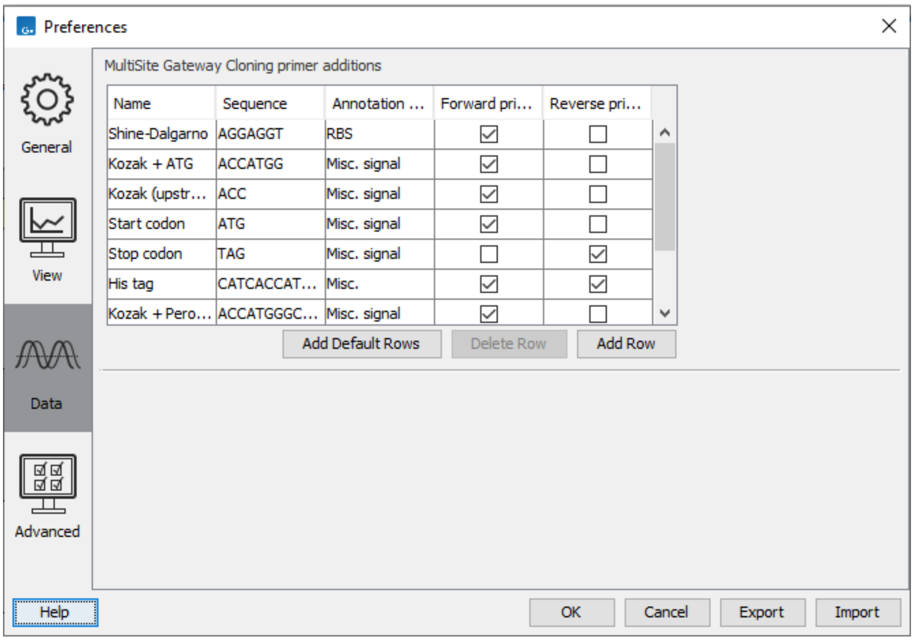
Annotation type The annotation type of the primer that is added to the fragment.

Forward primer addition Whether this addition should be visible in the list of additions for the forward primer.

Reverse primer addition Whether this addition should be visible in the list of additions for the reverse primer.

23.5.2 Create entry clones (BP)

The next step in the Gateway cloning work flow is to recombine the attB-flanked sequence of interest into a donor vector to create an entry clone. Before proceeding to this step, make



Preferences

MultiSite Gateway Cloning primer additions

Name	Sequence	Annotation ...	Forward pri...	Reverse pri...
Shine-Dalgarno	AGGAGGT	RBS	☑	☐
Kozak + ATG	ACCATGG	Misc. signal	☑	☐
Kozak (upstr...	ACC	Misc. signal	☑	☐
Start codon	ATG	Misc. signal	☑	☐
Stop codon	TAG	Misc. signal	☐	☑
His tag	CATCACCAT...	Misc.	☑	☑
Kozak + Pero...	ACCATGGGC...	Misc. signal	☑	☐

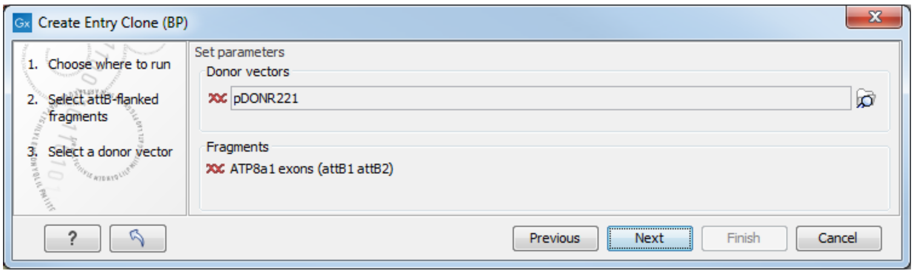
Figure 23.42: *Configuring the list of primer additions available when adding attB sites.*

sure that the sequence of the destination vector was saved in the Navigation Area: find the relevant vector sequence on the Thermo Fisher Scientific's website, copy it, and paste it in in the field that opens when you choose **New | Sequence** in the workbench. Fill in additional information appropriately (enter a "Name", check the "Circular" option) and save the sequence in the Navigation Area.

Tools | **Cloning** ( )| **Gateway Cloning** ( ) | **Create Entry Clone** ( )

In the first wizard window, select one or more sequences to be recombined into your donor vector. Note that the sequences you select should be flanked with attB sites (see section 23.5.1). You can select more than one sequence as input, and the corresponding number of entry clones will be created.

In the following dialog (figure 23.43), you can specify a donor vector.



Create Entry Clone (BP)

1. Choose where to run
2. Select attB-flanked fragments
3. Select a donor vector

Set parameters

Donor vectors: pDONR221

Fragments: ATP8a1 exons (attB1 attB2)

Figure 23.43: *Selecting one or more donor vectors.*

Once the vector is selected, a preview of the fragments selected and the attB sites that they contain is shown. This can be used to get an overview of which entry clones should be used and check that the right attB sites have been added to the fragments. Also note that the workbench looks for the attP sites (see how to change the definition of sites in appendix E), but it does not check that they correspond to the attB sites of the selected fragments at this step. If the right combination of attB and attP sites is not found, no entry clones will be produced.

The output is one entry clone per sequence selected. The attB and attP sites have been used for the recombination, and the entry clone is now equipped with attL sites as shown in figure 23.44.

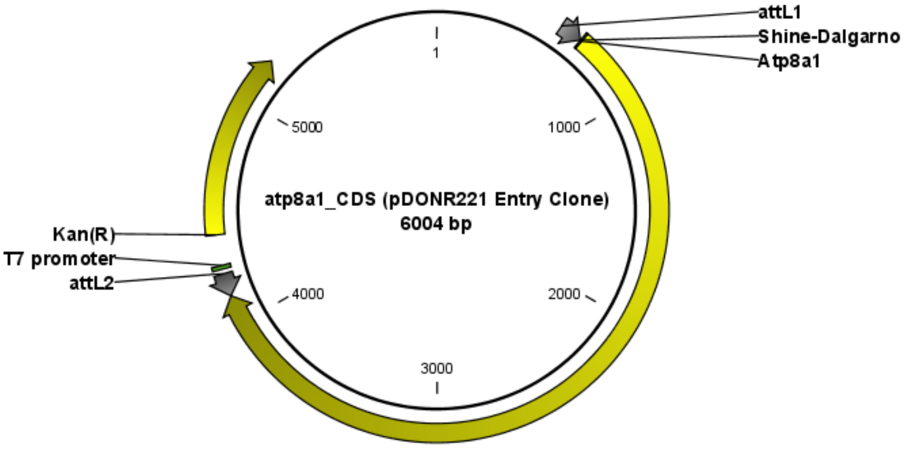


Figure 23.44: *The resulting entry vector opened in a circular view.*

Note that the bi-product of the recombination is not part of the output.

23.5.3 Create expression clones (LR)

The final step in the Gateway cloning work flow is to recombine the entry clone into a destination vector to create an expression clone. Before proceeding to this step, make sure that the sequence of the destination vector was saved in the Navigation Area: find the relevant vector sequence on the Thermo Fisher Scientific's website, copy it, and paste it in in the field that opens when you choose **New | Sequence** in the workbench. Fill in additional information appropriately (enter a "Name", check the "Circular" option) and save the sequence in the Navigation Area.

Note also that for a destination vector to be recognized, it must contain appropriate att sites and the *ccdB* gene. This gene must be present either as a 'ccdB' annotation, or as the exact sequence:

ATGCAGTTTAAGGTTTACACCTATAAAAGAGAGAGCCGTTATCGTCTGTTTGTGGATGTACAGAGTGATATT
ATTGACACGCCCGGGCGACGGATGGTGATCCCCCTGGCCAGTGCACGTCTGCTGTCAGATAAAGTCTCC
CGTGAACTTTACCCGGTGGTGCATATCGGGGATGAAAGCTGGCGCATGATGACCACCGATATGGCCAGT
GTGCCGGTCTCCGTTATCGGGGAAGAAGTGGCTGATCTCAGCCACCGCGAAAATGACATCAAAAACGCC
ATTAACCTGATGTTCTGGGGAATATAA

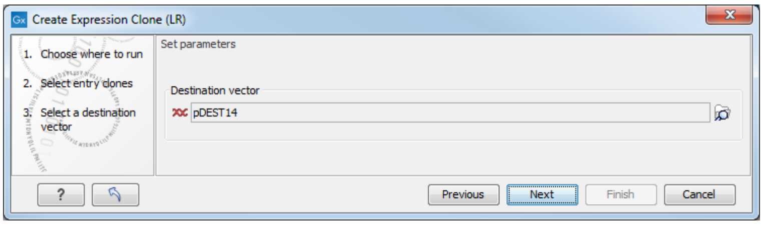
If the *ccdB* gene is not present or if the sequence is not identical to the above, a solution is to simply add a 'ccdB' annotation. Select part of the vector sequence, right-click and choose 'Add Annotation'. Name the annotation 'ccdB'.

You can now start the tool:

Tools | **Cloning** ( )| **Gateway Cloning** ( ) | **Create Expression Clone** ( )

In the first step, select one or more entry clones (see how to create an entry clone in section 23.5.2). If you wish to perform separate LR reactions with multiple entry clones, you should run the **Create Expression Clone** in batch mode (see section 11.3).

In the second step, select the destination vector that was previously saved in the Navigation Area (fig 23.45).



Create Expression Clone (LR)

1. Choose where to run
2. Select entry clones
3. Select a destination vector

Set parameters

Destination vector: pDEST14

Figure 23.45: *Selecting one or more destination vectors.*

Note that the workbench looks for the specific sequences of the attR sites in the sequences that you select in this dialog (see how to change the definition of sites in appendix E), but it does not check that they correspond to the attL sites of the selected fragments. If the right combination of attL and attR sites is not found, no entry clones will be produced.

When performing multi-site gateway cloning, *CLC Main Workbench* will insert the fragments (contained in entry clones) by matching the sites that are compatible. If the sites have been defined correctly, an expression clone containing all the fragments will be created. You can find an explanation of the multi-site gateway system at https://www.thermofisher.com/dk/en/home/life-science/cloning/gateway-cloning/multisite-gateway-technology.html?SID=fr-gwcloning-3

The output is a number of expression clones depending on how many entry clones and destination vectors that you selected. The attL and attR sites have been used for the recombination, and the expression clone is now equipped with attB sites as shown in figure 23.46.

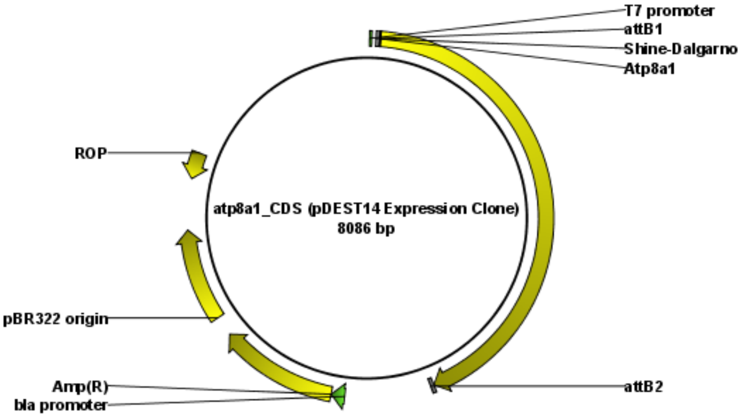


Figure 23.46: *The resulting expression clone opened in a circular view.*

You can choose to create a sequence list with the bi-products as well.

23.6 Gel electrophoresis

CLC Main Workbench enables the user to simulate the separation of nucleotide sequences on a gel. This feature is useful when designing an experiment which will allow the differentiation of a successful and an unsuccessful cloning experiment on the basis of a restriction map.

There are several ways to simulate gel separation of nucleotide sequences:

- When using the **Restriction Site Analysis** tool, available under the Tools menu, you can choose to create a restriction map which can be shown as a gel (see section 23.1.2).
- From all the graphical views of sequences, you can right-click the name of the sequence and choose **Digest and Create Restriction Map (▤)**. The sequence will be digested with the enzymes that are selected in the Side Panel. The views where this option is available are listed below:
  - Circular view (see section 14.2.5).
  - Ordinary sequence view (see section 14.2).
  - Graphical view of sequence lists (see section 14.1).
  - Cloning editor (see section 23.3).
  - Primer designer (see section 22.3).
- **Separate sequences on gel**: To separate sequences without restriction enzyme digestion, first create a sequence list of the sequences in question, then click the **Gel** button (▤) at the bottom of the view of the sequence list (figure 23.47).

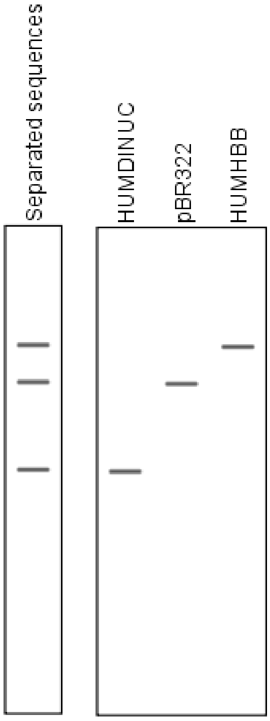
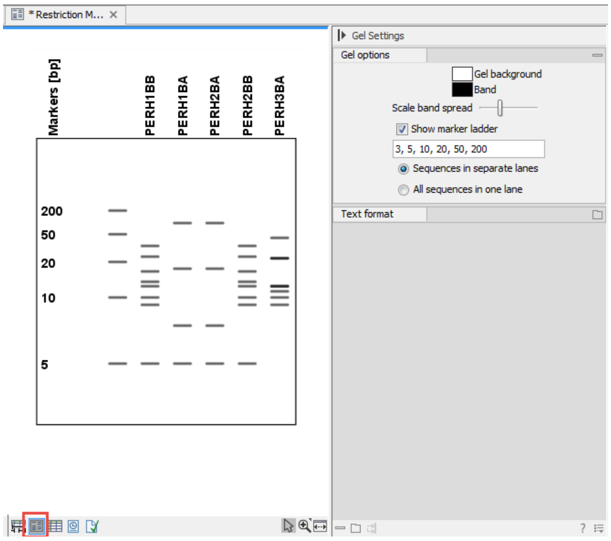


Figure 23.47: *A sequence list shown as a gel.*

23.6.1 Gel view

In figure 23.48 you can see a simulation of a gel with its Side Panel to the right.

Information on bands / fragments You can get information about the individual bands by hovering the mouse cursor on the band of interest. This will display a tool tip with the following information:



Gel Settings

Gel options

Gel background

Band

Scale band spread

☑ Show marker ladder

3, 5, 10, 20, 50, 200

● Sequences in separate lanes

○ All sequences in one lane

Text format

Figure 23.48: *Five lanes showing fragments of five sequences cut with restriction enzymes.*

- Fragment length
- Fragment region on the original sequence
- Enzymes cutting at the left and right ends, respectively

For gels comparing whole sequences, you will see the sequence name and the length of the sequence.

Note! You have to be in **Selection** ( ) or **Pan** ( ) mode in order to get this information.

It can be useful to add markers to the gel which enables you to compare the sizes of the bands. This is done by clicking **Show marker ladder** in the **Side Panel**.

Markers can be entered into the text field, separated by commas.

Modifying the layout The background of the lane and the colors of the bands can be changed in the Side Panel. Click the colored box to display a dialog for picking a color. The slider **Scale band spread** can be used to adjust the effective time of separation on the gel, i.e. how much the bands will be spread over the lane. In a real electrophoresis experiment this property will be determined by several factors including time of separation, voltage and gel density.

You can also choose how many lanes should be displayed:

- **Sequences in separate lanes**. This simulates that a gel is run for each sequence.
- **All sequences in one lane**. This simulates that one gel is run for all sequences.

You can also modify the layout of the view by zooming in or out. Click **Zoom in** ( ) or **Zoom out** ( ) in the Toolbar and click the view.

Finally, you can modify the format of the text heading each lane in the Text format preferences in the Side Panel.

Chapter 24

RNA structure

Contents

Ribonucleic acid (RNA) is a nucleic acid polymer that plays several important roles in the cell.

As for proteins, the three dimensional shape of an RNA molecule is important for its molecular function. A number of tertiary RNA structures are know from crystallography but de novo prediction of tertiary structures is not possible with current methods. However, as for proteins RNA tertiary structures can be characterized by secondary structural elements which are hydrogen bonds within the molecule that form several recognizable "domains" of secondary structure like stems, hairpin loops, bulges and internal loops. A large part of the functional information is thus contained in the secondary structure of the RNA molecule, as shown by the high degree of base-pair conservation observed in the evolution of RNA molecules.

Computational prediction of RNA secondary structure is a well defined problem and a large body of work has been done to refine prediction algorithms and to experimentally estimate the relevant biological parameters.

In *CLC Main Workbench* we offer the user a number of tools for analyzing and displaying RNA structures. These include:

- Secondary structure prediction using state-of-the-art algorithms and parameters
- Calculation of full partition function to assign probabilities to structural elements and hypotheses
- Scanning of large sequences to find local structure signal
- Inclusion of experimental constraints to the folding process
- Advanced viewing and editing of secondary structures and structure information

24.1 RNA secondary structure prediction

CLC Main Workbench uses a minimum free energy (MFE) approach to predict RNA secondary structure. Here, the stability of a given secondary structure is defined by the amount of free energy used (or released) by its formation. The more negative free energy a structure has, the more likely is its formation since more stored energy is released by the event.

Free energy contributions are considered additive, so the total free energy of a secondary structure can be calculated by adding the free energies of the individual structural elements. Hence, the task of the prediction algorithm is to find the secondary structure with the minimum free energy. As input to the algorithm empirical energy parameters are used. These parameters summarize the free energy contribution associated with a large number of structural elements. A detailed structure overview can be found in section 24.5.

In *CLC Main Workbench*, structures are predicted by a modified version of Professor Michael Zukers well known algorithm [Zuker, 1989b] which is the algorithm behind a number of RNA-folding packages including MFOLD. Our algorithm is a dynamic programming algorithm for free energy minimization which includes free energy increments for coaxial stacking of stems when they are either adjacent or separated by a single mismatch. The thermodynamic energy parameters used are from Mfold version 3. Read about Mfold here http://www.unafold.org/.

24.1.1 Selecting sequences for prediction

To start the **Predict Secondary Structure** tool, go to:

Tools | RNA Structure ( )| Predict Secondary Structure ( )

This opens the dialog shown in figure 24.1.

If you have selected sequences before running the tool, those sequences will be listed in the **Selected Elements** pane of the dialog. Use the arrows to add or remove sequences or sequence lists from the selected elements. You can use both DNA and RNA sequences - DNA will be folded as if it were RNA.

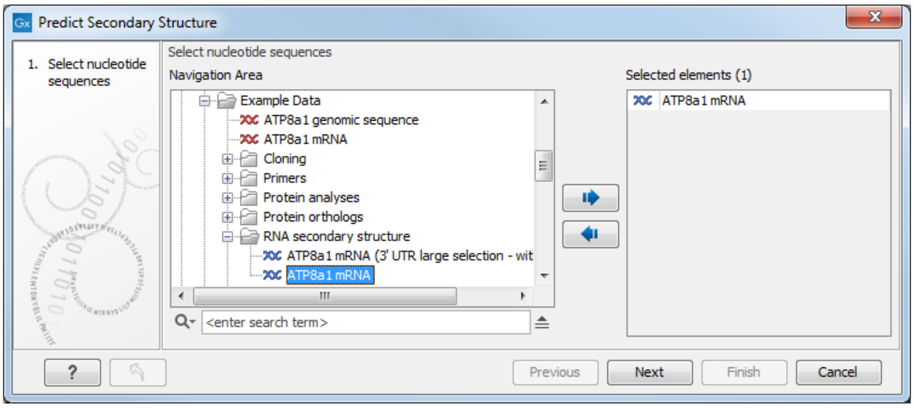
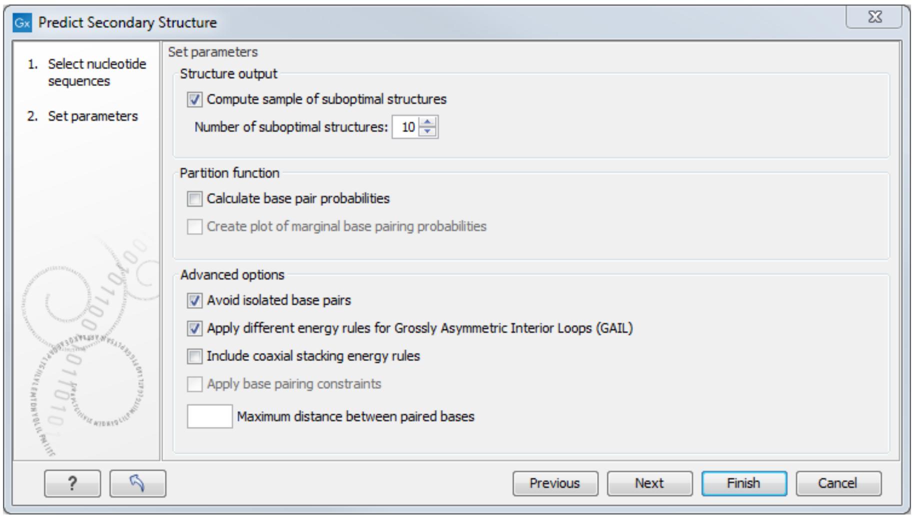


Figure 24.1: *Selecting RNA or DNA sequences for structure prediction (DNA is folded as if it was RNA).*

24.1.2 Secondary structure prediction parameters

Click **Next** to adjust secondary structure prediction parameters (figure 24.2).



Structure output

☑ Compute sample of suboptimal structures

Number of suboptimal structures: 10

Partition function

□ Calculate base pair probabilities

□ Create plot of marginal base pairing probabilities

Advanced options

☑ Avoid isolated base pairs

☑ Apply different energy rules for Grossly Asymmetric Interior Loops (GAIL)

□ Include coaxial stacking energy rules

□ Apply base pairing constraints

Maximum distance between paired bases

Figure 24.2: *Adjusting parameters for secondary structure prediction.*

Structure output

The predict secondary structure algorithm always calculates the minimum free energy structure of the input sequence. In addition to this, it is also possible to compute a sample of suboptimal structures by ticking the checkbox **Compute sample of suboptimal structures**.

Subsequently, you can specify how many structures to include in the output. The algorithm then iterates over all permissible canonical base pairs and computes the minimum free energy and associated secondary structure constrained to contain a specified base pair. These structures are then sorted by their minimum free energy and the most optimal are reported given the specified number of structures. Note that two different sub-optimal structures can have the same minimum free energy. Further information about suboptimal folding can be found in [Zuker, 1989a].

Partition function

The predicted minimum free energy structure gives a point-estimate of the structural conformation of an RNA molecule. However, this procedure implicitly assumes that the secondary structure is at equilibrium, that there is only a single accessible structure conformation, and that the parameters and model of the energy calculation are free of errors.

Obvious deviations from these assumptions make it clear that the predicted MFE structure may deviate somewhat from the actual structure assumed by the molecule. This means that rather than looking at the MFE structure it may be informative to inspect statistical properties of the structural landscape to look for general structural properties which seem to be robust to minor variations in the total free energy of the structure (see [Mathews et al., 2004]).

To this end *CLC Main Workbench* allows the user to calculate the complete secondary structure partition function using the algorithm described in [Mathews et al., 2004] which is an extension of the seminal work by [McCaskill, 1990].

There are two options regarding the partition function calculation:

- **Calculate base pair probabilities.** This option invokes the partition function calculation and calculates the marginal probabilities of all possible base pairs and the marginal probability that any single base is unpaired.
- **Create plot of marginal base pairing probabilities.** This creates a plot of the marginal base pair probability of all possible base pairs as shown in figure 24.3.

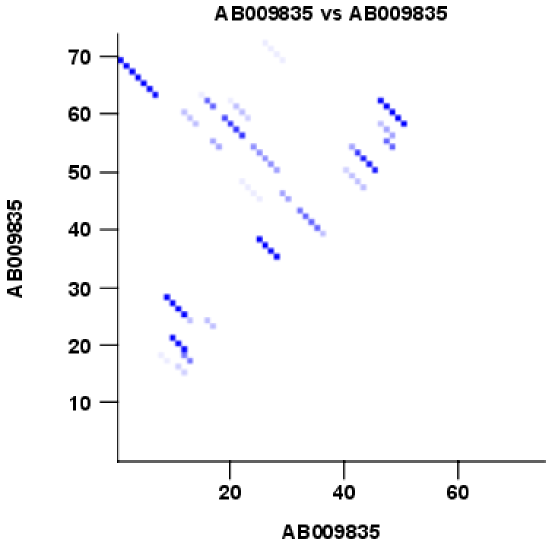
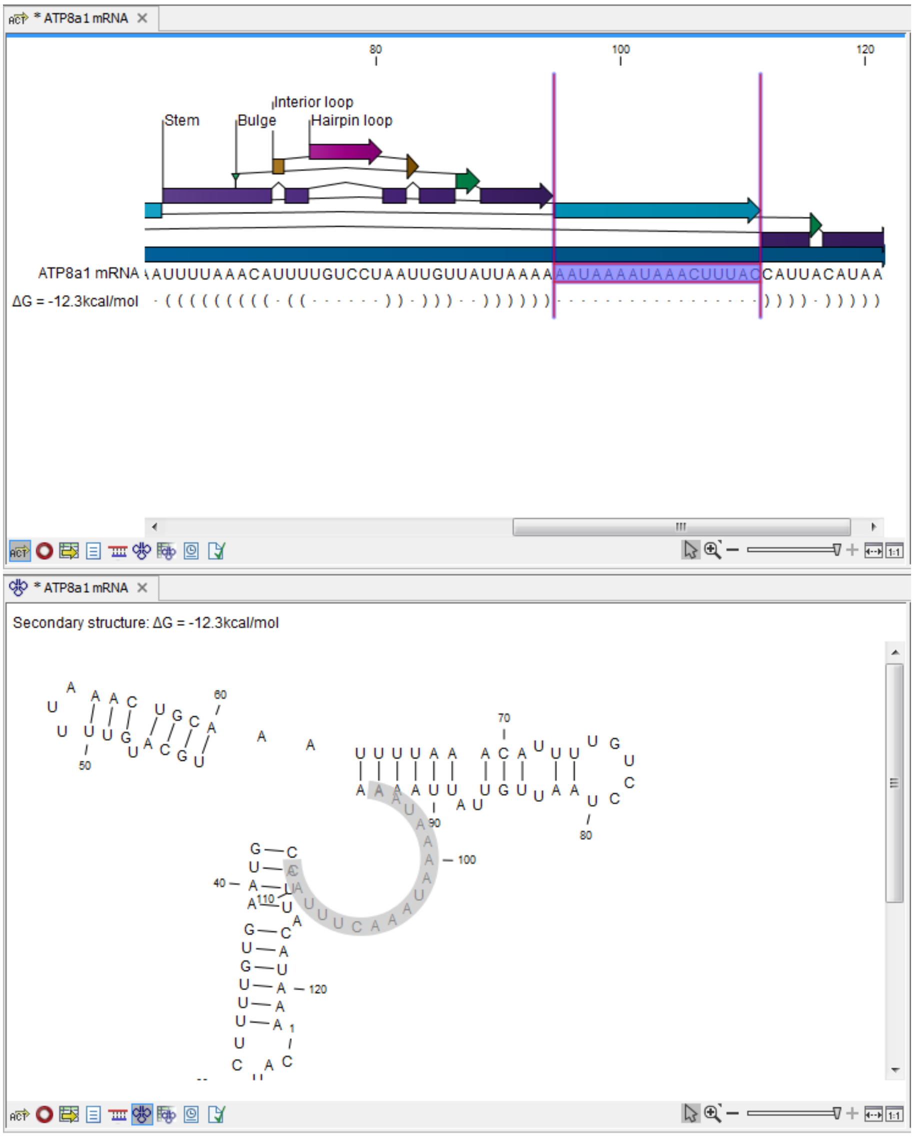


Figure 24.3: *The marginal base pair probability of all possible base pairs.*

The marginal probabilities of base pairs and of bases being unpaired are distinguished by colors which can be displayed in the normal sequence view using the **Side Panel** - see section 24.2.3 and also in the secondary structure view. An example is shown in figure 24.4. Furthermore, the marginal probabilities are accessible from tooltips when hovering over the relevant parts of the structure.



```
ATP8a1 mRNA  AUUUUAAACAUUUUGUCCUAAUUGUUAUUAAAAAAUAAAAUAAACUUUACCAUUACAUAA
ΔG = -12.3kcal/mol  ·(((((((((·((······))·)))··))))))·················))))·)))))
```

Secondary structure: ΔG = -12.3kcal/mol

Figure 24.4: *Marginal probability of base pairs shown in linear view (top) and marginal probability of being unpaired shown in the secondary structure 2D view (bottom).*

Advanced options

The free energy minimization algorithm includes a number of advanced options:

- **Avoid isolated base pairs.** The algorithm filters out isolated base pairs (i.e. stems of length 1).
- **Apply different energy rules for Grossly Asymmetric Interior Loops (GAIL).** Compute the minimum free energy applying different rules for Grossly Asymmetry Interior Loops (GAIL). A Grossly Asymmetry Interior Loop (GAIL) is an interior loop that is $1 \times n$ or $n \times 1$ where $n > 2$ (see http://www.unafold.org/doc/mfold-manual/node5.php).
- **Include coaxial stacking energy rules.** Include free energy increments of coaxial stacking for adjacent helices [Mathews et al., 2004].
- **Apply base pairing constraints.** With base pairing constraints, you can easily add

experimental constraints to your folding algorithm. When you are computing suboptimal structures, it is not possible to apply base pair constraints. The possible base pairing constraints are:

- Force two equal length intervals to form a stem.
- Prohibit two equal length intervals to form a stem.
- Prohibit all nucleotides in a selected region to be a part of a base pair.

Base pairing constraints have to be added to the sequence before you can use this option - see below.

- **Maximum distance between paired bases.** Forces the algorithms to only consider RNA structures of a given upper length by setting a maximum distance between the base pair that opens a structure.

Specifying structure constraints

Structure constraints can serve two purposes in *CLC Main Workbench*: they can act as experimental constraints imposed on the MFE structure prediction algorithm or they can form a structure hypothesis to be evaluated using the partition function (see section 24.1.2).

To *force* two regions to form a stem, open a normal sequence view and:

> **Select the two regions you want to force by pressing Ctrl while selecting - (use ⌘ on Mac) | right-click the selection | Add Structure Prediction Constraints| Force Stem Here**

This will add an annotation labeled "Forced Stem" to the sequence (see figure 24.5).

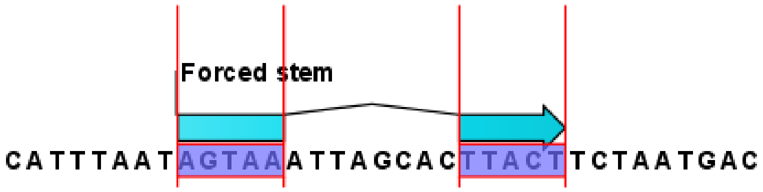


Figure 24.5: *Force a stem of the selected bases.*

Using this procedure to add base pairing constraints will force the algorithm to compute minimum free energy and structure with a stem in the selected region. The two regions must be of equal length.

To *prohibit* two regions to form a stem, open the sequence and:

> **Select the two regions you want to prohibit by pressing Ctrl while selecting - (use ⌘ on Mac) | right-click the selection | Add Structure Prediction Constraints | Prohibit Stem Here**

This will add an annotation labeled "Prohibited Stem" to the sequence (see figure 24.6).

Using this procedure to add base pairing constraints will force the algorithm to compute minimum free energy and structure without a stem in the selected region. Again, the two selected regions must be of equal length.

To prohibit a region to be part of *any* base pair, open the sequence and:

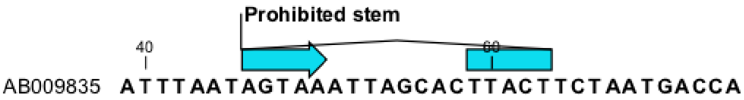


Figure 24.6: *Prohibit the selected bases from forming a stem.*

Select the bases you don't want to base pair | right-click the selection | Add Structure Prediction Constraints | Prohibit From Forming Base Pairs

This will add an annotation labeled "No base pairs" to the sequence, see 24.7.

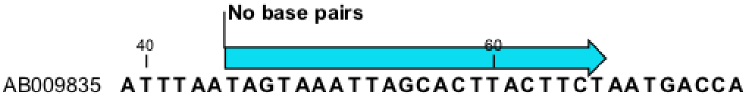


Figure 24.7: *Prohibiting any of the selected base from pairing with other bases.*

Using this procedure to add base pairing constraints will force the algorithm to compute minimum free energy and structure without a base pair containing any residues in the selected region.

When you click **Predict secondary structure** (⚘) and click **Next**, check **Apply base pairing constraints** in order to force or prohibit stem regions or prohibit regions from forming base pairs.

You can add multiple base pairing constraints, e.g. simultaneously adding forced stem regions and prohibited stem regions and prohibit regions from forming base pairs.

24.1.3 Structure as annotation

You can choose to add the elements of the best structure as annotations (see figure 24.8).

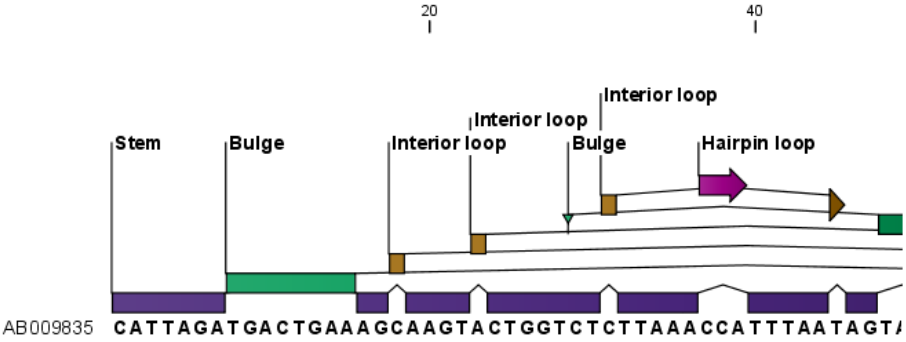


Figure 24.8: *Annotations added for each structure element.*

This makes it possible to use the structure information in other analysis in the *CLC Main Workbench*. You can e.g. align different sequences and compare their structure predictions.

Note that possibly existing structure annotation will be removed when a new structure is calculated and added as annotations.

If you generate multiple structures, only the best structure will be added as annotations. If you wish to add one of the sub-optimal structures as annotations, this can be done from the **Show Secondary Structure Table** (▦) described in section 24.2.2.

24.2 View and edit secondary structures

When you predict RNA secondary structure (see section 24.1), the resulting predictions are attached to the sequence and can be shown as:

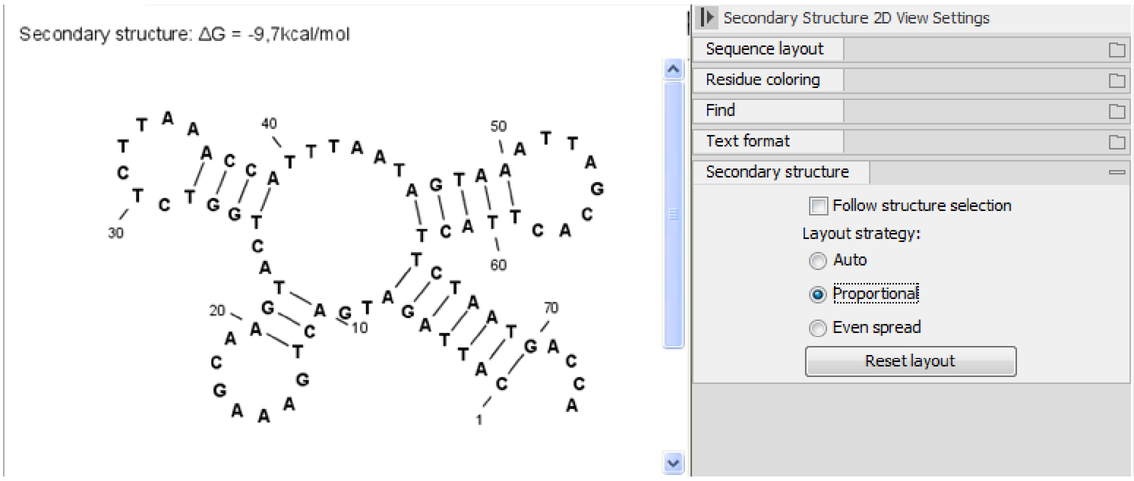
- Annotations in the ordinary sequence views (Linear sequence view (), Annotation table () etc. This is only possible if this has been chosen in the dialog in figure 24.2. See an example in figure 24.8.
- Symbolic representation below the sequence (see section 24.2.3).
- A graphical view of the secondary structure (see section 24.2.1).
- A tabular view of the energy contributions of the elements in the structure. If more than one structure have been predicted, the table is also used to switch between the structures shown in the graphical view. The table is described in section 24.2.2.

24.2.1 Graphical view and editing of secondary structure

To show the secondary view of an already open sequence, click the **Show Secondary Structure 2D View** () button at the bottom of the sequence view.

If the sequence is not open, click **Show** () and select **Secondary Structure 2D View** ().

This will open a view similar to the one shown in figure 24.9.



Secondary structure: ΔG = -9,7kcal/mol

Secondary Structure 2D View Settings

Sequence layout

Residue coloring

Find

Text format

Secondary structure

□ Follow structure selection

Layout strategy:

○ Auto

● Proportional

○ Even spread

Reset layout

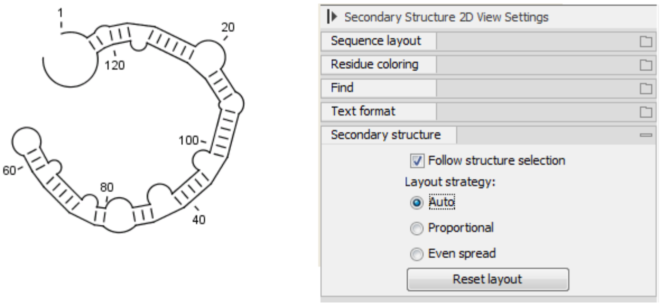
Figure 24.9: *The secondary structure view of an RNA sequence zoomed in.*

Like the normal sequence view, you can use **Zoom in** () and **Zoom out** (). Zooming in will reveal the residues of the structure as shown in figure 24.9. For large structures, zooming out will give you an overview of the whole structure.

Side Panel settings

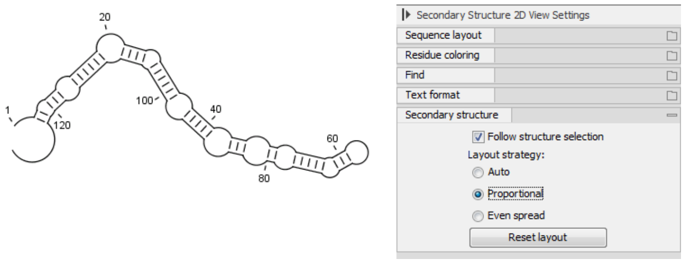
The settings in the **Side Panel** are a subset of the settings in the normal sequence view described in section 14.2.1. However, there are two additional groups of settings unique to the secondary structure 2D view: **Secondary structure**.

- **Follow structure selection.** This setting pertains to the connection between the structures in the secondary structure table (). If this option is checked, the structure displayed in the secondary structure 2D view will follow the structure selections made in this table. See section 24.2.2 for more information.
- **Layout strategy**. Specify the strategy used for the layout of the structure. In addition to these strategies, you can also modify the layout manually as explained in the next section.
  - **Auto.** The layout is adjusted to minimize overlapping structure elements [Han et al., 1999]. This is the default setting (see figure 24.10).
  - **Proportional.** Arc lengths are proportional to the number of residues (see figure 24.11). Nothing is done to prevent overlap.
  - **Even spread.** Stems are spread evenly around loops as shown in figure 24.12.
- **Reset layout.** If you have manually modified the layout of the structure, clicking this button will reset the structure to the way it was laid out when it was created.



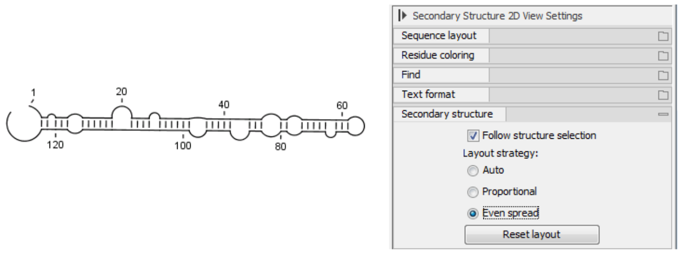
Secondary Structure 2D View Settings
Sequence layout
Residue coloring
Find
Text format
Secondary structure
☑ Follow structure selection
Layout strategy:
● Auto
○ Proportional
○ Even spread
Reset layout

Figure 24.10: *Auto layout. Overlaps are minimized.*



Secondary Structure 2D View Settings
Sequence layout
Residue coloring
Find
Text format
Secondary structure
☑ Follow structure selection
Layout strategy:
○ Auto
● Proportional
○ Even spread
Reset layout

Figure 24.11: *Proportional layout. Length of the arc is proportional to the number of residues in the arc.*



Secondary Structure 2D View Settings
Sequence layout
Residue coloring
Find
Text format
Secondary structure
☑ Follow structure selection
Layout strategy:
○ Auto
○ Proportional
● Even spread
Reset layout

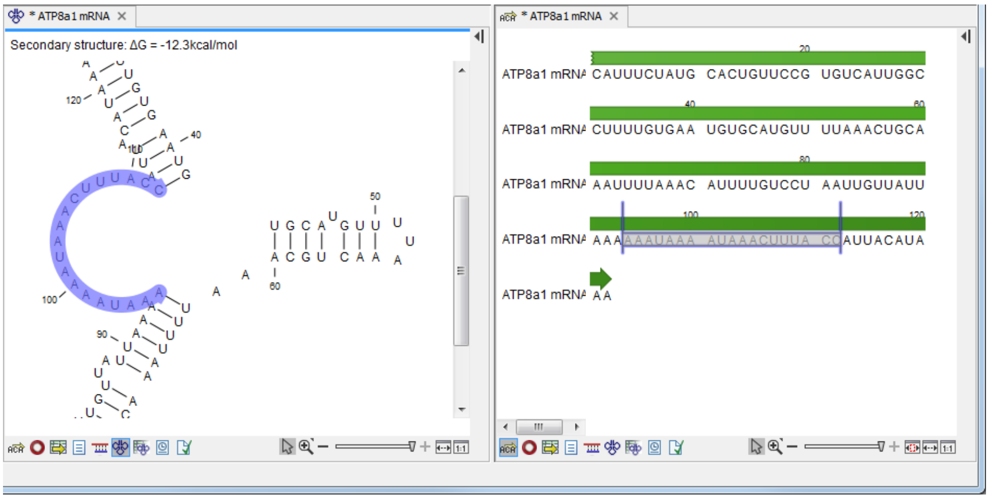
Figure 24.12: *Even spread. Stems are spread evenly around loops.*

Selecting and editing

When you are in **Selection mode** (), you can select parts of the structure like in a normal sequence view:

Press down the mouse button where the selection should start | move the mouse cursor to where the selection should end | release the mouse button

One of the advantages of the secondary structure 2D view is that it is integrated with other views of the same sequence. This means that any selection made in this view will be reflected in other views (see figure 24.13).



```
ATP8a1 mRN CAUUUCUAUG CACUGUUCCG UGUCAUUGGC
ATP8a1 mRN CUUUUGUGAA UGUGCAUGUU UUAAACUGCA
ATP8a1 mRN AAUUUUAAAC AUUUUGUCCU AAUUGUUAUU
ATP8a1 mRN AAAAAAUAAA AUAAACUUUA CCAUUACAUA
ATP8a1 mRN AA
```

Figure 24.13: *A split view of the secondary structure view and a linear sequence view.*

If you make a selection in another sequence view, this will will also be reflected in the secondary structure view.

The *CLC Main Workbench* seeks to produce a layout of the structure where none of the elements overlap. However, it may be desirable to manually edit the layout of a structure for ease of understanding or for the purpose of publication.

To edit a structure, first select the **Pan** (✋) mode in the Tool bar (right-click on the zoom icon below the View Area). Now place the mouse cursor on the opening of a stem, and a visual indication of the anchor point for turning the substructure will be shown (see figure 24.14).

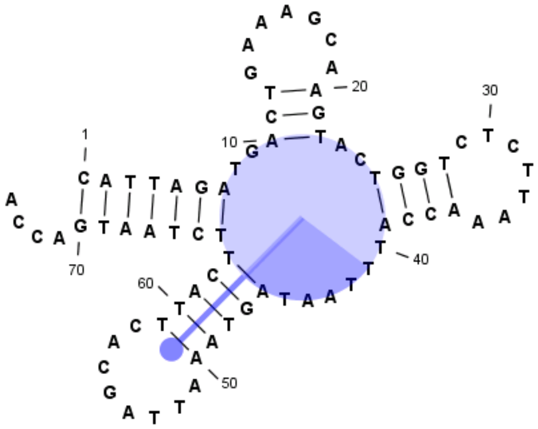


Figure 24.14: *The blue circle represents the anchor point for rotating the substructure.*

Click and drag to rotate the part of the structure represented by the line going from the anchor point. In order to keep the bases in a relatively sequential arrangement, there is a restriction on how much the substructure can be rotated. The highlighted part of the circle represents the angle where rotating is allowed.

In figure 24.15, the structure shown in figure 24.14 has been modified by dragging with the mouse.

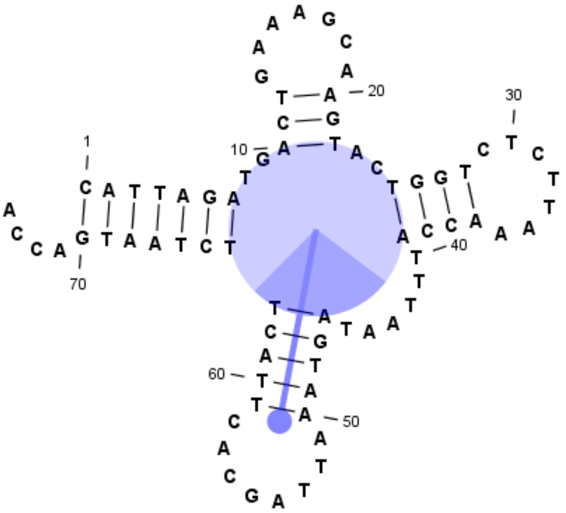


Figure 24.15: *The structure has now been rotated.*

Press **Reset layout** in the **Side Panel** to reset the layout to the way it looked when the structure was predicted.

24.2.2 Tabular view of structures and energy contributions

There are three main reasons to use the **Secondary structure table**:

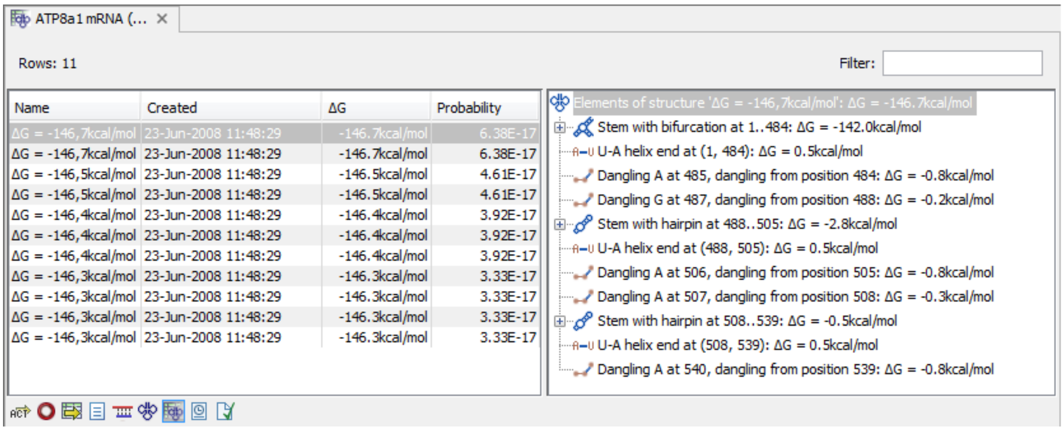
- If more than one structure is predicted (see section 24.1), the table provides an overview of all the structures which have been predicted.
- With multiple structures you can use the table to determine which structure should be displayed in the Secondary structure 2D view (see section 24.2.1).
- The table contains a hierarchical display of the elements in the structure with detailed information about each element's energy contribution.

To show the secondary structure table of an already open sequence, click the **Show Secondary Structure Table** () button at the bottom of the sequence view.

If the sequence is not open, click **Show** () and select **Secondary Structure Table** ().

This will open a view similar to the one shown in figure 24.16.

On the left side, all computed structures are listed with the information about structure name, when the structure was created, the free energy of the structure and the probability of the structure if the partition function was calculated. Selecting a row (equivalent: a structure) will display a tree of the contained substructures with their contributions to the total structure free energy. Each substructure contains a union of nested structure elements and other substructures (see a detailed description of the different structure elements in section 24.5.2). Each substructure



Rows: 11

Filter:

Name	Created	ΔG	Probability
ΔG = -146,7kcal/mol	23-Jun-2008 11:48:29	-146.7kcal/mol	6.38E-17
ΔG = -146,7kcal/mol	23-Jun-2008 11:48:29	-146.7kcal/mol	6.38E-17
ΔG = -146,5kcal/mol	23-Jun-2008 11:48:29	-146.5kcal/mol	4.61E-17
ΔG = -146,5kcal/mol	23-Jun-2008 11:48:29	-146.5kcal/mol	4.61E-17
ΔG = -146,4kcal/mol	23-Jun-2008 11:48:29	-146.4kcal/mol	3.92E-17
ΔG = -146,4kcal/mol	23-Jun-2008 11:48:29	-146.4kcal/mol	3.92E-17
ΔG = -146,4kcal/mol	23-Jun-2008 11:48:29	-146.4kcal/mol	3.92E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17

Elements of structure 'ΔG = -146,7kcal/mol': ΔG = -146.7kcal/mol
- Stem with bifurcation at 1..484: ΔG = -142.0kcal/mol
- U-A helix end at (1, 484): ΔG = 0.5kcal/mol
- Dangling A at 485, dangling from position 484: ΔG = -0.8kcal/mol
- Dangling G at 487, dangling from position 488: ΔG = -0.2kcal/mol
- Stem with hairpin at 488..505: ΔG = -2.8kcal/mol
- U-A helix end at (488, 505): ΔG = 0.5kcal/mol
- Dangling A at 506, dangling from position 505: ΔG = -0.8kcal/mol
- Dangling A at 507, dangling from position 508: ΔG = -0.3kcal/mol
- Stem with hairpin at 508..539: ΔG = -0.5kcal/mol
- U-A helix end at (508, 539): ΔG = 0.5kcal/mol
- Dangling A at 540, dangling from position 539: ΔG = -0.8kcal/mol

Figure 24.16: *The secondary structure table with the list of structures to the left, and to the right the substructures of the selected structure.*

contributes a free energy given by the sum of its nested substructure energies and energies of its nested structure elements.

The substructure elements to the right are ordered after their occurrence in the sequence; they are described by a region (the sequence positions covered by this substructure) and an energy contribution. Three examples of mixed substructure elements are "Stem base pairs", "Stem with bifurcation" and "Stem with hairpin".

The "Stem base pairs"-substructure is simply a union of stacking elements. It is given by a joined set of base pair positions and an energy contribution displaying the sum of all stacking element-energies.

The "Stem with bifurcation"-substructure defines a substructure enclosed by a specified base pair with and with energy contribution ΔG. The substructure contains a "Stem base pairs"-substructure and a nested bifurcated substructure (multi loop). Also bulge and interior loops can occur separating stem regions.

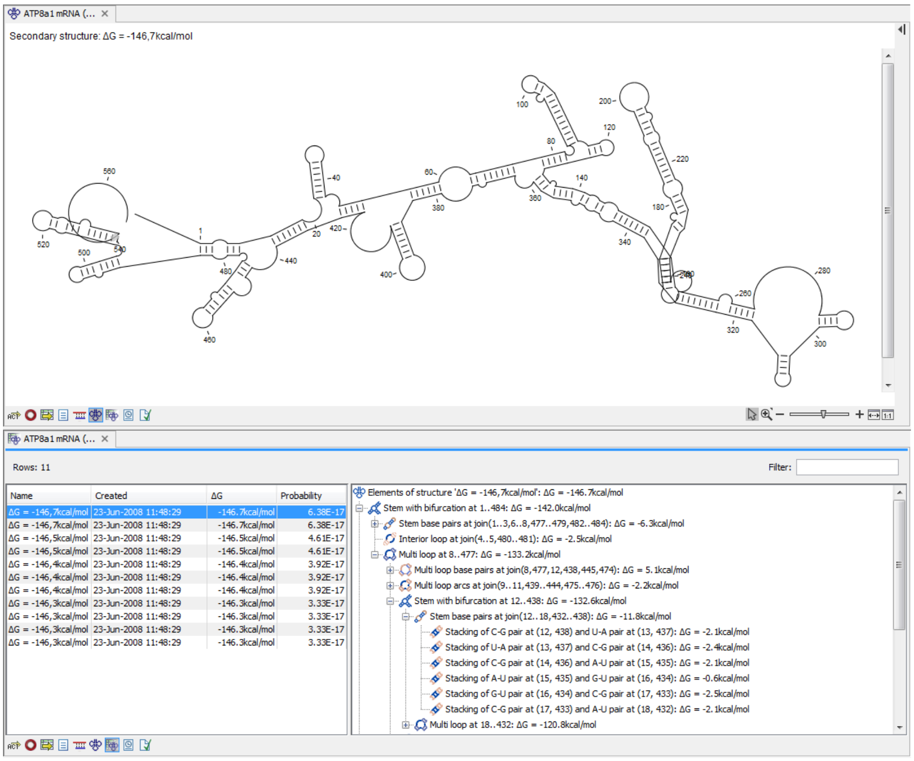
The "Stem with hairpin"-substructure defines a substructure starting at a specified base pair with an enclosed substructure-energy given by ΔG. The substructure contains a "Stem base pairs"-substructure and a hairpin loop. Also bulge and interior loops can occur, separating stem regions.

In order to describe the tree ordering of different substructures, we use an example as a starting point (see figure 24.17).

The structure is a (disjoint) nested union of a "Stem with bifurcation"-substructure and a dangling nucleotide. The nested substructure energies add up to the total energy. The "Stem with bifurcation"-substructure is again a (disjoint) union of a "Stem base pairs"-substructure joining position *1-7* with *64-70* and a multi loop structure element opened at base pair*(7,64)*. To see these structure elements, simply expand the "Stem with bifurcation" node (see figure 24.18).

The multi loop structure element is a union of three "Stem with hairpin"-substructures and contributions to the multi loop opening considering multi loop base pairs and multi loop arcs.

Selecting an element in the table to the right will make a corresponding selection in the **Show Secondary Structure 2D View** (☘) if this is also open and if the "Follow structure selection" has been set in the editors side panel. In figure 24.18 the "Stem with bifurcation" is selected in the table, and this part of the structure is high-lighted in the Secondary Structure 2D view.



Secondary structure: ΔG = -146,7kcal/mol

Rows: 11

Filter:

Name	Created	ΔG	Probability
ΔG = -146,7kcal/mol	23-Jun-2008 11:48:29	-146.7kcal/mol	6.38E-17
ΔG = -146,7kcal/mol	23-Jun-2008 11:48:29	-146.7kcal/mol	6.38E-17
ΔG = -146,5kcal/mol	23-Jun-2008 11:48:29	-146.5kcal/mol	4.61E-17
ΔG = -146,5kcal/mol	23-Jun-2008 11:48:29	-146.5kcal/mol	4.61E-17
ΔG = -146,4kcal/mol	23-Jun-2008 11:48:29	-146.4kcal/mol	3.92E-17
ΔG = -146,4kcal/mol	23-Jun-2008 11:48:29	-146.4kcal/mol	3.92E-17
ΔG = -146,4kcal/mol	23-Jun-2008 11:48:29	-146.4kcal/mol	3.92E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17

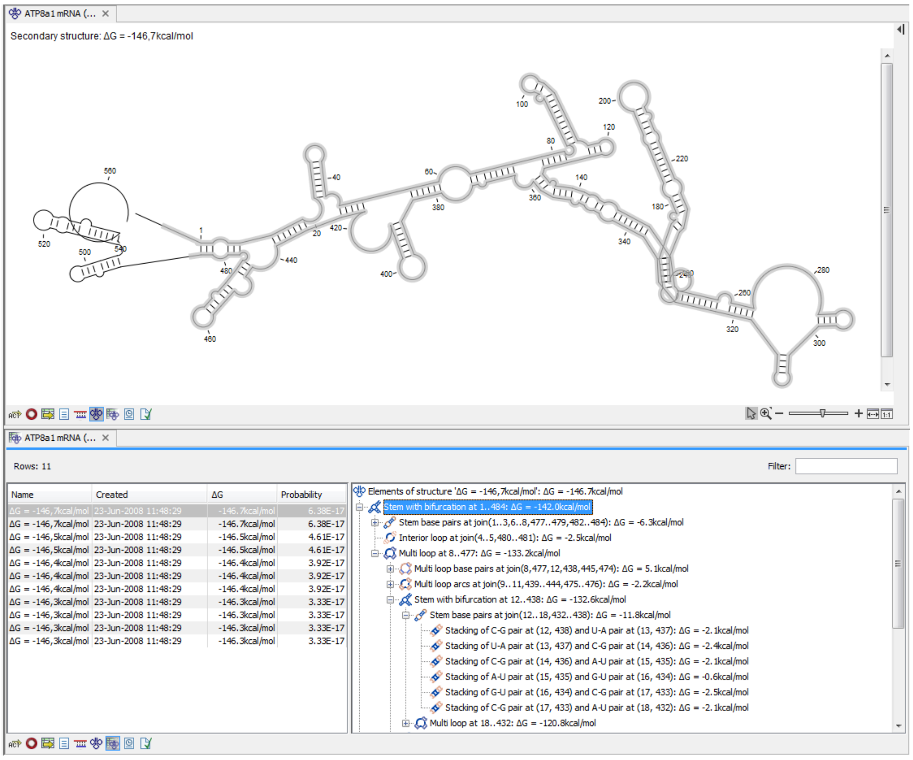
```
Elements of structure 'ΔG = -146,7kcal/mol': ΔG = -146.7kcal/mol
  Stem with bifurcation at 1..484: ΔG = -142.0kcal/mol
    Stem base pairs at join(1..3,6..8,477..479,482..484): ΔG = -6.3kcal/mol
    Interior loop at join(4..5,480..481): ΔG = -2.5kcal/mol
    Multi loop at 8..477: ΔG = -133.2kcal/mol
      Multi loop base pairs at join(8,477,12,438,445,474): ΔG = 5.1kcal/mol
      Multi loop arcs at join(9..11,439..444,475..476): ΔG = -2.2kcal/mol
      Stem with bifurcation at 12..438: ΔG = -132.6kcal/mol
        Stem base pairs at join(12..18,432..438): ΔG = -11.8kcal/mol
          Stacking of C-G pair at (12, 438) and U-A pair at (13, 437): ΔG = -2.1kcal/mol
          Stacking of U-A pair at (13, 437) and C-G pair at (14, 436): ΔG = -2.4kcal/mol
          Stacking of C-G pair at (14, 436) and A-U pair at (15, 435): ΔG = -2.1kcal/mol
          Stacking of A-U pair at (15, 435) and G-U pair at (16, 434): ΔG = -0.6kcal/mol
          Stacking of G-U pair at (16, 434) and C-G pair at (17, 433): ΔG = -2.5kcal/mol
          Stacking of C-G pair at (17, 433) and A-U pair at (18, 432): ΔG = -2.1kcal/mol
        Multi loop at 18..432: ΔG = -120.8kcal/mol
```

Figure 24.17: *A split view showing a structure table to the right and the secondary structure 2D view to the left.*

The correspondence between the table and the structure editor makes it easy to inspect the thermodynamic details of the structure while keeping a visual overview as shown in the above figures.

