



CLC **Main** Workbench

USER MANUAL

Manual for
CLC Main Workbench 7.6.7
Windows, Mac OS X and Linux

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This software is for research purposes only.

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Part I

Introduction

Chapter 1

Introduction to *CLC Main Workbench*

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Welcome to *CLC Main Workbench 7.6.7* – a software package supporting your daily bioinformatics work.

We strongly encourage you to read this user manual in order to get the best possible basis for working with the software package.

This software is for research purposes only.

1.1 Contact information

The *CLC Main Workbench* is developed by:

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Prismet
8000 Aarhus C
Denmark

<http://www.clcbio.com>

<http://www.qiagenbioinformatics.com>

VAT no.: DK 28 30 50 87

Email: support-clcbio@qiagen.com

Telephone: +45 70 22 32 44

If you have questions or comments regarding the program, you can contact us through the support team as described here: http://www.clcsupport.com/clcgenomicsworkbench/current/index.php?manual=Getting_help.html.

1.2 Download and installation

The *CLC Main Workbench* is developed for Windows, Mac OS X and Linux. The software for either platform can be downloaded from <http://www.clcbio.com/download>.

1.2.1 Program download

The program is available for download on <http://www.clcbio.com/download>.

Before you download the program you are asked to fill in the **Download** dialog.

In the dialog you must choose:

- Which operating system you use
- Whether you would like to receive information about future releases

Depending on your operating system and your Internet browser, you are taken through some download options.

When the download of the installer (an application which facilitates the installation of the program) is complete, follow the platform specific instructions below to complete the installation procedure. ¹

1.2.2 Installation on Microsoft Windows

Starting the installation process is done in one of the following ways:

¹ You must be connected to the Internet throughout the installation process.

When you have downloaded an installer:

Locate the downloaded installer and double-click the icon.

The default location for downloaded files is your desktop.

Installing the program is done in the following steps:

- On the welcome screen, click **Next**.
- Read and accept the License agreement and click **Next**.
- Choose where you would like to install the application and click **Next**.
- Choose a name for the Start Menu folder used to launch *CLC Main Workbench* and click **Next**.
- Choose if *CLC Main Workbench* should be used to open CLC files and click **Next**.
- Choose where you would like to create shortcuts for launching *CLC Main Workbench* and click **Next**.
- Choose if you would like to associate .clc files to *CLC Main Workbench*. If you check this option, double-clicking a file with a "clc" extension will open the *CLC Main Workbench*.
- Wait for the installation process to complete, choose whether you would like to launch *CLC Main Workbench* right away, and click **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 Mac OS X

Starting the installation process is done in the following way:

When you have downloaded an installer:

Locate the downloaded installer and double-click the icon.

The default location for downloaded files is your desktop.

Launch the installer by double-clicking on the "*CLC Main Workbench*" icon.

Installing the program is done in the following steps:

- On the welcome screen, click **Next**.
- Read and accept the License agreement and click **Next**.
- Choose where you would like to install the application and click **Next**.
- Choose if *CLC Main Workbench* should be used to open CLC files and click **Next**.
- Choose whether you would like to create desktop icon for launching *CLC Main Workbench* and click **Next**.

- Choose if you would like to associate `.clc` files to *CLC Main Workbench*. If you check this option, double-clicking a file with a `"clc"` extension will open the *CLC Main Workbench*.
- Wait for the installation process to complete, choose whether you would like to launch *CLC Main Workbench* right away, and click **Finish**.

When the installation is complete the program can be launched from your Applications folder, or from the desktop shortcut you chose to create. If you like, you can drag the application icon to the dock for easy access.

1.2.4 Installation on Linux with an installer

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

```
# sh CLCMainWorkbench_7_6_7_64.sh.sh
```

Installing the program is done in the following steps:

- On the welcome screen, click **Next**.
- Read and accept the License agreement and click **Next**.
- Choose where you would like to install the application and click **Next**.
For a system-wide installation you can choose for example `/opt` or `/usr/local`. If you do not have root privileges you can choose to install in your home directory.
- Choose where you would like to create symbolic links to the program
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 **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:

```
# clcmainwb7
```

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

```
# ./clcmainwb7
```


1.2.5 Installation on Linux with an RPM-package

Navigate to the directory containing the rpm-package and install it using the rpm-tool by running a command similar to:

```
# rpm -ivh CLCMainWorkbench_7_6_7_64.sh.rpm
```

Installation of RPM-packages usually requires root-privileges.

When the installation process is finished the program can be executed by running the command:

```
# clcmainwb7
```

1.3 System requirements

The system requirements of *CLC Main Workbench* are these:

- Windows Vista, Windows 7, Windows 8, Windows 10, Windows Server 2008, or Windows Server 2012
- Mac OS X 10.7 or later
- Linux: RHEL 5.0 or later. SUSE 10.2 or later. Fedora 6 or later
- 32 or 64 bit
- 1 GB RAM required
- 2 GB RAM recommended
- 1024 x 768 display required
- 1600 x 1200 display recommended

Special requirements for the 3D Molecule Viewer

- **System requirements**
 - A graphics card capable of supporting OpenGL 2.0.
 - Updated graphics drivers. Please make sure the latest driver for the graphics card is installed .
- **System Recommendations**
 - A discrete graphics card from either Nvidia or AMD/ATI. Modern integrated graphics cards (such as the Intel HD Graphics series) may also be used, but these are usually slower than the discrete cards.
 - A 64-bit workbench version is recommended for working with large complexes.

1.4 Workbench Licenses

When you have installed the *CLC Main Workbench*, and start it for the first time or after installing a new major release, you will meet the license assistant, shown in figure 1.1. The **License Manager** can also be accessed from the menu bar in the Workbench:

Help | License Manager

This can be useful if you wish to use a different license or want to view information about the license(s) the Workbench is currently using. The **License Manager** is described in detail in section 1.4.5 and can be seen in figure 1.23.

To install a license, you must be running the program in administrative mode ².

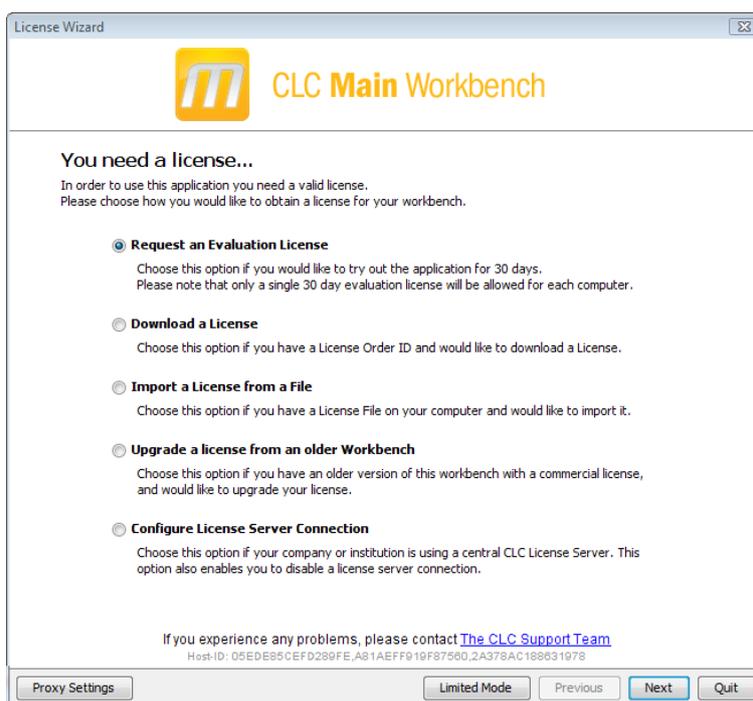


Figure 1.1: The license assistant showing you the options for getting started.

The following options are available. They are described in detail in the sections that follow.

- **Request an evaluation license.** Request a fully functional, time-limited license (see below).
- **Download a license.** Use the license order ID received when you purchase the software to download and install a license file.
- **Import a license from a file.** Import an existing license file, for example a file downloaded from the web-based licensing system.
- **Upgrade license.** 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 license file.

²How to do this differs for different operating systems. To run the program in administrator mode on Windows Vista, or 7, right-click the program shortcut and choose "Run as Administrator."

- **Configure license server connection.** If your organization has a CLC License Server, select this option to configure the connection to it.

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

To use the Download option in the License Manager, your machine must be able to access the external network. If this is not the case, please see section 1.4.6.

If for some reason you don't have a license order ID or access to a license, you can click the **Limited Mode** button (see section 1.4.7).

1.4.1 Request an evaluation license

We offer a fully functional version of the *CLC Main Workbench* for evaluation purposes, free of charge.

Each user is entitled to a 30-day trial of *CLC Main Workbench*.

If you are unable to complete your assessment in the available time, please send an email to bioinformaticssales@qiagen.com to request an additional evaluation period.

When you choose the option **Request an evaluation license**, you will see the dialog shown in figure 1.2.

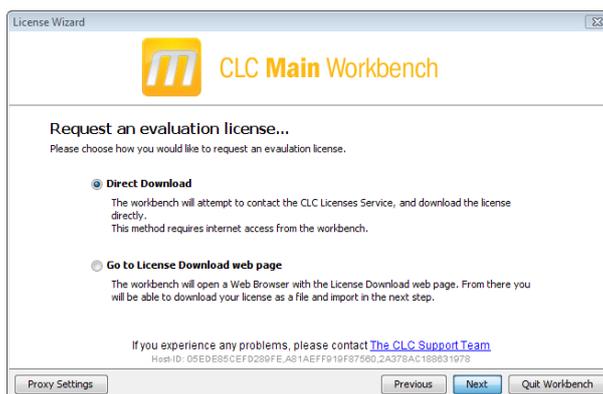


Figure 1.2: Choosing between direct download or going to the license download web page.

In this dialog, there are two options:

- **Direct download.** Download the license directly from CLC bio. This method requires that the Workbench has access to the external network.
- **Go to license download web page.** In a browser window, show the license download web page, which can be used to download a license file. This option is suitable in situations where, for example, you are working behind a proxy, so that the Workbench does not have direct access to the CLC Licenses Service.

If you select the option to download a license directly and it turns out that the Workbench does not have direct access to the external network, (because of a firewall, proxy server etc.), you can click **Previous** button to try the other method.

After selection on 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**, the dialog shown in figure 1.3 appears.

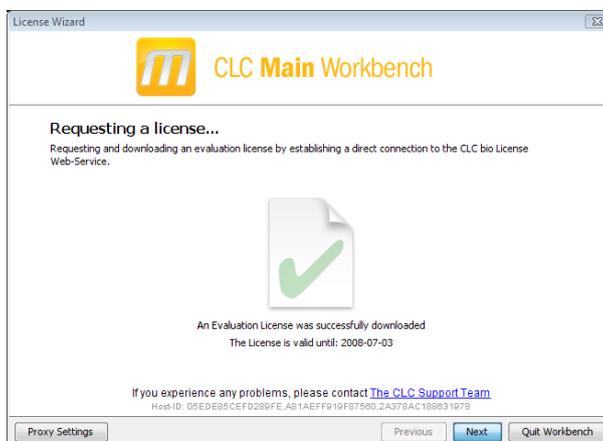


Figure 1.3: A license has been downloaded.

A progress for getting the license is shown, and when the license is downloaded, you will be able to click **Next**.

Go to license download web page

After choosing the *Go to license download web page* option and clicking on the button labeled **Next**, the license download web page appears in a browser window, as shown in 1.4.

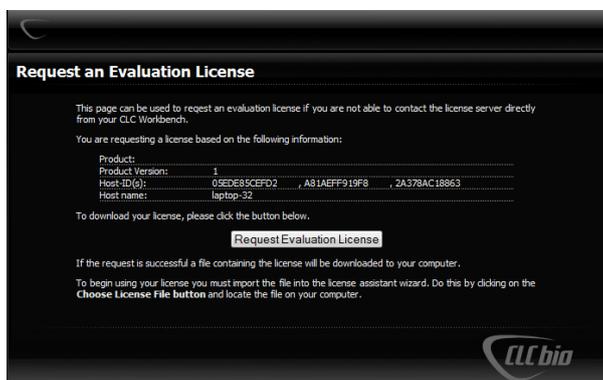


Figure 1.4: The license download web page.

Click the **Request Evaluation License** button. You can then save the license on your system.

Back in the Workbench window, you will now see the dialog shown in 1.5.

Click the **Choose License File** button and browse to find the license file you saved. When you have selected the file, click on the button labeled **Next**.

Accepting the license agreement

Part of the installation of the license involves checking and accepting the end user license agreement (EULA). You should now see the a window like that in figure 1.6.

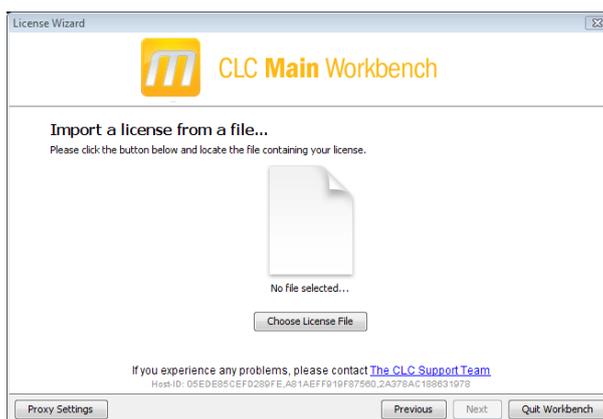


Figure 1.5: Importing the license file downloaded from the web page.

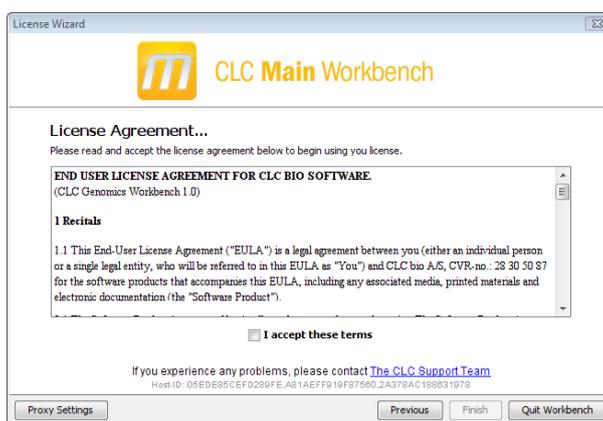


Figure 1.6: Read the license agreement carefully.

Please read the EULA text carefully before clicking in the box next to the text **I accept these terms** to accept, and then clicking on the button labeled **Finish**.

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 **Next** button, you will see the dialog shown in 1.7. Enter your license order ID into the text field under the title License Order-ID. (The ID can be pasted into the box after copying it and then using menus or key combinations like Ctrl+V on some system or ⌘ + V on Mac).

In this dialog, there are two options:

- **Direct download.** Download the license directly from CLC bio. This method requires that the Workbench has access to the external network.
- **Go to license download web page.** In a browser window, show the license download web page, which can be used to download a license file. This option is suitable in situations where, for example, you are working behind a proxy, so that the Workbench does not have direct access to the CLC Licenses Service.

If you select the option to download a license directly and it turns out that the Workbench does

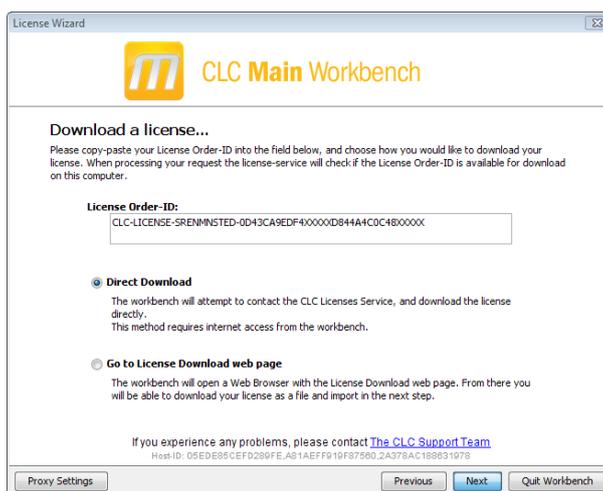


Figure 1.7: Enter a license order ID for the software.

not have direct access to the external network, (because of a firewall, proxy server etc.), you can click **Previous** button to try the other method.

After selection on 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**, the dialog shown in figure 1.8 appears.

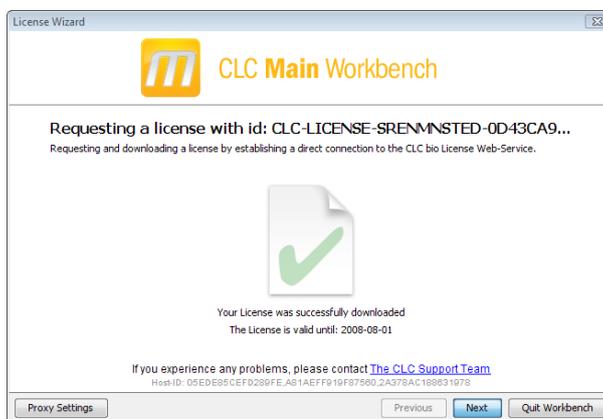


Figure 1.8: A license has been downloaded.

A progress for getting the license is shown, and when the license is downloaded, you will be able to click **Next**.

Go to license download web page

After choosing the *Go to license download web page* option and clicking on the button labeled **Next**, the license download web page appears in a browser window, as shown in 1.9.

Click the **Request Evaluation License** button. You can then save the license on your system.

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



Figure 1.9: The license download web page.

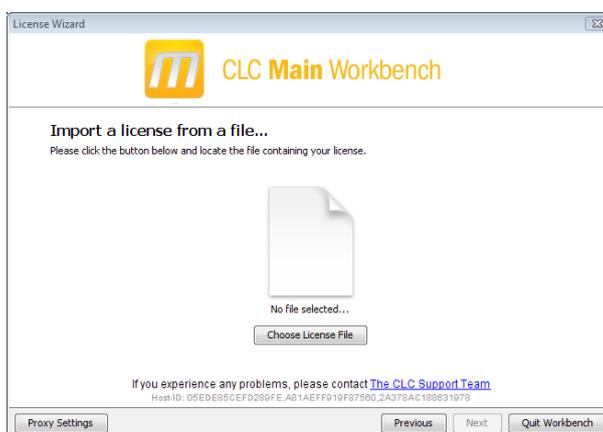


Figure 1.10: Importing the license file downloaded from the web page.

Click the **Choose License File** button and browse to find the license file you saved. When you have selected the file, click on the button labeled **Next**.

Accepting the license agreement

Part of the installation of the license involves checking and accepting the end user license agreement (EULA). You should now see the a window like that in figure 1.11.

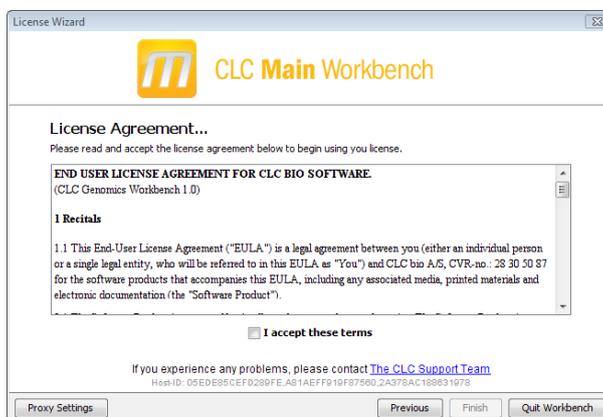


Figure 1.11: Read the license agreement carefully.

Please read the EULA text carefully before clicking in the box next to the text **I accept these**

terms to accept, and then clicking on the button labeled **Finish**.

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 1.12.

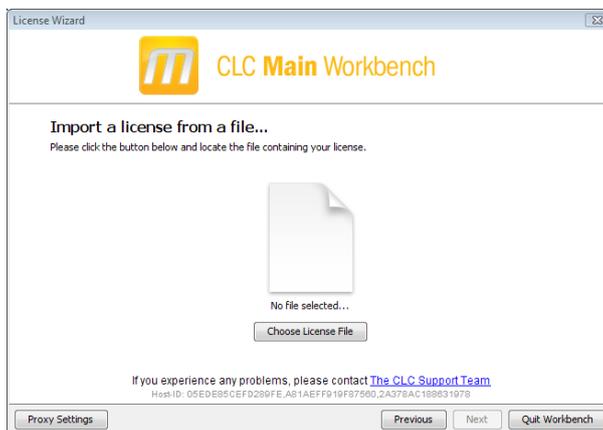


Figure 1.12: Selecting a license file .

Click the **Choose License File** button and browse to find the license file. When you have selected the file, click on the **Next** button.

Accepting the license agreement

Part of the installation of the license involves checking and accepting the end user license agreement (EULA). You should now see the a window like that in figure 1.13.

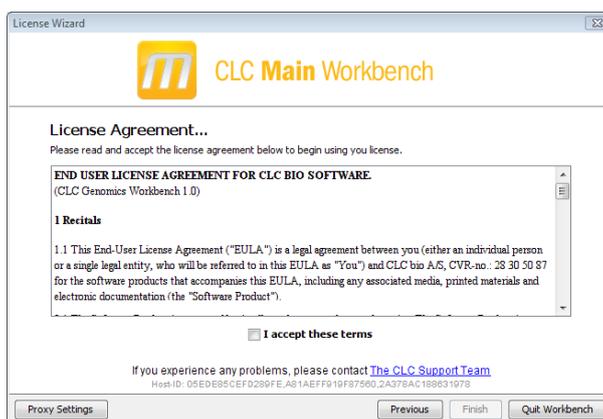


Figure 1.13: Read the license agreement carefully.

Please read the EULA text carefully before clicking in the box next to the text **I accept these terms** to accept, and then clicking on the button labeled **Finish**.

1.4.4 Upgrade license

This option is used when you already have used a previous version of *CLC Main Workbench*, and you are entitled to upgrade to a new major version. The Workbench will need direct access to the external network to use this option.

When you click on the **Next** button, the Workbench will search for a previous installation of *CLC Main Workbench*. It will then locate the old license.

If the Workbench finds an existing license file, the next dialog will look like figure 1.14.

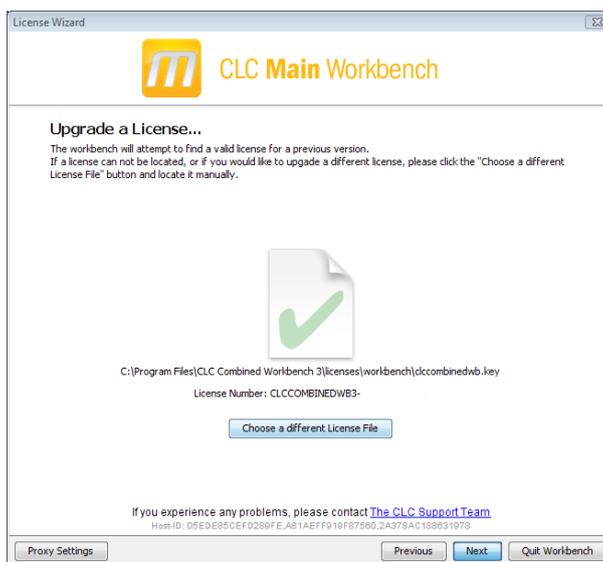


Figure 1.14: An license from an older installation is found.

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

If the Workbench cannot connect to the external network directly, please see the section on downloading a license for non-networked machines. You will need your license order ID for this.

Your license must be covered by our Maintenance, Upgrades and Support (MUS) program to be eligible to upgrade your license. If the license is covered for upgrades and there are any problems with this, please contact licenses@clcbio.com.

In this dialog, there are two options:

- **Direct download.** Download the license directly from CLC bio. This method requires that the Workbench has access to the external network.
- **Go to license download web page.** In a browser window, show the license download web page, which can be used to download a license file. This option is suitable in situations where, for example, you are working behind a proxy, so that the Workbench does not have direct access to the CLC Licenses Service.

If you select the option to download a license directly and it turns out that the Workbench does not have direct access to the external network, (because of a firewall, proxy server etc.), you can click **Previous** button to try the other method.

After selection on 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**, the dialog shown in figure 1.15 appears.

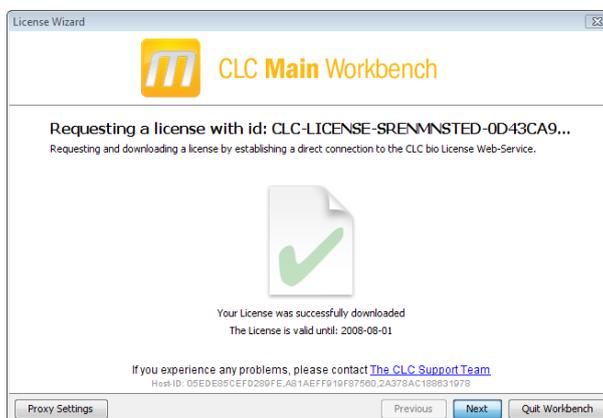


Figure 1.15: A license has been downloaded.

A progress for getting the license is shown, and when the license is downloaded, you will be able to click **Next**.

Go to license download web page

After choosing the *Go to license download web page* option and clicking on the button labeled **Next**, the license download web page appears in a browser window, as shown in 1.16.



Figure 1.16: The license download web page.

Click the **Request Evaluation License** button. You can then save the license on your system.

Back in the Workbench window, you will now see the dialog shown in 1.17.

Click the **Choose License File** button and browse to find the license file you saved. When you have selected the file, click on the button labeled **Next**.

Accepting the license agreement

Part of the installation of the license involves checking and accepting the end user license agreement (EULA). You should now see the a window like that in figure 1.18.

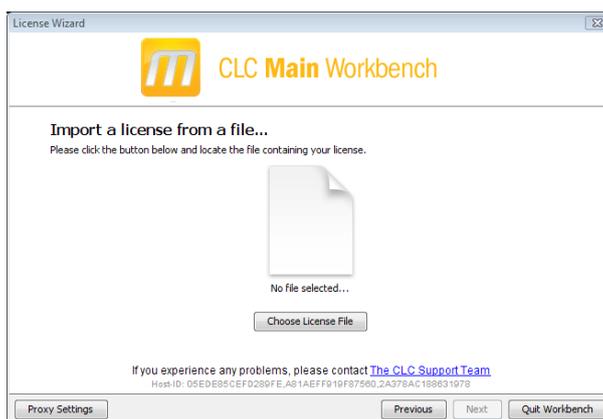


Figure 1.17: Importing the license file downloaded from the web page.

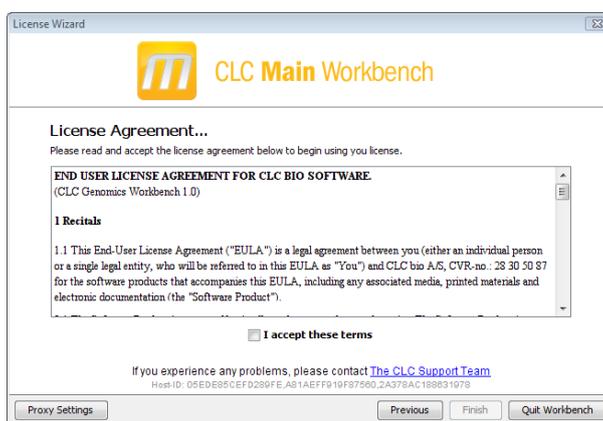


Figure 1.18: Read the license agreement carefully.

Please read the EULA text carefully before clicking in the box next to the text **I accept these terms** to accept, and then clicking on the button labeled **Finish**.

1.4.5 Configure license server connection

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

To do this, select this option and click on the **Next** button. A dialog like that shown in figure 1.19 then appears. Here, you configure how to connect to the CLC License Server.

- **Enable license server connection.** This box must be checked for the Workbench is to contact the CLC License Server to get a license for *CLC Main Workbench*.
- **Automatically detect license server.** By checking this option the Workbench will look for a CLC License Server accessible from the Workbench³.

³Automatic server discovery sends UDP broadcasts from the Workbench on a fixed port, 6200. Available license servers respond to the broadcast. The Workbench then uses TCP communication for to get a license, assuming 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 License server manually instead.

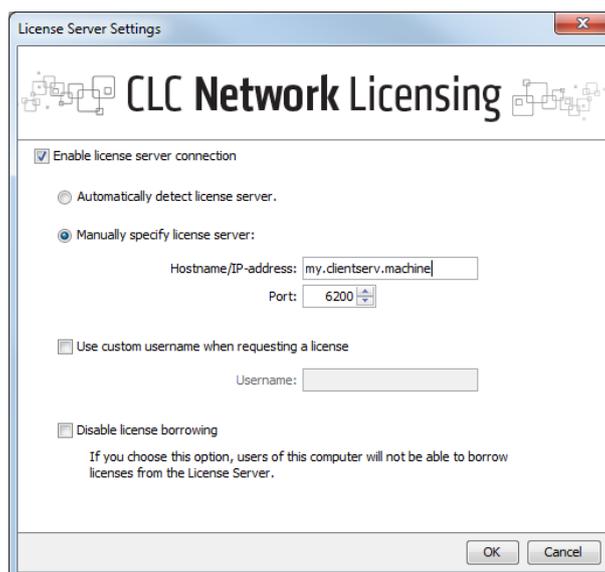


Figure 1.19: Connecting to a CLC License Server.

- **Manually specify license server.** If there are technical limitations such that the CLC License Server cannot be detected automatically, use this option to provides details of machine the CLC License Server software is on, and the port used by the software to receive requests. After selecting this option, please enter:
 - **Host name.** The address for the machine the CLC Licenser Server software is running on.
 - **Port.** The port used by the CLC License Server to receive requests.
- **Use custom username when requesting a license.** A username entered here will be passed to the CLC License Server instead of the username of your account this machine.
- **Disable license borrowing on this computer.** If you do not want users of the computer to borrow a license from the set of licenses available, then (see section 1.4.5), select this option.

Borrowing a license

A network license can only be used when you are connected to the a license server. If you wish to use the *CLC Main Workbench* when you are not connected to the CLC License Server, you can *borrow* an available license for a period of time. During this time, there will be one less network license available on the for other users. The Workbench must have a connection to the CLC License Server at the point in time when you wish to borrow a license.

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 dialog shown in figure 1.20.
3. Use the checkboxes at the right hand side of the table in the License overview section of the window to select the license(s) that you wish to borrow.

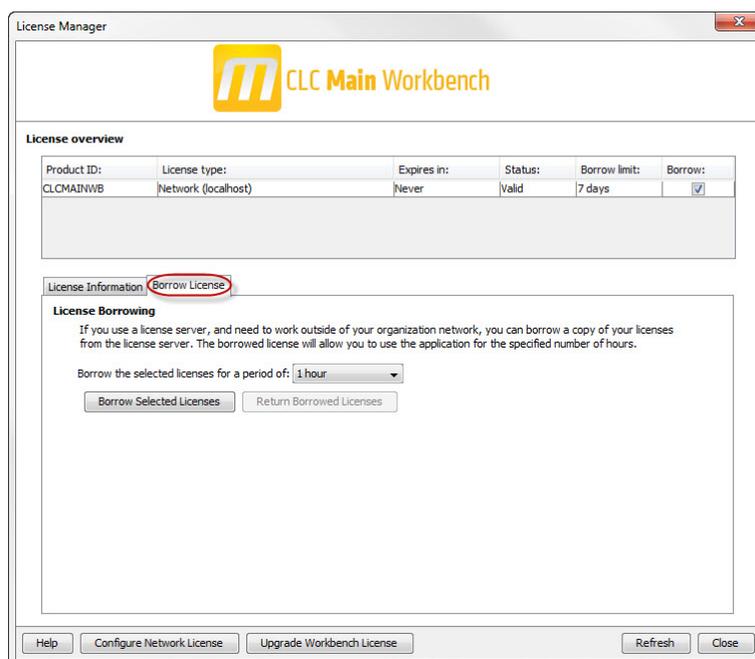


Figure 1.20: Borrow a license.

4. Select the length of time you wish to borrow the license(s).
5. Click on the button labeled **Borrow Licenses**.
6. Close the License Manager when you are done.

You can now go offline and work with the *CLC Main Workbench*. When the time period you borrowed the license for has elapsed, the network license you borrowed is made available again for other users to access. To continue using the *CLC Main Workbench* with a license, you will need to connect the Workbench to the network again so it can contact the CLC License Server to obtain one.

Note! Your CLC License Server administrator can choose to disable the option allowing the borrowing of licenses. If this has been done, you will not be able to borrow a network license using your Workbench.

Common issues when using a network license

No license available at the moment If all the network licenses or *CLC Main Workbench* are in use, you will see a dialog like that shown in figure 1.21 when you start up the Workbench.

This means others are using the network licenses. You will need to wait for them to return their licenses before you can continue to work with a fully functional copy of software. If this is a frequent issue, you may wish to discuss this with your CLC License Server administrator.

Clicking on the **Limited Mode** button in the dialog allows you to start the Workbench with functionality equivalent to the CLC Sequence Viewer. This includes the ability to access your CLC data.

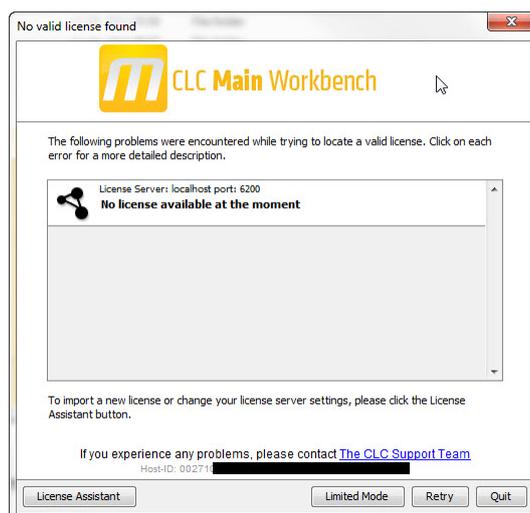


Figure 1.21: This window appears when there are no available network licenses for the software you are running.

Lost connection to the CLC License Server If the Workbench connection to the CLC License Server is lost, you will see a dialog as shown in figure 1.22.

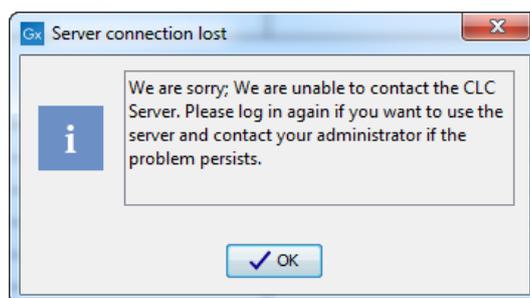


Figure 1.22: This message appears if the Workbench is unable to establish a connection to a CLC License server.

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

If you have successfully contacted the CLC License Server from your Workbench previously, please consider discussing this issue with your CLC License Server administrator or your local IT support, to make sure that the CLC License Server is running and that your Workbench can connect to it. There may be situations where you wish to use a different license or view information about the license(s) the Workbench is currently using. To do this, open the License Manager using the menu option:

Help | License Manager (📄)

The license manager is shown in figure 1.23.

This dialog can be used to:

- See information about the license (e.g. what kind of license, when it expires)

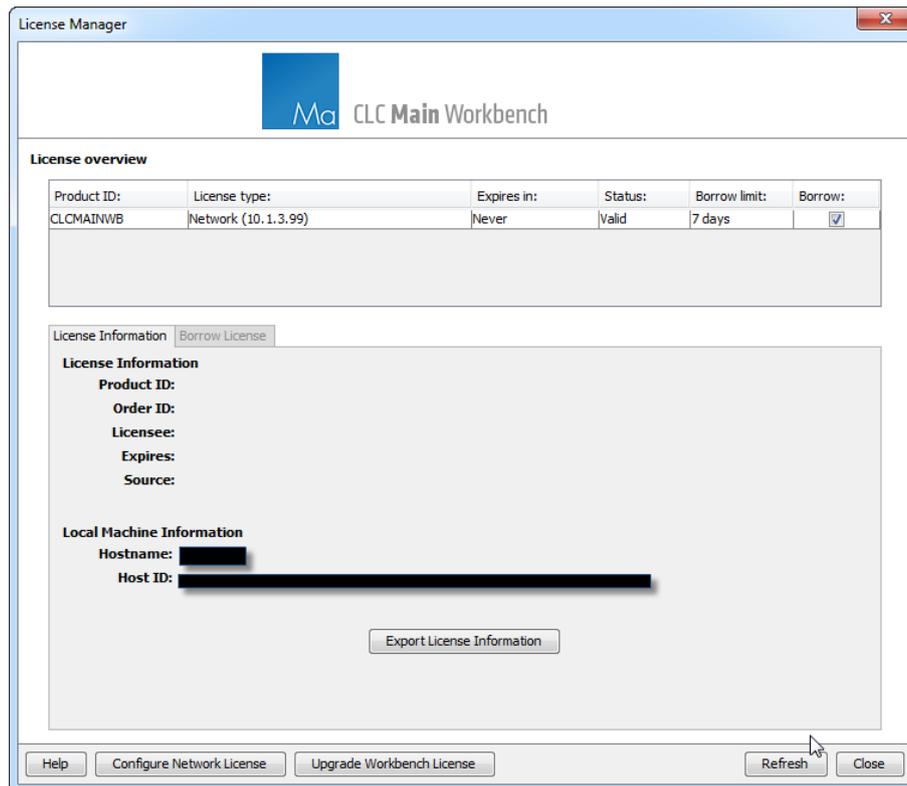


Figure 1.23: The license manager.

- Configure how to connect to a license server (**Configure License Server** the button at the lower left corner). Clicking this button will display a dialog similar to figure 1.19.
- Upgrade from an evaluation license by clicking the **Upgrade license** button. This will display the dialog shown in figure 1.1.
- Export license information to a text file.
- Borrow a license

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

1.4.6 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 the machine reported at the bottom of the License Manager window in grey text.
- 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 relevant network license download web page:
- For Workbenches released from January 2013 and later, (e.g. the Genomics Workbench version 6.0 or higher, and the Main Workbench, version 6.8 or higher), please go to:
<https://secure.clcbio.com/LmxWSv3/GetLicenseFile>
For earlier Workbenches, including any DNA, Protein or RNA Workbench, please go to:
<http://licensing.clcbio.com/LmxWSv1/GetLicenseFile>
It is vital that you choose the license download page appropriate to the version of the software you plan to run.
- Paste in your license order ID and the host ID that you noted down in the relevant boxes on the webpage.
- Click '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 'choose license file' to browse the location of 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 Help menu and choosing License Manager.
- Click on the **Next** button and go through the remaining steps of the license manager wizard.

1.4.7 Limited mode

We have created the limited mode to prevent a situation where you are unable to access your data because you do not have a license. When you run in limited mode, a lot of the tools in the Workbench are not available, but you still have access to your data (also when stored in a *CLC Bioinformatics Database*). When running in limited mode, the functionality is equivalent to the *CLC Sequence Viewer* (see section A). To get out of the limited mode and run the Workbench normally, restart the Workbench. When you restart the Workbench will try to find a proper license and if it does, it will start up normally. If it can't find a license, you will again have the option of running in limited mode.

1.5 About CLC Workbenches

In November 2005 CLC bio released two Workbenches: *CLC Free Workbench* and *CLC Protein Workbench*. *CLC Protein Workbench* is developed from the free version, giving it the well-tested user friendliness and look & feel. However, the *CLC Protein Workbench* includes a range of more advanced analyses.

In March 2006, *CLC DNA Workbench* (formerly *CLC Gene Workbench*) and *CLC Main Workbench* were added to the product portfolio of CLC bio. Like *CLC Protein Workbench*, *CLC DNA Workbench* builds on *CLC Free Workbench*. It shares some of the advanced product features of *CLC Protein Workbench*, and it has additional advanced features. *CLC Main Workbench* holds all basic and advanced features of the *CLC Workbenches*.

In June 2007, *CLC RNA Workbench* was released as a sister product of *CLC Protein Workbench* and *CLC DNA Workbench*. *CLC Main Workbench* now also includes all the features of *CLC RNA Workbench*.

In March 2008, the *CLC Free Workbench* changed name to *CLC Sequence Viewer*.

In June 2008, the first version of the *CLC Genomics Workbench* was released due to an extraordinary demand for software capable of handling sequencing data from all new high-throughput sequencing platforms such as Roche-454, Illumina and SOLiD in addition to Sanger reads and hybrid data.

For an overview of which features all the applications include, see <http://www.clcbio.com/features>.

In December 2006, CLC bio released a **Software Developer Kit** which makes it possible for anybody with a knowledge of programming in Java to develop plugins. The plugins are fully integrated with the CLC Workbenches and the Viewer and provide an easy way to customize and extend their functionalities.