Handling multiple structures The table to the left offers a number of tools for working with structures. Select a structure, right-click, and the following menu items will be available:

- **Open Secondary Structure in 2D View ( ).** This will open the selected structure in the Secondary structure 2D view.
- **Annotate Sequence with Secondary Structure.** This will add the structure elements as annotations to the sequence. Note that existing structure annotations will be removed.
- **Rename Secondary Structure.** This will allow you to specify a name for the structure to be displayed in the table.
- **Delete Secondary Structure.** This will delete the selected structure.
- **Delete All Secondary Structures.** This will delete all the selected structures. Note that once you save and close the view, this operation is irreversible. As long as the view is open, you can **Undo** ( ) the operation.



Secondary structure: ΔG = -146,7kcal/mol

ATP8a1 mRNA (...

Rows: 11

Filter:

Name	Created	ΔG	Probability
ΔG = -146,7kcal/mol	23-Jun-2008 11:48:29	-146.7kcal/mol	6.38E-17
ΔG = -146,7kcal/mol	23-Jun-2008 11:48:29	-146.7kcal/mol	6.38E-17
ΔG = -146,5kcal/mol	23-Jun-2008 11:48:29	-146.5kcal/mol	4.61E-17
ΔG = -146,5kcal/mol	23-Jun-2008 11:48:29	-146.5kcal/mol	4.61E-17
ΔG = -146,4kcal/mol	23-Jun-2008 11:48:29	-146.4kcal/mol	3.92E-17
ΔG = -146,4kcal/mol	23-Jun-2008 11:48:29	-146.4kcal/mol	3.92E-17
ΔG = -146,4kcal/mol	23-Jun-2008 11:48:29	-146.4kcal/mol	3.92E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17
ΔG = -146,3kcal/mol	23-Jun-2008 11:48:29	-146.3kcal/mol	3.33E-17

```
Elements of structure 'ΔG = -146,7kcal/mol': ΔG = -146.7kcal/mol
⊟ Stem with bifurcation at 1..484: ΔG = -142.0kcal/mol
    ⊞ Stem base pairs at join(1..3,6..8,477..479,482..484): ΔG = -6.3kcal/mol
      Interior loop at join(4..5,480..481): ΔG = -2.5kcal/mol
    ⊟ Multi loop at 8..477: ΔG = -133.2kcal/mol
        ⊞ Multi loop base pairs at join(8,477,12,438,445,474): ΔG = 5.1kcal/mol
        ⊞ Multi loop arcs at join(9..11,439..444,475..476): ΔG = -2.2kcal/mol
        ⊟ Stem with bifurcation at 12..438: ΔG = -132.6kcal/mol
            ⊟ Stem base pairs at join(12..18,432..438): ΔG = -11.8kcal/mol
                  Stacking of C-G pair at (12, 438) and U-A pair at (13, 437): ΔG = -2.1kcal/mol
                  Stacking of U-A pair at (13, 437) and C-G pair at (14, 436): ΔG = -2.4kcal/mol
                  Stacking of C-G pair at (14, 436) and A-U pair at (15, 435): ΔG = -2.1kcal/mol
                  Stacking of A-U pair at (15, 435) and G-U pair at (16, 434): ΔG = -0.6kcal/mol
                  Stacking of G-U pair at (16, 434) and C-G pair at (17, 433): ΔG = -2.5kcal/mol
                  Stacking of C-G pair at (17, 433) and A-U pair at (18, 432): ΔG = -2.1kcal/mol
            ⊞ Multi loop at 18..432: ΔG = -120.8kcal/mol
```

Figure 24.18: *Now the "Stem with bifurcation" node has been selected in the table and a corresponding selection has been made in the view of the secondary structure to the left.*

24.2.3 Symbolic representation in sequence view

In the **Side Panel** of normal sequence views (ACT), you will find an extra group under **Nucleotide info** called **Secondary Structure**. This is used to display a symbolic representation of the secondary structure along the sequence (see figure 24.19).

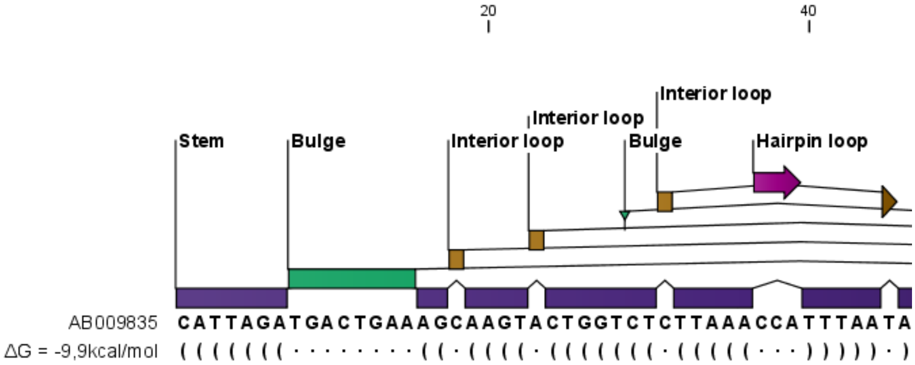


Figure 24.19: *The secondary structure visualized below the sequence and with annotations shown above.*

The following options can be set:

- **Show all structures.** If more than one structure is predicted, this option can be used if all the structures should be displayed.

- **Show first.** If not all structures are shown, this can be used to determine the number of structures to be shown.
- **Sort by.** When you select to display e.g. four out of eight structures, this option determines which the "first four" should be.
    - Sort by ΔG.
    - Sort by name.
    - Sort by time of creation.

  If these three options do not provide enough control, you can rename the structures in a meaningful alphabetical way so that you can use the "name" to display the desired ones.
- **Base pair symbol.** How a base pair should be represented (see figure 24.19).
- **Unpaired symbol.** How bases which are not part of a base pair should be represented (see figure 24.19).
- **Height.** When you zoom out, this option determines the height of the symbols as shown in figure 24.20 (when zoomed in, there is no need for specifying the height).
- **Base pair probability.** See section 24.2.4 below).

When you zoom in and out, the appearance of the symbols change. In figure 24.19, the view is zoomed in. In figure 24.20 you see the same sequence zoomed out to fit the width of the sequence.

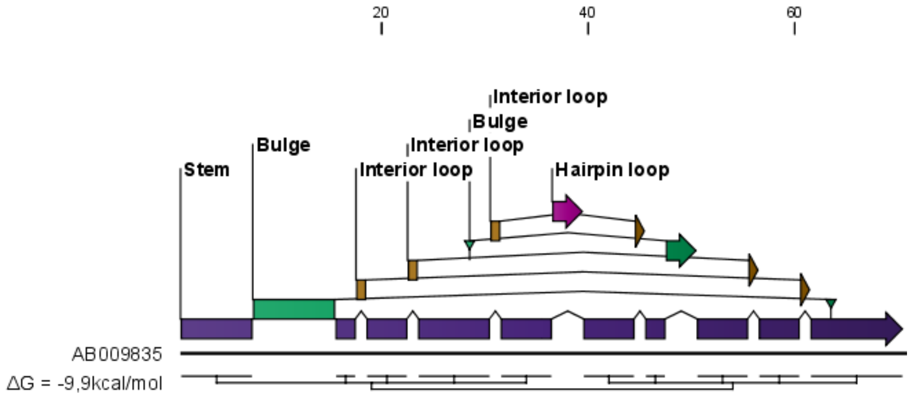


Figure 24.20: *The secondary structure visualized below the sequence and with annotations shown above. The view is zoomed out to fit the width of the sequence.*

24.2.4 Probability-based coloring

In the **Side Panel** of both linear and secondary structure 2D views, you can choose to color structure symbols and sequence residues according to the probability of base pairing / not base pairing, as shown in figure 24.4.

In the linear sequence view (), this is found in **Nucleotide info** under **Secondary structure**, and in the secondary structure 2D view (), it is found under **Residue coloring**.

For both paired and unpaired bases, you can set the foreground color and the background color to a gradient with the color at the left side indicating a probability of 0, and the color at the right side indicating a probability of 1.

Note that you have to **Zoom to 100%** (1:1) in order to see the coloring.

24.3 Evaluate structure hypothesis

Hypotheses about an RNA structure can be tested using *CLC Main Workbench*. A structure hypothesis *H* is formulated using the structural constraint annotations described in section 24.1.2. By adding several annotations complex structural hypotheses can be formulated (see 24.21).

Given the set *S* of all possible structures, only a subset of these S_H will comply with the formulated hypotheses. We can now find the probability of *H* as:

$$P(H) = \frac{\sum\limits_{s_H \in S_H} P(s_H)}{\sum\limits_{s \in S} P(s)} = \frac{PF_H}{PF_{\text{full}}},$$

where PF_H is the partition function calculated for all structures permissible by *H* (S_H) and PF_{full} is the full partition function. Calculating the probability can thus be done with two passes of the partition function calculation, one with structural constraints, and one without. 24.21.

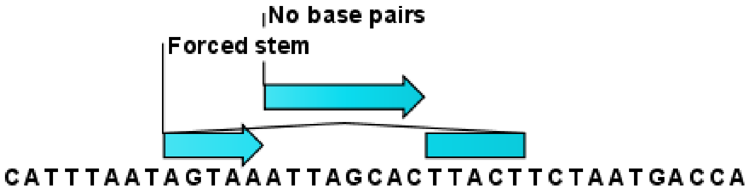


Figure 24.21: *Two constraints defining a structural hypothesis.*

24.3.1 Selecting sequences for evaluation

Start the **Evaluate Structure Hypothesis** tool by going to:

Tools | **RNA Structure** ( )| **Evaluate Structure Hypothesis** ( )

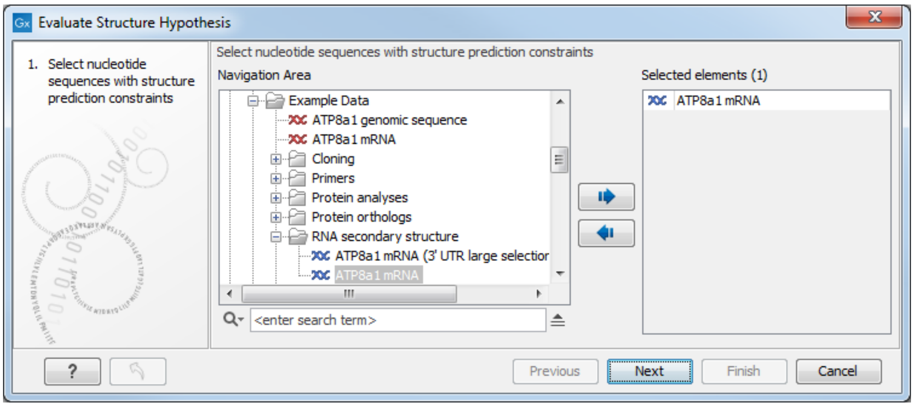
This opens the dialog shown in figure 24.22.

If you had selected sequences before running the tool, those sequences will be listed in the **Selected Elements** pane of the dialog. Use the arrows to add or remove sequences or sequence lists from the selected elements. Note that the selected sequences must contain a structure hypothesis in the form of manually added constraint annotations.

Click **Next** to adjust evaluation parameters (see figure 24.23).

The partition function algorithm includes a number of advanced options:

- **Avoid isolated base pairs**. The algorithm filters out isolated base pairs (i.e. stems of length 1).



Evaluate Structure Hypothesis

1. Select nucleotide sequences with structure prediction constraints

Select nucleotide sequences with structure prediction constraints

Navigation Area

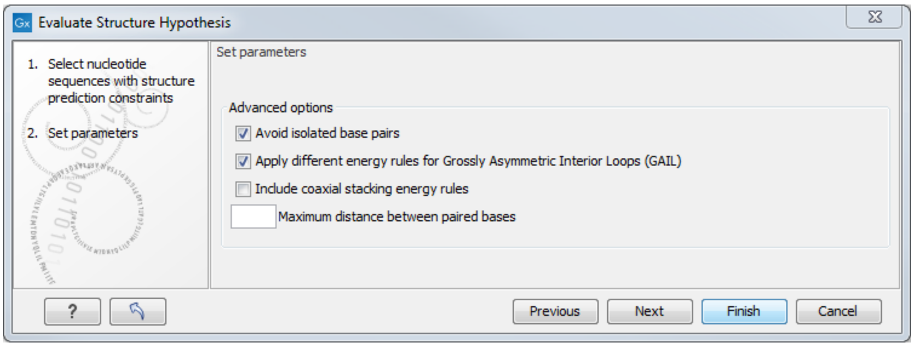
- Example Data
  - ATP8a1 genomic sequence
  - ATP8a1 mRNA
  - Cloning
  - Primers
  - Protein analyses
  - Protein orthologs
  - RNA secondary structure
    - ATP8a1 mRNA (3' UTR large selectior
    - ATP8a1 mRNA

<enter search term>

Selected elements (1)

ATP8a1 mRNA

Figure 24.22: *Selecting RNA or DNA sequences for evaluating structure hypothesis.*



Evaluate Structure Hypothesis

1. Select nucleotide sequences with structure prediction constraints
2. Set parameters

Set parameters

Advanced options

☑ Avoid isolated base pairs

☑ Apply different energy rules for Grossly Asymmetric Interior Loops (GAIL)

□ Include coaxial stacking energy rules

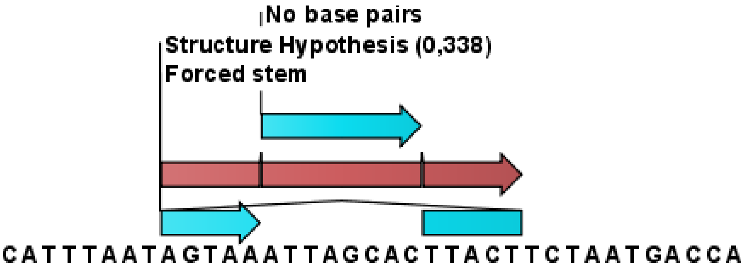
Maximum distance between paired bases

Figure 24.23: *Adjusting parameters for hypothesis evaluation.*

- **Apply different energy rules for Grossly Asymmetric Interior Loops (GAIL).** Compute the minimum free energy applying different rules for Grossly Asymmetry Interior Loops (GAIL). A Grossly Asymmetry Interior Loop (GAIL) is an interior loop that is $1 \times n$ or $n \times 1$ where $n > 2$ (see http://mfold.rna.albany.edu/doc/mfold-manual/node5.php)
- **Include coaxial stacking energy rules.** Include free energy increments of coaxial stacking for adjacent helices [Mathews et al., 2004].

24.3.2 Probabilities

After evaluation of the structure hypothesis an annotation is added to the input sequence. This annotation covers the same region as the annotations that constituted the hypothesis and contains information about the probability of the evaluated hypothesis (see figure 24.24).



No base pairs
Structure Hypothesis (0,338)
Forced stem
CATTTAATAGTAAATTAGCACTTACTTCTAATGACCA

Figure 24.24: *This hypothesis has a probability of 0.338 as shown in the annotation.*

24.4 Structure scanning plot

In *CLC Main Workbench* it is possible to scan larger sequences for the existence of local conserved RNA structures. The structure scanning approach is similar in spirit to the works of [Workman and Krogh, 1999] and [Clote et al., 2005]. The idea is that if natural selection is operating to maintain a stable local structure in a given region, then the minimum free energy of the region will be markedly lower than the minimum free energy found when the nucleotides of the subsequence are distributed in random order.

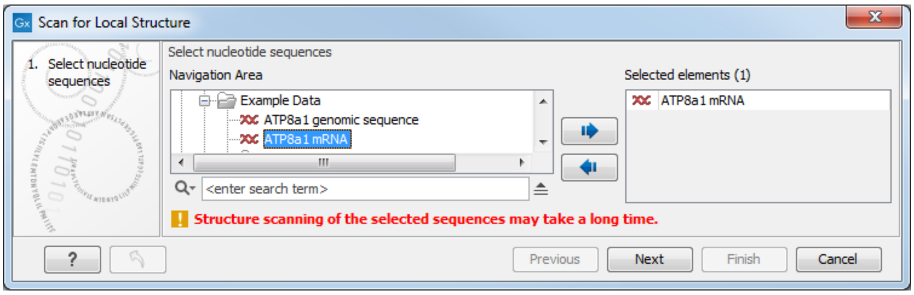
The algorithm works by sliding a window along the sequence. Within the window, the minimum free energy of the subsequence is calculated. To evaluate the significance of the local structure signal its minimum free energy is compared to a background distribution of minimum free energies obtained from shuffled sequences, using Z-scores [Rivas and Eddy, 2000]. The Z-score statistics corresponds to the number of standard deviations by which the minimum free energy of the original sequence deviates from the average energy of the shuffled sequences. For a given Z-score, the statistical significance is evaluated as the probability of observing a more extreme Z-score under the assumption that Z-scores are normally distributed [Rivas and Eddy, 2000].

24.4.1 Selecting sequences for scanning

To run the **Evaluate Structure Hypothesis** tool, go to:

Tools | **RNA Structure** ( )| **Evaluate Structure Hypothesis** ( )

This opens the dialog shown in figure 24.25.



Structure scanning of the selected sequences may take a long time.

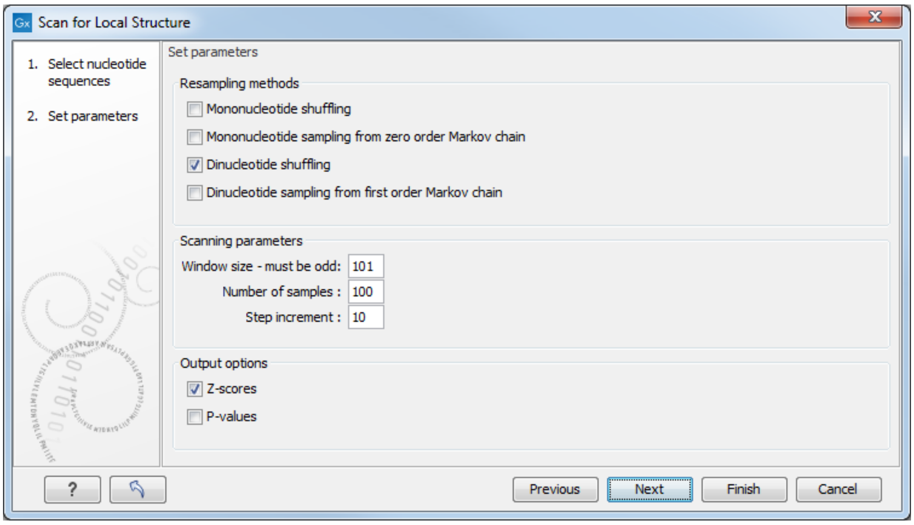
Figure 24.25: *Selecting RNA or DNA sequences for structure scanning.*

If you have selected sequences before running the tool, those sequences will be listed in the **Selected Elements** pane of the dialog. Use the arrows to add or remove sequences or sequence lists from the selected elements.

Click **Next** to adjust scanning parameters (see figure 24.26).

The first group of parameters pertain to the methods of sequence resampling. There are four ways of resampling, all described in detail in [Clote et al., 2005]:

- **Mononucleotide shuffling.** Shuffle method generating a sequence of the exact same mononucleotide frequency
- **Dinucleotide shuffling.** Shuffle method generating a sequence of the exact same dinucleotide frequency
- **Mononucleotide sampling from zero order Markov chain.** Resampling method generating a sequence of the same expected mononucleotide frequency.



Scan for Local Structure

1. Select nucleotide sequences
2. Set parameters

Set parameters

Resampling methods

□ Mononucleotide shuffling

□ Mononucleotide sampling from zero order Markov chain

☑ Dinucleotide shuffling

□ Dinucleotide sampling from first order Markov chain

Scanning parameters

Window size - must be odd: 101

Number of samples : 100

Step increment : 10

Output options

☑ Z-scores

□ P-values

Figure 24.26: *Adjusting parameters for structure scanning.*

- **Dinucleotide sampling from first order Markov chain.** Resampling method generating a sequence of the same expected dinucleotide frequency.

The second group of parameters pertain to the scanning settings and include:

- **Window size.** The width of the sliding window.
- **Number of samples.** The number of times the sequence is resampled to produce the background distribution.
- **Step increment.** Step increment when plotting sequence positions against scoring values.

The third parameter group contains the output options:

- **Z-scores.** Create a plot of Z-scores as a function of sequence position.
- **P-values.** Create a plot of the statistical significance of the structure signal as a function of sequence position.

24.4.2 The structure scanning result

The output of the analysis are plots of Z-scores and probabilities as a function of sequence position. A strong propensity for local structure can be seen as spikes in the graphs (see figure 24.27).

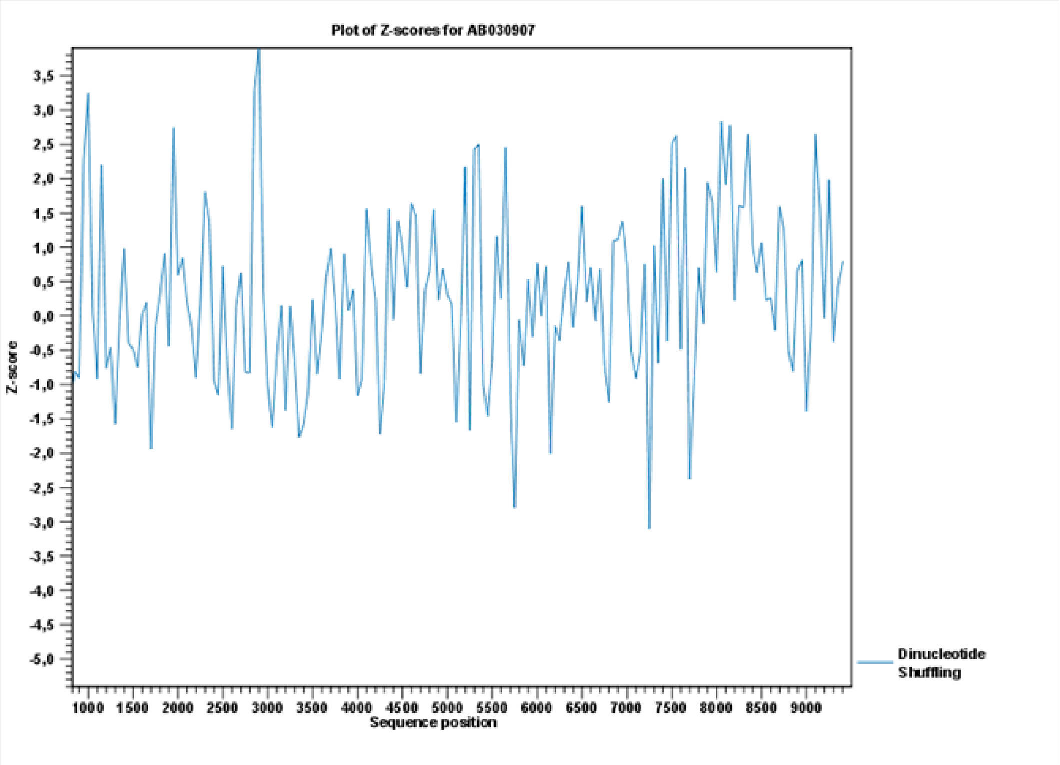


Figure 24.27: *A plot of the Z-scores produced by sliding a window along a sequence.*

24.5 Bioinformatics explained: RNA structure prediction by minimum free energy minimization

RNA molecules are hugely important in the biology of the cell. Besides their rather simple role as an intermediate messenger between DNA and protein, RNA molecules can have a plethora of biologic functions. Well known examples of this are the infrastructural RNAs such as tRNAs, rRNAs and snRNAs, but the existence and functionality of several other groups of non-coding RNAs are currently being discovered. These include micro- (miRNA), small interfering- (siRNA), Piwi interacting- (piRNA) and small modulatory RNAs (smRNA) [Costa, 2007].

A common feature of many of these non-coding RNAs is that the molecular structure is important for the biological function of the molecule.

Ideally, biological function is best interpreted against a 3D structure of an RNA molecule. However, 3D structure determination of RNA molecules is time-consuming, expensive, and difficult [Shapiro et al., 2007] and there is therefore a great disparity between the number of known RNA sequences and the number of known RNA 3D structures.

However, as it is the case for proteins, RNA tertiary structures can be characterized by secondary structural elements. These are defined by hydrogen bonds within the molecule that form several recognizable "domains" of secondary structure like stems, hairpin loops, bulges and internal loops (see below). Furthermore, the high degree of base-pair conservation observed in the evolution of RNA molecules shows that a large part of the functional information is actually contained in the secondary structure of the RNA molecule.

Fortunately, RNA secondary structure can be computationally predicted from sequence data allowing researchers to map sequence information to functional information. The subject of this paper is to describe a very popular way of doing this, namely free energy minimization. For an in-depth review of algorithmic details, we refer the reader to Mathews and Turner, 2006.

24.5.1 The algorithm

Consider an RNA molecule and one of its possible structures S_1. In a stable solution there will be an equilibrium between unstructured RNA strands and RNA strands folded into S_1. The propensity of a strand to leave a structure such as S_1 (the stability of S_1), is determined by the free energy change involved in its formation. The structure with the lowest free energy (S_{min}) is the most stable and will also be the most represented structure at equilibrium. The objective of minimum free energy (MFE) folding is therefore to identify S_{min} amongst all possible structures.

In the following, we only consider structures without pseudoknots, i.e. structures that do not contain any non-nested base pairs.

Under this assumption, a sequence can be folded into a single coherent structure or several sequential structures that are joined by unstructured regions. Each of these structures is a union of well described structure elements (see below for a description of these). The free energy for a given structure is calculated by an additive nearest neighbor model. Additive, means that the total free energy of a secondary structure is the sum of the free energies of its individual structural elements. Nearest neighbor, means that the free energy of each structure element depends only on the residues it contains and on the most adjacent Watson-Crick base pairs.

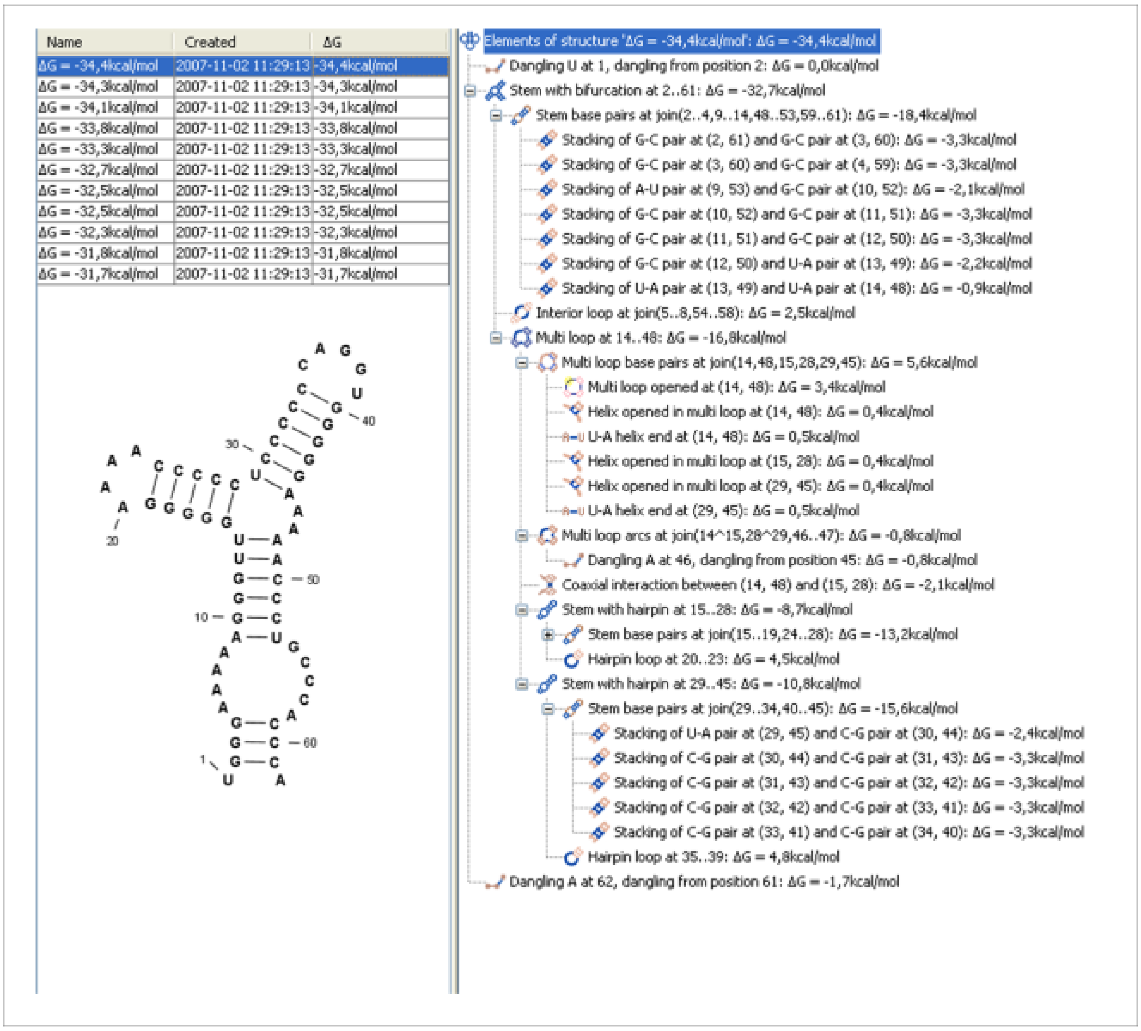
The simplest method to identify S_{min} would be to explicitly generate all possible structures, but it can be shown that the number of possible structures for a sequence grows exponentially with the sequence length [Zuker and Sankoff, 1984] leaving this approach unfeasible. Fortunately, a two step algorithm can be constructed which implicitly surveys all possible structures without explicitly generating the structures [Zuker and Stiegler, 1981]: The first step determines the free energy for each possible sequence fragment starting with the shortest fragments. Here, the lowest free energy for longer fragments can be expediently calculated from the free energies of the smaller sub-sequences they contain. When this process reaches the longest fragment, i.e., the complete sequence, the MFE of the entire molecule is known. The second step is called traceback, and uses all the free energies computed in the first step to determine S_{min} - the exact structure associated with the MFE. Acceptable calculation speed is achieved by using *dynamic programming* where sub-sequence results are saved to avoid recalculation. However, this comes at the price of a higher requirement for computer memory.

The structure element energies that are used in the recursions of these two steps, are derived from empirical calorimetric experiments performed on small molecules see e.g. [Mathews et al., 1999].

Suboptimal structures determination A number of known factors violate the assumptions that are implicit in MFE structure prediction. [Schroeder et al., 1999] and [Chen et al., 2004] have shown experimental indications that the thermodynamic parameters are sequence dependent. Moreover, [Longfellow et al., 1990] and [Kierzek et al., 1999], have demonstrated that some structural elements show non-nearest neighbor effects. Finally, single stranded nucleotides in multi loops are known to influence stability [Mathews and Turner, 2002].

These phenomena can be expected to limit the accuracy of RNA secondary structure prediction by free energy minimization and it should be clear that the predicted MFE structure may deviate somewhat from the actual preferred structure of the molecule. This means that it may be informative to inspect the landscape of suboptimal structures which surround the MFE structure to look for general structural properties which seem to be robust to minor variations in the total free energy of the structure.

An effective procedure for generating a sample of suboptimal structures is given in [Zuker, 1989a]. This algorithm works by going through all possible Watson-Crick base pair in the molecule. For each of these base pairs, the algorithm computes the most optimal structure among all the structures that contain this pair, see figure 24.28.



Name	Created	ΔG
ΔG = -34,4kcal/mol	2007-11-02 11:29:13	-34,4kcal/mol
ΔG = -34,3kcal/mol	2007-11-02 11:29:13	-34,3kcal/mol
ΔG = -34,1kcal/mol	2007-11-02 11:29:13	-34,1kcal/mol
ΔG = -33,8kcal/mol	2007-11-02 11:29:13	-33,8kcal/mol
ΔG = -33,3kcal/mol	2007-11-02 11:29:13	-33,3kcal/mol
ΔG = -32,7kcal/mol	2007-11-02 11:29:13	-32,7kcal/mol
ΔG = -32,5kcal/mol	2007-11-02 11:29:13	-32,5kcal/mol
ΔG = -32,5kcal/mol	2007-11-02 11:29:13	-32,5kcal/mol
ΔG = -32,3kcal/mol	2007-11-02 11:29:13	-32,3kcal/mol
ΔG = -31,8kcal/mol	2007-11-02 11:29:13	-31,8kcal/mol
ΔG = -31,7kcal/mol	2007-11-02 11:29:13	-31,7kcal/mol

- Elements of structure 'ΔG = -34,4kcal/mol': ΔG = -34,4kcal/mol
  - Dangling U at 1, dangling from position 2: ΔG = 0,0kcal/mol
  - Stem with bifurcation at 2..61: ΔG = -32,7kcal/mol
    - Stem base pairs at join(2..4,9..14,48..53,59..61): ΔG = -18,4kcal/mol
      - Stacking of G-C pair at (2, 61) and G-C pair at (3, 60): ΔG = -3,3kcal/mol
      - Stacking of G-C pair at (3, 60) and G-C pair at (4, 59): ΔG = -3,3kcal/mol
      - Stacking of A-U pair at (9, 53) and G-C pair at (10, 52): ΔG = -2,1kcal/mol
      - Stacking of G-C pair at (10, 52) and G-C pair at (11, 51): ΔG = -3,3kcal/mol
      - Stacking of G-C pair at (11, 51) and G-C pair at (12, 50): ΔG = -3,3kcal/mol
      - Stacking of G-C pair at (12, 50) and U-A pair at (13, 49): ΔG = -2,2kcal/mol
      - Stacking of U-A pair at (13, 49) and U-A pair at (14, 48): ΔG = -0,9kcal/mol
    - Interior loop at join(5..8,54..58): ΔG = 2,5kcal/mol
    - Multi loop at 14..48: ΔG = -16,8kcal/mol
      - Multi loop base pairs at join(14,48,15,28,29,45): ΔG = 5,6kcal/mol
        - Multi loop opened at (14, 48): ΔG = 3,4kcal/mol
        - Helix opened in multi loop at (14, 48): ΔG = 0,4kcal/mol
        - U-A helix end at (14, 48): ΔG = 0,5kcal/mol
        - Helix opened in multi loop at (15, 28): ΔG = 0,4kcal/mol
        - Helix opened in multi loop at (29, 45): ΔG = 0,4kcal/mol
        - U-A helix end at (29, 45): ΔG = 0,5kcal/mol
      - Multi loop arcs at join(14^15,28^29,46..47): ΔG = -0,8kcal/mol
        - Dangling A at 46, dangling from position 45: ΔG = -0,8kcal/mol
      - Coaxial interaction between (14, 48) and (15, 28): ΔG = -2,1kcal/mol
      - Stem with hairpin at 15..28: ΔG = -8,7kcal/mol
        - Stem base pairs at join(15..19,24..28): ΔG = -13,2kcal/mol
        - Hairpin loop at 20..23: ΔG = 4,5kcal/mol
      - Stem with hairpin at 29..45: ΔG = -10,8kcal/mol
        - Stem base pairs at join(29..34,40..45): ΔG = -15,6kcal/mol
          - Stacking of U-A pair at (29, 45) and C-G pair at (30, 44): ΔG = -2,4kcal/mol
          - Stacking of C-G pair at (30, 44) and C-G pair at (31, 43): ΔG = -3,3kcal/mol
          - Stacking of C-G pair at (31, 43) and C-G pair at (32, 42): ΔG = -3,3kcal/mol
          - Stacking of C-G pair at (32, 42) and C-G pair at (33, 41): ΔG = -3,3kcal/mol
          - Stacking of C-G pair at (33, 41) and C-G pair at (34, 40): ΔG = -3,3kcal/mol
        - Hairpin loop at 35..39: ΔG = 4,8kcal/mol
  - Dangling A at 62, dangling from position 61: ΔG = -1,7kcal/mol

Figure 24.28: *A number of suboptimal structures have been predicted using* **CLC Main Workbench** *and are listed at the top left. At the right hand side, the structural components of the selected structure are listed in a hierarchical structure and on the left hand side the structure is displayed.*

24.5.2 Structure elements and their energy contribution

In this section, we classify the structure elements defining a secondary structure and describe their energy contribution.

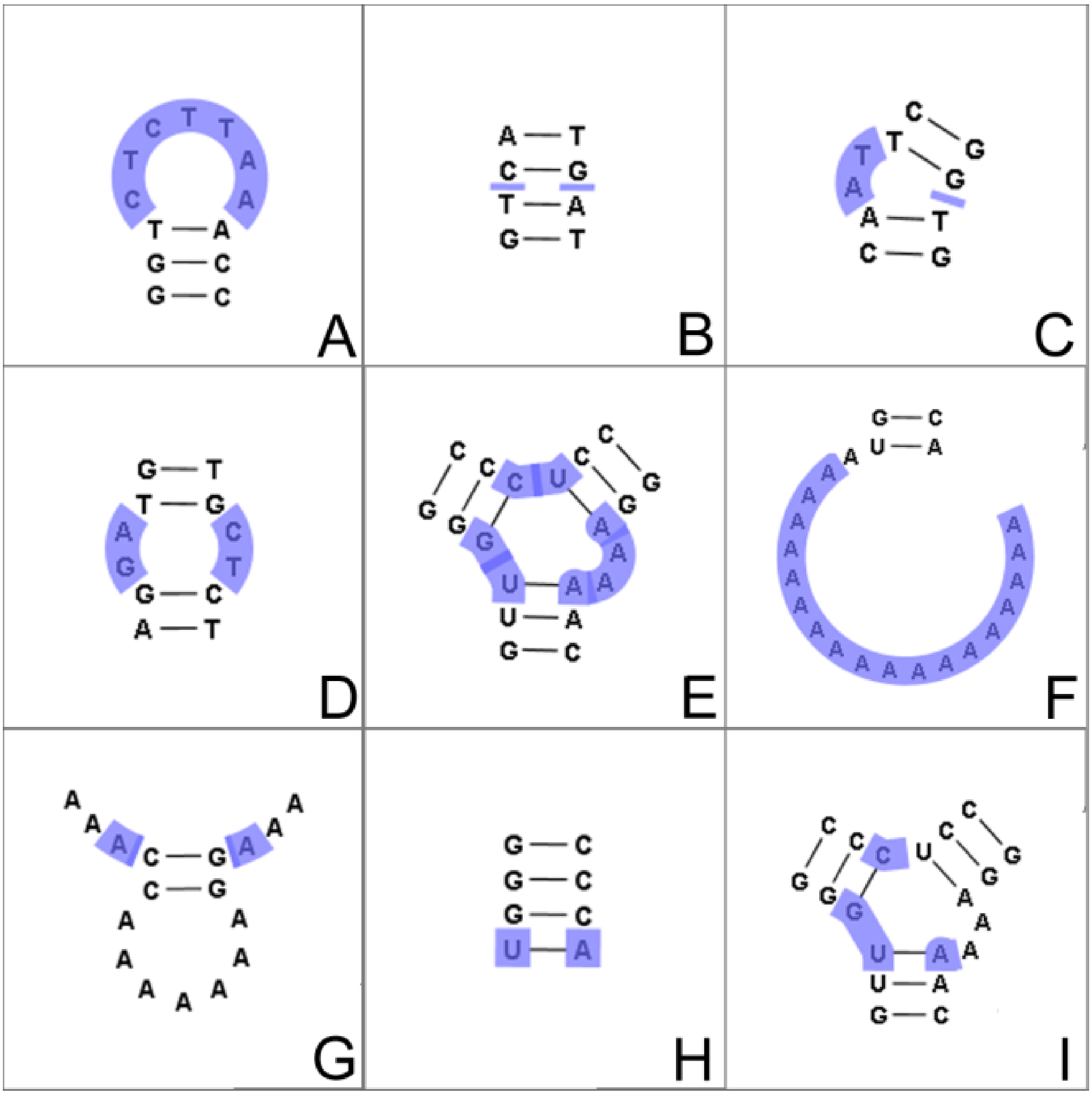


Figure 24.29: *The different structure elements of RNA secondary structures predicted with the free energy minimization algorithm in* **CLC Main Workbench**. *See text for a detailed description.*

Nested structure elements The structure elements involving nested base pairs can be classified by a given base pair and the other base pairs that are nested and *accessible* from this pair. For a more elaborate description we refer the reader to [Sankoff et al., 1983] and [Zuker and Sankoff, 1984].

If the nucleotides with position number (i, j) form a base pair and $i < k, l < j$, then we say that the base pair (k, l) is **accessible** from (i, j) if there is no intermediate base pair (i', j') such that $i < i' < k, l < j' < j$. This means that (k, l) is nested within the pair i, j and there is no other base pair in between.

Using the number of accessible pase pairs, we can define the following distinct structure elements:

1. **Hairpin loop ( )**. A base pair with 0 other accessible base pairs forms a *hairpin loop*. The energy contribution of a hairpin is determined by the length of the unpaired (loop) region

and the two bases adjacent to the closing base pair which is termed a terminal mismatch (see figure 24.29A).

2. A base pair with 1 accessible base pair can give rise to three distinct structure elements:

   - **Stacking of base pairs ( ).** A *stacking* of two consecutive pairs occur if $i' - i = 1 = j - j'$. Only canonical base pairs ($A - U$ or $G - C$ or $G - U$) are allowed (see figure 24.29B). The energy contribution is determined by the type and order of the two base pairs.
   - **Bulge ( ).** A *bulge loop* occurs if $i' - i > 1$ or $j - j' > 1$, but not both. This means that the two base pairs enclose an unpaired region of length 0 on one side and an unpaired region of length ≥ 1 on the other side (see figure 24.29C). The energy contribution of a bulge is determined by the length of the unpaired (loop) region and the two closing base pairs.
   - **Interior loop ( ).**An *interior loop* occurs if both $i' - i > 1$ and $i - j' > 1$ This means that the two base pairs enclose an unpaired region of length ≥ 1 on both sides (see figure 24.29D). The energy contribution of an interior loop is determined by the length of the unpaired (loop) region and the four unpaired bases adjacent to the opening- and the closing base pair.

3. **Multi loop opened ( ).** A base pair with more than two accessible base pairs gives rise to a *multi loop*, a loop from which three or more stems are opened (see figure 24.29E). The energy contribution of a multi loop depends on the number of **Stems opened in multi-loop ( )** that protrude from the loop.

Other structure elements

- A collection of single stranded bases not accessible from any base pair is called an *exterior (or external) loop* (see figure 24.29F). These regions do not contribute to the total free energy.

- **Dangling nucleotide ( ).** A *dangling nucleotide* is a single stranded nucleotide that forms a stacking interaction with an adjacent base pair. A dangling nucleotide can be a $3'$ or $5'$-dangling nucleotide depending on the orientation (see figure 24.29G). The energy contribution is determined by the single stranded nucleotide, its orientation and on the adjacent base pair.

- **Non-GC terminating stem (A–U).** If a base pair other than a G-C pair is found at the end of a stem, an energy penalty is assigned (see figure 24.29H).

- **Coaxial interaction ( ).** Coaxial stacking is a favorable interaction of two stems where the base pairs at the ends can form a stacking interaction. This can occur between stems in a multi loop and between the stems of two different sequential structures. Coaxial stacking can occur between stems with no intervening nucleotides (adjacent stems) and between stems with one intervening nucleotide from each strand (see figure 24.29I). The energy contribution is determined by the adjacent base pairs and the intervening nucleotides.

Experimental constraints A number of techniques are available for probing RNA structures. These techniques can determine individual components of an existing structure such as the existence of a given base pair. It is possible to add such experimental constraints to the secondary structure prediction based on free energy minimization (see figure 24.30) and it has been shown that this can dramatically increase the fidelity of the secondary structure prediction [Mathews and Turner, 2006].

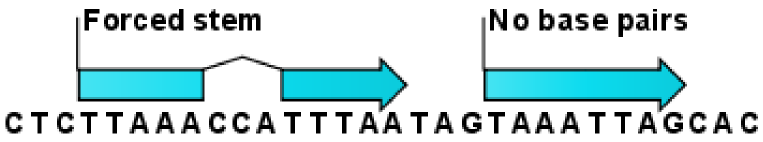


Figure 24.30: *Known structural features can be added as constraints to the secondary structure prediction algorithm in* **CLC Main Workbench***.*

Chapter 25

Expression analysis

Contents

This section focuses on analysing expression data from sources such as microarrays using tools found under the **Expresson Analysis** (**)** folder of the Tools menu. This includes tools for performing quality control of the data, transformation and normalization, statistical analysis to measure differential expression and annotation-based tests. A number of visualization tools such as volcano plots, MA plots, scatter plots, box plots, and heat maps are also available to aid interpretation of the results.

Tools for analysing RNA-Seq and small RNA data are available in the CLC Genomics Workbench.

25.1 Experimental design

Central to expression analysis in the *CLC Main Workbench*is the concept of the **sample**. For microarray data, a sample would typically consist of the expression values from one array. Within a sample, there are a number of **features**, usually genes, and their associated expression levels.

Importing expression data into the Workbench as samples is described in appendix section I.

The first step towards analyzing this expression data is to create an **Experiment**, which contains information about which samples belong to which groups.

25.1.1 Setting up a microarray experiment

To analyze differential expression, the Workbench must know which samples belong to which groups. This information is provided in an **Experiment**. Statistical analyses can then be carried out using the information in the Experiment to investigate differential expression. The Experiment element is also where things like t-test results and clustering information are stored.

The statistics created using this tool is for microarray data, RNA-Seq differential expression are better handled by the tools in the folder **Tools** | **Expression Analysis** (**)**

To set up an experiment:

Tools | **Expression Analysis** (**)**| **Set Up Microarray Experiment** (**)**

Select the samples that you wish to use by double-clicking or selecting and pressing the **Add** () button (see figure 25.1).

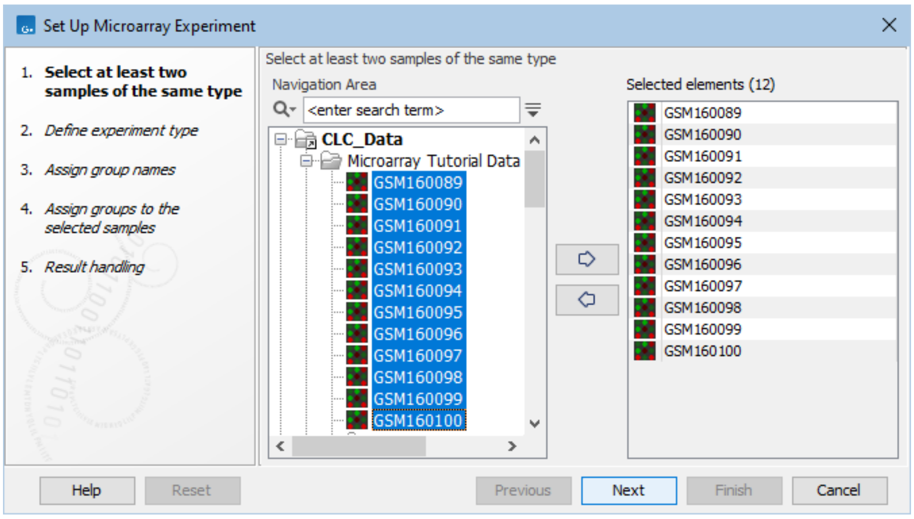
Clicking **Next** shows the dialog in figure 25.2.

Here you define the experiment type and the number of groups in the experiment.

The options are:

- **Experiment.** At the top you can select a two-group experiment, and below you can select a multi-group experiment and define the number of groups.

  Note that you can also specify if the samples are paired. Pairing is relevant if you have samples from the same individual under different conditions, e.g. before and after treatment, or at times 0, 2, and 4 hours after treatment. In this case statistical analysis becomes more efficient if effects of the individuals are taken into account, and comparisons are carried out not simply by considering *raw* group means but by considering these *corrected*



Set Up Microarray Experiment

1. **Select at least two samples of the same type**
2. *Define experiment type*
3. *Assign group names*
4. *Assign groups to the selected samples*
5. *Result handling*

Select at least two samples of the same type

Navigation Area

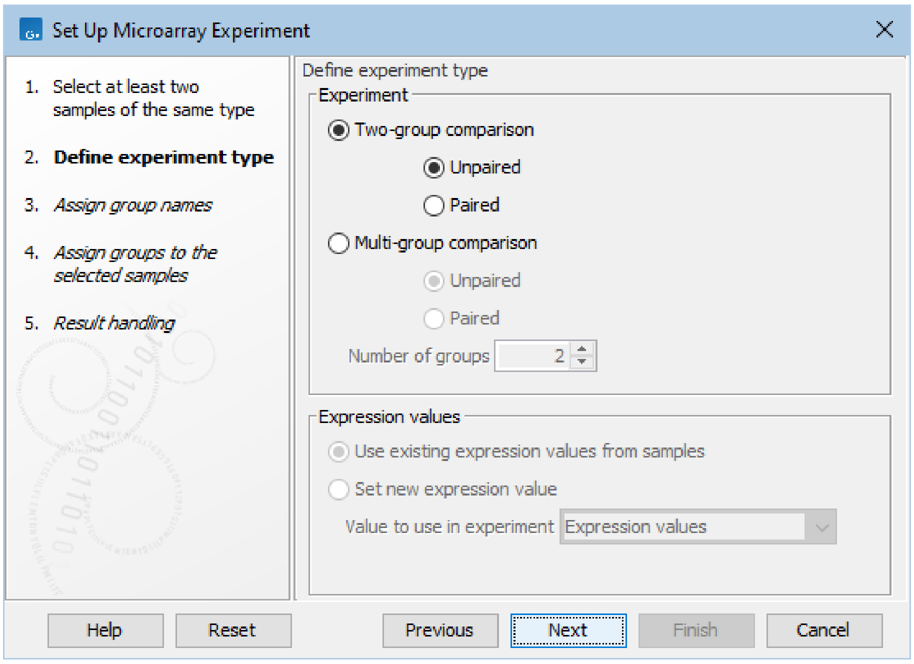
<enter search term>

- CLC_Data
  - Microarray Tutorial Data
    - GSM160089
    - GSM160090
    - GSM160091
    - GSM160092
    - GSM160093
    - GSM160094
    - GSM160095
    - GSM160096
    - GSM160097
    - GSM160098
    - GSM160099
    - GSM160100

Selected elements (12)

- GSM160089
- GSM160090
- GSM160091
- GSM160092
- GSM160093
- GSM160094
- GSM160095
- GSM160096
- GSM160097
- GSM160098
- GSM160099
- GSM160100

Figure 25.1: *Select the samples to use for setting up the experiment.*



Set Up Microarray Experiment

1. Select at least two samples of the same type
2. **Define experiment type**
3. *Assign group names*
4. *Assign groups to the selected samples*
5. *Result handling*

Define experiment type

Experiment

● Two-group comparison

● Unpaired

○ Paired

○ Multi-group comparison

○ Unpaired

○ Paired

Number of groups: 2

Expression values

○ Use existing expression values from samples

○ Set new expression value

Value to use in experiment: Expression values

Figure 25.2: *Defining the number of groups and expression value type.*

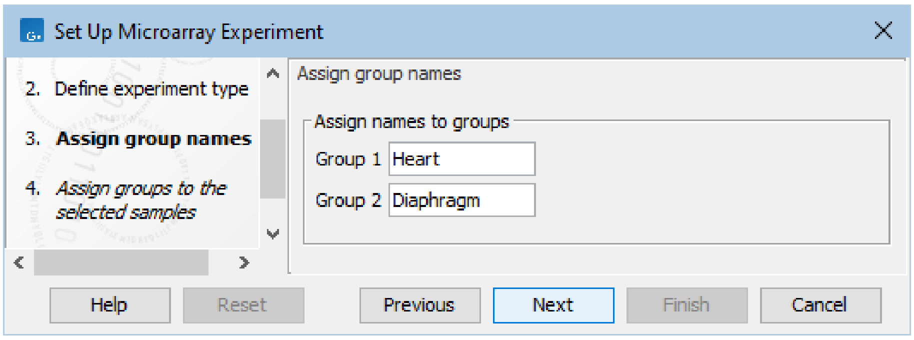
for effects of the individual. If **Paired** is selected, a paired rather than a standard t-test will be carried out for two group comparisons. For multiple group comparisons a repeated measures rather than a standard ANOVA will be used.

- **Expression values.** If you choose to **Set new expression value** you can choose between the following options depending on whether you look at the gene or transcript level:
  - **Genes: Unique exon reads.** The number of reads that match uniquely to the exons (including the exon-exon and exon-intron junctions).
  - **Genes: Unique gene reads.** This is the number of reads that match uniquely to the gene.
  - **Genes: Total exon reads.** Number of reads mapped to this gene that fall entirely within an exon or in exon-exon or exon-intron junctions. As for the "Total gene reads"

this includes both uniquely mapped reads and reads with multiple matches that were assigned to an exon of this gene.

- **Genes: Total gene reads.** This is all the reads that are mapped to this gene, i.e., both reads that map uniquely to the gene and reads that matched to more positions in the reference (but fewer than the "Maximum number of hits for a read" parameter) which were assigned to this gene.
- **Genes: RPKM.** This is the expression value measured in RPKM [Mortazavi et al., 2008]: $RPKM = \frac{\textit{total exon reads}}{\textit{mapped reads(millions)} \times \textit{exon length (KB)}}$. See exact definition below. Even if you have chosen the RPKM values to be used in the **Expression values** column, they will also be stored in a separate column. This is useful to store the RPKM if you switch the expression measure.
- **Transcripts: Unique transcript reads.** This is the number of reads in the mapping for the gene that are uniquely assignable to the transcript. This number is calculated after the reads have been mapped and both single and multi-hit reads from the read mapping may be unique transcript reads.
- **Transcripts: Total transcript reads.** Once the "Unique transcript read's" have been identified and their counts calculated for each transcript, the remaining (non-unique) transcript reads are assigned randomly to one of the transcripts to which they match. The "Total transcript reads" counts are the total number of reads that are assigned to the transcript once this random assignment has been done. As for the random assignment of reads among genes, the random assignment of reads within a gene but among transcripts, is done proportionally to the "unique transcript counts" normalized by transcript length, that is, using the RPKM. Unique transcript counts of 0 are not replaced by 1 for this proportional assignment of non-unique reads among transcripts.
- **Transcripts: RPKM.** The RPKM value for the transcript, that is, the number of reads assigned to the transcript divided by the transcript length and normalized by "Mapped reads" (see below).

Clicking **Next** shows the dialog in figure 25.3.



Set Up Microarray Experiment

2. Define experiment type
3. **Assign group names**
4. *Assign groups to the selected samples*

Assign group names

Assign names to groups

Group 1 Heart

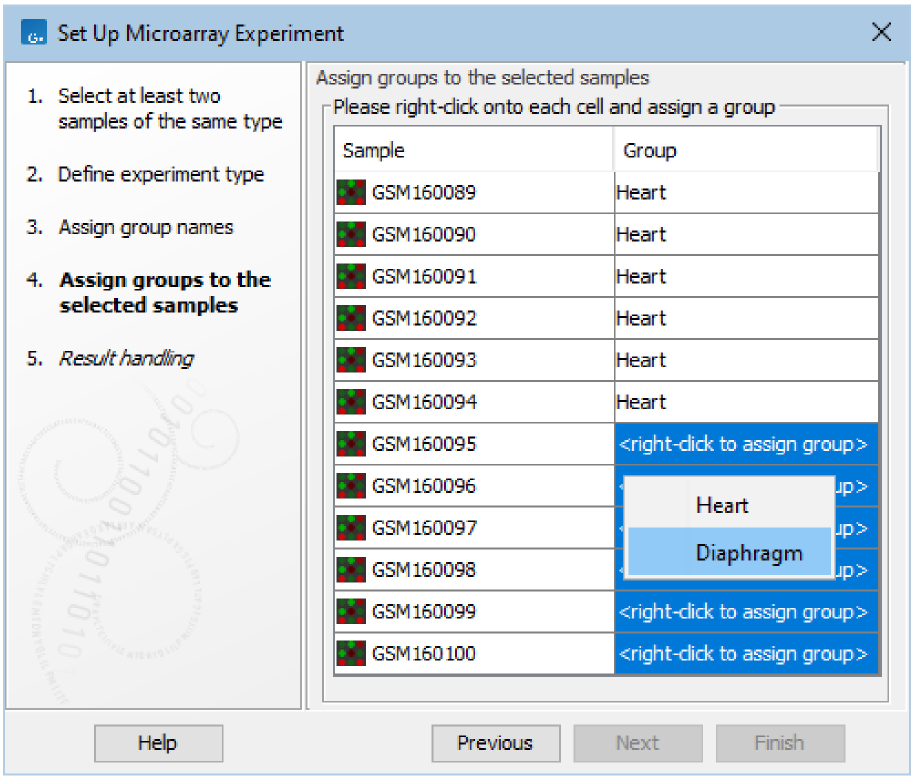
Group 2 Diaphragm

Figure 25.3: *Naming the groups.*

Depending on the number of groups selected in figure 25.2, you will see a list of groups with text fields where you can enter an appropriate name for that group.

For multi-group experiments, if you find out that you have too many groups, click the **Delete** (☒) button. If you need more groups, simply click **Add New Group**.

Click **Next** when you have named the groups, and you will see figure 25.4.


Set Up Microarray Experiment
1. Select at least two samples of the same type
2. Define experiment type
3. Assign group names
4. Assign groups to the selected samples
5. Result handling
Assign groups to the selected samples
Please right-click onto each cell and assign a group
Heart
Diaphragm
Help
Previous
Next
Finish

Sample	Group
GSM160089	Heart
GSM160090	Heart
GSM160091	Heart
GSM160092	Heart
GSM160093	Heart
GSM160094	Heart
GSM160095	<right-click to assign group>
GSM160096	up>
GSM160097	up>
GSM160098	up>
GSM160099	<right-click to assign group>
GSM160100	<right-click to assign group>

Figure 25.4: *Putting the samples into groups.*

This is where you define which group the individual sample belongs to. Simply select one or more samples (by clicking and dragging the mouse), right-click (Ctrl-click on Mac) and select the appropriate group.

Note that the samples are sorted alphabetically based on their names.

If you have chosen **Paired** in figure 25.2, there will be an extra column where you define which samples belong together. Just as when defining the group membership, you select one or more samples, right-click in the pairing column and select a pair.

Click **Finish** to start the tool.

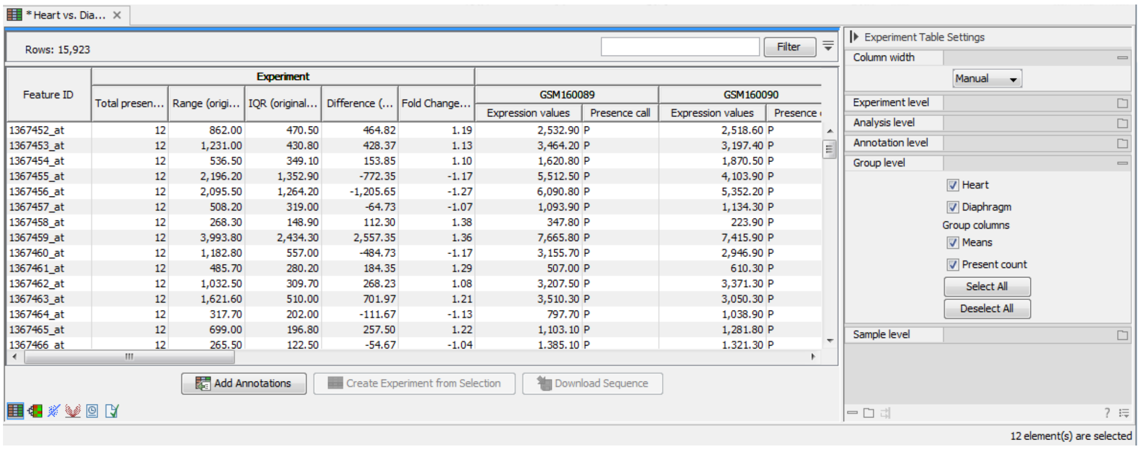
25.1.2 Organization of the experiment table

The resulting experiment includes all the expression values and other information from the samples (the values are copied - the original samples are not affected and can thus be deleted with no effect on the experiment). In addition it includes a number of summaries of the values across all, or a subset of, the samples for each feature. Which values are included is described in the sections below.

When you open it, it is shown in the experiment table (see figure 25.5).

For a general introduction to table features like sorting and filtering, see section 9.

Unlike other tables in *CLC Main Workbench*, the experiment table has a hierarchical grouping of the columns. This is done to reflect the structure of the data in the experiment. The **Side Panel** is divided into a number of groups corresponding to the structure of the table. These are described below. Note that you can customize and save the settings of the **Side Panel** (see section 4.6).



*Heart vs. Dia...

Rows: 15,923

Feature ID	Experiment								
						GSM160089		GSM160090	
	Total presen...	Range (origi...	IQR (original...	Difference (...	Fold Change...	Expression values	Presence call	Expression values	Presence
1367452_at	12	862.00	470.50	464.82	1.19	2,532.90	P	2,518.60	P
1367453_at	12	1,231.00	430.80	428.37	1.13	3,464.20	P	3,197.40	P
1367454_at	12	536.50	349.10	153.85	1.10	1,620.80	P	1,870.50	P
1367455_at	12	2,196.20	1,352.90	-772.35	-1.17	5,512.50	P	4,103.90	P
1367456_at	12	2,095.50	1,264.20	-1,205.65	-1.27	6,090.80	P	5,352.20	P
1367457_at	12	508.20	319.00	-64.73	-1.07	1,093.90	P	1,134.30	P
1367458_at	12	268.30	148.90	112.30	1.38	347.80	P	223.90	P
1367459_at	12	3,993.80	2,434.30	2,557.35	1.36	7,665.80	P	7,415.90	P
1367460_at	12	1,182.80	557.00	-484.73	-1.17	3,155.70	P	2,946.90	P
1367461_at	12	485.70	280.20	184.35	1.29	507.00	P	610.30	P
1367462_at	12	1,032.50	309.70	268.23	1.08	3,207.50	P	3,371.30	P
1367463_at	12	1,621.60	510.00	701.97	1.21	3,510.30	P	3,050.30	P
1367464_at	12	317.70	202.00	-111.67	-1.13	797.70	P	1,038.90	P
1367465_at	12	699.00	196.80	257.50	1.22	1,103.10	P	1,281.80	P
1367466_at	12	265.50	122.50	-54.67	-1.04	1,385.10	P	1,321.30	P

Experiment Table Settings

Column width: Manual

Experiment level

Analysis level

Annotation level

Group level

☑ Heart

☑ Diaphragm

Group columns

☑ Means

☑ Present count

Sample level

12 element(s) are selected

Figure 25.5: *Opening the experiment.*

Whenever you perform analyses like normalization, transformation, statistical analysis etc, new columns will be added to the experiment. You can at any time **Export** (📤) all the data in the experiment in csv or Excel format or **Copy** (❐) the full table or parts of it.

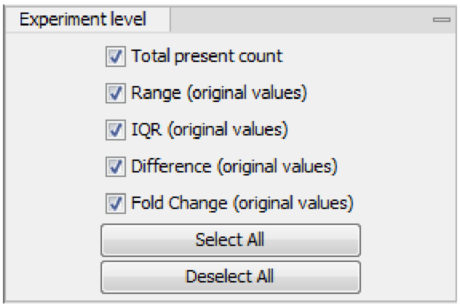
Column width

There are two options to specify the width of the columns and also the entire table:

- **Automatic**. This will fit the entire table into the width of the view. This is useful if you only have a few columns.
- **Manual**. This will adjust the width of all columns evenly, and it will make the table as wide as it needs to be to display all the columns. This is useful if you have many columns. In this case there will be a scroll bar at the bottom, and you can manually adjust the width by dragging the column separators.

Experiment level

The rest of the **Side Panel** is devoted to different levels of information on the values in the experiment. The experiment part contains a number of columns that, for each feature ID, provide summaries of the values across all the samples in the experiment (see figure 25.6).



Experiment level

☑ Total present count

☑ Range (original values)

☑ IQR (original values)

☑ Difference (original values)

☑ Fold Change (original values)

Figure 25.6: *The initial view of the experiment level for a two-group experiment.*

Initially, it has one header for the whole **Experiment**:

- **Range (original values)**. The 'Range' column contains the difference between the highest and the lowest expression value for the feature over all the samples. If a feature has the value NaN in one or more of the samples the range value is NaN.
- **IQR (original values)**. The 'IQR' column contains the inter-quantile range of the values for a feature across the samples, that is, the difference between the 75 %-ile value and the 25 %-ile value. For the IQR values, only the numeric values are considered when percentiles are calculated (that is, NaN and +Inf or -Inf values are ignored), and if there are fewer than four samples with numeric values for a feature, the IQR is set to be the difference between the highest and lowest of these.
- **Difference (original values)**. For a two-group experiment the 'Difference' column contains the difference between the mean of the expression values across the samples assigned to group 2 and the mean of the expression values across the samples assigned to group 1. Thus, if the mean expression level in group 2 is higher than that of group 1 the 'Difference' is positive, and if it is lower the 'Difference' is negative. For experiments with more than two groups the 'Difference' contains the difference between the maximum and minimum of the mean expression values of the groups, multiplied by -1 if the group with the maximum mean expression value occurs before the group with the minimum mean expression value (with the ordering: group 1, group 2, ...).
- **Fold Change (original values)**. For a two-group experiment the 'Fold Change' tells you how many times bigger the mean expression value in group 2 is relative to that of group 1. If the mean expression value in group 2 is bigger than that in group 1 this value is the mean expression value in group 2 divided by that in group 1. If the mean expression value in group 2 is smaller than that in group 1 the fold change is the mean expression value in group 1 divided by that in group 2 with a negative sign. Thus, if the mean expression levels in group 1 and group 2 are 10 and 50 respectively, the fold change is 5, and if the and if the mean expression levels in group 1 and group 2 are 50 and 10 respectively, the fold change is -5. Entries of plus or minus infinity in the 'Fold Change' columns of the Experiment area represent those where one of the expression values in the calculation is a 0. For experiments with more than two groups, the 'Fold Change' column contains the ratio of the maximum of the mean expression values of the groups to the minimum of the mean expression values of the groups, multiplied by -1 if the group with the maximum mean expression value occurs before the group with the minimum mean expression value (with the ordering: group 1, group 2, ...).

Thus, the sign of the values in the 'Difference' and 'Fold change' columns give the direction of the trend across the groups, going from group 1 to group 2, etc.

If the samples used are Affymetrix GeneChips samples and have 'Present calls' there will also be a 'Total present count' column containing the number of present calls for all samples.

The columns under the 'Experiment' header are useful for filtering purposes, e.g. you may wish to ignore features that differ too little in expression levels to be confirmed e.g. by qPCR by filtering on the values in the 'Difference', 'IQR' or 'Fold Change' columns or you may wish to ignore features that do not differ at all by filtering on the 'Range' column.

If you have performed normalization or transformation (see sections 25.2.3 and 25.2.2, respectively), the IQR of the normalized and transformed values will also appear. Also, if you later choose to transform or normalize your experiment, columns will be added for the transformed or normalized values.

Note! It is very common to filter features on fold change values in expression analysis and fold change values are also used in volcano plots, see section 25.5.4. There are different definitions of 'Fold Change' in the literature. The definition that is used typically depends on the original scale of the data that is analyzed. For data whose original scale is *not* the log scale the standard definition is the ratio of the group means [Tusher et al., 2001]. This is the value you find in the 'Fold Change' column of the experiment. However, for data whose original *is* the log scale, the difference of the mean expression levels is sometimes referred to as the fold change [Guo et al., 2006], and if you want to filter on fold change for these data you should filter on the values in the 'Difference' column. Your data's original scale will e.g. be the log scale if you have imported Affymetrix expression values which have been created by running the RMA algorithm on the probe-intensities.

Analysis level

The results of each statistical test performed are in the columns listed in this area. In the table, a heading is given for each test. Information about the results of statistical tests are described in the statistical analysis section (see section 25.5).

An example of Analysis level settings is shown in figure 25.7.

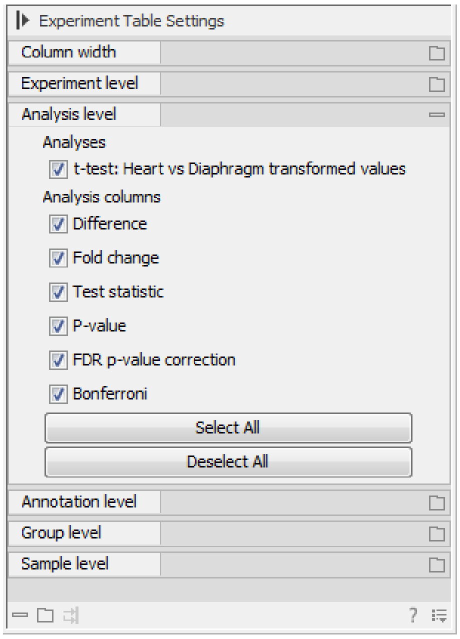


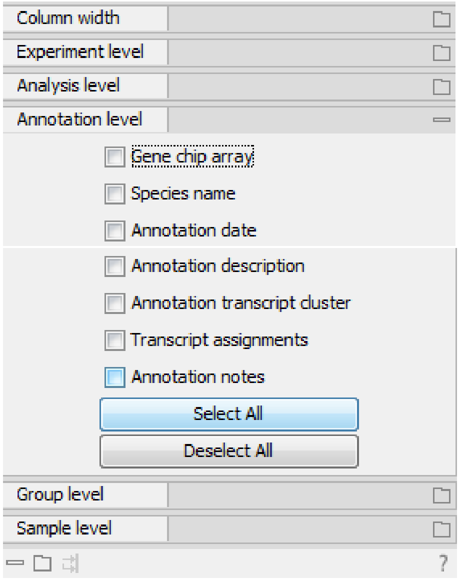
Figure 25.7: *An example of columns available under the Analysis level section.*

Note: Some column names here are the same as ones under the Experiment level, but the results here are from statistical tests, while those under the Experiment level section are calculations carried out directly on the expression levels.

Annotation level

If your experiment is annotated (see section 25.1.3), the annotations will be listed in the **Annotation level** group as shown in figure 25.8.

In order to avoid too much detail and cluttering the table, only a few of the columns are shown



Column width

Experiment level

Analysis level

Annotation level

□ Gene chip array

□ Species name

□ Annotation date

□ Annotation description

□ Annotation transcript cluster

□ Transcript assignments

□ Annotation notes

Group level

Sample level

Figure 25.8: *An annotated experiment.*

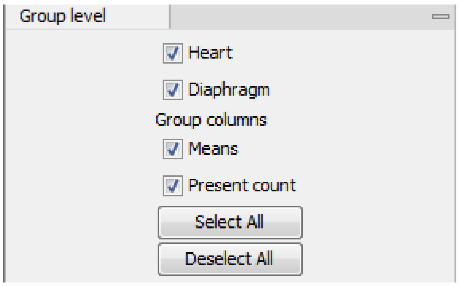
per default.

Note that if you wish a different set of annotations to be displayed each time you open an experiment, you need to save the settings of the **Side Panel** (see section 4.6).

Group level

At the group level, you can show/hide entire groups (*Heart* and *Diaphragm* in figure 25.5). This will show/hide everything under the group's header. Furthermore, you can show/hide group-level information like the group means and present count within a group. If you have performed normalization or transformation (see sections 25.2.3 and 25.2.2, respectively), the means of the normalized and transformed values will also appear.

An example is shown in figure 25.9.



Group level

☑ Heart

☑ Diaphragm

Group columns

☑ Means

☑ Present count

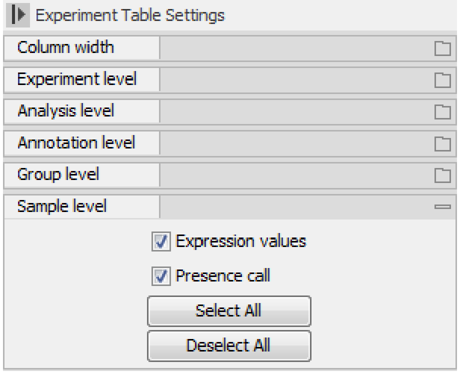
Figure 25.9: *Group level .*

Sample level

In this part of the side panel, you can control which columns to be displayed for each sample. Initially this is the all the columns in the samples.

If you have performed normalization or transformation (see sections 25.2.3 and 25.2.2, respectively), the normalized and transformed values will also appear.

An example is shown in figure 25.10.



Experiment Table Settings

Column width

Experiment level

Analysis level

Annotation level

Group level

Sample level

☑ Expression values

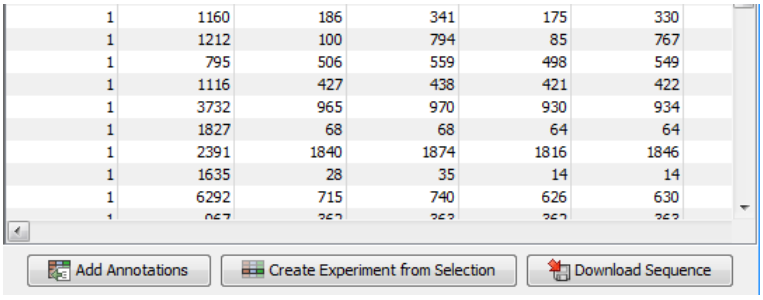
☑ Presence call

Figure 25.10: *Sample level when transformation and normalization has been performed.*

Creating a sub-experiment from a selection

If you have identified a list of genes that you believe are differentially expressed, you can create a subset of the experiment. (Note that the filtering and sorting may come in handy in this situation, see section 9).

To create a sub-experiment, first select the relevant features (rows). If you have applied a filter and wish to select all the visible features, press Ctrl + A (⌘ + A on Mac). Next, press the **Create Experiment from Selection** (▦) button at the bottom of the table (see figure 25.11).



1	1160	186	341	175	330
1	1212	100	794	85	767
1	795	506	559	498	549
1	1116	427	438	421	422
1	3732	965	970	930	934
1	1827	68	68	64	64
1	2391	1840	1874	1816	1846
1	1635	28	35	14	14
1	6292	715	740	626	630

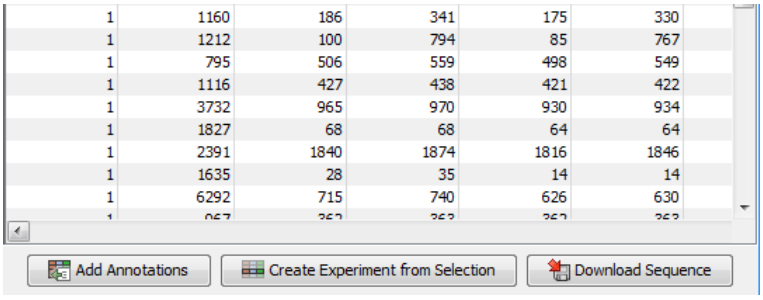
Figure 25.11: *Create a subset of the experiment by clicking the button at the bottom of the experiment table.*

This will create a new experiment that has the same information as the existing one but with less features.

Downloading sequences from the experiment table

If your experiment is annotated, you will be able to download the GenBank sequence for features which have a GenBank accession number in the 'Public identifier tag' annotation column. To do this, select a number of features (rows) in the experiment and then click **Download Sequence** (💾) (see figure 25.12).

This will open a dialog where you specify where the sequences should be saved. You can learn more about opening and viewing sequences in chapter 14. You can now use the downloaded sequences for further analysis in the Workbench.



1	1160	186	341	175	330
1	1212	100	794	85	767
1	795	506	559	498	549
1	1116	427	438	421	422
1	3732	965	970	930	934
1	1827	68	68	64	64
1	2391	1840	1874	1816	1846
1	1635	28	35	14	14
1	6292	715	740	626	630

Figure 25.12: *Select sequences and press the download button.*

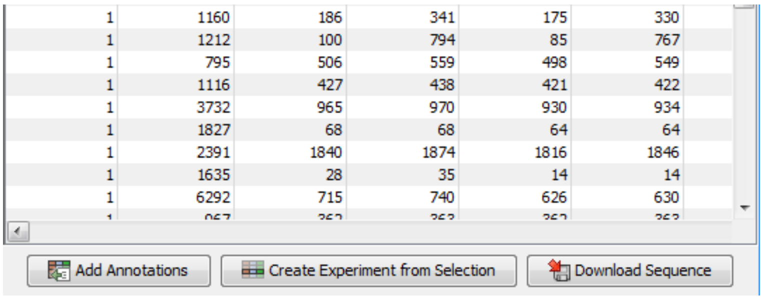
25.1.3 Adding annotations to an experiment

Annotation files provide additional information about each feature. This information could be which GO categories the protein belongs to, which pathways, various transcript and protein identifiers etc. See section I for information about the different annotation file formats that are supported *CLC Main Workbench*.

The annotation file can be imported into the Workbench and will get a special icon (). See an overview of annotation formats supported by *CLC Main Workbench* in section I. In order to associate an annotation file with an experiment, either select the annotation file when you set up the experiment (see section 25.1.1), or click:

Tools | **Expression Analysis** ()| **Annotation Test** () | **Add Annotations** ()

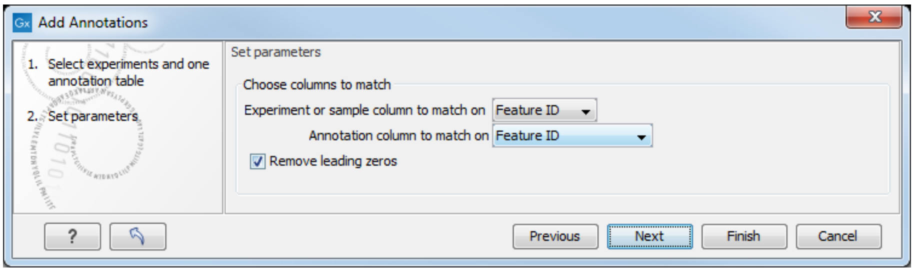
Select the experiment () and the annotation file () and click **Finish**. You will now be able to see the annotations in the experiment as described in section 25.1.2. You can also add annotations by pressing the **Add Annotations** () button at the bottom of the table (see figure 25.13).



1	1160	186	341	175	330
1	1212	100	794	85	767
1	795	506	559	498	549
1	1116	427	438	421	422
1	3732	965	970	930	934
1	1827	68	68	64	64
1	2391	1840	1874	1816	1846
1	1635	28	35	14	14
1	6292	715	740	626	630

Figure 25.13: *Adding annotations by clicking the button at the bottom of the experiment table.*

This will bring up a dialog where you can select the annotation file that you have imported together with the experiment you wish to annotate. Click **Next** to specify settings as shown in figure 25.14).



Add Annotations

1. Select experiments and one annotation table
2. Set parameters

Set parameters

Choose columns to match

Experiment or sample column to match on: Feature ID

Annotation column to match on: Feature ID

☑ Remove leading zeros

Figure 25.14: *Choosing how to match annotations with samples.*

In this dialog, you can specify how to match the annotations to the features in the sample. The Workbench looks at the columns in the annotation file and lets you choose which column that should be used for matching to the feature IDs in the experimental data (experiment or sample) as well as for the annotations. Usually the default is right, but for some annotation files, you need to select another column.

Some annotation files have leading zeros in the identifier which you can remove by checking the **Remove leading zeros** box.

Note! Existing annotations on the experiment will be overwritten.

25.1.4 Scatter plot view of an experiment

At the bottom of the experiment table, you can switch between different views of the experiment (see figure 25.15).

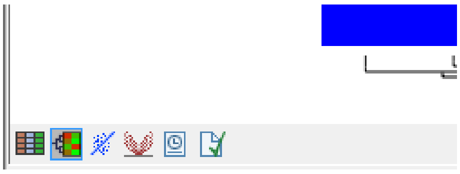


Figure 25.15: *An experiment can be viewed in several ways.*

One of the views is the **Scatter Plot** (). The scatter plot can be adjusted to show e.g. the group means for two groups (see more about how to adjust this below).

An example of a scatter plot is shown in figure 25.16.

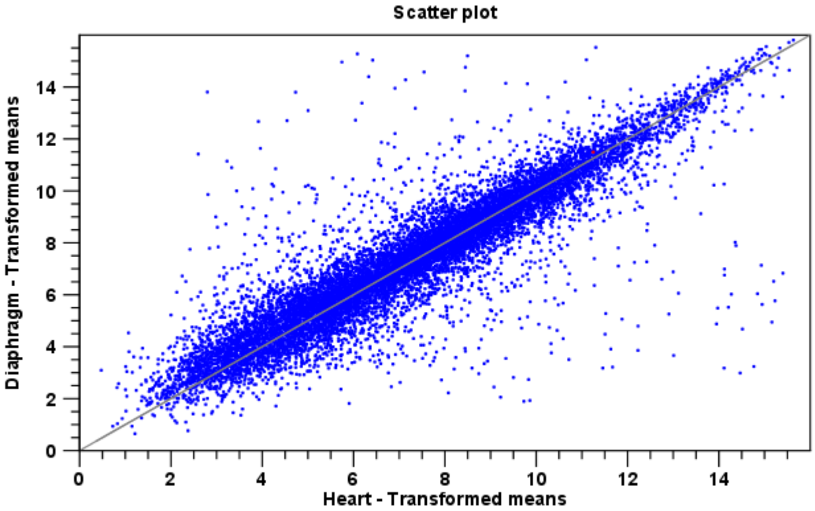


Figure 25.16: *A scatter plot of group means for two groups (transformed expression values).*

In the **Side Panel** to the left, there are a number of options to adjust this view. Under **Graph preferences**, you can adjust the general properties of the scatter plot:

- **Lock axes** This will always show the axes even though the plot is zoomed to a detailed level.

- **Frame** Shows a frame around the graph.
- **Show legends** Shows the data legends.
- **Tick type** Determine whether tick lines should be shown outside or inside the frame.
- **Tick lines at** Choosing Major ticks will show a grid behind the graph.
- **Horizontal axis range** Sets the range of the horizontal axis (x axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **Vertical axis range** Sets the range of the vertical axis (y axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **Draw x = y axis**. This will draw a diagonal line across the plot. This line is shown per default.
- **Line width** Thin, Medium or Wide
- **Line type** None, Line, Long dash or Short dash
- **Line color** Click the color box to select a color.
- Show Pearson correlation When checked, the Pearson correlation coefficient (r) is displayed on the plot.

Below the general preferences, you find the **Dot properties** preferences, where you can adjust coloring and appearance of the dots:

- **Dot type** Can be None, Cross, Plus, Square, Diamond, Circle, Triangle, Reverse triangle, or Dot.
- **Dot color** Click the color box to select a color.

Finally, the group at the bottom - **Values to plot** - is where you choose the values to be displayed in the graph. The default for a two-group experiment is to plot the group means.

Note that if you wish to use the same settings next time you open a scatter plot, you need to save the settings of the **Side Panel** (see section 4.6).

25.1.5 Cross-view selections

There are a number of different ways of looking at an experiment as shown in figure 25.17).

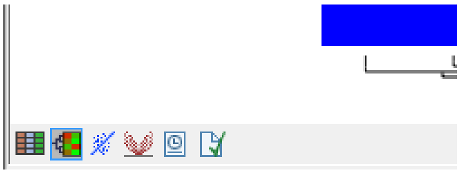
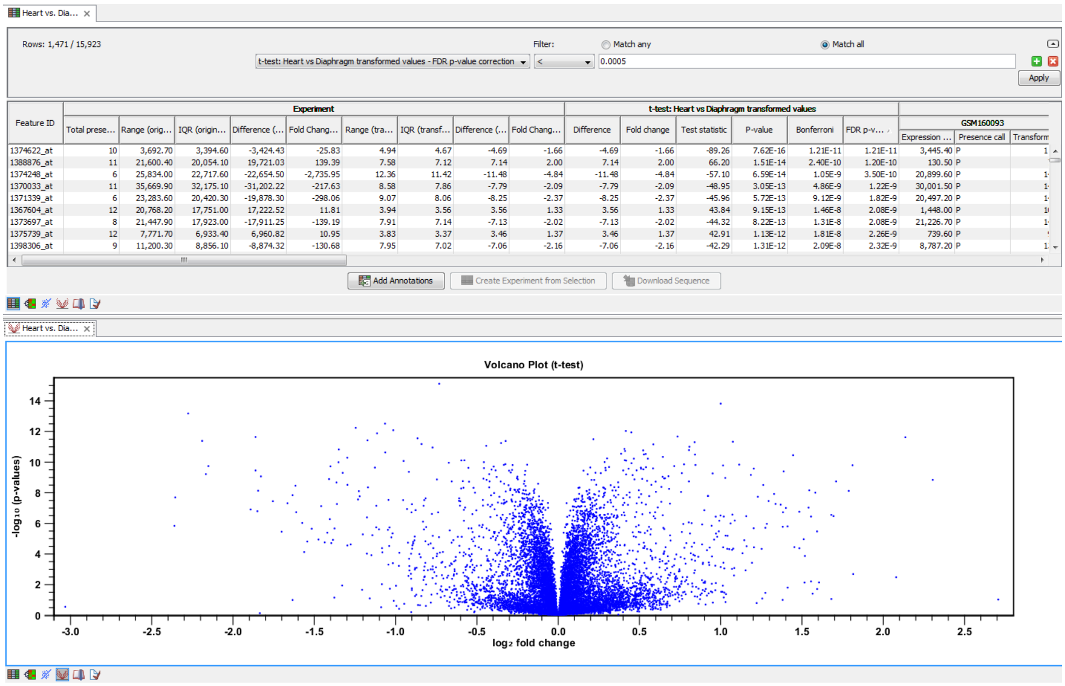


Figure 25.17: *An experiment can be viewed in several ways.*

Beside the **Experiment table** (▦) which is the default view, the views are: **Scatter plot** ( ), **Volcano plot** ( ) and the **Heat map** ( ). By pressing and holding the Ctrl (⌘ on Mac) button while you click one of the view buttons in figure 25.17, you can make a split view. This will make it possible to see e.g. the experiment table in one view and the volcano plot in another view.

An example of such a split view is shown in figure 25.18.



Rows: 1,471 / 15,923

Filter: ○ Match any ● Match all

t-test: Heart vs Diaphragm transformed values - FDR p-value correction < 0.0005

Apply

	Experiment									t-test: Heart vs Diaphragm transformed values						GSM160093		
Feature ID	Total prese...	Range (orig...	IQR (origin...	Difference (...	Fold Chang...	Range (tra...	IQR (transf...	Difference (...	Fold Chang...	Difference	Fold change	Test statistic	P-value	Bonferroni	FDR p-v...	Expression ...	Presence call	Transform
1374622_at	10	3,692.70	3,394.60	-3,424.43	-25.83	4.94	4.67	-4.69	-1.66	-4.69	-1.66	-89.26	7.62E-16	1.21E-11	1.21E-11	3,445.40	P	1
1388876_at	11	21,600.40	20,054.10	19,721.03	139.39	7.58	7.12	7.14	2.00	7.14	2.00	66.20	1.51E-14	2.40E-10	1.20E-10	130.50	P	
1374248_at	6	25,834.00	22,717.60	-22,654.50	-2,735.95	12.36	11.42	-11.48	-4.84	-11.48	-4.84	-57.10	6.59E-14	1.05E-9	3.50E-10	20,899.60	P	1
1370033_at	11	35,669.90	32,175.10	-31,202.22	-217.63	8.58	7.86	-7.79	-2.09	-7.79	-2.09	-48.95	3.05E-13	4.86E-9	1.22E-9	30,001.50	P	1
1371339_at	6	23,283.60	20,420.30	-19,878.30	-298.06	9.07	8.06	-8.25	-2.37	-8.25	-2.37	-45.96	5.72E-13	9.12E-9	1.82E-9	20,497.20	P	1
1367604_at	12	20,768.20	17,751.00	17,222.52	11.81	3.94	3.56	3.56	1.33	3.56	1.33	43.84	9.15E-13	1.46E-8	2.08E-9	1,448.00	P	1
1373697_at	8	21,447.90	17,923.00	-17,911.25	-139.19	7.91	7.14	-7.13	-2.02	-7.13	-2.02	-44.32	8.22E-13	1.31E-8	2.08E-9	21,226.70	P	1
1375739_at	12	7,771.70	6,933.40	6,960.82	10.95	3.83	3.37	3.46	1.37	3.46	1.37	42.91	1.13E-12	1.81E-8	2.26E-9	739.60	P	
1398306_at	9	11,200.30	8,856.10	-8,874.32	-130.68	7.95	7.02	-7.06	-2.16	-7.06	-2.16	-42.29	1.31E-12	2.09E-8	2.32E-9	8,787.20	P	1

Figure 25.18: *A split view showing an experiment table at the top and a volcano plot at the bottom (note that you need to perform statistical analysis to show a volcano plot, see section 25.5).*

Selections are shared between all these different views of an experiment. This means that if you select a number of rows in the table, the corresponding dots in the scatter plot, volcano plot or heatmap will also be selected. The selection can be made in any view, also the heat map, and all other open views will reflect the selection.

A common use of the split views is where you have an experiment and have performed a statistical analysis. You filter the experiment to identify all genes that have an FDR corrected p-value below 0.05 and a fold change for the test above say, 2. You can select all the rows in the experiment table satisfying these filters by holding down the Cntrl button and clicking 'a'. If you have a split view of the experiment and the volcano plot all points in the volcano plot corresponding to the selected features will be red. Note that the volcano plot allows two sets of values in the columns under the test you are considering to be displayed on the x-axis: the 'Fold change's and the 'Difference's. You control which to plot in the side panel. If you have filtered on 'Fold change' you will typically want to choose 'Fold change' in the side panel. If you have filtered on 'Difference' (e.g. because your original data is on the log scale, see the note on fold change in 25.1.2) you typically want to choose 'Difference'.

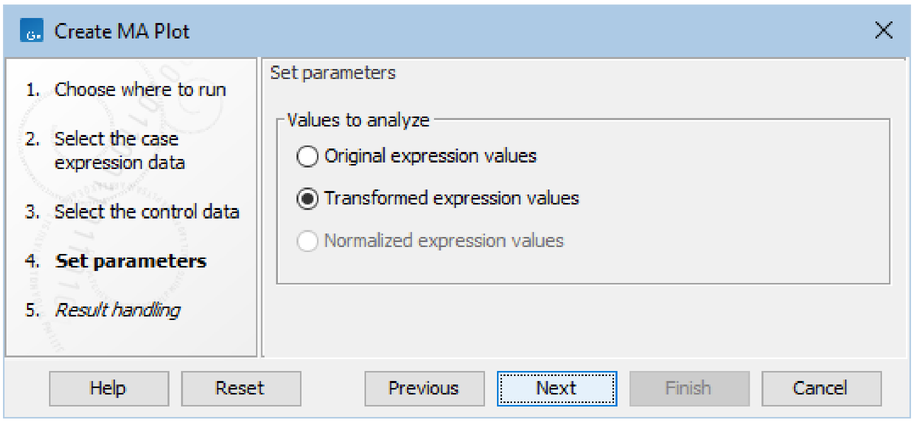
25.2 Transformation and normalization

The original expression values often need to be transformed and/or normalized in order to ensure that samples are comparable and assumptions on the data for analysis are met [Allison et al., 2006]. These are essential requirements for carrying out a meaningful analysis. The raw expression values often exhibit a strong dependency of the variance on the mean, and it may be preferable to remove this by log-transforming the data. Furthermore, the sets of expression values in the different samples in an experiment may exhibit systematic differences that are likely due to differences in sample preparation and array processing, rather being the result of the underlying biology. These noise effects should be removed before statistical analysis is carried out.

When you perform transformation and normalization, the original expression values will be kept, and the new values will be added. If you select an experiment (), the new values will be added to the experiment (not the original samples). And likewise if you select a sample (() or ()) - in this case the new values will be added to the sample (the original values are still kept on the sample).

25.2.1 Selecting transformed and normalized values for analysis

A number of the tools for Expression Analysis use the following expression level values: *Original*, *Transformed* and *Normalized* (figure 25.19).



Create MA Plot

1. Choose where to run
2. Select the case expression data
3. Select the control data
4. **Set parameters**
5. *Result handling*

Set parameters

Values to analyze

○ Original expression values

● Transformed expression values

○ Normalized expression values

Figure 25.19: *Selecting which version of the expression values to analyze. In this case, the values have not been normalized, so it is not possible to select normalized values.*

In this case, the values have not been normalized, so it is not possible to select normalized values.

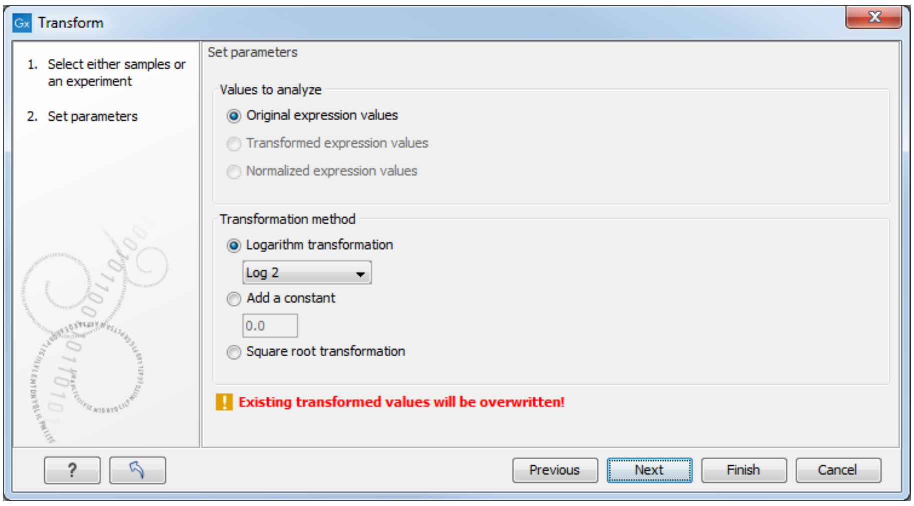
25.2.2 Transformation

The *CLC Main Workbench* lets you transform expression values based on logarithm and adding a constant:

Tools | Expression Analysis ()| Transformation and Normalization | Transform ()

Select a number of samples (() or ()) or an experiment () and click **Next**.

This will display a dialog as shown in figure 25.20.



1. Select either samples or an experiment
2. Set parameters

Set parameters

Values to analyze

● Original expression values

○ Transformed expression values

○ Normalized expression values

Transformation method

● Logarithm transformation

Log 2

○ Add a constant

0.0

○ Square root transformation

Existing transformed values will be overwritten!

Figure 25.20: *Transforming expression values.*

At the top, you can select which values to transform (see section 25.2.1).

Next, you can choose three kinds of transformation:

- **Logarithm transformation**. Transformed expression values will be calculated by taking the logarithm (of the specified type) of the values you have chosen to transform.
  - **10**.
  - **2**.
  - **Natural logarithm**.
- **Adding a constant**. Transformed expression values will be calculated by adding the specified constant to the values you have chosen to transform.
- **Square root transformation**.

Click **Finish** to start the tool.

25.2.3 Normalization

The *CLC Main Workbench* lets you normalize expression values.

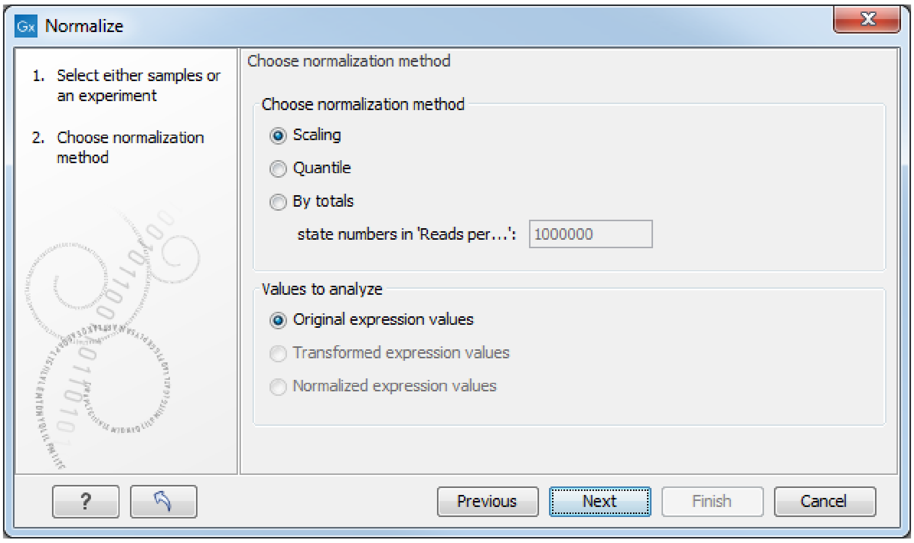
To start the normalization:

Tools | **Expression Analysis ( )**| **Transformation and Normalization** | **Normalize ( )**

Select a number of samples (( ) or ( )) or an experiment ( ) and click **Next**.

This will display a dialog as shown in figure 25.21.

At the top, you can choose three kinds of normalization (for mathematical descriptions see [Bolstad et al., 2003]):



Normalize

1. Select either samples or an experiment
2. Choose normalization method

Choose normalization method

Choose normalization method

● Scaling
○ Quantile
○ By totals

state numbers in 'Reads per...': 1000000

Values to analyze

● Original expression values
○ Transformed expression values
○ Normalized expression values

Figure 25.21: *Choosing normalization method.*

- **Scaling**. The sets of the expression values for the samples will be multiplied by a constant so that the sets of normalized values for the samples have the same 'target' value (see description of the **Normalization value** below).
- **Quantile**. The empirical distributions of the sets of expression values for the samples are used to calculate a common target distribution, which is used to calculate normalized sets of expression values for the samples.
- **By totals**. This option is intended to be used with count-based data, i.e. data from small RNA or expression profiling by tags. A sum is calculated for the expression values in a sample. The transformed value are generated by dividing the input values by the sample sum and multiplying by the factor (e.g. per '1,000,000').

Figures 25.22 and 25.23 show the effect on the distribution of expression values when using scaling or quantile normalization, respectively.

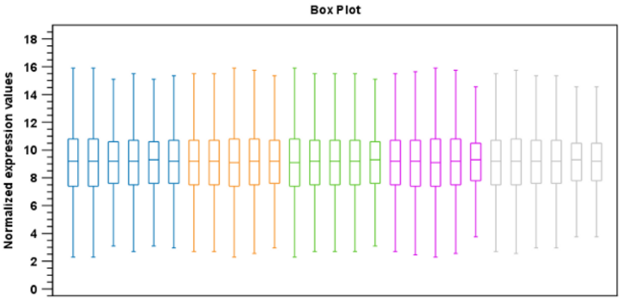


Figure 25.22: *Box plot after scaling normalization.*

At the bottom of the dialog in figure 25.21, you can select which values to normalize (see section 25.2.1).

Clicking **Next** will display a dialog as shown in figure 25.24.

The following parameters can be set:

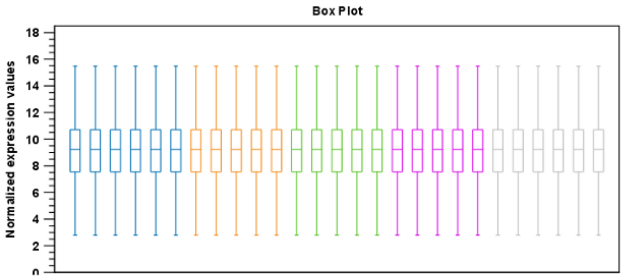
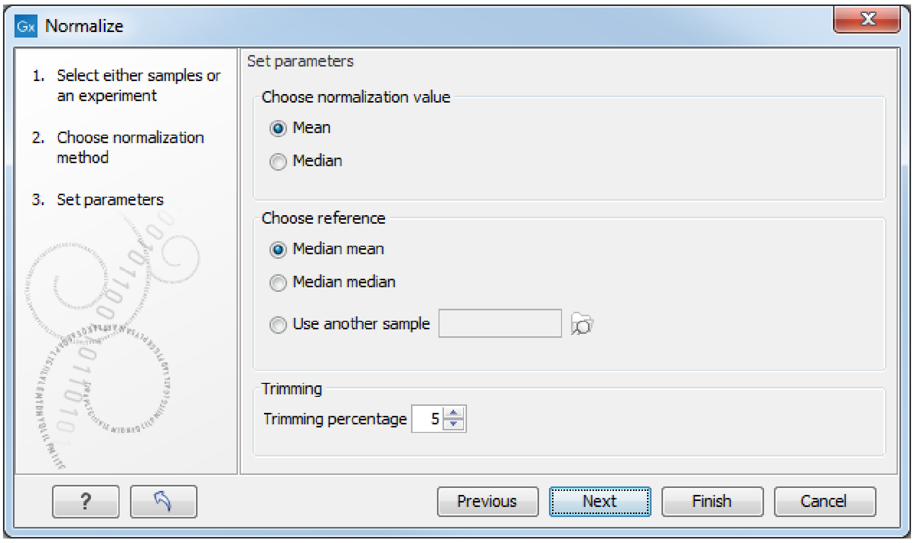


Figure 25.23: *Box plot after quantile normalization.*


Normalize
1. Select either samples or an experiment
2. Choose normalization method
3. Set parameters
?
Previous
Next
Finish
Cancel

Set parameters

Choose normalization value

● Mean

○ Median

Choose reference

● Median mean

○ Median median

○ Use another sample

Trimming

Trimming percentage 5

Figure 25.24: *Normalization settings.*

- **Normalization value.** The type of value of the samples which you want to ensure are equal for the normalized expression values
  - **Mean.**
  - **Median.**
- **Reference.** The specific value that you want the normalized value to be after normalization.
  - **Median mean.**
  - **Median median.**
  - **Use another sample.**
- **Trimming percentage.** Expression values that lie below the value of this percentile, or above 100 minus the value of this percentile, in the empirical distribution of the expression values in a sample will be excluded when calculating the normalization and reference values.

Click **Finish** to start the tool.

25.3 Quality control

The *CLC Main Workbench* includes a number of tools for quality control. These allow visual inspection of the overall distributions, variability and similarity of the sets of expression values in samples, and may be used to spot unwanted systematic differences between samples, outlying samples and samples of poor quality, that you may want to exclude.

25.3.1 Create Box Plot

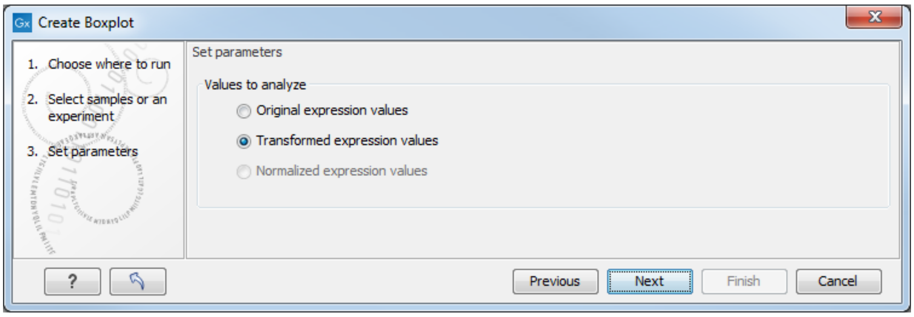
In most cases you expect the majority of genes to behave similarly under the conditions considered, and only a smaller proportion to behave differently. Thus, at an overall level you would expect the distributions of the sets of expression values in samples in a study to be similar. A boxplot provides a visual presentation of the distributions of expression values in samples. For each sample the distribution of it's values is presented by a line representing a center, a box representing the middle part, and whiskers representing the tails of the distribution. Differences in the overall distributions of the samples in a study may indicate that normalization is required before the samples are comparable. An atypical distribution for a single sample (or a few samples), relative to the remaining samples in a study, could be due to imperfections in the preparation and processing of the sample, and may lead you to reconsider using the sample(s).

To create a box plot:

> **Tools** | **Expression Analysis ()**| **Quality Control** () | **Create Box Plot ()**

Select a number of samples (() or () or an experiment () and click **Next**.

This will display a dialog as shown in figure 25.25.



Create Boxplot

1. Choose where to run
2. Select samples or an experiment
3. Set parameters

Set parameters

Values to analyze

○ Original expression values

● Transformed expression values

○ Normalized expression values

Figure 25.25: *Choosing values to analyze for the box plot.*

Here you select which values to use in the box plot (see section 25.2.1).

Click **Finish** to start the tool.

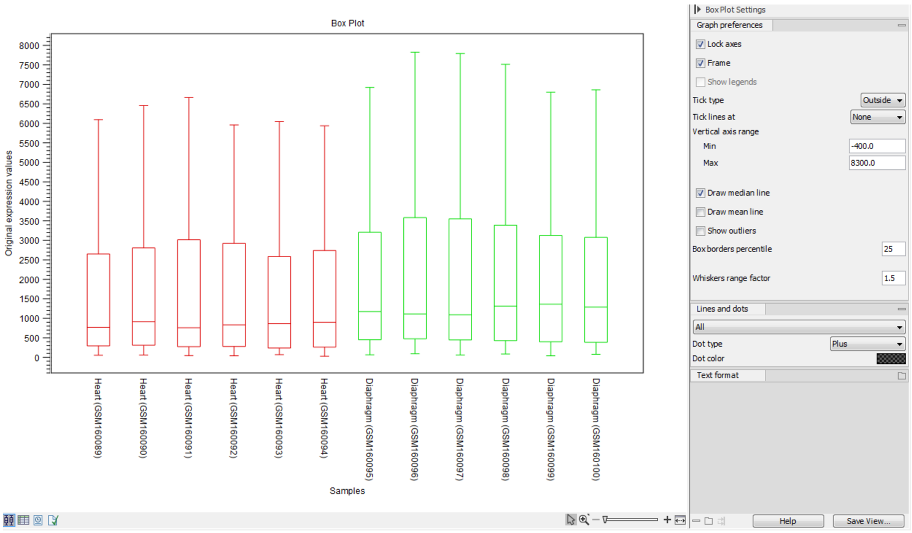
Viewing box plots

An example of a box plot of a two-group experiment with 12 samples is shown in figure 25.26.

Note that the boxes are colored according to their group relationship. At the bottom you find the names of the samples, and the y-axis shows the expression values. The box also includes the IQR values (from the lower to the upper quartile) and the median is displayed as a line in the box. The ends of the boxplot whiskers are the lowest data point within 1.5 times the inter quartile range (IQR) of the lower quartile and the highest data point within 1.5 IQR of the upper quartile.

It is possible to change the default value of 1.5 using the side panel option "Whiskers range factor".

In the **Side Panel** to the left, there is a number of options to adjust this view. Under **Graph**



Box Plot Settings

Graph preferences

☑ Lock axes

☑ Frame

□ Show legends

Tick type: Outside

Tick lines at: None

Vertical axis range

Min: -400.0

Max: 8300.0

☑ Draw median line

□ Draw mean line

□ Show outliers

Box borders percentile: 25

Whiskers range factor: 1.5

Lines and dots

All

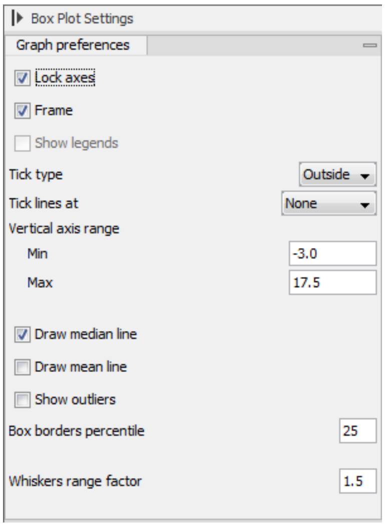
Dot type: Plus

Dot color

Text format

Figure 25.26: *A box plot of 12 samples in a two-group experiment, colored by group.*

preferences, you can adjust the general properties of the box plot (see figure 25.27).



Box Plot Settings

Graph preferences

☑ Lock axes

☑ Frame

□ Show legends

Tick type: Outside

Tick lines at: None

Vertical axis range

Min: -3.0

Max: 17.5

☑ Draw median line

□ Draw mean line

□ Show outliers

Box borders percentile: 25

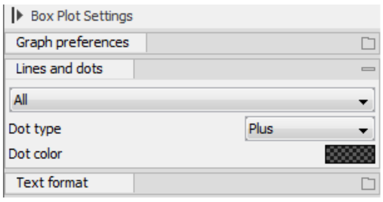
Whiskers range factor: 1.5

Figure 25.27: *Graph preferences for a box plot.*

- **Lock axes** This will always show the axes even though the plot is zoomed to a detailed level.
- **Frame** Shows a frame around the graph.
- **Show legends** Shows the data legends.
- **Tick type** Determine whether tick lines should be shown outside or inside the frame.
- **Tick lines at** Choosing Major ticks will show a grid behind the graph.
- **Vertical axis range** Sets the range of the vertical axis (y axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **Draw median line**. This is the default - the median is drawn as a line in the box.

- **Draw mean line**. Alternatively, you can also display the mean value as a line.
- **Show outliers**. The values outside the whiskers range are called outliers. Per default they are not shown. Note that the dot type that can be set below only takes effect when outliers are shown. When you select and deselect the **Show outliers**, the vertical axis range is automatically re-calculated to accommodate the new values.

Below the general preferences, you find the **Lines and dots** preferences, where you can adjust coloring and appearance (see figure 25.28).



Dot type Plus

Dot color ▨

Figure 25.28: *Lines and dot preferences for a box plot.*

- **Select sample or group**. When you wish to adjust the properties below, first select an item in this drop-down menu. That will apply the changes below to this item. If your plot is based on an experiment, the drop-down menu includes both group names and sample names, as well as an entry for selecting "All". If your plot is based on single elements, only sample names will be visible. Note that there are sometimes "mixed states" when you select a group where two of the samples e.g. have different colors. Selecting a new color in this case will erase the differences.
- **Dot type** Can be None, Cross, Plus, Square, Diamond, Circle, Triangle, Reverse triangle, or Dot.
- **Dot color** Click the color box to select a color.

Note that if you wish to use the same settings next time you open a box plot, you need to save the settings of the **Side Panel** (see section 4.6).

Interpreting box plots

This section will show how to interpret a box plot through a few examples.

First, if you look at figure 25.29, you can see a box plot for an experiment with 5 groups and 27 samples.

None of the samples stand out as having distributions that are atypical: the boxes and whiskers ranges are about equally sized. The locations of the distributions however, differ some, and indicate that normalization may be required. Figure 25.30 shows a box plot for the same experiment after quantile normalization: the distributions have been brought into par.

In figure 25.31 a box plot for a two group experiment with 5 samples in each group is shown.

The distribution of values in the second sample from the left is quite different from those of other samples, and could indicate that the sample should not be used.

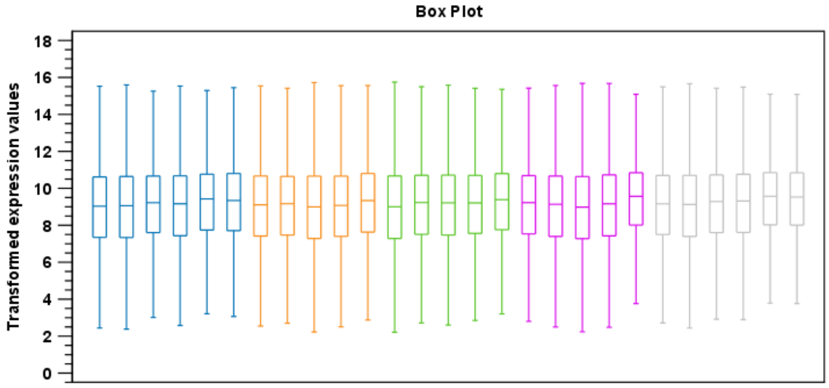


Figure 25.29: *Box plot for an experiment with 5 groups and 27 samples.*

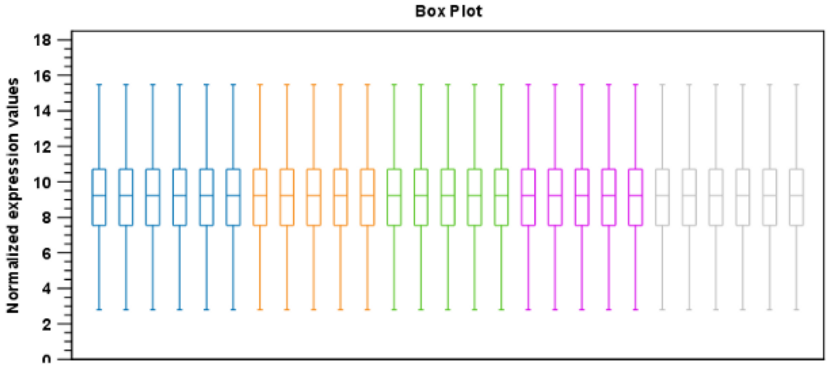


Figure 25.30: *Box plot after quantile normalization.*

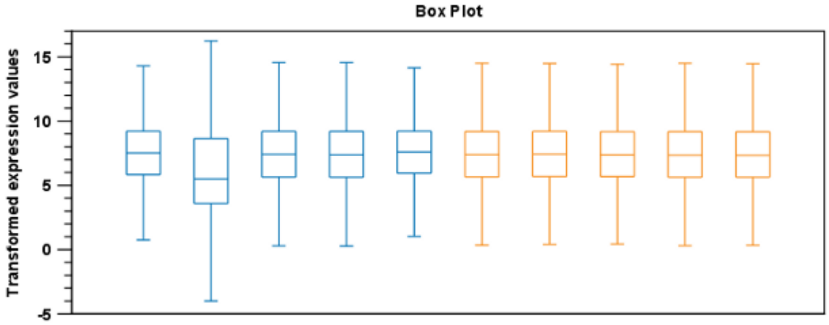


Figure 25.31: *Box plot for a two-group experiment with 5 samples.*

25.3.2 Hierarchical Clustering of Samples

A hierarchical clustering of samples is a tree representation of their relative similarity.

The tree structure is generated by

1. letting each sample be a cluster
2. calculating pairwise distances between all clusters
3. joining the two closest clusters into one new cluster

4. iterating 2-3 until there is only one cluster left (which will contain all samples).

The tree is drawn so that the distances between clusters are reflected by the lengths of the branches in the tree. Thus, features with expression profiles that closely resemble each other have short distances between them, those that are more different, are placed further apart.

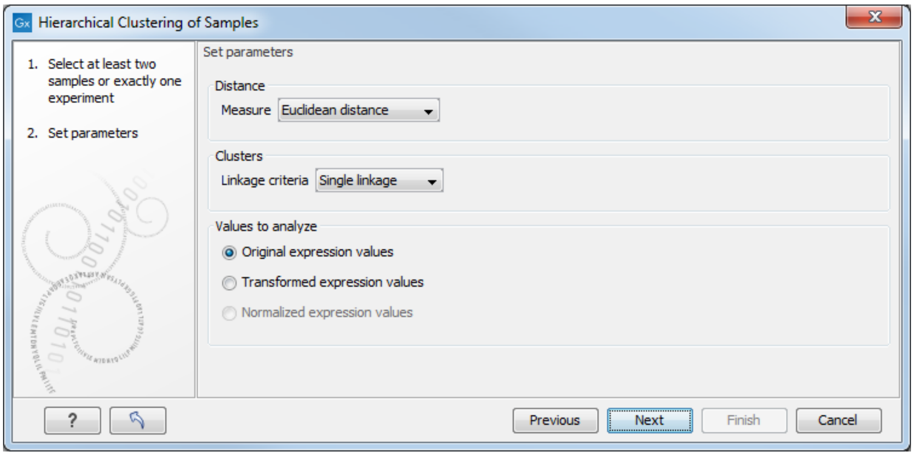
(See [Eisen et al., 1998] for a classical example of application of a hierarchical clustering algorithm in microarray analysis. The example is on features rather than samples).

To start the clustering:

Tools | **Expression Analysis ()**| **Quality Control** () | **Hierarchical Clustering of Samples ()**

Select a number of samples (() or ()) or an experiment () and click **Next**.