In April 2012, *CLC Protein Workbench*, *CLC DNA Workbench* and *CLC RNA Workbench* were discontinued. All customers with a valid license for any of these products were offered an upgrade to the *CLC Main Workbench*.

In February 2014, CLC bio expanded the product repertoire with the release of *CLC Drug Discovery Workbench*, a product that enables studies of protein-ligand interactions for drug discovery.

In April 2014, CLC bio released the *CLC Cancer Research Workbench*, a product that containing streamlined data analysis workflows with integrated trimming and quality control tailored to meet the requirements of clinicians and researchers working within the cancer field.

In April 2015, the *CLC Cancer Research Workbench* was renamed to *Biomedical Genomics Workbench* to reflect the inclusion of tools addressing the requirements of clinicians and researchers working within the hereditary disease field in addition to the tools designed for those working within the cancer field.

1.5.1 New program feature request

The CLC team is continuously improving the *CLC Main Workbench* with our users' 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. To contact us via the Workbench, please go to the menu option:

Help | Contact Support

1.5.2 Getting help

If you encounter a problem or need help understanding how the *CLC Main Workbench* works, and the license you are using is covered by our Maintenance, Upgrades and Support (MUS) program (<https://www.clcbio.com/support/maintenance-support-program/>), you can contact our customer support via the Workbench by going to the menu option:

Help | Contact Support

This will open a dialog to enter your contact information and a text field for entering the question

or problem you have.

You can also attach small datasets, if this helps explain the problem or you believe it will help in troubleshooting the problem.

When you send a support request this way, it will include technical information about your installation that usually helps when troubleshooting. It also includes your license information so that you do not have to look this up yourself. Our support staff will reply to you by email.

Further information about Maintenance, Upgrades and Support (MUS) program can be found online at <https://www.clcbio.com/support/maintenance-support-program/>.

Information about how to find your license information is included in the licenses section of our Frequently Asked Questions (FAQ) area: <https://secure.clcbio.com/helpspot/index.php?pg=kb>

Information about MUS cover on particular licenses can be found by <https://secure.clcbio.com/myclc/login>.

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).

1.5.3 CLC Sequence Viewer vs. Workbenches

The *CLC Sequence Viewer* is a user friendly application offering basic bioinformatics analyses. The *CLC Sequence Viewer* can be used to view outputs from many analyses of the CLC commercial workbenches, with notable exceptions being workflows and track-based data, which can only be viewed using our commercial Workbench offerings.

Track-based outputs can be viewed using the *CLC Genomics Workbench* and *CLC Main Workbench*, while workflows can be viewed in all commercial CLC workbenches, including the *CLC Main Workbench*, *CLC Genomics Workbench* and the *CLC Drug Discovery Workbench*.

The CLC Workbenches and the *CLC Sequence Viewer* are developed for Windows, Mac and Linux platforms. Data can be exported/imported between the different platforms in the same easy way as when exporting/importing between two computers with e.g. Windows.

1.6 When the program is installed: Getting started

CLC Main Workbench includes an extensive **Help** function, which can be found in the **Help** menu of the program's **Menu bar**. The **Help** can also be shown by pressing F1. The help topics are sorted in a table of contents and the topics can be searched.

Tutorials describing hands-on examples of how to use the individual tools and features of the *CLC Main Workbench* can be found at <http://www.clcbio.com/support/tutorials/>. We

also recommend our **Online presentations** where a product specialist from CLC bio demonstrates our software. This is a very easy way to get started using the program. Read more about video tutorials and other online presentations here: <http://www.clcbio.tv/>.

1.6.1 Quick start

When the program opens for the first time, the background of the workspace is visible. In the background are three quick start shortcuts, which will help you getting started. These can be seen in figure 1.24.



Figure 1.24: Quick start shortcuts, available in the background of the workspace.

The function of the quick start shortcuts is explained here:

- **Import data.** Opens the **Import** dialog, which you let you browse for, and import data from your file system.
- **New sequence.** Opens a dialog which allows you to enter your own sequence.
- **Read tutorials.** Opens the tutorials menu with a number of tutorials. These are also available from the **Help** menu in the **Menu bar**.

Below these three quick start shortcuts, you will see a text: "Looking for more features?" Clicking this text will take you to a page on <http://www.clcbio.com> where you can read more about how to get more functionalities into *CLC Main Workbench*.

1.6.2 Import of example data

It might be easier to understand the logic of the program by trying to do simple operations on existing data. Therefore *CLC Main Workbench* includes an example data set.

When downloading *CLC Main Workbench* you are asked if you would like to import the example data set. If you accept, the data is downloaded automatically and saved in the program. If you didn't download the data, or for some other reason need to download the data again, you have two options:

You can click **Import Example Data** (📁) in the **Help** menu of the program. This imports the data automatically. You can also go to <http://www.clcbio.com/download> and download the example data from there.

If you download the file from the website, you need to import it into the program. See chapter 7 for more about importing data.

1.7 Plugins

When you install *CLC Main Workbench*, it has a standard set of features. However, you can upgrade and customize the program using a variety of plugins.

As the range of plugins is continuously updated and expanded, they will not be listed here. Instead we refer to <http://www.clcbio.com/plugins> for a full list of plugins with descriptions of their functionalities.

1.7.1 Installing plugins

Plugins are installed using the plugin manager⁴:

Help in the Menu Bar | Plugins and Resources... ()

or **Plugins** () **in the Toolbar**

The plugin manager has three tabs at the top:

- **Manage Plugins.** This is an overview of plugins that are installed.
- **Download Plugins.** This is an overview of available plugins on CLC bio's server.
- **Manage Resources.** This is an overview of resources that are installed.

To install a plugin, click the **Download Plugins** tab. This will display an overview of the plugins that are available for download and installation (see figure 1.25).

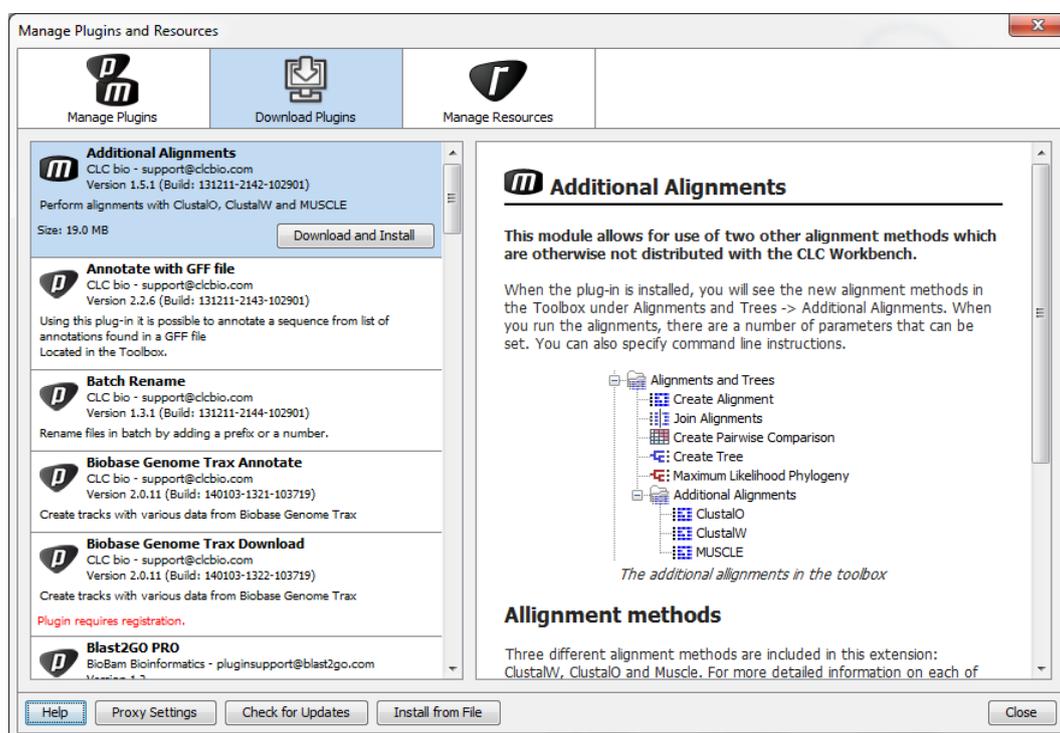


Figure 1.25: The plugins that are available for download.

⁴In order to install plugins on Windows, the Workbench must be run in administrator mode: Right-click the program shortcut and choose "Run as Administrator". Then follow the procedure described below.

Clicking a plugin will display additional information at the right side of the dialog. This will also display a button: **Download and Install**.

Click the plugin and press **Download and Install**. A dialog displaying progress is now shown, and the plugin is downloaded and installed.

If the plugin is not shown on the server, and you have it on your computer (e.g. if you have downloaded it from our web-site), you can install it by clicking the **Install from File** button at the bottom of the dialog. This will open a dialog where you can browse for the plugin. The plugin file should be a file of the type ".cpa".

When you close the dialog, you will be asked whether you wish to restart the *CLC Main Workbench*. The plugin will not be ready for use until you have restarted.

1.7.2 Uninstalling plugins

Plugins are uninstalled using the plugin manager:

Help in the Menu Bar | Plugins and Resources... ()

or **Plugins () in the Toolbar**

This will open the dialog shown in figure 1.26.

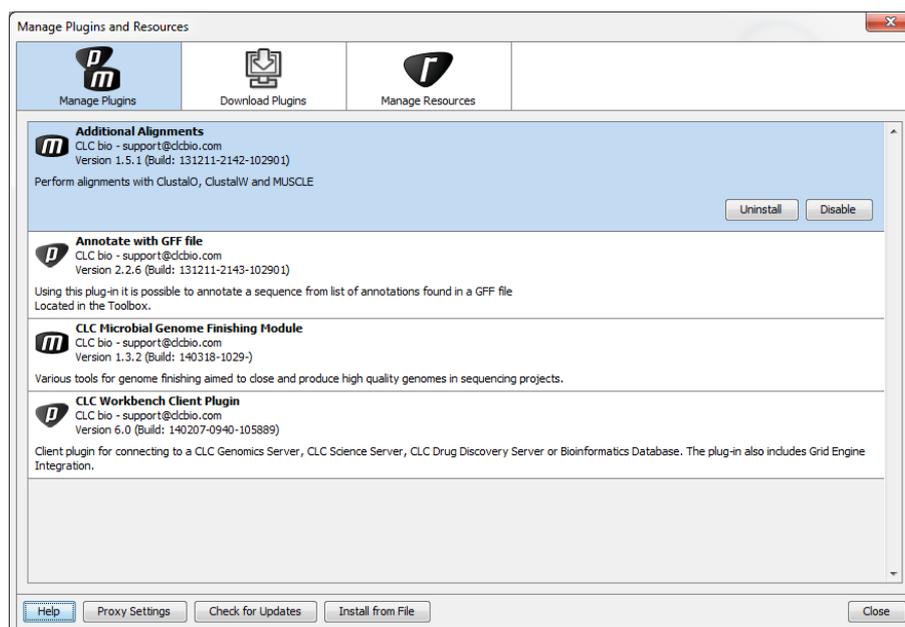


Figure 1.26: The plugin manager with plugins installed.

The installed plugins are shown in this dialog. To uninstall:

Click the plugin | Uninstall

If you do not wish to completely uninstall the plugin but you don't 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.

1.7.3 Updating plugins

If a new version of a plugin is available, you will get a notification during start-up as shown in figure 1.27.

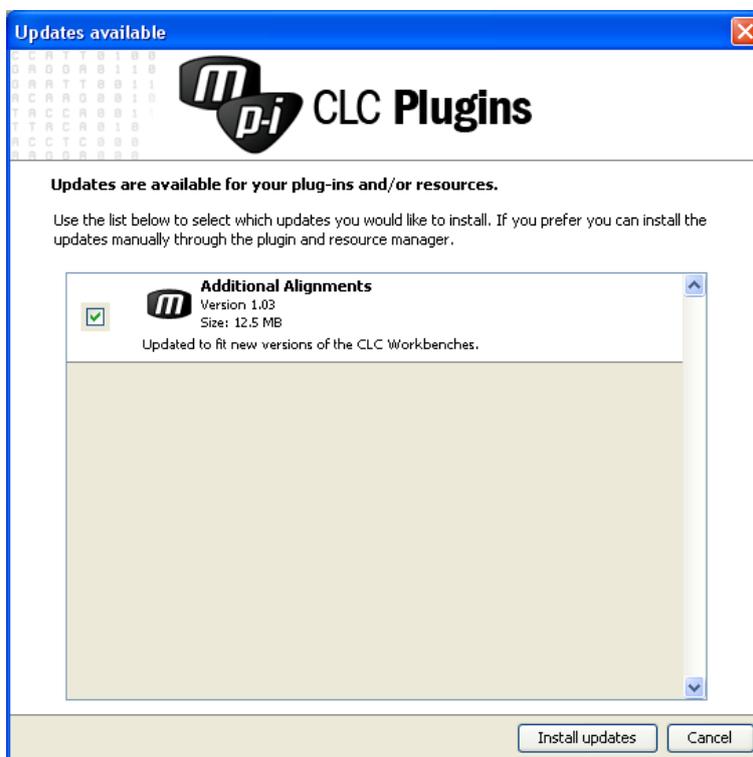


Figure 1.27: Plugin updates.

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 1.26).

1.7.4 Resources

Resources can be manually installed and un-installed the same way as plugins.

1.8 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 (e.g. searching GenBank).

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

To configure your proxy settings, open *CLC Main Workbench*, and go to the **Advanced**-tab of the **Preferences** dialog (figure 1.28) and enter the appropriate information. The **Preferences** dialog is opened from the **Edit** menu.

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

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

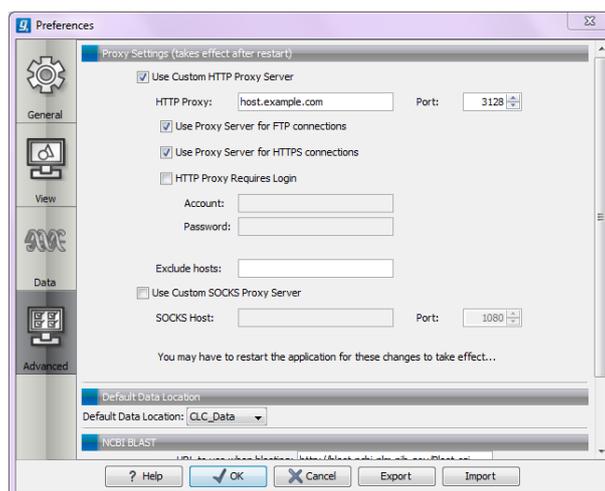


Figure 1.28: Adjusting proxy preferences.

Exclude hosts can be used if there are some hosts that should be contacted directly and not through the proxy server. The value can be a list of hosts, each separated by a |, and in addition a wildcard character * can be used for matching. For example: *.foo.com|localhost.

If you have any problems with these settings you should contact your systems administrator.

1.9 The format of the user manual

This user manual offers support to Windows, Mac OS X and Linux users. The software is very similar on these operating systems. In areas where differences exist, these will be described separately. However, the term "right-click" is used throughout the manual, but some Mac users may have to use Ctrl+click in order to perform a "right-click" (if they have a single-button mouse).

The most recent version of the user manuals can be downloaded from <http://www.clcbio.com/usermanuals>.

The user manual consists of four parts.

- The **first part** includes the introduction to the *CLC Main Workbench*.
- The **second part** describes in detail how to operate all the program's basic functionalities.
- The **third part** digs deeper into some of the molecular modeling and bioinformatic features of the program. In this part, you will also find our "Bioinformatics explained" sections. These sections elaborate on the algorithms and analyses of *CLC Main Workbench* and provide more general knowledge of molecular modeling and bioinformatic concepts.
- The **fourth part** is the Appendix and Index.

Each chapter includes a short table of contents.

1.9.1 Text formats

In order to produce a clearly laid-out content in this manual, different formats are applied:

- A feature in the program is in bold starting with capital letters. (Example: **Navigation Area**)
- An explanation of how a particular function is activated, is illustrated by "|" and bold. (E.g.: **select the element | Edit | Rename**)

1.10 Latest improvements

CLC Main Workbench is under constant development and improvement. A detailed list that includes a description of new features, improvements, bugfixes, and changes for the current version of *CLC Main Workbench* can be found at:

<http://www.clcbio.com/products/latest-improvements-main-workbench/>

Chapter 2

Tutorials

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2.1 Getting Started

This brief tutorial will take you through the most basic steps of working with the CLC Workbenches. The tutorial introduces the user interface, shows how to create a folder, and demonstrates how to import your own existing data into the program. The *CLC Main Workbench* will be used to illustrate these functions.

When you open *CLC Main Workbench* for the first time, the user interface looks like figure 2.1.

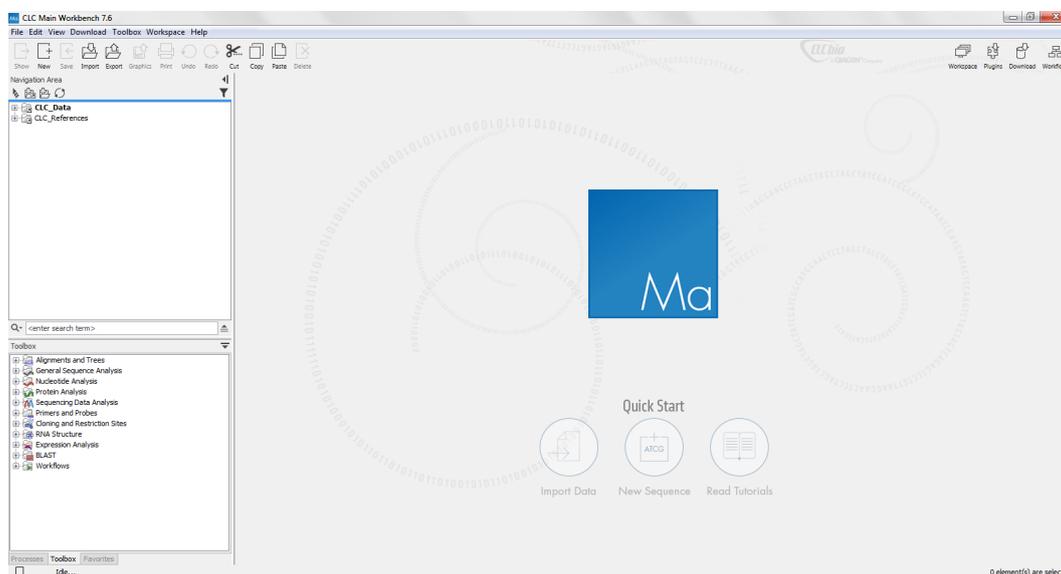


Figure 2.1: The user interface as it looks when you start the program for the first time. (Mac version of **CLC Main Workbench**. The interface is similar for Windows and Linux.)

At this stage, the important issues are the **Navigation Area** and the **View Area**.

The **Navigation Area** to the left is where you keep all your data for use in the program. Most analyses of *CLC Main Workbench* require that the data is saved in the **Navigation Area**. There are several ways to get data into the **Navigation Area**, and this tutorial describes how to import existing data.

The **View Area** is the main area to the right. This is where the data can be 'viewed'. In general, a **View** is a display of a piece of data, and the **View Area** can include several **Views**. The **Views** are represented by tabs, and can be organized e.g. by using 'drag and drop'.

2.1.1 Creating a folder

When *CLC Main Workbench* is started there is one element in the **Navigation Area** called **CLC_Data**¹. This element is a **Location**. A location points to a folder on your computer where your data for use with *CLC Main Workbench* is stored.

The data in the location can be organized into folders. Create a folder:

File | New | Folder (📁)
or **Ctrl + Shift + N** (⌘ + Shift + N on Mac)

Name the folder 'My folder' and press **Enter**.

2.1.2 Import data

As an example, first generation sequence data as well as high throughput sequencing data can be downloaded from <http://www.clcbio.com/downloads> under "EXAMPLE DATA", Roche/454 pyrosequencing genome data from E. coli commensal strain K-12. The NC_010473.gbk (GenBank format) can be imported by all types of CLC Workbenches, while import of the high throughput sequencing data requires specialised import actions. (This "EXAMPLE DATA" file is chosen for demonstration purposes only - you may have another file on your desktop, which you can use to follow this tutorial. You can import all kinds of files.)

The sequence data is imported into the folder that was selected in the **Navigation Area**, before you clicked **Import**. Double-click the sequence in the **Navigation Area** to view it. The NC_010473.gbk (GenBank format) result looks like figure 2.2 while the high throughput data looks like figure 2.3.

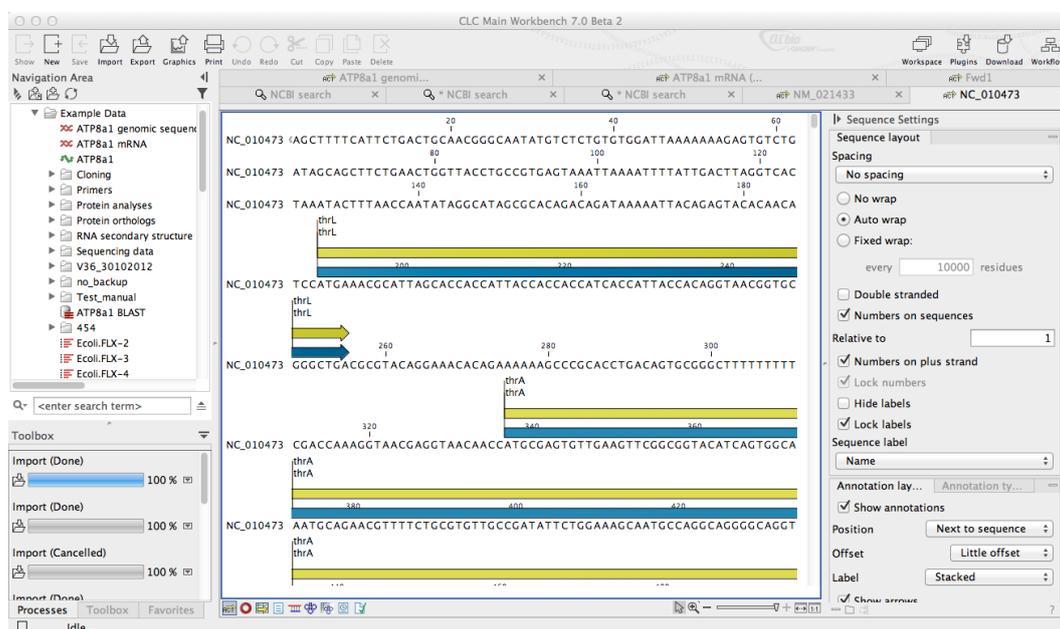


Figure 2.2: The NC_010473.gbk (GenBank format) file is imported and opened.

The sequence is imported into the folder that was selected in the **Navigation Area**, before you clicked **Import**. Double-click the sequence in the **Navigation Area** to view it.

¹If you have downloaded the example data, this will be placed as a folder in *CLC_Data*

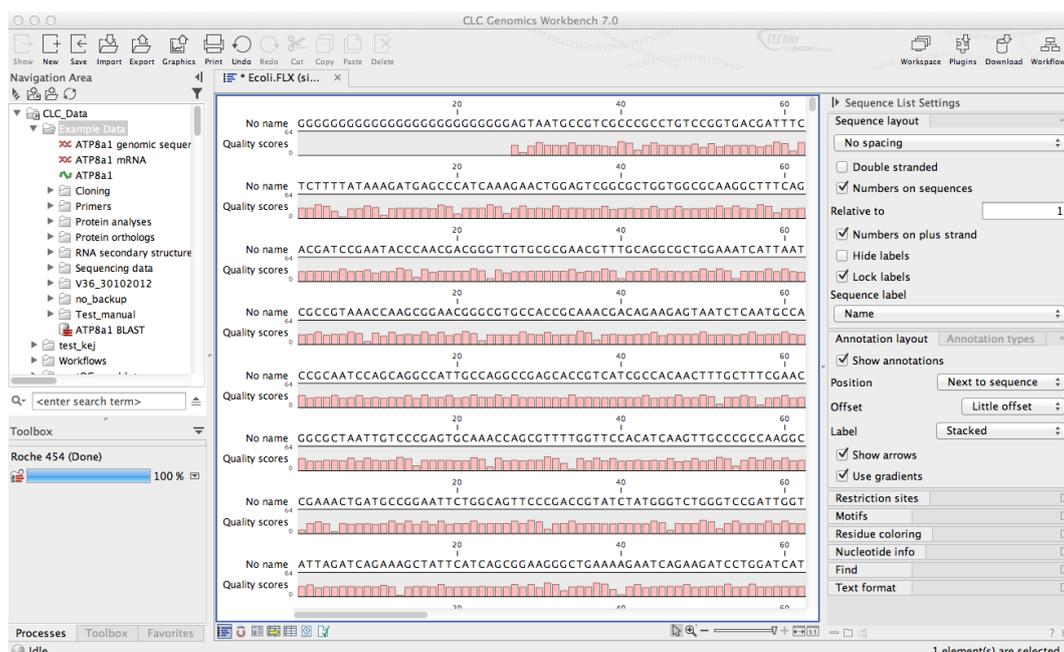


Figure 2.3: The NC_010473 high throughput data imported and opened.

2.2 View a DNA Sequence

This brief tutorial will take you through some different ways to display a sequence in the program. The tutorial introduces zooming on a sequence, dragging tabs, and opening selection in new view.

We will be working with the sequence called *pcDNA3-atp8a1* located in the 'Cloning' folder in the Example data. Double-click the sequence in the **Navigation Area** to open it. The sequence is displayed with annotations above it. (See figure 2.4).

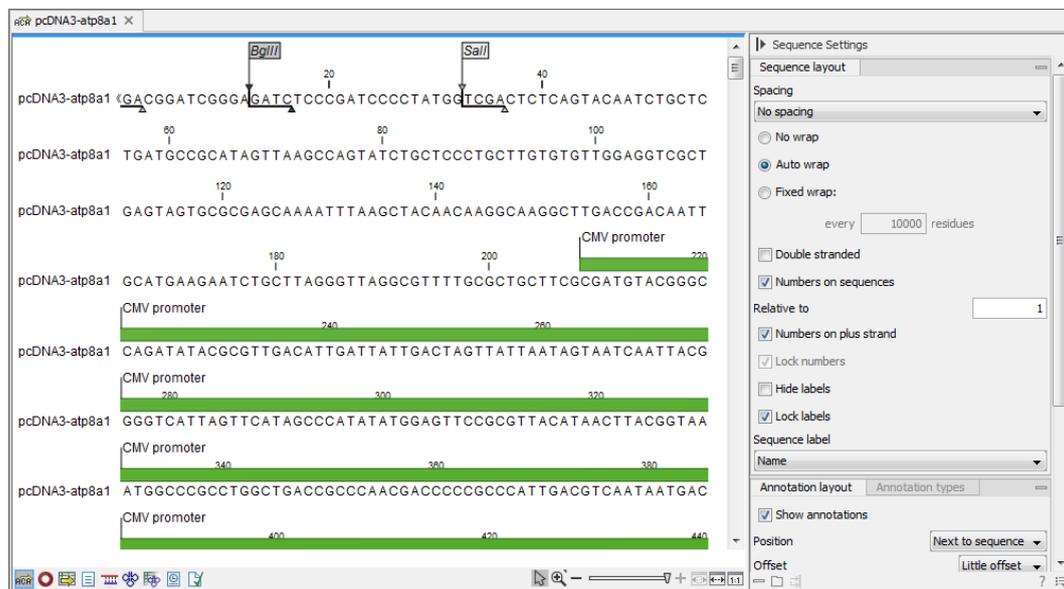


Figure 2.4: Sequence *pcDNA3-atp8a1* opened in a view.

As default, CLC Main Workbench displays a sequence with annotations (colored arrows on the

sequence like the green promoter region annotation in figure 2.4) and zoomed to see the residues.

In this tutorial we want to have an overview of the whole sequence. Hence;

click Zoom Out (🔍) in the Toolbar | click the sequence until you can see the whole sequence

This sequence is circular, which is indicated by << and >> at the beginning and the end of the sequence.

In the following we will show how the same sequence can be displayed in two different views - one linear view and one circular view. First, zoom in to see the residues again by using the **Zoom In** (🔍) or the **100%** (1:1). Then we make a split view by:

press and hold the Ctrl-button on the keyboard (⌘ on Mac) | click Show as Circular (🔄) at the bottom of the view

This opens an additional view of the vector with a circular display, as can be seen in figure 2.5.

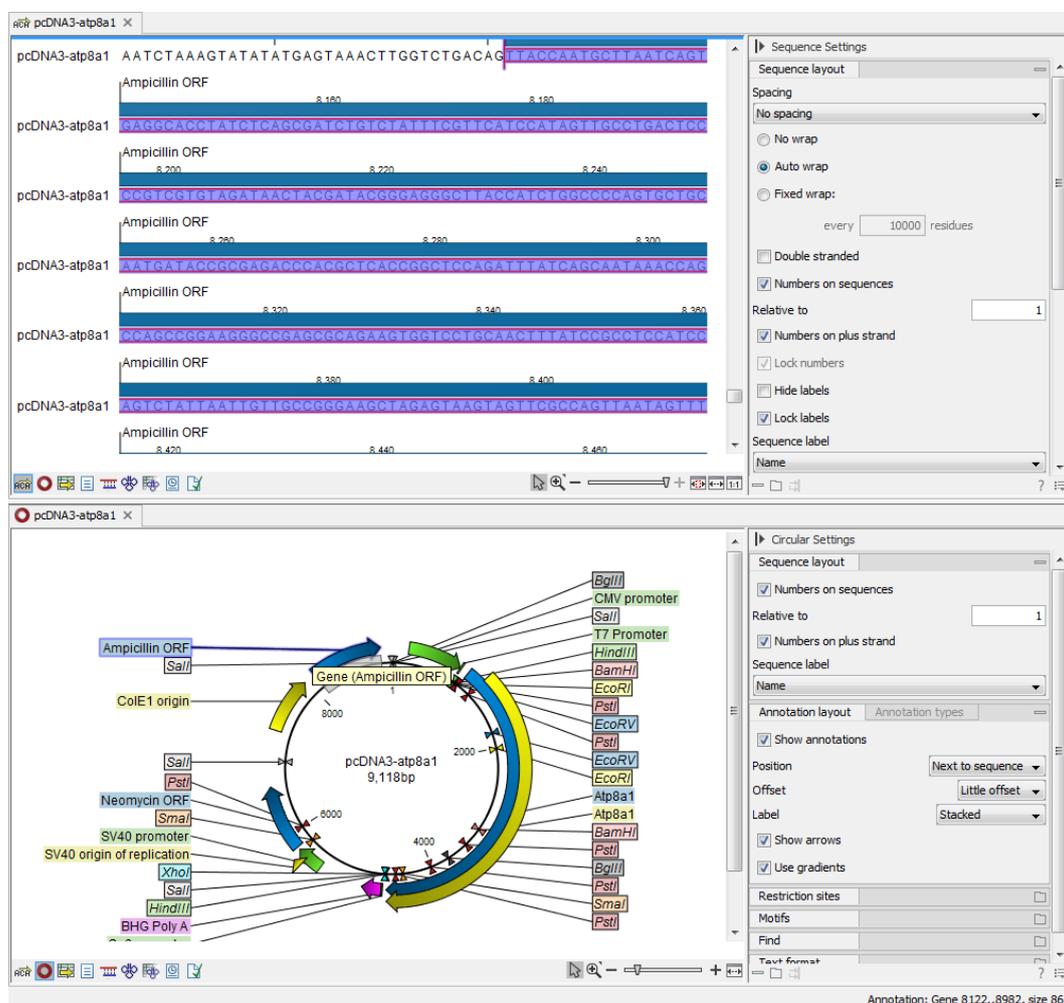


Figure 2.5: The resulting two views which are split horizontally.

You may want to change the text size in the top panel to see more of the sequence. Scroll down

in the **Sequence Settings** panel to **Text format** and change text size to **Tiny** or **Small**. You can also resize the panels relative to each other by clicking and dragging the separator line between them.

Make a selection on the circular sequence (remember to switch to the **Selection** () tool in the tool bar). Note that this selection is also reflected in the linear view above, and that the selection coordinates appear at the bottom right corner of the screen (in figure 2.5 the **Ampicillin ORF** was selected).

You can open a third view of just the selected part of the sequence by right-clicking anywhere in the highlighted sequence text in the top panel and choosing **Open Selection in New View** as shown in figure 2.6.

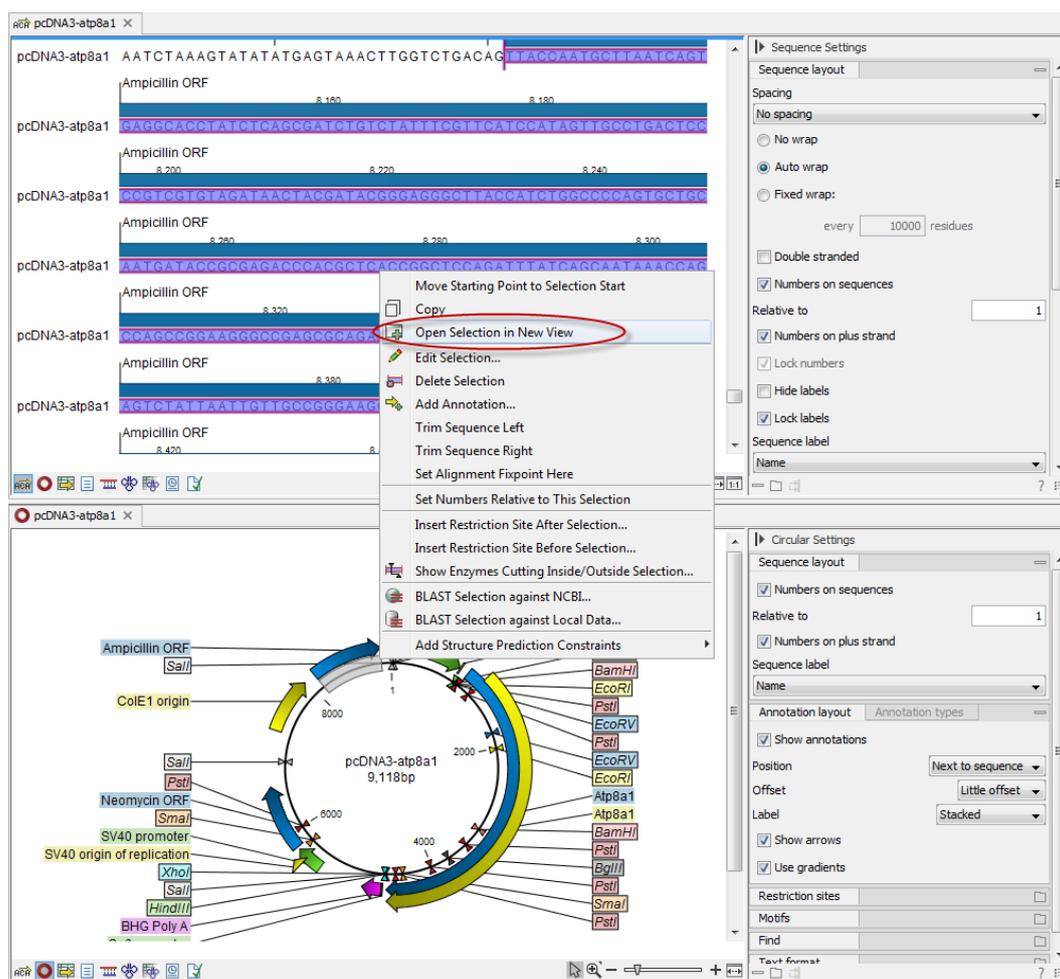


Figure 2.6: Creating a new view panel for just the selected sequence.

Click and drag the new tab from the bottom panel to the top one, next to the existing linear view.

2.3 Side Panel Settings

This brief tutorial will show you how to use the **Side Panel** to change the way your sequences, alignments and other data are shown. You will also see how to save the changes that you made in the **Side Panel**.

Open the protein alignment located under *Protein orthologs* in the **Example data**. The initial view of the alignment has colored the residues according to the Rasmol color scheme, and the alignment is automatically wrapped to fit the width of the view (shown in figure 2.7).

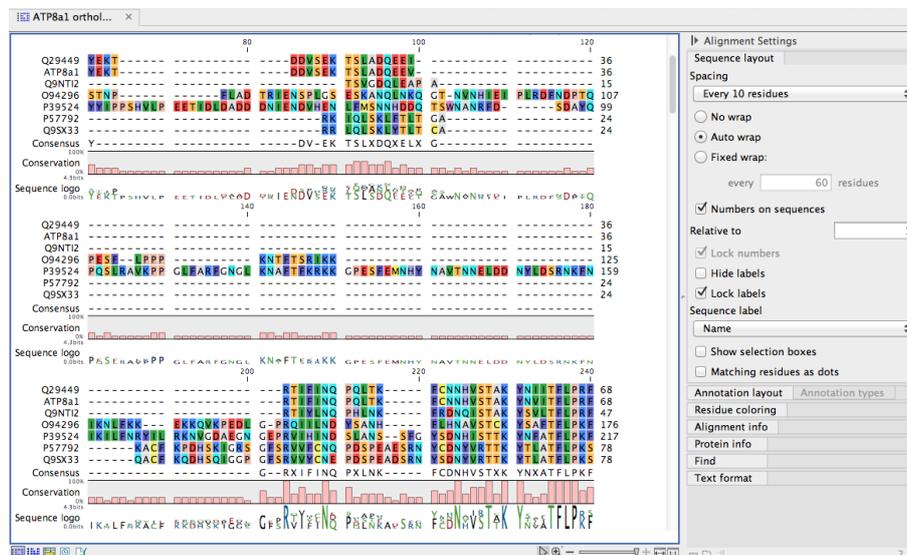


Figure 2.7: The protein alignment as it looks when you open it with background color according to the Rasmol color scheme and automatically wrapped.

Now, we are going to modify how this alignment is displayed. For this, we use the settings in the **Side Panel** to the right. All the settings are organized into groups, which can be expanded / collapsed by clicking the name of the group. The first group is **Sequence Layout** which is expanded by default.

First, select **No wrap** in the **Sequence Layout**. This means that each sequence in the alignment is kept on the same line. To see more of the alignment, you now have to scroll horizontally.

Next, expand the **Annotation Layout** group and select **Show Annotations**. Set the **Offset** to "More offset" and set the **Label** to "Stacked".

Click on the **Annotation Types** tab. Here you will see a list of the types annotation that are carried by the sequences in the alignment (see figure 2.8).

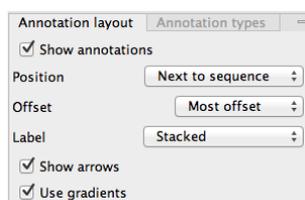


Figure 2.8: The Annotation Layout and the Annotation Types tabs in the Side Panel.

Check the "Region" annotation type, and you will see the regions as red annotations on the sequences.

Next, we will change the way the residues are colored. Click the **Alignment Info** group and under **Conservation**, check "Background color". This will use a gradient as background color for the residues. You can adjust the coloring by dragging the small arrows above the color box.

2.3.1 Saving the settings in the Side Panel

Now the alignment should look similar to figure 2.9.

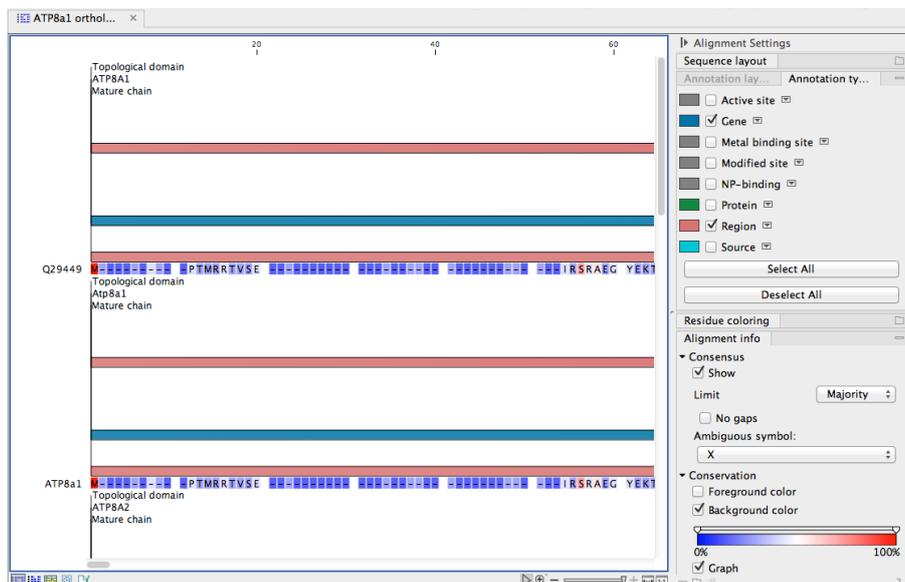


Figure 2.9: The alignment when all the above settings have been changed.