This will display a dialog as shown in figure 25.32. The hierarchical clustering algorithm requires that you specify a distance measure and a cluster linkage. The similarity measure is used to specify how distances between two samples should be calculated. The cluster distance metric specifies how you want the distance between two clusters, each consisting of a number of samples, to be calculated.


Hierarchical Clustering of Samples

1. Select at least two samples or exactly one experiment
2. Set parameters

Set parameters

Distance
Measure: Euclidean distance

Clusters
Linkage criteria: Single linkage

Values to analyze
● Original expression values
○ Transformed expression values
○ Normalized expression values

Figure 25.32: *Parameters for hierarchical clustering of samples.*

There are three kinds of **distance measures**:

- **Euclidean distance**. The length of the segment connecting two points. If $u = (u_1, u_2, \ldots, u_n)$ and $v = (v_1, v_2, \ldots, v_n)$, then the Euclidean distance between u and v is

$$|u - v| = \sqrt{\sum_{i=1}^{n} (u_i - v_i)^2}.$$

- **Manhattan distance.** The distance between two points measured along axes at right angles. If $u = (u_1, u_2, \ldots, u_n)$ and $v = (v_1, v_2, \ldots, v_n)$, then the Manhattan distance between u and v is

$$|u - v| = \sum_{i=1}^{n} |u_i - v_i|.$$

- **1 - Pearson correlation.** The Pearson correlation coefficient between $x = (x_1, x_2, ..., x_n)$ and $y = (y_1, y_2, ..., y_n)$ is defined as

  $$r = \frac{1}{n-1}\sum_{i=1}^{n}\left(\frac{x_i - \overline{x}}{s_x}\right)\cdot\left(\frac{y_i - \overline{y}}{s_y}\right)$$

  where $\overline{x}/\overline{y}$ and s_x/s_y are the average and sample standard deviation, respectively, of the values in x/y values.

  The Pearson correlation coefficient ranges from -1 to 1, with high absolute values indicating strong correlation, and values near 0 suggesting little to no relationship between the elements.

  Using 1 - | Pearson correlation | as the distance measure ensures that highly correlated elements have a shorter distance, while elements with low correlation are farther apart.

The distance between two clusters is determined using one of the following linkage types:

- **Single linkage**. The distance between the two closest elements in the two clusters.
- **Average linkage**. The average distance between elements in the first cluster and elements in the second cluster.
- **Complete linkage**. The distance between the two farthest elements in the two clusters.

At the bottom, you can select which values to cluster (see section 25.2.1).

Click **Finish** to start the tool.

Note: To be run on a server, the tool has to be included in a workflow, and the results will be displayed in a a stand-alone new heat map rather than added into the input experiment table.

Result of hierarchical clustering of samples

The result of a sample clustering is shown in figure 25.33.

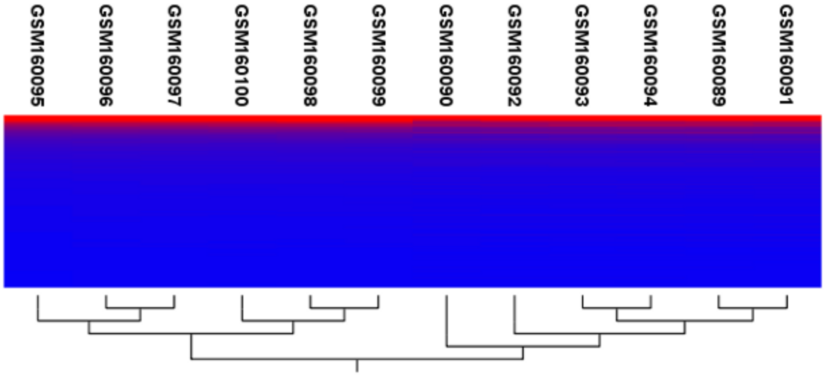


Figure 25.33: *Sample clustering.*

If you have used an **experiment** (▦) and ran the non-workflow version of the tool, the clustering is added to the experiment and will be saved when you save the experiment. It can be viewed by clicking the **Show Heat Map** (▦) button at the bottom of the view (see figure 25.34).

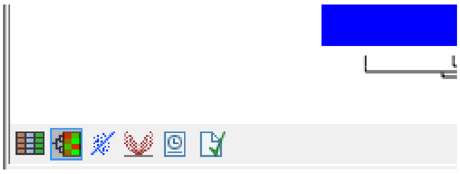


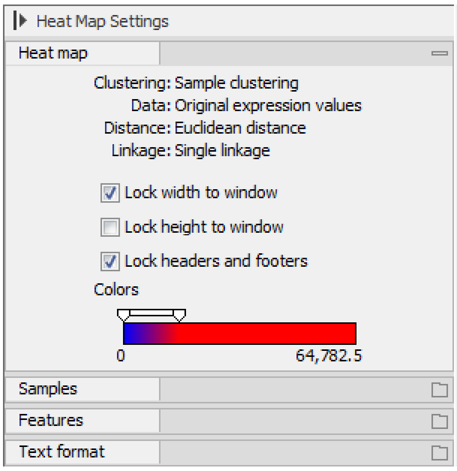
Figure 25.34: *Showing the hierarchical clustering of an experiment.*

If you have run the workflow version of the tool, or selected a number of **samples** ((▦) or (☰)) as input, a new element will be created that has to be saved separately.

Regardless of the input, the view of the clustering is the same. As you can see in figure 25.33, there is a tree at the bottom of the view to visualize the clustering. The names of the samples are listed at the top. The features are represented as horizontal lines, colored according to the expression level. If you place the mouse on one of the lines, you will see the names of the feature to the left. The features are sorted by their expression level in the first sample (in order to cluster the features, see section 25.4.1).

Researchers often have a priori knowledge of which samples in a study should be similar (e.g. samples from the same experimental condition) and which should be different (samples from biological distinct conditions). Thus, researches have expectations about how they should cluster. Samples that are placed unexpectedly in the hierarchical clustering tree may be samples that have been wrongly allocated to a group, samples of unintended or unclean tissue composition or samples for which the processing has gone wrong. Unexpectedly placed samples, of course, could also be highly interesting samples.

There are a number of options to change the appearance of the heat map. At the top of the **Side Panel**, you find the **Heat map** preference group (see figure 25.35).



Heat Map Settings

Heat map

Clustering: Sample clustering
Data: Original expression values
Distance: Euclidean distance
Linkage: Single linkage

☑ Lock width to window

☐ Lock height to window

☑ Lock headers and footers

Colors

Samples

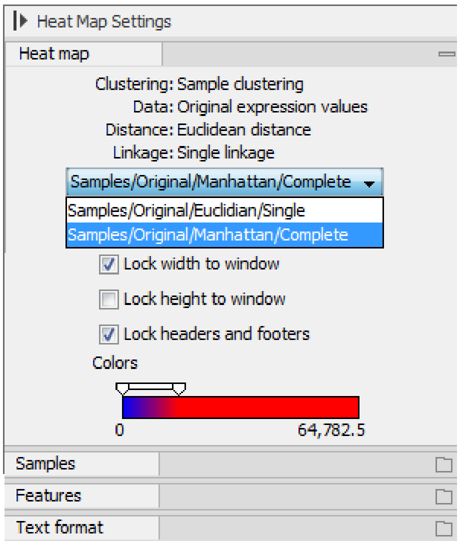
Features

Text format

Figure 25.35: *Side Panel of heat map.*

At the top, there is information about the heat map currently displayed. The information regards type of clustering, expression value used together with distance and linkage information. If you have performed more than one clustering, you can choose between the resulting heat maps in a drop-down box (see figure 25.36).

Note that if you perform an identical clustering, the existing heat map will simply be replaced. Below this box, there is a number of settings for displaying the heat map.



Heat Map Settings

Heat map

Clustering: Sample clustering
Data: Original expression values
Distance: Euclidean distance
Linkage: Single linkage

Samples/Original/Manhattan/Complete
Samples/Original/Euclidian/Single
Samples/Original/Manhattan/Complete

☑ Lock width to window

☐ Lock height to window

☑ Lock headers and footers

Colors

Samples

Features

Text format

Figure 25.36: *When more than one clustering has been performed, there will be a list of heat maps to choose from.*

- **Lock width to window**. When you zoom in the heat map, you will per default only zoom in on the vertical level. This is because the width of the heat map is locked to the window. If you uncheck this option, you will zoom both vertically and horizontally. Since you always have more features than samples, it is useful to lock the width since you then have all the samples in view all the time.

- **Lock height to window**. This is the corresponding option for the height. Note that if you check both options, you will not be able to zoom at all, since both the width and the height is fixed.

- **Lock headers and footers**. This will ensure that you are always able to see the sample and feature names and the trees when you zoom in.

- **Colors**. The expression levels are visualized using a gradient color scheme, where the right side color is used for high expression levels and the left side color is used for low expression levels. You can change the coloring by clicking the box, and you can change the relative coloring of the values by dragging the two knobs on the white slider above.

Below you find the **Samples** and **Features** groups. They contain options to show names, legend, and tree above or below the heat map. Note that for clustering of samples, you find the tree options in the **Samples** group, and for clustering of features, you find the tree options in the **Features** group. With the tree options, you can also control the **Tree size**, from tiny to very large, and the option of showing the full tree, no matter how much space it will use.

For clustering of features, the **Features** group has an option to "Optimize tree layout". This attempts to reorder the features, consistently with the tree, such that the most expressed features form a diagonal from the top-left to the bottom-right of the heat map.

The **Samples** group contains an "Order by:" dropdown that allows re-ordering of the columns of the heat map. When clustering by samples it is possible to choose between using the "Tree" to determine the sample ordering, and showing the "Samples" in the order they were input to the tool. When clustering by features, only the "Samples" input order is available.

Note that if you wish to use the same settings next time you open a heat map, you need to save the settings of the **Side Panel** (see section 4.6).

25.3.3 Principal Component Analysis

A principal component analysis is a mathematical analysis that identifies and quantifies the directions of variability in the data. For a set of samples, e.g. an experiment, this can be done either by finding the eigenvectors and eigenvalues of the *covariance matrix* of the samples or the *correlation matrix* of the samples (the correlation matrix is a 'normalized' version of the covariance matrix: the entries in the covariance matrix look like this $Cov(X, Y)$, and those in the correlation matrix like this: $Cov(X, Y)/(sd(X) * sd(Y))$. A covariance maybe any value, but a correlation is always between -1 and 1).

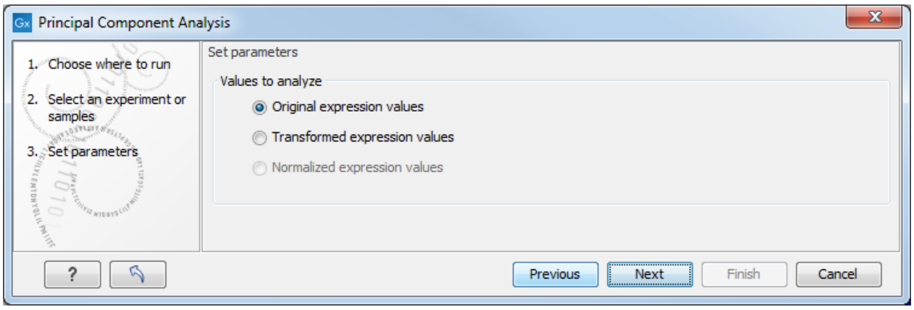
The eigenvectors are orthogonal. The first principal component is the eigenvector with the largest eigenvalue, and specifies the direction with the largest variability in the data. The second principal component is the eigenvector with the second largest eigenvalue, and specifies the direction with the second largest variability. Similarly for the third, etc. The data can be projected onto the space spanned by the eigenvectors. A plot of the data in the space spanned by the first and second principal component will show a simplified version of the data with variability in other directions than the two major directions of variability ignored.

To start the analysis:

> **Tools** | **Expression Analysis ( )**| **Quality Control** ( ) | **Principal Component Analysis ( )**

Select a number of samples (( ) or ( )) or an experiment ( ) and click **Next**.

This will display a dialog as shown in figure 25.37.



Principal Component Analysis

1. Choose where to run
2. Select an experiment or samples
3. Set parameters

Set parameters

Values to analyze

● Original expression values

○ Transformed expression values

○ Normalized expression values

Figure 25.37: *Selecting which values the principal component analysis should be based on.*

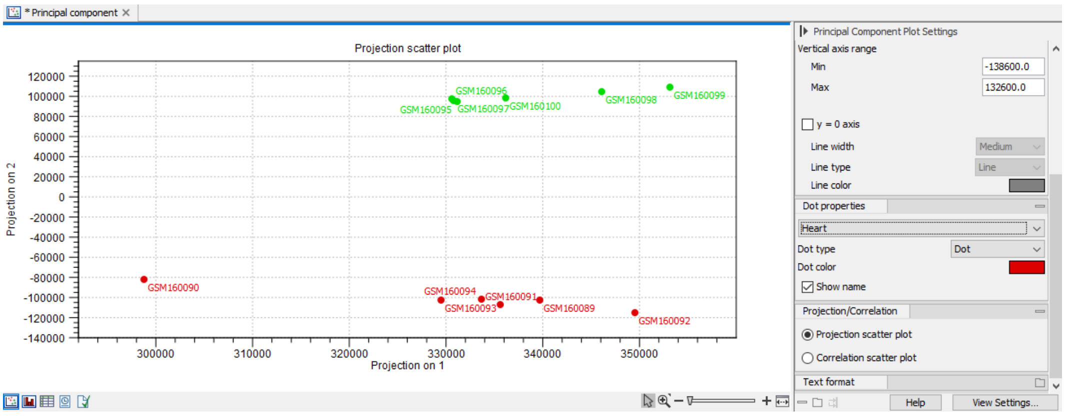
In this dialog, you select the values to be used for the principal component analysis (see section 25.2.1).

Click **Finish** to start the tool.

Principal component analysis plot

This will create a principal component plot as shown in figure 25.38.

The plot shows the projection of the samples onto the two-dimensional space spanned by the first and second principal component of the covariance matrix. In the bottom part of the side-panel, the 'Projection/Correlation' part, you can change to show the projection onto the *correlation*



Vertical axis range

Min -138600.0

Max 132600.0

□ y = 0 axis

Line width Medium

Line type Line

Line color

Dot properties

Heart

Dot type Dot

Dot color

☑ Show name

Projection/Correlation

● Projection scatter plot

○ Correlation scatter plot

Text format

Figure 25.38: *A principal component analysis.*

matrix rather than the *covariance* matrix by choosing 'Correlation scatter plot'. Both plots will show how the samples separate along the two directions between which the samples exhibit the largest amount of variation. For the 'projection scatter plot' this variation is measured in absolute terms, and depends on the units in which you have measured your samples. The correlation scatter plot is a normalized version of the projection scatter plot, which makes it possible to compare principal component analysis between experiments, even when these have not been done using the same units (e.g an experiment that uses 'original' scale data and another one that uses 'log-scale' data).

The plot in figure 25.38 is based on a two-group experiment. The group relationships are indicated by color. We expect the samples from within a group to exhibit less variability when compared, than samples from different groups. Thus samples should cluster according to groups and this is what we see. The PCA plot is thus helpful in identifying outlying samples and samples that have been wrongly assigned to a group.

In the **Side Panel** to the left, there is a number of options to adjust the view. Under **Graph preferences**, you can adjust the general properties of the plot.

- **Lock axes** This will always show the axes even though the plot is zoomed to a detailed level.
- **Frame** Shows a frame around the graph.
- **Show legends** Shows the data legends.
- **Tick type** Determine whether tick lines should be shown outside or inside the frame.
- **Tick lines at** Choosing Major ticks will show a grid behind the graph.
- **Horizontal axis range** Sets the range of the horizontal axis (x axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **Vertical axis range** Sets the range of the vertical axis (y axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **y = 0 axis.** Draws a line where y = 0. Below there are some options to control the appearance of the line:

  - **Line width** Thin, Medium or Wide
  - **Line type** None, Line, Long dash or Short dash
  - **Line color** Click the color box to select a color.

Below the general preferences, you find the **Dot properties**:

- **Select sample or group**. When you wish to adjust the properties below, first select an item in this drop-down menu. That will apply the changes below to this item. If your plot is based on an experiment, the drop-down menu includes both group names and sample names, as well as an entry for selecting "All". If your plot is based on single elements, only sample names will be visible. Note that there are sometimes "mixed states" when you select a group where two of the samples e.g. have different colors. Selecting a new color in this case will erase the differences.
- **Dot type** Can be None, Cross, Plus, Square, Diamond, Circle, Triangle, Reverse triangle, or Dot.
- **Dot color** Click the color box to select a color.
- **Show name**. This will show a label with the name of the sample next to the dot. Note that the labels quickly get crowded, so that is why the names are not put on per default.

Note that if you wish to use the same settings next time you open a principal component plot, you need to save the settings of the **Side Panel** (see section 4.6).

Scree plot

Besides the view shown in figure 25.38, the result of the principal component can also be viewed as a scree plot by clicking the **Show Scree Plot** (📊) button at the bottom of the view. The scree plot shows the proportion of variation in the data explained by each of the principal components. The first principal component accounts for the largest part of the variability.

In the **Side Panel** to the left, there is a number of options to adjust the view. Under **Graph preferences**, you can adjust the general properties of the plot.

- **Lock axes** This will always show the axes even though the plot is zoomed to a detailed level.
- **Frame** Shows a frame around the graph.
- **Show legends** Shows the data legends.
- **Tick type** Determine whether tick lines should be shown outside or inside the frame.
- **Tick lines at** Choosing Major ticks will show a grid behind the graph.
- **Horizontal axis range** Sets the range of the horizontal axis (x axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.

- **Vertical axis range** Sets the range of the vertical axis (y axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.

The **Lines and plots** below contains the following parameters:

- **Dot type** Can be None, Cross, Plus, Square, Diamond, Circle, Triangle, Reverse triangle, or Dot.
- **Dot color** Click the color box to select a color.
- **Line width** Thin, Medium or Wide
- **Line type** None, Line, Long dash or Short dash
- **Line color** Click the color box to select a color.

Note that the graph title and the axes titles can be edited simply by clicking them with the mouse. These changes will be saved when you **Save** ( ) the graph - whereas the changes in the **Side Panel** need to be saved explicitly (see section 4.6).

25.4 Feature clustering

Feature clustering is used to identify and cluster together features with similar expression patterns over samples (or experimental groups). Features that cluster together may be involved in the same biological process or be co-regulated. Also, by examining annotations of genes within a cluster, one may learn about the underlying biological processes involved in the experiment studied.

25.4.1 Hierarchical clustering of features

A hierarchical clustering of features is a tree presentation of the similarity in expression profiles of the features over a set of samples (or groups).

The tree structure is generated by

1. letting each feature be a cluster
2. calculating pairwise distances between all clusters
3. joining the two closest clusters into one new cluster
4. iterating 2-3 until there is only one cluster left (which will contain all samples).

The tree is drawn so that the distances between clusters are reflected by the lengths of the branches in the tree. Thus, features with expression profiles that closely resemble each other have short distances between them, those that are more different, are placed further apart.

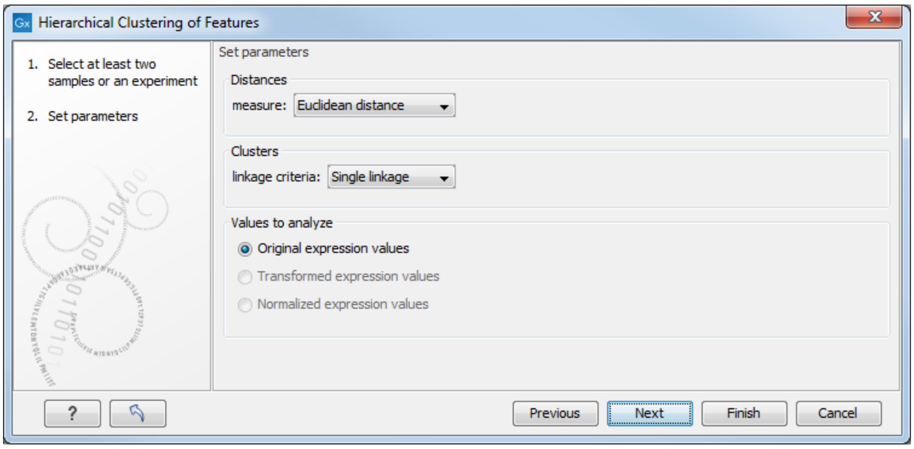
To start the clustering of features:

Tools | **Expression Analysis** ( )| **Feature Clustering** ( ) | **Hierarchical Clustering of Features** ( )

Select at least two samples ((▦) or (⇄)) or an experiment (▦).

Note! If your data contains many features, the clustering will take very long time and could make your computer unresponsive. It is recommended to perform this analysis on a subset of the data (which also makes it easier to make sense of the clustering. Typically, you will want to filter away the features that are thought to represent only noise, e.g. those with mostly low values, or with little difference between the samples). See how to create a sub-experiment in section 25.1.2.

Clicking **Next** will display a dialog as shown in figure 25.39. The hierarchical clustering algorithm requires that you specify a distance measure and a cluster linkage. The distance measure is used specify how distances between two features should be calculated. The cluster linkage specifies how you want the distance between two clusters, each consisting of a number of features, to be calculated.



Hierarchical Clustering of Features

1. Select at least two samples or an experiment
2. Set parameters

Set parameters

Distances

measure: Euclidean distance

Clusters

linkage criteria: Single linkage

Values to analyze

● Original expression values

○ Transformed expression values

○ Normalized expression values

Figure 25.39: *Parameters for hierarchical clustering of features.*

There are three kinds of **distance measures**:

- **Euclidean distance**. The length of the segment connecting two points. If $u = (u_1, u_2, \ldots, u_n)$ and $v = (v_1, v_2, \ldots, v_n)$, then the Euclidean distance between u and v is

$$|u - v| = \sqrt{\sum_{i=1}^{n} (u_i - v_i)^2}.$$

- **Manhattan distance.** The distance between two points measured along axes at right angles. If $u = (u_1, u_2, \ldots, u_n)$ and $v = (v_1, v_2, \ldots, v_n)$, then the Manhattan distance between u and v is

$$|u - v| = \sum_{i=1}^{n} |u_i - v_i|.$$

- **1 - Pearson correlation.** The Pearson correlation coefficient between $x = (x_1, x_2, ..., x_n)$ and $y = (y_1, y_2, ..., y_n)$ is defined as

$$r = \frac{1}{n-1} \sum_{i=1}^{n} \left(\frac{x_i - \overline{x}}{s_x} \right) \cdot \left(\frac{y_i - \overline{y}}{s_y} \right)$$

where $\overline{x}/\overline{y}$ and s_x/s_y are the average and sample standard deviation, respectively, of the values in x/y values.

> The Pearson correlation coefficient ranges from -1 to 1, with high absolute values indicating strong correlation, and values near 0 suggesting little to no relationship between the elements.
>
> Using 1 - | Pearson correlation | as the distance measure ensures that highly correlated elements have a shorter distance, while elements with low correlation are farther apart.

The distance between two clusters is determined using one of the following linkage types:

- **Single linkage**. The distance between the two closest elements in the two clusters.
- **Average linkage**. The average distance between elements in the first cluster and elements in the second cluster.
- **Complete linkage**. The distance between the two farthest elements in the two clusters.

At the bottom, you can select which values to cluster (see section 25.2.1).

Click **Finish** to start the tool.

Result of hierarchical clustering of features

The result of a feature clustering is shown in figure 25.40.

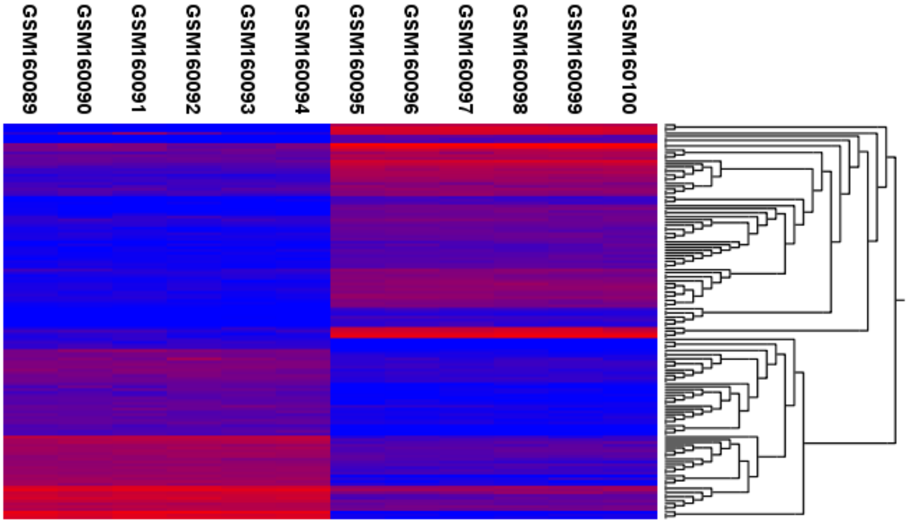


Figure 25.40: *Hierarchical clustering of features.*

If you have used an **experiment** () as input, the clustering is added to the experiment and will be saved when you save the experiment. It can be viewed by clicking the **Show Heat Map** () button at the bottom of the view (see figure 25.41).

If you have selected a number of **samples** (() or ()) as input, a new element will be created that has to be saved separately.

Regardless of the input, a hierarchical tree view with associated heatmap is produced (figure 25.40). In the heatmap each row corresponds to a feature and each column to a sample. The

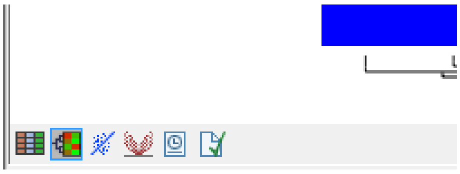
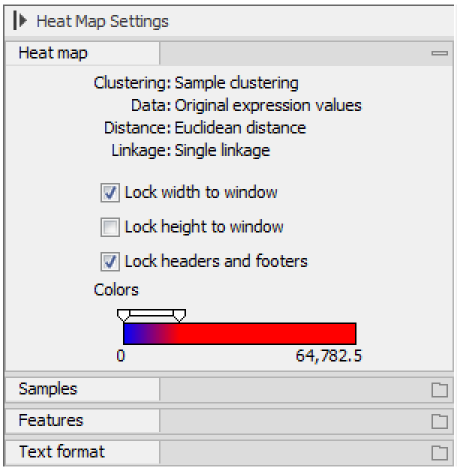


Figure 25.41: *Showing the hierarchical clustering of an experiment.*

color in the i'th row and j'th column reflects the expression level of feature i in sample j (the color scale can be set in the side panel). The order of the rows in the heatmap are determined by the hierarchical clustering. If you place the mouse on one of the rows, you will see the name of the corresponding feature to the left. The order of the columns (that is, samples) is determined by their input order or (if defined) experimental grouping. The names of the samples are listed at the top of the heatmap and the samples are organized into groups.

There are a number of options to change the appearance of the heat map. At the top of the **Side Panel**, you find the **Heat map** preference group (see figure 25.42).



Heat Map Settings

Heat map

Clustering: Sample clustering
Data: Original expression values
Distance: Euclidean distance
Linkage: Single linkage

☑ Lock width to window

□ Lock height to window

☑ Lock headers and footers

Colors

Samples

Features

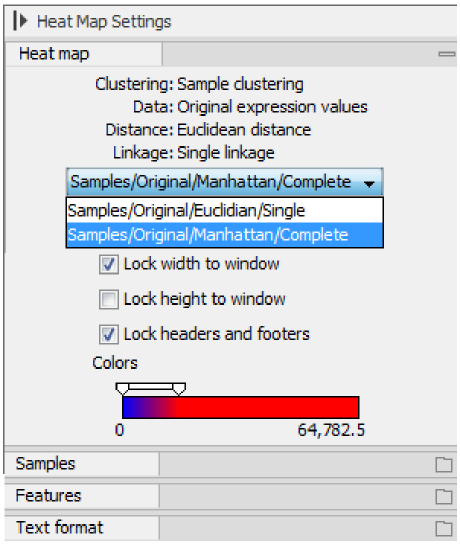
Text format

Figure 25.42: *Side Panel of heat map.*

At the top, there is information about the heat map currently displayed. The information regards type of clustering, expression value used together with distance and linkage information. If you have performed more than one clustering, you can choose between the resulting heat maps in a drop-down box (see figure 25.43).

Note that if you perform an identical clustering, the existing heat map will simply be replaced. Below this box, there is a number of settings for displaying the heat map.

- **Lock width to window**. When you zoom in the heat map, you will per default only zoom in on the vertical level. This is because the width of the heat map is locked to the window. If you uncheck this option, you will zoom both vertically and horizontally. Since you always have more features than samples, it is useful to lock the width since you then have all the samples in view all the time.
- **Lock height to window**. This is the corresponding option for the height. Note that if you check both options, you will not be able to zoom at all, since both the width and the height is fixed.
- **Lock headers and footers**. This will ensure that you are always able to see the sample and



Heat Map Settings

Heat map

Clustering: Sample clustering
Data: Original expression values
Distance: Euclidean distance
Linkage: Single linkage

Samples/Original/Manhattan/Complete
Samples/Original/Euclidian/Single
Samples/Original/Manhattan/Complete

☑ Lock width to window
□ Lock height to window
☑ Lock headers and footers

Colors
0 64,782.5

Samples
Features
Text format

Figure 25.43: *When more than one clustering has been performed, there will be a list of heat maps to choose from.*

feature names and the trees when you zoom in.

- **Colors**. The expression levels are visualized using a gradient color scheme, where the right side color is used for high expression levels and the left side color is used for low expression levels. You can change the coloring by clicking the box, and you can change the relative coloring of the values by dragging the two knobs on the white slider above.

Below you find the **Samples** and **Features** groups. They contain options to show names, legend, and tree above or below the heat map. Note that for clustering of samples, you find the tree options in the **Samples** group, and for clustering of features, you find the tree options in the **Features** group. With the tree options, you can also control the **Tree size**, from tiny to very large, and the option of showing the full tree, no matter how much space it will use.

For clustering of features, the **Features** group has an option to "Optimize tree layout". This attempts to reorder the features, consistently with the tree, such that the most expressed features form a diagonal from the top-left to the bottom-right of the heat map.

The **Samples** group contains an "Order by:" dropdown that allows re-ordering of the columns of the heat map. When clustering by samples it is possible to choose between using the "Tree" to determine the sample ordering, and showing the "Samples" in the order they were input to the tool. When clustering by features, only the "Samples" input order is available.

Note that if you wish to use the same settings next time you open a heat map, you need to save the settings of the **Side Panel** (see section 4.6).

25.4.2 K-means/medoids clustering

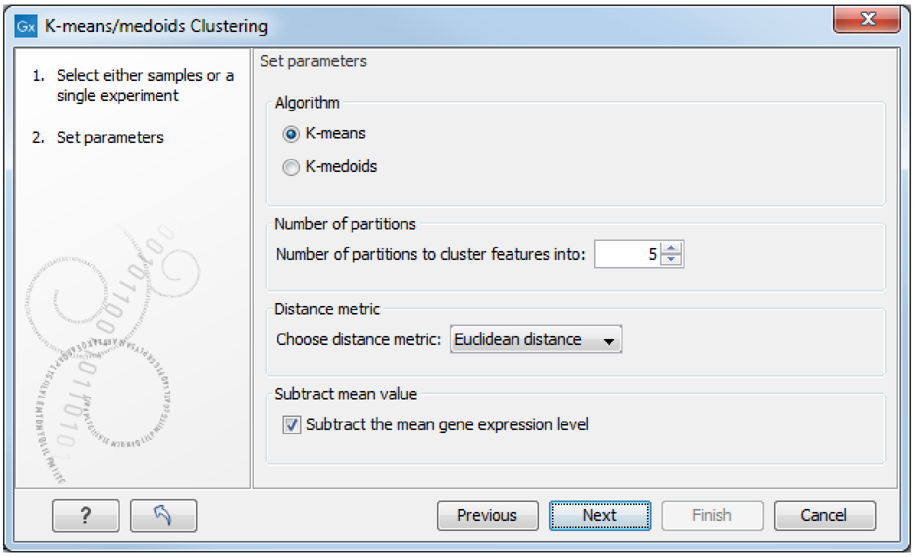
In a k-means or medoids clustering, features are clustered into k separate clusters. The procedures seek to find an assignment of features to clusters, for which the distances between features within the cluster is small, while distances between clusters are large.

Tools | **Expression Analysis** ()| **Feature Clustering** () | **K-means/medoids Clustering** ()

Select at least two samples ((▦) or (▦)) or an experiment (▦).

Note! If your data contains many features, the clustering will take very long time and could make your computer unresponsive. It is recommended to perform this analysis on a subset of the data (which also makes it easier to make sense of the clustering). See how to create a sub-experiment in section 25.1.2.

Clicking **Next** will display a dialog as shown in figure 25.44.



K-means/medoids Clustering

1. Select either samples or a single experiment
2. Set parameters

Set parameters

Algorithm

● K-means

○ K-medoids

Number of partitions

Number of partitions to cluster features into: 5

Distance metric

Choose distance metric: Euclidean distance

Subtract mean value

☑ Subtract the mean gene expression level

Figure 25.44: *Parameters for k-means/medoids clustering.*

The parameters are:

- **Algorithm**. You can choose between two clustering methods:
  - **K-means**. K-means clustering assigns each point to the cluster whose center is nearest. The center/centroid of a cluster is defined as the average of all points in the cluster. If a data set has three dimensions and the cluster has two points $X = (x_1, x_2, x_3)$ and $Y = (y_1, y_2, y_3)$, then the centroid Z becomes $Z = (z_1, z_2, z_3)$, where $z_i = (x_i + y_i)/2$ for $i = 1, 2, 3$. The algorithm attempts to minimize the intra-cluster variance defined by:

    $$V = \sum_{i=1}^{k} \sum_{x_j \in S_i} (x_j - \mu_i)^2$$

    where there are k clusters $S_i, i = 1, 2, \ldots, k$ and μ_i is the centroid of all points $x_j \in S_i$. The detailed algorithm can be found in [Lloyd, 1982].
  - **K-medoids**. K-medoids clustering is computed using the PAM-algorithm (PAM is short for Partitioning Around Medoids). It chooses datapoints as centers in contrast to the K-means algorithm. The PAM-algorithm is based on the search for k representatives (called medoids) among all elements of the dataset. When having found k representatives k clusters are now generated by assigning each element to its nearest medoid. The algorithm first looks for a good initial set of medoids (the BUILD phase). Then it

finds a local minimum for the objective function:

$$V = \sum_{i=1}^{k} \sum_{x_j \in S_i} (x_j - c_i)^2$$

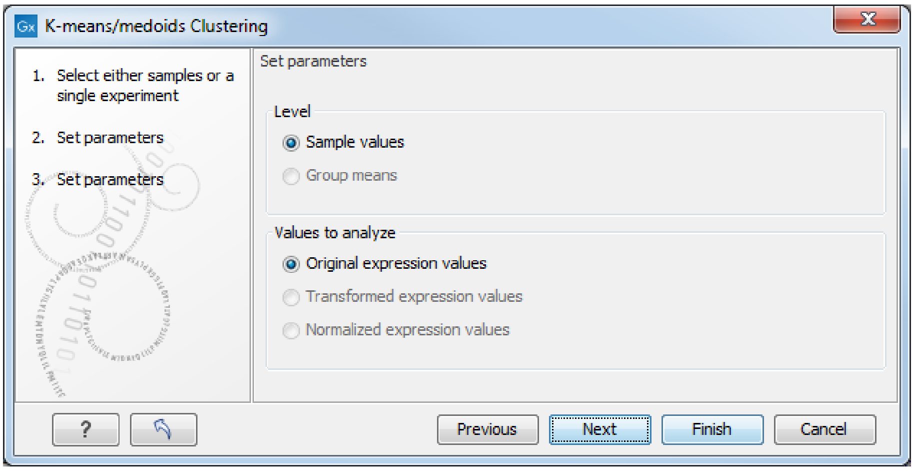
where there are k clusters $S_i, i = 1, 2, \ldots, k$ and c_i is the medoid of S_i. This solution implies that there is no single switch of an object with a medoid that will decrease the objective (this is called the SWAP phase). The PAM-agorithm is described in [Kaufman and Rousseeuw, 1990].

- **Number of partitions.** The maximum number of partitions to cluster features into: the final number of clusters can be smaller than that.
- **Distance metric**. The metric to compute distance between data points.
  - **Euclidean distance.** The ordinary distance between two elements - the length of the segment connecting them. If $u = (u_1, u_2, \ldots, u_n)$ and $v = (v_1, v_2, \ldots, v_n)$, then the Euclidean distance between u and v is

    $$|u - v| = \sqrt{\sum_{i=1}^{n} (u_i - v_i)^2}.$$
  - **Manhattan distance.** The Manhattan distance between two elements is the distance measured along axes at right angles. If $u = (u_1, u_2, \ldots, u_n)$ and $v = (v_1, v_2, \ldots, v_n)$, then the Manhattan distance between u and v is

    $$|u - v| = \sum_{i=1}^{n} |u_i - v_i|.$$
- **Subtract mean value**. For each gene, subtract the mean gene expression value over all input samples.

Clicking **Next** will display a dialog as shown in figure 25.45.



K-means/medoids Clustering
1. Select either samples or a single experiment
2. Set parameters
3. Set parameters
?
Previous
Next
Finish
Cancel

Set parameters

Level

● Sample values
○ Group means

Values to analyze

● Original expression values
○ Transformed expression values
○ Normalized expression values

Figure 25.45: *Parameters for k-means/medoids clustering.*

At the top, you can choose the **Level** to use. Choosing 'sample values' means that distances will be calculated using all the individual values of the samples. When 'group means' are chosen, distances are calculated using the group means.

At the bottom, you can select which values to cluster (see section 25.2.1).

Click **Finish** to start the tool.

The k-means implementation first assigns each feature to a cluster at random. Then, at each iteration, it reassigns features to the centroid of the nearest cluster. During this reassignment, it can happen that one or more of the clusters becomes empty, explaining why the final number of clusters might be smaller than the one specified in "number of partitions". Note that the initial assignment of features to clusters is random, so results can differ when the algorithm is run again.

Viewing the result of k-means/medoids clustering

The result of the clustering is a number of graphs. The number depends on the number of partitions chosen (figure 25.44) - there is one graph per cluster. Using drag and drop as explained in section 2.1.4, you can arrange the views to see more than one graph at the time.

Figure 25.46 shows an example where four clusters have been arranged side-by-side.

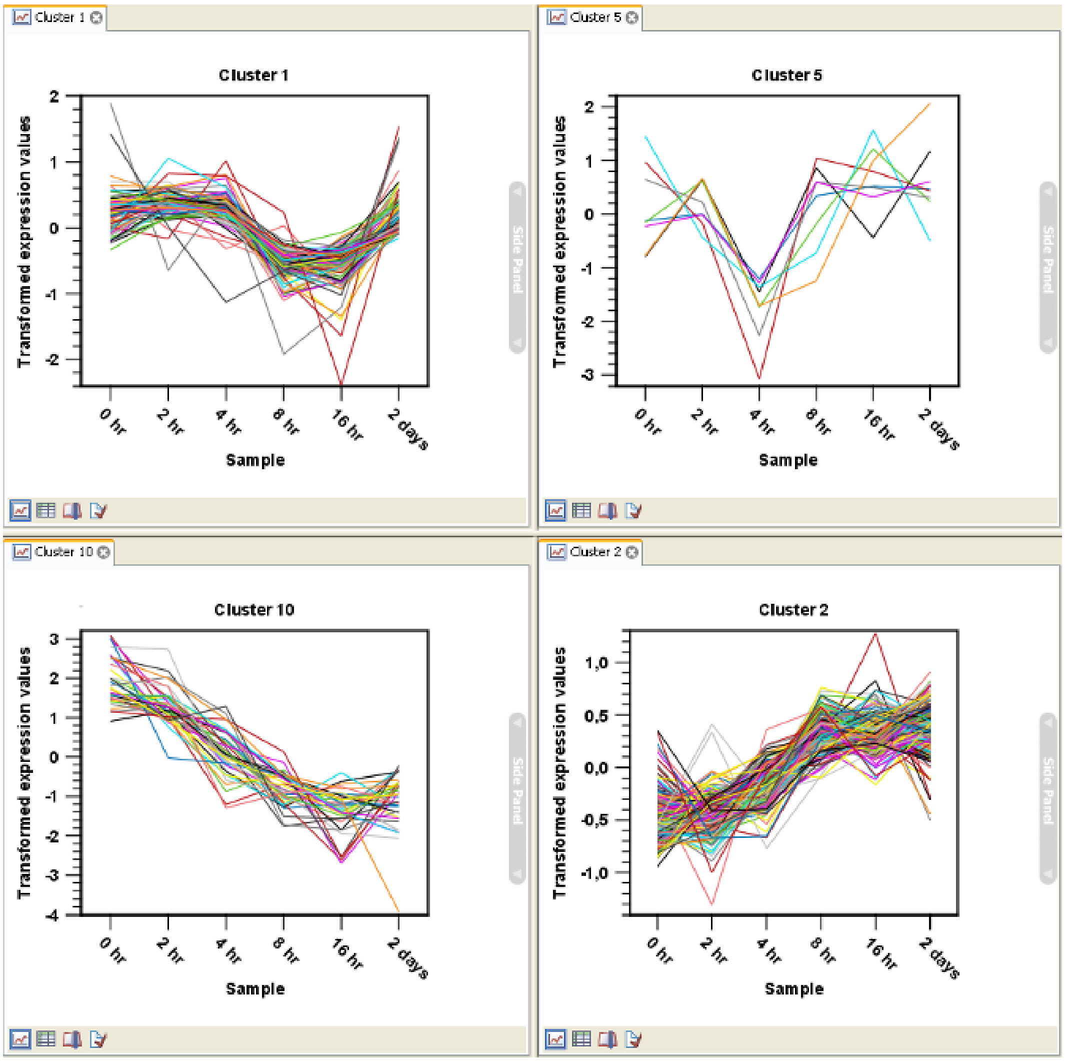


Figure 25.46: *Four clusters created by k-means/medoids clustering.*

The samples used are from a time-series experiment, and you can see that the expression levels for each cluster have a distinct pattern. The two clusters at the bottom have falling and rising expression levels, respectively, and the two clusters at the top both fall at the beginning but then rise again (the one to the right starts to rise earlier that the other one).

Having inspected the graphs, you may wish to take a closer look at the features represented in each cluster. In the experiment table, the clustering has added an extra column with the name of the cluster that the feature belongs to. In this way you can filter the table to see only features from a specific cluster. This also means that you can select the feature of this cluster in a volcano or scatter plot as described in section 25.1.5.

25.5 Statistical analysis - identifying differential expression

The *CLC Main Workbench* is designed to help you identify differential expression.

You have a choice of a number of standard statistical tests, that are suitable for different data types and different types of experimental settings. There are two main types of tests: tests that assume that data consists of counts and compare these or their proportions (described in section 25.5.1) and tests that assume that the data is real-valued, has Gaussian distributions and compare means (described in section 25.5.2).

To run the statistical analysis:

Expression Analysis ( )| Statistical Analysis ( ) | Proportion-based Statistical Analysis ( )

or **Expression Analysis ( )| Statistical Analysis ( ) | Gaussian Statistical Analysis ( )**

For all kinds of statistical analyses, you first select the experiment ( ) that you wish to use and click **Next** (learn more about setting up experiments in section 25.1.1).

The first part of the explanation of how to proceed and perform the statistical analysis is divided into three, depending on whether you are doing tests on proportions or Gaussian-based tests. The last part has an explanation of the options regarding corrected p-values which applies to all tests.

25.5.1 Tests on proportions

The proportions-based tests are applicable in situations where your data samples consists of counts of a number of 'types' of data. This could e.g. be in a study where gene expression levels are measured by tag profiling for example. Here the different 'types' could correspond to the different 'genes' in a reference genome, and the counts could be the numbers of reads matching each of these genes. The tests compare counts by considering the proportions that they make up the total sum of counts in each sample. By comparing the expression levels at the level of proportions rather than raw counts, the data is corrected for sample size.

There are two tests available for comparing proportions: the test of [Kal et al., 1999] and the test of [Baggerly et al., 2003]. Both tests compare pairs of groups. If you have a multi-group experiment (see section 25.1.1), you may choose either to have tests produced for all pairs of groups (by clicking the 'All pairs' button) or to have a test produced for each group compared to a specified reference group (by clicking the 'Against reference' button). In the last case you must specify which of the groups you want to use as reference (the default is to use the group you specified as Group 1 when you set up the experiment).

Note that the proportion-based tests use the total sample counts (that is, the sum over all expression values). If one (or more) of the counts are NaN, the sum will be NaN and all the test statistics will be NaN. As a consequence all p-values will also be NaN. You can avoid this by filtering your experiment and creating a new experiment so that no NaN values are present, before you apply the tests.

Kal et al.'s test (Z-test)

Kal et al.'s test [Kal et al., 1999] compares a single sample against another single sample, and thus requires that each group in you experiment has only one sample. The test relies on an approximation of the binomial distribution by the normal distribution [Kal et al., 1999]. Considering proportions rather than raw counts the test is also suitable in situations where the sum of counts is different between the samples.

When Kal's test is run on an experiment four columns will be added to the experiment table for each pair of groups that are analyzed. The 'Proportions difference' column contains the difference between the proportion in group 2 and the proportion in group 1. The 'Fold Change' column tells you how many times bigger the proportion in group 2 is relative to that of group 1. If the proportion in group 2 is bigger than that in group 1 this value is the proportion in group 2 divided by that in group 1. If the proportion in group 2 is smaller than that in group 1 the fold change is the proportion in group 1 divided by that in group 2 with a negative sign. The 'Test statistic' column holds that value of the test statistic, and the 'P-value' holds the two-sided p-value for the test. Up to two more columns may be added if the options to calculate Bonferroni and FDR corrected p-values were chosen.

Baggerley et al.'s test (Beta-binomial)

Baggerley et al.'s test [Baggerly et al., 2003] compares the proportions of counts in a group of samples against those of another group of samples, and is suited to cases where replicates are available in the groups. The samples are given different weights depending on their sizes (total counts). The weights are obtained by assuming a Beta distribution on the proportions in a group, and estimating these, along with the proportion of a binomial distribution, by the method of moments. The result is a weighted t-type test statistic.

When Baggerley's test is run on an experiment four columns will be added to the experiment table for each pair of groups that are analyzed. The 'Weighted proportions difference' column contains the difference between the mean of the weighted proportions across the samples assigned to group 2 and the mean of the weighted proportions across the samples assigned to group 1. The 'Weighted proportions fold change' column tells you how many times bigger the mean of the weighted proportions in group 2 is relative to that of group 1. If the mean of the weighted proportions in group 2 is bigger than that in group 1 this value is the mean of the weighted proportions in group 2 divided by that in group 1. If the mean of the weighted proportions in group 2 is smaller than that in group 1 the fold change is the mean of the weighted proportions in group 1 divided by that in group 2 with a negative sign. The 'Test statistic' column holds that value of the test statistic, and the 'P-value' holds the two-sided p-value for the test. Up to two more columns may be added if the options to calculate Bonferroni and FDR corrected p-values were chosen.

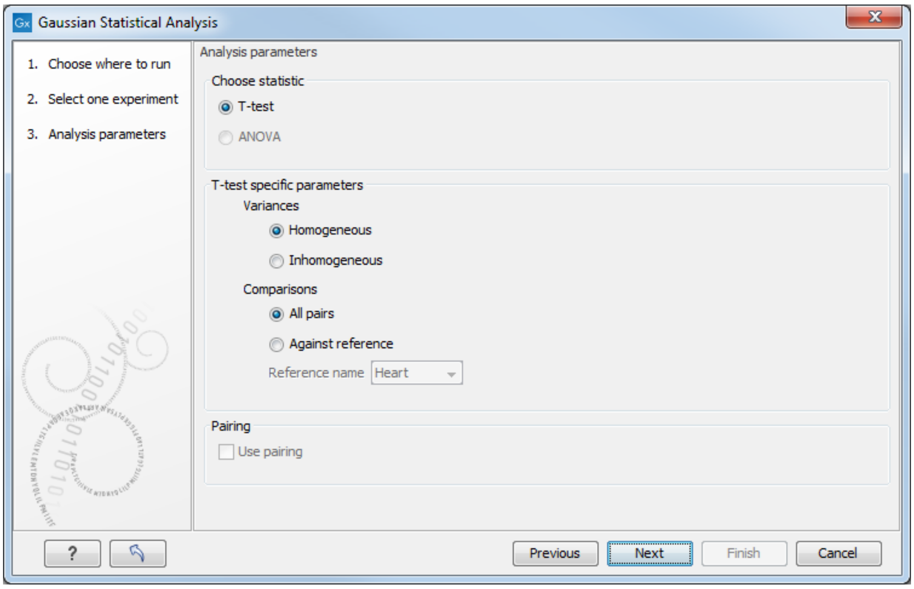
25.5.2 Gaussian-based tests

The tests based on the Gaussian distribution essentially compare the mean expression level in the experimental groups in the study, and evaluates the significance of the difference relative to the variance (or 'spread') of the data within the groups. The details of the formula used for calculating the test statistics vary according to the experimental setup and the assumptions you make about the data (read more about this in the sections on t-test and ANOVA below). The explanation of how to proceed is divided into two, depending on how many groups there are in your experiment. First comes the explanation for t-tests which is the only analysis available for two-group experimental setups (t-tests can also be used for pairwise comparison of groups in multi-group experiments). Next comes an explanation of the ANOVA test which can be used for multi-group experiments.

Note that the test statistics for the t-test and ANOVA analysis use the estimated group variances in their denominators. If all expression values in a group are identical the estimated variance for that group will be zero. If the estimated variances for both (or all) groups are zero the denominator of the test statistic will be zero. The numerator's value depends on the difference of the group means. If this is zero, the numerator is zero and the test statistic will be 0/0 which is NaN. If the numerator is different from zero the test statistic will be + or - infinity, depending on which group mean is bigger. If all values in all groups are identical the test statistic is set to zero.

T-tests

For experiments with two groups you can, among the Gaussian tests, only choose a **T-test** as shown in figure 25.47.



Gaussian Statistical Analysis

1. Choose where to run
2. Select one experiment
3. Analysis parameters

Analysis parameters

Choose statistic
- ● T-test
- ○ ANOVA

T-test specific parameters

Variances
- ● Homogeneous
- ○ Inhomogeneous

Comparisons
- ● All pairs
- ○ Against reference

Reference name: Heart

Pairing
- □ Use pairing

Figure 25.47: *Selecting a t-test.*

There are different types of t-tests, depending on the assumption you make about the variances in the groups. By selecting 'Homogeneous' (the default) calculations are done assuming that the groups have equal variances. When 'In-homogeneous' is selected, this assumption is not made.

The t-test can also be chosen if you have a multi-group experiment. In this case you may choose either to have t-tests produced for all pairs of groups (by clicking the 'All pairs' button) or to have a t-test produced for each group compared to a specified reference group (by clicking the 'Against reference' button). In the last case you must specify which of the groups you want to use as reference (the default is to use the group you specified as Group 1 when you set up the experiment).

If a experiment with pairing was set up (see section 25.1.1) the **Use pairing** tick box is active. If ticked, paired t-tests will be calculated, if not, the formula for the standard t-test will be used.

When a t-test is run on an experiment four columns will be added to the experiment table for each pair of groups that are analyzed. The 'Difference' column contains the difference between the mean of the expression values across the samples assigned to group 2 and the mean of the expression values across the samples assigned to group 1. The 'Fold Change' column tells you how many times bigger the mean expression value in group 2 is relative to that of group 1. If the mean expression value in group 2 is bigger than that in group 1 this value is the mean expression value in group 2 divided by that in group 1. If the mean expression value in group 2 is smaller than that in group 1 the fold change is the mean expression value in group 1 divided by that in group 2 with a negative sign. The 'Test statistic' column holds that value of the test statistic, and the 'P-value' holds the two-sided p-value for the test. Up to two more columns may be added if the options to calculate Bonferroni and FDR corrected p-values were chosen.

ANOVA

For experiments with more than two groups you can choose **T-test**, see section 25.5.2, or **ANOVA**.

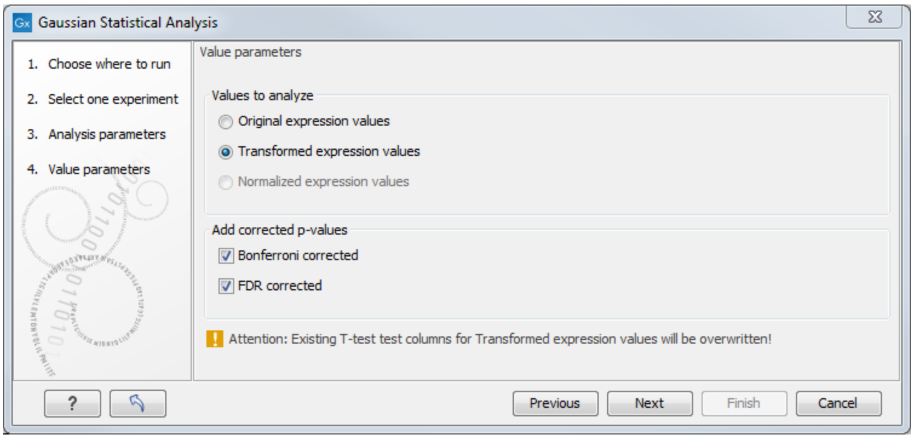
The ANOVA method allows analysis of an experiment with one factor and a number of groups, e.g. different types of tissues, or time points. In the analysis, the variance within groups is compared to the variance between groups. You get a significant result (that is, a small ANOVA p-value) if the difference you see between groups relative to that within groups, is larger than what you would expect, if the data were really drawn from groups with equal means.

If an experiment with pairing was set up (see section 25.1.1) the **Use pairing** tick box is active. If ticked, a repeated measures one-way ANOVA test will be calculated, if not, the formula for the standard one-way ANOVA will be used.

When an ANOVA analysis is run on an experiment four columns will be added to the experiment table for each pair of groups that are analyzed. The 'Max difference' column contains the difference between the maximum and minimum of the mean expression values of the groups, multiplied by -1 if the group with the maximum mean expression value occurs before the group with the minimum mean expression value (with the ordering: group 1, group 2, ...). The 'Max fold change' column contains the ratio of the maximum of the mean expression values of the groups to the minimum of the mean expression values of the groups, multiplied by -1 if the group with the maximum mean expression value occurs before the group with the minimum mean expression value (with the ordering: group 1, group 2, ...). The 'Test statistic' column holds the value of the test statistic, and the 'P-value' holds the two-sided p-value for the test. Up to two more columns may be added if the options to calculate Bonferroni and FDR corrected p-values were chosen.

25.5.3 Corrected p-values

Clicking **Next** will display a dialog as shown in figure 25.48.



1. Choose where to run
2. Select one experiment
3. Analysis parameters
4. Value parameters

Value parameters

Values to analyze

○ Original expression values
● Transformed expression values
○ Normalized expression values

Add corrected p-values

☑ Bonferroni corrected
☑ FDR corrected

Attention: Existing T-test test columns for Transformed expression values will be overwritten!

Figure 25.48: *Additional settings for the statistical analysis.*

At the top, you can select which values to analyze (see section 25.2.1).

Below you can select to add two kinds of corrected p-values to the analysis (in addition to the standard p-value produced for the test statistic):

- **Bonferroni corrected**.
- **FDR corrected**.

Both are calculated from the original p-values, and aim in different ways to take into account the issue of multiple testing [Dudoit et al., 2003]. The problem of multiple testing arises because the original p-values are related to a single test: the p-value is the probability of observing a more extreme value than that observed in the test carried out. If the p-value is 0.04, we would expect an as extreme value as that observed in 4 out of 100 tests carried out among groups with no difference in means. Popularly speaking, if we carry out 10000 tests and select the features with original p-values below 0.05, we will expect about 0.05 times 10000 = 500 to be false positives.

The Bonferroni corrected p-values handle the multiple testing problem by controlling the 'family-wise error rate': the probability of making at least one false positive call. They are calculated by multiplying the original p-values by the number of tests performed. The probability of having at least one false positive among the set of features with Bonferroni corrected p-values below 0.05, is less than 5%. The Bonferroni correction is conservative: there may be many genes that are differentially expressed among the genes with Bonferroni corrected p-values above 0.05, that will be missed if this correction is applied.

Instead of controlling the family-wise error rate we can control the false discovery rate: FDR. The false discovery rate is the proportion of false positives among all those declared positive. We expect 5 % of the features with FDR corrected p-values below 0.05 to be false positive. There are many methods for controlling the FDR - the method used in *CLC Main Workbench* is that of [Benjamini and Hochberg, 1995].

Click **Finish** to start the tool.

Note that if you have already performed statistical analysis on the same values, the existing one will be overwritten.

25.5.4 Volcano plots - inspecting the result of the statistical analysis

The results of the statistical analysis are added to the experiment and can be shown in the experiment table, see section 25.1.2. Typically columns containing the differences (or weighted differences) of the mean group values and the fold changes (or weighted fold changes) of the mean group values will be added along with a column of p-values. Also, columns with FDR or Bonferroni corrected p-values will be added if these were calculated. This added information allows features to be sorted and filtered to exclude the ones without sufficient proof of differential expression (learn more in section 9).

If you want a more visual approach to the results of the statistical analysis, you can click the **Show Volcano Plot** ( ) button at the bottom of the experiment table view. In the same way as the scatter plot (see section 25.1.4 for details), the volcano plot is yet another view on the experiment. Because it uses the p-values and mean differences produced by the statistical analysis, the plot is only available once a statistical analysis has been performed on the experiment.

An example of a volcano plot is shown in figure 25.49.

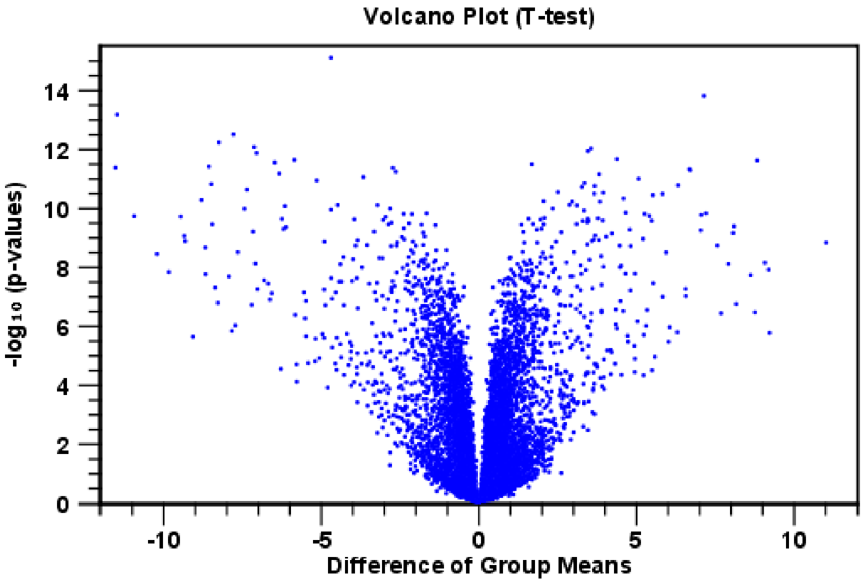


Figure 25.49: *Volcano plot.*

The volcano plot shows the relationship between the p-values of a statistical test and the magnitude of the difference in expression values of the samples in the groups. On the y-axis the $-\log_{10}$ p-values are plotted. For the x-axis you may choose between two sets of values by choosing either 'Fold change' or 'Difference' in the volcano plot side panel's 'Values' part. If you choose 'Fold change' the log of the values in the 'fold change' (or 'Weighted fold change') column for the test will be displayed. If you choose 'Difference' the values in the 'Difference' (or 'Weighted difference') column will be used. Which values you wish to display will depend upon the scale of you data (Read the note on fold change in section 25.1.2).

The larger the difference in expression of a feature, the more extreme it's point will lie on the X-axis. The more significant the difference, the smaller the p-value and thus the higher the $-\log_{10}(p)$ value. Thus, points for features with highly significant differences will lie high in the plot. Features of interest are typically those which change significantly and by a certain magnitude. These are the points in the upper left and upper right hand parts of the volcano plot.

If you have performed different tests or you have an experiment with multiple groups you need to specify for which test and which group comparison you want the volcano plot to be shown. You do this in the 'Test' and 'Values' parts of the volcano plot side panel.

Options for the volcano plot are described in further detail when describing the **Side Panel** below.

If you place your mouse on one of the dots, a small text box will tell the name of the feature. Note that you can zoom in and out on the plot (see section 2.2).

In the **Side Panel** to the right, there is a number of options to adjust the view of the volcano plot. Under **Graph preferences**, you can adjust the general properties of the volcano plot

- **Lock axes** This will always show the axes even though the plot is zoomed to a detailed level.
- **Frame** Shows a frame around the graph.
- **Show legends** Shows the data legends.
- **Tick type** Determine whether tick lines should be shown outside or inside the frame.
- **Tick lines at** Choosing Major ticks will show a grid behind the graph.
- **Horizontal axis range** Sets the range of the horizontal axis (x axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **Vertical axis range** Sets the range of the vertical axis (y axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.

Below the general preferences, you find the **Dot properties**, where you can adjust coloring and appearance of the dots.

- **Dot type** Can be None, Cross, Plus, Square, Diamond, Circle, Triangle, Reverse triangle, or Dot.
- **Dot color** Click the color box to select a color.

At the very bottom, you find two groups for choosing which values to display:

- **Test**. In this group, you can select which kind of test you want the volcano plot to be shown for.
- **Values**. Under **Values**, you can select which values to plot. If you have multi-group experiments, you can select which groups to compare. You can also select whether to plot **Difference** or **Fold change** on the x-axis. Read the note on fold change in section 25.1.2.

Note that if you wish to use the same settings next time you open a box plot, you need to save the settings of the **Side Panel** (see section 4.6).

25.6 Annotation tests

The annotation tests are tools for detecting significant patterns among features (e.g. genes) of experiments, based on their annotations. This may help in interpreting the analysis of the large numbers of features in an experiment in a biological context. Which biological context, depends on which annotation you choose to examine, and could e.g. be biological process, molecular function or pathway as specified by the Gene Ontology or KEGG. The annotation testing tools of course require that the features in the experiment you want to analyze are annotated. Learn how to annotate an experiment in section 25.1.3.

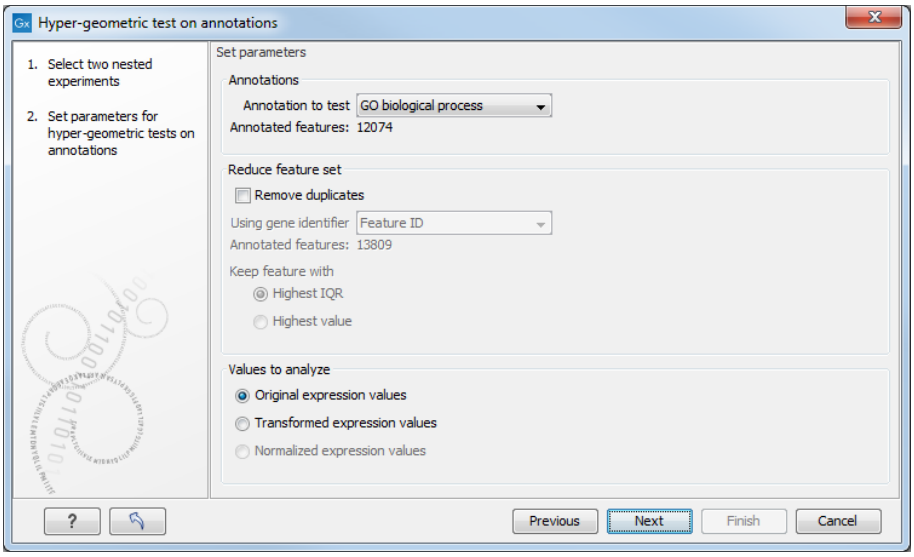
25.6.1 Hypergeometric Tests on Annotations

The first approach to using annotations to extract biological information is the hypergeometric annotation test. This test measures the extent to which the annotation categories of features in a smaller gene list, 'A', are over or under-represented relative to those of the features in larger gene list 'B', of which 'A' is a sub-list. Gene list B is often the features of the full experiment, possibly with features which are thought to represent only noise, filtered away. Gene list A is a sub-experiment of the full experiment where most features have been filtered away and only those that seem of interest are kept. Typically gene list A will consist of a list of candidate differentially expressed genes. This could be the gene list obtained after carrying out a statistical analysis on the experiment, and choosing to keep only those features with FDR corrected p-values <0.05 and a fold change larger than 2 in absolute value. The hyper geometric test procedure implemented is similar to the unconditional GOstats test of [Falcon and Gentleman, 2007].

> **Tools** | **Expression Analysis ()**| **Annotation Test** () | **Hypergeometric Tests on Annotations ()**

This will show a dialog where you can select the two experiments - the larger experiment, e.g. the original experiment including the full list of features - and a sub-experiment (see how to create a sub-experiment in section 25.1.2).

Click **Next**. This will display the dialog shown in figure 25.50.



Hyper-geometric test on annotations

1. Select two nested experiments
2. Set parameters for hyper-geometric tests on annotations

Set parameters

Annotations

Annotation to test: GO biological process

Annotated features: 12074

Reduce feature set

□ Remove duplicates

Using gene identifier: Feature ID

Annotated features: 13809

Keep feature with

● Highest IQR

○ Highest value

Values to analyze

● Original expression values

○ Transformed expression values

○ Normalized expression values

Figure 25.50: *Parameters for performing a hypergeometric test on annotations.*

At the top, you select which annotation to use for testing. You can select from all the annotations available on the experiment, but it is of course only a few that are biologically relevant. Once you have selected an annotation, you will see the number of features carrying this annotation below.

Annotations are typically given at the gene level. Often a gene is represented by more than one feature in an experiment. If this is not taken into account it may lead to a biased result. The standard way to deal with this is to reduce the set of features considered, so that each gene is represented only once. In the next step, **Remove duplicates**, you can choose the basis on which the feature set will be reduced:

- **Using gene identifier**.
- **Keep feature with**:
  - **Highest IQR**. The feature with the highest interquartile range (IQR) is kept.
  - **Highest value**. The feature with the highest expression value is kept.

First you specify which annotation you want to use as gene identifier. Once you have selected this, you will see the number of features carrying this annotation below. Next you specify which feature you want to keep for each gene. This may be either the feature with the highest inter-quartile range or the highest value.

At the bottom, you can select which values to analyze (see section 25.2.1). Only features that have a numerical value assigned to them will be used for the analysis. That is, any feature which has a value of plus infinity, minus infinity or NaN will not be included in the feature list taken into the test. Thus, the choice of value at this step can affect the features that are taken forward into the test in two ways:

- If there are features with values of plus infinity, minus infinity or NaN, those features will not be taken forward into the test. This can be a consideration when choosing transformed values, where the mathematical manipulations involved may lead to such values.
- If you chose to remove duplicates, then the value type you choose here is the value used for checking the highest IQR or value to determine which feature is taken forward into the test.

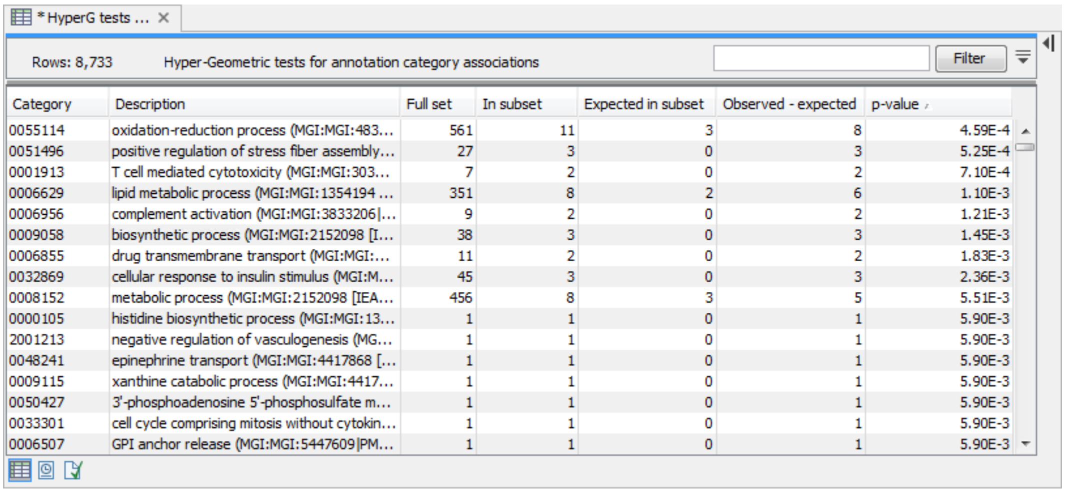
Click **Finish** to start the tool.

The final number of features used for the test is reported in this history view of the test results.

Result of hypergeometric tests on annotations The result of performing hypergeometric tests on annotations using GO biological process is shown in figure 25.51.

The table shows the following information:

- **Category**. This is the identifier for the category.
- **Description**. This is the description belonging to the category. Both of these are simply extracted from the annotations.



Rows: 8,733 Hyper-Geometric tests for annotation category associations

Category	Description	Full set	In subset	Expected in subset	Observed - expected	p-value
0055114	oxidation-reduction process (MGI:MGI:483...	561	11	3	8	4.59E-4
0051496	positive regulation of stress fiber assembly...	27	3	0	3	5.25E-4
0001913	T cell mediated cytotoxicity (MGI:MGI:303...	7	2	0	2	7.10E-4
0006629	lipid metabolic process (MGI:MGI:1354194 ...	351	8	2	6	1.10E-3
0006956	complement activation (MGI:MGI:3833206|...	9	2	0	2	1.21E-3
0009058	biosynthetic process (MGI:MGI:2152098 [I...	38	3	0	3	1.45E-3
0006855	drug transmembrane transport (MGI:MGI:...	11	2	0	2	1.83E-3
0032869	cellular response to insulin stimulus (MGI:M...	45	3	0	3	2.36E-3
0008152	metabolic process (MGI:MGI:2152098 [IEA...	456	8	3	5	5.51E-3
0000105	histidine biosynthetic process (MGI:MGI:13...	1	1	0	1	5.90E-3
2001213	negative regulation of vasculogenesis (MG...	1	1	0	1	5.90E-3
0048241	epinephrine transport (MGI:MGI:4417868 [...	1	1	0	1	5.90E-3
0009115	xanthine catabolic process (MGI:MGI:4417...	1	1	0	1	5.90E-3
0050427	3'-phosphoadenosine 5'-phosphosulfate m...	1	1	0	1	5.90E-3
0033301	cell cycle comprising mitosis without cytokin...	1	1	0	1	5.90E-3
0006507	GPI anchor release (MGI:MGI:5447609|PM...	1	1	0	1	5.90E-3

Figure 25.51: *The result of testing on GO biological process.*

- **Full set.** The number of features in the original experiment (not the subset) with this category. (Note that this is after removal of duplicates).
- **In subset.** The number of features in the subset with this category. (Note that this is after removal of duplicates).
- **Expected in subset.** The number of features we would have expected to find with this annotation category in the subset, if the subset was a random draw from the full set.
- **Observed - expected.** 'In subset' - 'Expected in subset'
- **p-value.** The tail probability of the hyper geometric distribution This is the value used for sorting the table.

Categories with small p-values are over-represented on the features in the subset relative to the full set.

GO terms are organized in a hierarchical structure. For example, the term "GO:0033151 V(D)J recombination" from the Gene Ontology [Ashburner et al., 2000,The Gene Ontology Consortium, 2019] (`https://geneontology.org/`) is a descendant of "GO:0006259 DNA metabolic process".

When testing for the significance of a particular GO term, all features linked to descendant GO terms are included in the test. This can lead to a higher number of detected genes in the output table, compared to the number of genes linked to the tested GO term.

Due to the hierarchical structure, GO terms are not independent of one another, and the p-values provided in the table should be interpreted with caution.

25.6.2 Gene Set Enrichment Analysis

When carrying out a hypergeometric test on annotations you typically compare the annotations of the genes in a subset containing 'the significantly differentially expressed genes' to those of the total set of genes in the experiment. Which, and how many, genes are included in the subset is somewhat arbitrary - using a larger or smaller p-value cut-off will result in including more or less. Also, the magnitudes of differential expression of the genes is not considered.

The Gene Set Enrichment Analysis (GSEA) does NOT take a sublist of differentially expressed genes and compare it to the full list - it takes a single gene list (a single experiment). The idea behind GSEA is to consider a measure of association between the genes and phenotype of interest (e.g. test statistic for differential expression) and rank the genes according to this measure of association. A test is then carried out for each annotation category, for whether the ranks of the genes in the category are evenly spread throughout the ranked list, or tend to occur at the top or bottom of the list.

The GSEA test implemented here is that of [Tian et al., 2005]. The test implicitly calculates and uses a standard t-test statistic for two-group experiments, and ANOVA statistic for multiple group experiments for each feature, as measures of association. For each category, the test statistics for the features in than category are summed and a category based test statistic is calculated as this sum divided by the square root of the number of features in the category. Note that if a feature has the value NaN in one of the samples, the t-test statistic for the feature will be NaN. Consequently, the combined statistic for each of the categories in which the feature is included will be NaN. Thus, it is advisable to filter out any feature that has a NaN value before applying GSEA.

The p-values for the GSEA test statistics are calculated by permutation: The original test statistics for the features are permuted and new test statistics are calculated for each category, based on the permuted feature test statistics. This is done the number of times specified by the user in the wizard. For each category, the lower and upper tail probabilities are calculated by comparing the original category test statistics to the distribution of the permutation-based test statistics for that category. The lower and higher tail probabilities are the number of these that are lower and higher, respectively, than the observed value, divided by the number of permutations.

As the p-values are based on permutations you may some times see results where category x's test statistic is lower than that of category y and the categories are of equal size, but where the lower tail probability of category x is higher than that of category y. This is due to imprecision in the estimations of the tail probabilities from the permutations. The higher the number of permutations, the more stable the estimation.

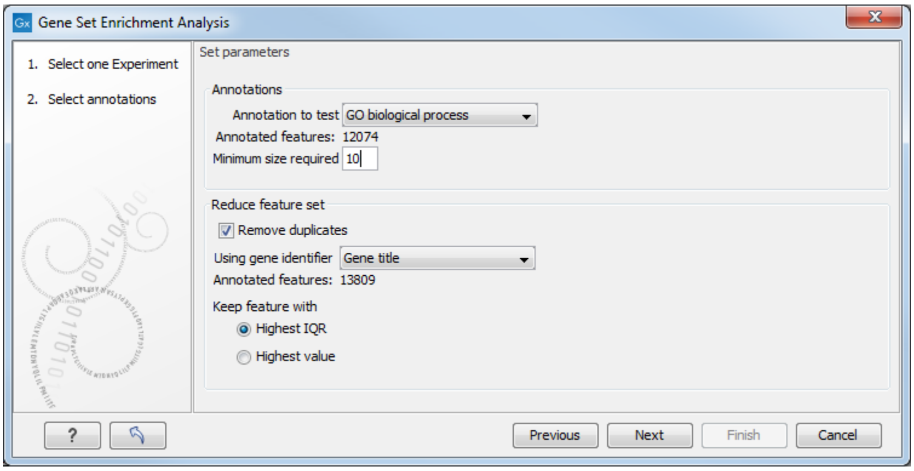
You may run a GSEA on a full experiment, or on a sub-experiment where you have filtered away features that you think are un-informative and represent only noise. Typically you will remove features that are constant across samples (those for which the value in the 'Range' column is zero' — these will have a t-test statistic of zero) and/or those for which the inter-quantile range is small. As the GSEA algorithm calculates and ranks genes on p-values from a test of differential expression, it will generally not make sense to filter the experiment on p-values produced in an analysis if differential expression, prior to running GSEA on it.

> **Tools** | **Expression Analysis ( )**| **Annotation Test** ( ) | **Gene Set Enrichment Analysis (GSEA) ( )**

Select an experiment and click **Next**.

Click **Next**. This will display the dialog shown in figure 25.52.

At the top, you select which annotation to use for testing. You can select from all the annotations available on the experiment, but it is of course only a few that are biologically relevant. Once you have selected an annotation, you will see the number of features carrying this annotation below.



Gene Set Enrichment Analysis

1. Select one Experiment
2. Select annotations

Set parameters

Annotations

Annotation to test: GO biological process

Annotated features: 12074

Minimum size required: 10

Reduce feature set

☑ Remove duplicates

Using gene identifier: Gene title

Annotated features: 13809

Keep feature with

● Highest IQR

○ Highest value

Figure 25.52: *Gene set enrichment analysis on GO biological process.*

In addition, you can set a filter: **Minimum size required**. Only categories with more genes (i.e. features) than the specified number will be considered. Excluding categories with small numbers of genes may lead to more robust results.

Annotations are typically given at the gene level. Often a gene is represented by more than one feature in an experiment. If this is not taken into account it may lead to a biased result. The standard way to deal with this is to reduce the set of features considered, so that each gene is represented only once. Check the **Remove duplicates** check box to reduce the feature set, and you can choose how you want this to be done:

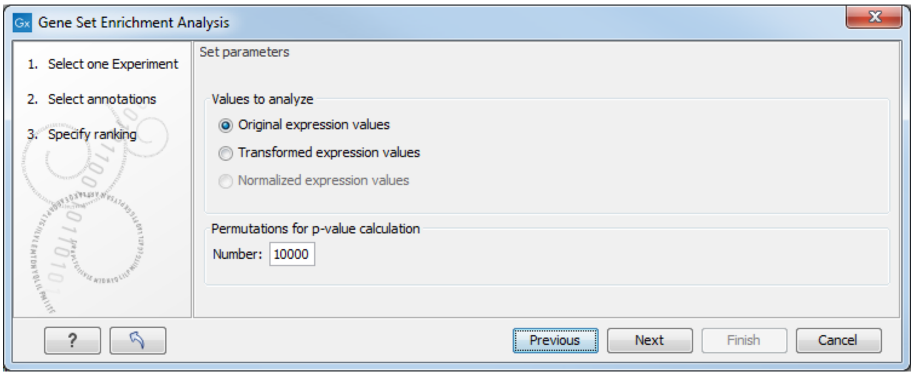
- **Using gene identifier**.
- **Keep feature with**:
  - **Highest IQR**. The feature with the highest interquartile range (IQR) is kept.
  - **Highest value**. The feature with the highest expression value is kept.

First you specify which annotation you want to use as gene identifier. Once you have selected this, you will see the number of features carrying this annotation below. Next you specify which feature you want to keep for each gene. This may be either the feature with the highest inter-quartile range or the highest value.

Clicking **Next** will display the dialog shown in figure 25.53.

At the top, you can select which values to analyze (see section 25.2.1).

Below, you can set the **Permutations for p-value calculation**. For the GSEA test a p-value is calculated by permutation: p permuted data sets are generated, each consisting of the original features, but with the test statistics permuted. The GSEA test is run on each of the permuted data sets. The test statistic is calculated on the original data, and the resulting value is compared to the distribution of the values obtained for the permuted data sets. The permutation based p-value is the number of permutation based test statistics above (or below) the value of the test statistic for the original data, divided by the number of permuted data sets. For reliable permutation-based p-value calculation a large number of permutations is required (100 is the default).



Gene Set Enrichment Analysis

1. Select one Experiment
2. Select annotations
3. Specify ranking

Set parameters

Values to analyze

- ● Original expression values
- ○ Transformed expression values
- ○ Normalized expression values

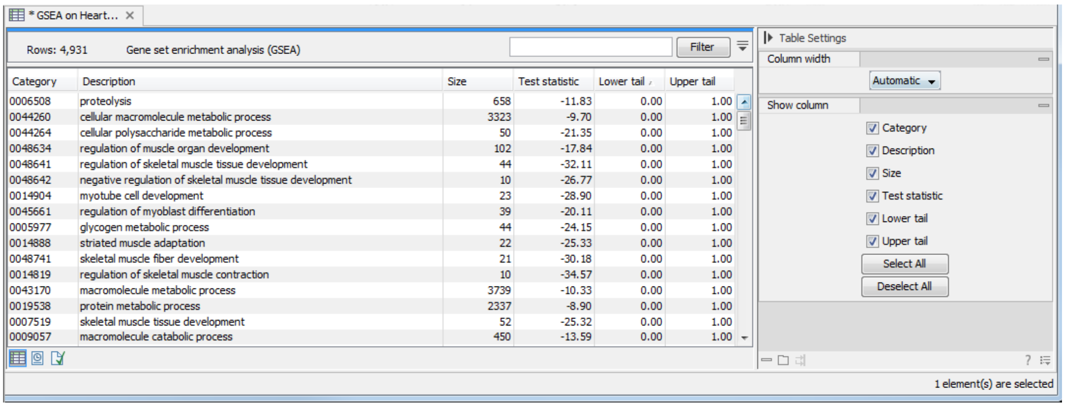
Permutations for p-value calculation

Number: 10000

Figure 25.53: *Gene set enrichment analysis parameters.*

Click **Finish** to start the tool.

Result of gene set enrichment analysis The result of performing gene set enrichment analysis using GO biological process is shown in figure 25.54.



* GSEA on Heart...

Rows: 4,931 Gene set enrichment analysis (GSEA)

Category	Description	Size	Test statistic	Lower tail	Upper tail
0006508	proteolysis	658	-11.83	0.00	1.00
0044260	cellular macromolecule metabolic process	3323	-9.70	0.00	1.00
0044264	cellular polysaccharide metabolic process	50	-21.35	0.00	1.00
0048634	regulation of muscle organ development	102	-17.84	0.00	1.00
0048641	regulation of skeletal muscle tissue development	44	-32.11	0.00	1.00
0048642	negative regulation of skeletal muscle tissue development	10	-26.77	0.00	1.00
0014904	myotube cell development	23	-28.90	0.00	1.00
0045661	regulation of myoblast differentiation	39	-20.11	0.00	1.00
0005977	glycogen metabolic process	44	-24.15	0.00	1.00
0014888	striated muscle adaptation	22	-25.33	0.00	1.00
0048741	skeletal muscle fiber development	21	-30.18	0.00	1.00
0014819	regulation of skeletal muscle contraction	10	-34.57	0.00	1.00
0043170	macromolecule metabolic process	3739	-10.33	0.00	1.00
0019538	protein metabolic process	2337	-8.90	0.00	1.00
0007519	skeletal muscle tissue development	52	-25.32	0.00	1.00
0009057	macromolecule catabolic process	450	-13.59	0.00	1.00

Table Settings

Column width: Automatic

Show column

- ☑ Category
- ☑ Description
- ☑ Size
- ☑ Test statistic
- ☑ Lower tail
- ☑ Upper tail

1 element(s) are selected

Figure 25.54: *The result of gene set enrichment analysis on GO biological process.*

The table shows the following information:

- **Category**. This is the identifier for the category.
- **Description**. This is the description belonging to the category. Both of these are simply extracted from the annotations.
- **Size**. The number of features with this category. (Note that this is after removal of duplicates).
- **Test statistic**. This is the GSEA test statistic.
- **Lower tail**. This is the mass in the permutation based p-value distribution below the value of the test statistic.
- **Upper tail**. This is the mass in the permutation based p-value distribution above the value of the test statistic.

A small lower (or upper) tail p-value for an annotation category is an indication that features in this category viewed as a whole are perturbed among the groups in the experiment considered.

GO terms are organized in a hierarchical structure. For example, the term "GO:0033151 V(D)J recombination" from the Gene Ontology [Ashburner et al., 2000, The Gene Ontology Consortium, 2019] (`https://geneontology.org/`) is a descendant of "GO:0006259 DNA metabolic process".

When testing for the significance of a particular GO term, all features linked to descendant GO terms are included in the test. This can lead to a higher number of detected genes in the output table, compared to the number of genes linked to the tested GO term.

25.7 General plots

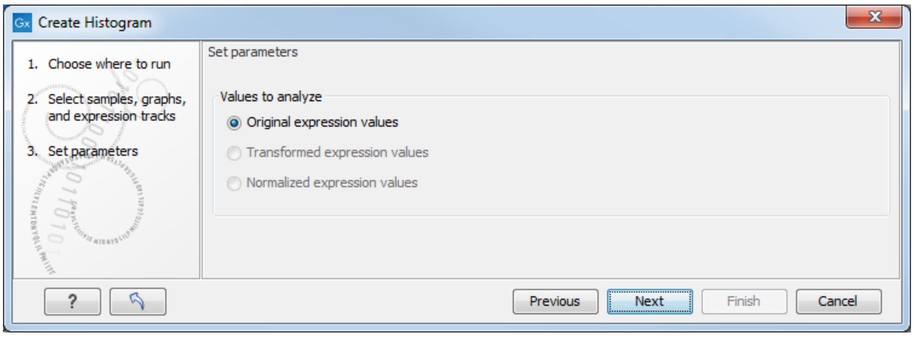
In the **General Plots** folder, you find three general plots that may be useful at various point of your analysis work flow. The plots are explained in detail below.

25.7.1 Histogram

A histogram shows a distribution of a set of values. Histograms are often used for examining and comparing distributions, e.g. of expression values of different samples, in the quality control step of an analysis. You can create a histogram showing the distribution of expression value for a sample:

> **Tools | Expression Analysis ( )| General Plots** ( ) | **Create Histogram ( )**

Select a number of samples (( ), ( ), ( )) or a graph track. When you have selected more than one sample, a histogram will be created for each one. Clicking **Next** will display a dialog as shown in figure 25.55.



Create Histogram

1. Choose where to run
2. Select samples, graphs, and expression tracks
3. Set parameters

Set parameters

Values to analyze

● Original expression values

○ Transformed expression values

○ Normalized expression values

Figure 25.55: *Selecting which values the histogram should be based on.*

In this dialog, you select the values to be used for creating the histogram (see section 25.2.1).

Click **Finish** to start the tool.

Viewing histograms

The resulting histogram is shown in a figure 25.56

The histogram shows the expression value on the x axis (in the case of figure 25.56 the transformed expression values) and the counts of these values on the y axis.

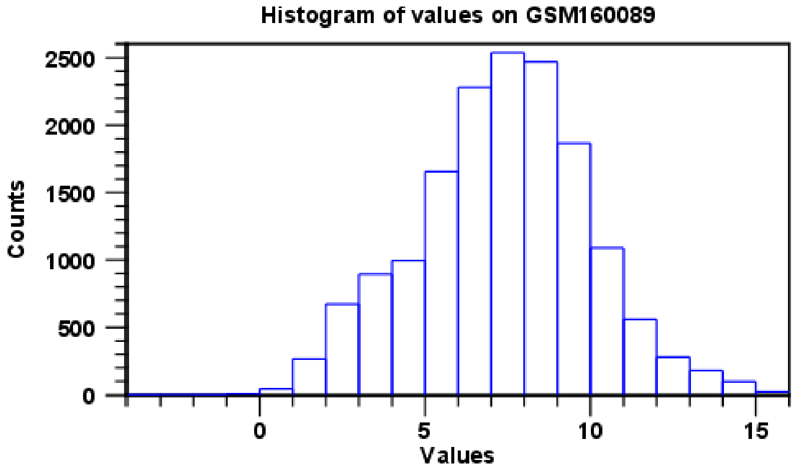


Figure 25.56: *Histogram showing the distribution of transformed expression values.*

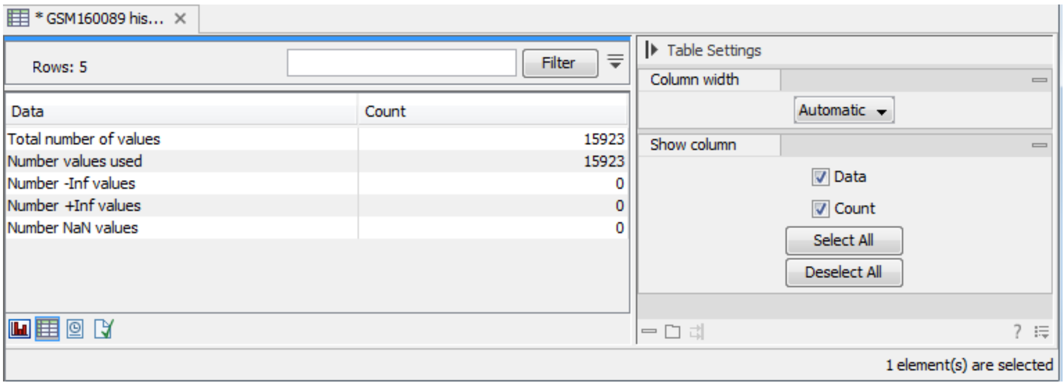
In the **Side Panel** to the left, there is a number of options to adjust the view. Under **Graph preferences**, you can adjust the general properties of the plot.

- **Lock axes** This will always show the axes even though the plot is zoomed to a detailed level.
- **Frame** Shows a frame around the graph.
- **Show legends** Shows the data legends.
- **Tick type** Determine whether tick lines should be shown outside or inside the frame.
- **Tick lines at** Choosing Major ticks will show a grid behind the graph.
- **Horizontal axis range** Sets the range of the horizontal axis (x axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **Vertical axis range** Sets the range of the vertical axis (y axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **Break points**. Determines where the bars in the histogram should be:
  - **Sturges method**. This is the default. The number of bars is calculated from the range of values by Sturges formula [Sturges, 1926].
  - **Equi-distanced bars**. This will show bars from **Start** to **End** and with a width of **Sep**.
  - **Number of bars**. This will simply create a number of bars starting at the lowest value and ending at the highest value.

Below the graph preferences, you find **Line color.** Allows you to choose between many different colors. Click the color box to select a color.

Note that if you wish to use the same settings next time you open a principal component plot, you need to save the settings of the **Side Panel** (see section 4.6).

Besides the histogram view itself, the histogram can also be shown in a table, summarizing key properties of the expression values. An example is shown in figure 25.57.



Data	Count
Total number of values	15923
Number values used	15923
Number -Inf values	0
Number +Inf values	0
Number NaN values	0

Figure 25.57: *Table view of a histogram.*

The table lists the following properties:

- **Number +Inf values**
- **Number -Inf values**
- **Number NaN values**
- **Number values used**
- **Total number of values**

25.7.2 MA plot

The MA plot is a scatter rotated by 45°. For two samples of expression values it plots for each gene the difference in expression against the mean expression level. MA plots are often used for quality control, in particular, to assess whether normalization and/or transformation is required.

You can create an MA plot comparing two samples:

Tools | **Expression Analysis** ( )| **General Plots** ( ) | **Create MA Plot** ( )

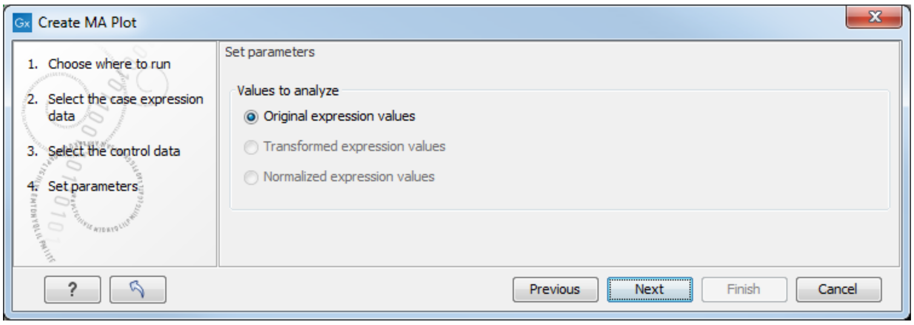
In the first two dialogs, select two samples ( ), ( ) or ( ): the first must be the case expression data, and the second the control data. Clicking **Next** will display a dialog as shown in figure 25.58.

In this dialog, you select the values to be used for creating the MA plot (see section 25.2.1).

Click **Finish** to start the tool.

Viewing MA plots

The resulting plot is shown in a figure 25.59.



Create MA Plot

1. Choose where to run
2. Select the case expression data
3. Select the control data
4. Set parameters

Set parameters

Values to analyze

● Original expression values

○ Transformed expression values

○ Normalized expression values

Figure 25.58: *Selecting which values the MA plot should be based on.*

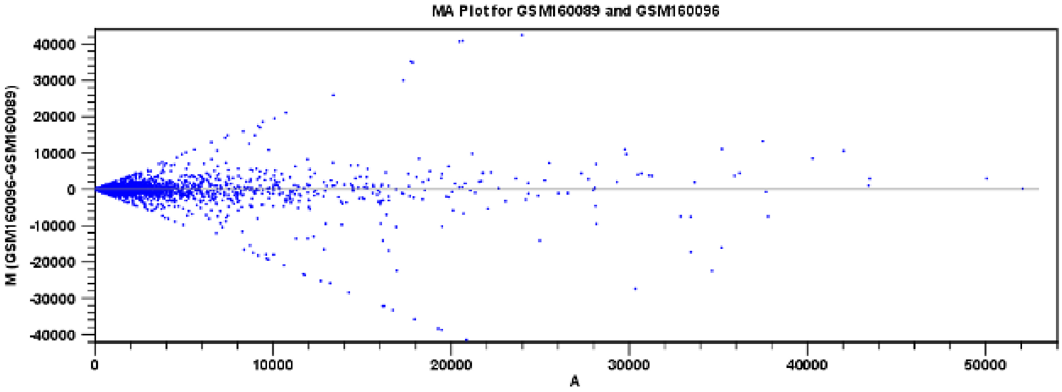


Figure 25.59: *MA plot based on original expression values.*

The X axis shows the mean expression level of a feature on the two samples and the Y axis shows the difference in expression levels for a feature on the two samples. From the plot shown in figure 25.59 it is clear that the variance increases with the mean. With an MA plot like this, you will often choose to transform the expression values (see section 25.2.2).

Figure 25.60 shows the same two samples where the MA plot has been created using log2 transformed values.

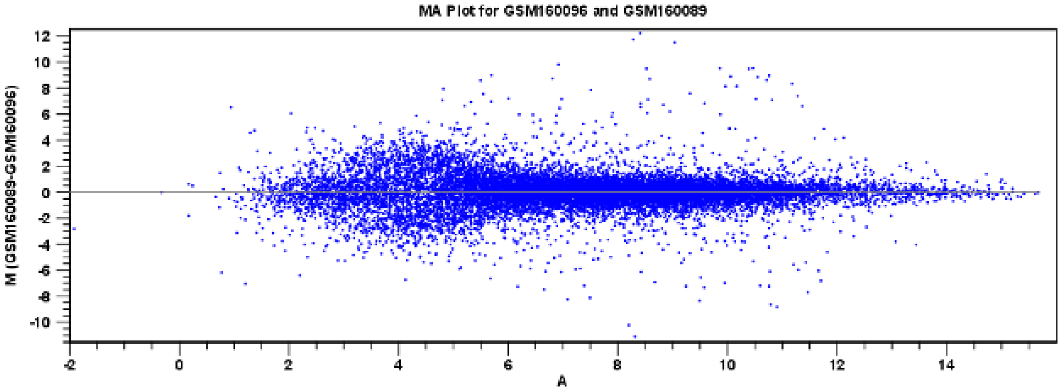


Figure 25.60: *MA plot based on transformed expression values.*

The much more symmetric and even spread indicates that the dependance of the variance on the mean is not as strong as it was before transformation.

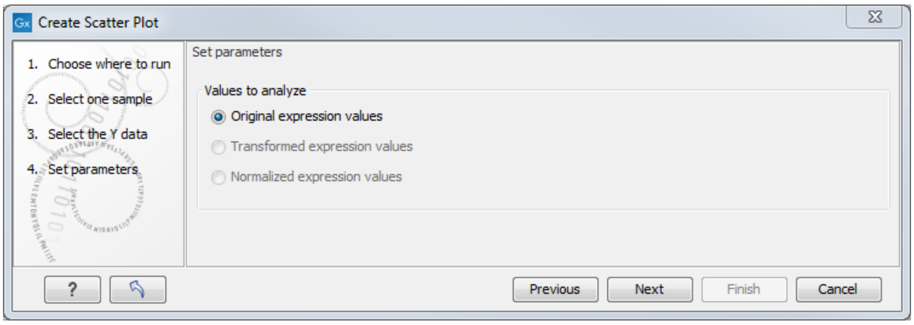
In the **Side Panel** to the left, there is a number of options to adjust the view. Under **Graph preferences**, you can adjust the general properties of the plot.

- **Lock axes** This will always show the axes even though the plot is zoomed to a detailed level.
- **Frame** Shows a frame around the graph.
- **Show legends** Shows the data legends.
- **Tick type** Determine whether tick lines should be shown outside or inside the frame.
- **Tick lines at** Choosing Major ticks will show a grid behind the graph.
- **Horizontal axis range** Sets the range of the horizontal axis (x axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **Vertical axis range** Sets the range of the vertical axis (y axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **y = 0 axis**. Draws a line where y = 0. Below there are some options to control the appearance of the line:
  - **Line width** Thin, Medium or Wide
  - **Line type** None, Line, Long dash or Short dash
  - **Line color** Click the color box to select a color.
- **Line width** Thin, Medium or Wide
- **Line type** None, Line, Long dash or Short dash
- **Line color** Click the color box to select a color.

Below the general preferences, you find the **Dot properties** preferences, where you can adjust coloring and appearance of the dots:

- **Dot type** Can be None, Cross, Plus, Square, Diamond, Circle, Triangle, Reverse triangle, or Dot.
- **Dot color** Click the color box to select a color.

Note that if you wish to use the same settings next time you open a scatter plot, you need to save the settings of the **Side Panel** (see section 4.6).


Create Scatter Plot

1. Choose where to run
2. Select one sample
3. Select the Y data
4. Set parameters

Set parameters

Values to analyze

● Original expression values
○ Transformed expression values
○ Normalized expression values

Figure 25.61: *Selecting which values the scatter plot should be based on.*

25.7.3 Scatter plot

As described in section 25.1.4, an experiment can be viewed as a scatter plot. However, you can also create a "stand-alone" scatter plot of two samples:

Tools | **Expression Analysis ( )**| **General Plots** ( ) | **Create Scatter Plot ( )**

In the first two dialogs, select two samples (( ), ( ) or ( )): the first is the sample that will be plotted on the X axis of the plot, the second the one that will define the Y axis. Clicking **Next** will display a dialog as shown in figure 25.61.

In this dialog, you select the values to be used for creating the scatter plot (see section 25.2.1).

Click **Finish** to start the tool.

For more information about the scatter plot view and how to interpret it, please see section 25.1.4.

Chapter 26

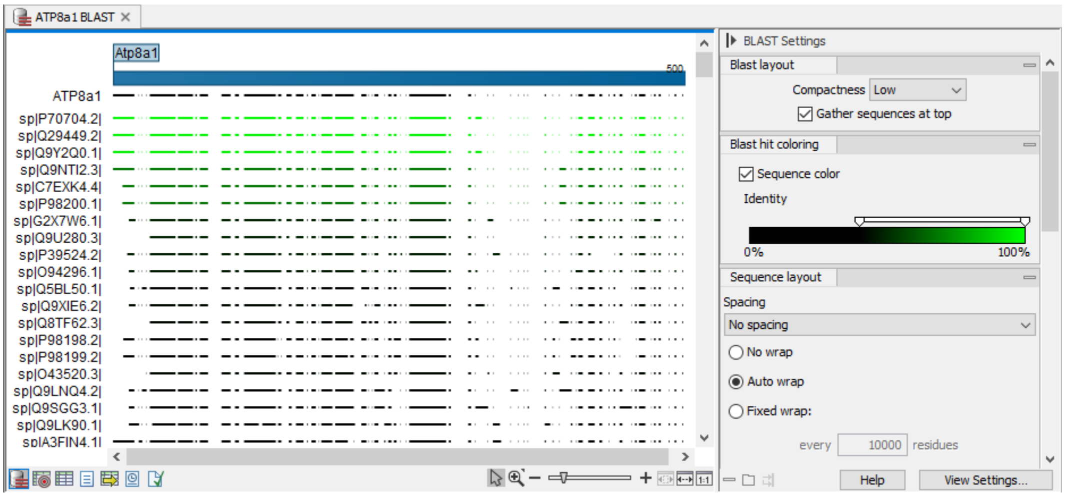
BLAST search

Contents

CLC Main Workbench offers to conduct BLAST searches on protein and DNA sequences. In short, a BLAST search identifies homologous sequences between your input (query) query sequence and a database of sequences [McGinnis and Madden, 2004]. BLAST (Basic Local Alignment Search Tool), identifies homologous sequences using a heuristic method which finds short matches between two sequences. After initial match BLAST attempts to start local alignments from these initial matches.

If you are interested in the bioinformatics behind BLAST, there is an easy-to-read explanation of this in section 26.5.

Figure 26.1 shows an example of a BLAST result in the *CLC Main Workbench*.



Blast layout

Compactness Low

☑ Gather sequences at top

Blast hit coloring

☑ Sequence color

Identity

0% 100%

Sequence layout

Spacing

No spacing

○ No wrap

● Auto wrap

○ Fixed wrap:

every 10000 residues

Figure 26.1: *Display of the output of a BLAST search. At the top is there a graphical representation of BLAST hits with tool-tips showing additional information on individual hits. Below is a tabular form of the BLAST results.*

26.1 Running BLAST searches

With the *CLC Main Workbench* there are two ways of performing BLAST searches: You can either have the BLAST process run on NCBI's BLAST servers (`https://blast.ncbi.nlm.nih.gov/Blast.cgi`) or you can perform the BLAST search on your own computer.

The advantage of running the BLAST search on NCBI servers is that you have readily access to the popular, and often very large, BLAST databases without having to download them to your own computer. The advantages of running BLAST on your own computer include that you can use your own sequence collections as blast databases, and that running big batch BLAST jobs can be faster and more reliable when done locally.

26.1.1 BLAST at NCBI

When running a BLAST search at the NCBI, the Workbench sends the sequences you select to the NCBI's BLAST servers. When the results are ready, they will be automatically downloaded and displayed in the Workbench. When you enter a large number of sequences for searching with BLAST, the Workbench automatically splits the sequences up into smaller subsets and sends one subset at the time to NCBI. This is to avoid exceeding any internal limits the NCBI places on the number of sequences that can be submitted to them for BLAST searching. The size of the subset created in the CLC software depends both on the number and size of the sequences.

To start a BLAST job to search your sequences against databases held at the NCBI, go to:

Tools | BLAST ( )| BLAST at NCBI ( )

Alternatively, use the keyboard shortcut: Ctrl+Shift+B for Windows and ⌘ +Shift+B on Mac OS.

This opens the dialog seen in figure 26.2

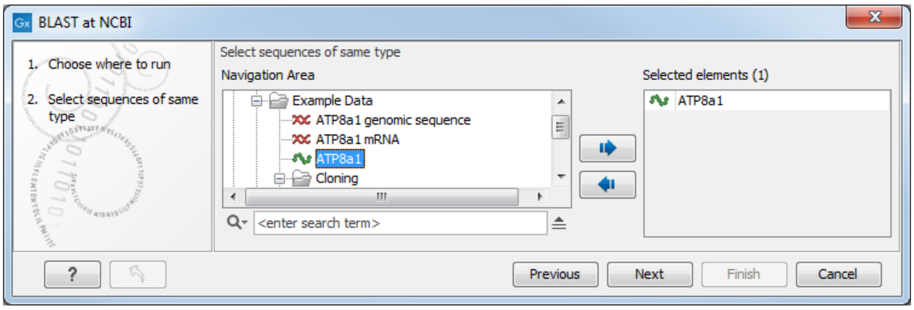
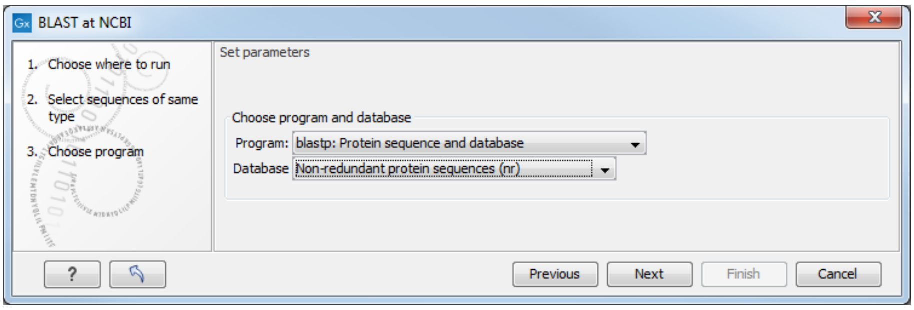


Figure 26.2: *Choose one or more sequences to conduct a BLAST search with.*

Select one or more sequences of the same type (either DNA or protein) and click **Next**.

In this dialog, you choose which type of BLAST search to conduct, and which database to search against (figure 26.3). The databases at the NCBI listed in the dropdown box will correspond to the query sequence type you have, DNA or protein, and the type of blast search you can chose among to run. A complete list of these databases can be found in Appendix B. Here you can also read how to add additional databases available the NCBI to the list provided in the dropdown menu.



Program: blastp: Protein sequence and database

Database Non-redundant protein sequences (nr)

Figure 26.3: *Choose a BLAST Program and a database for the search.*

BLAST programs for DNA query sequences:

- **blastn: DNA sequence against a DNA database.** Searches for DNA sequences with homologous regions to your nucleotide query sequence.
- **blastx: Translated DNA sequence against a Protein database.** Automatic translation of your DNA query sequence in six frames; these translated sequences are then used to search a protein database.
- **tblastx: Translated DNA sequence against a Translated DNA database.** Automatic translation of your DNA query sequence and the DNA database, in six frames. The resulting peptide query sequences are used to search the resulting peptide database. Note that this type of search is computationally intensive.

BLAST programs for protein query sequences:

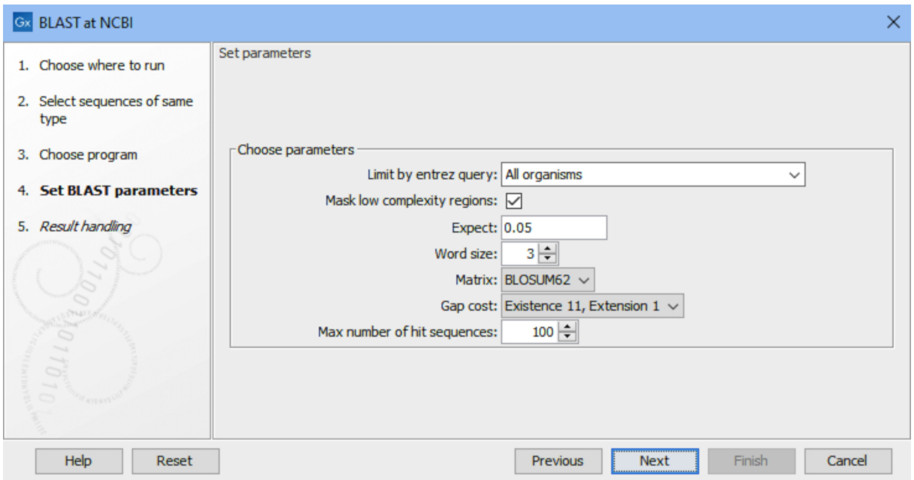
- **blastp: Protein sequence against Protein database.** Used to look for peptide sequences with homologous regions to your peptide query sequence.

- **tblastn: Protein sequence against Translated DNA database.** Peptide query sequences are searched against an automatically translated, in six frames, DNA database.

If you search against the **Protein Data Bank protein** database homologous sequences are found to the query sequence, these can be downloaded and opened with the 3D view.

Click **Next**.

This window, see figure 26.4, allows you to choose parameters to tune your BLAST search, to meet your requirements.



Limit by entrez query: All organisms
Mask low complexity regions: ☑
Expect: 0.05
Word size: 3
Matrix: BLOSUM62
Gap cost: Existence 11, Extension 1
Max number of hit sequences: 100

Figure 26.4: *Parameters that can be set before submitting a BLAST search.*

When choosing blastx or tblastx to conduct a search, you get the option of selecting a translation table for the genetic code. The standard genetic code is set as default. This setting is particularly useful when working with organisms or organelles that have a genetic code different from the standard genetic code.

BLAST search parameters are described below. See https://blast.ncbi.nlm.nih.gov/doc/blast-topics/ for further details.

- **Limit by Entrez query**. BLAST searches can be limited to the results of an Entrez query against the database chosen. This can be used to limit searches to subsets of entries in the BLAST databases. Any terms can be entered that would normally be allowed in an Entrez search session. More information about Entrez queries can be found at https://www.ncbi.nlm.nih.gov/books/NBK3837/#EntrezHelp.Entrez_Searching_Options. The syntax described there is the same as would be accepted in the CLC interface. Some commonly used Entrez queries are pre-entered and can be chosen in the drop down menu.

- **Mask low complexity regions**. Mask off segments of the query sequence that have low compositional complexity.

- **Mask low complexity regions**. Mask off segments of the query sequence that have low compositional complexity. Filtering can eliminate statistically significant, but biologically uninteresting reports from the BLAST output (e.g. hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

- **Expect**. The threshold for reporting matches against database sequences. The Expect value (E-value) describes the number of hits one can expect to see matching a query by chance when searching against a database of a given size. If the E-value ascribed to a match is greater than the value entered in the Expect field, the match will not be reported. Details of how E-values are calculated can be found at the NCBI: `https://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html`. Lower thresholds are more stringent, leading to fewer chance matches being reported. Increasing the threshold results in more matches being reported, but many may just matching by chance, not due to any biological similarity. Values lower than 1 can be entered as decimals, or in scientific notiation. For example, 0.001, 1e-3 and 10e-4 would be equivalent and acceptable values.

- **Word Size**. BLAST is a heuristic that works by finding word-matches between the query and database sequences. You may think of this process as finding "hot-spots" that BLAST can then use to initiate extensions that might lead to full-blown alignments. For nucleotide-nucleotide searches (i.e. "BLASTn") an exact match of the entire word is required before an extension is initiated, so that you normally regulate the sensitivity and speed of the search by increasing or decreasing the wordsize. For other BLAST searches non-exact word matches are taken into account based upon the similarity between words. The amount of similarity can be varied so that you normally uses just the wordsizes 2 and 3 for these searches.

- **Match/mismatch**. A key element in evaluating the quality of a pairwise sequence alignment is the "substitution matrix", which assigns a score for aligning any possible pair of residues. The matrix used in a BLAST search can be changed depending on the type of sequences you are searching with (see the BLAST Frequently Asked Questions). Only applicable for protein sequences or translated DNA sequences.

- **Gap Cost**. The pull down menu shows the Gap Costs (Penalty to open Gap and penalty to extend Gap). Increasing the Gap Costs and Lambda ratio will result in alignments which decrease the number of Gaps introduced.

- **Max number of hit sequences**. The maximum number of database sequences, where BLAST found matches to your query sequence, to be included in the BLAST report.

The parameters you choose will affect how long BLAST takes to run. A search of a small database, requesting only hits that meet stringent criteria will generally be quite quick. Searching large databases, or allowing for very remote matches, will of course take longer.

Click **Finish** to start the tool.

BLAST a partial sequence against NCBI You can search a database using only a part of a sequence directly from the sequence view:

> **select the sequence region to send to BLAST | right-click the selection | BLAST Selection Against NCBI ()**

This will go directly to the dialog shown in figure 26.3 and the rest of the options are the same as when performing a BLAST search with a full sequence.

26.1.2 BLAST against local data

Running BLAST searches on your local machine can have several advantages over running the searches remotely at the NCBI:

- It can be faster.
- It does not rely on having a stable internet connection.
- It does not depend on the availability of the NCBI BLAST servers.
- You can use longer query sequences.
- You use your own data sets to search against.

On a technical level, *CLC Main Workbench* uses the NCBI's blast+ software (see `ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/`). Thus, the results of using a particular data set to search the same database with the same search parameters would give the same results, whether run locally or at the NCBI.

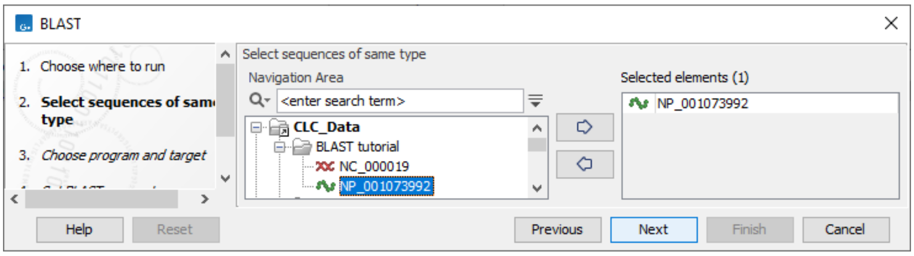
There are a number of options for what you can search against:

- You can create a database based on data already imported into your Workbench (see section 26.3.3)
- You can add pre-formatted databases (see section 26.3.2)
- You can use sequence data from the **Navigation Area** directly, without creating a database first.

To conduct a local BLAST search, go to:

Tools | **BLAST** ( )| **BLAST** ( )

This opens the dialog seen in figure 26.5:



BLAST

1. Choose where to run
2. **Select sequences of same type**
3. *Choose program and target*

Select sequences of same type

Navigation Area

<enter search term>

- CLC_Data
  - BLAST tutorial
    - NC_000019
    - NP_001073992

Selected elements (1)

NP_001073992

Figure 26.5: *Choose one or more sequences to conduct a BLAST search.*

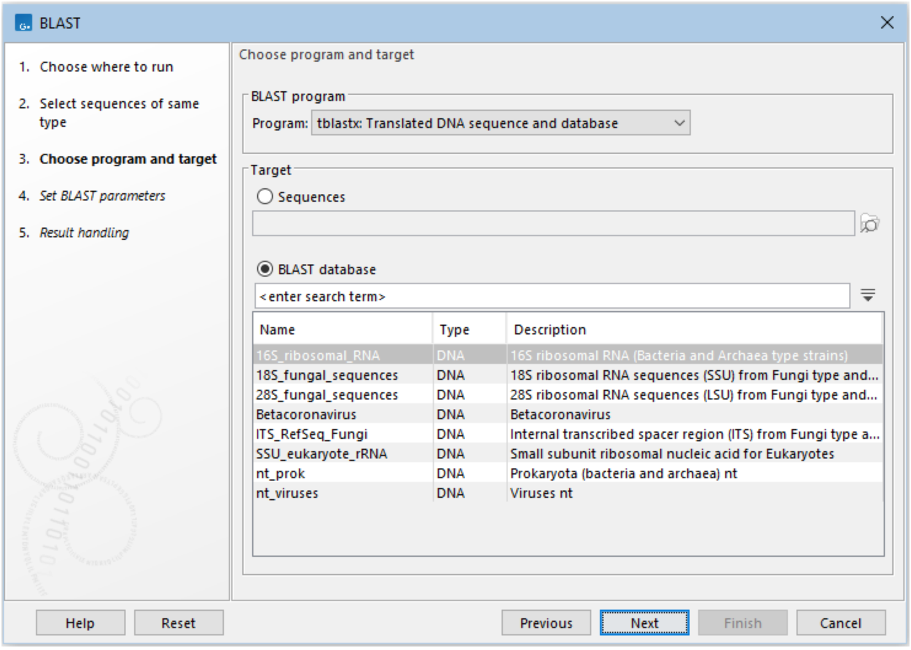
Select one or more sequences of the same type (DNA or protein) and click **Next**.

This opens the dialog seen in figure 26.6:

At the top, you can choose between different BLAST programs.

BLAST programs for DNA query sequences:

- **blastn: DNA sequence against a DNA database.** Searches for DNA sequences with homologous regions to your nucleotide query sequence.



1. Choose where to run
2. Select sequences of same type
3. **Choose program and target**
4. *Set BLAST parameters*
5. *Result handling*

Choose program and target

BLAST program

Program: tblastx: Translated DNA sequence and database

Target

○ Sequences

● BLAST database

<enter search term>

Name	Type	Description
16S_ribosomal_RNA	DNA	16S ribosomal RNA (Bacteria and Archaea type strains)
18S_fungal_sequences	DNA	18S ribosomal RNA sequences (SSU) from Fungi type and...
28S_fungal_sequences	DNA	28S ribosomal RNA sequences (LSU) from Fungi type and...
Betacoronavirus	DNA	Betacoronavirus
ITS_RefSeq_Fungi	DNA	Internal transcribed spacer region (ITS) from Fungi type a...
SSU_eukaryote_rRNA	DNA	Small subunit ribosomal nucleic acid for Eukaryotes
nt_prok	DNA	Prokaryota (bacteria and archaea) nt
nt_viruses	DNA	Viruses nt

Figure 26.6: *Choose a BLAST program and a target database.*

- **blastx: Translated DNA sequence against a Protein database.** Automatic translation of your DNA query sequence in six frames; these translated sequences are then used to search a protein database.
- **tblastx: Translated DNA sequence against a Translated DNA database.** Automatic translation of your DNA query sequence and the DNA database, in six frames. The resulting peptide query sequences are used to search the resulting peptide database. Note that this type of search is computationally intensive.

BLAST programs for protein query sequences:

- **blastp: Protein sequence against Protein database.** Used to look for peptide sequences with homologous regions to your peptide query sequence.
- **tblastn: Protein sequence against Translated DNA database.** Peptide query sequences are searched against an automatically translated, in six frames, DNA database.