At this point, if you just close the view, the changes made to the **Side Panel** will not be saved. This means that you would have to perform the changes again next time you open the alignment. To save the changes to the **Side Panel**, click the **Save/Restore Settings** button (⚙️) at the bottom of the **Side Panel** and click **Save Alignment View Settings** (see figure 2.10).

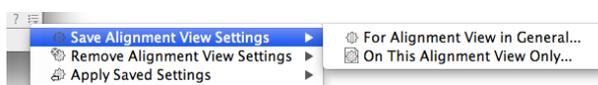


Figure 2.10: Saving the settings of the Side Panel either generally or this particular alignment only.

This will open the dialog shown in figure 2.11.

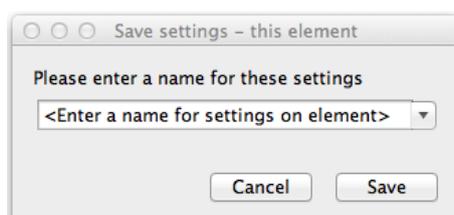


Figure 2.11: Dialog for saving the settings of the Side Panel.

In this way you can save the current state of the settings in the **Side Panel** so that you can apply them to alignments later on. If you check **Always apply these settings**, these settings will be applied every time you open a view of the alignment.

Type "My settings" in the dialog and click **Save**.

2.3.2 Remove alignment view settings

When you click the **Save/Restore Settings** button () and select **Remove Alignments View Settings**, you can choose whether this should be applied generally or on this alignment view only (see figure 2.12).



Figure 2.12: Menu for removing saved settings.

This will open the dialog shown in figure 2.13 and allow you to remove specific settings.

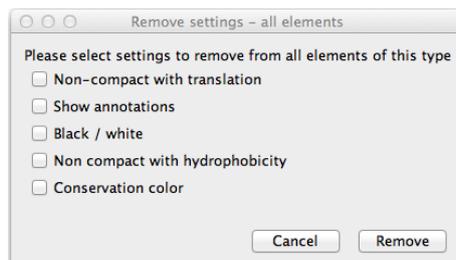


Figure 2.13: Menu for removing saved settings.

2.3.3 Applying saved settings

When you click the **Save/Restore Settings** button () again and select **Apply Saved Settings**, you will see "My settings" in the menu together with some pre-defined settings that the *CLC Main Workbench* has created for you (see figure 2.14).

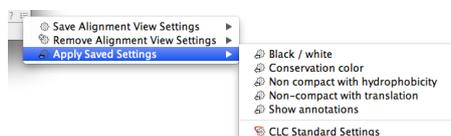


Figure 2.14: Menu for applying saved settings.

Whenever you open an alignment, you will be able to apply these settings. Each kind of view has its own list of settings that can be applied.

At the bottom of the list you will see the "CLC Standard Settings" which are the default settings for the view.

2.4 Microarray-Based Expression Analysis Part I: Getting Started

This tutorial is the first part of a series of tutorials about expression analysis. Expression analysis often requires advanced skills in statistics, but this tutorial is intended to show a straight-forward example of how to identify and interpret the differentially expressed genes in samples from two different tissues. If you are familiar with the statistical concepts and issues within expression analysis, you may find this tutorial too simplistic, but we have favored a simple and quick introduction over an exhaustive and more "correct" explanation.

The data comes from a study of gene expression in tissues from cardiac left ventricle and diaphragm muscle of rats [[van Lunteren et al., 2008](#)]. During this series of tutorials, you will

see how to import and set up the data in an experiment with two groups (part I), to perform quality checks on the data (part II), to perform statistics and clustering to identify and visualize differentially expressed genes (part III), and finally to use annotations to categorize and interpret patterns among the differentially expressed genes in a biological context (part IV).

2.4.1 Importing array data

First, import the data set which can be downloaded from the Gene Expression Omnibus (GEO) database at NCBI: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6943&targ=gsm&form=text&view=data>.

After download, click **Import** (📁) in the Tool bar and choose the "Standard import" option. Select the file called "GSE6943" and choose where you want to save it in the Navigation Area of your workbench. You will now have 12 arrays in your **Navigation Area** as shown in figure 2.15.

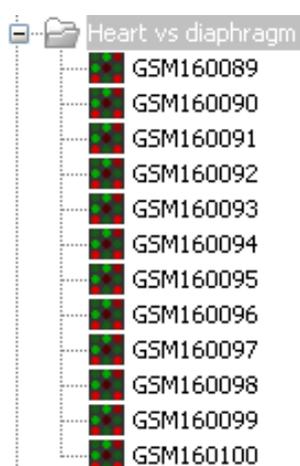


Figure 2.15: 12 microarrays have been imported.

2.4.2 Grouping the samples

The next step is to tell the workbench how the 12 samples are related.

This is done by setting up an **Experiment** (📊). An **Experiment** is the central data type when analyzing expression data in the *CLC Main Workbench*. It includes a set of samples and information about how the samples are related (which groups they belong to). The **Experiment** is also used to accumulate calculations like t-tests and clustering.

1. To set up the experiment:

Toolbox | Transcriptomics Analysis (📁) | Set Up Experiment (📊)

2. Select the 12 arrays that you have imported (see figure 2.16).

Note that we use "samples" as the general term for both microarray-based expression values and sequencing-based expression values. Click **Next**

3. In the next wizard window, you can define the number of groups in the experiment. Since we compare heart tissue with diaphragm tissue, we use a two-group comparison. Leave the option as **Unpaired** as in figure 2.17. Click on the button labeled **Next**.

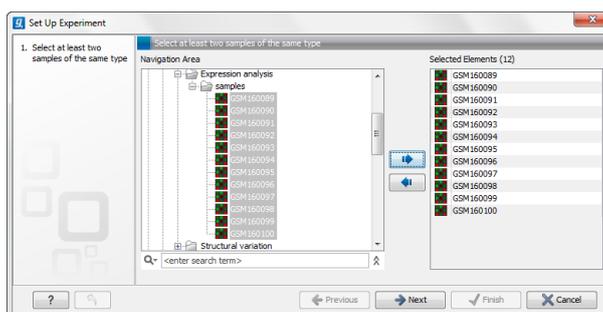


Figure 2.16: Select the 12 microarrays that have been imported.

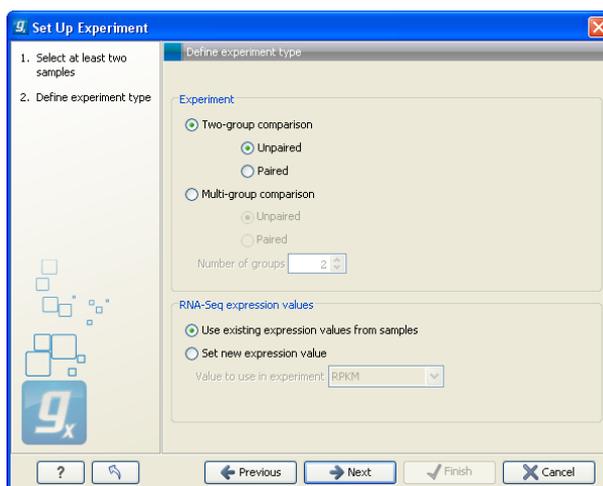


Figure 2.17: Defining the number of groups.

4. Name the first group **Heart** and the second group **Diaphragm** (figure 2.18) and click **Next**.

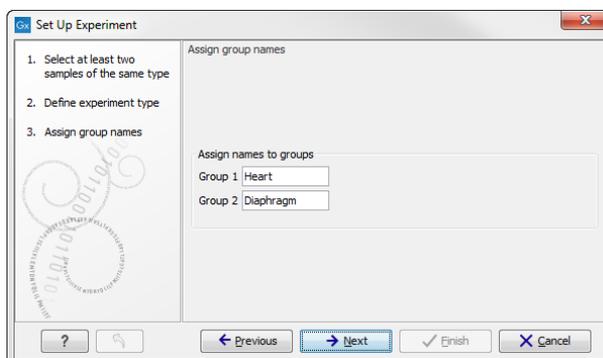


Figure 2.18: Naming the groups.

5. Here you see a list of all the samples preselected (figure 2.19). Select the first 6 samples (by clicking in the group column of the first sample and while holding down the mouse button you drag and select the other five samples), right-click and select **Heart**. Select the last 6 samples, right-click and select **Diaphragm**. In this way you define which group each sample belongs to. Click **Next**.
6. Chose to **Save** the experiment, indicate where in your Navigation Area you would like to save it and click **Finish**.

The experiment is created. Note that the information from samples located in the **Navigation**

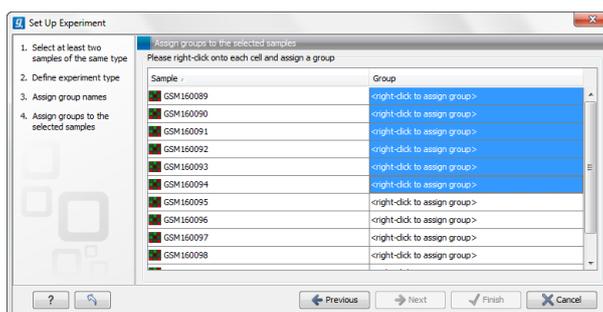


Figure 2.19: Assigning the samples to groups.

Area is copied into the experiment, so they now exist independently of each other.

2.4.3 The experiment table

Once it is created, the experiment can be opened in a table as shown in figure 2.20.

| Feature ID | Tot... | Experiment | | | | GSM160089 | | GSM160090 | | G... |
|------------|--------|-------------------------|-----------------------|------------------------------|-------------------------------|-------------------|---------------|-------------------|---------------|------|
| | | Range (original values) | IQR (original values) | Difference (original values) | Fold Change (original values) | Expression values | Presence call | Expression values | Presence call | |
| 1367452_at | 12 | 862.00 | 470.50 | 464.82 | 1.19 | 2,532.90 P | 2,518.60 P | 2,518.60 P | 2,... | |
| 1367453_at | 12 | 1,231.00 | 430.80 | 428.37 | 1.13 | 3,464.20 P | 3,197.40 P | 3,197.40 P | 3,... | |
| 1367454_at | 12 | 536.50 | 349.10 | 153.85 | 1.10 | 1,620.80 P | 1,870.50 P | 1,870.50 P | 1,... | |
| 1367455_at | 12 | 2,196.20 | 1,352.90 | -772.35 | -1.17 | 5,512.50 P | 4,103.90 P | 4,103.90 P | 5,... | |
| 1367456_at | 12 | 2,095.50 | 1,354.20 | -1,205.65 | -1.27 | 6,090.80 P | 5,352.20 P | 5,352.20 P | 5,... | |
| 1367457_at | 12 | 508.20 | 319.00 | -64.73 | -1.07 | 1,093.90 P | 1,134.30 P | 1,134.30 P | 1,... | |
| 1367458_at | 12 | 268.30 | 148.90 | 112.30 | 1.38 | 347.80 P | 223.90 P | 223.90 P | 1,... | |
| 1367459_at | 12 | 3,993.80 | 2,434.30 | 2,557.35 | 1.36 | 7,665.80 P | 7,415.90 P | 7,415.90 P | 7,... | |
| 1367460_at | 12 | 1,182.80 | 557.00 | -484.73 | -1.17 | 3,155.70 P | 2,946.90 P | 2,946.90 P | 3,... | |
| 1367461_at | 12 | 485.70 | 280.20 | 184.35 | 1.29 | 807.00 P | 610.30 P | 610.30 P | 3,... | |
| 1367462_at | 12 | 1,032.50 | 509.70 | 268.23 | 1.08 | 3,207.50 P | 3,371.30 P | 3,371.30 P | 3,... | |
| 1367463_at | 12 | 1,621.60 | 910.00 | 701.97 | 1.21 | 5,510.30 P | 3,050.30 P | 3,050.30 P | 3,... | |
| 1367464_at | 12 | 317.70 | 202.00 | -111.67 | -1.13 | 797.70 P | 1,038.90 P | 1,038.90 P | 1,... | |
| 1367465_at | 12 | 699.00 | 196.80 | 257.50 | 1.22 | 1,103.10 P | 1,281.80 P | 1,281.80 P | 1,... | |
| 1367466_at | 12 | 265.50 | 122.50 | -54.67 | -1.04 | 1,385.10 P | 1,321.30 P | 1,321.30 P | 1,... | |

Figure 2.20: The experiment table.

The table includes the expression values for each sample and in addition a few extra values have been calculated such as the range, the IQR (Interquartile Range), fold change and difference values and the present counts for the whole experiment and the individual groups (note that absent/present calls are not available on all kind of data).

Save the experiment and you are ready to proceed to the expression analysis tutorial part II.

2.5 Microarray-Based Expression Analysis Part II: Quality Control

This tutorial is the second part of a series of tutorials about expression analysis. We continue working with the data set introduced in the first tutorial.

In this tutorial we will examine various methods to perform quality control of the data.

2.5.1 Transformation

First we inspect to what extent the variance in expression values depends on the mean.

1. For this we create an **MA Plot**:

Toolbox | Transcriptomics Analysis () | **General Plots | Create MA Plot** ()

2. Since the MA plot compares two samples, we will start out selecting any two of the 12 arrays. Leave the parameters as default and click **Finish**.
3. This will show a plot similar to the one shown in figure 2.21.

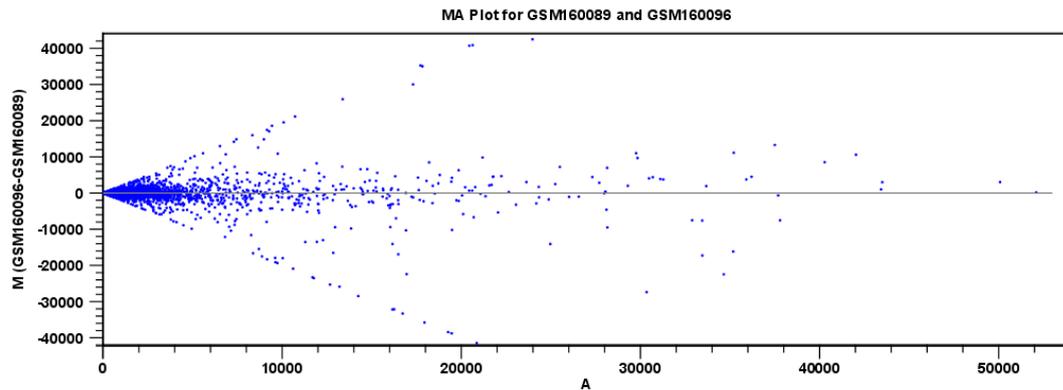


Figure 2.21: MA plot before transformation.

The X axis shows the mean expression level of a feature on the two arrays and the Y axis shows the difference in expression levels for a feature on the two arrays. From the plot shown in figure 2.21 it is clear that the variance increases with the mean.

To remove some of the dependency, we want to **transform** the data.

1. Go to:

Toolbox | Transcriptomics Analysis (📄) | Transformation and Normalization | Transform (📄)

2. Select the same two arrays as used for the plot and click **Next**.
3. Leave the "Values to analyze" as "Original expression values" but change the "Logarithm transformation" to **Log 2**. Click **Finish**.
4. Now create an MA plot again as described above and using the samples you just transformed. Click **Next**
5. In the set parameters window, you can see that you now also have the option to choose **Transformed expression values** as parameters (see figure 2.22).



Figure 2.22: Select the transformed expression values.

Original, Transformed and Normalized expression values are used several places when expression values are used in a calculation. Select the "transformed expression values" option and click on **Finish**.

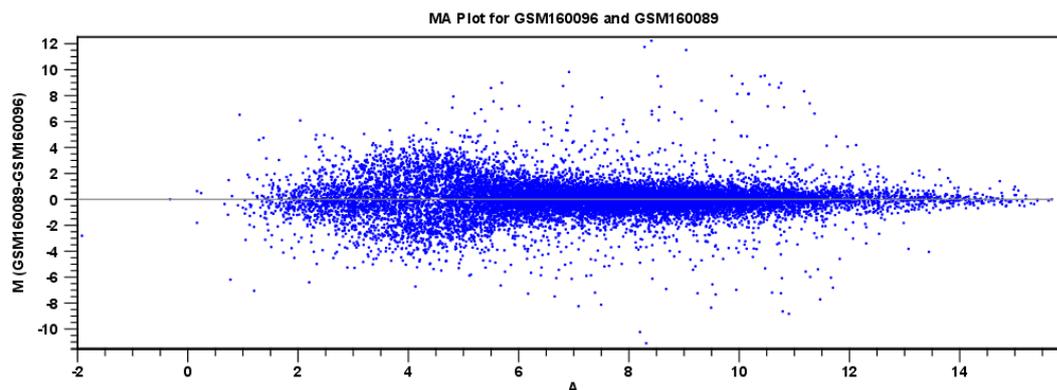


Figure 2.23: MA plot after transformation.

6. This will result in a quite different plot as shown in figure 2.23.

The much more symmetric and even spread indicates that the dependence of the variance on the mean is not as strong as it was before transformation.

7. We have now only transformed the values of the two samples used for the MA plot. Hence, we need to transform the expression values within the entire experiment, as we will use the transformed data in the further analysis. To transform the entire experiment:

Toolbox | Transcriptomics Analysis (📁) | Transformation and Normalization | Transform (📄)

8. Select the experiment created in the first part of this tutorial series, click **Next**, choose **Log 2** transformation and click **Finish**.

If you open the table, you will see that all the samples have an extra column with transformed expression values (figure 2.24).

| Feature ID | Experiment | | | GSM160089 | | | GSM160090 | | |
|------------|----------------|----------------|----------------|----------------|---------------|---------------|----------------|---------------|-----|
| | Total prese... | IQR - Expre... | IQR - Trans... | Expression ... | Presence call | Transforme... | Expression ... | Presence call | Tra |
| 1367452_at | 12 | 470,50 | 0,08 | 2.532,90 P | | 3,40 | 2.518,60 P | | |
| 1367453_at | 12 | 430,80 | 0,05 | 3.464,20 P | | 3,54 | 3.197,40 P | | |
| 1367454_at | 12 | 349,10 | 0,09 | 1.620,80 P | | 3,21 | 1.870,50 P | | |
| 1367455_at | 12 | 1.352,90 | 0,12 | 5.512,50 P | | 3,74 | 4.103,90 P | | |
| 1367456_at | 12 | 1.264,20 | 0,11 | 6.090,80 P | | 3,78 | 5.352,20 P | | |
| 1367457_at | 12 | 319,00 | 0,15 | 1.093,90 P | | 3,04 | 1.134,30 P | | |
| 1367458_at | 12 | 148,90 | 0,19 | 347,80 P | | 2,54 | 223,90 P | | |
| 1367459_at | 12 | 2.434,30 | 0,12 | 7.665,80 P | | 3,88 | 7.415,90 P | | |
| 1367460_at | 12 | 557,00 | 0,08 | 3.155,70 P | | 3,50 | 2.946,90 P | | |
| 1367461_at | 12 | 280,20 | 0,16 | 507,00 P | | 2,71 | 610,30 P | | |
| 1367462_at | 12 | 309,70 | 0,04 | 3.207,50 P | | 3,51 | 3.371,30 P | | |
| 1367463_at | 12 | 510,00 | 0,06 | 3.510,30 P | | 3,55 | 3.050,30 P | | |
| 1367464_at | 12 | 202,00 | 0,10 | 797,70 P | | 2,90 | 1.038,90 P | | |

Figure 2.24: Transformed expression values have been added to the table.

There is also an extra column for transformed group means and transformed IQR.

2.5.2 Comparing spread and distribution

In order to perform meaningful statistical analysis and inferences from the data, you need to ensure that the samples are comparable. Systematic differences between the samples that are likely to be due to noise (e.g. differences in sample preparation and processing) rather than true biological variability should be removed. To examine and compare the overall distribution of the transformed expression values in the samples you may use a **Box plot** (📊).

1. Go to: **Toolbox | Transcriptomics Analysis** (📁) | **Quality Control | Create Box Plot** (📊)
2. Select the experiment and click **Next**.
3. Choose the option **Transformed expression values** and click on the button labeled **Next**.
4. Chose to open your results before clicking on **Finish**.

The box plot is shown in figure 2.25.

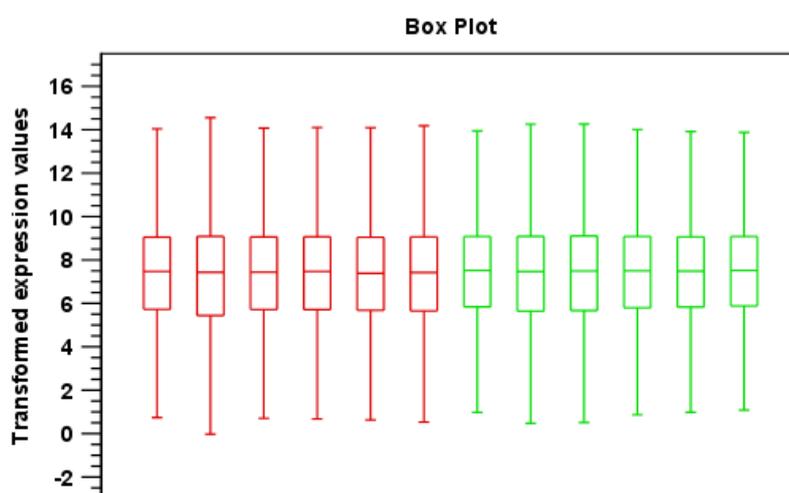


Figure 2.25: A box plot of the 12 samples in the experiment, colored by group.

This plot looks very good because none of the samples stands out from the rest. If you compare this plot to the one shown in figure 2.26 from another data set, you can see the difference.

The second sample from the left has a distribution that is quite different from the others. If you have a data set like this, then you should consider removing the bad quality sample.

2.5.3 Group differentiation

The next step in the quality control is to check whether the overall variability of the samples reflect their grouping. In other words we want the replicates to be relatively homogenous and distinguishable from the samples of the other group.

1. First, we perform a **Principal Component Analysis (PCA)**:

Toolbox | Transcriptomics Analysis (📁) | **Quality Control | Principal Component Analysis** (📊)

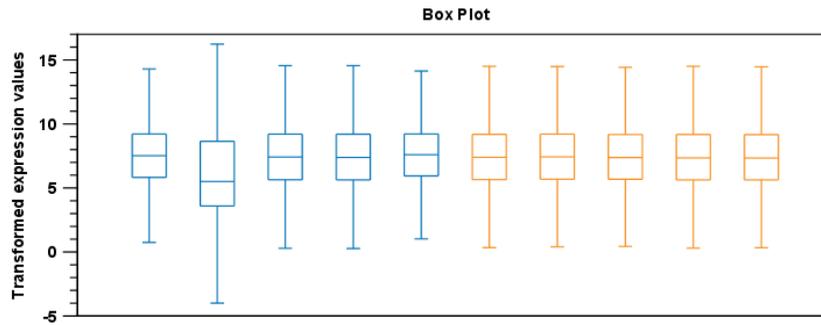


Figure 2.26: A box plot showing one sample that stands out from the rest.

2. Select the experiment and click **Next**. Keep the parameters as set by default to "Original expression values" and **Save** the data before clicking on **Finish**. This will create a PCA plot as shown in figure 2.27).

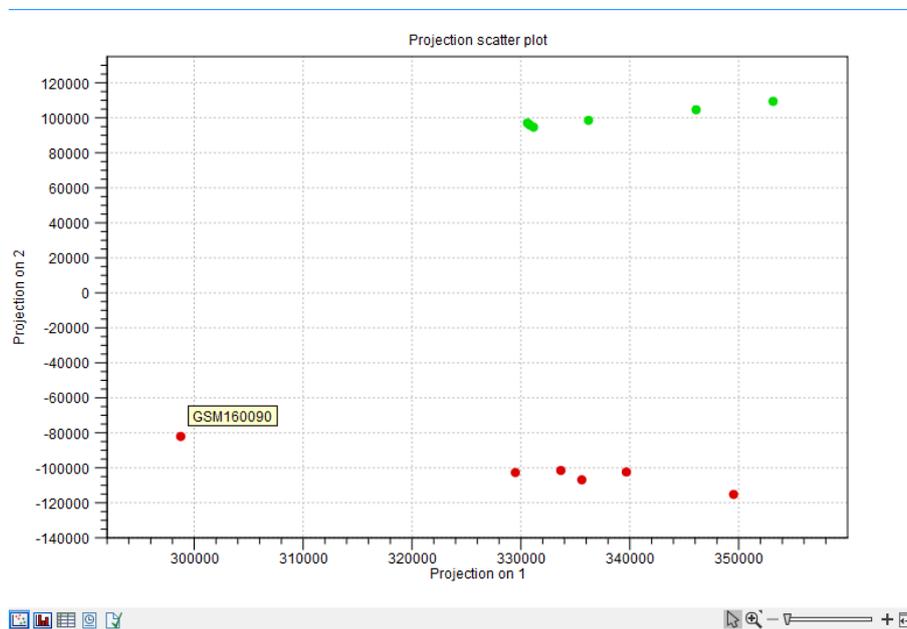


Figure 2.27: A principal component analysis colored by group.

The plot shows the projection of the samples onto the two-dimensional space spanned by the first and second principal components (these are the orthogonal directions in which the data exhibits the largest and second-largest variability).

3. The dots are colored according to the groups, and the plot shows that they cluster together. There is only one outlier - to see which sample it is, place the mouse cursor on the dot for a second, and you will see that it is the *GSM160090* from the *Heart* group.
4. You can display this information in the plot using the settings in the **Side Panel** to the right of the view:

Dot properties | select **GSM160090** in the drop-down box | **Show names**

In this way you can control the coloring and dot types of the different samples and groups (see figure 2.28).

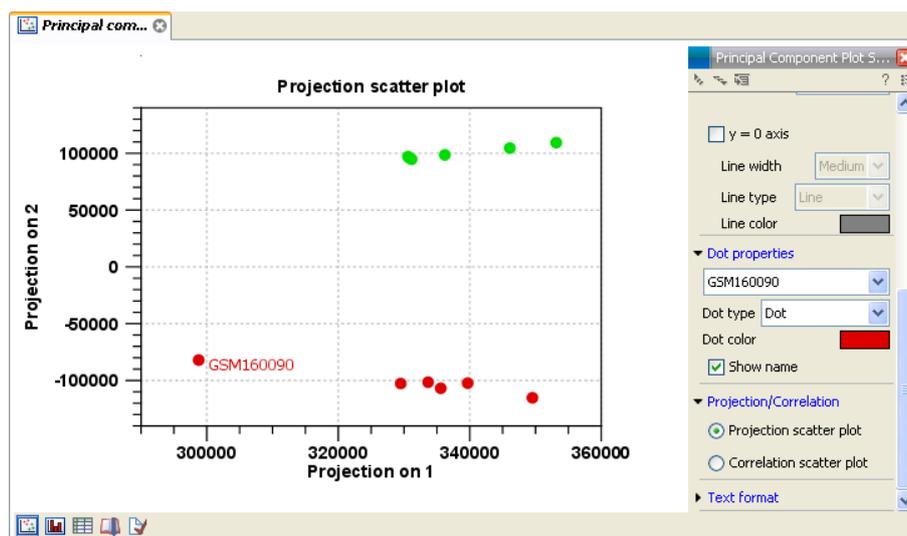


Figure 2.28: Naming the outlier.

- In order to complement the principal component analysis, we will also do a hierarchical clustering of the samples to see if the samples cluster in the groups we expect:

Toolbox | Transcriptomics Analysis (📁) | Quality Control | Hierarchical Clustering of Samples (📁)

- Select the experiment and click **Next**. Leave the parameters at their default as seen in figure 2.29) and click **Finish**.

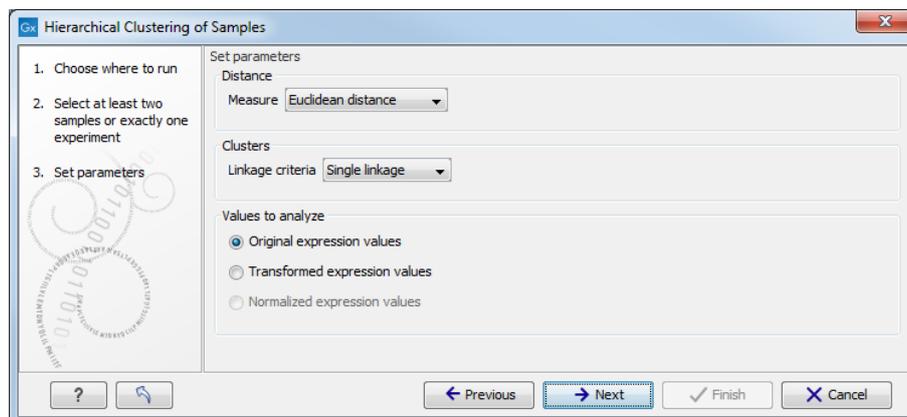


Figure 2.29: Set parameters for the Hierarchical Clustering of Samples tool.

This will display a heat map showing the clustering of samples at the bottom (see figure 2.30).

The two overall groups formed are identical to the grouping in the experiment. You can double-check by placing your mouse on the name of the sample - that will show which group it belongs to.

Since both the principal component analysis and the hierarchical clustering confirms the grouping of the samples, we have no reason to be sceptical about the quality of the samples and we conclude that the data is OK.

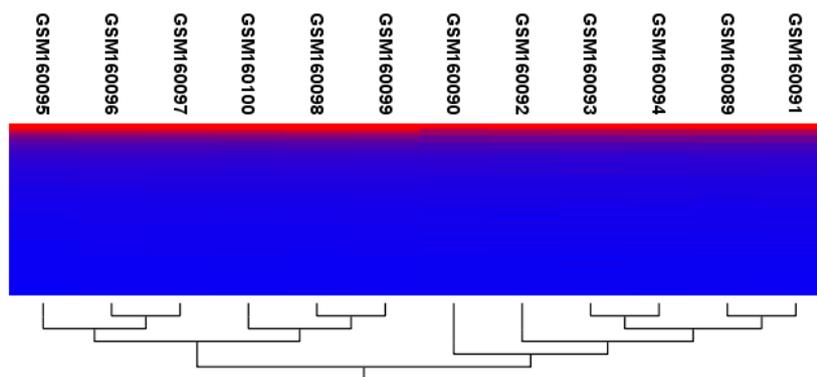


Figure 2.30: Sample clustering.

Note that the heat map is not a new element to be stored in the **Navigation Area** - it is just another way of looking at the experiment. You can use the buttons at the bottom of the editor to switch between different views in figure 2.31.



Figure 2.31: Different views on an experiment.

To summarize this part about quality control, it looks like the data have good quality, and we are now ready to proceed to the next step where we do some statistical analysis to see which genes are differentially expressed. In part III of the tutorial series we will be looking into the different views in more detail.

2.6 Microarray-Based Expression Analysis Part III: Differentially Expressed Genes

This tutorial is the third part of a series of tutorials about expression analysis. We continue working with the data set introduced in the first tutorial.

In this tutorial we will identify and investigate the genes that are differentially expressed.

2.6.1 Statistical analysis

First we will carry out some statistical tests that we will use to identify the genes that are differentially expressed between the two groups.

1. Go to:

Toolbox | Transcriptomics Analysis (📁) | Statistical Analysis | Gaussian Statistical Analysis (📊)

2. Select the experiment created in part I of the tutorial and click **Next**.
3. Leave the parameters at the default as shown in figure 2.32 and click **Next** again.

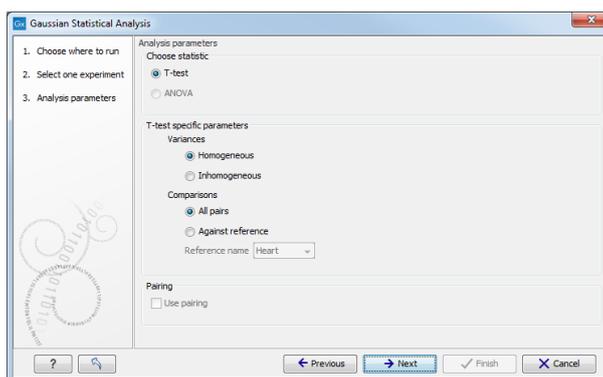


Figure 2.32: Set parameters for the Gaussian Statistical Analysis tool.

4. Select the "transformed expression values" option and check the two corrected p-values as well (figure 2.33). You can read more about what they mean by clicking the **Help** (?) button in the dialog.

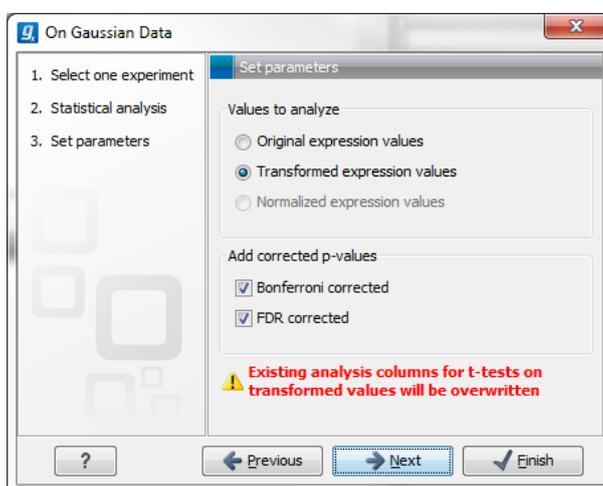


Figure 2.33: Statistical analysis.

5. Save the results (it will be saved in the existing table) and click on **Finish**

A number of extra columns will be added to your experiment (see figure 2.34). For this analysis we will use the FDR p-value which is a measure that allows us to control how big a proportion of false positives (genes that we think are differentially expressed but really are not) we are willing to accept.

Click the **FDR p-value correction** column to sort it with the lowest values at the top. If you scroll down to values around 5E-4 you can clearly see the difference between using the FDR p-value and the Bonferroni-corrected p-value which is much stricter (p-values approaching 1 -).

2.6.2 Filtering p-values

To do a more refined selection of the genes that we believe to be differentially expressed, we use the advanced filter located at the top of the experiment table. Click the **Advanced Filter** (🔍) button and you will see that the simple text-based filter is now replaced with a more advanced filter. Select **t-test: Heart vs Diaphragm transformed values - FDR p-value correction** in the

Figure 2.34: FDR p -values compared to Bonferroni-corrected p -values.

first drop-down box, select \leftarrow in the next, and enter 0.0005 (or 0,0005 depending on your locale settings). Click **Apply** or press **Enter**.

This will filter the table so that only values below 0.0005 are shown (see figure 2.35).

Figure 2.35: Filtering on FDR p -values.

You can see that 1471 genes fulfilled this criterion (marked with a red circle).

2.6.3 Inspecting the volcano plot

Another way of looking at this data is to click the **Volcano Plot**  at the bottom of the view. Press and hold the Ctrl key while you click (\mathbb{C} on Mac).

This will make a split view of the experiment table and the volcano plot as shown in figure 2.36.

The volcano plot shows the difference between the means of the two groups on the X axis and the $-\log_{10}$ p -values on the Y axis.

If you now select the genes in the table (click in the table and press Ctrl + A / \mathbb{C} + A on Mac), you can see that the corresponding dots are selected in the volcano plot (see figure 2.37).

2.6.4 Filtering absent/present calls and fold change

Besides filtering on low p -values, we may also take the absent/present status of the features into consideration. The absent/present status is assigned by the Affymetrix software. There can

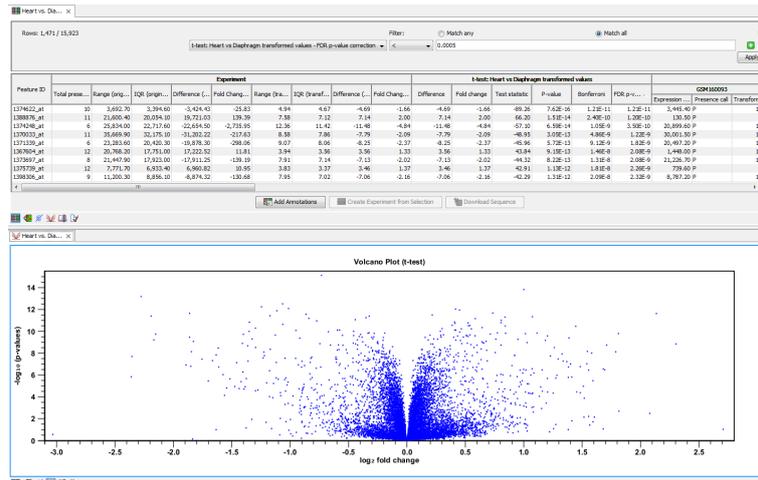


Figure 2.36: Split view of volcano plot and experiment table.

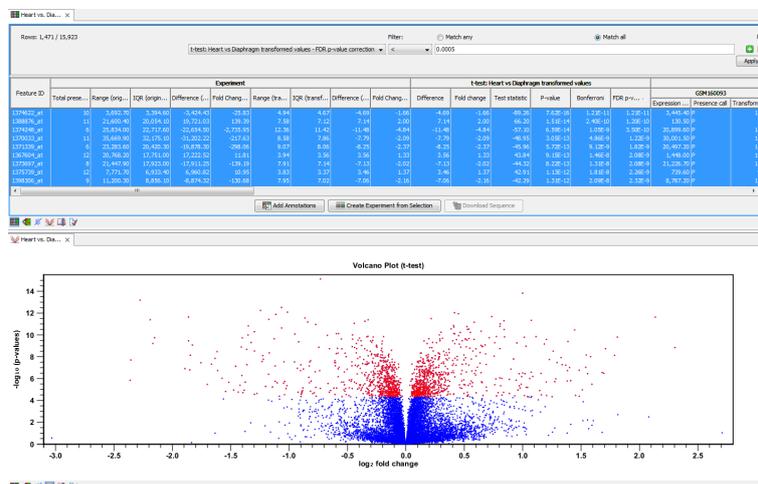


Figure 2.37: Volcano plot where selected dots are colored red.

be a number of reasons why a gene is called *absent*, and sometimes it is simply because the signal is very weak. When a gene is called absent, we may not wish to include it in the list of differentially expressed genes, so we want to filter these out as well.

This can be done in several ways - in our approach we say that for any gene there must not be more than one absent call in each group. Thus, we add more criteria to the filter by clicking the **Add search criterion** (+) button twice and enter the limit for present calls as shown in figure 2.38.

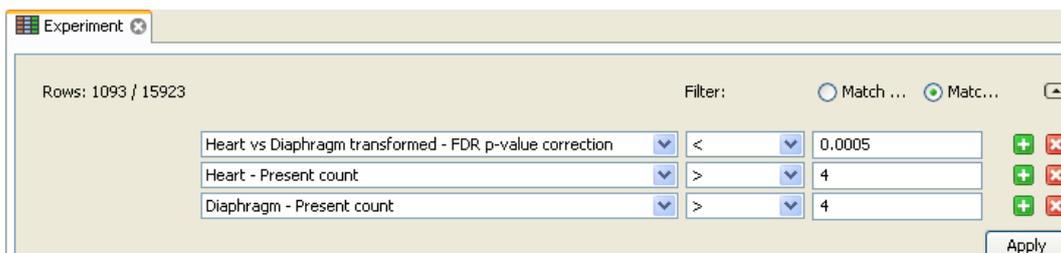


Figure 2.38: Filtering genes where at least 5 out of 6 calls in each group are present.

Before applying this filter, 1471 genes were selected, and this list is now reduced to 1093.

Often the results of microarray experiments are verified using other methods such as QPCR, and then we may want to filter out genes that exhibit differences in expression that are so small that we will not be able to verify them with another method. This is done by adding one last criterion to the filter: Difference should have an absolute value higher than 2 (as we are working with log transformed data, the group mean difference is really the *fold change*, so this filter means that we require a fold change above 2).

This final filtering is shown in figure 2.39.

The screenshot shows a filtering interface with the following details:

- Rows: 142 / 15.923
- Filter: Match any Match all
- Filter 1: t-test: Heart vs Diaphragm transformed values - FDR p-value correction < 0.0005
- Filter 2: Heart - Present count > 4
- Filter 3: Diaphragm - Present count > 4
- Filter 4: t-test: Heart vs Diaphragm transformed values - Difference abs value > 2
- Buttons: +, x for each filter; Apply at the bottom right.