In cases where you have selected blastx or tblastx to conduct a search, you will get the option of selecting a translation table for the genetic code. The standard genetic code is set as default. This setting is particularly useful when working with organisms or organelles that have a genetic code that differs from the standard genetic code.

If you search against the **Protein Data Bank** database and homologous sequences are found to the query sequence, these can be downloaded and opened with the **3D Molecule Viewer** (see section 15.1.3).

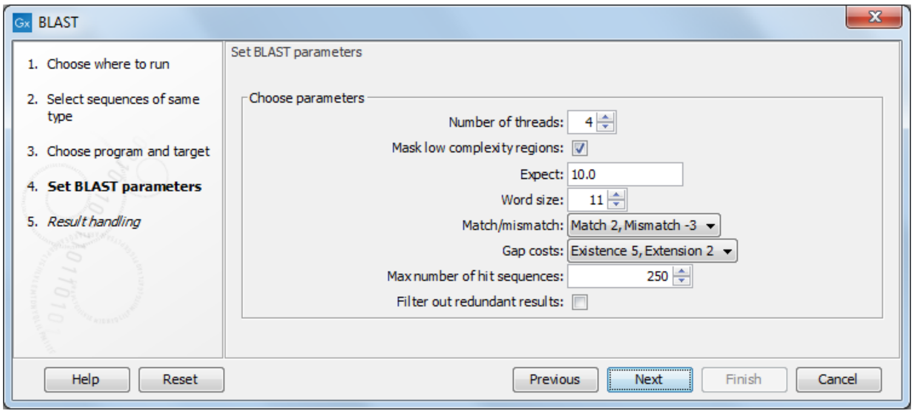
You then specify the target database to use:

- **Sequences**. When you choose this option, you can use sequence data from the **Navigation Area** as database by clicking the **Browse and select** icon (🔍). A temporary BLAST database will be created from these sequences and used for the BLAST search. It is deleted afterwards. If you want to be able to click in the BLAST result to retrieve the hit sequences from the BLAST database at a later point, you should *not* use this option; create a create a BLAST database first, see section 26.3.3.

- **BLAST Database**. Select a database already available in one of your designated BLAST database folders. Read more in section 26.4.

When a database or a set of sequences has been selected, click **Next**.

The next dialog allows you to adjust the parameters to meet the requirements of your BLAST search (figure 26.7).


BLAST

1. Choose where to run
2. Select sequences of same type
3. Choose program and target
4. **Set BLAST parameters**
5. *Result handling*

Set BLAST parameters

Choose parameters

Number of threads: 4
Mask low complexity regions: ☑
Expect: 10.0
Word size: 11
Match/mismatch: Match 2, Mismatch -3
Gap costs: Existence 5, Extension 2
Max number of hit sequences: 250
Filter out redundant results: □

Figure 26.7: *Parameters that can be set before submitting a local BLAST search.*

- **Number of threads**. You can specify the number of threads, which should be used if your Workbench is installed on a multi-threaded system.
- **Mask low complexity regions**. Mask off segments of the query sequence that have low compositional complexity. Filtering can eliminate statistically significant, but biologically uninteresting reports from the BLAST output (e.g. hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.
- **Expect**. The threshold for reporting matches against database sequences. The Expect value (E-value) describes the number of hits one can expect to see matching a query by chance when searching against a database of a given size. If the E-value ascribed to a match is greater than the value entered in the Expect field, the match will not be reported. Details of how E-values are calculated can be found at the NCBI: `https://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html`. Lower thresholds are more stringent, leading to fewer chance matches being reported. Increasing the threshold results in more matches being reported, but many may just matching by chance, not due to any biological similarity. Values lower than 1 can be entered as decimals, or in scientific notiation. For example, 0.001, 1e-3 and 10e-4 would be equivalent and acceptable values.
- **Word Size**. BLAST is a heuristic that works by finding word-matches between the query and database sequences. You may think of this process as finding "hot-spots" that BLAST can then use to initiate extensions that might lead to full-blown alignments. For nucleotide-nucleotide searches (i.e. "BLASTn") an exact match of the entire word is required before

an extension is initiated, so that you normally regulate the sensitivity and speed of the search by increasing or decreasing the wordsize. For other BLAST searches non-exact word matches are taken into account based upon the similarity between words. The amount of similarity can be varied so that you normally uses just the wordsizes 2 and 3 for these searches.

- **Match/mismatch**. A key element in evaluating the quality of a pairwise sequence alignment is the "substitution matrix", which assigns a score for aligning any possible pair of residues. The matrix used in a BLAST search can be changed depending on the type of sequences you are searching with (see the BLAST Frequently Asked Questions). Only applicable for protein sequences or translated DNA sequences.
- **Gap Cost**. The pull down menu shows the Gap Costs (Penalty to open Gap and penalty to extend Gap). Increasing the Gap Costs and Lambda ratio will result in alignments which decrease the number of Gaps introduced.
- **Max number of hit sequences**. The maximum number of database sequences, where BLAST found matches to your query sequence, to be included in the BLAST report.
- **Filter out redundant results**. This option culls HSPs on a per subject sequence basis by removing HSPs that are completely enveloped by another HSP.

BLAST a partial sequence against a local database You can search a database using only a part of a sequence directly from the sequence view:

> **select the region that you wish to BLAST | right-click the selection | BLAST Selection Against Local Database ( )**

This will go directly to the dialog shown in figure 26.6 and the rest of the options are the same as when performing a BLAST search with a full sequence.

26.2 Output from BLAST searches

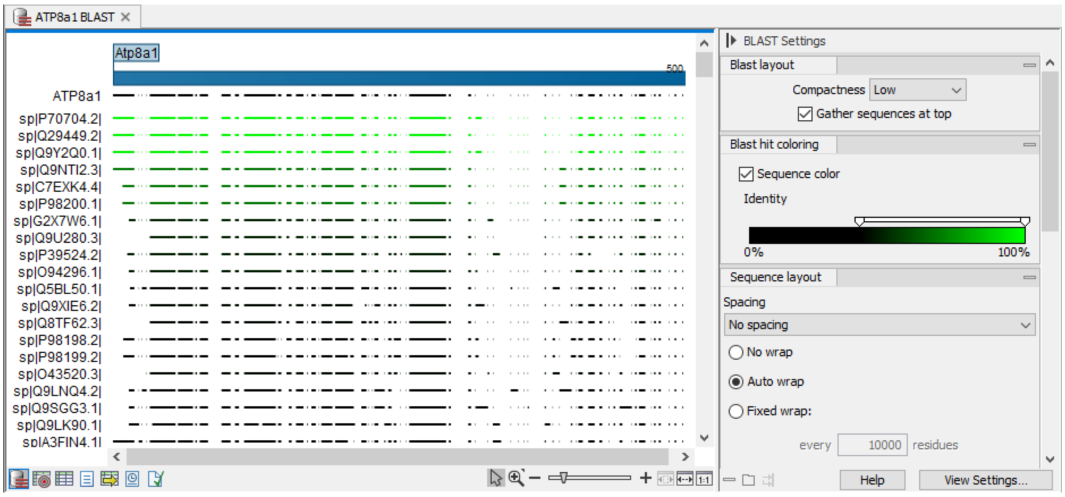
The output of a BLAST search is similar whether you have chosen to run your search locally or at the NCBI.

If a **single query** sequence was used, then the results will show the hits and High-Scoring Segment Pairs (HSPs) found in that database with that single sequence. If **more than one query** sequence was used, the default view of the results is a summary table, where the description of the top match found for each query sequence and the number of matches found is reported. The summary table is described in detail in section 26.2.2.

26.2.1 Graphical overview for each query sequence

Double clicking on a given row of a tabular blast table opens a graphical overview of the blast results for a particular query sequence, as shown in figure figure 26.8. In cases where only one sequence was entered into a BLAST search, such a graphical overview is the default output.

Figure 26.8 shows an example of a BLAST result for an individual query sequence in the *CLC Main Workbench*.



Compactness: Low

☑ Gather sequences at top

☑ Sequence color

Spacing: No spacing

○ No wrap

● Auto wrap

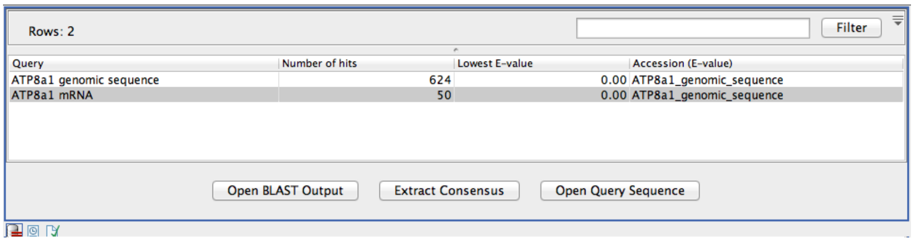
○ Fixed wrap: every 10000 residues

Figure 26.8: *Default display of the output of a BLAST search for one query sequence. At the top is there a graphical representation of BLAST hits with tooltips showing additional information on individual hits.*

Detailed descriptions of the overview BLAST table and the graphical BLAST results view are described below.

26.2.2 Overview BLAST table

In the overview BLAST table for a multi-sequence blast search, as shown in figure 26.9, there is one row for each query sequence. Each row represents the BLAST result for this query sequence.



Rows: 2

Query	Number of hits	Lowest E-value	Accession (E-value)
ATP8a1 genomic sequence	624	0.00	ATP8a1_genomic_sequence
ATP8a1 mRNA	50	0.00	ATP8a1_genomic_sequence

Figure 26.9: *An overview BLAST table summarizing the results for a number of query sequences.*

Double-clicking a row will open the BLAST result for this query sequence, allowing more detailed investigation of the result. You can also select one or more rows and click the **Open BLAST Output** button at the bottom of the view. Consensus sequence can be extracted by clicking the **Extract Consensus** button at the bottom. Clicking the **Open Query Sequence** will open a sequence list with the selected query sequences. This can be useful in work flows where BLAST is used as a filtering mechanism where you can filter the table to include e.g. sequences that have a certain top hit and then extract those.

In the overview table, the following information is shown:

- Query: Since this table displays information about several query sequences, the first column is the name of the query sequence.

- Number of HSPs: The number of High-scoring Segment Pairs (HSPs) for this query sequence.
- For the following list, the value of the best HSP is displayed together with accession number and description of this HSP, with respect to E-value, identity or positive value, hit length or bit score.
  - Lowest E-value
  - Accession (E-value)
  - Description (E-value)
  - Greatest identity %
  - Accession (identity %)
  - Description (identity %)
  - Greatest positive %
  - Accession (positive %)
  - Description (positive %)
  - Greatest HSPs length
  - Accession (HSP length)
  - Description (HSP length)
  - Greatest bit score
  - Accession (bit score)
  - Description (bit score)

If you wish to save some of the BLAST results as individual elements in the **Navigation Area**, open them and click **Save As** in the **File** menu.

26.2.3 BLAST graphics

The **BLAST editor** shows the sequences hits which were found in the BLAST search. The hit sequences are represented by colored horizontal lines, and when hovering the mouse pointer over a BLAST hit sequence, a tooltip appears, listing the characteristics of the sequence. As default, the query sequence is fitted to the window width, but it is possible to zoom in the windows and see the actual sequence alignments returned from the BLAST server.

There are several settings available in the **BLAST Settings** side panel.

- **Blast layout.** You can control the level of **Compactness** for displaying sequences:
  - **Not compact.** Full detail and spaces between the sequences.
  - **Low.** The normal settings where the residues are visible (when zoomed in) but with no extra spaces between.
  - **Medium.** The sequences are represented as lines and the residues are not visible. There is some space between the sequences.
  - **Compact.** Even less space between the sequences.

You can also choose to **Gather sequences at top**. Enabling this option affects the view that is shown when scrolling horizontally along a BLAST result. If selected, the sequence hits which did not contribute to the visible part of the BLAST graphics will be omitted whereas the found BLAST hits will automatically be placed right below the query sequence.

- **BLAST hit coloring.** You can choose whether to color hit sequences and adjust the coloring scale for visualisation of identity level.

The remaining View preferences for BLAST Graphics are the same as those of alignments. See section 14.2.

Some of the information available in the tooltips when hovering over a particular hit sequence is:

- **Name of sequence.** Here is shown some additional information of the sequence which was found. This line corresponds to the description line in GenBank (if the search was conducted on the nr database).
- **Score.** This shows the bit score of the local alignment generated through the BLAST search.
- **Expect.** Also known as the E-value. A low value indicates a homologous sequence. Higher E-values indicate that BLAST found a less homologous sequence.
- **Identities.** This number shows the number of identical residues or nucleotides in the obtained alignment.
- **Gaps.** This number shows whether the alignment has gaps or not.
- **Strand.** This is only valid for nucleotide sequences and show the direction of the aligned strands. Minus indicate a complementary strand.

The numbers of the query and subject sequences refer to the sequence positions in the submitted and found sequences. If the subject sequence has number 59 in front of the sequence, this means that 58 residues are found upstream of this position, but these are not included in the alignment.

By right clicking the sequence name in the Graphical BLAST output it is possible to download the full hits sequence from NCBI with accompanying annotations and information. It is also possible to just open the actual hit sequence in a new view.

26.2.4 BLAST HSP table

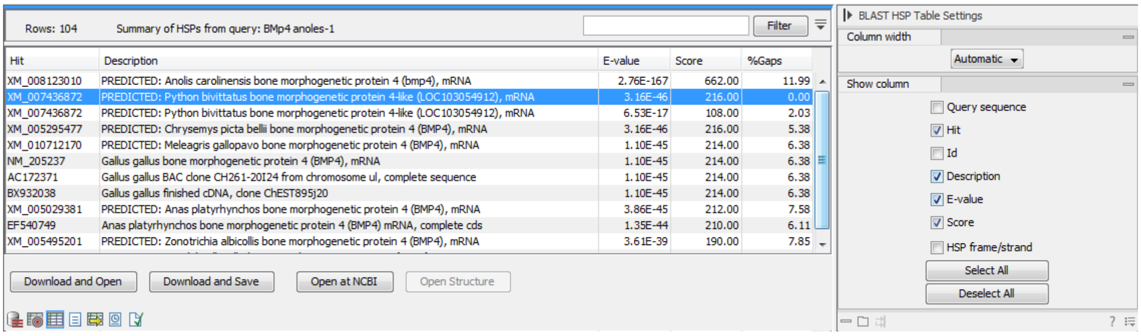
In addition to the graphical display of a BLAST result, it is possible to view the BLAST results in a tabular view. In the tabular view, one can get a quick and fast overview of the results. Here you can also select multiple sequences and download or open all of these in one single step. Moreover, there is a link from each sequence to the sequence at NCBI. These possibilities are either available through a right-click with the mouse or by using the buttons below the table.

The **BLAST table** view can be shown in the following way:

Click the Show BLAST HSP Table button (▦) at the bottom of the view

Figure 26.10 is an example of a BLAST HSP Table.

The BLAST HSP Table includes the following information:



Rows: 104 Summary of HSPs from query: BMp4 anoles-1

Hit	Description	E-value	Score	%Gaps
XM_008123010	PREDICTED: Anolis carolinensis bone morphogenetic protein 4 (bmp4), mRNA	2.76E-167	662.00	11.99
XM_007436872	PREDICTED: Python bivittatus bone morphogenetic protein 4-like (LOC103054912), mRNA	3.16E-46	216.00	0.00
XM_007436872	PREDICTED: Python bivittatus bone morphogenetic protein 4-like (LOC103054912), mRNA	6.53E-17	108.00	2.03
XM_005295477	PREDICTED: Chrysemys picta bellii bone morphogenetic protein 4 (BMP4), mRNA	3.16E-46	216.00	5.38
XM_010712170	PREDICTED: Meleagris gallopavo bone morphogenetic protein 4 (BMP4), mRNA	1.10E-45	214.00	6.38
NM_205237	Gallus gallus bone morphogenetic protein 4 (BMP4), mRNA	1.10E-45	214.00	6.38
AC172371	Gallus gallus BAC clone CH261-20I24 from chromosome ul, complete sequence	1.10E-45	214.00	6.38
BX932038	Gallus gallus finished cDNA, clone ChEST895j20	1.10E-45	214.00	6.38
XM_005029381	PREDICTED: Anas platyrhynchos bone morphogenetic protein 4 (BMP4), mRNA	3.86E-45	212.00	7.58
EF540749	Anas platyrhynchos bone morphogenetic protein 4 (BMP4) mRNA, complete cds	1.35E-44	210.00	6.11
XM_005495201	PREDICTED: Zonotrichia albicollis bone morphogenetic protein 4 (BMP4), mRNA	3.61E-39	190.00	7.85

BLAST HSP Table Settings

Column width: Automatic

Show column

□ Query sequence
☑ Hit
□ Id
☑ Description
☑ E-value
☑ Score
□ HSP frame/strand

Figure 26.10: *BLAST HSP Table. The HSPs can be sorted by the different columns, simply by clicking the column heading.*

- **Query sequence.** The sequence which was used for the search.
- **HSP.** The Name of the sequences found in the BLAST search.
- **Id.** GenBank ID.
- **Description.** Text from NCBI describing the sequence.
- **E-value.** Measure of quality of the match. Higher E-values indicate that BLAST found a less homologous sequence.
- **Score.** This shows the score of the local alignment generated through the BLAST search.
- **Bit score.** This shows the bit score of the local alignment generated through the BLAST search. Bit scores are normalized, which means that the bit scores from different alignments can be compared, even if different scoring matrices have been used.
- **HSP start.** Shows the start position in the HSP sequence.
- **HSP end.** Shows the end position in the HSP sequence.
- **HSP length.** The length of the HSP.
- **Query start.** Shows the start position in the query sequence.
- **Query end.** Shows the end position in the query sequence.
- **Overlap.** Display a percentage value for the overlap of the query sequence and HSP sequence. Only the length of the local alignment is taken into account and not the full length query sequence.
- **Identity.** Shows the number of identical residues in the query and HSP sequence.
- **%Identity.** Shows the percentage of identical residues in the query and HSP sequence.
- **Positive.** Shows the number of similar but not necessarily identical residues in the query and HSP sequence.
- **%Positive.** Shows the percentage of similar but not necessarily identical residues in the query and HSP sequence.
- **Gaps.** Shows the number of gaps in the query and HSP sequence.

- **%Gaps.** Shows the percentage of gaps in the query and HSP sequence.
- **Query Frame/Strand.** Shows the frame or strand of the query sequence.
- **HSP Frame/Strand.** Shows the frame or strand of the HSP sequence.

In the **BLAST table** view you can handle the HSP sequences. Select one or more sequences from the table, and apply one of the following functions.

- **Download and Open.** Download the full sequence from NCBI and opens it. If multiple sequences are selected, they will all open (if the same sequence is listed several times, only one copy of the sequence is downloaded and opened).
- **Download and Save.** Download the full sequence from NCBI and save it. When you click the button, there will be a save dialog letting you specify a folder to save the sequences. If multiple sequences are selected, they will all open (if the same sequence is listed several times, only one copy of the sequence is downloaded and opened).
- **Open at NCBI.** Opens the corresponding sequence(s) at GenBank at NCBI. Here is stored additional information regarding the selected sequence(s). The default Internet browser is used for this purpose.
- **Open structure.** If the HSP sequence contain structure information, the sequence is opened in a text view or a 3D view. Note that the 3D view has special system requirements, see section 1.3.

The HSPs can be sorted by the different columns, simply by clicking the column heading. In cases where individual rows have been selected in the table, the selected rows will still be selected after sorting the data.

You can do a text-based search in the information in the BLAST table by using the filter at the upper right part of the view. In this way you can search for e.g. species or other information which is typically included in the "Description" field.

The table is integrated with the graphical view described in section 26.2.3 so that selecting a HSP in the table will make a selection on the corresponding sequence in the graphical view.

26.2.5 BLAST hit table

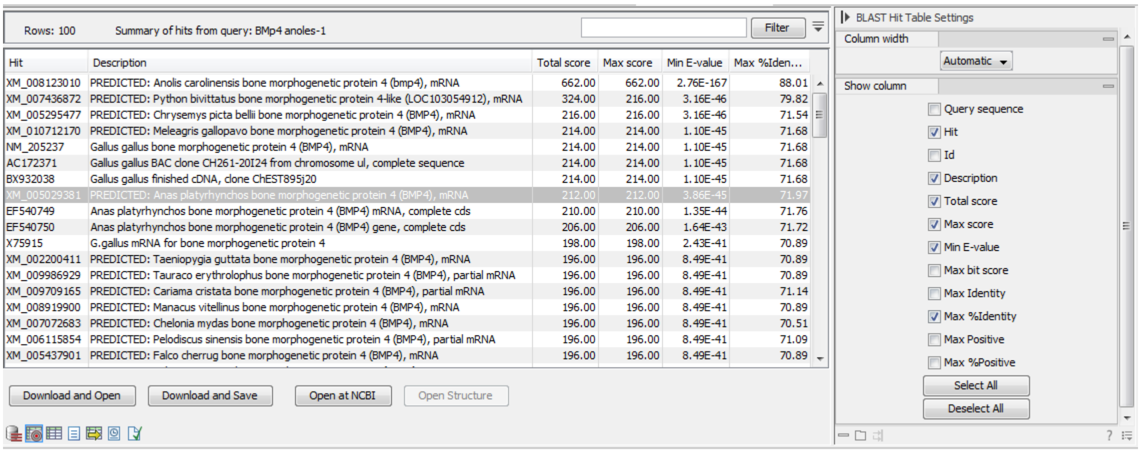
The **BLAST Hit table** view can be shown in the following way:

Click the Show BLAST Hit Table button () at the bottom of the view

Figure 26.11 is an example of a BLAST Hit Table.

The BLAST Hit Table includes the following information:

- **Query sequence.** The sequence which was used for the search.
- **Hit.** The Name of the sequences found in the BLAST search.
- **Id.** GenBank ID.



Rows: 100 Summary of hits from query: BMp4 anoles-1

Hit	Description	Total score	Max score	Min E-value	Max %Iden...
XM_008123010	PREDICTED: Anolis carolinensis bone morphogenetic protein 4 (bmp4), mRNA	662.00	662.00	2.76E-167	88.01
XM_007436872	PREDICTED: Python bivittatus bone morphogenetic protein 4-like (LOC103054912), mRNA	324.00	216.00	3.16E-46	79.82
XM_005295477	PREDICTED: Chrysemys picta bellii bone morphogenetic protein 4 (BMP4), mRNA	216.00	216.00	3.16E-46	71.54
XM_010712170	PREDICTED: Meleagris gallopavo bone morphogenetic protein 4 (BMP4), mRNA	214.00	214.00	1.10E-45	71.68
NM_205237	Gallus gallus bone morphogenetic protein 4 (BMP4), mRNA	214.00	214.00	1.10E-45	71.68
AC172371	Gallus gallus BAC clone CH261-20I24 from chromosome ul, complete sequence	214.00	214.00	1.10E-45	71.68
BX932038	Gallus gallus finished cDNA, clone ChEST895j20	214.00	214.00	1.10E-45	71.68
XM_005029381	PREDICTED: Anas platyrhynchos bone morphogenetic protein 4 (BMP4), mRNA	212.00	212.00	3.86E-45	71.97
EF540749	Anas platyrhynchos bone morphogenetic protein 4 (BMP4) mRNA, complete cds	210.00	210.00	1.35E-44	71.76
EF540750	Anas platyrhynchos bone morphogenetic protein 4 (BMP4) gene, complete cds	206.00	206.00	1.64E-43	71.72
X75915	G.gallus mRNA for bone morphogenetic protein 4	198.00	198.00	2.43E-41	70.89
XM_002200411	PREDICTED: Taeniopygia guttata bone morphogenetic protein 4 (BMP4), mRNA	196.00	196.00	8.49E-41	70.89
XM_009986929	PREDICTED: Tauraco erythrolophus bone morphogenetic protein 4 (BMP4), partial mRNA	196.00	196.00	8.49E-41	70.89
XM_009709165	PREDICTED: Cariama cristata bone morphogenetic protein 4 (BMP4), partial mRNA	196.00	196.00	8.49E-41	71.14
XM_008919900	PREDICTED: Manacus vitellinus bone morphogenetic protein 4 (BMP4), mRNA	196.00	196.00	8.49E-41	70.89
XM_007072683	PREDICTED: Chelonia mydas bone morphogenetic protein 4 (BMP4), mRNA	196.00	196.00	8.49E-41	70.51
XM_006115854	PREDICTED: Pelodiscus sinensis bone morphogenetic protein 4 (BMP4), partial mRNA	196.00	196.00	8.49E-41	71.09
XM_005437901	PREDICTED: Falco cherrug bone morphogenetic protein 4 (BMP4), mRNA	196.00	196.00	8.49E-41	70.89

Column width: Automatic

Show column
- □ Query sequence
- ☑ Hit
- □ Id
- ☑ Description
- ☑ Total score
- ☑ Max score
- ☑ Min E-value
- □ Max bit score
- □ Max Identity
- ☑ Max %Identity
- □ Max Positive
- □ Max %Positive

Figure 26.11: *BLAST Hit Table. The hits can be sorted by the different columns, simply by clicking the column heading.*

- **Description.** Text from NCBI describing the sequence.
- **Total Score.** Total score for all HSPs.
- **Max Score.** Maximum score of all HSPs.
- **Min E-value.** Minimum e-value of all HSPs.
- **Max Bit score.** Maximum Bit score of all HSPs.
- **Max Identity.** Shows the maximum number of identical residues in the query and Hit sequence.
- **Max %Identity.** Shows the percentage of maximum identical residues in the query and Hit sequence.
- **Max Positive.** Shows the maximum number of similar but not necessarily identical residues in the query and Hit sequence.
- **Max %Positive.** Shows the percentage of maximum similar but not necessarily identical residues in the query and Hit sequence.

26.2.6 Extracting a consensus sequence from a BLAST result

A consensus sequence can be extracted from nucleotide BLAST results, as described in section 21.10. That section focuses on working with read mappings, but the same underlying tool is used to extract consensus sequences from nucleotide BLAST results.

26.3 Local BLAST databases

Databases can be made available for **BLAST** searches in several ways:

- Download pre-formatted BLAST databases from the NCBI using **Download BLAST Databases** (see section 26.3.1).

- Specify locations where BLAST databases are stored at your site using **Manage Blast Databases** (see section 26.4).
- Use **Create BLAST Database** to create databases using sequences or sequence lists selected from the Workbench Navigation Area (see section 26.3.3).

For BLAST searches against a small amount of sequence data, a sequence list can be specified instead of a database when launching the **BLAST** tool (see section 26.1.2). A database for those sequences will be created as part of that job. This adds to the overall execution time, so if those sequences will be used for multiple searches, creating a BLAST database and referring to that when launching searches is likely to be preferable.

26.3.1 Download NCBI pre-formatted BLAST databases

Using **Download BLAST Databases**, many pre-formatted BLAST databases can be downloaded from the NCBI. The source of these databases is https://ftp.ncbi.nlm.nih.gov/blast/db/.

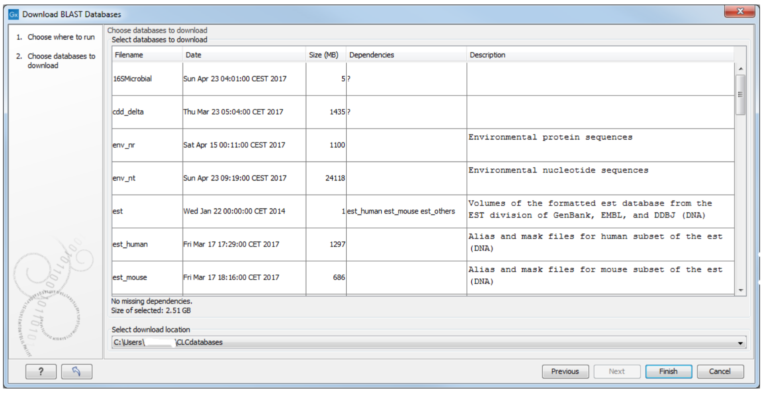
Your Workbench must be connected to the internet to use this tool.

To download a BLAST database from the NCBI, go to:

Tools | **BLAST** ( )| **Download BLAST Databases** ( )

A dialog listing the available databases will open (figure 26.12). The date each database was made available for download on the NCBI site, the size of the files associated with that database, and a brief description of each database is provided.

Note: Many of the databases listed are very large. If you are working on a shared system, we recommend discussing your download plans with others that make use of, or who administer, that system.



Choose databases to download

Select databases to download

Filename	Date	Size (MB)	Dependencies	Description
16SMicrobial	Sun Apr 23 04:01:00 CEST 2017	5	?	
cdd_delta	Thu Mar 23 05:04:00 CET 2017	1435	?	
env_nr	Sat Apr 15 00:11:00 CEST 2017	1100		Environmental protein sequences
env_nt	Sun Apr 23 09:19:00 CEST 2017	24118		Environmental nucleotide sequences
est	Wed Jan 22 00:00:00 CET 2014	1	est_human est_mouse est_others	Volumes of the formatted est database from the EST division of GenBank, EMBL, and DDBJ (DNA)
est_human	Fri Mar 17 17:29:00 CET 2017	1297		Alias and mask files for human subset of the est (DNA)
est_mouse	Fri Mar 17 18:16:00 CET 2017	686		Alias and mask files for mouse subset of the est (DNA)

No missing dependencies.
Size of selected: 2.51 GB

Select download location

C:\Users\ \CLCdatabases

Figure 26.12: *Choose from pre-formatted BLAST databases at the NCBI available for download.*

If there is more than one BLAST database location configured, you will be able to specify which one to store the BLAST database files in. See section 26.4 for details about adding BLAST database locations.

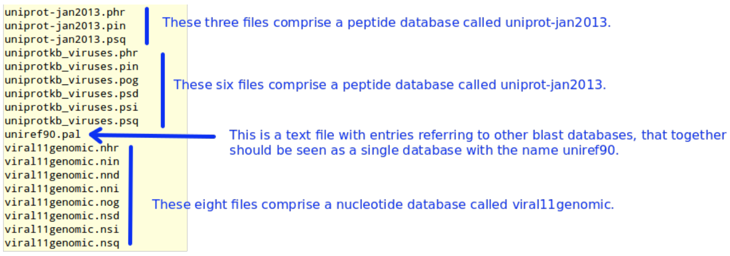
26.3.2 Make pre-formatted BLAST databases available

To use databases that have been downloaded or created outside the Workbench, you can either:

- Add the location where BLAST database files are stored as a BLAST database location (see section 26.4).

  OR

- Move the files that make up the BLAST database to a location already configured as a BLAST databse location. All the files that comprise a given BLAST database must be moved. This may be as few as three files, but can be more (figure 26.13).



```
uniprot-jan2013.phr
uniprot-jan2013.pin
uniprot-jan2013.psq
uniprotkb_viruses.phr
uniprotkb_viruses.pin
uniprotkb_viruses.pog
uniprotkb_viruses.psd
uniprotkb_viruses.psi
uniprotkb_viruses.psq
uniref90.pal
viral11genomic.nhr
viral11genomic.nin
viral11genomic.nnd
viral11genomic.nni
viral11genomic.nog
viral11genomic.nsd
viral11genomic.nsi
viral11genomic.nsq
```

These three files comprise a peptide database called uniprot-jan2013.

These six files comprise a peptide database called uniprot-jan2013.

This is a text file with entries referring to other blast databases, that together should be seen as a single database with the name uniref90.

These eight files comprise a nucleotide database called viral11genomic.

Figure 26.13: *BLAST databases are made up of several files. The exact number varies. Large databases will be split into the number of volumes and there will be several files per volume.*

26.3.3 Create local BLAST databases

You can create a BLAST database from DNA, RNA, *or* protein sequences to use in local BLAST searches.

The BLAST database files will be saved to an area that is specified as a BLAST database location (see section 26.4). The BLAST databases found in these locations are listed when launching the **BLAST** tool (see section 26.1.2).

Making pre-existing BLAST databases available for searching using your Workbench is described in section 26.3.2.

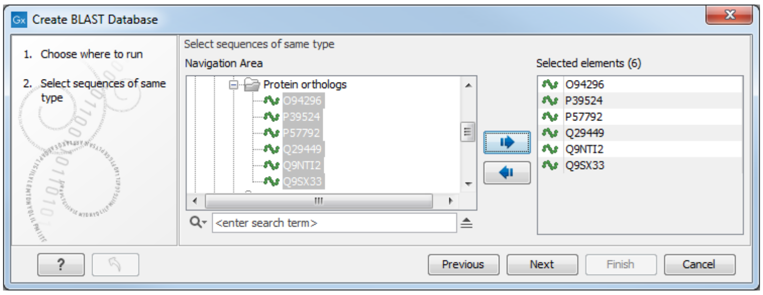
To create a BLAST database, go to:

Tools | **BLAST** ()| **Create BLAST Database** ()

This opens the dialog seen in figure 26.14.

After selecting the sequences or sequence lists to include in your database and clicking on **Next**, you provide information about the BLAST database being made figure 26.15:

- **Name.** The name of the BLAST database. This name will be used when running BLAST searches and also as the base file name for the BLAST database files.
- **Description.** A short description. This is displayed along with the database name in the list of available databases when launching a local BLAST search. If no description is entered, the creation date is used as the description.
- **Location.** The BLAST database location to save the BLAST database files to.



Select sequences of same type

Navigation Area

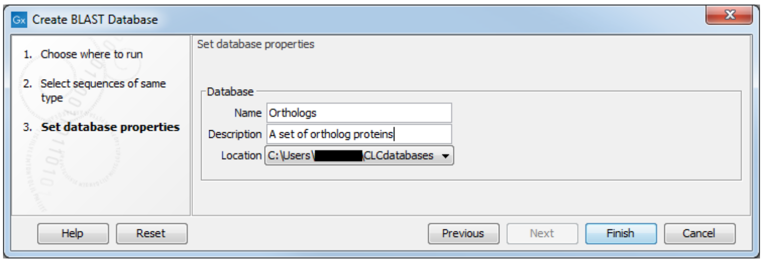
- Protein orthologs
  - O94296
  - P39524
  - P57792
  - Q29449
  - Q9NTI2
  - Q9SX33

<enter search term>

Selected elements (6)

- O94296
- P39524
- P57792
- Q29449
- Q9NTI2
- Q9SX33

Figure 26.14: *Select sequences to be used in the creation of a BLAST database.*



Set database properties

Database

Name: Orthologs

Description: A set of ortholog proteins

Location: C:\Users\[redacted]CLCdatabases

Figure 26.15: *Provide information about the BLAST database being created and specify where the files should be saved to.*

Click **Finish** to create the BLAST database. Once the process is complete, the new database will be available in the **Manage BLAST Databases** dialog, see section 26.4, and when launching IBLAST jobs (see section 26.1.2).

Create BLAST Database creates BLAST+ version 4 (dbV4) databases.

Sequence identifiers and BLAST databases

Restrictions on sequence identifier lengths, format, and duplicates present in the underlying BLAST+ program for making databases, *makeblastdb*, do not apply when making databases using **Create BLAST Database**.

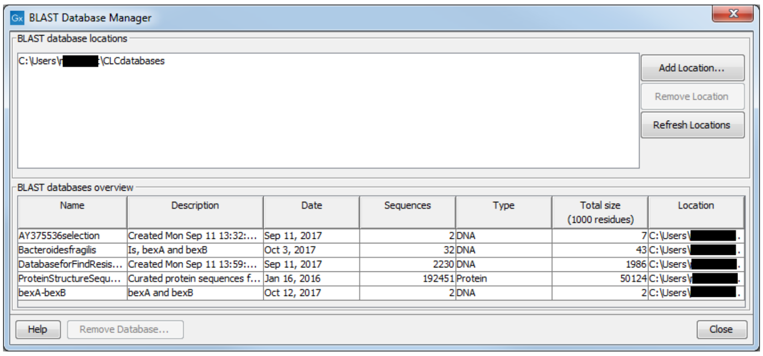
Internal handling of sequence names, introduced in version 21.0, allows this level of naming flexibility with newer versions of BLAST+. There should be no obvious effects of this internal handling of sequence names on local **BLAST** search results, including the names written to BLAST reports. For further details, see the FAQ **Can I search BLAST databases made using Create BLAST Databases with BLAST+ software?** at https://qiagen.my.salesforce-sites.com/KnowledgeBase/KnowledgeNavigatorPage?id=kA41i000000CjJ9CAK

26.4 Manage BLAST databases

The BLAST databases available to search using the **BLAST** tool can be managed using **Manage BLAST Databases** (figure 26.16), launched by going to:

Tools | **BLAST** ( )| **Manage BLAST Databases** ( )

At the top of the dialog, there is a list of the **BLAST database locations**. These locations are folders where the Workbench will look for valid BLAST databases. These can either be created



C:\Users\[redacted]\CLCdatabases

Name	Description	Date	Sequences	Type	Total size (1000 residues)	Location
AY375536selection	Created Mon Sep 11 13:32:...	Sep 11, 2017	2	DNA	7	C:\Users\[redacted].
Bacteroidesfragilis	Is, bexA and bexB	Oct 3, 2017	32	DNA	43	C:\Users\[redacted].
DatabaseforFindResis...	Created Mon Sep 11 13:59:...	Sep 11, 2017	2230	DNA	1986	C:\Users\[redacted].
ProteinStructureSequ...	Curated protein sequences f...	Jan 16, 2016	192451	Protein	50124	C:\Users\[redacted].
bexA-bexB	bexA and bexB	Oct 12, 2017	2	DNA	2	C:\Users\[redacted].

Figure 26.16: *BLAST databases are listed and can be managed using Manage BLAST Databases.*

from within the Workbench using the **Create BLAST Database tool**, see section 26.3.3, or they can be pre-formatted BLAST databases.

The list of locations can be modified using the **Add Location** and **Remove Location** buttons. Once the Workbench has scanned the locations, it will keep a cache of the databases (in order to improve performance). If you have added new databases that are not listed, you can press **Refresh Locations** to clear the cache and search the database locations again.

By default a BLAST database location will be added under your home area in a folder called CLCdatabases. This folder is scanned recursively, through all subfolders, to look for valid databases. All other folder locations are scanned only at the top level.

Below the list of locations, all the BLAST databases are listed with the following information:

- **Name.** The name of the BLAST database.
- **Description.** Detailed description of the contents of the database.
- **Date.** The date the database was created.
- **Sequences.** The number of sequences in the database.
- **Type.** The type can be either nucleotide (DNA) or protein.
- **Total size (1000 residues).** The number of residues in the database, either bases or amino acid.
- **Location.** The location of the database.

Below the list of BLAST databases, there is a button to **Remove Database**. This option will delete the database files belonging to the database selected.

26.5 Bioinformatics explained: BLAST

BLAST (Basic Local Alignment Search Tool) has become the *defacto* standard in search and alignment tools [Altschul et al., 1990]. The BLAST algorithm is still actively being developed and is one of the most cited papers ever written in this field of biology. Many researchers use BLAST as an initial screening of their sequence data from the laboratory and to get an idea of what they are working on. BLAST is far from being basic as the name indicates; it is a highly advanced algorithm which has become very popular due to availability, speed, and accuracy. In short, BLAST search programs look for potentially homologous sequences to your query sequences in databases, either locally held databases or those hosted elsewhere, such as at the NCBI (http://www.ncbi.nlm.nih.gov/) [McGinnis and Madden, 2004].

BLAST can be used for a lot of different purposes. Some of the most popular purposes are listed on the BLAST webpage at the NCBI: https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Searching for homology Most research projects involving sequencing of either DNA or protein have a requirement for obtaining biological information of the newly sequenced and maybe unknown sequence. If the researchers have no prior information of the sequence and biological content, valuable information can often be obtained using BLAST. The BLAST algorithm will search for homologous sequences in predefined and annotated databases of the users choice.

In an easy and fast way the researcher can gain knowledge of gene or protein function and find evolutionary relations between the newly sequenced DNA and well established data.

A BLAST search generates a report specifying the potentially homologous sequences found and their local alignments with the query sequence.

26.5.1 How does BLAST work?

BLAST identifies homologous sequences using a heuristic method which initially finds short matches between two sequences. After finding initial matches, BLAST attempts to build local alignments with the query sequence using these. Thus, BLAST does not guarantee the optimal alignment and some sequence hits may be missed. To find optimal alignments, the Smith-Waterman algorithm should be used (see below). Below, the BLAST algorithm is described in more detail.

Seeding When finding a match between a query sequence and a hit sequence, the starting point is the *words* that the two sequences have in common. A word is simply defined as a number of letters. For blastp the default word size is 3 *W=3*. If a query sequence has a QWRTG, the searched words are QWR, WRT, RTG. See figure 26.17 for an illustration of words in a protein sequence.

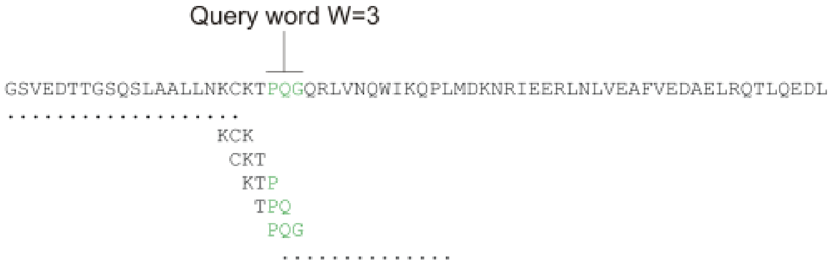

Query word W=3
GSVEDTTGSQSLAALLNKCKTPQGQRLVNQWIKQPLMDKNRIEERLNLVEAFVEDAELRQTLQEDL
KCK
CKT
KTP
TPQ
PQG

Figure 26.17: *Generation of exact BLAST words with a word size of W=3.*

During the initial BLAST seeding, the algorithm finds all common words between the query sequence and the hit sequence(s). Only regions with a word hit will be used to build on an alignment.

BLAST will start out by making words for the entire query sequence (see figure 26.17). For each word in the query sequence, a compilation of neighborhood words, which exceed the threshold of *T*, is also generated.

A neighborhood word is a word obtaining a score of at least *T* when comparing, using a selected scoring matrix (see figure 26.18). The default scoring matrix for blastp is BLOSUM62. The compilation of exact words and neighborhood words is then used to match against the database sequences.

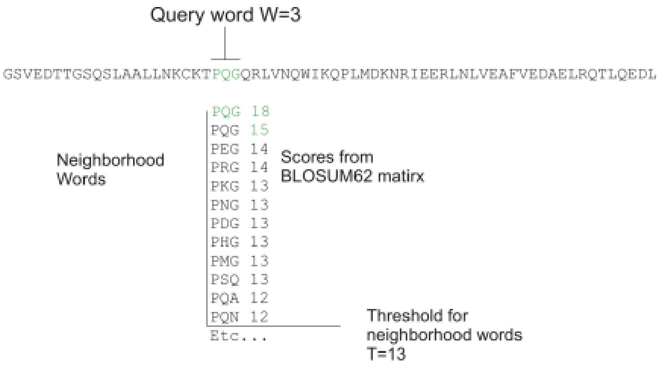
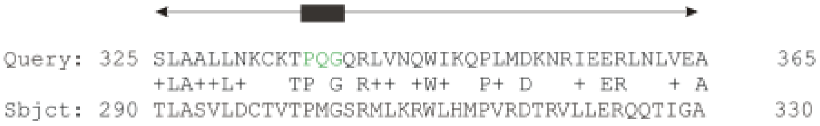

Query word W=3
GSVEDTTGSQSLAALLNKCKTPQGQRLVNQWIKQPLMDKNRIEERLNLVEAFVEDAELRQTLQEDL
Neighborhood Words
PQG 18
PQG 15
PEG 14
PRG 14
PKG 13
PNG 13
PDG 13
PHG 13
PMG 13
PSQ 13
PQA 12
PQN 12
Etc...
Scores from BLOSUM62 matirx
Threshold for neighborhood words T=13

Figure 26.18: *Neighborhood BLAST words based on the BLOSUM62 matrix. Only words where the threshold* T *exceeds 13 are included in the initial seeding.*

After the initial finding of words (seeding), the BLAST algorithm will extend the (only 3 residues long) alignment in both directions (see figure 26.19). Each time the alignment is extended, an alignment score is increases/decreased. When the alignment score drops below a predefined threshold, the extension of the alignment stops. This ensures that the alignment is not extended to regions where only very poor alignment between the query and hit sequence is possible. If the obtained alignment receives a score above a certain threshold, it will be included in the final BLAST result.



```
Query: 325 SLAALLNKCKTPQGQRLVNQWIKQPLMDKNRIEERLNLVEA     365
           +LA++L+    TP G R++ +W+  P+ D   + ER   + A
Sbjct: 290 TLASVLDCTVTPMGSRMLKRWLHMPVRDTRVLLERQQTIGA     330
```

Figure 26.19: *Blast aligning in both directions. The initial word match is marked green.*

By tweaking the word size *W* and the neighborhood word threshold *T*, it is possible to limit the search space. E.g. by increasing *T*, the number of neighboring words will drop and thus limit the search space as shown in figure 26.20.

This will increase the speed of BLAST significantly but may result in loss of sensitivity. Increasing the word size *W* will also increase the speed but again with a loss of sensitivity.

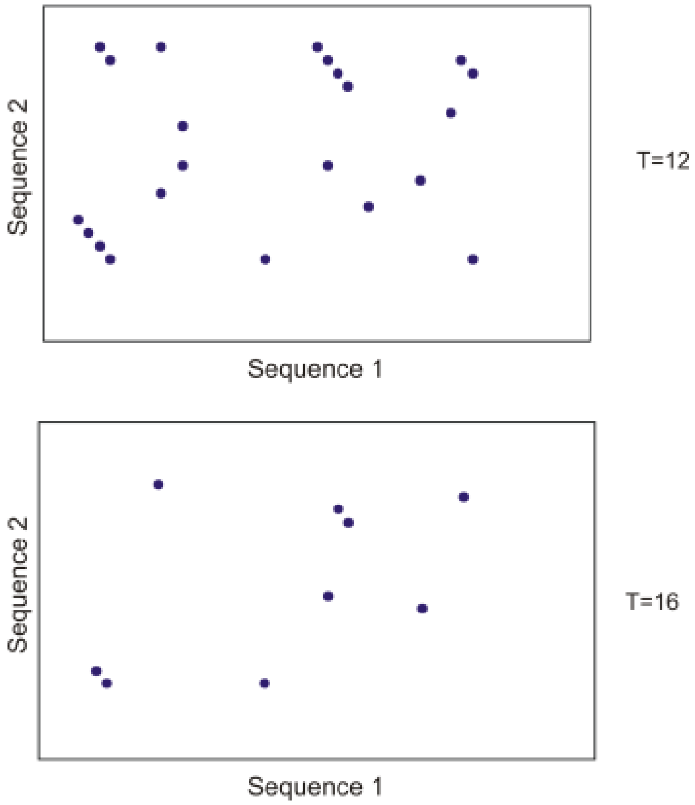


Figure 26.20: *Each dot represents a word match. Increasing the threshold of* T *limits the search space significantly.*

26.5.2 Which BLAST program should I use?

Depending on the nature of the sequence it is possible to use different BLAST programs for the database search. There are five versions of the BLAST program, blastn, blastp, blastx, tblastn, tblastx included in CLC software:

Option	**Query Type**	**DB Type**	**Comparison**	**Note**
blastn	Nucleotide	Nucleotide	Nucleotide-Nucleotide	
blastp	Protein	Protein	Protein-Protein	
tblastn	Protein	Nucleotide	Protein-Protein	The database is translated into protein
blastx	Nucleotide	Protein	Protein-Protein	The queries are translated into protein
tblastx	Nucleotide	Nucleotide	Protein-Protein	The queries and database are translated into protein

The most commonly used method is to BLAST a nucleotide sequence against a nucleotide database (blastn) or a protein sequence against a protein database (blastp). But often another BLAST program will produce more interesting hits. E.g. if a nucleotide sequence is translated before the search, it is more likely to find better and more accurate hits than just a blastn search. One of the reasons for this is that protein sequences are evolutionarily more conserved than nucleotide sequences. Another good reason for translating the query sequence before the search is that you get protein hits which are likely to be annotated. Thus you can directly see the protein function of the sequenced gene.

26.5.3 Which BLAST options should I change?

There are a number of options that can be configured when using BLAST search programs. Setting these options to relevant values can have a great impact on the search result. A few of the key settings are described briefly below.

The E-value The *expect value* (E-value) describes the number of hits one can expect to see matching the query by chance when searching against a database of a given size. An E-value of 1 can be interpreted as meaning that in a search like the one just run, you could expect to see 1 match of the same score by chance once. That is, a match that is not homologous to the query sequence. When looking for very similar sequences in a database, it is often beneficial to use very low E-values.

E-values depend on the query sequence length and the database size. Short identical sequence may have a high E-value and may be regarded as "false positive" hits. This is often seen if one searches for short primer regions, small domain regions etc. Below are some comments on what one could infer from results with E-values in particular ranges.

- **E-value < 10e-100** Identical sequences. You will get long alignments across the entire query and hit sequence.
- **10e-100 < E-value < 10e-50** Almost identical sequences. A long stretch of the query matches the hit sequence.
- **10e-50 < E-value < 10e-10** Closely related sequences, could be a domain match or similar.
- **10e-10 < E-value < 1** Could be a true homolog, but it is a gray area.
- **E-value > 1** Proteins are most likely not related
- **E-value > 10** Hits are most likely not related unless the query sequence is very short.

Gap costs For blastp it is possible to specify gap cost for the chosen substitution matrix. There is only a limited number of options for these parameters. The *open gap cost* is the price of introducing gaps in the alignment, and *extension gap cost* is the price of every extension past the initial opening gap. Increasing the gap costs will result in alignments with fewer gaps.

Filters It is possible to set different filter options before running a BLAST search. Low-complexity regions have a very simple composition compared to the rest of the sequence and may result in problems during the BLAST search [Wootton and Federhen, 1993]. A low complexity region of a protein can for example look like this 'fftfflllsss', which in this case is a region as part of a signal peptide. In the output of the BLAST search, low-complexity regions will be marked in lowercase gray characters (default setting). The low complexity region cannot be thought of as a significant match; thus, disabling the low complexity filter is likely to generate more hits to sequences which are not truly related.

Word size Changing the word size has a great impact on the seeded sequence space as described above. But one can change the word size to find sequence matches which would otherwise not be found using the default parameters. For instance the word size can be decreased when searching for primers or short nucleotides. For blastn a suitable setting would be to decrease the default word size of 11 to 7, increase the E-value significantly (1000) and turn off the complexity filtering.

For blastp a similar approach can be used. Decrease the word size to 2, increase the E-value and use a more stringent substitution matrix, e.g. a PAM30 matrix.

The BLAST search programs at the NCBI adjust settings automatically when short sequences are being used for searches, and there is a dedicated page, Primer-BLAST, for searching for primer sequences. https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Substitution matrix For protein BLAST searches, a default substitution matrix is provided. If you are looking at distantly related proteins, you should either choose a high-numbered PAM matrix or a low-numbered BLOSUM matrix. The default scoring matrix for blastp is BLOSUM62.

26.5.4 Where can I get the BLAST+ programs

The BLAST+ package can be downloaded for use on your own computer, institution computer cluster or similar from ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/. It is available for a wide range of different operating systems.

Pre-formatted databases are available from a dedicated BLAST ftp site ftp://ftp.ncbi.nlm.nih.gov/blast/db/. Most BLAST databases on the NCBI site are updated on a daily basis.

A few commercial software packages are available for searching your own data. The advantage of using a commercial program is obvious when BLAST is integrated with the existing tools of these programs. Furthermore, they let you perform BLAST searches and retain annotations on the query sequence (see figure 26.21). It is also much easier to batch download a selection of hit sequences for further inspection.

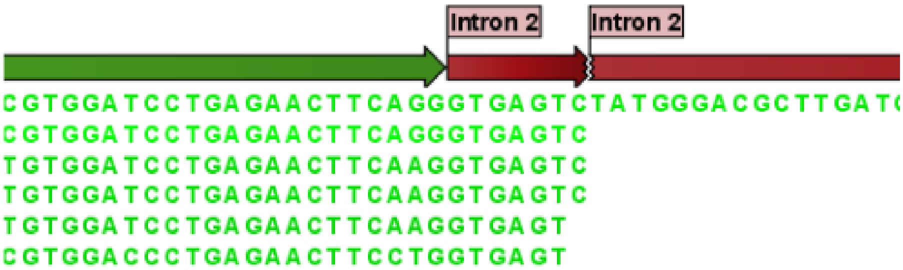

Intron 2
Intron 2
CGTGGATCCTGAGAACTTCAGGGTGAGTCTATGGGACGCTTGAT
CGTGGATCCTGAGAACTTCAGGGTGAGTC
TGTGGATCCTGAGAACTTCAAGGTGAGTC
TGTGGATCCTGAGAACTTCAAGGTGAGTC
TGTGGATCCTGAGAACTTCAAGGTGAGT
CGTGGACCCTGAGAACTTCCTGGTGAGT

Figure 26.21: *Snippet of alignment view of BLAST results. Individual alignments are represented directly in a graphical view. The top sequence is the query sequence and is shown with a selection of annotations.*

26.5.5 What you cannot get out of BLAST

Don't expect BLAST to produce the best available alignment. BLAST is a heuristic method which does not guarantee the best results, and therefore you cannot rely on BLAST if you wish to find *all* the hits in the database.

Instead, use the Smith-Waterman algorithm for obtaining the best possible local alignments [Smith and Waterman, 1981].

BLAST only makes local alignments. This means that a great but short hit in another sequence may not at all be related to the query sequence even though the sequences align well in a small region. It may be a domain or similar.

It is always a good idea to be cautious of the material in the database. For instance, the sequences may be wrongly annotated; hypothetical proteins are often simple translations of a found ORF on a sequenced nucleotide sequence and may not represent a true protein.

Don't expect to see the best result using the default settings. As described above, the settings should be adjusted according to the what kind of query sequence is used, and what kind of results you want. It is a good idea to perform the same BLAST search with different settings to get an idea of how they work. There is not a final answer on how to adjust the settings for your particular sequence.

26.5.6 Other useful resources

The NCBI BLAST web page
`https://blast.ncbi.nlm.nih.gov/Blast.cgi`

The latest BLAST+ release
`ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST`

Download pages for pre-formatted BLAST databases
`ftp://ftp.ncbi.nlm.nih.gov/blast/db/`

O'Reilly book on BLAST
`http://www.oreilly.com/catalog/blast/`

Chapter 27

Utility tools

Contents

27.1 Extract Annotated Regions

Using **Extract Annotated Regions**, parts of a sequence (or several sequences) can be extracted based on annotations. Lengths of flanking regions can be specified if desired. The output is a sequence list that contains sequences carrying the annotation specified.

Some examples of the use of this tool:

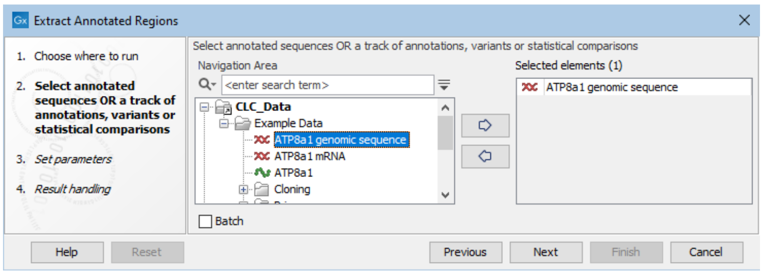
- Extract all tRNA gene sequences from a genome.
- Extract sequences for regions where annotations contain particular text

To launch **Extract Annotated Regions**, go to:

Tools | **Utility Tools** ( ) | **Extract Annotated Regions** ( )

This opens the wizard. In the first step (figure 27.1) you can select one or more sequences to extract annotated regions from.

Note: With the *CLC Main Workbench*, annotated sequences are expected as input. Options relating to track-based input are intended for the *CLC Genomics Workbench*, where this tool is also present.



Extract Annotated Regions

1. Choose where to run
2. **Select annotated sequences OR a track of annotations, variants or statistical comparisons**
3. *Set parameters*
4. *Result handling*

Select annotated sequences OR a track of annotations, variants or statistical comparisons

Navigation Area

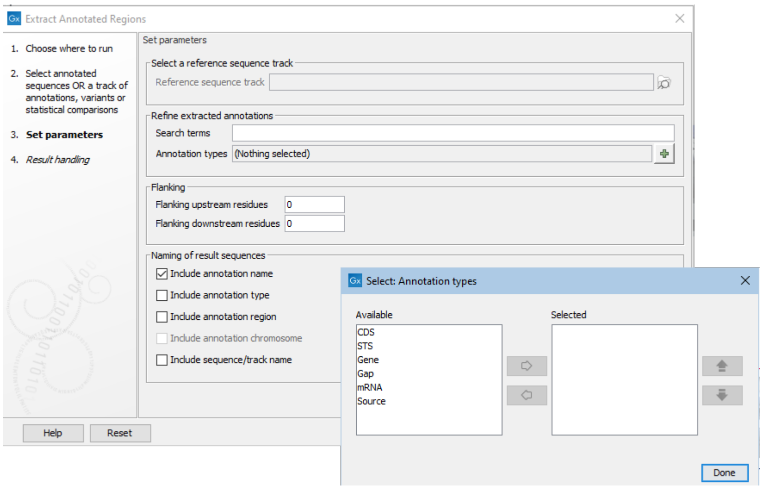
Selected elements (1)

ATP8a1 genomic sequence

□ Batch

Figure 27.1: *Select one or more sequences to extract annotated regions from.*

At the top of the next dialog step (figure 27.2) you can specify which annotations to use.



Extract Annotated Regions

1. Choose where to run
2. Select annotated sequences OR a track of annotations, variants or statistical comparisons
3. **Set parameters**
4. *Result handling*

Set parameters

Select a reference sequence track

Reference sequence track

Refine extracted annotations

Search terms

Annotation types (Nothing selected)

Flanking

Flanking upstream residues 0

Flanking downstream residues 0

Naming of result sequences

☑ Include annotation name

□ Include annotation type

□ Include annotation region

□ Include annotation chromosome

□ Include sequence/track name

Select: Annotation types

Available: CDS, STS, Gene, Gap, mRNA, Source

Selected

Figure 27.2: *Configure the regions for sequence extraction.*

- **Search terms** All annotations and attached information for each annotation will be searched for the entered term. It can be used to make general searches for search terms such as "Gene" or "Exon", or it can be used to make more specific searches. For example, if you have a gene annotation called "MLH1" and another called "MLH3", you can extract both annotations by entering "MLH" in the search term field. If you wish to enter more specific search terms, separate them with commas: "MLH1, Human" will find annotations where both "MLH1" and "Human" are included.
- **Annotation types** If only certain types of annotations should be extracted, this can be specified here.

The sequence of interest can be extracted with flanking sequences:

- **Flanking upstream residues** The output will include this number of extra residues at the 5' end of the annotation.
- **Flanking downstream residues** The output will include this number of extra residues at the 3' end of the annotation.

The sequences that are created can be named after the annotation name, type, etc:

- **Include annotation name** This will use the name of the annotation in the name of the extracted sequence.
- **Include annotation type** This corresponds to the type chosen above and will put this information in the name of the resulting sequences. This is useful information if you have chosen to extract "All" types of annotations.
- **Include annotation region** The region covered by the annotation on the original sequence (i.e. not including flanking regions) will be included in the name.
- **Include sequence/track name** If you have selected more than one sequence as input, this option enables you to discern the origin of the resulting sequences in the list by putting the name of the original sequence into the name of the resulting sequences.

Click **Finish** to start the tool.

27.2 Combine Reports

Combine Reports takes reports as input and summarizes their contents. A single summary report is generated. The report content and its order is configurable.

Creating a combined report

To run **Combine Reports**, go to:

Tools | **Utility Tools** ( ) | **Reports** ( ) | **Combine Reports** ( )

To see a list of tools that produce reports that can be used as input, click on the (ⓘ) icon in the top right corner of the input selection wizard (figure 27.3).

One section is included in the combined report for each report type provided as input, with the type determining the title of the corresponding section. See **Report types and combined report content** below for further details.

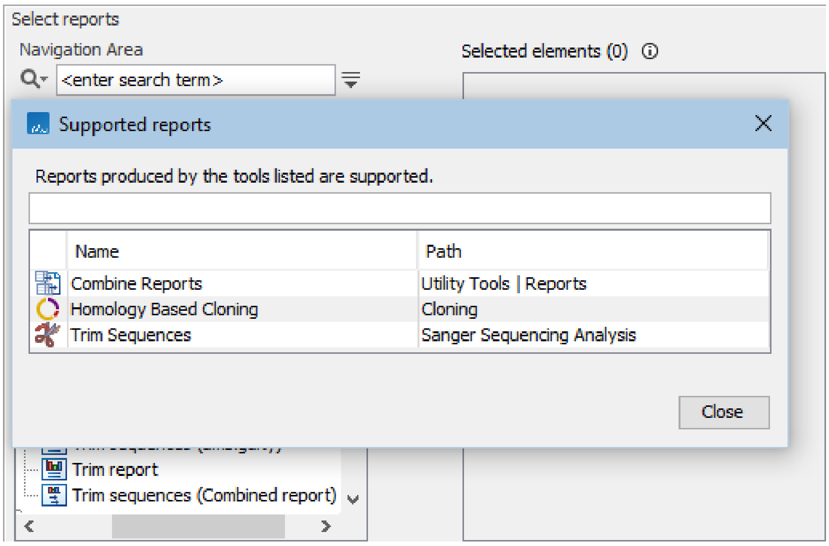
Specifying the order of the sections in the report

When more than one report type is provided as input, the order of the sections in the output report is configurable in the "Set order" wizard step:

- **Order of inputs** Use the order that the input reports were specified in.
- **Define order** Explicitly define the section order by moving items up and down in the listing.

  Defining the order is recommended when the tool is being launched in batch mode with folders of reports provided as the batch units. Doing this avoids reliance on the order of the elements within the folders being the same.

When **Combine Reports** is included in a workflow, sections are ordered according to the order of the inputs. See section 13.1.3 for information about ordering inputs in workflows.



Select reports

Navigation Area

<enter search term>

Selected elements (0)

Supported reports

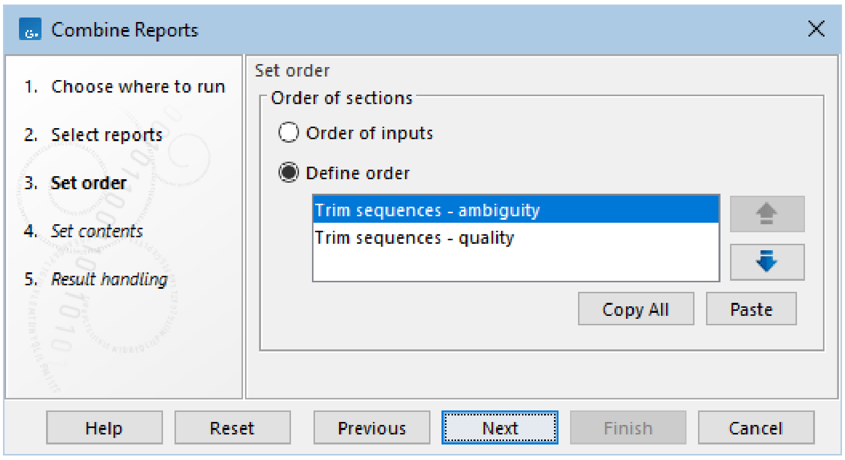
Reports produced by the tools listed are supported.

Name	Path
Combine Reports	Utility Tools | Reports
Homology Based Cloning	Cloning
Trim Sequences	Sanger Sequencing Analysis

Trim report

Trim sequences (Combined report)

Figure 27.3: *Clicking on the info icon at the top right corner of the input selection wizard opens a window showing a list of tools that produce supported reports. Text entered in the field at the top limits the list to just tools with names containing the search term.*



Combine Reports

1. Choose where to run
2. Select reports
3. **Set order**
4. *Set contents*
5. *Result handling*

Set order

Order of sections

○ Order of inputs

● Define order

Trim sequences - ambiguity

Trim sequences - quality

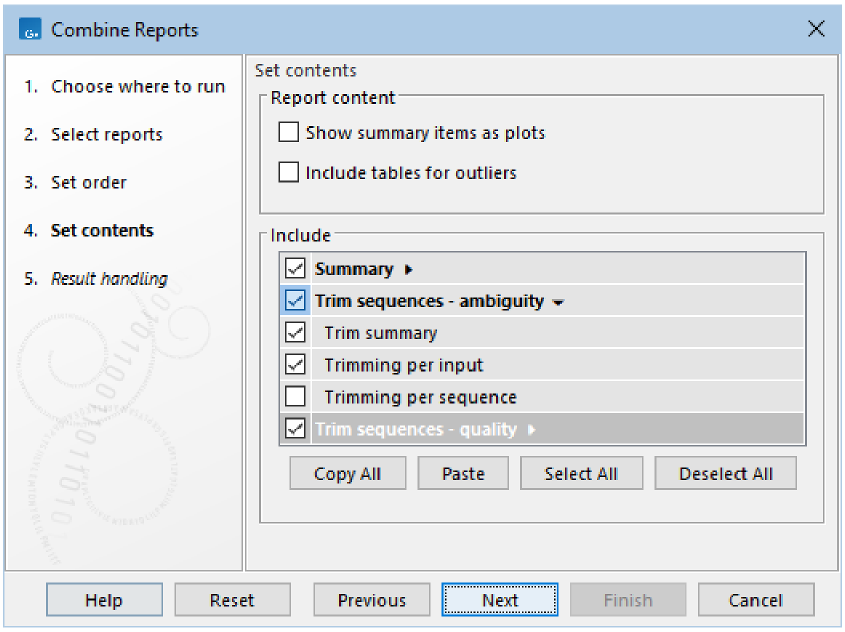
Figure 27.4: *When more than one report type is provided as input, the order of the sections can be configured in the "Set order" wizard step.*

Specifying the content of the report

The content of the report is configured in the "Set contents" wizard step (figure 27.5):

- Under "Report contents", the following options are available:
  - **Show summary items as plots** When checked, summary items are displayed as box plots instead of tables, where possible.
  - **Include tables for outliers** When checked, samples detected as outliers for each table/box plot are added to an outliers table, which is printed under the table/box plot.
- Under "Include", the sections to be added to the report are specified. The combined report contains a general summary section that appears at the top of the report when included.

Where available, individual subsections and summary items can also be specified. Where only some subsections or summary items are excluded, the checkbox for the parent section(s) are highlighted for visibility.



Report content

□ Show summary items as plots

□ Include tables for outliers

Include

☑ Summary ▸

☑ Trim sequences - ambiguity ▾

☑ Trim summary

☑ Trimming per input

□ Trimming per sequence

☑ Trim sequences - quality ▸

Figure 27.5: *The content of the combined report is configured in the "Set contents" wizard step. Sections with a check in the box are included, while those without a check are excluded from the combined report. For visibility, sections where some contents have been excluded have checkboxes highlighted.*

See section 27.2.1 for information about the generated report.

Note: The options under "Report contents" and the summary section are most relevant for reports produced by tools in the *CLC Genomics Workbench*.

Reusing configurations

Configurations defined previously can be used in subsequent runs.

Configurations can be copied in two ways:

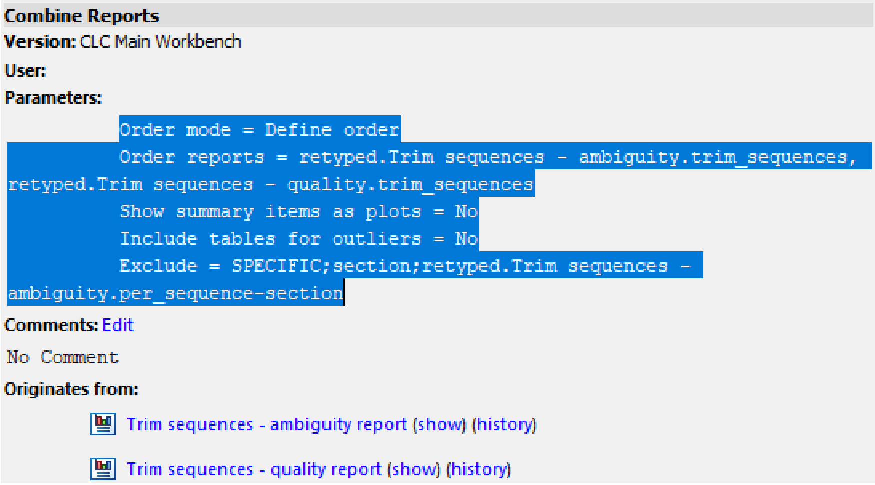
- Copy the configuration defined in the relevant wizard step using the **Copy all** button.
- Copy the configuration used in previous runs of the tool from the History ( ) view of the output, described further below.

Copied configurations can be pasted into a text file for later use.

A copied configuration can be pasted into the wizard step using the **Paste** button.

Any existing settings in that wizard step will be overwritten.

The history of a report output by **Combine Reports** contains both the order of the sections (Order reports) and the excluded sections/subsections/summary items (Exclude) (figure 27.6). These can be selected, copied, and then pasted into the "Set order"/"Set contents" wizard steps, respectively, in a subsequent run. Alternatively, the entire history can be selected, copied, and then pasted in each wizard step. Only the relevant configuration is pasted into each step.



Combine Reports
Version: CLC Main Workbench
User:
Parameters:

```
          Order mode = Define order
          Order reports = retyped.Trim sequences - ambiguity.trim_sequences,
retyped.Trim sequences - quality.trim_sequences
          Show summary items as plots = No
          Include tables for outliers = No
          Exclude = SPECIFIC;section;retyped.Trim sequences -
ambiguity.per_sequence-section
```

Comments: Edit

No Comment

Originates from:

Trim sequences - ambiguity report (show) (history)

Trim sequences - quality report (show) (history)

Figure 27.6: *The history of a report output by Combine Reports with the parameters selected, ready to be copied.*

Report types and combined report content

Reports that can be supplied as input have a report type. The type of a report affects the placement of its summary information in the output. Specifically:

- Reports with the *same type* that are generated by *the same tool* are summarized into a single section, named according to the type.

  This is useful when the aim is to compare the values from those reports, for example results from different samples or different analysis runs. However, if a particular tool has been used more than once in an analysis, for different purposes, then placing the summary of these results in different sections may be desirable. This can be done by editing the report type in some of the reports (see section 27.4.).

- Reports with *different types* are summarized in separate sections, named according to the types.

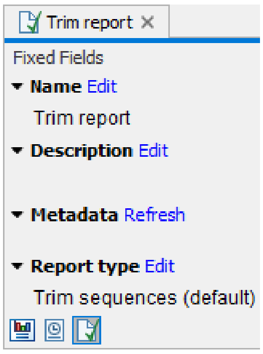
  The report type assigned by a particular tool is unique, so reports generated by different tools have different types.

  If reports generated by different tools are later modified so their report types are the same, those reports will still be summarized in different sections, although each of these sections will have the same name.

The type of a report can be seen in the Element Info ( ) view for that report.

27.2.1 Combine Reports output

Combine Reports generates a single report ( ) containing summary items and other selected information from the reports provided as input.



Trim report ✕

Fixed Fields

▾ **Name** Edit

Trim report

▾ **Description** Edit

▾ **Metadata** Refresh

▾ **Report type** Edit

Trim sequences (default)

Figure 27.7: *The type of a report can be found in the Element Info view of reports that are supported as input for tools that summarize reports.*

The report contains one section per input report type, as described in section 27.2. Summary items are displayed in table format.

Note: The summaries for reports produced by **Trim Sequences** do not follow the format described below.

The tables contain one row per input report and one column per summary item. The last rows, shaded in pale gray, report the minimum, median, maximum, mean and standard deviation for all numeric summary items (figure 27.8).

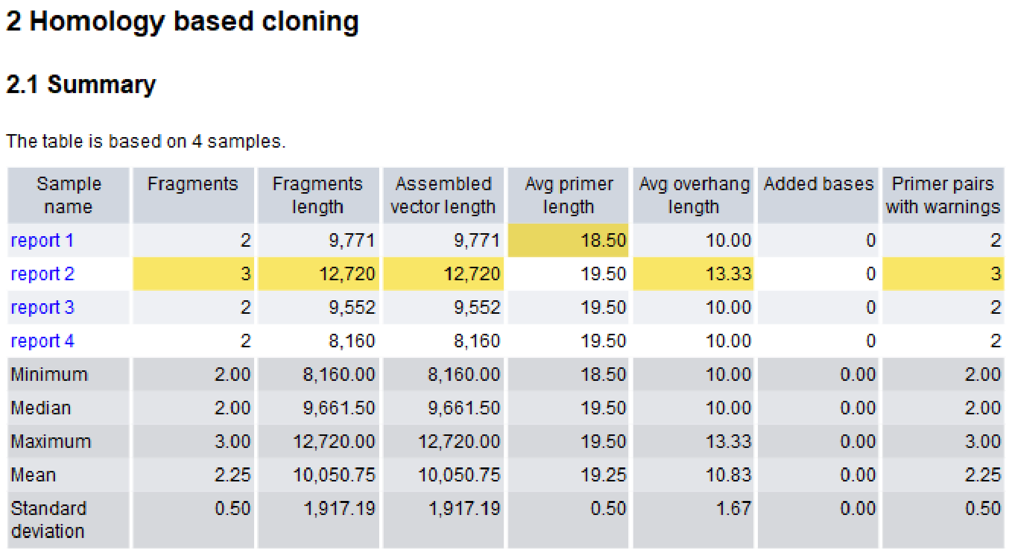
The first column indicates the sample name, i.e. the name of the input report. The combined report contains links to the input reports and clicking on the sample name selects the corresponding report in the Navigation Area.

Highlighted cells

Table cells are highlighted in yellow if they are detected as outliers (figure 27.8). For each numeric summary item, the lower quartile - 1.5 IQR (interquartile range) to upper quartile + 1.5 IQR range is calculated using all the values for the summary item. Samples with values outside this range are considered outliers.

Summary section

By default, combined reports contain a summary section, offering a quick overview of samples that have been identified as outliers and/or problematic. The summary section is only present if it was included when configuring the report content (see section 27.2) and it only contains summaries those sections/subsections/summary items that are also included in the combined report.



2 Homology based cloning

2.1 Summary

The table is based on 4 samples.

Sample name	Fragments	Fragments length	Assembled vector length	Avg primer length	Avg overhang length	Added bases	Primer pairs with warnings
report 1	2	9,771	9,771	18.50	10.00	0	2
report 2	3	12,720	12,720	19.50	13.33	0	3
report 3	2	9,552	9,552	19.50	10.00	0	2
report 4	2	8,160	8,160	19.50	10.00	0	2
Minimum	2.00	8,160.00	8,160.00	18.50	10.00	0.00	2.00
Median	2.00	9,661.50	9,661.50	19.50	10.00	0.00	2.00
Maximum	3.00	12,720.00	12,720.00	19.50	13.33	0.00	3.00
Mean	2.25	10,050.75	10,050.75	19.25	10.83	0.00	2.25
Standard deviation	0.50	1,917.19	1,917.19	0.50	1.67	0.00	0.50

Figure 27.8: *Summary items are reported in tables. Cells are highlighted in yellow when identified as outliers.*

27.3 Create Report from Table

The **Create Report from Table** tool allows any element that can be exported to "Tab delimited text" to be turned into a report with a single table.

Many element types are accepted as input, including:

- variant track ()
- annotation track ()
- expression track ()
- statistical comparison track ()

Note that tables with many rows will create very long reports. Consider filtering the table first. Filtering can be done manually when in table view, see section 9.2.

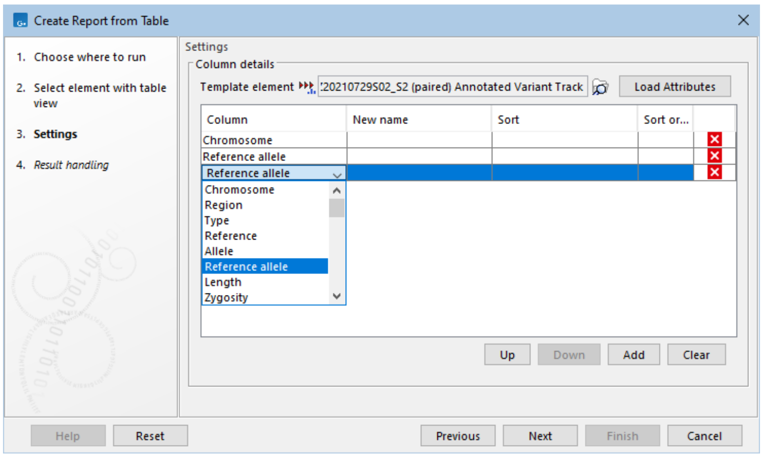
To run **Create Report from Table**, go to:

Tools | **Utility Tools** () | **Reports** () | **Create Report from Table** ()

After selecting the input element, the columns to include in the report must be defined. Column definitions consist of four parts:

- **Column.** The original name of the column in the table.
- **New name.** The name this column should have in the report. Shorter names are often preferred in reports, because PDF exports have limited width. If the name is left blank, the column will not be renamed.
- **Sort.** Whether the column in the table should be sorted in ascending or descending order. When left blank, the sorting will match that of the input table.

- **Sort order.** The order in which sorting should be applied (only relevant when sorting on multiple columns). Sorting is applied to columns in order from smallest to largest sort order i.e., column with sort order 1 is sorted on before column with sort order 2, and so on. It is not possible to manually enter a sort order. Instead the order is populated automatically according to the order in which columns are chosen for sorting. To change an existing sort order, toggle sorting of the affected columns off and on, such that they receive new sort orders.



Create Report from Table

1. Choose where to run
2. Select element with table view
3. **Settings**
4. *Result handling*

Settings

Column details

Template element: '20210729S02_S2 (paired) Annotated Variant Track

Column	New name	Sort	Sort or...	
Chromosome				☒
Reference allele				☒
Reference allele				☒

Figure 27.9: *Define the columns to be included in the report.*

To make defining columns easier, the **Load Attributes** button can be used to populate a dropdown list of the columns found in the element selected in the **Template element** field (figure 27.9). By default, the tool's input is preselected. Use the browse ( ) button to select a different element.

If a template element is not used for populating a dropdown list, columns can be entered by typing directly in the **Column** field.

The **Add** button adds additional columns while pressing the X (☒) button to the right of a column removes it. It is possible to reorder columns using the **Up** and **Down** buttons. The **Clear** button removes all defined columns.

If a column is defined that is not present in the input element, then an empty column with that name will be placed in the report.

Combined reports

Report sections from this tool cannot be used in **Combine Reports**, because the contents of the tables may be specific to each sample.

27.4 Modify Report Type

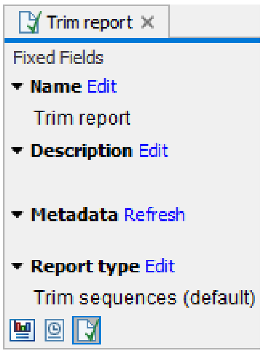
Report types affect the placement of information in reports generated by tools designed to summarize other reports. For information about the effect of report type on summary report content see: section 27.2.

A report's type can be edited directly in the Element Info ( ) tab or the **Modify Report Type** tool can be used. Both options are described in this section. Note that report types are case sensitive. E.g. 'Trim by Quality' and 'Trim by quality' are interpreted as different types.

The report type assigned by a particular tool is unique, so reports generated by different tools have different types. The term "(default)" at the end of a report type suggests the type has not been modified since the report was created.

Directly updating a report type

The report type is shown in the Element Info ( ) view of that report (figure 27.10). It can be edited in this view by clicking on "Edit".



Trim report ×

Fixed Fields

Name Edit

Trim report

Description Edit

Metadata Refresh

Report type Edit

Trim sequences (default)

Figure 27.10: *Reports types can be seen in the Element Info view of a report.*

Running the Modify Report Type tool

To run **Modify Report Type**, go to:

Tools | **Utility Tools** ( ) | **Reports** ( ) | **Modify Report Type** ( )

To see a list of tools that produce supported report types, click on the (ⓘ) icon in the top right corner of the input selection wizard (figure 27.11).

The type of the report is set in the **Report type** text field in the "Parameters" wizard step. The type specified here is used for the title of the corresponding section of summary reports generated using this report as input. If left blank, the report type reverts back to the original "(default)" report type.

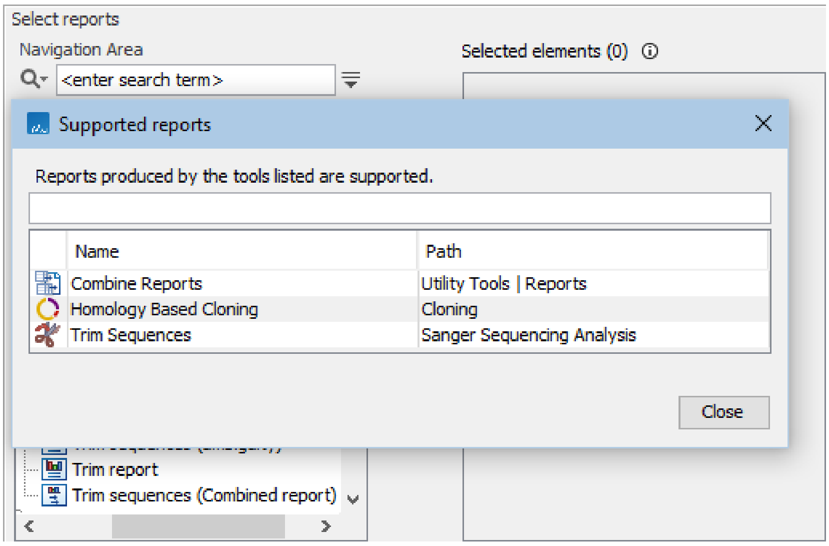
Modify Report Type outputs a report with the updated report type, but that is otherwise identical to the original report.

27.4.1 Modifying report types in workflows

Modify Report Type can be added to workflows, where it can be particularly useful in cases where the same tool is present in the workflow multiple times.

Consider the example workflow in figure 27.13.

- Two **Trim Sequences** workflow elements, named "Trim by Quality" and "Trim by Ambiguous", to reflect the type of trimming performed.

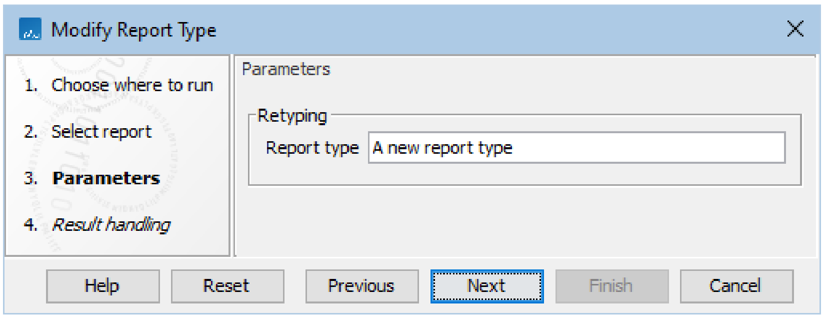


Supported reports

Reports produced by the tools listed are supported.

Name	Path
Combine Reports	Utility Tools | Reports
Homology Based Cloning	Cloning
Trim Sequences	Sanger Sequencing Analysis

Figure 27.11: *Clicking on the info icon at the top right corner of the input selection wizard opens a window showing a list of tools that produce supported report types. Text entered in the field at the top limits the list to just tools with names containing the search term.*



Report type: A new report type

Figure 27.12: *Enter the report type to assign in the "Report type" field.*

- Two **Modify Report Type** workflow elements, named "Modify Report Type to Trim by Quality" and "Modify Report Type to Trim by Ambiguous", to reflect which reports it modifies and the type it sets.
- One **Combine Reports** workflow element, which uses the two trim reports with modified types.

Considerations when designing workflows

- **Links within summary reports to input reports**

  Where report types are modified, we recommend that the individual reports are output after the report type modification has been done, i.e. link an output element to the **Modify Report Type** element, as illustrated in figure 27.13. This preserves the links found within the summary reports to the input reports with modified types, see section 27.2.1.

- **Content of summary reports**

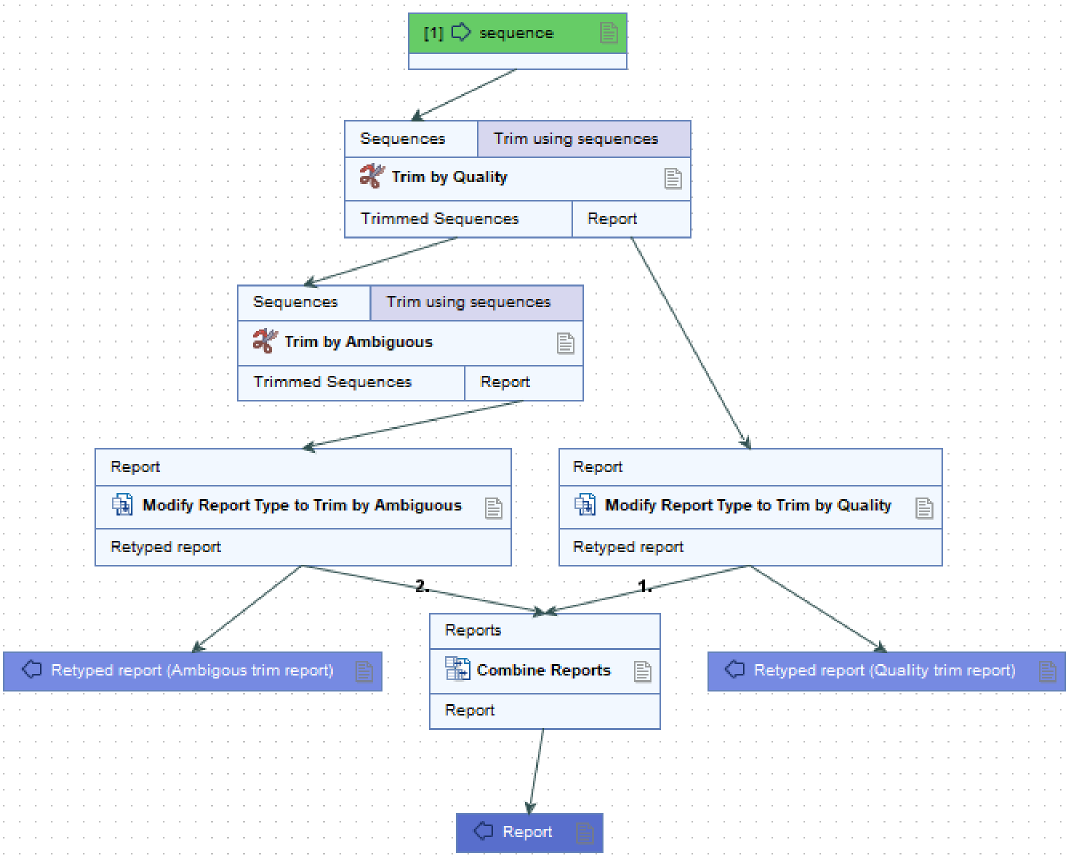
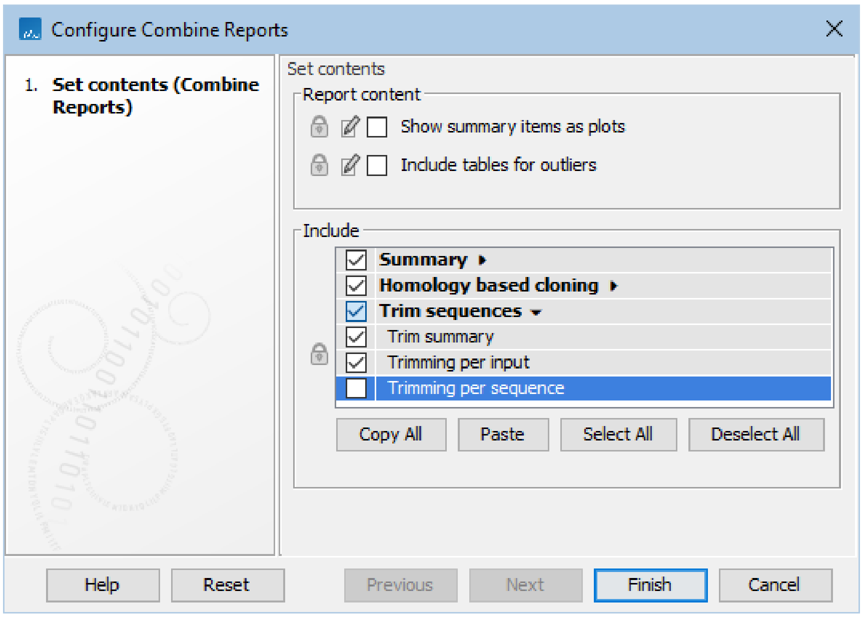


Figure 27.13: *An example workflow running two trimming jobs. The name of each trim element is different but the underlying tool is the same, so the reports generated have the same type. The report types are then modified, and reports with the modified type are used as input to the next step.*

The content of the summary reports can only be defined for the default report types and applies too all reports, even those with a modified type, that originally had that report type (figure 27.14).



Set contents

Report content

☐ Show summary items as plots

☐ Include tables for outliers

Include

☑ Summary

☑ Homology based cloning

☑ Trim sequences

☑ Trim summary

☑ Trimming per input

☐ Trimming per sequence

Figure 27.14: *Defining the contents for trimming applies to all reports produced by the trimming tool, regardless of their report type.*

27.5 Create Sequence List

For information on creating sequence lists, please see section 14.1.

27.6 Update Sequence Attributes in Lists

Update Sequence Attributes in Lists updates information about sequences in a Sequence List. Information can be added to existing attributes and new attribute types can be added.

The tool takes attribute information from an Excel file (`.xls/xlsx`), a comma separated text file (`.csv`), or a tab separated text file (`.tsv`). Each sequence is updated with the relevant information by matching the content of a particular column in the file, specified when launching the tool, with the contents of a column of the same name in the Sequence List.

The columns to take information from are specified when launching the tool. Column names are used as attribute names. If a column name matches an existing attribute in the Sequence List, the information from that column can be added to the existing attribute (details below). When a column name does not match an existing attribute, a new attribute is added to the Sequence List.

Additional notes:

- This tool is recommended when updating information for many sequences. However, attributes can also be updated individually, either directly in the Table view of the Sequence List (see section 14.1.3), or, by opening the sequence from the Sequence List and editing attributes in its Element info view. Opening a sequence can be done from the Sequence List view (right-click on the sequence name and choose "Open Sequence") or from the Table view (right-click in the row for that sequence and choose the option "Open This Sequence").
- Attributes relating to characteristics of the sequence itself, such as its length or the start

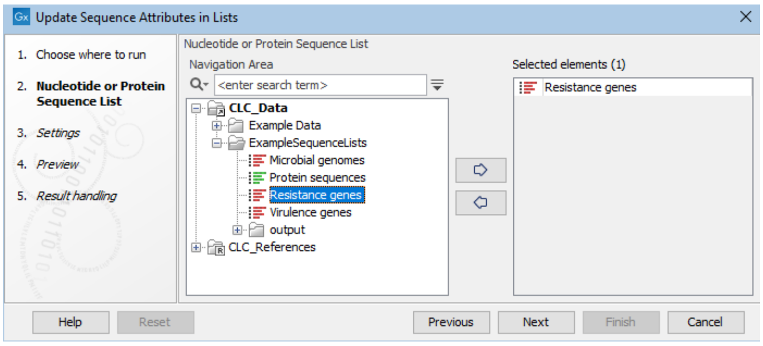
of the sequence, cannot be updated using this tool, nor by directly editing the Sequence List.

To launch the **Update Sequence Attributes in Lists** tool, go to:

Tools | **Utility Tools** () | **Sequence Lists** () |**Update Sequence Attributes in Lists** ()

and select one or more Sequence Lists of the same type (nucleotide *or* peptide) as input (figure 27.15).

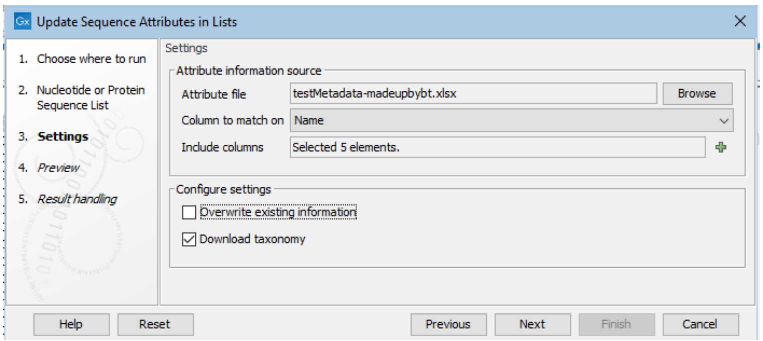
Note: Sequences in all inputs provided will be worked upon as a single entity. A single Sequence List containing all sequences is output.



Selected elements (1)
Resistance genes

Figure 27.15: *Select one or more Sequence Lists as input.*

In the Settings wizard step, the file containing attribute information is specified, along with details about how to handle that information (figure 27.16).



Attribute file: testMetadata-madeupbybt.xlsx
Column to match on: Name
Include columns: Selected 5 elements.

☐ Overwrite existing information
☑ Download taxonomy

Figure 27.16: *Information in the attribute file will be matched with the relevant sequence based on contents of the Name column in the file and in the Sequence List. Five columns containing relevant attribute information have been selected. The option to overwrite existing information has been left unchecked.*

Attribute information source fields

- **Attribute file** Select an Excel file (`.xls/xlsx`), a comma separated text file (`.csv`), or a tab separated text file (`.tsv`) containing attribute information. Column names are used as attribute names, so a header row is required. One column in the file must contain information that can be matched with information already present in the Sequence List (see "Column to match on", below).

- **Column to match on** Specify the column in the attribute file to use to match each row with the relevant sequence(s) in the Sequence List. When a value in this column matches a value in the column *of the same name* in the Sequence List, information from that row in the file is added to the attribute information for that sequence. Only information from specified columns will be added (see "Include columns", below.)

  When matching based on sequence names, the column in the file containing the names must be called `Name`.

- **Include columns** Select the columns in the file containing the information to be updated or added to the Sequence List as well as the column specified in the "Column to match on" field.

  When the name of a column does not match existing attribute name in the Sequence List, a new attribute will be added.

Configure settings checkboxes

- **Overwrite existing information** When this option is checked, existing sequence attribute values will be overwritten by values for the corresponding attributes in the attribute file. When no corresponding value is present in the attributes file, no change is made to the value in the Sequence List.

  When left unchecked, existing attribute values in the Sequence List are not overwritten with new information from the file.

- **Download taxonomy** Check this box to download a 7-step taxonomy from the NCBI into an attribute called "Taxonomy". To use this option, there must be a column in the attributes file called `TaxID` containing valid taxonomic identifiers. See the "Column headings and value validation" section below for further details.

  The "Taxonomy" attribute will be listed in the Preview wizard step, alongside the columns selected for inclusion.

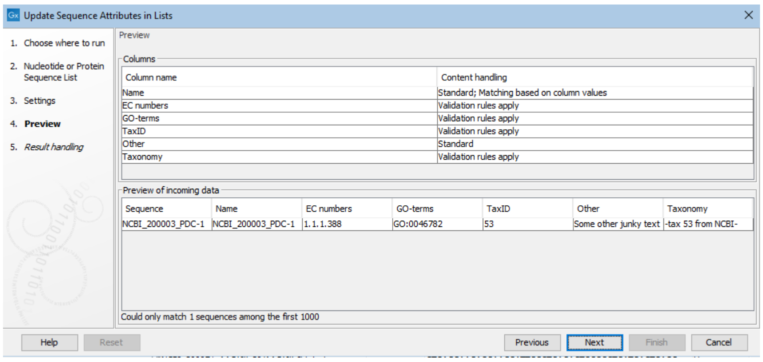
The result of the choices made in the Settings step are reflected in the Preview wizard step (figure 27.17). In the upper pane is a list of the attribute types to be updated or added, as well as the attribute to be used to match sequences with the relevant information. How particular columns will be handled is indicated in the "Content handling" column, including whether validation will be applied. The columns subject to validation checks are described later in this section.

Shown in the lower pane is a small subset of the incoming information from the attribute file, based on the choices made in the Settings wizard step. Click on the "Previous" button to go back to that step if anything needs to be adjusted.

Column headings and value validation

Certain column names are recognized by the software and validation rules are applied to these. When the contents pass the validation checks, entries in those columns may be further processed.

In most cases, this further processing involves adding hyperlinks to online data resources. However, the contents of columns with the following names trigger different handling:



Column name	Content handling
Name	Standard; Matching based on column values
EC numbers	Validation rules apply
GO-terms	Validation rules apply
TaxID	Validation rules apply
Other	Standard
Taxonomy	Validation rules apply

Sequence	Name	EC numbers	GO-terms	TaxID	Other	Taxonomy
NCBI_200003_PDC-1	NCBI_200003_PDC-1	1.1.1.388	GO:0046782	53	Some other junky text	-tax 53 from NCBI-

Could only match 1 sequences among the first 1000

Figure 27.17: *The Preview wizard steps shows information about how columns from the attribute file will be handled, and whether any problems were detected. Where validation checks are carried out, if any had failed, a yellow exclamation mark in the bottom pane would be shown for that column. Here, all entries pass. The "Other" column is not subject to validation checks. Only one sequence in the list is being updated in this example.*

- **TaxID** When valid taxonomic identifiers are found in a column called `TaxID`, and the **Download taxonomy** checkbox was checked in the Settings wizard step, then a 7-step taxonomy is downloaded from the NCBI.

  Examples of valid identifiers for TaxID attribute are those found in `/db_xref="taxon` fields in Genbank entries. For example, for `/db_xref="taxon:5833`, the expected value in the TaxID column would be `5833`.

  If a given sequence has an value already set for the Taxonomy attribute, then that existing value remains in place unless the "Overwrite existing information" box was checked in the Settings wizard step.

- **Gene ID** The following identifiers in a Gene ID column are added as attribute values and hyperlinked to the relevant online database:

  - Ensembl Gene IDs
  - HUGO Gene IDs
  - VFDB Gene IDs

  Any other values in a Gene ID column are added as attributes to the relevant sequences, but are not hyperlinked to an online data resource. Note that this is different to how other non-validated attribute values are handled, as described below.

  Multiple identifiers in a given cell, separated by commas, will be added as multiple Gene ID attributes for the relevant sequence. If any one of those identifiers is not recognized as one of the above types, then none will be hyperlinked.

Other columns where contents are validated are those with the headings listed below. If a value in such a column cannot be validated, it is **not** added nor used to update attributes.

If you wish to add information of this type but do not want this level of validation applied, use a heading other than the ones listed below.

- **COG-terms** COG identifiers

- **Compound AROs** Antibiotic Resistance Ontology identifiers
- **Compound Class AROs** Antibiotic Resistance Ontology identifiers
- **Confers-resistance-to ARO** Antibiotic Resistance Ontology identifiers
- **Drug ARO** Antibiotic Resistance Ontology identifiers
- **Drug Class ARO** Antibiotic Resistance Ontology identifiers
- **EC numbers** EC identifiers
- **GenBank accession** Genbank accession numbers
- **Gene ARO** Antibiotic Resistance Ontology identifiers
- **GO-terms** Gene Ontology (GO) identifiers
- **KO-terms** KEGG Orthology (KO) identifiers
- **Pfam domains** PFAM domain identifiers
- **Phenotype ARO** Antibiotic Resistance Ontology identifiers
- **PubMed IDs** Pubmed identifiers
- **TIGRFAM-terms** TIGRFAM identifiers
- **Virulence factor ID** Virulence Factors of Pathogenic Bacteria identifiers

Updating Location-specific attributes

Location-specific attributes can be created, which are the present for all elements created in that CLC File Location. Such attributes can be updated using the **Update Sequence Attributes in Lists** tool.

Of note when working with such attributes:

- Because these attributes are tied to the Location, they will not appear until the updated Sequence List has been saved.
- The updated Sequence List must be saved to the same File Location as the input for these attributes and their values to appear.
- If this tool is run on an unsaved Sequence List , or using inputs from more than one File Location at the same time, Location-specific attributes will not be updated. Information in the preview pane reflects this.

Location-specific attributes are described in section 3.3.

27.7 Split Sequence List

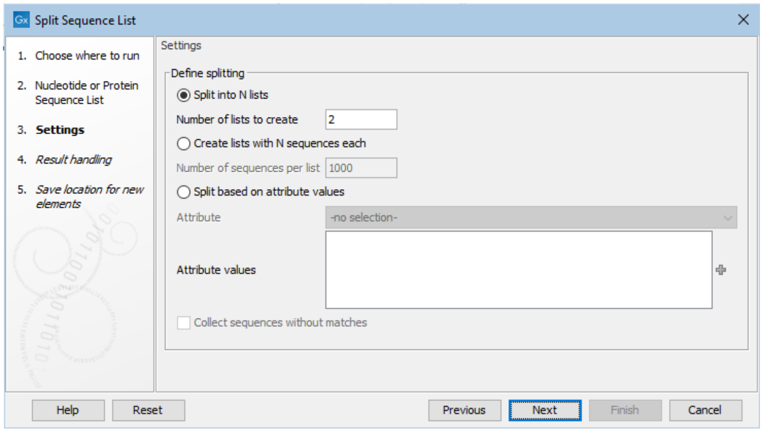
Split Sequence List can split up nucleotide or peptide sequence lists. The output can be a specified number of lists, lists containing a specified number of sequences, or lists containing sequences with particular attribute values, such as terms in the description,

To launch the **Split Sequence List** tool, go to:

Tools | **Utility Tools** ( ) | **Sequence Lists** ( ) |**Split Sequence List** ( )

Select one or more sequence lists as input. If multiple lists are selected, then to process each input list separately, check the "Batch" checkbox. When unchecked, sequences in all the lists are considered together as a single input.

In the next wizard step, the basis upon which to do the splitting and any necessary settings for that option are specified (figure 27.18).



Settings

Define splitting

● Split into N lists

Number of lists to create: 2

○ Create lists with N sequences each

Number of sequences per list: 1000

○ Split based on attribute values

Attribute: -no selection-

Attribute values

□ Collect sequences without matches

Figure 27.18: *Sequence lists can be split into a set number of groups, or into lists containing particular numbers of sequences, or split based on attribute values.*

The options are:

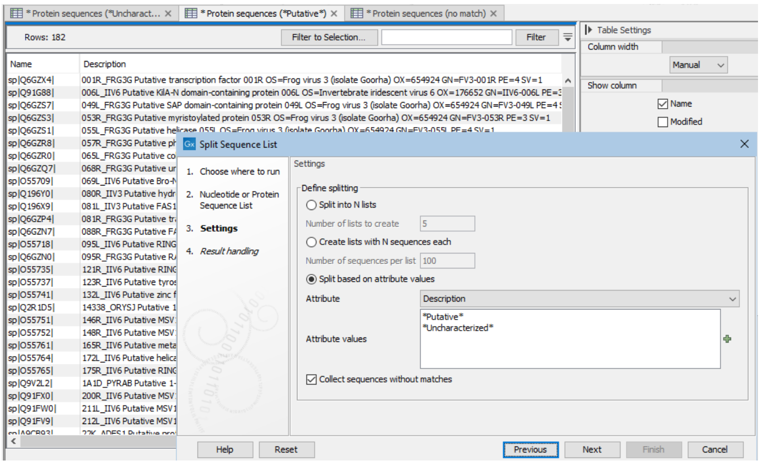
- **Split into N lists** In the "Number of lists to create" box, enter the number of lists to split the input into.
- **Create lists with N sequences each** In the "Number of sequences per list" box, enter the relevant number. The final sequence list in the set created may contain fewer than this number.
- **Split based on attribute values** Specify the attribute to split upon from the drop-down list. Columns in the table view of a sequence list equate to the attributes that the list can be split upon.

  If no information is entered into the "Attribute values" field, a sequence list is created for each unique value of the specified attribute. If values are provided, a sequence list is created for each of these where at least one sequence has that attribute value. For example, if 3 values are specified, and sequences were found with attributes matching each of these values, 3 sequence lists would be created. If no sequences were found containing 1 of those attribute values, then only 2 sequence lists would be created. Check the "Collect sequences without matches" box to additionally produce a sequence list containing the sequences where no match to a specified value was identified.

Attribute values should be added in a new-line separated list (figure 27.19).

Matching of values is case specific. An asterisk should be added before and/or after the term if the value is part of a longer entry (figure 27.19).

Attribute values are matched in the order they are listed. For example, if the attribute values shown in figure 27.19 were used, and a sequence had a description with both terms in it, that sequence would be placed in the list containing sequences with "Putative" in their descriptions. This is because *Putative* is listed first in the list of values provided to the tool.



Settings

Define splitting

○ Split into N lists

Number of lists to create: 5

○ Create lists with N sequences each

Number of sequences per list: 100

● Split based on attribute values

Attribute: Description

Attribute values:
Putative
Uncharacterized

☑ Collect sequences without matches

Figure 27.19: *With the settings shown here, 3 sequence lists were created. These lists are open in the background tabs shown. One contains sequences with descriptions that include the term "Putative", one contains sequences with descriptions that include the term "Uncharacterized", and one contains sequences containing neither term in the desccription.*

27.8 Rename Elements

Using **Rename Elements**, you can add or remove characters from element names. You can also replace parts of names, optionally using regular expressions to define the sections to replace.

Replacing spaces or other special characters in element names can be particularly useful before exporting to systems where filenames with those characters are problematic to work with.

Other methods of changing or controlling element names exist: Individual elements can be renamed directly in the Navigation Area by doing a slow double-click on the name, or selecting the element and choosing the menu item Edit | Rename. In workflows, the naming of outputs can be configured, as described in section 13.2.4.

To launch the **Rename Elements** tool, go to:

Tools | **Utility Tools** ( ) | **Renaming** ( ) |**Rename Elements** ( )

In the first wizard step, select the elements to rename (figure 27.20).

Alternatively, you can right-click and select "Add folder contents" or, select "Add folder contents (recursively)" to rename all elements in a folder structure recursively. (figure 27.21).

When multiple elements are supplied as input, each will be renamed. There is no need to check the Batch checkbox for this. However, if you wish to rename only certain elements from the full

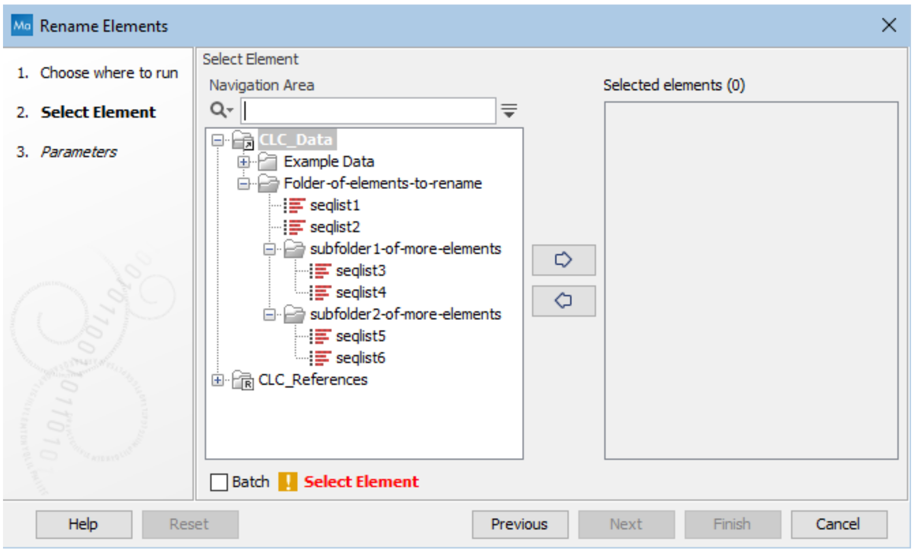


Figure 27.20: *Select the elements or folders to be renamed.*

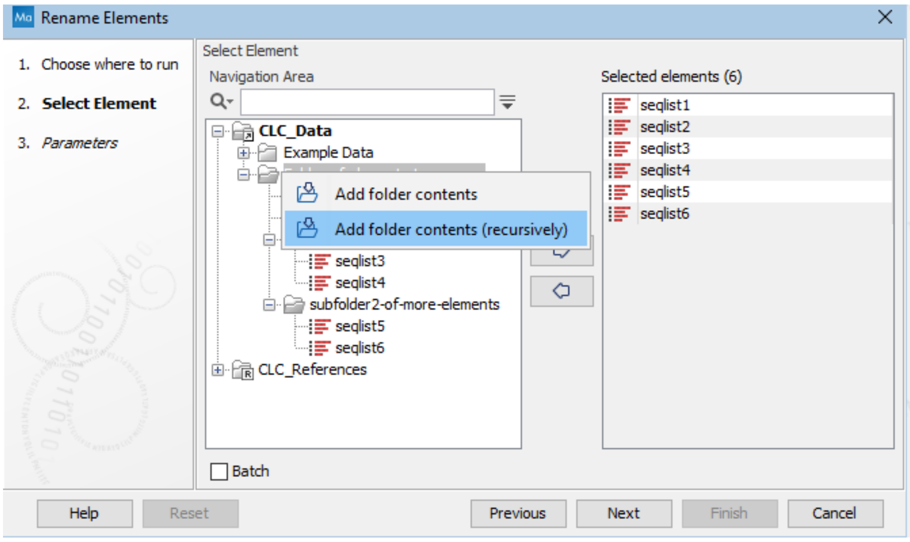


Figure 27.21: *Right-click for options to add the contents of folders as inputs. Here, the "Add folder contents (recursively)" option was selected. If "Add folder contents" had been selected, only the elements seqlist1 and seqlist2 would have been added to the Selected elements list on the right.*

selection, then checking the Batch box provides the opportunity to do that. When checked, the next wizard step shows the batch overview, where elements can be explicitly included or excluded from those to be renamed, based on text patterns. This step is described in more detail at section 11.3).

Checking the Batch checkbox for this tool also has the following effect when a folder is selected as input:

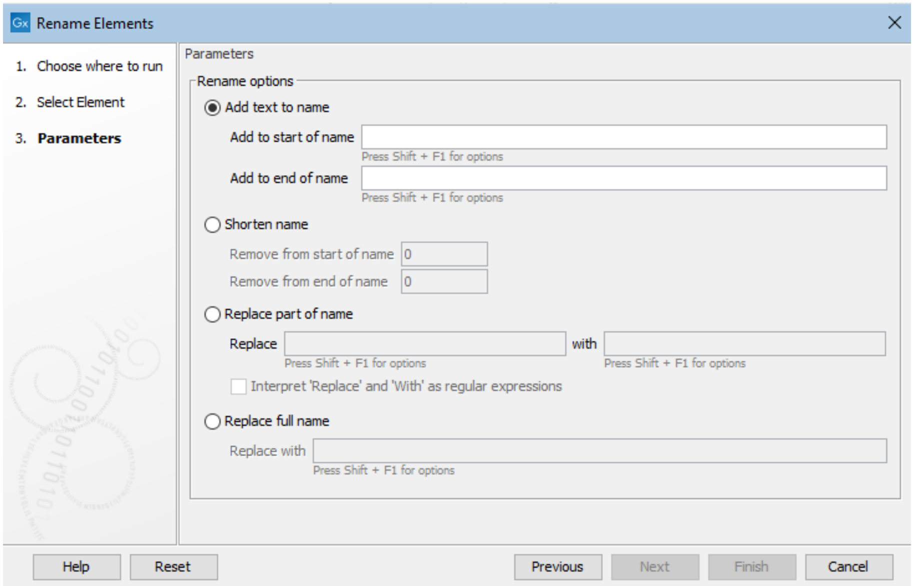
- With the Batch option checked, the top level contents of that folder will be renamed.
- With the Batch option unchecked, the folder itself will be renamed.

Important notes about renaming elements

- Renaming elements **cannot be undone**. To alter the names further, the elements must be renamed again.
- The renaming action is recorded in the **History** ( ) for the element, but the "Originates from" entries lists the changed element name, rather than the original element name.

Renaming options

This wizard step presents various options for the renaming action (figure 27.22). The **Rename Elements** is used for illustration in this section, but the options are the same for the **Rename Sequences in Lists** tool.



Parameters

Rename options

● Add text to name

Add to start of name

Press Shift + F1 for options

Add to end of name

Press Shift + F1 for options

○ Shorten name

Remove from start of name 0

Remove from end of name 0

○ Replace part of name

Replace with

Press Shift + F1 for options Press Shift + F1 for options

□ Interpret 'Replace' and 'With' as regular expressions

○ Replace full name

Replace with

Press Shift + F1 for options

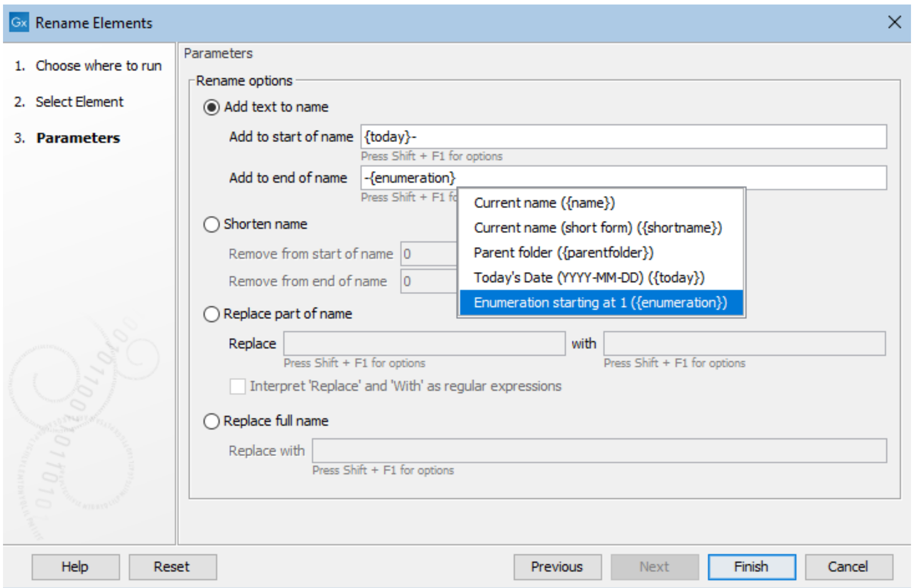
Figure 27.22: *Text can be added, removed or replaced in the existing names.*

- **Add text to name** Select this option to add text at the beginning or the end of the existing name.

  You can add text directly to these fields, and you can also include placeholders to indicate certain types of information should be added. Multiple placeholders can be used, in combination with other text if desired (figure 27.23). The available placeholders are:

  - `{name}` The current name of the element. Usually used when defining a new naming pattern for replacing the full name of elements.
  - `{shortname}` Truncates the original name to 10 characters. Usually used when replacing the full names of elements.
  - `{Parent folder}` The name of the folder containing the element.
  - `{today}` Today's date in the form YYYY-MM-DD
  - `{enumeration}` Adds a number to the name. This is intended for use when multiple elements are selected as input. Each is assigned a number, starting with the number 1, (added as 0000001) for the first element that was selected, 2 (added as 0000002) for the second element selected, and so on.

Click in a field and use Shift + F1 (Shift + Fn + F1 on Mac) to show the list of available placeholders, as shown in figure 27.23. Click on a placeholder in that list to have it entered into the field.



Rename Elements

1. Choose where to run
2. Select Element
3. **Parameters**

Parameters

Rename options

● Add text to name

Add to start of name: {today}-

Press Shift + F1 for options

Add to end of name: -{enumeration}

Press Shift + F1 for options

○ Shorten name

Remove from start of name: 0

Remove from end of name: 0

○ Replace part of name

Replace: with:

Press Shift + F1 for options

□ Interpret 'Replace' and 'With' as regular expressions

○ Replace full name

Replace with:

Press Shift + F1 for options

Current name ({name})
Current name (short form) ({shortname})
Parent folder ({parentfolder})
Today's Date (YYYY-MM-DD) ({today})
Enumeration starting at 1 ({enumeration})
Help
Reset
Previous
Next
Finish
Cancel

Figure 27.23: *Click on Shift+F1 (Shift + Fn + F1 on Mac) to reveal a drop-down list of placeholders that can be used. Here, today's date and a hypen would be prepended, and a hyphen and ascending numeric value appended, to the existing names.*

- **Shorten name** Select this option to shorten a name by removing a specified number of characters from the start and/or end of the name.

- **Replace part of name** Select this option to specify text or regular expressions to define parts of the element names to be replaced. By default, the text entered in the fields is interpreted literally. Check the "Interpret 'Replace' as regular expression" option to indicate that the terms provided in the "Replace" field should be treated as regular expressions. Information on regular expressions can be found at `https://docs.oracle.com/javase/tutorial/essential/regex/`.