Figure 2.39: The absolute value of group mean difference should be larger than 2.

Note that the **abs value >** is important because the difference could be negative as well as positive.

The result is that we end up with a list of 142 genes that are likely candidates to exhibit differential expression in the two groups.

Click one of the rows and press Ctrl + A (⌘ +A on Mac) to select the 142 genes. You can now inspect the selection in the volcano plot below as shown in figure 2.40.

2.6.5 Saving the gene list

Before we proceed to the final part of the tutorials, we save the list of genes; click on **Create Experiment from Selection** (📄).

This will create a new experiment based on the selection. **Save** (📁) the new experiment next to the old one in your Navigation Area.

2.7 Microarray-Based Expression Analysis Part IV: Annotation Test

This tutorial is the fourth and final part of a series of tutorials about expression analysis. We continue working with the data set introduced in the first tutorial and analyzed in part two and three.

In this tutorial we will annotate the gene list and use the annotations to see if there is a pattern in the biological annotations of the genes in the list of candidate differentially expressed genes.

We use two different methods for annotation testing: Hypergeometric Tests on Annotations and Gene Set Enrichment Analysis (GSEA).

2.7.1 Importing and adding the annotations

First step is to import an annotation file used to annotate the arrays.

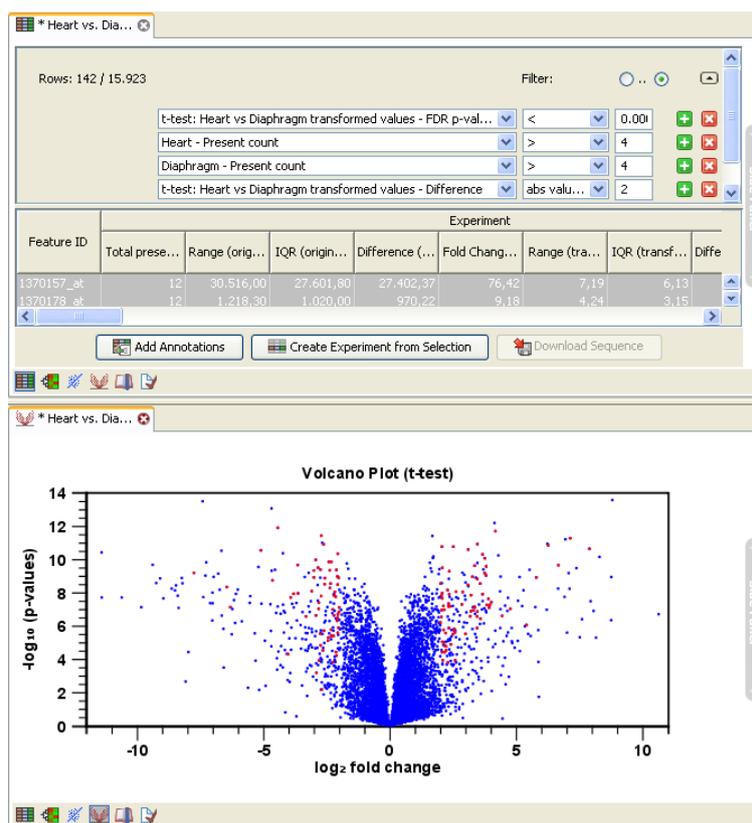


Figure 2.40: 142 genes out of 15923 selected.

1. In this case, the data were produced using an Affymetrix chip, and the annotation file can be downloaded from the web site <http://www.affymetrix.com/support/technical/annotationfilesmain.affx>. You can access the file by search for **RAE230A** (search using the Ctrl + F command of your browser and not the search field from the Affymetrix website). Note that this is a free service but you still have to sign up in order to download the file.
2. To import the annotation file in the workbench, click **Import** (📄) | **Standard Import** in the Tool bar and select the "RAE230A.na35.annot.csv" zip file.
3. Next, annotate the experiment with the annotation tool:
Toolbox | Transcriptomics Analysis (📁) | Annotation Test | Add Annotations (📄)
4. Select the experiment created in the previous tutorial and the annotation file (📄) (figure 2.41) and click **Next**.

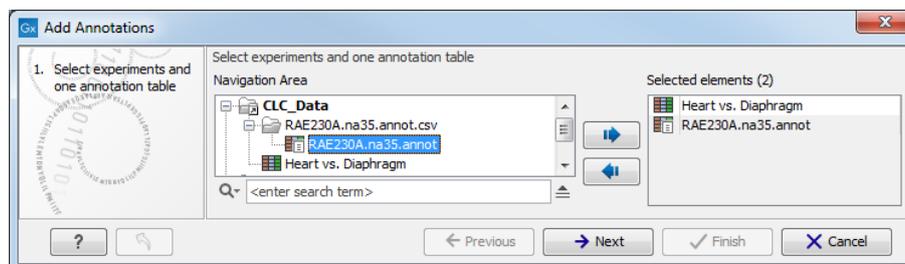


Figure 2.41: Annotating the experiment.

5. Leave the parameters as default (figure 2.42) and click **Next**.

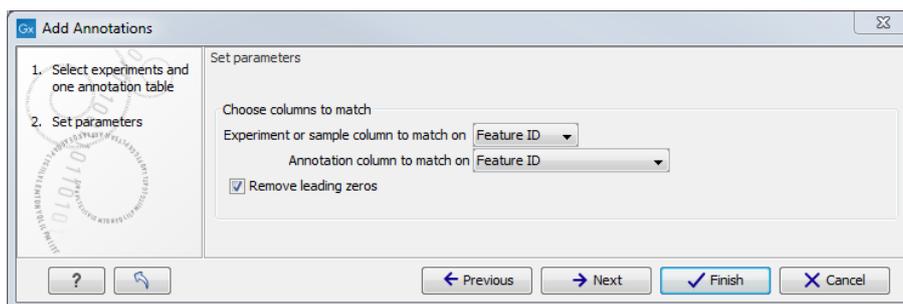


Figure 2.42: Parameters of the Add Annotations tool.

6. **Open** the results and click on **Finish**.

When you look in the **Side Panel** of the experiment, there are a lot of options to show and hide columns in the table. This can be done on several levels. At the **Annotation level** you find a list of all the annotations. Some are shown per default, others you will have to click to show.

An important annotation is the **Gene title** which describes the gene and is much more informative than the Feature ID.

Further down the list you find the annotation type **GO biological process**. We will use this annotation in the next two analyses.

2.7.2 Processes that are over or under represented in the small list

The first annotation test will show whether any of the GO biological processes are over-represented in our small list of 142 differentially expressed genes relate to the full set of genes measured.

1. Go to:

Toolbox | Transcriptomics Analysis (📁) | Annotation Test | Hypergeometric Tests on Annotations (🌐)

2. Select the two experiments (the original full experiment and the small subset of 142 genes) and click **Next** (figure 2.43).

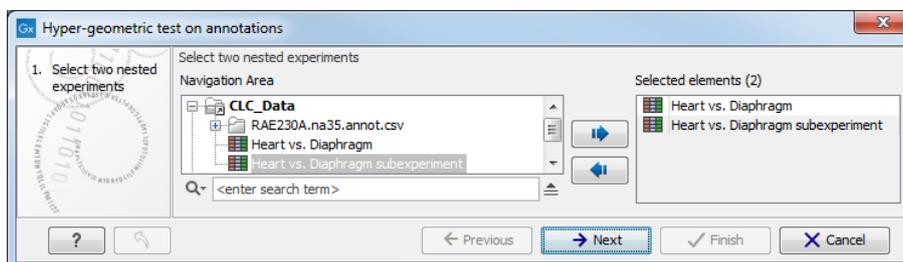


Figure 2.43: Selecting an experiment and a nested sub-experiment.

3. Select **GO biological process** and **Transformed expression values** (see figure 2.44). Click on **Next**.
4. Click **Save** and **Finish** to perform the test.

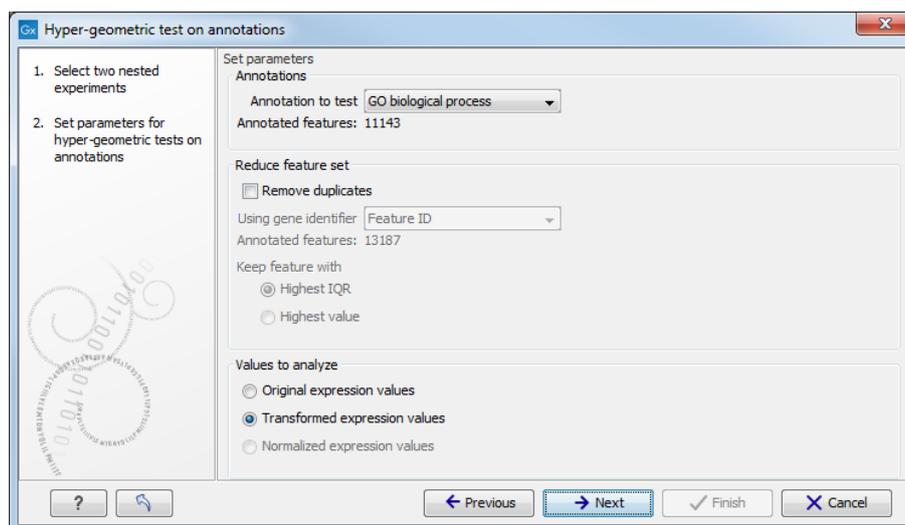


Figure 2.44: Testing on GO biological process.

| Category | Description | Full set | In subset | Expected in subset | Observed - expected | p-value |
|----------|---|----------|-----------|--------------------|---------------------|---------|
| 0006810 | transport | 1053 | 10 | 9 | 1 | 0.43 |
| 0005975 | carbohydrate metabolic process | 135 | 7 | 1 | 6 | 1.60E-4 |
| 0005977 | glycogen metabolic process | 30 | 7 | 0 | 7 | 4.88E-9 |
| 0016310 | phosphorylation | 463 | 7 | 4 | 3 | 0.11 |
| 0007165 | signal transduction | 763 | 6 | 7 | -1 | 0.66 |
| 0042493 | response to drug | 439 | 5 | 4 | 1 | 0.33 |
| 0006468 | protein phosphorylation | 431 | 5 | 4 | 1 | 0.32 |
| 0043066 | negative regulation of apoptotic process | 367 | 5 | 3 | 2 | 0.21 |
| 0015031 | protein transport | 383 | 5 | 3 | 2 | 0.24 |
| 0006874 | cellular calcium ion homeostasis | 67 | 5 | 1 | 4 | 2.74E-4 |
| 0006355 | regulation of transcription, DNA-templated | 868 | 4 | 8 | -4 | 0.95 |
| 0007519 | skeletal muscle tissue development | 46 | 4 | 0 | 4 | 6.48E-4 |
| 0007275 | multicellular organismal development | 386 | 4 | 3 | 1 | 0.43 |
| 0055085 | transmembrane transport | 352 | 4 | 3 | 1 | 0.36 |
| 0006915 | apoptotic process | 330 | 4 | 3 | 1 | 0.32 |
| 0060048 | cardiac muscle contraction | 40 | 4 | 0 | 4 | 3.78E-4 |
| 0000122 | negative regulation of transcription from RNA poly... | 412 | 3 | 4 | -1 | 0.70 |
| 0007507 | heart development | 162 | 3 | 1 | 2 | 0.17 |

Figure 2.45: The result of testing on GO biological process.

The result is shown in figure 2.45.

This table lists the GO categories. Click on the header "In subset" twice to sort values according to how many genes in each categories were in the subset. If you take the first row, there are 1053 genes in this category in the full set, and 10 were expected in the subset. The analysis found 9 in the subset, so this biological process does not seem to be over-represented. On the other hand, on the second row, 7 genes related to "carbohydrate metabolic process" were expected in the subset (based on having 135 in the full set). Only 1 was found, so this process is under-represented and given a p-value of 1.60E-4. to find easily over and under represented processes, click on the header "Observed - expected" and check processes at both end of the list.

2.7.3 Gene Set Enrichment Analysis (GSEA)

The hypergeometric tests on annotations uses a pre-defined subset of differentially expressed genes as a starting point and compares the annotations in this list to those of the genes in the full experiment. The exact limit for this subset is somewhat arbitrary - in our case we could have chosen a p-value less than 0.005 instead of 0.0005 and it would lead to a different result.

Furthermore, only the most apparently differentially expressed genes are used in the subset - one could easily imagine that other categories would be significant based on more genes with e.g., lower fold change or higher p-values.

The Gene Set Enrichment Analysis (GSEA) does not take an *a priori* defined list of differentially expressed genes and compares it to the full list - it uses a single experiment. It ranks the genes on p-value and analyzes whether there are some categories that are over-represented in the top of the list.

1. Go to: **Toolbox | Transcriptomics Analysis (📁) | Annotation Test | Gene Set Enrichment Analysis (GSEA) (🖱️)**
2. Select the original full experiment and click **Next**.
3. In the next step, make sure the **GO biological process** is chosen, and check the option to **remove duplicates** using "gene symbol" as identifier (see figure 2.46).

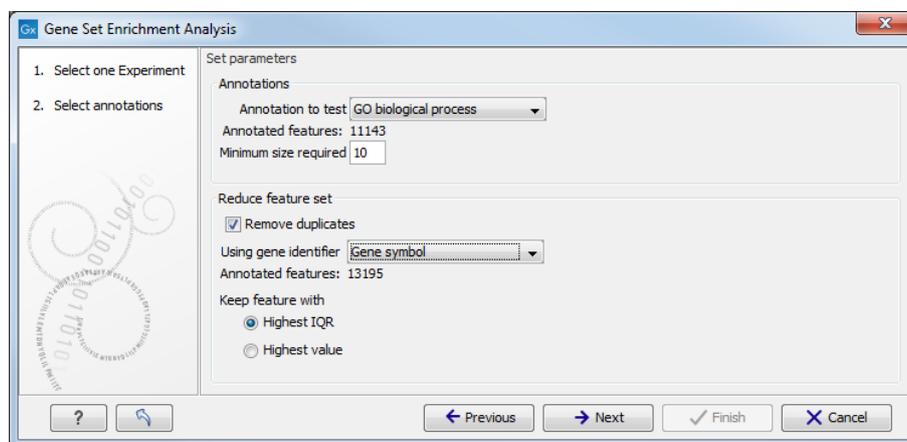


Figure 2.46: Gene set enrichment analysis based on GO biological process.

4. Select the **Transformed expression values**. The number of permutations for p-value calculation is left at 10 000. Click **Next**.
5. Chose to **Open** the result and click on **Finish**.

The result is shown in figure 2.47.

The table is sorted on the lower tail so that the GO categories where up-regulated genes in the first group are over-represented are placed at the top, and the GO categories where up-regulated genes in the second group are over-represented are placed at the bottom.

Note that we could have chosen to filter away genes with less reliable measurements from the experiment (as shown in the previous tutorial) before subjecting it to the GSEA analysis in order to limit noise and aim for a more robust result.

2.8 Visualization of Phylogenetic Trees and Meta Data

This tutorial briefly introduces the reconstruction of phylogenetic trees and visualization using the tree viewer. The main focus in this tutorial is the visualization of metadata as a powerful tool for analyzing your data.

| Gene set enrichment analysis (GSEA) | | | | | |
|-------------------------------------|--|------|----------------|------------|------------|
| Category | Description | Size | Test statistic | Lower tail | Upper tail |
| 0007519 | skeletal muscle tissue development | 46 | -31.43 | 0.00 | 1.00 |
| 0006936 | muscle contraction | 38 | -41.25 | 0.00 | 1.00 |
| 0003009 | skeletal muscle contraction | 23 | -34.67 | 1.00E-4 | 1.00 |
| 0006412 | translation | 205 | -16.34 | 1.00E-4 | 1.00 |
| 0035329 | hippo signaling | 14 | -26.38 | 1.00E-4 | 1.00 |
| 0001702 | gastrulation with mouth forming second | 20 | -23.95 | 3.00E-4 | 1.00 |
| 0006413 | translational initiation | 48 | -17.99 | 6.00E-4 | 1.00 |
| 0043536 | positive regulation of blood vessel endothelial cell migration | 12 | -26.02 | 1.00E-3 | 1.00 |
| 0005978 | glycogen biosynthetic process | 18 | -21.53 | 1.20E-3 | 1.00 |
| 0006937 | regulation of muscle contraction | 19 | -21.29 | 1.30E-3 | 1.00 |
| 0005977 | glycogen metabolic process | 30 | -17.51 | 1.50E-3 | 1.00 |
| 0006094 | gluconeogenesis | 24 | -18.68 | 1.70E-3 | 1.00 |
| 0006414 | translational elongation | 23 | -17.67 | 1.80E-3 | 1.00 |
| 0016525 | negative regulation of angiogenesis | 47 | -15.05 | 2.10E-3 | 1.00 |
| 0007029 | endoplasmic reticulum organization | 17 | -17.32 | 2.20E-3 | 1.00 |
| 0045663 | positive regulation of myoblast differentiation | 13 | -18.03 | 2.20E-3 | 1.00 |
| 0001501 | skeletal system development | 68 | -13.93 | 2.80E-3 | 1.00 |
| 0030334 | regulation of cell migration | 41 | -13.92 | 3.10E-3 | 1.00 |

Figure 2.47: The result of a gene set enrichment analysis based on GO biological process.

The features demonstrated in this tutorial include:

- Alignment of sequences
- Reconstruction of phylogenetic trees
- Introduction to the tree viewer
- Metadata and visualization of these
- Grouping of nodes
- Labeling of subtrees

Visualizing metadata on phylogenetic trees is an easy and flexible way to view different types of metadata in context. The phylogenetic tree viewer provides many features for customizing tree visualization such as:

- Circular and radial layouts
- Adjustment of node size and branch width
- Curved branches
- Grouping of nodes and visualization of these
- Visualization of metadata

Example Dataset

This tutorial involves a dataset that contains sequences and metadata from the viral hemorrhagic septicemia virus (VHSV). This virus is a fish novirhabdovirus (negative stranded RNA virus) with an unusually broad host spectra: it has been isolated from more than 80 fish species in locations around the Northern hemisphere. This dataset contains two files. One contains sequences encoding the viral surface glycoprotein of VHSV in fasta format. The other contains key metadata information in an excel spreadsheet.

Sequence data:

http://download.clcbio.com/testdata/phylogeny_module_tutorial_data/Phylogeny_

```
module_example_data.fa
```

Metadata:

```
http://download.clcbio.com/testdata/phylogeny_module_tutorial_data/Phylogeny_module_tutorial_meta_data.xls
```

The metadata includes the following categories, which are used later in this tutorial.

- **Sequence** - The nucleotide sequence of a surface glycoprotein.
- **Strain** - The virus strain.
- **Host** - The fish from which the sample was obtained.
- **Water** - The type of water the fish lives in.
- **Country** - The country where the fish was caught.
- **ACCNo** - The accession number of the virus genome.
- **Year** - The year in which the sample was obtained.

Importing the Sequence Data

To download the example data, click the two links above. The sequence data can then be imported into the CLC Workbench by following these steps:

1. Start up the CLC Workbench.
2. Use the import tool at:
File | Import (📄) | Standard Import (📄)
3. Choose the file "phylogeny_module_example_data.fa". Ensure the import type under **Options** is set to **Automatic import**.
4. Click on the button labeled **Next** See figure 2.48.
5. Select the location where you want to store the imported sequences.
6. Click on the button labeled **Finish**.

2.8.1 Aligning Sequences

Most phylogenetic reconstruction methods require a multiple alignment of the input sequences, which is used to reconstruct the corresponding phylogenetic tree.

It is, however, possible to reconstruct a phylogenetic tree without first creating a multiple alignment of the input sequences. For example, the **K-mer Based Tree Construction** tool available in the Workbench utilizes a kmer- based approach to estimate pair-wise distances between the input sequences. This distance estimate can then be used to reconstruct the tree using either the UPGMA or Neighbor-Joining algorithms. If the input dataset is large and/or contain very long sequences, it can be an advantage to use this tool for reconstructing trees to avoid the time consuming task of creating a multiple alignment.

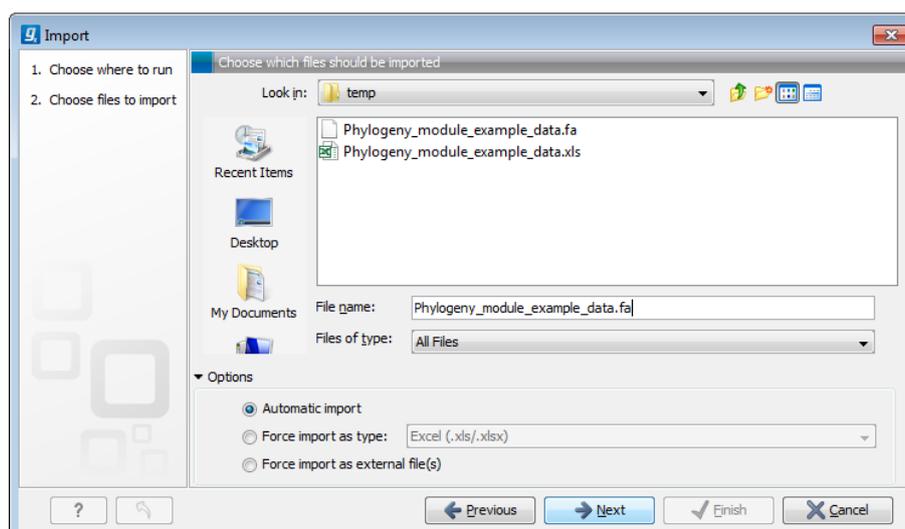


Figure 2.48: *Import of the virus sequence data.*

In this tutorial, we will create a multiple alignment, on which the tree will be based. To create an alignment of the imported sequences:

1. Start the **Create alignment** tool by going to:

Toolbox | Classical Sequence Analysis (🗄️) | **Alignments and Trees** (📄) | **Create Alignment** (🔍)

2. Select the imported sequences **Phylogeny_module_example_data**.
3. Click on the button labeled **Next**.
4. Use the default gap cost settings.
5. Select the **Less accurate (fast)** option in the Alignment section See figure 2.49.
6. Click on the button labeled **Next**.
7. Choose the option **Save** and select the location where you wish to store the alignment.
8. Click on the button labeled **Finish**.

2.8.2 Reconstructing the Tree

A phylogenetic tree can now be reconstructed Using the multiple sequence alignment created in the previous step. There are two tools that can be used for this in the Workbench. Both are found under the Alignments and Trees section of the Toolbox. They are:

- **Create Tree** - This tool constructs trees using one of two distance based methods:
 - UPGMA
 - Neighbor-Joining
- **Maximum Likelihood Phylogeny** - This tool reconstructs phylogenetic trees using a maximum likelihood approach.

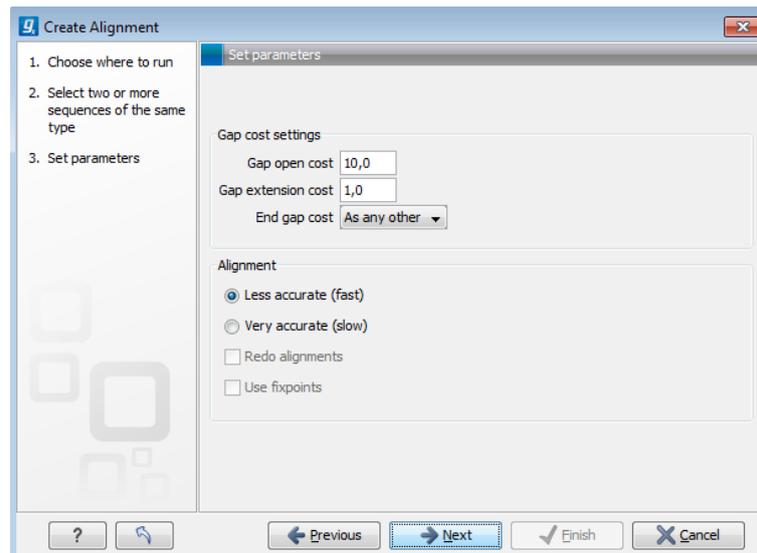


Figure 2.49: Creating a multiple alignment of the sequence data.

In this tutorial we will use the Neighbor-Joining method. This is a fast and fairly accurate method for phylogenetic reconstruction.

1. Start the **Create Tree** tool:

Toolbox | Classical Sequence Analysis (🗄️) | **Alignments and Trees** (📄) | **Create Tree** (🌳)

2. Select the multiple alignment made in the previous section.
3. Work through the Wizard, leaving all options set to the default values. See figure 2.50.
4. Choose to save the tree.

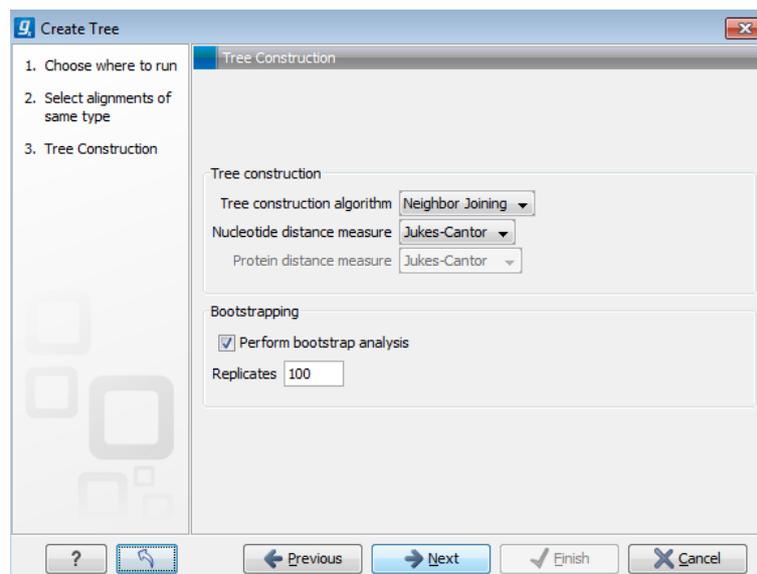


Figure 2.50: Reconstruct a neighbor-joining tree with 100 bootstrap replicates.

It can take some time to reconstruct the tree because the default parameters include bootstrapping with 100 replicates. That is, using this option, the tree is reconstructed 101 times and the results are used to test the confidence of each internal node in the resulting tree.

2.8.3 Visualizing the Tree

To open a tree in the tree viewer, double click the tree object (figure 2.51) in the **Navigation Area**. The viewer can display the tree in five different layouts:

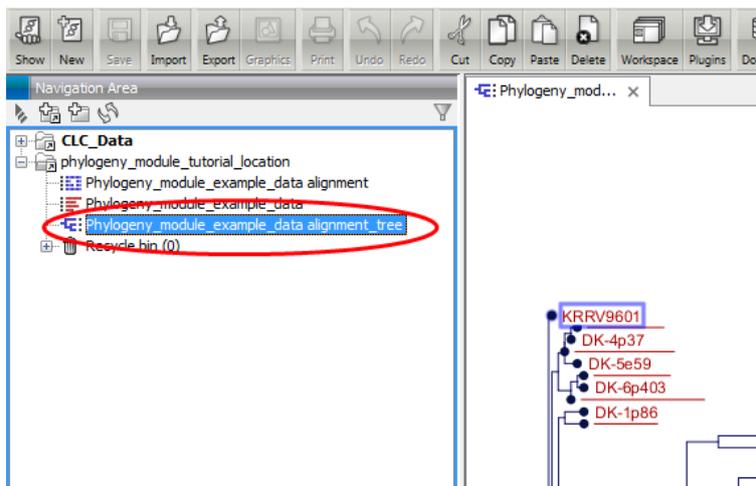


Figure 2.51: Double click the tree object (in the red circle) to open the tree in the View Area.

- **Phylogram** - The tree is displayed as a rooted tree where branch lengths correspond to the computed lengths.
- **Cladogram** - The tree is displayed as a rooted tree where branch lengths are ignored and all leaves are aligned to the right.
- **Circular Phylogram** - Same as a phylogram but with the leaves in a circular layout.
- **Circular Cladogram** - Same as a cladogram but with the leaves in a circular layout.
- **Radial** - The tree is displayed as an unrooted tree where branch lengths correspond to the computed lengths.

In this tutorial we will primarily use the phylogram layout, but we would encourage you to try the different layouts while going through this tutorial.

Zooming and scrolling in the tree view work in the same way as other viewers/editors in the Workbench. When zooming in on a large tree, one can easily lose orientation. Hence, in order to reduce the need for zooming out again, the new tree viewer includes a small minimap in the right side panel (figure 2.52). The minimap shows the full tree, with a grey rectangle highlighting the area of the tree that currently is shown in the View Area. The grey rectangle can be dragged around in the minimap with the mouse, and as this is done, the location highlighted in the minimap and the part of the tree shown in the View Area are synchronized. The minimap is particularly useful for seeing what section of a large tree is visible the View Area.

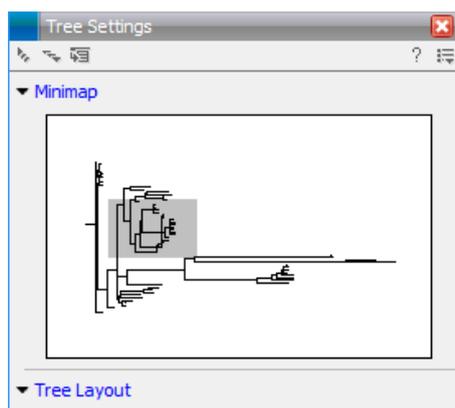


Figure 2.52: The minimap. The grey rectangle represents the area of the tree that is visible in the View Area.

Try clicking on the minimap and dragging the grey rectangle around to see how the tree is displayed in the View Area.

The right side panel contains options for related to how the tree and associated information is displayed visualization. For example, using the options in the side panel, you can adjust the tree colors, node shape, node size and the shape of branches. For more details on these options, please refer to the manual section:

http://www.clcsupport.com/clcgenomicsworkbench/current/index.php?manual=Tree_Settings.html

Bootstrap Values

In the right side panel under **Bootstrap settings** enable the option **Show bootstrap values** (figure 2.53). Now each internal node is labeled with a value between 0 and 100. These values represent confidence levels, where a high confidence indicates a clade strongly supported by the data from which the tree was constructed. Bootstrap values are useful for identifying clades in the tree where the topology (and branch lengths) should not be trusted.

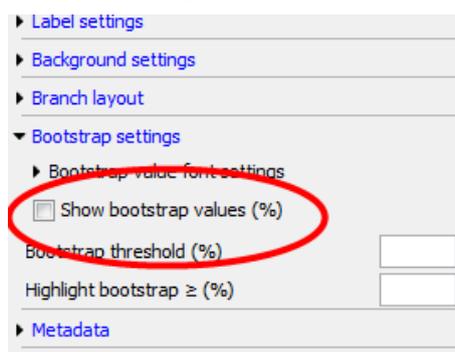


Figure 2.53: Enabling bootstrap values.

To help get an overview of the confidence values we can highlight all branches that lead to a node with a bootstrap value of e.g. $\geq 95\%$. To do this enter "95" in the field labeled **Highlight bootstrap above** under **Bootstrap settings** in the right side panel (figure 2.54). Figure 2.55 shows an example of this.

Another way to visualize bootstrap values is to collapse internal nodes with bootstrap values under a certain threshold. After removing a node the child nodes will be connected to the parent

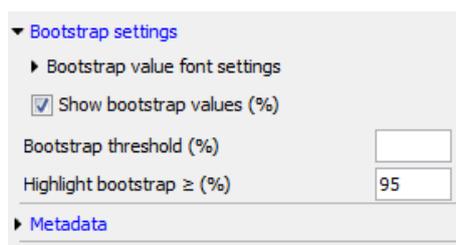


Figure 2.54: Highlighting edges with bootstrap values equal to or larger than a threshold.

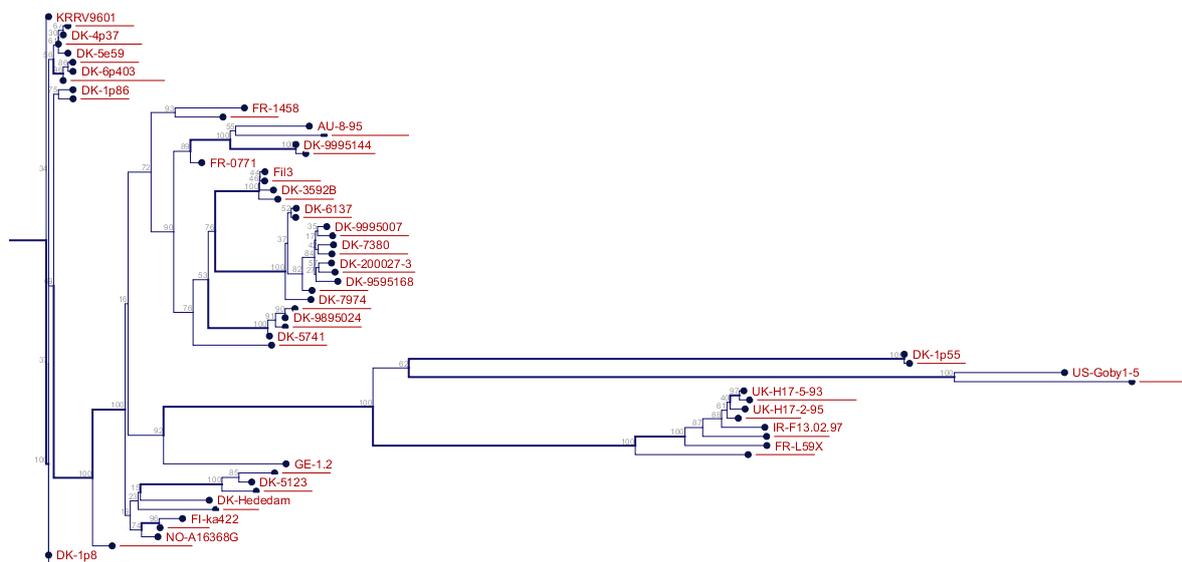


Figure 2.55: The tree where edges corresponding to nodes with bootstrap values $\geq 95\%$ are highlighted.

of the collapsed node. This creates a multifurcating tree containing only high confidence nodes. To enable this visualization enter a threshold (e.g. 95%) in the field labeled **Bootstrap threshold** under **Bootstrap settings** in the right side panel. Figure 2.56 shows an example of this.

Metadata

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. Examples of mandatory metadata fields are:

- **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 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 a table of metadata associated with a tree click the **Show Table** button at the bottom of the View Area. (See figure 2.57). This opens a table view. Each row represents a node. The

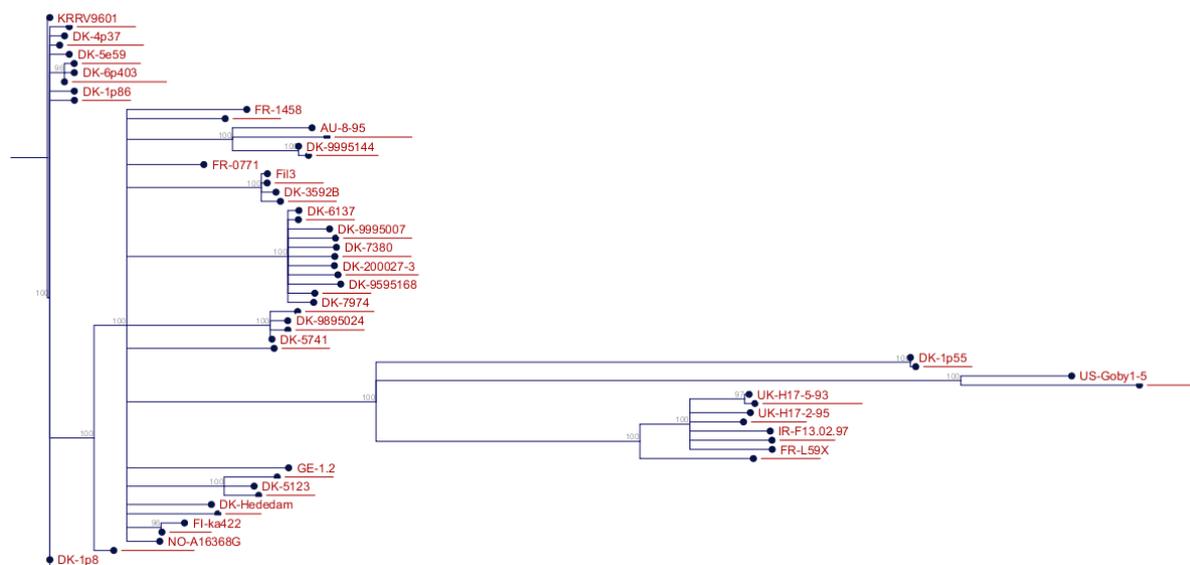


Figure 2.56: The tree where edges corresponding to nodes with bootstrap values <95% are collapsed.

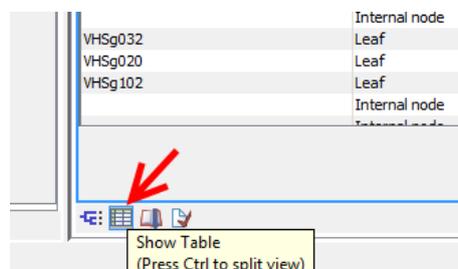


Figure 2.57: The metadata table button.

columns contain different categories of metadata. To open the metadata table in a split view, such that both the tree and the table are visible at the same time and the table information is linked to the tree view, click the **Show Table** button while holding down the Ctrl key (or ⌘ for Mac) See figure 2.58. When the table and tree views are open and linked in this way, clicking on a row in the table highlights the relevant location in the tree.

Importing Metadata

Additional metadata can be imported by clicking the **Import Metadata** button below at the bottom of the table view. See figure 2.58. The file "Phylogeny_module_example_meta_data.xls" you downloaded earlier is an Excel format file containing seven categories of metadata. These categories are listed near the beginning of this tutorial. To import the metadata contained in this file:

1. Click the **Import Metadata** button.
2. Click on the folder icon (📁) next to the field in the Import section.
3. Select the file "Phylogeny_module_example_meta_data.xls", which you downloaded earlier.
4. Use the default settings. The **Named columns** option should be checked. Information just below that option describes the mapping of categories to particular columns in the file. One column must be assigned type Name to allow the importer to associate metadata with

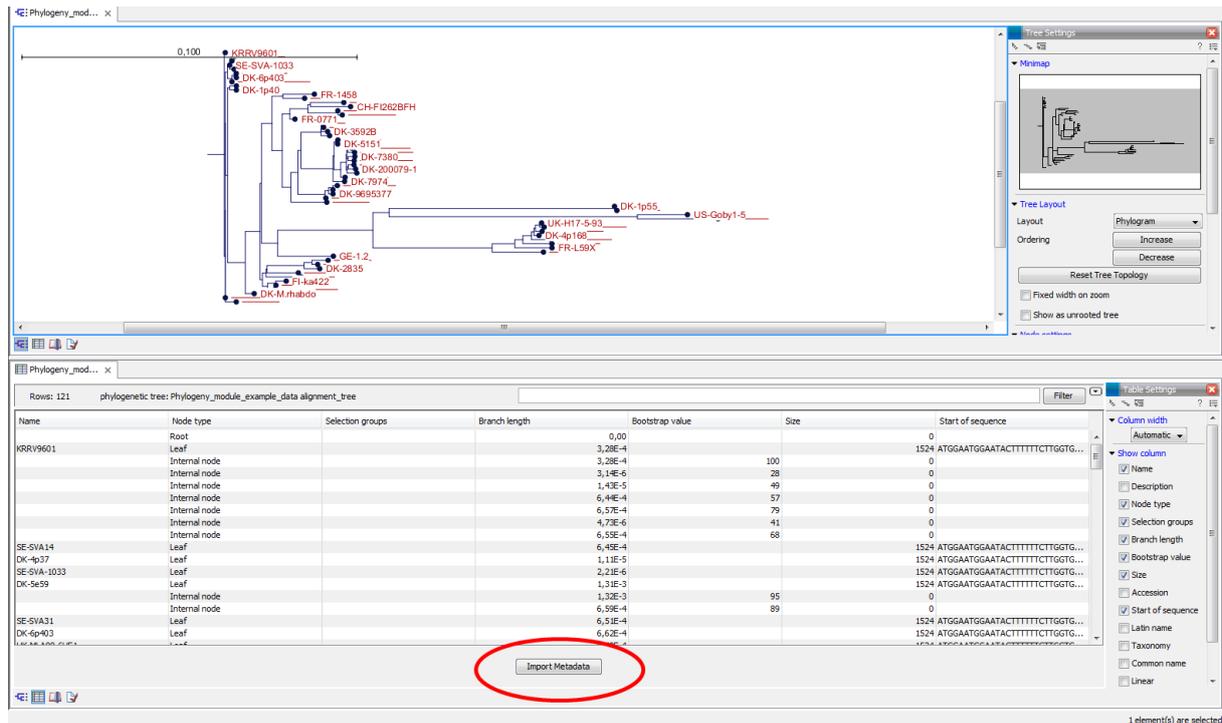


Figure 2.58: Metadata table and tree viewer in split view mode. The red circle indicate the button for importing additional metadata.

nodes in the tree. In this case, a column in the original file called Strain will be mapped to the Name category. This means that the identifiers in the Strain column of the Excel sheet are used to link the information in a particular row of the file with the relevant nodes in the tree. See figure 2.59.