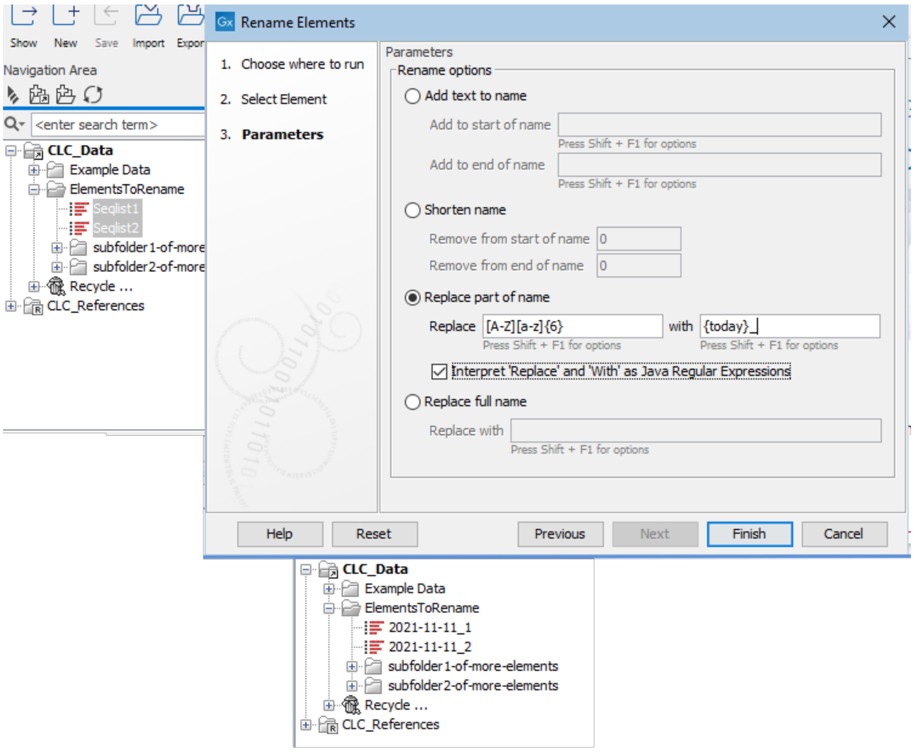
  By clicking in either the "Replace" or "with" field and pressing Shift + F1 (Shift + Fn + F1 on Mac), a drop down list of renaming possibilities is presented. The options listed for the Replace field are some commonly used regular expressions. Other standard regular expressions are also admissible in this field. The placeholders described above for adding text to names are available for use in the "with" field. **Note:** We recommend caution when using these placeholders in combination with regular expressions in the Replace field. Please run a small test to ensure it works as you intend.

- **Replace full name** Select this option to replace the full element name. Text and placeholders can be used in this field. The placeholders described above for adding text to names are available for use. Use Shift + F1 (Shift + Fn + F1 on Mac) to see a list.

Examples using regular expressions to replace parts of element names

The following are examples to illustrate using regular expressions for renaming. The same principles apply for renaming using the **Rename Elements** and **Rename Sequences in Lists** tools.

- Replacing part of an element's name with today's date and an underscore. Details are shown in figure 27.24.



Rename Elements

1. Choose where to run
2. Select Element
3. **Parameters**

Parameters

Rename options

○ Add text to name

Add to start of name

Add to end of name

○ Shorten name

Remove from start of name 0

Remove from end of name 0

● Replace part of name

Replace [A-Z][a-z]{6} with {today}_

☑ Interpret 'Replace' and 'With' as Java Regular Expressions

○ Replace full name

Replace with

Figure 27.24: *Elements with names Seqlist1 and Seqlist2 each start with a capital letter, followed by 6 small letters. Using the settings shown, their names are updated to be the date the renaming was done, followed by a hypen, and the remaining parts of the original name, here, the integer at the end of each name.*

- Rename using the first 4 non-whitespace characters from names that start with 2 characters, then have a space, then have multiple characters following, such as `1N R1\_0001`.
  - Check the box beside "Interpret 'Replace' and 'with' as Java regular expressions".
  - Enter `([\w]{2})\s([\w]{2}).*` into the "Replaces" field.
  - Enter `$1$2` into the "with" field.

- Keep only the last 4 characters of the name.
  - Check the box beside "Interpret 'Replace' and 'with' as Java regular expressions".
  - Enter `(.*)(.{4}$)` into the "Replaces" field.
  - Enter `$2` into the "with" field.

- Replace a set pattern of text with the name of the parent folder. Here, we start with the name `p140101034_1R_AMR` and replace the first letter and 9 numbers with the parent folder name.
  - Check the box beside "Interpret 'Replace' and 'with' as Java regular expressions".
  - Enter `([a-z]\d{9})(.*)` into the "Replaces" field.
  - Enter `{parentfolder}$2` into the "with" field.

- Rename using just the text between the first and second underscores in `1234_sample-code_5678`.
  - Check the box beside "Interpret 'Replace' and 'with' as Java regular expressions".
  - Enter `(^[^_]+)_([^_]+)_(.*)` into the "Replaces" field.
  - Enter `$2` into the "with" field.

27.9 Rename Sequences in Lists

Using **Rename Sequences in Lists**, you can add or remove characters from the names of sequences within sequence lists. You can also replace parts of sequence names, optionally using regular expressions to define the sections to replace.

Individual sequences in a sequence list can also be renamed manually. Open the sequence list, right-click on the sequence name to change, and select the "Rename Sequence..." option.

To launch the **Rename Sequences in Lists** tool, go to:

Tools | **Utility Tools** ( ) | **Renaming** ( ) | **Rename Sequences in Lists** ( )

In the first wizard step, select a sequence list. To rename sequences in multiple sequence lists using the same renaming pattern, check the Batch checkbox (figure 27.25).

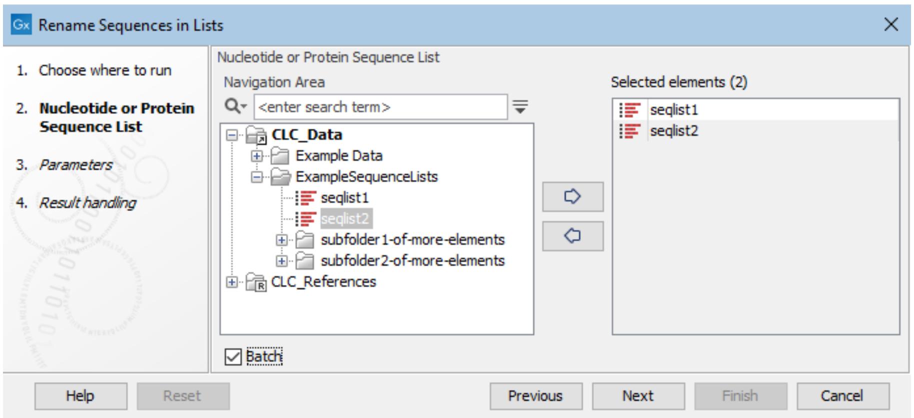


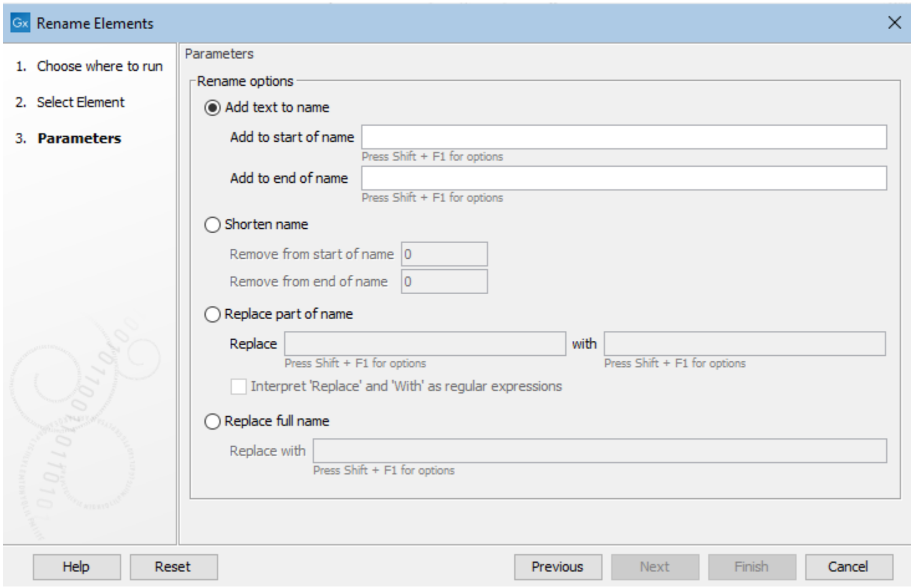
Figure 27.25: *When the sequences in more than one list should be renamed, check the Batch checkbox.*

The text "Renamed" is added within parentheses to the name of sequence lists output by this tool. E.g. with an input called "seqlist2", the sequence list containing the renamed sequences will be called "seqlist2 (Renamed)".

Renaming options

This wizard step presents various options for the renaming action (figure 27.26). The **Rename Elements** is used for illustration in this section, but the options are the same for the **Rename Sequences in Lists** tool.

- **Add text to name** Select this option to add text at the beginning or the end of the existing name.



Rename Elements

1. Choose where to run
2. Select Element
3. **Parameters**

Parameters

Rename options

● Add text to name

Add to start of name Press Shift + F1 for options

Add to end of name Press Shift + F1 for options

○ Shorten name

Remove from start of name 0

Remove from end of name 0

○ Replace part of name

Replace Press Shift + F1 for options with Press Shift + F1 for options

□ Interpret 'Replace' and 'With' as regular expressions

○ Replace full name

Replace with Press Shift + F1 for options

Figure 27.26: *Text can be added, removed or replaced in the existing names.*

You can add text directly to these fields, and you can also include placeholders to indicate certain types of information should be added. Multiple placeholders can be used, in combination with other text if desired (figure 27.27). The available placeholders are:

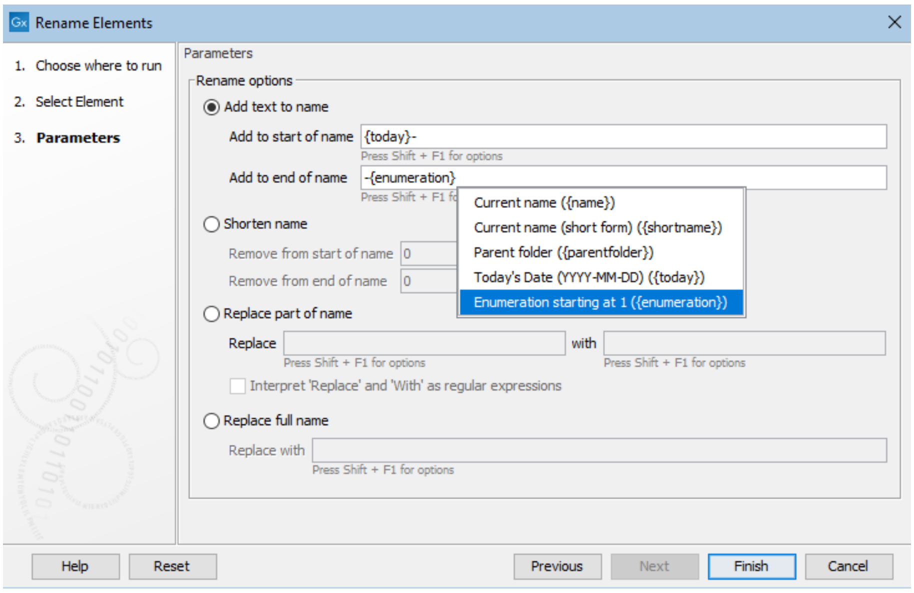
- `{name}` The current name of the element. Usually used when defining a new naming pattern for replacing the full name of elements.
- `{shortname}` Truncates the original name to 10 characters. Usually used when replacing the full names of elements.
- `{Parent folder}` The name of the folder containing the element.
- `{today}` Today's date in the form YYYY-MM-DD
- `{enumeration}` Adds a number to the name. This is intended for use when multiple elements are selected as input. Each is assigned a number, starting with the number 1, (added as 0000001) for the first element that was selected, 2 (added as 0000002) for the second element selected, and so on.

Click in a field and use Shift + F1 (Shift + Fn + F1 on Mac) to show the list of available placeholders, as shown in figure 27.27. Click on a placeholder in that list to have it entered into the field.

- **Shorten name** Select this option to shorten a name by removing a specified number of characters from the start and/or end of the name.

- **Replace part of name** Select this option to specify text or regular expressions to define parts of the element names to be replaced. By default, the text entered in the fields is interpreted literally. Check the "Interpret ’Replace’ as regular expression" option to indicate that the terms provided in the "Replace" field should be treated as regular expressions. Information on regular expressions can be found at `https://docs.oracle.com/javase/tutorial/essential/regex/`.

  By clicking in either the "Replace" or "with" field and pressing Shift + F1 (Shift + Fn + F1 on Mac), a drop down list of renaming possibilities is presented. The options listed for



Rename Elements

1. Choose where to run
2. Select Element
3. **Parameters**

Parameters

Rename options

● Add text to name

Add to start of name: {today}-

Add to end of name: -{enumeration}

- Current name ({name})
- Current name (short form) ({shortname})
- Parent folder ({parentfolder})
- Today's Date (YYYY-MM-DD) ({today})
- Enumeration starting at 1 ({enumeration})

○ Shorten name

Remove from start of name: 0

Remove from end of name: 0

○ Replace part of name

Replace: with:

□ Interpret 'Replace' and 'With' as regular expressions

○ Replace full name

Replace with:

Figure 27.27: *Click on Shift+F1 (Shift + Fn + F1 on Mac) to reveal a drop-down list of placeholders that can be used. Here, today's date and a hypen would be prepended, and a hyphen and ascending numeric value appended, to the existing names.*

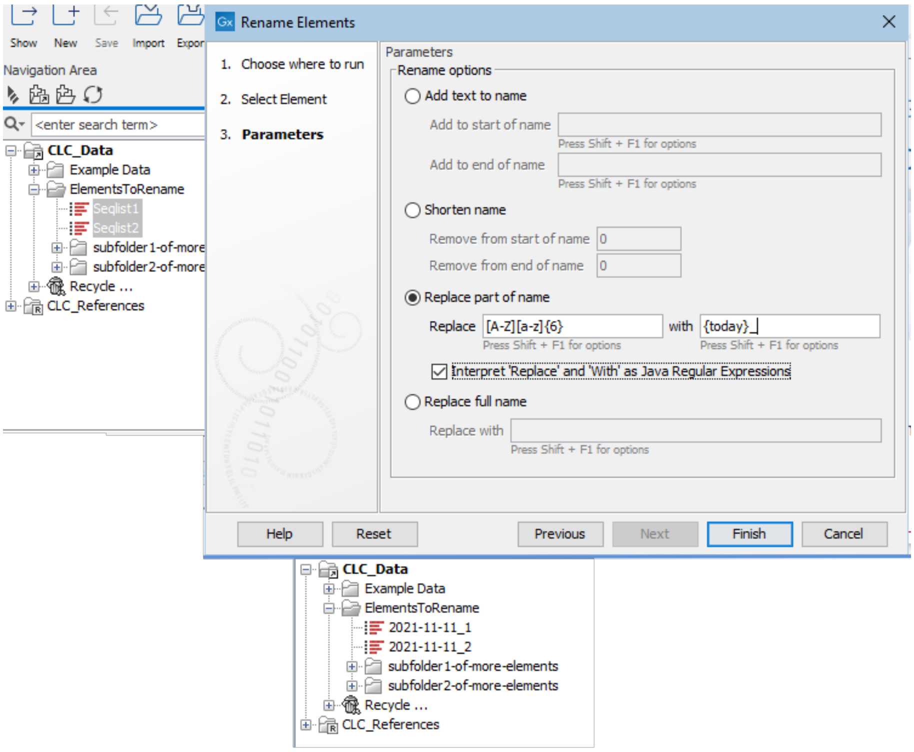
the Replace field are some commonly used regular expressions. Other standard regular expressions are also admissible in this field. The placeholders described above for adding text to names are available for use in the "with" field. **Note:** We recommend caution when using these placeholders in combination with regular expressions in the Replace field. Please run a small test to ensure it works as you intend.

- **Replace full name** Select this option to replace the full element name. Text and placeholders can be used in this field. The placeholders described above for adding text to names are available for use. Use Shift + F1 (Shift + Fn + F1 on Mac) to see a list.

Examples using regular expressions to replace parts of element names

The following are examples to illustrate using regular expressions for renaming. The same principles apply for renaming using the **Rename Elements** and **Rename Sequences in Lists** tools.

- Replacing part of an element's name with today's date and an underscore. Details are shown in figure 27.28.
- Rename using the first 4 non-whitespace characters from names that start with 2 characters, then have a space, then have multiple characters following, such as `1N R1\_0001`.
  - Check the box beside "Interpret 'Replace' and 'with' as Java regular expressions".
  - Enter `([\w]{2})\s([\w]{2}).*` into the "Replaces" field.
  - Enter `$1$2` into the "with" field.
- Keep only the last 4 characters of the name.
  - Check the box beside "Interpret 'Replace' and 'with' as Java regular expressions".



Parameters

Rename options

○ Add text to name

Add to start of name

Press Shift + F1 for options

Add to end of name

Press Shift + F1 for options

○ Shorten name

Remove from start of name 0

Remove from end of name 0

● Replace part of name

Replace [A-Z][a-z]{6} with {today}_

Press Shift + F1 for options Press Shift + F1 for options

☑ Interpret 'Replace' and 'With' as Java Regular Expressions

○ Replace full name

Replace with

Press Shift + F1 for options

Figure 27.28: *Elements with names Seqlist1 and Seqlist2 each start with a capital letter, followed by 6 small letters. Using the settings shown, their names are updated to be the date the renaming was done, followed by a hypen, and the remaining parts of the original name, here, the integer at the end of each name.*

- Enter `(.*)(.{4}$)` into the "Replaces" field.
- Enter `$2` into the "with" field.

- Replace a set pattern of text with the name of the parent folder. Here, we start with the name `p140101034_1R_AMR` and replace the first letter and 9 numbers with the parent folder name.
  - Check the box beside "Interpret 'Replace' and 'with' as Java regular expressions".
  - Enter `([a-z]\d{9})(.*)` into the "Replaces" field.
  - Enter `{parentfolder}$2` into the "with" field.

- Rename using just the text between the first and second underscores in `1234_sample-code_5678`.
  - Check the box beside "Interpret 'Replace' and 'with' as Java regular expressions".
  - Enter `(^[^_]+)_([^_]+)_(.*)` into the "Replaces" field.
  - Enter `$2` into the "with" field.

Part IV

Appendix

Appendix A

Graph preferences

This section explains the view settings of graphs. The **Graph preferences** at the top of the **Side Panel** includes the following settings:

- **Lock axes** This will always show the axes even though the plot is zoomed to a detailed level.
- **Frame** Shows a frame around the graph.
- **Show legends** Shows the data legends.
- **Tick type** Determine whether tick lines should be shown outside or inside the frame.
- **Tick lines at** Choosing Major ticks will show a grid behind the graph.
- **Horizontal axis range** Sets the range of the horizontal axis (x axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.
- **Vertical axis range** Sets the range of the vertical axis (y axis). Enter a value in **Min** and **Max**, and press Enter. This will update the view. If you wait a few seconds without pressing Enter, the view will also be updated.

Certain types of graphs can additionally contain the following settings:

- **X-axis at zero**. This will draw the x axis at y = 0. Note that the axis range will not be changed.
- **Y-axis at zero**. This will draw the y axis at x = 0. Note that the axis range will not be changed.
- **Show as histogram**. For some data-series it is possible to see the graph as a histogram rather than a line plot.

The representation of the data is configured in the bottom area, e.g. line widths, dot types, colors, etc. For graphs of multiple data series, the series to apply the settings to can be selected from a drop down list.

- **Dot type** Can be None, Cross, Plus, Square, Diamond, Circle, Triangle, Reverse triangle, or Dot.
- **Dot color** Click the color box to select a color.
- **Line width** Thin, Medium or Wide
- **Line type** None, Line, Long dash or Short dash
- **Line color** Click the color box to select a color.

The graph and axes titles can be edited simply by clicking with the mouse. These changes will be saved when you **Save** (⇤) the graph - whereas the changes in the **Side Panel** need to be saved explicitly (see section 4.6).

Appendix B

BLAST databases

Several databases are available at NCBI, which can be selected to narrow down the possible BLAST hits.

B.1 Peptide sequence databases

- **nr.** Non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF, excluding those in env_nr.
- **refseq.** Protein sequences from NCBI Reference Sequence project https://www.ncbi.nlm.nih.gov/RefSeq/.
- **swissprot.** Last major release of the SWISS-PROT protein sequence database (no incremental updates).
- **pat.** Proteins from the Patent division of GenBank.
- **pdb.** Sequences derived from the 3-dimensional structure records from the Protein Data Bank.
- **env_nr.** Non-redundant CDS translations from env_nt entries.
- **tsa_nr.** Transcriptome Shotgun Assembly db proteins. (NCBI BLAST only)
- **month.** All new or revised GenBank CDS translations + PDB + SwissProt + PIR + PRF released in the last 30 days. (Create Protein Report only)

B.2 Nucleotide sequence databases

- **nr.** All GenBank + EMBL + DDBJ + PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences). No longer "non-redundant" due to computational cost.
- **Human G+T.** Human genomic and transcript sequences
- **Mouse G+T.** Mouse genomic and transcript sequences
- **refseq_rna.** mRNA sequences from NCBI Reference Sequence Project.

- **refseq_genomic.** Genomic sequences from NCBI Reference Sequence Project.
- **refseq_representative_genomes.** Representative sequences from NCBI Reference Sequence Project.
- **est.** Database of GenBank + EMBL + DDBJ sequences from EST division.
- **est_human.** Human subset of est.
- **est_mouse.** Mouse subset of est.
- **est_others.** Subset of est other than human or mouse.
- **gss.** Genome Survey Sequence, includes single-pass genomic data, exon-trapped sequences, and Alu PCR sequences.
- **htgs.** Unfinished High Throughput Genomic Sequences: phases 0, 1 and 2. Finished, phase 3 HTG sequences are in nr.
- **pat.** Nucleotides from the Patent division of GenBank.
- **pdb.** Sequences derived from the 3-dimensional structure records from Protein Data Bank. They are NOT the coding sequences for the corresponding proteins found in the same PDB record.
- **alu.** Select Alu repeats from REPBASE, suitable for masking Alu repeats from query sequences. See "Alu alert" by Claverie and Makalowski, Nature 371: 752 (1994).
- **dbsts.** Database of Sequence Tag Site entries from the STS division of GenBank + EMBL + DDBJ.
- **chromosome.** Complete genomes and complete chromosomes from the NCBI Reference Sequence project. It overlaps with refseq_genomic.
- **env_nt.** Sequences from environmental samples, such as uncultured bacterial samples isolated from soil or marine samples. The largest single source is Sagarsso Sea project. This does overlap with nucleotide nr.
- **tsa_nt.** Transcriptome Shotgun Assembly database.
- **prokaryotic_16S_ribosomal_RNA.** 16S ribomsal RNA sequences.
- **Betacoronavirus.** NCBI database of betacoronavirus sequences.

B.3 Adding more databases

Besides the databases that are part of the default configuration, you can add more databases located at NCBI by configuring files in the Workbench installation directory.

The list of databases that can be added is here: https://web.archive.org/web/20120409025527/http://www.ncbi.nlm.nih.gov/staff/tao/URLAPI/remote_blastdblist.html.

In order to add a new database, find the `settings` folder in the Workbench installation directory (e.g. `C:\Program files\CLC Genomics Workbench 4`). Download unzip and place the following files in this directory to replace the built-in list of databases:

- Nucleotide databases: https://resources.qiagenbioinformatics.com/wbsettings/NCBI_BlastNucleotideDatabases.zip
- Protein databases: https://resources.qiagenbioinformatics.com/wbsettings/NCBI_BlastProteinDatabases.zip

Open the file you have downloaded into the settings folder, e.g. NCBI_BlastProteinDatabases.proper in a text editor and you will see the contents look like this:

```
nr[clcdefault] = Non-redundant protein sequences
refseq_protein = Reference proteins
swissprot = Swiss-Prot protein sequences
pat = Patented protein sequences
pdb = Protein Data Bank proteins
env_nr = Environmental samples
month = New or revised GenBank sequences
```

Simply add another database as a new line with the first item being the database name taken from https://web.archive.org/web/20120409025527/http://www.ncbi.nlm.nih.gov/staff/tao/URLAPI/remote_blastdblist.html and the second part is the name to display in the Workbench. Restart the Workbench, and the new database will be visible in the BLAST dialog.

Appendix C

Proteolytic cleavage enzymes

Most proteolytic enzymes cleave at distinct patterns. Below is a compiled list of proteolytic enzymes used in *CLC Main Workbench*.

Name	P4	P3	P2	P1	P1’	P2’
Cyanogen bromide (CNBr)	-	-	-	M	-	-
Asp-N endopeptidase	-	-	-	-	D	-
Arg-C	-	-	-	R	-	-
Lys-C	-	-	-	K	-	-
Trypsin	-	-	-	K, R	not P	-
Trypsin	-	-	W	K	P	-
Trypsin	-	-	M	R	P	-
Trypsin*	-	-	C, D	K	D	-
Trypsin*	-	-	C	K	H, Y	-
Trypsin*	-	-	C	R	K	-
Trypsin*	-	-	R	R	H,R	-
Chymotrypsin-high spec.	-	-	-	F, Y	not P	-
Chymotrypsin-high spec.	-	-	-	W	not M, P	-
Chymotrypsin-low spec.	-	-	-	F, L, Y	not P	-
Chymotrypsin-low spec.	-	-	-	W	not M, P	-
Chymotrypsin-low spec.	-	-	-	M	not P, Y	-
Chymotrypsin-low spec.	-	-	-	H	not D, M, P, W	-
o-Iodosobenzoate	-	-	-	W	-	-
Thermolysin	-	-	-	not D, E	A, F, I, L, M or V	-
Post-Pro	-	-	H, K, R	P	not P	-
Glu-C	-	-	-	E	-	-
Asp-N	-	-	-	-	D	-
Proteinase K	-	-	-	A, E, F, I, L, T, V, W, Y	-	-
Factor Xa	A, F, G, I, L, T, V, M	D,E	G	R	-	-
Granzyme B	I	E	P	D	-	-
Thrombin	-	-	G	R	G	-
Thrombin	A, F, G, I, L, T, V, M	A, F, G, I, L, T, V, W, A	P	R	not D, E	not D, E
TEV (Tobacco Etch Virus)	-	Y	-	Q	G, S	-

Appendix D

Restriction enzymes database configuration

CLC Main Workbench uses enzymes from the **REBASE** restriction enzyme database at http://rebase.neb.com. If you wish to add enzymes to this list, you can do this by manually using the procedure described here.

Note! Please be aware that this process needs to be handled carefully, otherwise you may have to re-install the Workbench to get it to work.

First, download the following file: https://resources.qiagenbioinformatics.com/wbsettings/link_emboss_e_custom. In the Workbench installation folder under `settings`, create a folder named `rebase` and place the extracted `link_emboss_e_custom` file here.

Note that in macOS, the extension file "link_emboss_e_custom" will have a ".txt" extension in its filename and metadata that needs to be removed. Right click the file name, choose "Get info" and remove ".txt" from the "Name & extension" field.

Open the file in a text editor. The top of the file contains information about the format, and at the bottom there are two example enzymes that you should replace with your own.

Please note that the CLC Workbenches only support the addition of 2-cutter enzymes. Further details about how to format your entries accordingly are given within the file mentioned above.

After adding the above file, or making changes to it, you must restart the Workbench for changes take effect.

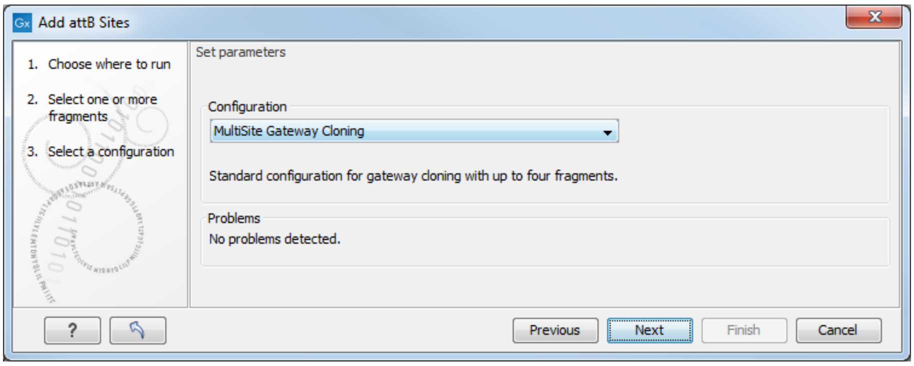
Appendix E

Technical information about modifying Gateway cloning sites

The *CLC Main Workbench* comes with a pre-defined list of Gateway recombination sites. These sites and the recombination logics can be modified by downloading and editing a properties file. Note that this is a technical procedure only needed if the built-in functionality is not sufficient for your needs.

The properties file can be downloaded from https://resources.qiagenbioinformatics.com/wbsettings/gatewaycloning.zip. Extract the file included in the zip archive and save it in the `settings` folder of the Workbench installation folder. The file you download contains the standard configuration. You should thus update the file to match your specific needs. See the comments in the file for more information.

The name of the properties file you download is `gatewaycloning.1.properties`. You can add several files with different configurations by giving them a different number, e.g. `gatewaycloning.2.properties` and so forth. When using the Gateway tools in the Workbench, you will be asked which configuration you want to use (see figure E.1).


Add attB Sites

1. Choose where to run
2. Select one or more fragments
3. Select a configuration

Set parameters

Configuration

MultiSite Gateway Cloning

Standard configuration for gateway cloning with up to four fragments.

Problems

No problems detected.

Figure E.1: *Selecting between different gateway cloning configurations.*

Appendix F

IUPAC codes for amino acids

(Single-letter codes based on International Union of Pure and Applied Chemistry)

The information is gathered from: https://www.insdc.org/documents/feature_table.html

One-letter abbreviation	Three-letter abbreviation	Description
A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartic acid
C	Cys	Cysteine
Q	Gln	Glutamine
E	Glu	Glutamic acid
G	Gly	Glycine
H	His	Histidine
J	Xle	Leucine or Isoleucineucine
L	Leu	Leucine
I	ILe	Isoleucine
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
O	Pyl	Pyrrolysine
U	Sec	Selenocysteine
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine
B	Asx	Aspartic acid or Asparagine Asparagine
Z	Glx	Glutamic acid or Glutamine Glutamine
X	Xaa	Any amino acid

Appendix G

IUPAC codes for nucleotides

(Single-letter codes based on International Union of Pure and Applied Chemistry)

The information is gathered from: `https://www.iupac.org` and `https://www.insdc.org/documents/feature_table.html`.

Code	Description
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil
R	Purine (A or G)
Y	Pyrimidine (C, T, or U)
M	C or A
K	T, U, or G
W	T, U, or A
S	C or G
B	C, T, U, or G (not A)
D	A, T, U, or G (not C)
H	A, T, U, or C (not G)
V	A, C, or G (not T, not U)
N	Any base (A, C, G, T, or U)

Appendix H

Formats for import and export

H.1 List of bioinformatic data formats

Below is a list of bioinformatic data formats, i.e. formats for importing and exporting molecule structures, sequences, alignments, trees, etc.

H.1.1 Sequence data formats

Sequence data formats that can be imported using **Standard Import** are listed in the table below.

File type	Suffix	Import	Export	Description
AB1	.ab1	X		See notes below
ABI	.abi	X		See notes below
CLC	.clc	X	X	Rich format including all information
Clone manager	.cm5	X		Clone manager sequence format
DNAstrider	.str/.strider	X	X	
DS Gene	.bsml	X		
EMBL	.emb/.embl	X	X	Rich information incl. annotations (nucs only)
FASTA	.fa/.fsa/.fasta	X	X	Simple format, name & description
GenBank	.gbk/.gb/.gp/.gbff	X	X	Rich information incl. annotations
Gene Construction Kit	.gck	X		
Lasergene	.pro/.seq	X		
Nexus	.nxs/.nexus	X	X	
Phred	.phd	X		See notes below
PIR (NBRF)	.pir	X	X	Simple format, name & description
Raw sequence	any	X		Only sequence (no name)
SCF2	.scf	X		See notes below
SCF3	.scf	X	X	See notes below
Sequence Comma separated values	.csv	X	X	Simple format. One seq per line: name, description(optional), sequence
Staden	.sdn	X		
Swiss-Prot	.swp	X	X	Rich information incl. annotations (only peptides)
Tab delimited text	.txt		X	Annotations in tab delimited text format

Additional notes about working with trace data

- When importing trace data, the called bases in the file are imported and the chromatogram information associated with the called bases is imported. If the base calls within the file have already been trimmed, the part of the chromatogram not associated with base calls will not be imported.
- The **Trim Sequences** tool, described in section 21.2, adds annotations to trimmed regions. When exporting to fasta format, there is an option to remove sequence ends covered by Trim annotations.

H.1.2 Contig formats

File type	Suffix	Import	Export	Description
ACE	.ace	X	X	No chromatogram or quality score
CLC	.clc	X	X	Rich format including all information

H.1.3 Alignment formats

File type	Suffix	Import	Export	Description
Aligned fasta	.fa/.fsa/.fasta	X	X	Simple fasta-based format with – for gaps
CLC	.clc	X	X	Rich format including all information
ClustalW	.aln	X	X	
GCG Alignment	.msf	X	X	
Nexus	.nxs/.nexus	X	X	
Phylip Alignment	.phy	X	X	

H.1.4 Tree formats

File type	Suffix	Import	Export	Description
CLC	.clc	X	X	Rich format including all information
Newick	.nwk	X	X	
Nexus	.nxs/.nexus	X	X	

H.1.5 Expression data formats

File type	Suffix	Import	Export	Description
Affymetrix CHP	.chp/.psi	X		Expression values and annotations, see section I.2.
Affymetrix pivot/metric	.txt/.csv	X		Gene-level expression value, see section I.2.
Affymetrix NetAffx	.csv	X		Annotations, see section I.2.
CLC	.clc	X	X	Rich format including all information
Excel	.xls/.xlsx		X	All tables and reports
Generic	.txt/.csv	X		Expression values
Generic	.txt/.csv	X		Annotations
GEO sample/series	.txt/.csv	X		Expression values, see section I.1
Illumina	.txt	X		Expression values and annotations, see section I.3
Table CSV	.csv		X	Samples and experiments, see section I.5
Tab delimited	.txt	X	X	Samples and experiments, see section I.5

H.1.6 Other formats

File type	Suffix	Import	Export	Description
CLC	.clc	X	X	Rich format including all information
PDB	.pdb	X		3D structure
RNA structures	.ct, .col, .rnaml/.xml	x		Secondary structure for RNA

H.1.7 Table and text formats

File type	Suffix	Import	Export	Description
Excel	.xls/.xlsx	X	X	All tables and reports
Table CSV	.csv	X	X	All tables
Tab delimited	.tsv	X		All tables
Tab delimited	.txt		X	All tables
Text	.txt	X	X	All data in a textual format
CLC	.clc	X	X	Rich format including all information
HTML	.html		X	All tables
PDF	.pdf		X	Export reports in Portable Document Format

Please see section H.1.5 for special cases of table imports.

H.1.8 File compression formats

File type	Suffix	Import	Export	Description
Zip export	.zip		X	Selected files in CLC format
Zip import	.zip/.gz/.tar	X		Contained files/folder structure (.tar and .zip not supported for NGS data)

Note: It is possible to import 'external' files into the *CLC Main Workbench* and view these in the **Navigation Area**, but it is only the above mentioned formats whose *contents* can be shown in the *CLC Main Workbench*.

H.2 List of graphics data formats

Below is a list of formats for exporting graphics. All data displayed in a graphical format can be exported using these formats. Data represented in lists and tables can only be exported in .pdf format (see section 8.2 for further details).

Format	Suffix	Type
Portable Network Graphics	.png	bitmap
JPEG	.jpg	bitmap
Tagged Image File	.tif	bitmap
PostScript	.ps	vector graphics
Encapsulated PostScript	.eps	vector graphics
Portable Document Format	.pdf	vector graphics
Scalable Vector Graphics	.svg	vector graphics

Appendix I

Gene expression annotation files and microarray data formats

The *CLC Main Workbench* supports analysis of one-color expression arrays. These may be imported from GEO soft sample- or series- file formats, or for Affymetrix arrays, tab-delimited pivot or metrics files, or from Illumina expression files. Expression array data from other platforms may be imported from tab, semi-colon or comma separated files containing the expression feature IDs and levels in a tabular format (see internalrefsec:customexpressiondataformatssectionGeneric expression and annotation data file formats).

The *CLC Main Workbench* assumes that expression values are given at the gene level, thus probe-level analysis of Affymetrix GeneChips and import of Affymetrix CEL and CDF files is currently not supported. However, the *CLC Main Workbench* allows import of txt files exported from R containing processed Affymetrix CEL-file data (see internalrefsec:AffymetrixGeneChipFormatssectionAffymetrix GeneChip).

Affymetrix NetAffx annotation files for expression GeneChips in csv format and Illumina annotation files can also be imported.

Also, you may import your own annotation data in tabular format (see internalrefsec:customexpressiondataforma expression and annotation data file formats).

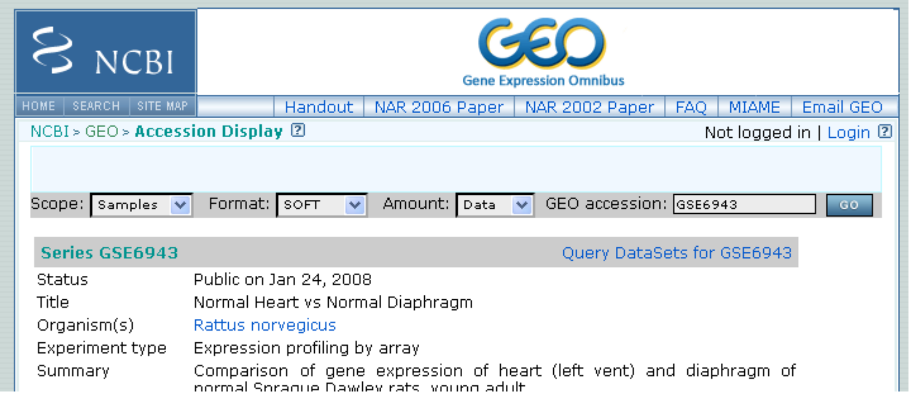
Below you find descriptions of the microarray data formats that are supported by *CLC Main Workbench*. Note that we for some platforms support both expression data and annotation data.

I.1 GEO (Gene Expression Omnibus)

The GEO (Gene Expression Omnibus) sample and series formats are supported. Figure I.1 shows how to download the data from GEO in the right format. GEO is located at https://www.ncbi.nlm.nih.gov/geo/.

The GEO sample files are tab-delimited .txt files. They have three required lines:

```
^SAMPLE = GSM21610
!sample_table_begin
...
!sample_table_end
```


NCBI
GEO
Gene Expression Omnibus
HOME | SEARCH | SITE MAP
Handout | NAR 2006 Paper | NAR 2002 Paper | FAQ | MIAME | Email GEO
NCBI > GEO > Accession Display
Not logged in | Login
GO
Query DataSets for GSE6943

Scope: Samples Format: SOFT Amount: Data GEO accession: GSE6943

Series GSE6943

Status	Public on Jan 24, 2008
Title	Normal Heart vs Normal Diaphragm
Organism(s)	Rattus norvegicus
Experiment type	Expression profiling by array
Summary	Comparison of gene expression of heart (left vent) and diaphragm of normal Sprague Dawley rats, young adult

Figure I.1: *Selecting Samples, SOFT and Data before clicking go will give you the format supported by the* **CLC Main Workbench***.*

The first line should start with `^SAMPLE =` followed by the sample name, the line `!sample_table_begin` and the line `!sample_table_end`. Between the `!sample_table_begin` and `!sample_table_end`, lines are the column contents of the sample.

Note that GEO sample importer will also work for concatenated GEO sample files — allowing multiple samples to be imported in one go. Download a sample file containing concatenated sample files here:
https://resources.qiagenbioinformatics.com/madata/GEOSampleFilesConcatenated.txt

Below you can find examples of the formatting of the GEO formats.

GEO sample file, simple

This format is very simple and includes two columns: one for feature id (e.g. gene name) and one for the expression value.

```
^SAMPLE = GSM21610
!sample_table_begin
ID_REF    VALUE
id1       105.8
id2       32
id3       50.4
id4       57.8
id5       2914.1
!sample_table_end
```

Download the sample file here:
https://resources.qiagenbioinformatics.com/madata/GEOSampleFileSimple.txt

GEO sample file, including present/absent calls

This format includes an extra column for absent/present calls that can also be imported.

```
^SAMPLE = GSM21610
```

```
!sample_table_begin
ID_REF    VALUE    ABS_CALL
id1       105.8    M
id2       32       A
id3       50.4     A
id4       57.8     A
id5       2914.1   P
!sample_table_end
```

Download the sample file here:
https://resources.qiagenbioinformatics.com/madata/GEOSampleFileAbsentPresent.txt

GEO sample file, including present/absent calls and p-values

This format includes two extra columns: one for absent/present calls and one for absent/present call p-values, that can also be imported.

```
^SAMPLE = GSM21610
!sample_table_begin
ID_REF     VALUE     ABS_CALL     DETECTION P-VALUE
id1        105.8     M            0.00227496
id2        32        A            0.354441
id3        50.4      A            0.904352
id4        57.8      A            0.937071
id5        2914.1    P            6.02111e-05
!sample_table_end
```

Download the sample file here:
https://resources.qiagenbioinformatics.com/madata/GEOSampleFileAbsentPresentCall
txt

GEO sample file: using absent/present call and p-value columns for sequence information

The *CLC Main Workbench* assumes that if there is a third column in the GEO sample file then it contains present/absent calls and that if there is a fourth column then it contains p-values for these calls. This means that the contents of the third column is assumed to be text and that of the fourth column a number. As long as these two basic requirements are met, the sample should be recognized and interpreted correctly.

You can thus use these two columns to carry additional information on your probes. The absent/present column can be used to carry additional information like e.g. sequence tags as shown below:

```
^SAMPLE = GSM21610
!sample_table_begin
ID_REF      VALUE     ABS_CALL
id1        105.8      AAA
id2        32         AAC
```

```
id3         50.4        ATA
id4         57.8        ATT
id5         2914.1      TTA
!sample_table_end
```

Download the sample file here:
https://resources.qiagenbioinformatics.com/madata/GEOSampleFileSimpleSequenceTag
txt

Or, if you have multiple probes per sequence you could use the present/absent column to hold the sequence name and the p-value column to hold the interrogation position of your probes:

```
^SAMPLE = GSM21610
!sample_table_begin
ID_REF     VALUE     ABS_CALL     DETECTION P-VALUE
probe1     755.07    seq1         1452
probe2     587.88    seq1         497
probe3     716.29    seq1         1447
probe4     1287.18   seq2         1899
!sample_table_end
```

Download the sample file here: https://resources.qiagenbioinformatics.com/madata/GEOSampleFileSimpleSequenceTagAndProbe.txt

GEO series file, simple

The series file includes expression values for multiple samples. Each of the samples in the file will be represented by its own element with the sample name. The first row lists the sample names.

```
!Series_title "Myb specificity determinants"
!series_matrix_table_begin
"ID_REF" "GSM21610" "GSM21611" "GSM21612"
"id1"       2541       1781.8     1804.8
"id2"       11.3       621.5     50.2
"id3"       61.2       149.1     22
"id4"       55.3       328.8     97.2
"id5"         183.8        378.3      423.2
!series_matrix_table_end
```

Download the sample file here: https://resources.qiagenbioinformatics.com/madata/GEOSeriesFile.txt

I.2 Affymetrix GeneChip

For Affymetrix, three types of files are currently supported: Affymetrix .CHP files, Affymetrix NetAffx annotation files and tab-delimited pivot or metrics files. Affymetrix .CEL files are currently not supported. However, the Bioconductor R package 'affy' allows you to preprocess the .CEL files and export a txt file containing a table of estimated probe-level log-transformed expression values in three lines of code:

```
library(affy) # loading Bioconductor library 'affy'
data=ReadAffy() # probe-level data import
eset=rma(data) # probe-level data pre-processing using 'rma'
write.exprs(eset,file="evals.txt") # writing log2 expression levels to 'evals.txt'
```

The exported txt file (evals.txt) can be imported into the *CLC Main Workbench* using the Generic expression data table format importer (see internalrefsec:customexpressiondataformatssectionGeneric expression and annotation data file formats; you can just 'drag-and-drop' it in). In R, you should have all the CEL files you wish to process in your working directory and the file 'evals.txt' will be written to that directory.

If multiple probes are present for the same gene, further processing may be required to merge them into a single gene-level expression.

Affymetrix CHP expression files

The Affymetrix scanner software produces a number of files when a GeneChip is scanned. Two of these are the .CHP and the .CEL files. These are binary files with native Affymetrix formats. The Affymetrix GeneChips contain a number of probes for each gene (typically between 22 and 40). The .CEL file contains the probe-level intensities, and the .CHP file contains the gene-level information. The gene-level information has been obtained by the scanner software through postprocessing and summarization of the probe-level intensities.

In order to interpret the probe-level information in the .CEL file, the .CDF file for the type of GeneChip that was used is required. Similarly for the .CHP file: in order to interpret the gene-level information in the .CHP file, the .PSI file for the type of GeneChip that was used is required.

In order to import a .CHP file it is required that the corresponding .PSI file is present in the same folder as the .CHP file you want to import, and furthermore, this must be the only .PSI file that is present there. There are no requirements for the name of the .PSI file. Note that the .PSI file itself will not be imported - it is only used to guide the import of the .CHP file which contains the expression values.

Download example .CHP and .PSI files here (note that these are binary files):
https://resources.qiagenbioinformatics.com/madata/AffymetrixCHPandPSI.zip

Affymetrix metrix files

The Affymetrix metrics or pivot files are tab-delimited files that may be exported from the Affymetrix scanner software. The metrics files have a lot of technical information that is only partly used in the *CLC Main Workbench*. The feature ids (Probe Set Name), expression values (Used Signal), absent/present call (Detection) and absent/present p-value (Detection p-value) are imported into the *CLC Main Workbench*.

Download a small example sample file here:
https://resources.qiagenbioinformatics.com/madata/AffymetrixMetrics.txt

Affymetrix NetAffx annotation files

The NetAffx annotation files for Whole-Transcript Expression Gene arrays and 3' IVT Expression Analysis Arrays can be imported and used to annotate experiments as shown in section 25.1.3.

Download a small example annotation file here which includes header information:
https://resources.qiagenbioinformatics.com/madata/AffymetrixNetAffxAnnotationFil
csv

I.3 Illumina BeadChip

Both BeadChip expression data files from Illumina's BeadStudio software and the corresponding BeadChip annotation files are supported by *CLC Main Workbench*. The formats of the BeadStudio and annotation files have changed somewhat over time and various formats are supported.

Illumina expression data, compact format

An example of this format is shown below:

```
TargetID          AVG_Signal        BEAD_STDEV       Detection
GI_10047089-S     112.5             4.2              0.16903226
GI_10047091-S     127.6             4.8              0.76774194
```

All this information is imported into the *CLC Main Workbench*. The `AVG_Signal` is used as the expression measure.

Download a small sample file here:
https://resources.qiagenbioinformatics.com/madata/IlluminaBeadChipCompact.txt

Illumina expression data, extended format

An example of this format is shown below:

```
TargetID        MIN_Signal  AVG_Signal  MAX_Signal  NARRAYS  ARRAY_STDEV  BEAD_STDEV  Avg_NBEADS  Detection
GI_10047089-S   73.7        73.7        73.7        1        NaN          3.4         53          0.05669084
GI_10047091-S   312.7       312.7       312.7       1        NaN          11.1        50          0.99604483
```

All this information is imported into the *CLC Main Workbench*. The `AVG_Signal` is used as the expression measure.

Download a small sample file here:
https://resources.qiagenbioinformatics.com/madata/IlluminaBeadChipExtended.txt

Illumina expression data, with annotations

An example of this format is shown below:

```
TargetID Accession Symbol Definition Synonym Signal-BG02 DCp32  Detection-BG02 DCp32
GI_10047089-S NM_014332.1 SMPX "Homo sapiens small muscle protein, X-linked (SMPX), mRNA."  -17.6  0.03559657
GI_10047091-S NM_013259.1 NP25 "Homo sapiens neuronal protein (NP25), mRNA." NP22  32.6  0.99604483
GI_10047093-S NM_016299.1 HSP70-4 "Homo sapiens likely ortholog of mouse heat shock protein, 70 kDa 4 (HSP70-4), mRNA."  228.1 1
```

Only the TargetID, Signal and Detection columns will be imported, the remaining columns will be ignored. This means that the annotations are not imported. The `Signal` is used as the expression measure.

Download a small example sample file here:
https://resources.qiagenbioinformatics.com/madata/IlluminaBeadStudioWithAnnotati
txt

Illumina expression data, multiple samples in one file

This file format has too much information to show it inline in the text. You can download a small example sample file here:
https://resources.qiagenbioinformatics.com/madata/IlluminaBeadStudioMultipleSamp
txt

This file contains data for 18 samples. Each sample has an expression value (the value in the `AVG_Signal` column), a detection p-value, a bead standard deviation and an average bead number column. The *CLC Main Workbench* recognizes the 18 samples and their columns.

Illumina annotation files

The *CLC Main Workbench* supports import of two types of Illumina BeadChip annotation files. These are either comma-separated or tab-delimited .txt files. They can be used to annotate experiments as shown in section 25.1.3.

This file format has too much information to show it inline in the text.

Download a small example annotation file of the first type here:
https://resources.qiagenbioinformatics.com/madata/IlluminaBeadChipAnnotation.txt

I.4 Gene ontology annotation files

The Gene ontology web site provides annotation files for a variety of species which can all be downloaded and imported into the *CLC Main Workbench*. They can be used to annotate experiments as shown in section 25.1.3. They can also be used with the Gene Set Test and Create Expression Browser tools.

Import GO annotation file using the Standard Import tool. For GO annotation files in GAF format, use the option "Force import as type: Gene Ontology Annotation file" from the drop down menu at the bottom of the Standard Import dialog.

See the list of available files at https://current.geneontology.org/products/pages/downloads.html.

I.5 Generic expression and annotation data file formats

If you have your expression or annotation data in Excel and can export the data as a txt file, or if you are able to do some scripting or other manipulations to format your data files, you will be able to import them into the *CLC Main Workbench* as a 'generic' expression or annotation data file. There are a few simple requirements that need to be fulfilled to do this as described below.

Generic expression data table format

The *CLC Main Workbench* will import a tab, semicolon or comma-separated .txt or .csv file as expression array samples if the following requirements are met:

1. the first non-empty line of the file contains text. All entries, except the first, will be used as sample names
2. the following (non-empty) lines contain the same number of entries as the first non-empty line. The requirements to these are that the first entry should be a string (this will be used as the feature ID) and the remaining entries should contain numbers (which will be used as expression values — one per sample). Empty entries are not allowed, but NaN values are allowed.
3. the file contains at least two samples.

An example of this format is shown below:

```
FeatureID;sample1;sample2;sample3
gene1;200;300;23
gene2;210;30;238
gene3;230;50;23
gene4;50;100;235
gene5;200;300;23
gene6;210;30;238
gene7;230;50;23
gene8;50;100;235
```

This will be imported as three samples with eight genes in each sample.

Download this example as a file here:
https://resources.qiagenbioinformatics.com/madata/CustomExpressionData.txt

Generic annotation file for expression data format

The *CLC Main Workbench* will import a tab, semicolon or comma-separated .txt or .csv file as an annotation file if the following is met:

1. It has a line which can serve as a valid header line. In order to do this, the line should have a number of headers where at least two are among the valid column headers in the **Column header** column below.
2. It contains one of the `PROBE_ID` headers (that is: 'Probe Set ID', 'Feature ID', 'ProbeID' or 'Probe_Id').

The importer will import an annotation table with a column for each of the valid column headers (those in the **Column header** column below). Columns with invalid headers will be ignored.

Note that some column headers are alternatives so that only one of the alternative columns headers should be used.

When adding annotations to an experiment, you can specify the column in your annotation file containing the relevant identifiers. These identifiers are matched to the feature ids already present in your experiment. When a match is found, the annotation is added to that entry in the experiment. In other words, at least one column in your annotation file must contain identfiers matching the feature identifiers in the experiment, for those annotations to be applied.

A simple example of an annotation file is shown here:

```
"Probe Set ID","Gene Symbol","Gene Ontology Biological Process"
"1367452_at","Sumo2","0006464 // protein modification process //  not recorded"
"1367453_at","Cdc37","0051726 // regulation of cell cycle //  not recorded"
"1367454_at","Copb2","0006810 // transport //  ///  0016044 // membrane organization // "
```

Download this example plus a more elaborate one here:
https://resources.qiagenbioinformatics.com/madata/SimpleCustomAnnotation.csv
https://resources.qiagenbioinformatics.com/madata/FullCustomAnnotation.csv

To meet requirements imposed by special functionalities in the *CLC Main Workbench*, there are a number of further restrictions on the contents in the entries of the columns:

Download sequence functionality In the experiment table, you can click a button to download sequence. This uses the contents of the PUBLIC_ID column, so this column must be present for the action to work and should contain the NCBI accession number.

Annotation tests The annotation tests can make use of several entries in a column as long as a certain format is used. The tests assume that entries are separated by /// and it interprets all that appears before // as the actual entry and all that appears after // within an entry as comments. Example:

```
/// 0000001 //  comment1  /// 0000008 // comment2 /// 0003746 //  comment3
```

The annotation tests will interpret this as three entries (0000001, 0000008, and 0003746) with the according comments.

The most common column headers are summarized below:

Column header in imported file (alternatives separated by commas)	Label in experiment table	Description (tool tip)
Probe Set ID, Feature ID, ProbeID, Probe_Id, transcript_cluster_id	Feature ID	Probe identifier tag
Representative Public ID, Public identifier tag, GenbankAccession	Public identifier tag	Representative public ID
Gene Symbol, GeneSymbol	Gene symbol	Gene symbol
Gene Ontology Biological Process, Ontology_Process, GO_biological_process	GO biological process	Gene Ontology biological process
Gene Ontology Cellular Component, Ontology_Component, GO_cellular_component	GO cellular component	Gene Ontology cellular component
Gene Ontology Molecular Function, Ontology_Function, GO_molecular_function	GO molecular function	Gene Ontology molecular function
Pathway	Pathway	Pathway

The full list of possible column headers:

Column header in imported file (alternatives separated by commas)	Label in experiment table	Description (tool tip)
Species Scientific Name, Species Name, Species	Species name	Scientific species name
GeneChip Array	Gene chip array	Gene Chip Array name
Annotation Date	Annotation date	Date of annotation
Sequence Type	Sequence type	Type of sequence
Sequence Source	Sequence source	Source from which sequence was obtained
Transcript ID(Array Design), Transcript	Transcript ID	Transcript identifier tag
Target Description	Target description	Target description
Archival UniGene Cluster	Archival UniGene cluster	Archival UniGene cluster
UniGene ID, UniGeneID, Unigene_ID, unigene	UniGene ID	UniGene identifier tag
Genome Version	Genome version	Version of genome on which annotation is based
Alignments	Alignments	Alignments
Gene Title	Gene title	Gene title
geng_assignments	Gene assignments	Gene assignments
Chromosomal Location	Chromosomal location	Chromosomal location
Unigene Cluster Type	UniGene cluster type	UniGene cluster type
Ensemble Ensembl	Ensembl	
Entrez Gene, EntrezGeneID, Entrez_Gene_ID	Entrez gene	Entrez gene
SwissProt	SwissProt	SwissProt
EC	EC	EC
OMIM	OMIM	Online Mendelian Inheritance in Man
RefSeq Protein ID	RefSeq protein ID	RefSeq protein identifier tag
RefSeq Transcript ID	RefSeq transcript ID	RefSeq transcript identifier tag
FlyBase	FlyBase	FlyBase
AGI	AGI	AGI
WormBase	WormBase	WormBase
MGI Name	MGI name	MGI name
RGD Name	RGD name	RGD name
SGD accession number	SGD accession number	SGD accession number
InterPro	InterPro	InterPro
Trans Membrane	Trans membrane	Trans membrane
QTL	QTL	QTL
Annotation Description	Annotation description	Annotation description
Annotation Transcript Cluster	Annotation transcript cluster	Annotation transcript cluster
Transcript Assignments	Transcript assignments	Trancript assignments
mrna_assignments	mRNA assignments	mRNA assignments
Annotation Notes	Annotation notes	Annotation notes
GO, Ontology	Go annotations	Go annotations
Cytoband	Cytoband	Cytoband
PrimaryAccession	Primary accession	Primary accession
RefSeqAccession	RefSeq accession	RefSeq accession
GeneName	Gene name	Gene name
TIGRID	TIGR Id	TIGR Id
Description	Description	Description
GenomicCoordinates	Genomic coordinates	Genomic coordinates
Search_key	Search key	Search key
Target	Target	Target
Gid, GI	Genbank identifier	Genbank identifier
Accession	GenBank accession	GenBank accession
Symbol	Gene symbol	Gene symbol
Probe_Type	Probe type	Probe type
crosshyb_type	Crosshyb type	Crosshyb type
category	category	category
Start, Probe_Start	Start	Start
Stop	Stop	Stop
Definition	Definition	Definition
Synonym, Synonyms	Synonym	Synonym
Source	Source	Source
Source_Reference_ID	Source reference id	Source reference id
RefSeq_ID	Reference sequence id	Reference sequence id
ILMN_Gene	Illumina Gene	Illumina Gene
Protein_Product	Protein product	Protein product
protein_domains	Protein domains	Protein domains
Array_Address_Id	Array adress id	Array adress id
Probe_Sequence	Sequence	Sequence
seqname	Seqname	Seqname
Chromosome	Chromosome	Chromosome
strand	Strand	Strand
Probe_Chr_Orientation	Probe chr orientation	Probe chr orientation
Probe_Coordinates	Probe coordinates	Probe coordinates
Obsolete_Probe_Id	Obsolete probe id	Obsolete probe id

Appendix J

Custom codon frequency tables

You can edit the list of codon frequency tables used by *CLC Main Workbench*.

Note! Please be aware that this process needs to be handled carefully, otherwise you may have to re-install the Workbench to get it to work.

In the Workbench installation folder under `res`, there is a folder named `codonfreq`. This folder contains all the codon frequency tables organized into subfolders in a hierarchy. In order to change the tables, you simply add, delete or rename folders and the files in the folders. If you wish to add new tables, please use the existing ones as template. In existing tables, the "_number" at the end of the ".cftbl" file name is the number of CDSs that were used for calculation, according to the `https://www.kazusa.or.jp/codon/` site.

When creating a custom table, it is not necessary to fill in all fields as only the codon information (e.g. 'GCG' in the example below) and the counts (e.g. 47869.00) are used when doing reverse translation:

Name: Rattus norvegicus GeneticCode: 1 Ala GCG 47869.00 6.86 0.10 Ala GCA 109203.00 15.64 0.23 ....

In particular, the amino acid type is not used: in order to use an alternative genetic code, it must be specified in the 'GeneticCode' line instead.

Restart the Workbench to have the changes take effect.

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