5. Click on the button labeled **Finish**

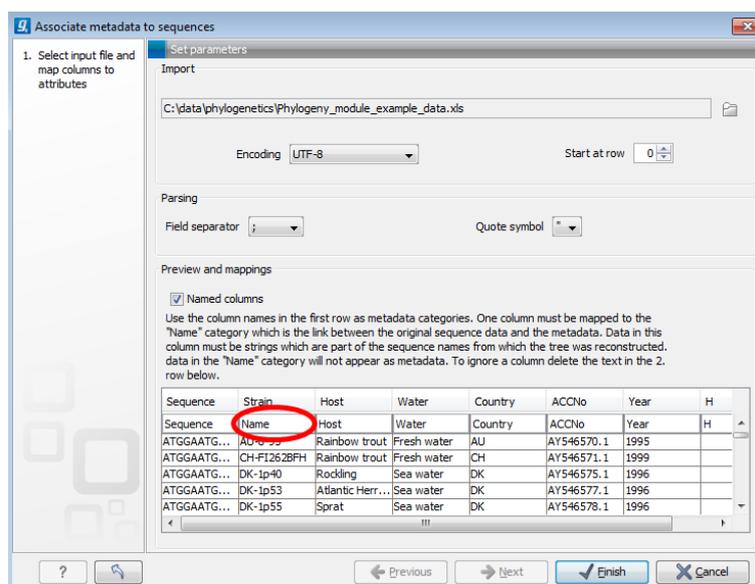


Figure 2.59: The metadata import wizard. In this example the "Strain" column is mapped to the "Name" category and hereby used to map metadata to the tree nodes.

| Name | Node type | Selection groups | Branch length | Bootstrap value | Size | Start of sequence | Sequence | Host | Water | Country | ACCNo | Year | H |
|-------------|---------------|------------------|---------------|-----------------|------|-------------------|-----------------|-------------------|-----------|---------|------------|------|---|
| | Root | | 0,00 | | 0 | | | | | | | | |
| KRIV9601 | Leaf | | 3,28E-4 | 100 | 0 | 1524 | ATGGAATGGAAT... | Japanese flounder | Sea water | JP | AB672614.1 | 1996 | |
| | Internal node | | 3,14E-6 | 28 | 0 | | | | | | | | |
| | Internal node | | 1,43E-5 | 49 | 0 | | | | | | | | |
| | Internal node | | 6,44E-4 | 57 | 0 | | | | | | | | |
| | Internal node | | 6,53E-4 | 79 | 0 | | | | | | | | |
| | Internal node | | 4,73E-6 | 41 | 0 | | | | | | | | |
| | Internal node | | 6,53E-4 | 68 | 0 | | | | | | | | |
| SE-SVA14 | Leaf | | 6,45E-4 | | 0 | 1524 | ATGGAATGGAAT... | Rainbow trout | Sea water | SE | AY546622.1 | 1998 | |
| DK-4p37 | Leaf | | 1,11E-5 | | 0 | 1524 | ATGGAATGGAAT... | Blue whiting | Sea water | DK | F3460590.1 | 1997 | |
| SE-SVA-1033 | Leaf | | 2,23E-6 | | 0 | 1524 | ATGGAATGGAAT... | Rainbow trout | Sea water | SE | FJ460591.1 | 2000 | |
| DK-5e99 | Leaf | | 1,31E-3 | | 0 | 1524 | ATGGAATGGAAT... | Dab | Sea water | DK | AY546583.1 | 1998 | |
| | Internal node | | 1,32E-3 | 95 | 0 | | | | | | | | |
| | Internal node | | 6,59E-4 | 89 | 0 | | | | | | | | |
| SE-SVA31 | Leaf | | 6,53E-4 | | 0 | 1524 | ATGGAATGGAAT... | Atlantic Herring | Sea water | SE | AY546626.1 | 2000 | |
| DK-6p403 | Leaf | | 6,63E-4 | | 0 | 1524 | ATGGAATGGAAT... | Atlantic Herring | Sea water | DK | AY546584.1 | 1999 | |

Figure 2.60: Content of the metadata table after additional metadata has been imported.

After the import is complete, a number of new metadata columns have appeared in the metadata table (figure 2.60). You can see a full list of the columns available in the right hand Table settings pane.

A quick way to see the metadata for a specific node in the tree is to hold the mouse over the node for a few seconds until a tooltip appears (figure 2.61).

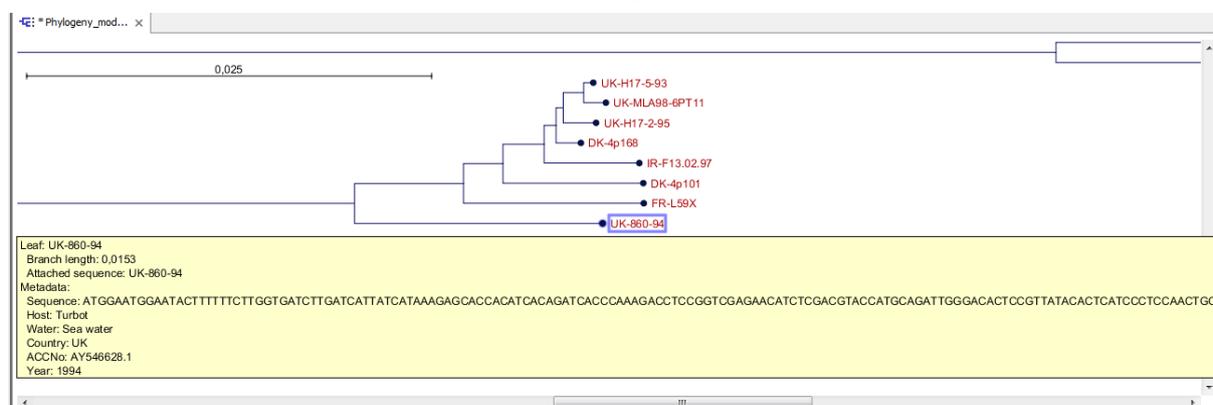


Figure 2.61: When holding the mouse over a node in the tree a tooltip shows all metadata for that node.

Visualization of Metadata Visualization of metadata is carried out using the viewing option under the **Metadata** section in the right side panel (see figure 2.62).

The tree viewer has several options for visualizing both textual and numeric metadata. Here we demonstrate three options.

Node color

1. Under the **Metadata** section in the right side panel click on the text **Node color**.
2. In the dropdown menu select **Host** (figure 2.63).

Nodes are now colored according to the host organism from which the virus sample was extracted. Random colors are assigned to each entry in the metadata category. To change a color go to **Node color** in the right side panel, left click on the color you want to change and choose a new color in the color chooser that pops up. See figure 2.64. If an entry does not contain any data, the corresponding node is assigned a default color. This default color is the first color in the legend and has the label **Unknown**. This default color can be adjusted just like any other color.

Node symbol size

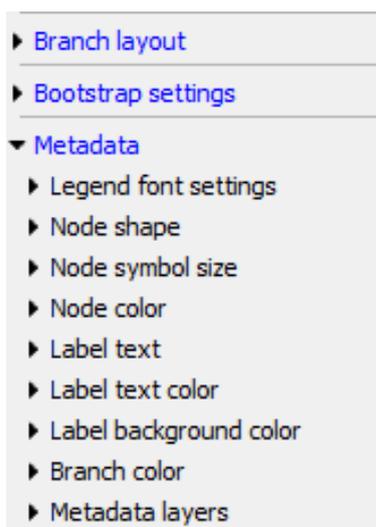


Figure 2.62: The metadata visualization options in the right side panel of the tree viewer.

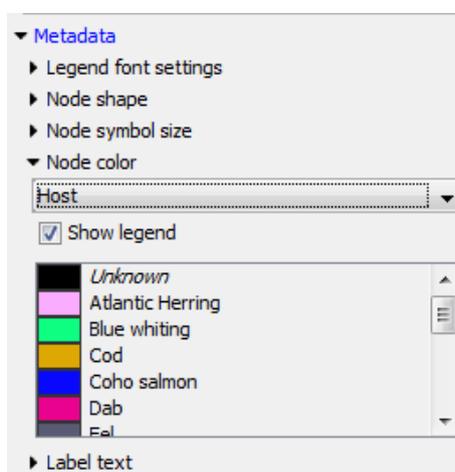


Figure 2.63: Color nodes by the host which the sample was extracted from.

1. Under **Metadata** in the right side panel click **Node symbol size**.
2. In the dropdown menu select **Water** (figure 2.65).

All nodes are now assigned sizes which symbolize the water type in which the host fish lives. Each type of water is automatically mapped to a node size. These sizes can be changed by using the sliders visible under **Node symbol size** (figure 2.65).

Metadata layers The metadata can be visualized on the tree as color-coded layers.

1. Under **Metadata** in the right side panel, select **Metadata layers** and then select **Metadata layer #1**.
2. In the dropdown menu select **Country** (figure 2.66).

The countries where each sample was obtained are now displayed using colors in the color-code layer as shown at the bottom of the tree view. See figure 2.67. Like the node color visualization, the color for each entry in the "Country" category can be changed under **Metadata layer #1**. It

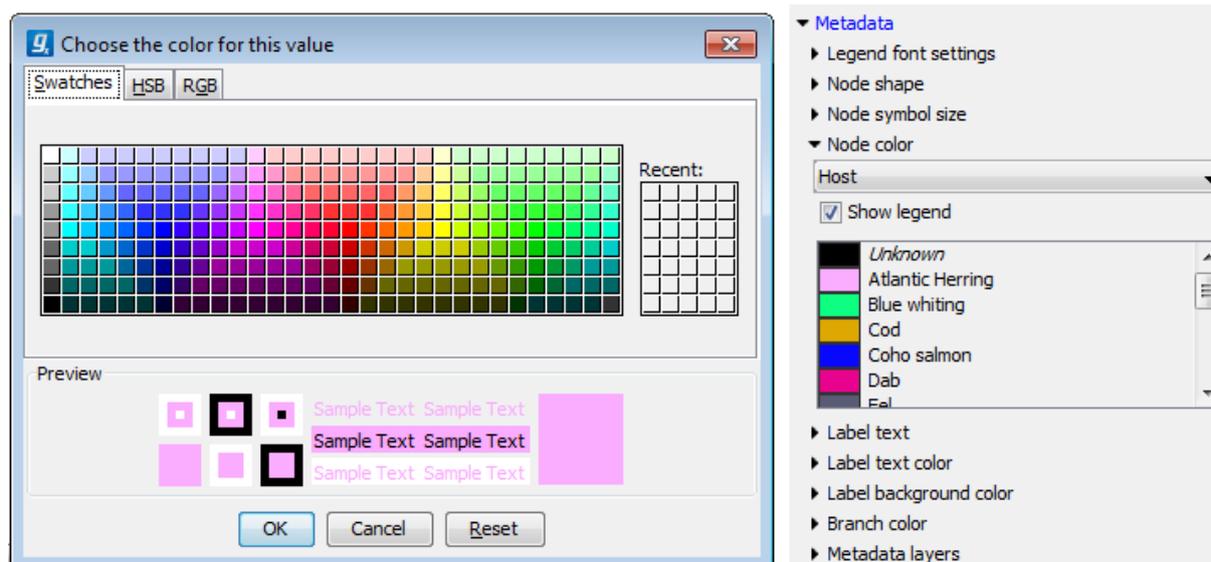


Figure 2.64: The color chooser can be used to select new colors for each metadata category.

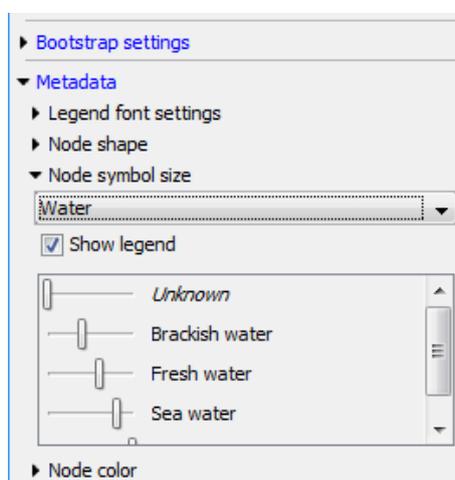


Figure 2.65: Visualization of the water type in which the host fish lives using node sizes.

is also possible to adjust the thickness of the color-code layer by using the slider labeled **Layer thickness** under **Metadata layer #1**. If the **Show layer names** option is enabled, there is a limit to how thin a layer can be. This is to ensure that the name of different color-code layers do not overlap. By disabling the **Show layer names** option, layers can be made as thin as a single pixel.

After adding the color-code layer, a new option appears under the **Metadata layers** section in the right side panel called **Metadata layer #2**. By selecting a metadata category for this option, a new color-code layer will be added on top of the first color-code layer. By visualising metadata using multiple color-code layers, users can get a quick overview of data in different metadata categories. This makes it possible to visualize complex correlations. Figure 2.68 shows an example where three metadata categories are visualized using color-code layers.

Create and Modify Metadata

Metadata do not have to be imported from an external file. It is possible to both create and modify metadata through the metadata table and the tree viewer. A new metadata category can be created using the tree view by following these steps:

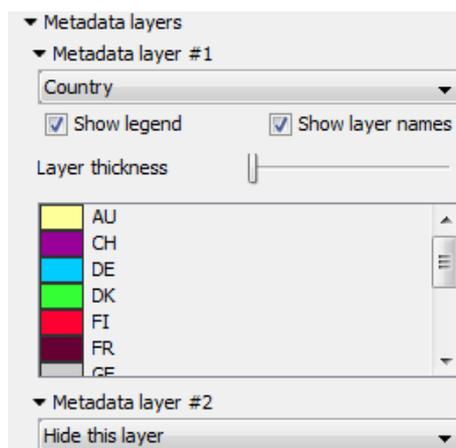


Figure 2.66: Visualization of the country in which the host fish was caught using a color-code layer.

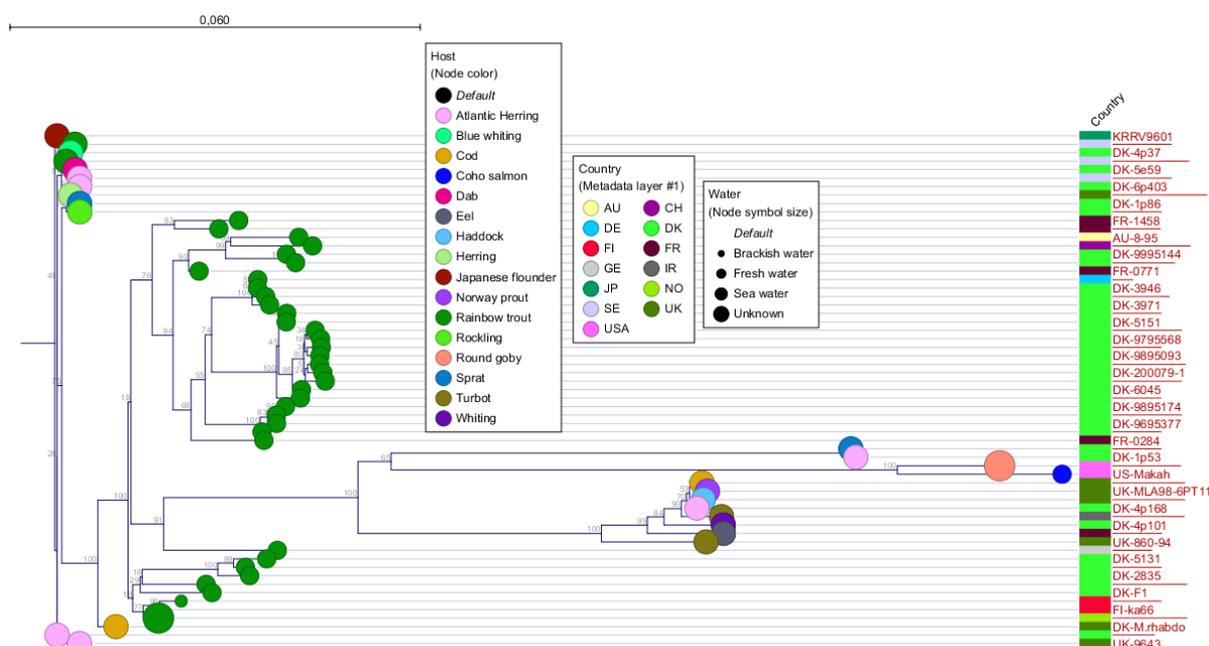


Figure 2.67: Tree where water type, host and country are visualized.

- Select one or more nodes in the tree view
- Right click one of the selected nodes and select **Assing metadata** (figure 2.69).
- Type in a name for metadata category, and use the **Value** field to assign a value to all selected nodes.
- Click on **Add**

Now a new metadata category has been created with values assigned to all selected nodes. To assign values in the same metadata category to other nodes, follow the steps above but instead of writing a new name for a metadata category, use the drop down menu in the **Name** field to select the newly created category (figure 2.70). The value entered in the **Value** field will now be assigned to the selected category for all selected nodes.

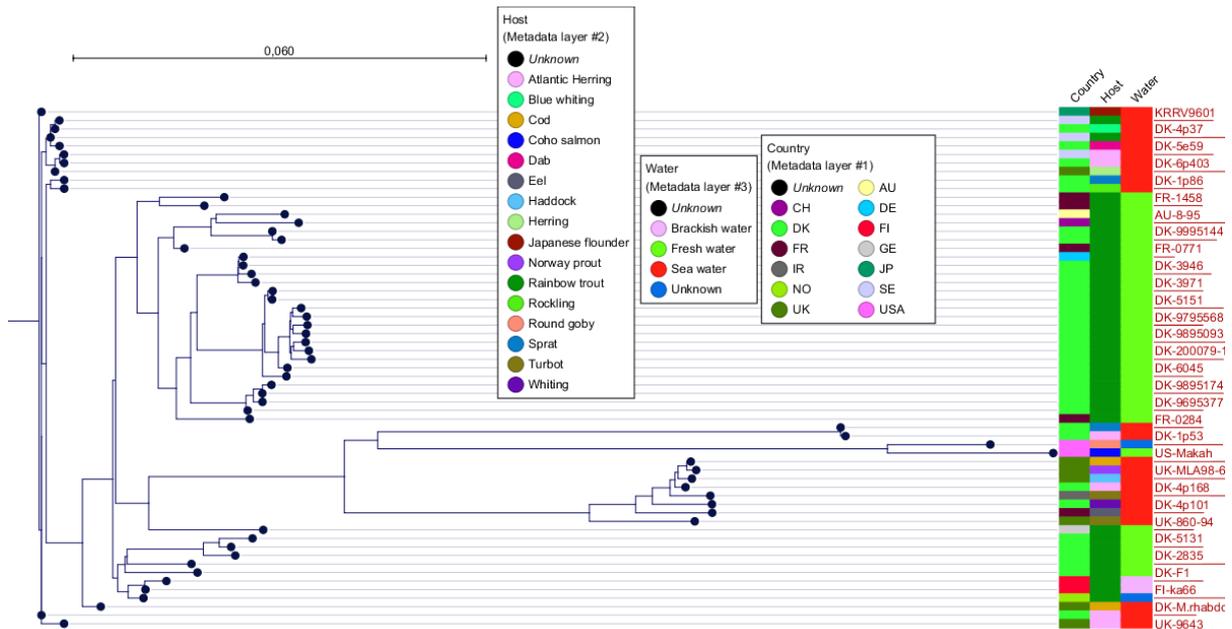


Figure 2.68: Tree where water type, host and country are all visualized with color-code layers.

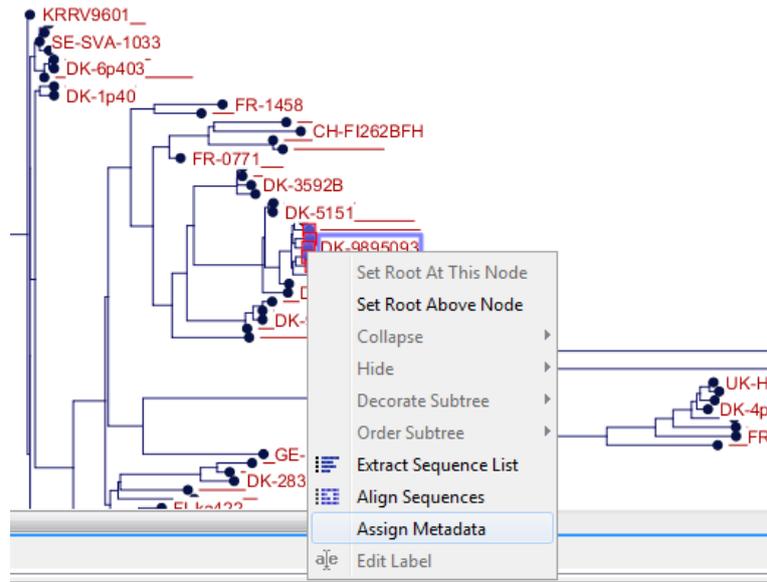


Figure 2.69: Creating a new metadata category using the right click context menu.

Manipulation of metadata via the metadata table is very similar to manipulating metadata via the tree view. The only difference is that you need to select one or more rows in the table instead of selecting nodes in the tree.

Manually created metadata categories can be visualized in the same way as any other metadata category. An example where a new metadata category called "Continents" has been created and where all leaf nodes have been assigned values in that category according to the continent where the sample was obtained, is shown in figure 2.71.

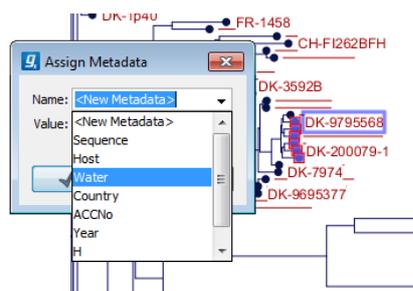


Figure 2.70: Modifying an existing metadata category.

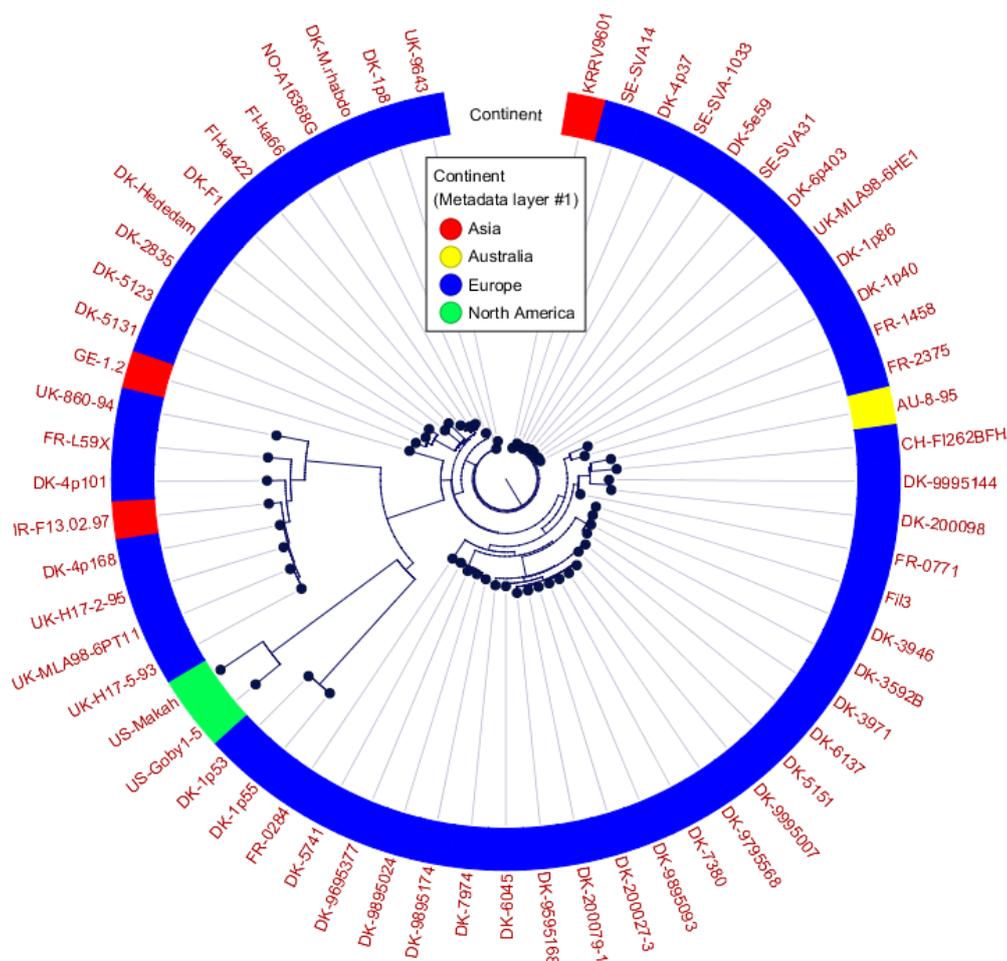


Figure 2.71: Visualization of the continents where the host fish was found. The circular phylogram layout is used here.

2.8.4 Subtree Labels

It is often convenient to put labels on subtrees to illustrate what the nodes in a subtree represents. This can be done in the following way:

1. Right click an inner node in the tree.
2. Select **Decorate Subtree** and then select **Set Subtree Label** (figure 2.72).

3. Enter a label text and select the line color.

The result is a labelled line at the bottom of a subtree where the inner node which was clicked in step 1 is the root. Figure 2.73 shows an example where different subtrees have been labeled.

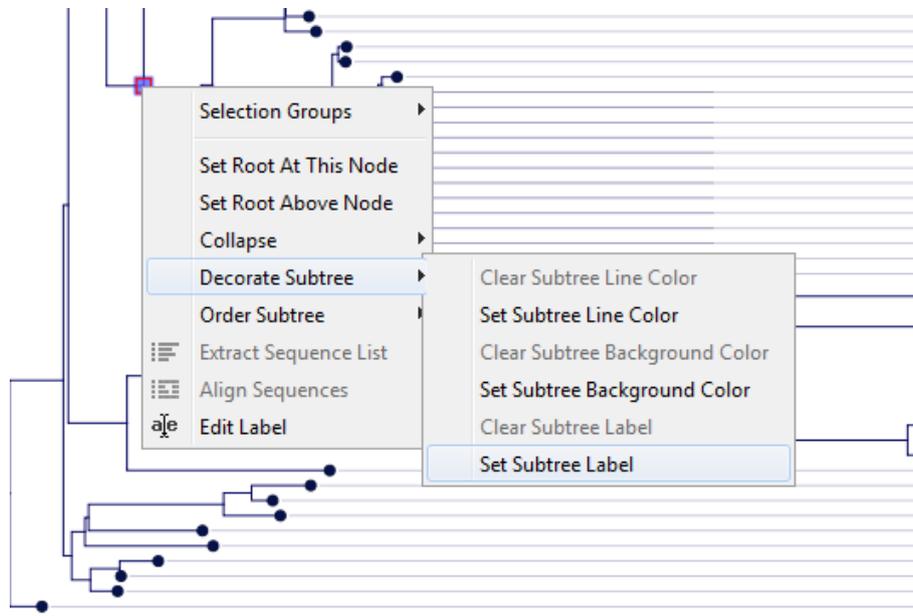


Figure 2.72: Creating a label for a subtree.

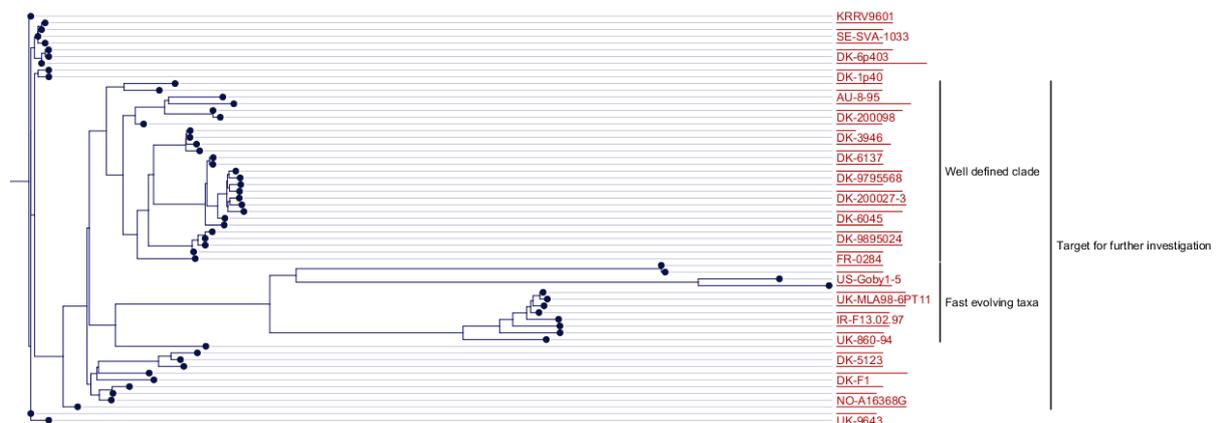


Figure 2.73: A tree with three labeled subtrees. One label is for a subtree in which both the other two labeled subtrees are contained.

2.9 Assemble to Reference

In this tutorial, you will see how to assemble data from automated sequencers into a contig and how to find and inspect any conflicts that may exist between different reads.

This tutorial shows how to assemble sequencing data generated by conventional "Sanger" sequencing techniques using the *CLC Main Workbench*. For high-throughput sequencing data, we refer to the *CLC Genomics Workbench* (see <http://www.clcbio.com/genomics>).

The data used in this tutorial are the sequence reads in the "Sequencing reads" folder in the "Sequencing data" folder of the **Example data** in the **Navigation Area**. If you do not have the example data, please go to the **Help** menu to import it.

2.9.1 Trimming the sequences

The first thing to do when analyzing sequencing data is to trim the sequences. Trimming serves a dual purpose: it both takes care of parts of the reads with poor quality, and it removes potential vector contamination. Trimming the sequencing data gives a better result in the further analysis.

Toolbox | Sequencing Data Analysis (🔍) | Trim Sequences (✂️)

Select the 9 sequences and click **Next**.

In this dialog, you will be able to specify how this trimming should be performed.

For this data, we wish to use a more stringent trimming, so we set the limit of the quality score trim to 0.02 (see figure 2.74).

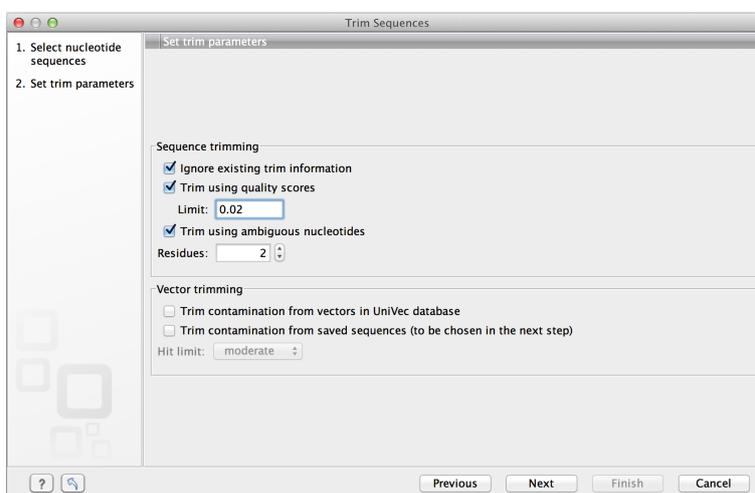


Figure 2.74: Specifying how sequences should be trimmed. A stringent trimming of 0.02 is used in this example.

There is no vector contamination in these data, so we only trim for poor quality.

If you place the mouse cursor on the parameters, you will see a brief explanation.

Click **Next** and choose to **Save** the results.

When the trimming is performed, the parts of the sequences that are trimmed are actually annotated, not removed (see figure 2.75). By choosing **Save**, the Trim annotations will be saved directly to the sequences, without opening them for you to view first.

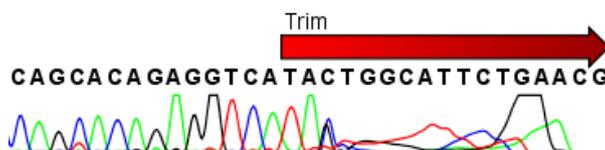


Figure 2.75: Trimming creates annotations on the regions that will be ignored in the assembly process.

These annotated parts of the sequences will be ignored in the subsequent assembly.

A natural question is: Why not simply delete the trimmed regions instead of annotating them? In some cases, deleting the regions would do no harm, but in other cases, these regions could potentially contain valuable information, and this information would be lost if the regions were deleted instead of annotated. We will see an example of this later in this tutorial.

2.9.2 Assembling the sequencing data

The next step is to assemble the sequences. This is the technical term for aligning the sequences where they overlap and reverse the reverse reads to make a contiguous sequence (also called a contig).

In this tutorial, we will use assembly to a reference sequence. This can be used when you have a reference sequence that you know is similar to your sequencing data.

Toolbox | Sequencing Data Analysis (AA) | Assemble Sequences to Reference (MW)

In the first dialog, select the nine sequencing reads and click **Next** to go to the second step of the assembly where you select the reference sequence.

Click the **Browse and select** button (🔍) and select the "ATP8a1 mRNA (reference)" from the "Sequencing data" folder (see figure 2.76). You can leave the other options in this window set to their defaults.

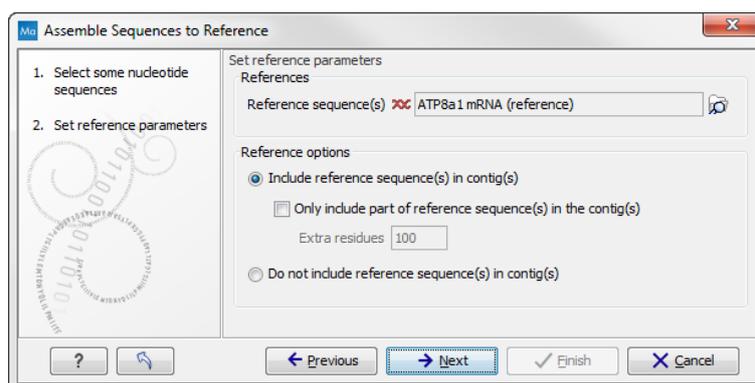


Figure 2.76: The "ATP8a1 mRNA (reference)" sequence selected as reference sequence for the assembly.

Click **Next** and choose under **Trimming options** to **Use existing trim information** (that you have just added). This is shown in figure 21.10.

Click on the button labeled **Next** and choose where you would like to save the results. Click **Finish** and the assembly process will begin.

2.9.3 Getting an overview of the contig

The result of the assembly is a **Contig** which is an alignment of the nine reads to the reference sequence. Click **Fit width** (↔) to see an overview of the contig. To help you determine the coverage, display a coverage graph (see figure 2.78). This can be done in the side panel under the tab **Alignment info**.

Alignment info in Side Panel | Coverage | Graph

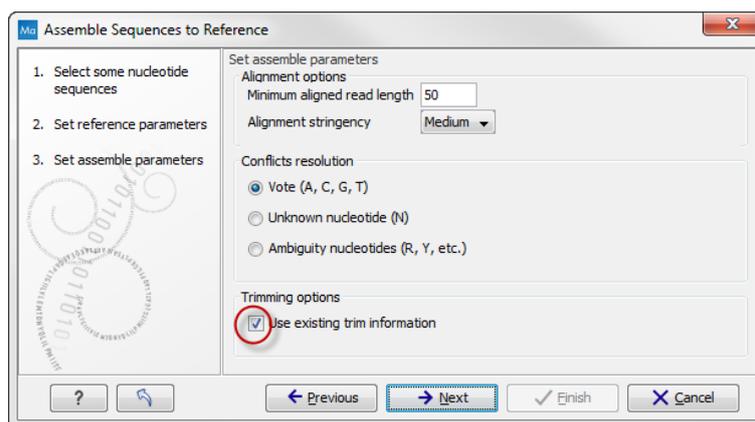


Figure 2.77: Use the default settings and tick the box "Use existing trim information".



Figure 2.78: An overview of the contig with the coverage graph.

This overview can be an aid in determining whether coverage is satisfactory, and if not, which regions a new sequencing effort should focus on. Next, we go into the details of the contig.

2.9.4 Finding and editing conflicts

Click **Zoom to 100%** () to zoom in on the residues at the beginning of the contig. Click the **Find Conflict** button at the top of the **Side Panel** or press the **Space** key to find the first position where there is disagreement between the reads; you can also use **'**, **'** and **' . '** keys to move

back and forth between conflicts (see figure 2.79).

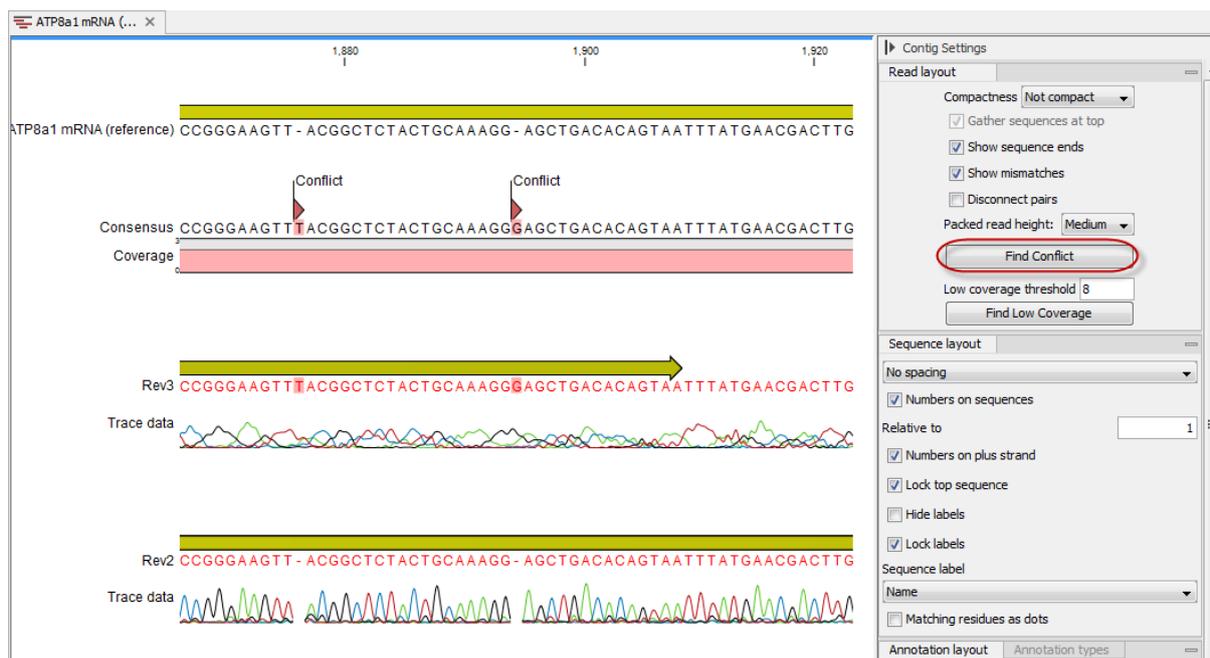


Figure 2.79: Using the *Find Conflict* button highlights conflicts.

In this example, the first read has a "T" (marked with a light-pink background color), whereas the second line has a gap. In order to determine which of the reads we should trust, we assess the quality of the read at this position.

A quick look at the regularity of the peaks of read "Rev2" compared to "Rev3" indicates that we should trust the "Rev2" read. In addition, you can see that we are close to the end of "Rev3", and the quality of the chromatogram traces is often low near the ends.

Based on this, we decide not to trust "Rev3". To correct the read, select the "T" in the "Rev3" sequence by placing the cursor to the left of it and dragging the cursor across the T. Press **Delete**. If a warning pops up with the text **Edit Warning**, press **OK**. This will resolve the conflict.

2.9.5 Including regions that have been trimmed off

Clicking the **Find Conflict** button again until you find the conflict shown in figure 2.80.

This is the beginning of a stretch with low coverage in the consensus sequence. This is because the reads have been trimmed at this position and only one sequence contribute to the consensus sequence. However, if you look at the read at the bottom, *Fwd2*, you can see that a lot of the peaks actually seem to be fine, so we could just as well include this information in the contig.

You can see where the trimmed region begins. To include part of the trimmed region of *Fwd2* in the contig, move the vertical slider around position 1980 to the left to position 1968 (see figure 21.13).

You will now see how the low coverage in the coverage graph is replaced by a higher coverage.

What we have demonstrated here is particularly relevant in situations where you have trimming that results in no sequences being left to contribute to the consensus sequence and as a result

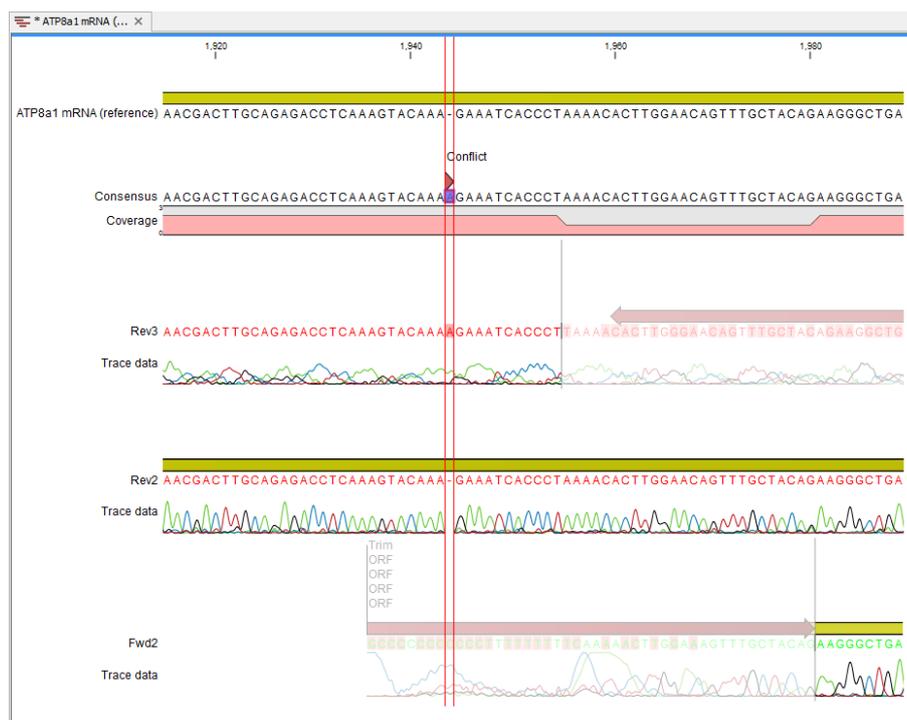


Figure 2.80: Dragging the edge of the trimmed region.

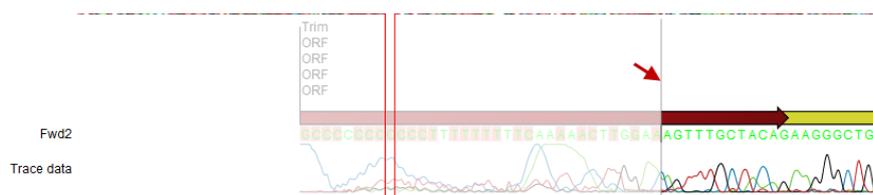


Figure 2.81: Dragging the edge of the trimmed region.

would leave a gap in the sequence.

Note that you can only move the sliders when you are zoomed in to see the sequence residues.

2.9.6 Inspecting the traces

Clicking the **Find Conflict** button again until you find the conflict shown in figure 2.82, which is at position 2564.

Here both reads are different than the reference sequence. We now inspect the traces in more detail. In order to see the details, we zoom in on this position:

Zoom in using the Tool Bar zoom function (🔍) | Click the selected base | Click again three times

Now you have zoomed in on the trace (see figure 2.82).

This gives more space between the residues, but if we would like to inspect the peaks even more, simply drag the peaks up and down with your mouse (see figure 21.2).

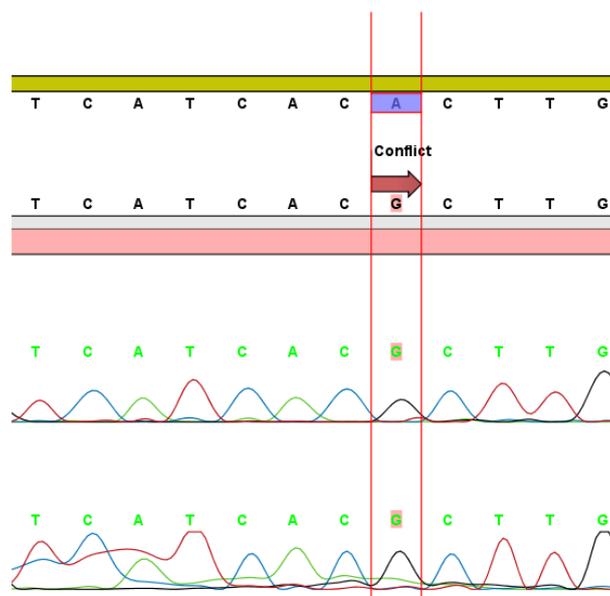


Figure 2.82: Now you can see all the details of the traces.

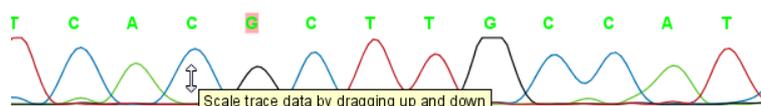


Figure 2.83: Grab the traces to scale.

2.9.7 Synonymous substitutions?

In this case we have sequenced the coding part of a gene. Often you want to know what a variation like this would mean on the protein level. To do this, show the translation along the contig:

Nucleotide info in the Side Panel | Translation | Show | Select ORF/CDS in the Frame box

The result is shown in figure 2.84.

You can see that the variation is on the third base of the codon coding for threonine, so this is a synonymous substitution. That is why the T is colored orange. If it was a non-synonymous substitution, it would be colored in red.

2.9.8 Getting an overview of the conflicts

Browsing the conflicts by clicking the **Find Conflict** button is useful in many cases, but you might also want to get an overview of all the conflicts in the entire contig. This is easily achieved by showing the contig in a table view:

Press and hold the Ctrl-button (⌘ on Mac) | Click Show Table (📄) at the bottom of the view

This will open a table showing the conflicts. You can right-click the **Note** field and enter your own comment. In this dialog, enter a new text in the **Name** and click **OK**.

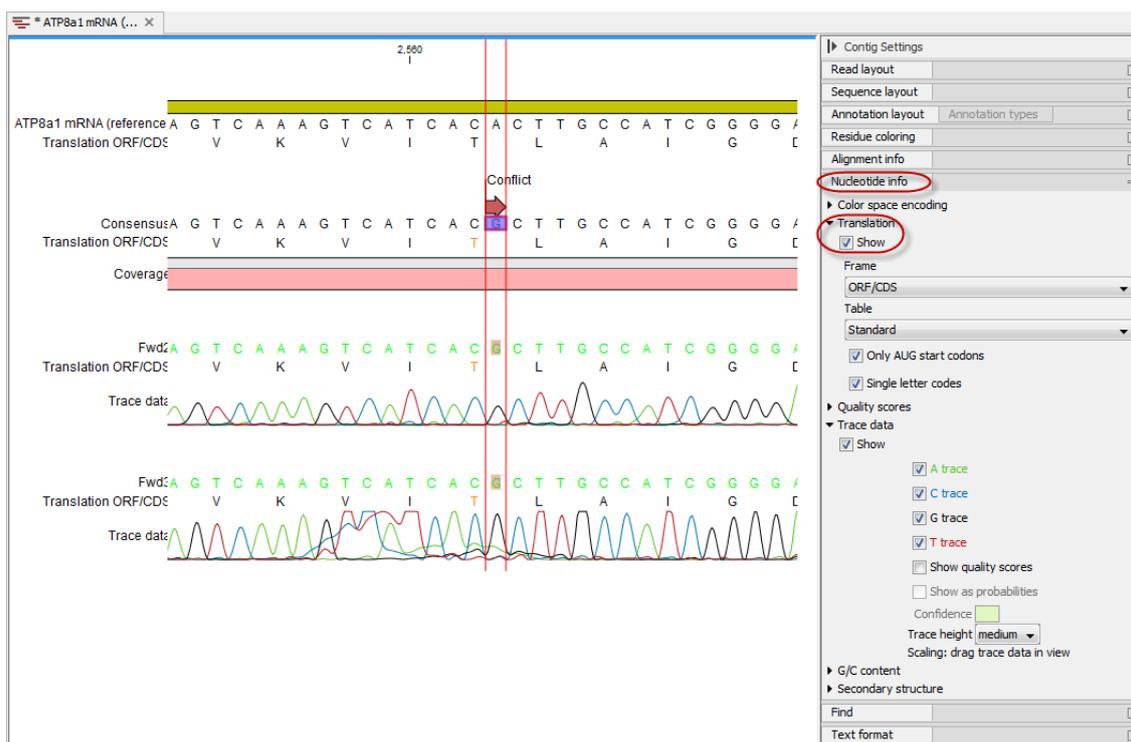


Figure 2.84: Showing the translation along the contig.

When you edit a comment, this is reflected in the conflict annotation on the consensus sequence. This means that when you use this sequence later on, you will easily be able to see the comments you have entered. The comment could be e.g. your interpretation of the conflict.

2.9.9 Documenting your changes

Whenever you make a change like deleting a "T", it will be noted in the contig's history. To open the history, click the **History** (🕒) icon at the bottom of the view.

In the history, you can see the details of each change (see figure 2.85).

2.9.10 Using the result for further analyses

When you have finished editing the contig, it can be saved, and you can also extract and save the consensus sequence:

Right-click the name "Consensus" | Open Sequence | Save (📁)

This will make it possible to use this sequence for further analyses in the *CLC Main Workbench*. All the conflict annotations are preserved, and in the sequence's history, you will find a reference to the original contig. As long as you also save the original contig, you will always be able to go back to it by choosing the Reference contig in the consensus sequence's history (see figure 2.86).

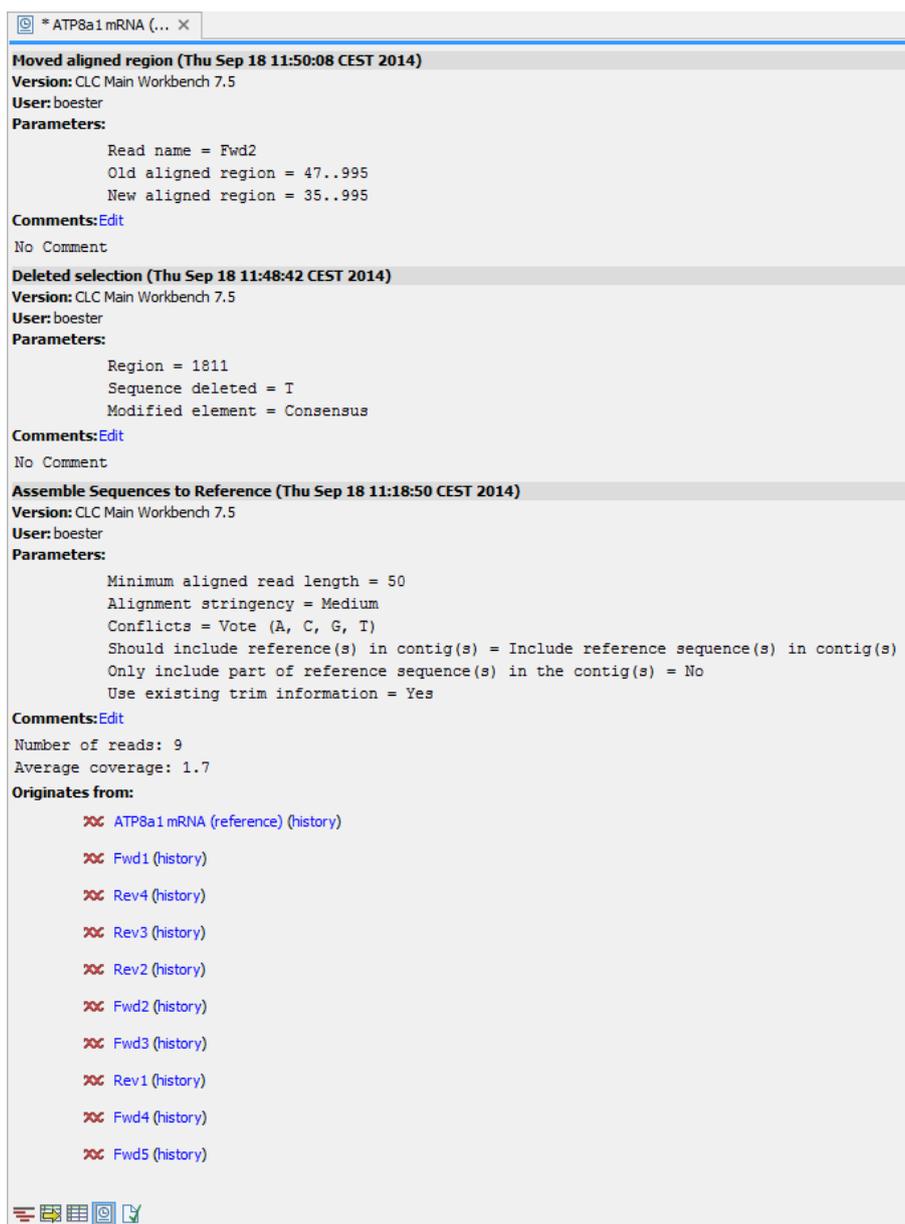


Figure 2.85: The history of the contig showing that a "T" has been deleted and that the aligned region has been moved.

2.10 In Silico Cloning Workflow

In this tutorial, the goal is to make a virtual PCR-amplification of a gene using primers with restriction sites at the 5' ends, and to insert the gene in a multiple cloning site of an expression vector. We start off with a set of primers, a DNA template sequence and an expression vector loaded into the Workbench.

This tutorial will guide you through the following steps:

1. Adding restriction sites to the primers
2. Simulating the effect of PCR by creating the fragment to use for cloning.
3. Specifying restriction sites to use for cloning, and inserting the fragment into the vector

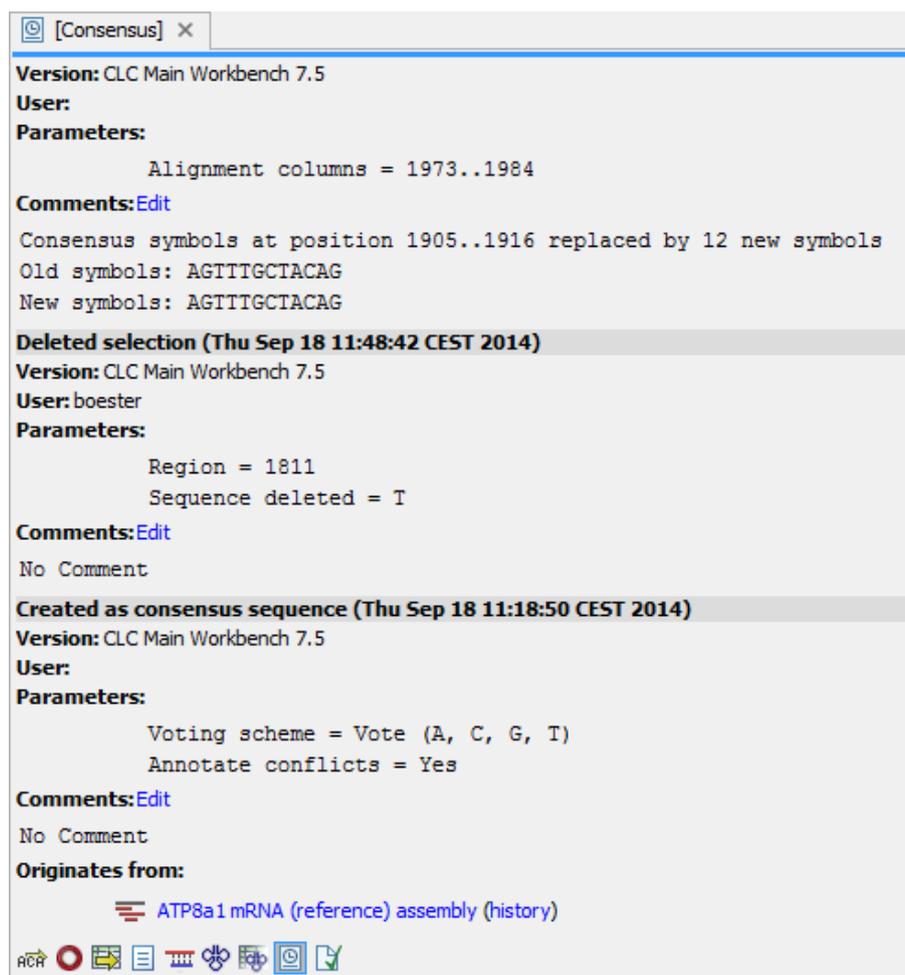


Figure 2.86: The history of the consensus sequence, which has been extracted from the contig. Clicking the blue text "Reference contig" will find and highlight the name of the saved contig in the Navigation Area. Clicking the blue text "history" to the right will open the history view of the earlier contig. From there, you can choose other views, such as the Read mapping view, of the contig.

2.10.1 Locating the data to use

Open the `Example data` folder in the **Navigation Area**. Open the `Cloning` folder, and inside this folder, open the `Primers` folder.

If you do not have the example data, please go to the **Help** menu to import it.

The data to use in these folders are shown in figure 2.87.

Double-click the `ATP8a1 mRNA` sequence and zoom to **Fit Width** () and you will see the yellow annotation, which is the coding part of the gene. This is the part that we want to insert into the `pcDNA4_TO` vector. The primers have already been designed using the primer design tool in *CLC Main Workbench* (to learn more about this, please refer to the Primer design tutorial).

2.10.2 Add restriction sites to primers

First, we add restriction sites to the primers. In order to see which restriction enzymes can be used, we create a split view of the vector and the fragment to insert. In this way we can easily

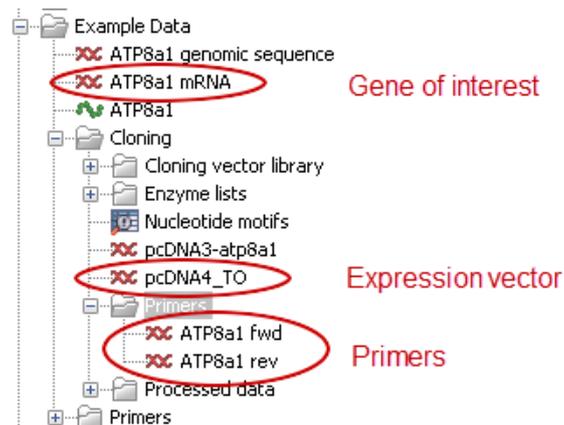


Figure 2.87: The data to use in this tutorial.

make a visual check to find enzymes from the multiple cloning site in the vector that do not cut in the gene of interest. To create the split view:

double-click the pcDNA4_TO sequence | View | Split Horizontally (☰)

Note that this can also be achieved by simply dragging the pcDNA4_TO sequence into the lower part of the open view.

Switch to the **Circular** (○) view at the bottom of the view.

Zoom in (🔍) on the multiple cloning site downstream of the green CMV promoter annotation. You should now have a view similar to the one shown in figure 2.88.

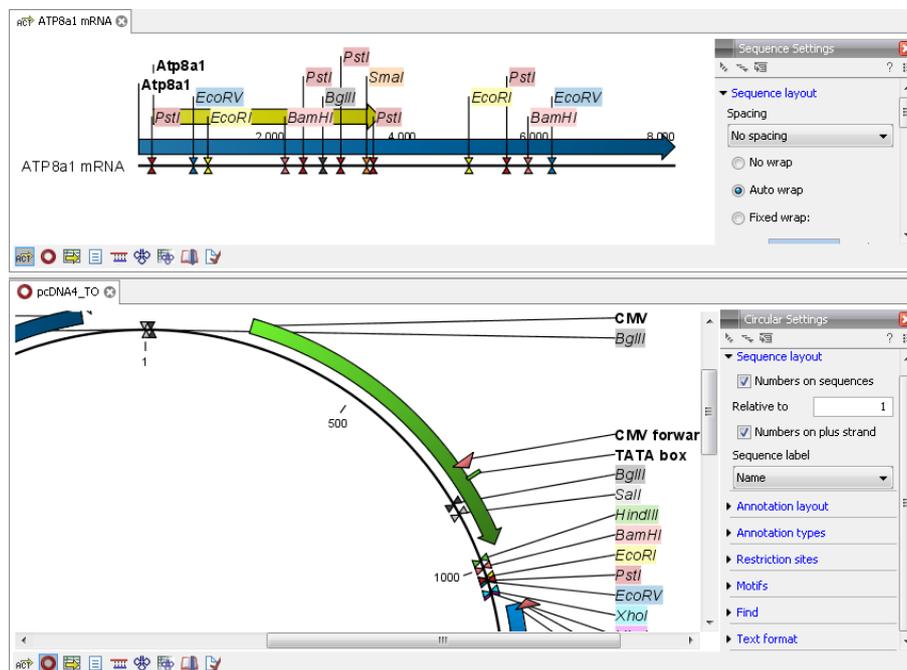


Figure 2.88: Check cut sites.

By looking at the enzymes we can see that both *HindIII* and *XhoI* cut in the multi-cloning site of the vector and not in the *Atp8a1* gene. Note that you can add more enzymes to the list in the **Side Panel** by clicking **Manage Enzymes** under the **Restriction Sites** group.

Close both views and open the `ATP8a1 fwd` primer sequence. When it opens, double-click the name of the sequence to make a selection of the full sequence. If you do not see the whole sequence turn purple, please make sure you have the Selection Tool chosen, and not one of the other tools available from the top right side of the Workbench (e.g. Pan, Zooming tools, etc.)

Once the sequence is selected, right-click and choose to **Insert Restriction Site Before Selection** as shown in figure 2.89.

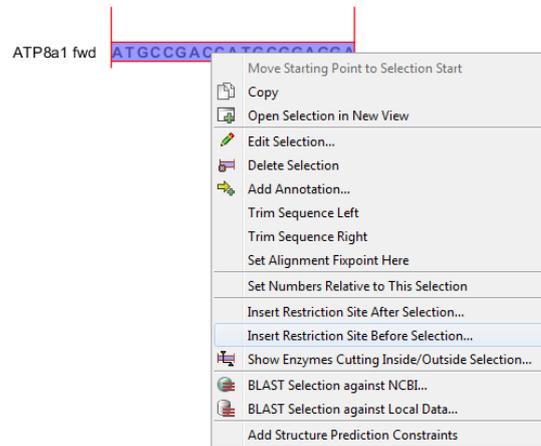


Figure 2.89: Adding restriction sites to a primer.

In the **Filter** box enter `HindIII` and click on it. At the bottom of the dialog, add a few extra bases 5' of the cut site (this is done to increase the efficiency of the enzyme) as shown figure 23.13.

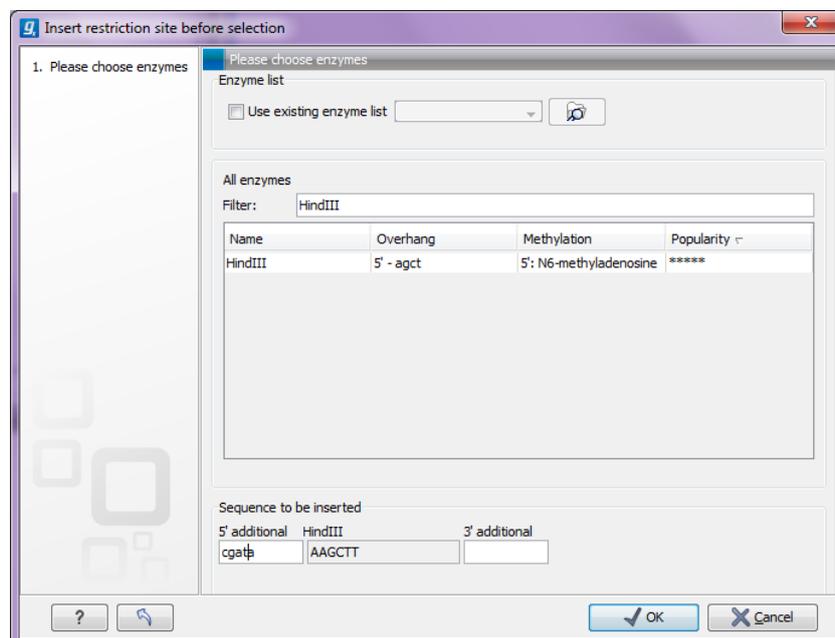


Figure 2.90: Adding restriction sites to a primer.

Click **OK** and the sequence will be inserted at the 5' end of the primer as shown in figure 2.91.

Perform the same process for the `ATP8a1 rev` primer, this time using `XhoI` instead. This time,



Figure 2.91: Adding restriction sites to a primer.

you should also add a few bases at the 5' end as was done in figure 2.13 when inserting the *HindIII* site.

Note! The *ATP8a1 rev* primer is designed to match the negative strand, so the restriction site should be added at the 5' end of this sequence as well (**Insert Restriction Site before Selection**).

Save (↵) the two primers and close the views and you are ready for next step.

2.10.3 Simulate PCR to create the fragment

Now, we want to extract the PCR product from the template *ATP8a1 mRNA* sequence using the two primers with restriction sites.

In the *CLC Main Workbench*:

Toolbox | Primers and Probes (📁) | Find Binding Sites and Create Fragments (🔍)

In the *CLC Genomics Workbench*:

Toolbox | Molecular Biology Tools (🔧) | Primers and Probes (📁) | Find Binding Sites and Create Fragments (🔍)

Select the *ATP8a1 mRNA* sequence and click **Next**. In this dialog, use the **Browse** (📁) button to select the two primer sequences. Click **Next** and adjust the output options as shown in figure 2.92.

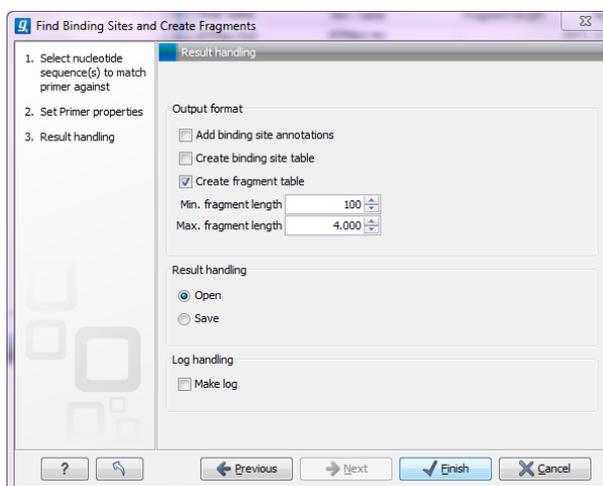


Figure 2.92: Creating the fragment table including fragments up to 4000 bp.

Click **Finish** and you will now see the fragment table displaying the PCR product.

In the **Side Panel** you can choose to show information about melting temperature for the primers.

Right-click the fragment and select **Open Fragment** as shown in figure 2.93.

This will create a new sequence representing the PCR product. **Save** (↵) the sequence in the *Cloning* folder and close the views. You do not need to save the fragment table.

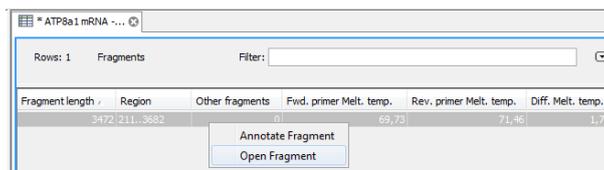


Figure 2.93: Opening the fragment as a sequence.

2.10.4 Specify restriction sites and perform cloning

The final step in this tutorial is to insert the fragment into the cloning vector:

Toolbox in the Menu Bar | Cloning and Restriction Sites (🔧) | Cloning (🔄)

Select the Fragment (ATP8a1 mRNA (ATP8a1 fwd - ATP8a1 rev)) sequence you just saved and also select the pcDNA4_TO cloning vector also located in the Cloning folder and click **Next**. In this dialog,

The cloning editor will now create a sequence list of the selected fragment and vector sequence as shown in figure 23.2. Select the vector sequence and specify it as vector by clicking the "Change to Current" button at the top of the editor (indicated with a red circle in figure 23.2)

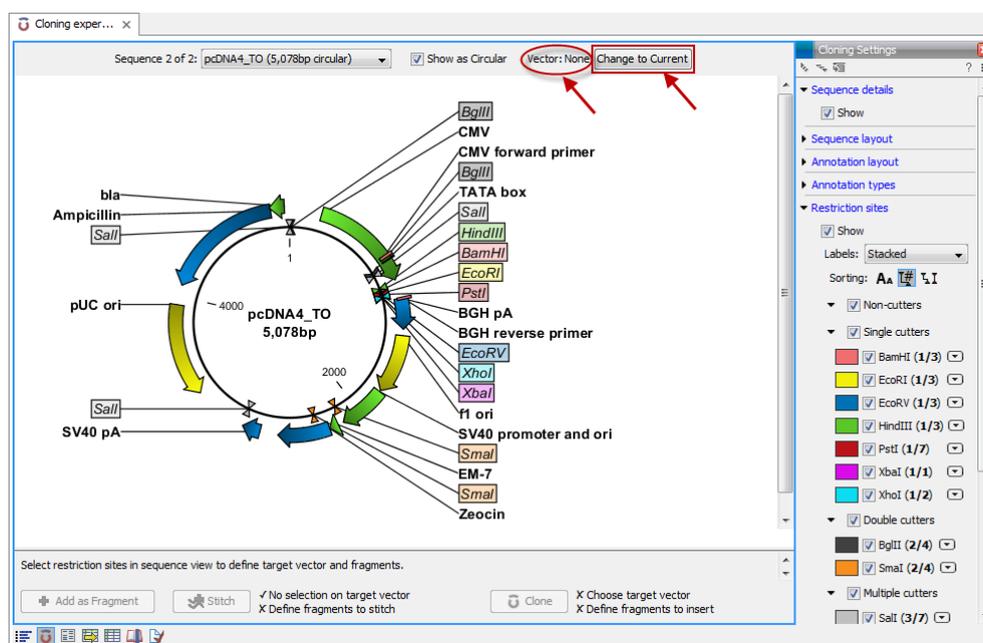


Figure 2.94: Cloning editor.

You will now see the cloning editor where you will see the pcDNA4_TO vector in a circular view. Press and hold the Ctrl (⌘ on Mac) key while you click first the *HindIII* site and next the *XhoI* site (see figure 2.95).

At the bottom of the view you can now see information about how the vector will be cut open. Since the vector has now been split into two fragments, you can decide which one to use as the target vector. If you selected first the *HindIII* site and next the *XhoI* site, the Workbench has already selected the right fragment as the target vector. If you click one of the vector fragments, the corresponding part of the sequence will be high-lighted.

Next step is to cut the fragment. At the top of the view you can switch between the sequences

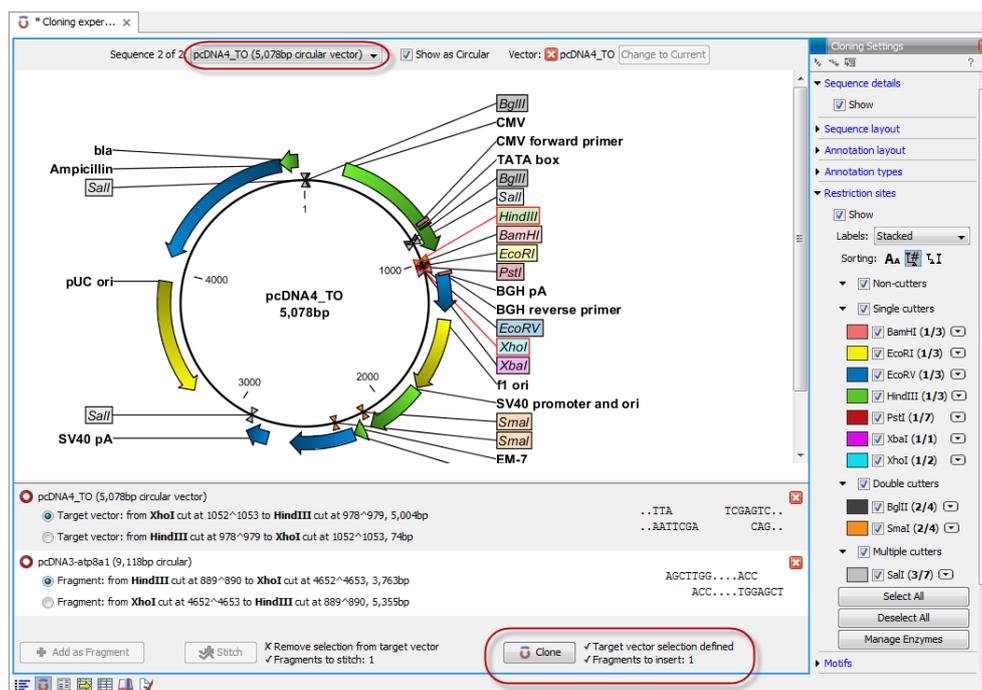


Figure 2.95: Press and hold the **Ctrl** key while you click first the **HindIII** site and next the **XhoI** site in the cloning vector.

used for cloning (at this point it says `pcDNA4_TO 5.078bp circular vector`). Switch to the fragment sequence and perform the same selection of cut sites as before while pressing the **Ctrl** (⌘ on Mac) key. You should now see a view identical to the one shown in figure 2.96.

When this is done, the **Clone** (🔗) button at the lower right corner of the view is active because there is now a valid selection of both fragment and target vector. Click the **Clone** (🔗) button and you will see the dialog shown in figure 2.97.

This dialog lets you inspect the overhangs of the cut site, showing the vector sequence on each side and the fragment in the middle. The fragment can be reverse complemented by clicking the **Reverse complement fragment** (↺) but this is not necessary in this case. Click **Finish** and your new construct will be opened.

When saving your work, there are two options:

- Save the Cloning Experiment. This is saved as a sequence list, including the specified cut sites. This is useful if you need to perform the same process again or double-check details.
- Save the construct shown in the circular view. This will only save the information on the particular sequence including details about how it was created (this can be shown in the **History** view).

You can, of course, save both. In that case, the history of the construct will point to the sequence list in its own history.

The construct is shown in figure 2.98.

Figure 2.96: Change the view to display the fragment sequence. Press and hold the Ctrl key while you click first the HindIII site and next the XhoI site.

Figure 2.97: Showing the insertion point of the vector

2.11 Gateway Cloning

This tutorial will show you how to use the Workbench tool for in silico Gateway®Cloning. With this you can perform both standard (1 fragment) and multi-site Gateway Cloning. Here we focus on the 1-fragment cloning approach, but we provide tips to help you move onto multisite Gateway Cloning easily.

For information on the Gateway Cloning system please visit:

<http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning.html>.

After you have the Gateway Cloning vectors imported into your Workbench (section 2.11.1), you work through the three Gateway Cloning tools, which imitate the steps of the Gateway Cloning

For further information about the vectors for MultiSite Gateway Cloning, please refer to Invitrogen's web site that can be found here <http://www.invitrogen.com/>.

If you choose which type of MultiSite Cloning you wish to perform (number of fragments), then the relevant vectors can be listed. Vector information can then be downloaded from the linked areas on the site.

2.11.2 Adding attB sites

This step mimics the lab PCR procedure of adding attB sites to the target fragment making it eligible for recombination into the donor vector. The output can also include a set of primers, although we do not do that in this tutorial.

Our target fragment is the coding sequence (CDS) of ATP8a1, the mRNA of which is found in the standard example data you can download into the Workbench. If you do not already have the example data go to the **Help** menu in the Workbench, and choose to **Import Example Data**.

Now:

1. Extract the CDS sequence from the ATP8a1 mRNA file. To do this,
 - Double click on the sequence object called **ATP8a1 mRNA**. This opens it in the viewing area of the Workbench.
 - Right-click the CDS annotation, which by default will be coloured in yellow, and chose **Open annotation in New View**
 - Save the new data object that has just opened in the new view. There are different ways to do this. Two options are:
 - (a) Right click on the tab at the top of the new view, and choose Save As... from the menu that appears, or
 - (b) Move the cursor over the tab at the top of the new view, press the left mouse button. Keeping the mouse button down, drag the tab over into a folder in the **Navigation Area** of the Workbench. The data will then be saved into that folder.
2. Add the attB sites. To do this,
 - Start the **Gateway Cloning** tool:
Toolbox | Cloning and Restriction Sites (📁) | Gateway Cloning | Add attB Sites (🔧)
 - In the wizard, select the ATP8a1 CDS fragment you just saved. When the wizard starts up, that fragment should be in the list of **Selected Elements**. If it is not, please select it in the wizard, and click on the right hand arrow to add it to the **Selected Elements** list.
 - Click on the button marked **Next**.
 - It is in this step of the wizard you choose the attB sites to be added to your fragment. This is shown in figure 2.99. The sites to choose depend on your choice of donor vector. We will be going with the default option of attB1 and attB2. This ensures compatibility with our downstream choice of donor vector, which will be **pDONR221**.
 - Click on the button marked **Next**.

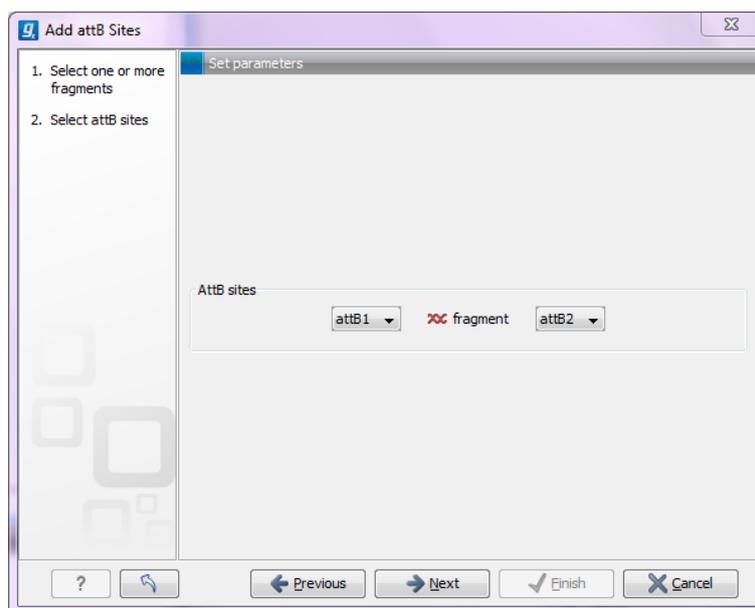


Figure 2.99: Adding attB sites.

If you have selected more than one fragment these will all be given the same combination of attB sites. If you wish to add different combination of sites you will have to run the tool once for each combination.

3. Next you will be given the option to insert additional elements downstream and upstream of the first and second attB site, respectively. That is, to insert elements immediately 5 prime and 3 prime of the fragment.

We will add a Shine-Dalgarno site 5 prime of ATP8a1. Click in the field **Forward primer additions** and press on the keys Shift+F1 (for Mac: Shift + Fn + F1) to bring up a list of elements you can choose from. Use either the mouse or the up and down arrow keys and enter to select the Shine-Dalgarno site. See figure 2.100.

4. Insert 8 random nucleotides after the Shine-Dalgarno sequence. This is done by simply typing in the bases. See figure 2.101. Doing this ensures that the spacing between the Shine-Dalgarno sequence and the initiation codon is correct.

You will find a preview of the final PCR product below. Any preset additional elements appear in green whereas the bases typed in manually are displayed in blue.

5. Click on the button marked **Next**.
6. In this step, you can choose the length of the template-specific part of your primers. See figure 2.102.

This template-specific fragment is joined with the attB site and any additional elements you choose to make up the primer. Here, we will just keep the defaults.

7. Click on the button marked **Next**.
8. Choose to **Save** the results. Uncheck the **create primer list** option as we will not be needing this in this tutorial. See figure 2.103.
9. Click on the button marked **Next** and choose where to **Save** the output.

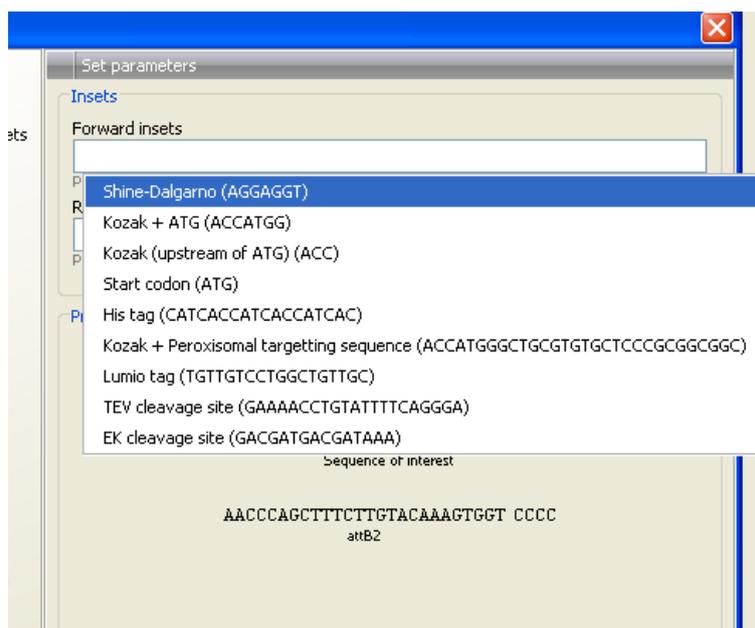


Figure 2.100: Choose Shine-Dalgarno.

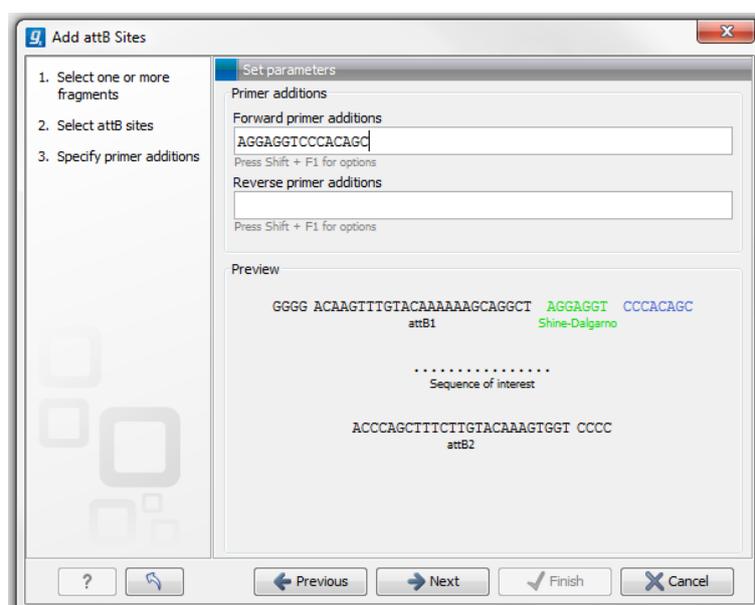


Figure 2.101: Add bases to ensure correct spacing between the Shine-Dalgarno sequence and the initiation codon.

10. Open up the result **ATP8a1 CDS (attB1 attB2)** to have a look. See figure 2.104.
11. In the side panel called **Sequence Settings**, find the section called **Annotation types**. Click on it to expand it.
12. Click on the box beside **RBS** to mark it, so that the Shine-Dalgarno ribosomal binding site annotation is displayed. Note also that you can see annotations for primer regions and the attB sites.

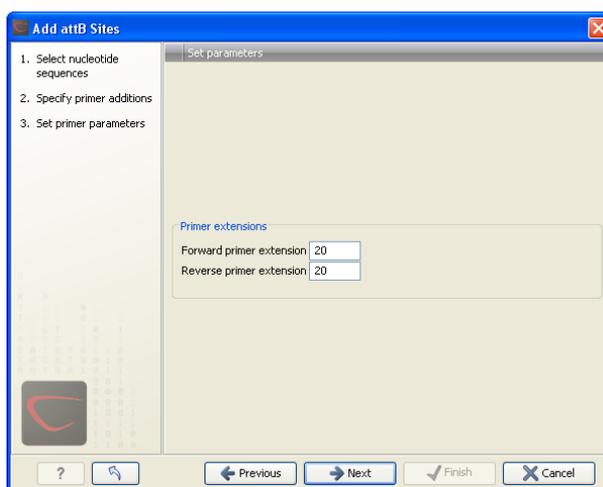


Figure 2.102: Set the appropriate primer lengths.

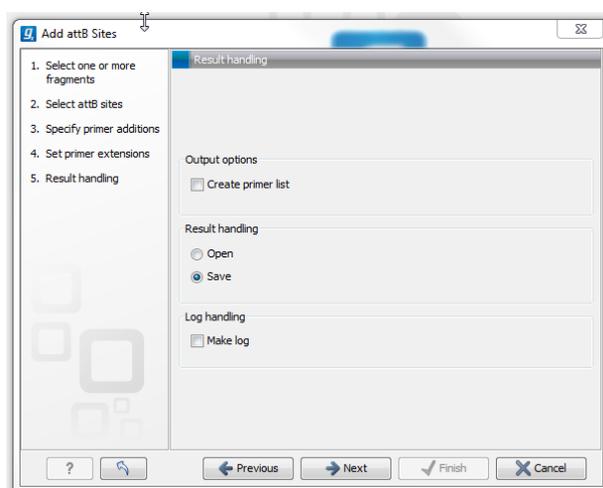


Figure 2.103: Choose to save your results.

2.11.3 Creation of an entry vector

This step recombines the attB-flanked ATP8a1 into the donor vector, which is **pDONR221**, resembling the BP-reaction carried out in the lab.

1. Choose **Create Entry Clone** from under the Gateway Cloning menu:
Toolbox | Cloning and Restriction Sites (📁) | Gateway Cloning | Create Entry Clone (🔄)
2. In the dialog select the result that you generated earlier: **ATP8a1 CDS (attB1 attB2)**. Then click on the button marked **Next**.
3. Click the **Browse button (📁)** and find the Gateway folder of vectors, which was previously imported.
4. Select donor vector **pDONR221** as shown in figure 2.105 and click on the button labelled **OK**.
5. Click on the button labelled **Next**.

Note: The Workbench only checks the chosen entry clone for valid attP sites. It does **not** check that these are compatible with the attB sites present in the fragment. You need to

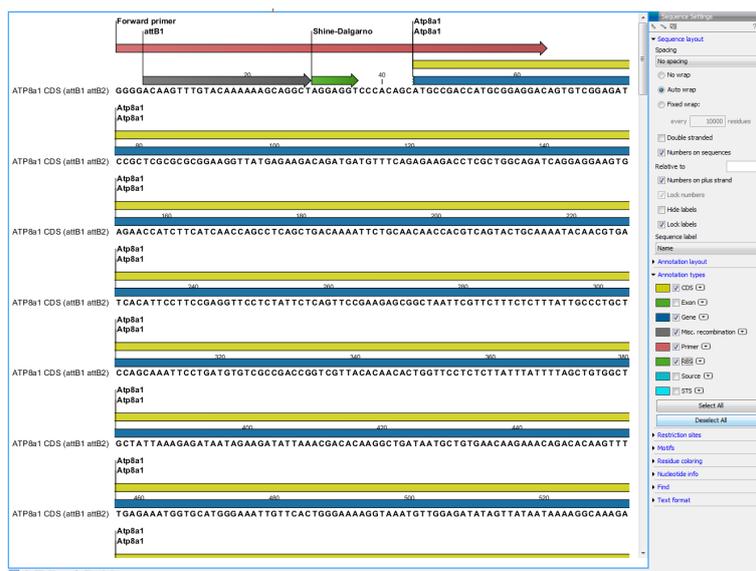


Figure 2.104: A view of the results: ATP8a1 CDS (attB1 attB2).

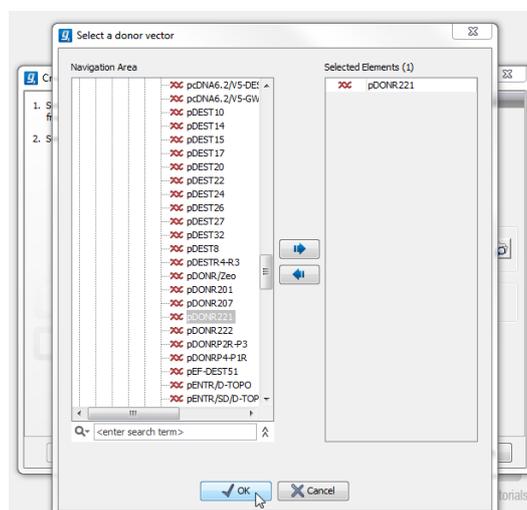


Figure 2.105: Select the donor vector, which in this case is pDONR221.

make sure that a valid entry vector is used for the attB-fragment of interest. If the right combination of attB and attP sites is not found, no entry clones will be produced.

6. Choose to **Save** the result and click on the button labelled **Next** to be able to specify where to save the results to.
7. Click on the button labelled **Finish**.
8. Open the output **pDONR221 (ATP8a1 CDS (attB1 attB2))** to have a look at it. To see a view like that shown in figure 2.106:
 - Go to circular view (🕒).
 - Check annotation type **RBS** in the side panel to get a better view. The attB/attP recombination event has resulted in the fragment of interest being inserted into the vector. The fragment is flanked by attL sites.

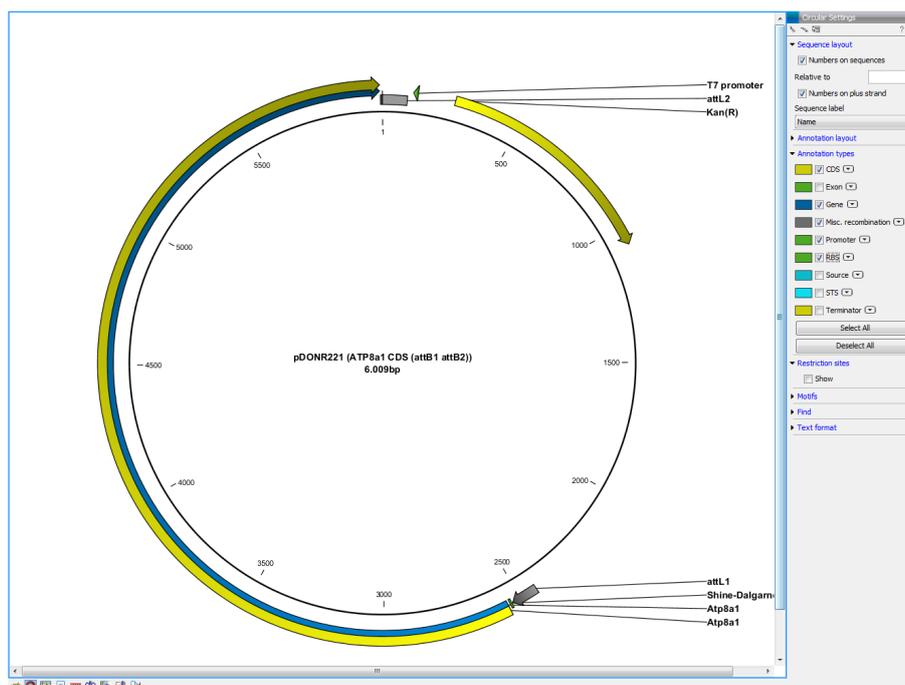


Figure 2.106: View of the entry clone, consisting of the donor vector, pDONR221, with the fragment of interest inserted by attB/attP recombination.

If several fragments were selected for input (using the batch option), the output would be one entry clone per fragment, all based on the same donor vector.

2.11.4 Creating an expression vector (LR)

This last step in a Gateway Cloning workflow mimics the lab LR recombination reaction. That is, the fragment of interest is recombined from the entry vector into a destination vector (the LR reaction) to create the final expression vector.

In this tutorial we are carrying out a standard, single fragment Gateway Cloning task. Thus we will choose only one entry clone as input during this step. For MultiSite Gateway Cloning, we would choose all the entry clones to be joined in the final expression vector. Note, that if you do this, you need to ensure that the entry clones contain a valid combination of attL sites. For more information on this please have a look at the MultiSite Gateway explanation on the Invitrogen webpage:

<http://tools.invitrogen.com/downloads/gateway-multisite-seminar.html>

The steps to create an expression clone are:

1. Choose **Create Expression Clone** from under the Gateway Cloning menu:

Toolbox | Cloning and Restriction Sites (🔧) | Gateway Cloning | Create Expression Clone (🔄)

2. In the dialog box select the entry clone you created in the previous step and click on the button marked **Next**.
3. In the next window, click the **Browse** button and navigate to the Gateway vector folder.

Select the destination vector: **pDEST14** and click on the button labelled **OK**.

4. Click on the button labelled **Next**.

The Workbench only checks for valid attR sites in the destination vectors. You need to ensure that the attL and attR sites are compatible. If not, no expression clone will be created.

5. In the next window choose to open the result. If you would like to also have a look at the byproduct, check the corresponding box. Click on the button labelled **Finish**.

The output is the result of the recombination between the attL and attR sites. The fragment of interest is now inserted in the expression vector flanked by attB sites, see figure 2.107

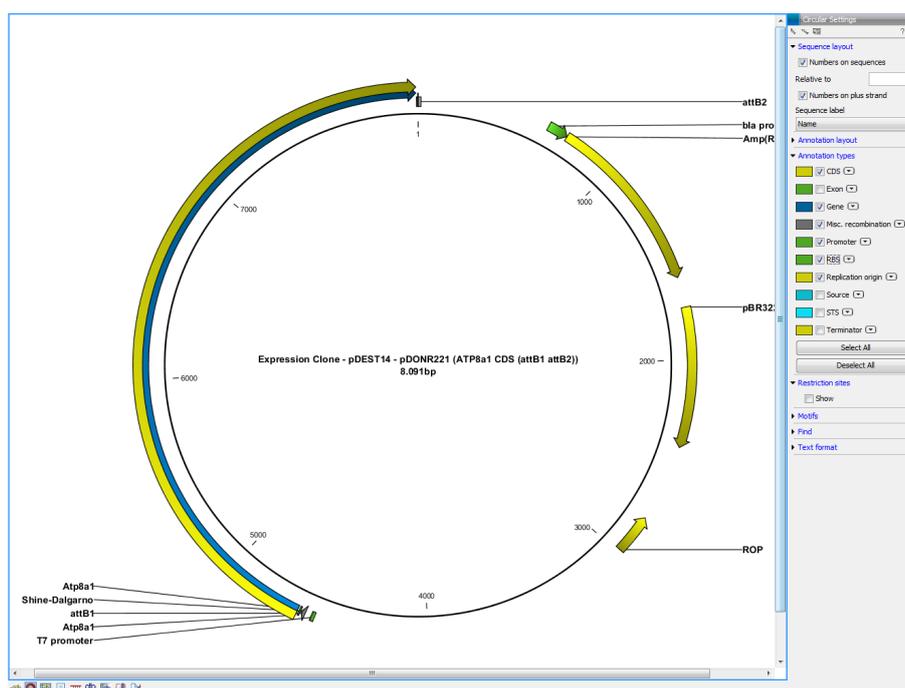


Figure 2.107: View the finished expression vector.

This concludes the Gateway Cloning tutorial. Below is a section outlining the general steps you could take in the case of MultiSite Gateway Cloning.

2.11.5 Short suggestion for a MultiSite Gateway Workflow using 2 fragments

The vectors mentioned in this section can be downloaded from Invitrogens web page:

<http://tools.invitrogen.com/downloads/Gatewayvectors.ma4>

1. Choose two fragments, which you would like to have in the final vector.
2. Create the following fragments:
 - Add attB1 and attB5r sites to sequence of interest 1 → sequence of interest 1 (attB1 attB5r)

- Add attB5 and attB2 sites to sequence of interest 2 → sequence of interest 2 (attB5 attB2)
3. Create two entry clones:
 - sequence of interest 1 (attB1 attB5r) + pDONR221-P1P5r
 - sequence of interest 2 (attB5 attB2) + pDONR221-P5P2
 4. Create the final expression vector by selecting:
 - the first entry clone: pDONR221-P1P5r (fragment of interest 1(attB1 attB5r)), and
 - the second entry clone: pDONR221-P5P2 (fragment of interest 2(attB5 attB2))
 - the destination vector: pDEST14

2.12 Primer Design

In this tutorial, you will see how to use the *CLC Main Workbench* to find primers for PCR amplification of a specific region.

We use the pcDNA3-atp8a1 sequence from the 'Primers' folder in the Example data. This sequence is the pcDNA3 vector with the atp8a1 gene inserted. In this tutorial, we wish to design primers that would allow us to generate a PCR product covering the insertion point of the gene. This would let us use PCR to check that the gene is inserted where we think it is.

First, open the sequence in the Primer Designer. You can do this in different ways:

Open the pcDNA3-atp8a1 sequence by double-clicking on the sequence name in the Navigation Area | Shift to the Primer Designer view by clicking on the icon () in the lower left corner of the View Area

or:

Toolbox | Primers and Probes () | Design Primers ()

This will open a wizard. Select the pcDNA3-atp8a1 sequence from the 'Primers' folder in the Example data by double-clicking on the sequence name or by clicking once on the sequence name and then on the arrow in the center of the wizard pointing to the right hand side. Click on the button labeled **OK**.

Now the sequence is opened and we are ready to begin designing primers (see figure 2.108).

2.12.1 Specifying a region for the forward primer

When opening the sequence in the Primer Designer view, the sequence is shown at single nucleotide resolution. To get an overview of the sequence, we will zoom out a bit by clicking on the **Fit Width** icon () that are found in the lower right corner of the **View Area**. You can now see the blue gene annotation labeled Atp8a1, and just before that there is the green CMV promoter (see figure 2.109). This may be hidden behind restriction site annotations. Remember that you can always choose not to Show these by altering the settings in the right hand pane.

In this tutorial, we want the forward primer to be in a region between positions 600 and 900 - just before the gene and we will zoom in () with the zoom in tool () found in the lower right corner to make the selection.

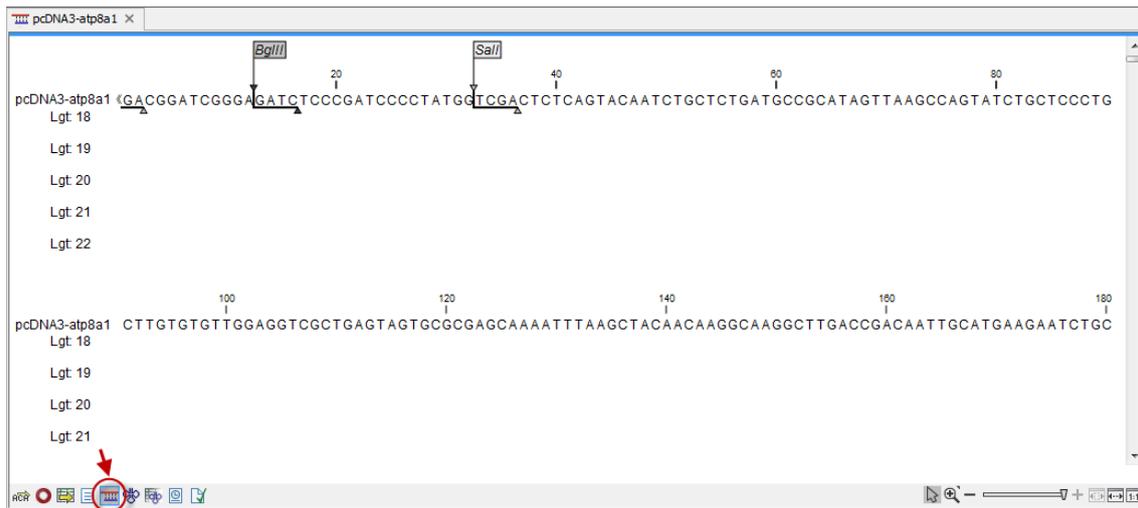


Figure 2.108: The sequence that you would like to design primers to. The sequence has been opened in the Primer Designer. The red arrow highlights the icon that symbolizes the Primer Designer view.

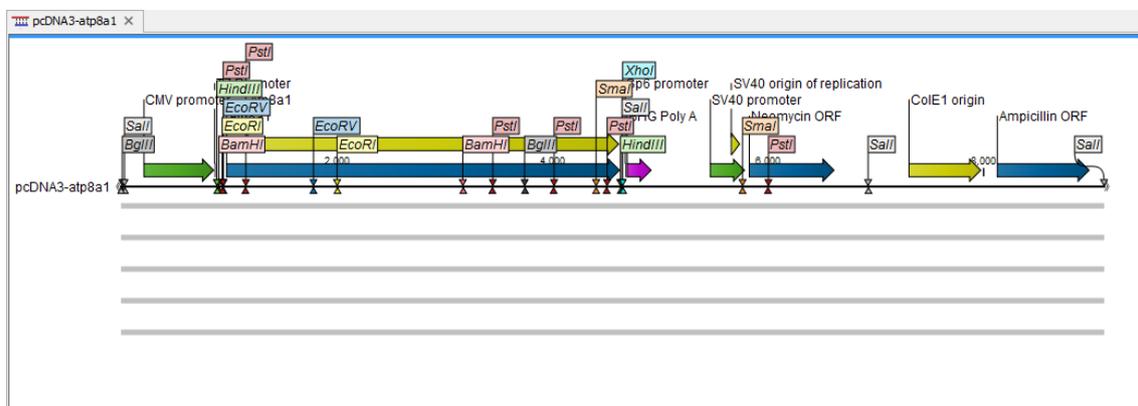


Figure 2.109: Zoom out to get an overview of the sequence.

Select this region, right-click and choose "Forward primer region here" (⇒) (see figure 2.110).

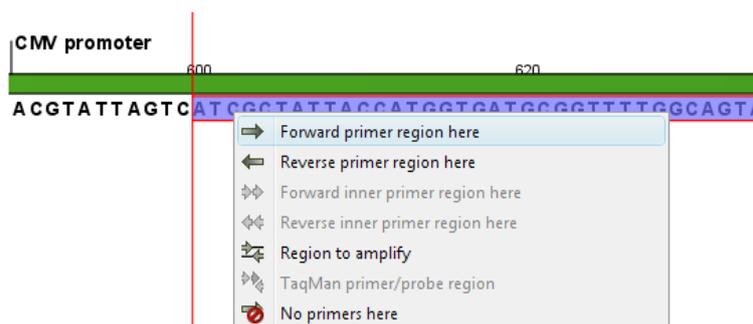


Figure 2.110: Right-clicking a selection and choosing "Forward primer region here".

This will add an annotation to this region, and five rows of red and green dots are seen below as shown in figure 2.111:

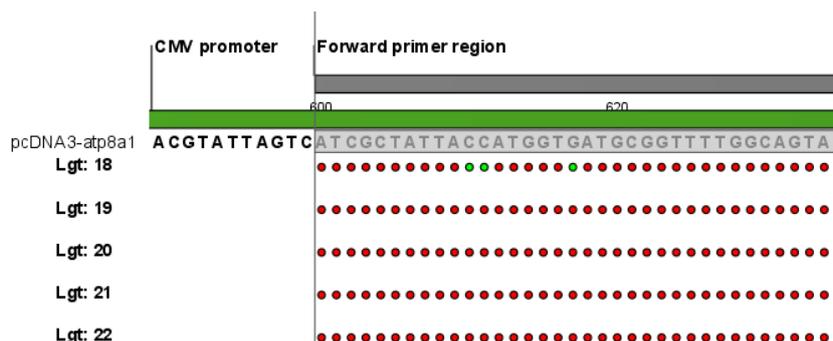


Figure 2.111: Five lines of dots representing primer suggestions. There is a line for each primer length - 18bp through to 22 bp.

2.12.2 Examining the primer suggestions

Each line consists of a number of dots, each representing the *starting point* of a possible primer. E.g. the first dot on the first line (primers of length 18) represents a primer starting at the dot's position and with a length of 18 nucleotides (shown as the white area in figure 2.112):



Figure 2.112: The first dot on line one represents the starting point of a primer that will anneal to the highlighted region.

Position the mouse cursor over a dot. A box will appear, providing data about this primer. Clicking the dot will select the region where that primer would anneal. (See figure 2.113):

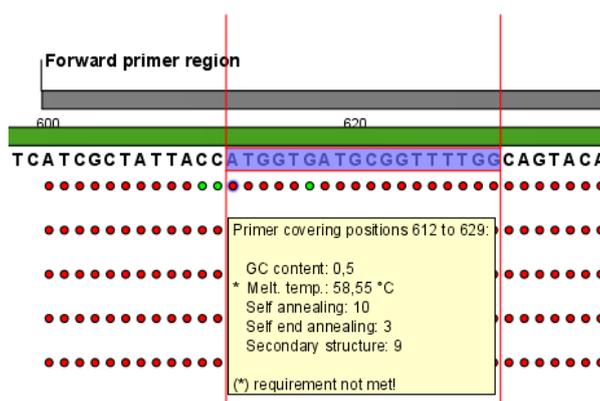


Figure 2.113: Clicking the dot will select the corresponding primer region. Hovering the cursor over the dot will bring up an information box containing details about that primer.

Note that some of the dots are colored red. This indicates that the primer represented by this dot does not meet the requirements set in the **Primer parameters** (see figure 2.114):

The default maximum melting temperature is 58. This is the reason why the primer in figure 2.113 with a melting temperature of 58.55 does not meet the requirements and is colored red.

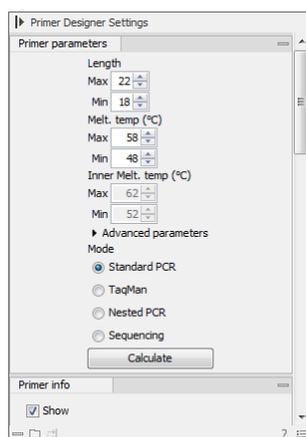


Figure 2.114: The Primer parameters.

If you raise the maximum melting temperature to 59, the primer will meet the requirements and the dot becomes green.

In figure 2.113 there is an asterisk (*) before the melting temperature. This indicates that this primer does not meet the requirements regarding melting temperature. In this way, you can easily see why a specific primer (represented by a dot) fails to meet the requirements.

By adjusting the **Primer parameters** you can define primers to meet your specific needs. Since the dots are dynamically updated, you can immediately see how a change in the primer parameters affects the number of red and green dots.

2.12.3 Calculating a primer pair

Until now, we have been looking at the forward primer. To mark a region for the reverse primer, make a selection from position 1200 to 1400 and:

Right-click the selection | Reverse primer region here (←)

The two regions should now be located as shown in figure 2.115:

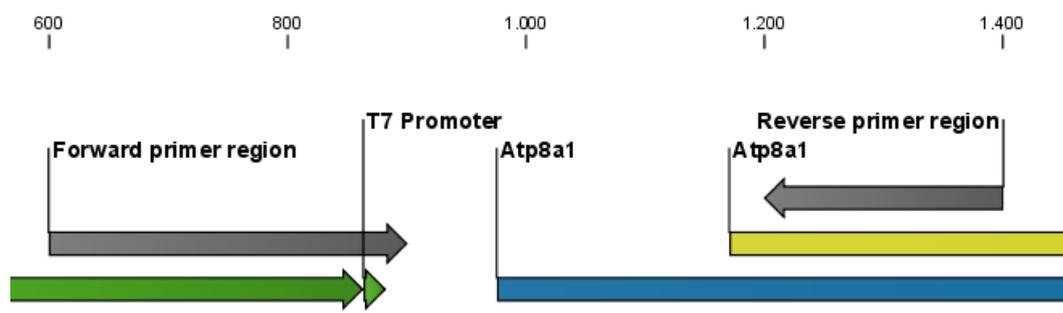


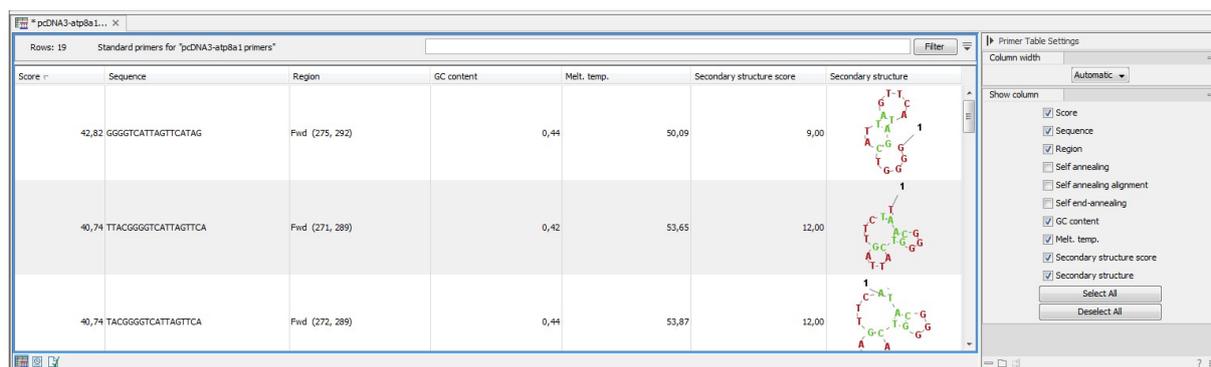
Figure 2.115: A forward and a reverse primer region.

Now, you can let *CLC Main Workbench* calculate all the possible primer pairs based on the **Primer parameters** that you have defined:

Click the Calculate button (right hand pane) | Modify parameters regarding the combination of the primers (for now, just leave them unchanged) | Calculate

This will open a table showing the possible combinations of primers. To the right, you can specify

the information you want to display, e.g. showing **Fragment length** (see figure 2.116):



| Score | Sequence | Region | GC content | Melt. temp. | Secondary structure score | Secondary structure |
|-------|--------------------|----------------|------------|-------------|---------------------------|---------------------|
| 42,82 | GGGGTCATTAGTCATAG | Fwd (275, 292) | | 0,44 | 50,09 | 9,00 |
| 40,74 | TTACGGGGTCATTAGTCA | Fwd (271, 289) | | 0,42 | 53,65 | 12,00 |
| 40,74 | TACGGGGTCATTAGTCA | Fwd (272, 289) | | 0,44 | 53,87 | 12,00 |

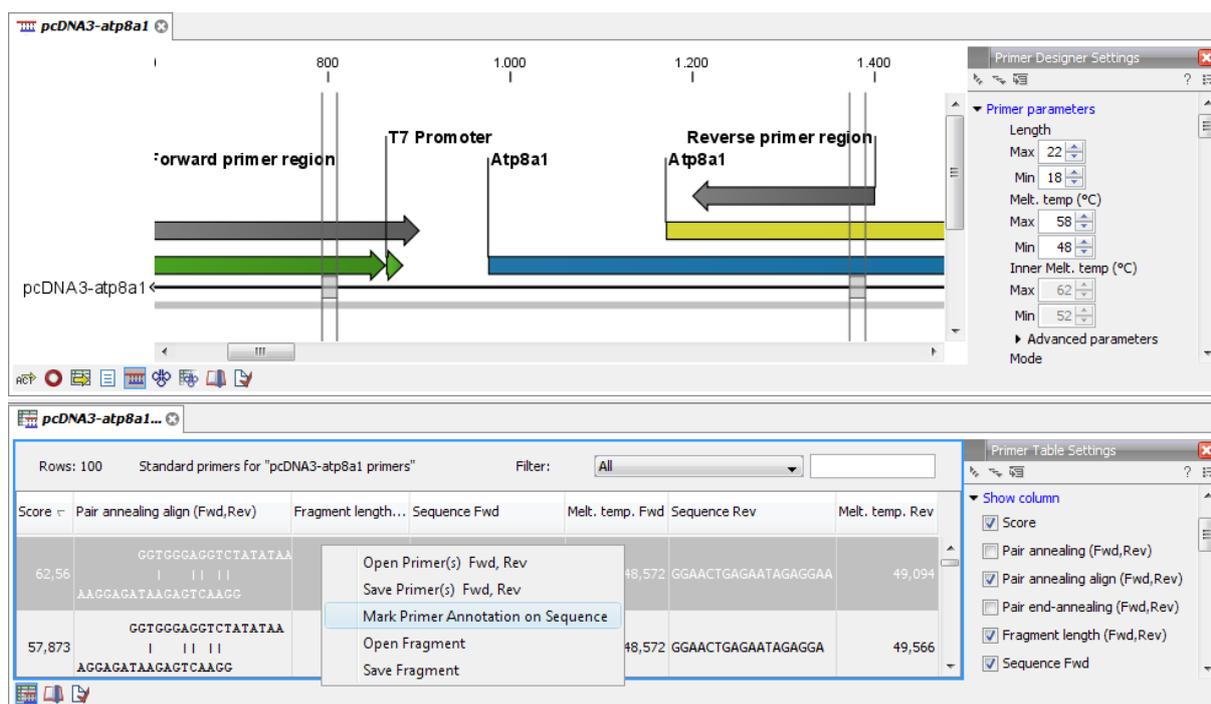
The side panel 'Primer Table Settings' shows the following 'Show column' options:

- Score
- Sequence
- Region
- Self annealing
- Self annealing alignment
- Self end-annealing
- GC content
- Melt. temp.
- Secondary structure score
- Secondary structure

Figure 2.116: A list of primers. To the right are the Side Panel showing the available choices of information to display.

Clicking a primer pair in the table will make a corresponding selection on the sequence in the view above. At this point, you can either settle on a specific primer pair or save the table for later. If you want to use e.g. the first primer pair for your experiment, right-click this primer pair in the table and save the primers.

You can also mark the position of the primers on the sequence by selecting **Mark primer annotation on sequence** in the right-click menu (see figure 2.117):



The top part of the image shows a sequence view for 'pcDNA3-atp8a1' with a T7 Promoter and Atp8a1 gene. Two primers are highlighted: a forward primer (green arrow) and a reverse primer (yellow arrow). The bottom part shows a table of primers with a right-click menu open over the first row. The menu options are:

- Open Primer(s) Fwd, Rev
- Save Primer(s) Fwd, Rev
- Mark Primer Annotation on Sequence
- Open Fragment
- Save Fragment

The 'Mark Primer Annotation on Sequence' option is highlighted. The table below shows the primer details:

| Score | Pair annealing align (Fwd,Rev) | Fragment length... | Sequence Fwd | Melt. temp. Fwd | Sequence Rev | Melt. temp. Rev |
|--------|---|--------------------|--------------|-----------------|---------------------|-----------------|
| 62,56 | CGTGGGAGGCTATATAA AAGGACATAAGACTCAAGG | | | 48,572 | GGAAGTGAAGATAGAGGAA | 49,094 |
| 57,873 | CGTGGGAGGCTATATAA AAGGACATAAGACTCAAGG | | | 48,572 | GGAAGTGAAGATAGAGGA | 49,566 |

Figure 2.117: The options available in the right-click menu. Here, "Mark primer annotation on sequence" has been chosen, resulting in two annotations on the sequence above (labeled "Oligo").

This tutorial has shown some of the many options of the primer design functionalities of *CLC Main Workbench*. You can read much more using the program's **Help** function (?) or in the *CLC Main Workbench* user manual, linked to on this webpage: <http://www.clcbio.com/download>.

2.13 Working with Annotations

Annotations are the basis of many of the analyses in the Workbench. Once an annotation has been added to a sequence, it stays there even when the sequence is transformed to be part of an alignment, a BLAST result or a sequence list.

Because annotations are so fundamental to many of the tasks performed in the Workbench, there are a number of ways to browse, search for, view and edit annotations. This tutorial takes you through a lot of different areas of the Workbench to show some of the places where you can work with annotations.

2.13.1 Browsing and viewing annotations in sequence views

Open sequence *ATP8a1 mRNA* from the Example data by double-clicking the sequence. Click **Annotation types** in the **Side Panel** and you will see a list of the types of annotations on this sequence (shown in figure 2.118).

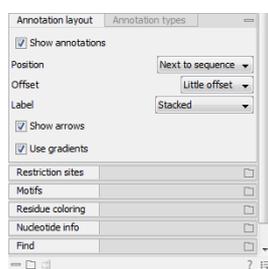


Figure 2.118: Annotation types of the *ATP8a1 mRNA* sequence.

Click the *Exon* annotation type. This will show exon annotations on the sequence. As this sequence is very long, you cannot see the whole sequence, and the exons may therefore not be visible. In this situation, you can either Zoom out to find the annotations, or you can use the browse button (🔍) next to the annotation type to jump directly to one of the annotations (see figure 2.119).

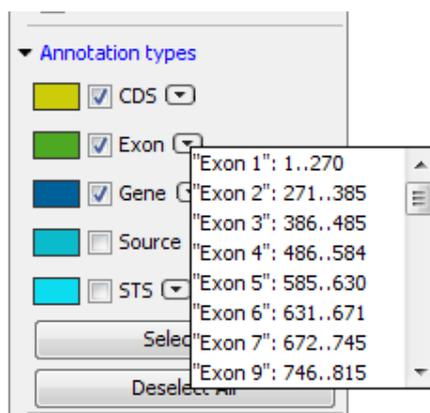


Figure 2.119: Click in the list to go directly to this annotation.

Click *Exon 2*, and the beginning of the annotation will be visible in the view (see figure 2.120).

Annotations are displayed as arrows, going from left to right on the positive strand, and going from right to left on the negative strand. Placing your mouse cursor on the arrow will show

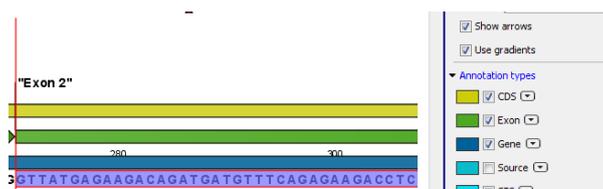


Figure 2.120: The view jumps to the beginning of exon 2.

additional information about the annotation. Try to place the mouse cursor on the blue gene annotation, and you can see more information about it (see figure 2.121).

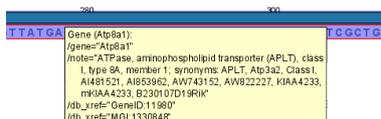


Figure 2.121: Information about the annotation.

This way of displaying and accessing annotations is similar for both circular views, alignments, and all other views displaying sequence residues.

2.13.2 Adding and editing annotations

You can add your own annotations to a sequence. A new annotation is most easily added if you first select a region on the sequence. In the upper view, select a region from residue 10 to 26 (see the status bar in the lower right corner of the Workbench). Then:

right-click the selection | Add Annotation (➡)

This will display a dialog where you can enter more information about the annotation. Enter *Test* as the name of the annotation (see figure 2.122).

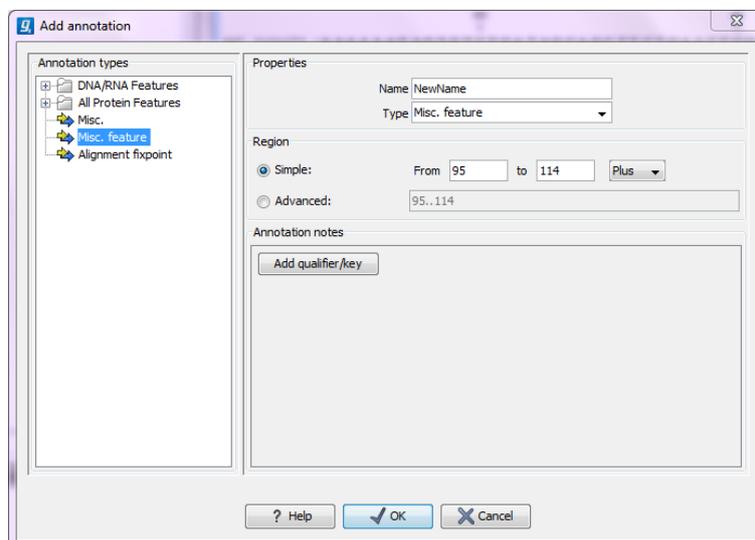


Figure 2.122: The Add Annotation dialog.

Click **OK** and the annotation is added to the sequence (see figure 2.123).

Now click the **Show Annotation Table** (📄) icon at the bottom of the view. This will display a list of all the annotations on the sequence.

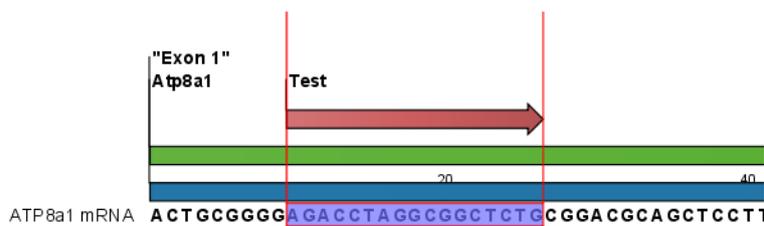


Figure 2.123: The annotation has been added.

Enter *Test* into the **Filter** in the annotation table, and your newly added annotation will appear.

You can now double-click any of the **Name**, **Type** or **Region** fields to edit the annotation. Double-click the **Type** which is now "Misc. feature" and enter *My type* and press **Enter**.

The table should now look as shown in figure 2.124.

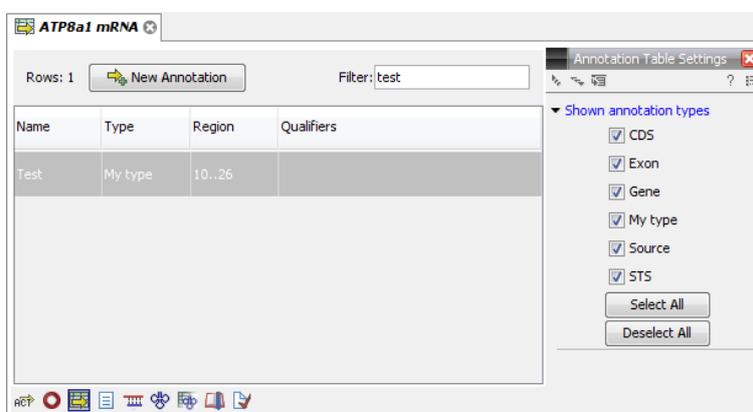


Figure 2.124: The type of the annotation is now *My type*.

In this way, you can quickly edit annotations. If you double-click the **Qualifiers** cell, a dialog similar to the one in figure 2.122 will be shown.

2.13.3 Copying annotations

One of the more advanced features of the Workbench is the ability to copy and paste annotations. Annotations can be copied from one sequence to another or you can create a duplicate of the annotation by pasting it back on the same sequence. Because it is very easy to edit the region of the annotation, this can be a quick way of adding a number of similar annotations.

Select the *Test* annotation in the table | Copy (⌘) | Paste (⌘)

Double-click the region of the new annotation and type 30..46 and press **Enter**. You now have two annotations as shown in figure 2.125.

2.14 BLAST

BLAST is an invaluable tool in bioinformatics. It has become central to identification of homologues and similar sequences, and can also be used for many other different purposes. This tutorial takes you through the steps of running a blast search in CLC Workbenches. If you plan to use BLAST for your research, we highly recommend that you read further about it.

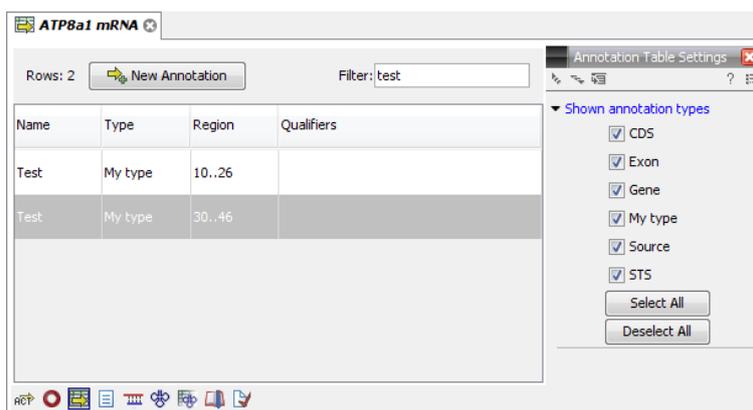


Figure 2.125: Two copies of the Test annotation.

Understanding how BLAST works is key to setting up meaningful and efficient searches.

Suppose you are working with the ATP8a1 protein sequence, which is a phospholipid-transporting ATPase expressed in the adult house mouse, *Mus musculus*. To obtain more information about this molecule, different nucleotide or protein BLAST query options can be performed against NCBI, in data subsets hereof or in local databases:

Toolbox | BLAST

- BLAST
- BLAST at NCBI
- Download BLAST Databases
- Create BLAST Database
- Manage BLAST Database

To obtain more information about this molecule you wish to query the peptides held in the Swiss-Prot* database to find homologous proteins in humans *Homo sapiens*, using the **Basic Local Alignment Search Tool** (BLAST) algorithm.

Please note: This tutorial involves running BLAST remotely using databases housed at the NCBI. Your computer must be connected to the internet to complete this tutorial.

2.14.1 Performing the BLAST search in own sequence data or own BLAST database

Select your query sequence(s), go to the Toolbox and select the BLAST search:

select e.g. ATP8a1 genomic sequence | Toolbox | BLAST

As the tool works with either nucleotide or protein query sequences, it is important to select the relevant BLAST search program:

BLAST programs for DNA query sequences:

- **blastn: DNA sequence against a DNA database.** Used to look for DNA sequences with homologous regions to your nucleotide query sequence.

- **tblastn: Translated DNA sequence against a Translated DNA database.** Automatic translations 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.
- **blastx: Automatic translation of your DNA query sequence in six frames;** these translated sequences are then used to search a protein database.

BLAST programs for protein query sequences:

- **tblastn: Protein sequence against Translated DNA database.** Peptide query sequences are searched against an automatically translated, in six frames, DNA database.
- **blastp: Protein sequence against Protein database.** Used to look for peptide sequences with homologous regions to your peptide query sequence.

The sequence data to be searched within is called the Target. This can either be locally stored sequences or a local database BLAST database (see subsequent sections on how to Create and Manage Local Blast Databases, respectively).

Note: Selected subsets of sequence will be used as a temporary database, and not be accessible afterwards.

Click **Next**.

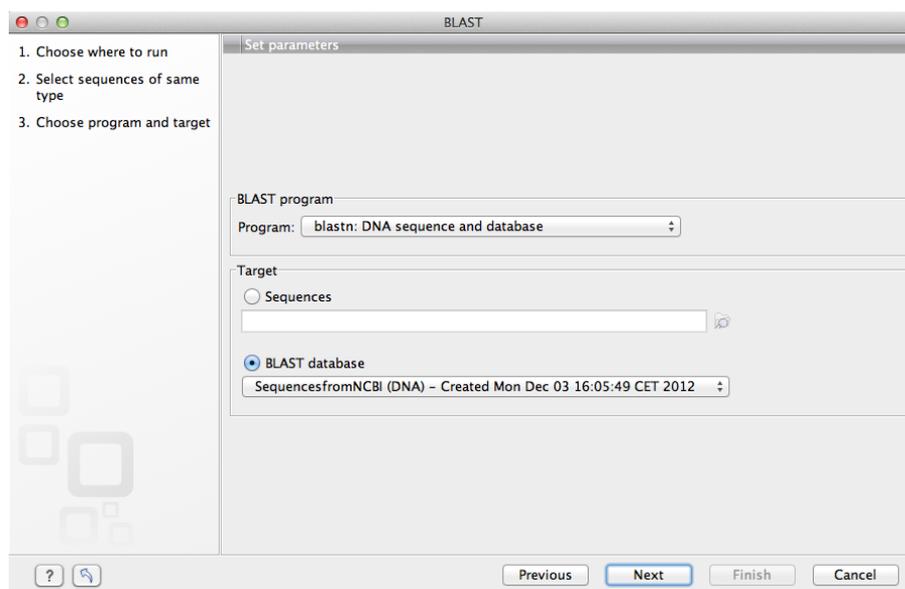


Figure 2.126: The outcome of the BLAST query search is dependent on the BLAST program selected as well as the Target data to search within (locally stored sequences or BLAST database).

A number of optional parameters can be set prior to performing the BLAST search (figure 2.127) e.g. number of threads, filter, size of expected E-value, word size, matrix, gap cost, and maximum number of hits to be shown. In the current and earlier versions of the Workbench, the number of hits being reported is as specified, however, all high-scoring segment pairs (HSPs) for those hits are provided in the results. Thus, in cases where some hits have more than one HSP, the hit table will have more rows than the number of hits requested.

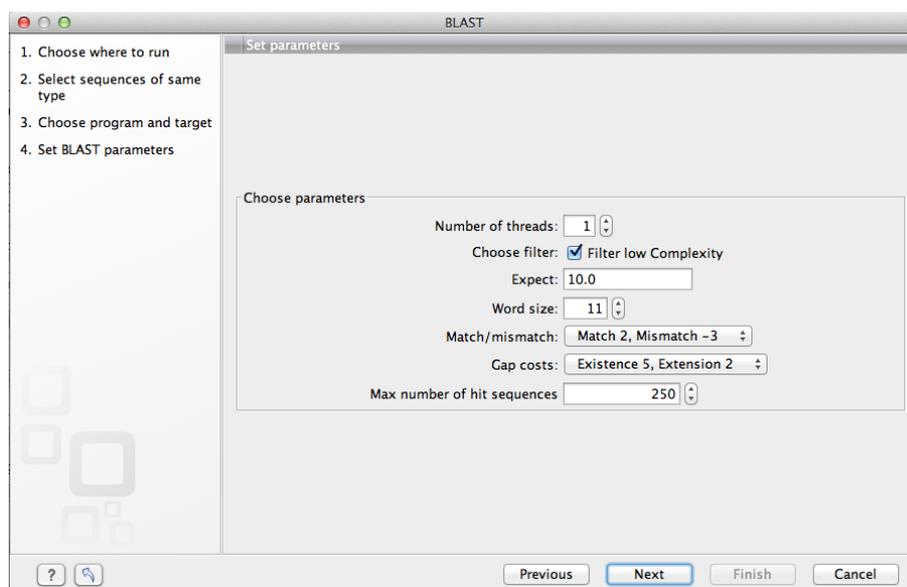


Figure 2.127: A number of parameters can be modified to optimize the selected BLAST search.

2.14.2 Create BLAST Database

BLAST databases can be created from DNA, RNA, and protein sequences located in the **Navigation Area**. Any given BLAST database can only include one molecule type. If you wish to use a pre-formatted BLAST database instead, see above section on Download BLAST Database.

To create a BLAST database, go to:

Toolbox | BLAST (📁) | Create BLAST Database (🛠️)

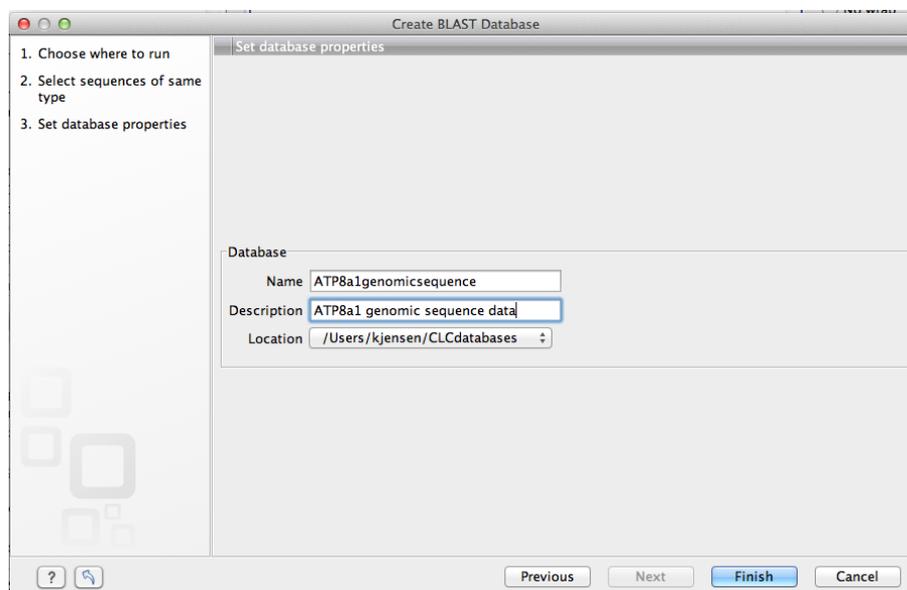


Figure 2.128: When using Create BLAST Database, a database of the specified sequences files (and/or sequence lists) will be created.

2.14.3 Download BLAST Database

The Download BLAST Database allows you to fetch copies of specific databases stored at NCBI FTP-site. Examples include 16S microbial sequences, environmental nucleotide or protein sequences, EST- or genomic data data from mouse, human, other organisms, ref sequences etc. (figure 2.129).

The download location can be specified, and downloaded databases can be managed subsequently using the Manage BLAST Databases (see following section).

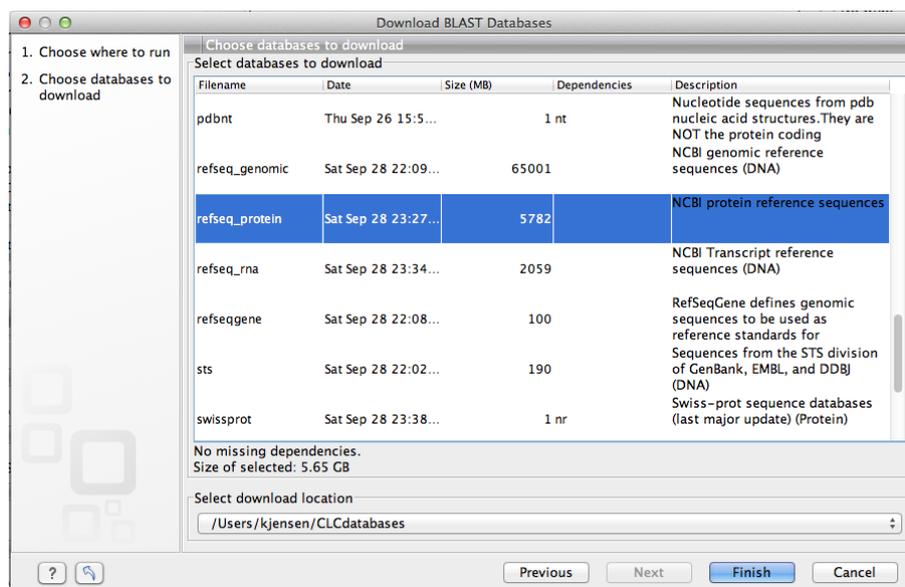


Figure 2.129: Topic specific databases can be downloaded from NCBI's FTP server, and stored locally.

2.14.4 BLAST Database Manager

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. Once downloaded, it is easy to get an overview of the local databases as well as to modify them (add- and refresh location as well as remove database, see figure 2.130).

2.14.5 Performing the BLAST search

Start out by:

select protein ATP8a1 | Toolbox | BLAST (🔍) | BLAST at NCBI (🌐)

In **Step 1** you can choose which sequence to use as query sequence. AS you have already selected a sequence, the sequence is already displayed in the **Selected Elements** list.

Click **Next**.

In **Step 2** (figure 2.131), choose the default BLAST program: **blastp: Protein sequence and database** and select the **Swiss-Prot** database in the **Database** drop down menu.

Click **Next**.

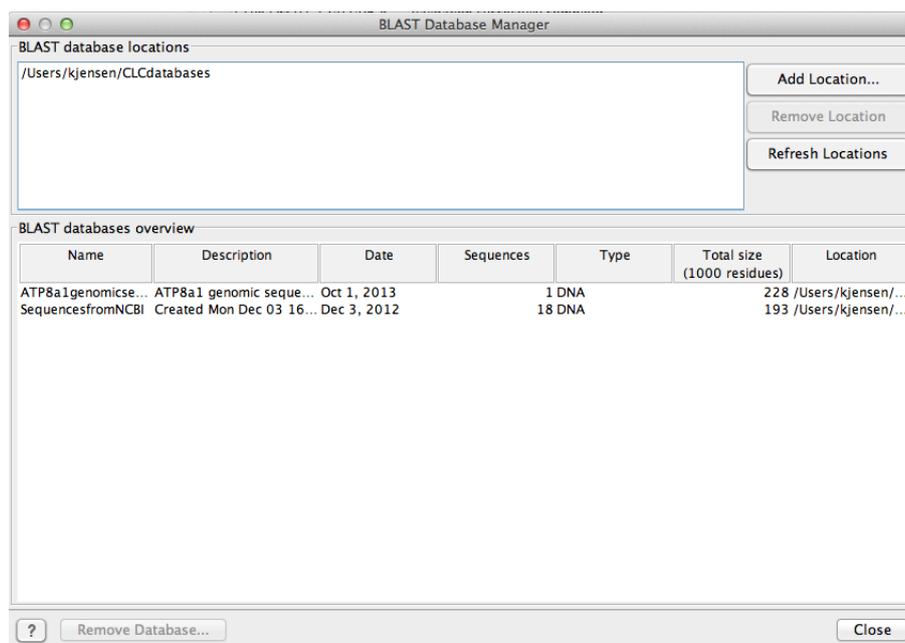


Figure 2.130: The BLAST Database Manager provides an overview of available BLAST databases, their description, size and location, as well as the option to change location.

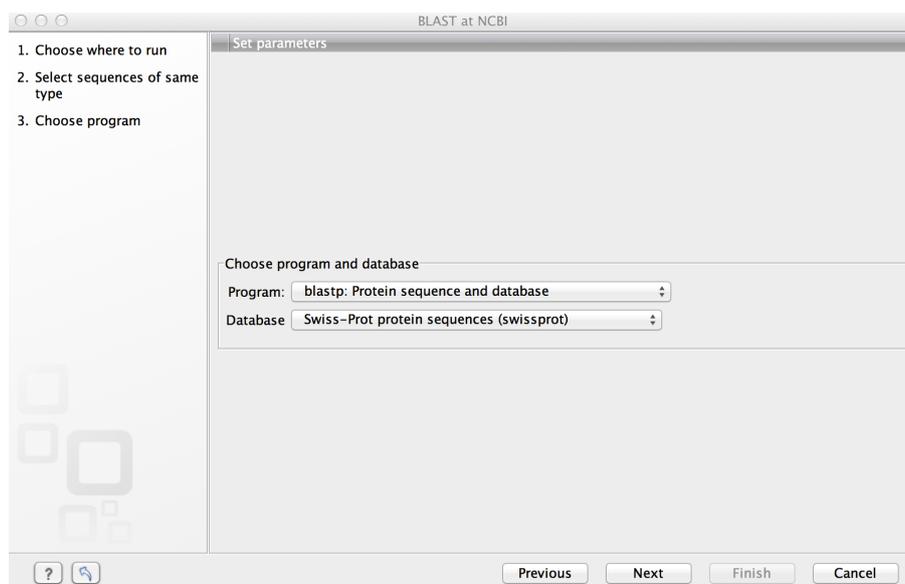


Figure 2.131: Choosing BLAST program and database.

In the **Limit by Entrez query** in **Step 3**, choose **Homo sapiens[ORGN]** from the drop down menu to arrive at the search configuration seen in figure 2.132. Including this term will limit the query to proteins of human origin.

Choose to **Open** your results.

Click **Finish** to accept the parameter settings and begin the BLAST search.

The computer now contacts NCBI and places your query in the BLAST search queue. After a short while the result should be received and opened in a new view.

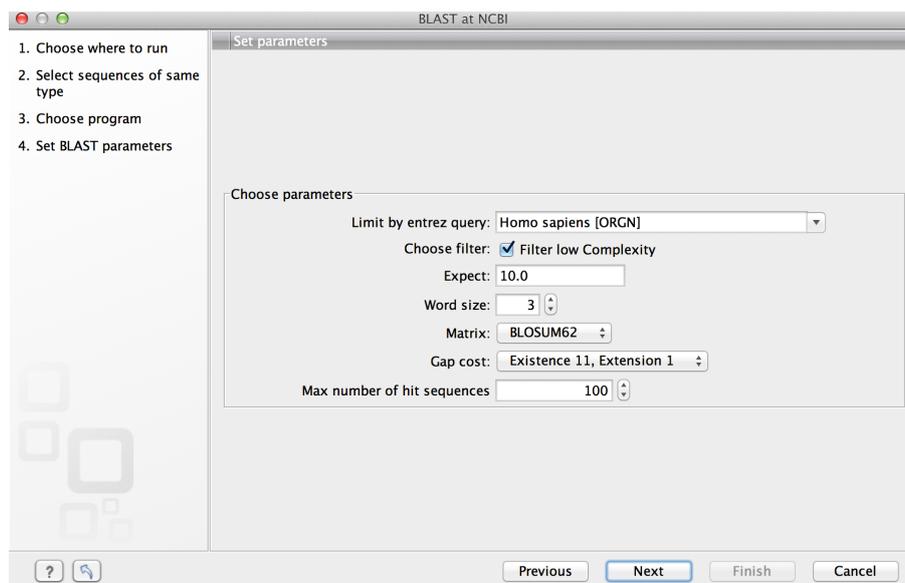


Figure 2.132: The BLAST search is limited to homo sapiens[ORGN]. The remaining parameters are left as default.

2.14.6 Inspecting the results

The output is shown in figure 2.133 and consists of a list of potential homologs that are sorted by their BLAST match-score and shown in descending order below the query sequence.



Figure 2.133: Output of a BLAST search. By holding the mouse pointer over the lines you can get information about the sequence.

Try placing your mouse cursor over a potential homologous sequence. You will see that a context box appears containing information about the sequence and the match-scores obtained from the BLAST algorithm.

The lines in the BLAST view are the actual sequences that have been downloaded. This means that you can zoom in and see the actual alignment:

Zoom in using the Tool Bar zoom function (🔍) | Click in the BLAST view a number of times until you see the residues

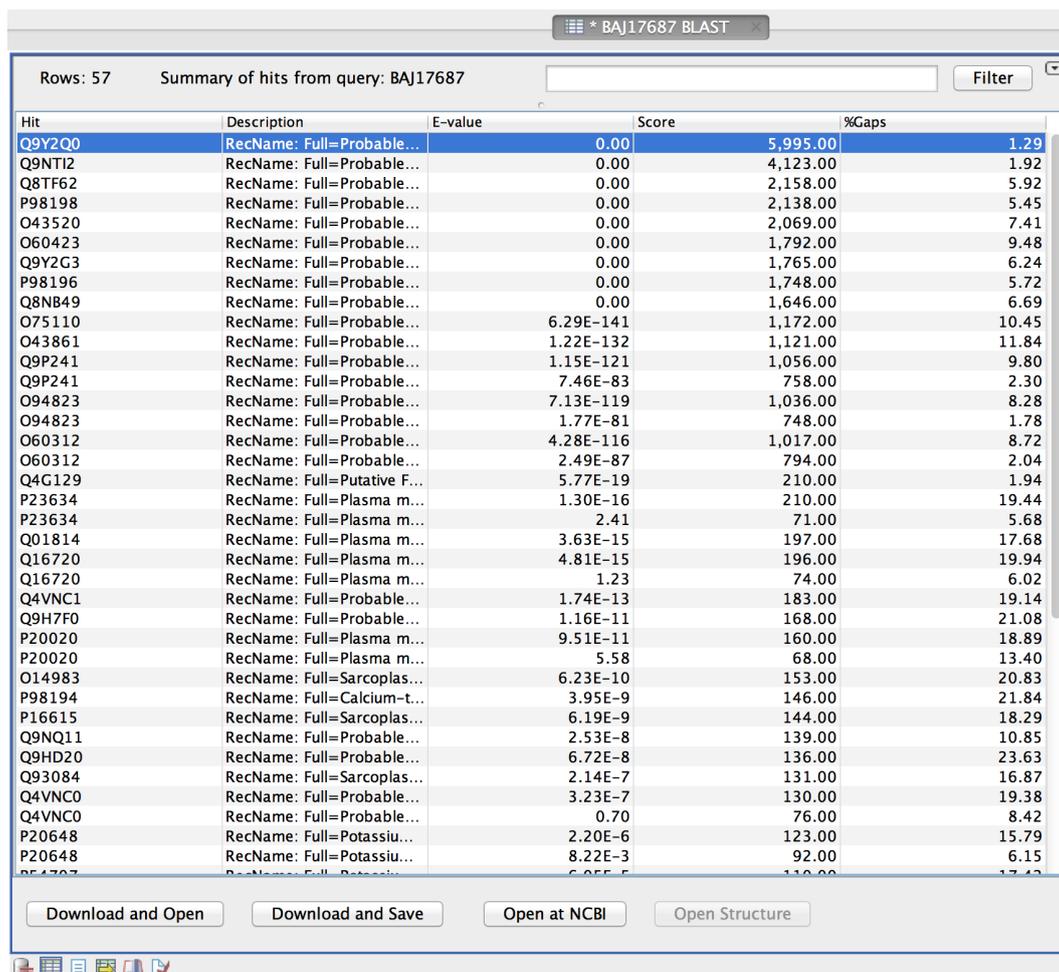
Now we will focus our attention on sequence Q9Y2Q0 - the BLAST hit that is at the top of the list. To download the full sequence:

right-click the line representing sequence Q9Y2Q0 | Download Full Hit Sequence from NCBI

This opens the sequence. However, the sequence is not saved yet. Drag and drop the sequence into the **Navigation Area** to save it. This homologous sequence is now stored in the *CLC Main Workbench* and you can use it to gain information about the query sequence by using the various tools of the workbench, e.g. by studying its textual information, by studying its annotation or by aligning it to the query sequence.

2.14.7 Using the BLAST table view

As an alternative to the graphic BLAST view, you can click the Table View  at the bottom. This will display a tabular view of the BLAST hits as shown in figure 2.134.



| Hit | Description | E-value | Score | %Gaps |
|--------|-----------------------------|-----------|----------|-------|
| Q9Y2Q0 | RecName: Full=Probable... | 0.00 | 5,995.00 | 1.29 |
| Q9NTI2 | RecName: Full=Probable... | 0.00 | 4,123.00 | 1.92 |
| Q8TF62 | RecName: Full=Probable... | 0.00 | 2,158.00 | 5.92 |
| P98198 | RecName: Full=Probable... | 0.00 | 2,138.00 | 5.45 |
| O43520 | RecName: Full=Probable... | 0.00 | 2,069.00 | 7.41 |
| O60423 | RecName: Full=Probable... | 0.00 | 1,792.00 | 9.48 |
| Q9Y2G3 | RecName: Full=Probable... | 0.00 | 1,765.00 | 6.24 |
| P98196 | RecName: Full=Probable... | 0.00 | 1,748.00 | 5.72 |
| Q8NB49 | RecName: Full=Probable... | 0.00 | 1,646.00 | 6.69 |
| O75110 | RecName: Full=Probable... | 6.29E-141 | 1,172.00 | 10.45 |
| O43861 | RecName: Full=Probable... | 1.22E-132 | 1,121.00 | 11.84 |
| Q9P241 | RecName: Full=Probable... | 1.15E-121 | 1,056.00 | 9.80 |
| Q9P241 | RecName: Full=Probable... | 7.46E-83 | 758.00 | 2.30 |
| O94823 | RecName: Full=Probable... | 7.13E-119 | 1,036.00 | 8.28 |
| O94823 | RecName: Full=Probable... | 1.77E-81 | 748.00 | 1.78 |
| O60312 | RecName: Full=Probable... | 4.28E-116 | 1,017.00 | 8.72 |
| O60312 | RecName: Full=Probable... | 2.49E-87 | 794.00 | 2.04 |
| Q4G129 | RecName: Full=Putative F... | 5.77E-19 | 210.00 | 1.94 |
| P23634 | RecName: Full=Plasma m... | 1.30E-16 | 210.00 | 19.44 |
| P23634 | RecName: Full=Plasma m... | 2.41 | 71.00 | 5.68 |
| Q01814 | RecName: Full=Plasma m... | 3.63E-15 | 197.00 | 17.68 |
| Q16720 | RecName: Full=Plasma m... | 4.81E-15 | 196.00 | 19.94 |
| Q16720 | RecName: Full=Plasma m... | 1.23 | 74.00 | 6.02 |
| Q4VNC1 | RecName: Full=Probable... | 1.74E-13 | 183.00 | 19.14 |
| Q9H7F0 | RecName: Full=Probable... | 1.16E-11 | 168.00 | 21.08 |
| P20020 | RecName: Full=Plasma m... | 9.51E-11 | 160.00 | 18.89 |
| P20020 | RecName: Full=Plasma m... | 5.58 | 68.00 | 13.40 |
| O14983 | RecName: Full=Sarcoplas... | 6.23E-10 | 153.00 | 20.83 |
| P98194 | RecName: Full=Calcium-t... | 3.95E-9 | 146.00 | 21.84 |
| P16615 | RecName: Full=Sarcoplas... | 6.19E-9 | 144.00 | 18.29 |
| Q9NQ11 | RecName: Full=Probable... | 2.53E-8 | 139.00 | 10.85 |
| Q9HD20 | RecName: Full=Probable... | 6.72E-8 | 136.00 | 23.63 |
| Q93084 | RecName: Full=Sarcoplas... | 2.14E-7 | 131.00 | 16.87 |
| Q4VNC0 | RecName: Full=Probable... | 3.23E-7 | 130.00 | 19.38 |
| Q4VNC0 | RecName: Full=Probable... | 0.70 | 76.00 | 8.42 |
| P20648 | RecName: Full=Potassiu... | 2.20E-6 | 123.00 | 15.79 |
| P20648 | RecName: Full=Potassiu... | 8.22E-3 | 92.00 | 6.15 |
| P51707 | RecName: Full=Potassiu... | 6.02E-5 | 110.00 | 17.42 |

Figure 2.134: Output of a BLAST search shown in a table.

This view provides more statistics about the hits, and you can use the filter to search for e.g. a specific type of protein etc. If you wish to download several of the hit sequences, this is easily done in this view. Simply select the relevant sequences and drag them into a folder in the **Navigation Area**.

2.15 Tips for Specialized BLAST Searches

Here, you will learn how to:

- Use BLAST to find the gene coding for a protein in a genomic sequence.
- Find primer binding sites on genomic sequences.

This tutorial requires some experience with the Workbench, so if you get stuck at some point, we recommend that you go through the more basic tutorials first.

2.15.1 Locate a protein sequence on the chromosome

If you have a protein sequence but want to see the actual location on the chromosome this is easy to do using BLAST.

In this example we wish to map the protein sequence of the human arrestin domain-containing protein 5 (ARRDC5) protein to a chromosome. We know in advance that the ARRDC5 is located somewhere on chromosome 19.

Data used in this example can be downloaded from GenBank:

Download | **Search for Sequences at NCBI** ()

Human chromosome 19 (NC_000019) consists of 59128983 nucleotides and the ARRDC5 (NP_001073992) protein has 342 amino acids.

BLAST configuration

Next, conduct a local BLAST search:

Toolbox | **BLAST** () | **BLAST** ()

Select the protein sequence as query sequence and click **Next**. Since you wish to BLAST a protein sequence against a nucleotide sequence, select **tblastn**, which will automatically translate the selected nucleotide sequence to database format.

Select the just downloaded chr19 sequence *NC_000019* as target. If you are used to BLAST, you will know that you usually have to create a BLAST database before BLASTing. However, the Workbench does this "on the fly" when one or more sequences have been selected.

Click **Next**, leave the parameters at their default, click **Next** again, and then **Finish**.

Inspect BLAST result

When the BLAST result appears make a split view so that both the table and graphical view is visible (see figure 2.135). This is done by pressing Ctrl ( on Mac) while clicking the table view () at the bottom of the view.

In the side panel under BLAST table settings, select the same settings as shown in figure 2.135 by checking the relevant parameters under "Show column".

Now, sort the BLAST table view by clicking twice on the column header "% Positive". Then, press and hold the Ctrl button ( on Mac) and click the header "Query start". You have now sorted the table first on % Positive hits and then on the start position of the query sequence. In the table you can see three hits with 97 - 100% positive (=similar residues) at different locations on the chromosome sequence (see figure 2.135).

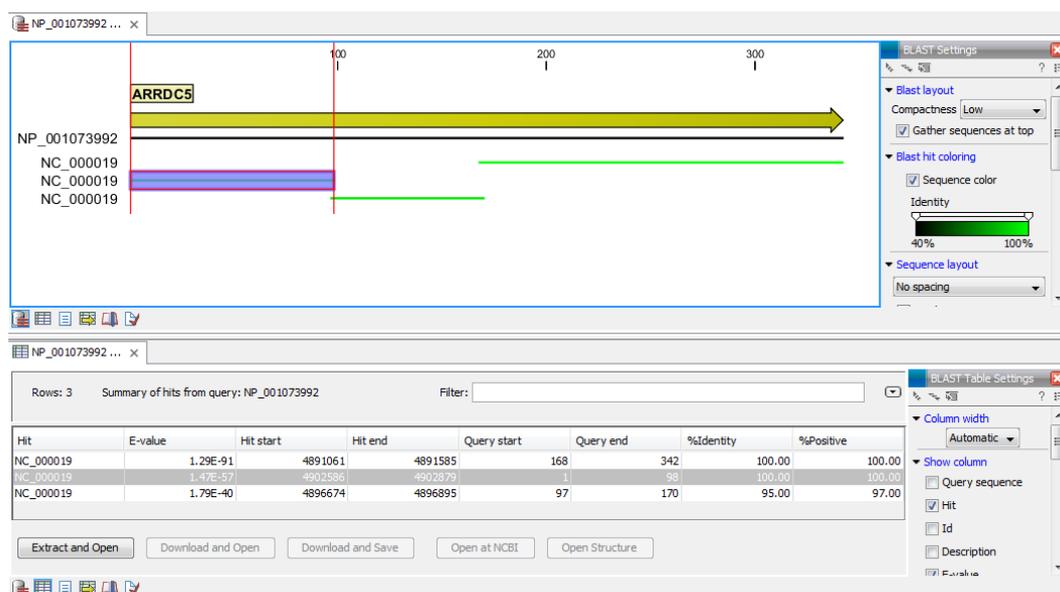


Figure 2.135: Placement of translated nucleotide sequence hits on the Human ARRDC5 protein.

Why did we find, on the protein level, three identical regions between our query protein sequence and nucleotide database?

The ARRDC5 gene is known to have three exons, which is in agreement with the three hits in the BLAST search. Each translated exon will hit the corresponding sequence on the chromosome.

If you place the mouse cursor on the sequence hits in the graphical view, you can see the reading frame, which is -1, -3 and -3 for the three hits, respectively.

Verify the result

Open NC_000019 in a view, and go to Hit position (4,902,879) and zoom to see the blue gene annotation. You can now see the exon structure of the ARRDC5 gene showing the three exons on the reverse strand (see figure 2.136).

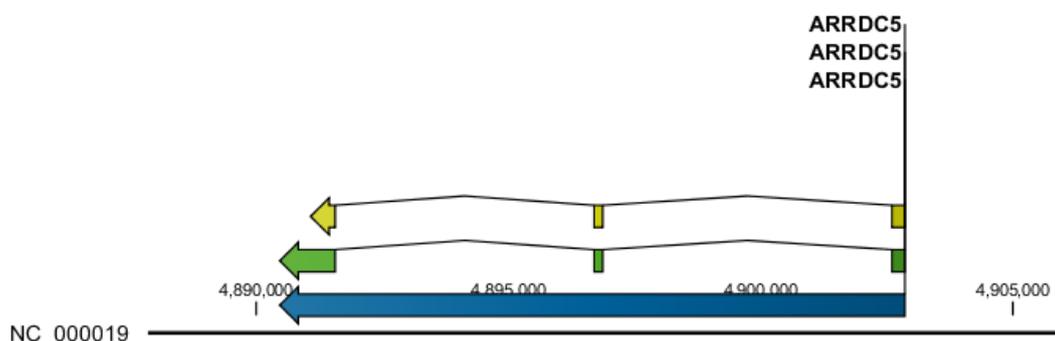


Figure 2.136: ARRDC5 exon view.

If you wish to verify the result, make a selection covering the gene region and open it in a new view:

right-click | Open Selection in New View (📄) | Save (💾)

Save the sequence and perform a new BLAST search:

- Use the new sequence as query.
- Use BLASTx
- Use the protein sequence, NP_001073992, as target sequence

Tip: In stead of using the protein sequence NP_001073992 as target sequence you can also use it as database. To do this, you first have to create a database:

Toolbox | BLAST (📄) | **Create BLAST database** (👤)

Using the genomic sequence as query, the mapping of the protein sequence to the exons is visually very clear as shown in figure 2.137.

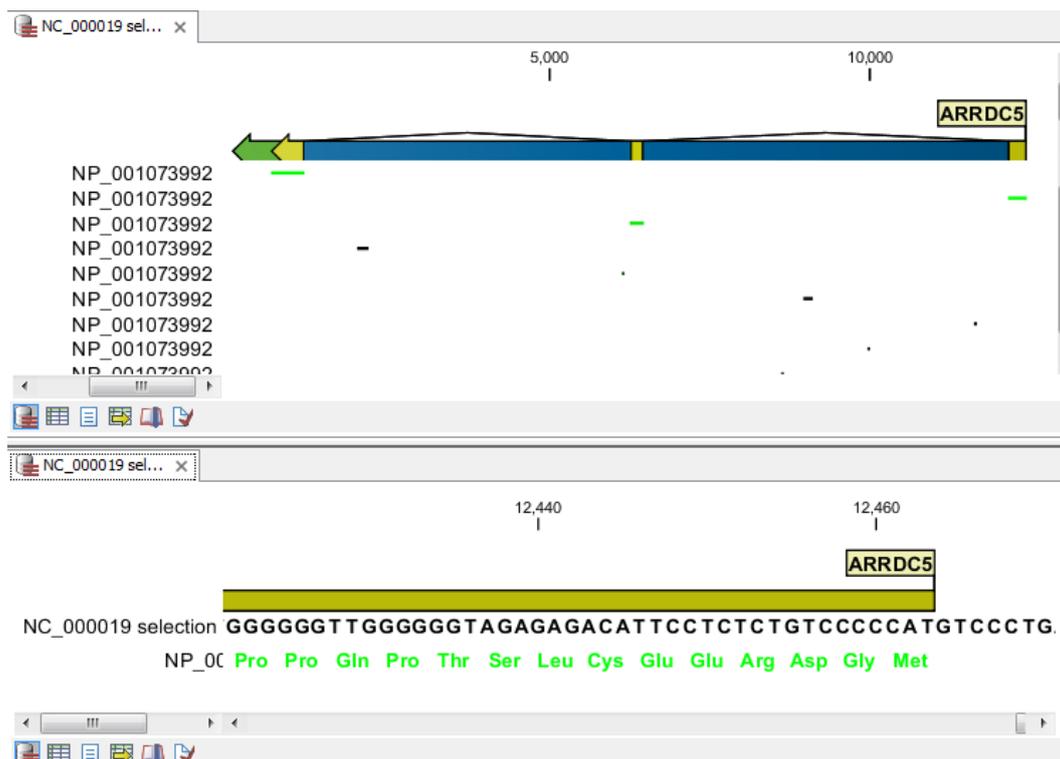


Figure 2.137: Verification of the result: at the top a view of the whole BLAST result. At the bottom the same view zoomed in on exon 3 to show the amino acids.

In theory you could use the chromosome sequence as query, but the performance would not be optimal: it would take a long time, and the computer might run out of memory.

In this example you have used well-annotated sequences where you could have searched for the name of the gene instead of using BLAST. However, there are other situations where you either do not know the name of the gene, or the genomic sequence is poorly annotated. In these cases, the approach described in this tutorial can be very productive.

2.15.2 BLAST for primer binding sites

You can adjust the BLAST parameters so it becomes possible to match short primer sequences against a larger sequence. Then it is easy to examine whether already existing lab primers can be reused for other purposes, or if the primers you designed are specific.

| Purpose | Program | Word size | Low complexity filter | Expect value |
|----------------|---------|-----------|-----------------------|--------------|
| Standard BLAST | blastn | 11 | On | 10 |
| Primer search | blastn | 7 | Off | 1000 |

These settings are shown in figure 2.138.

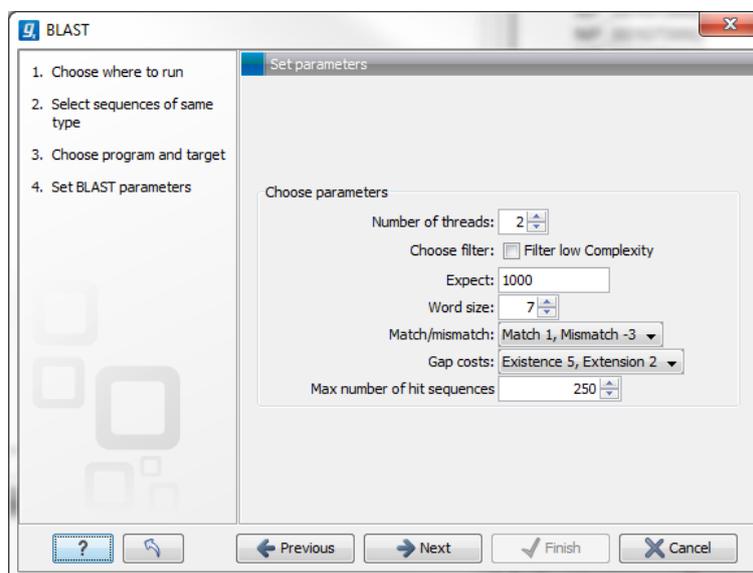


Figure 2.138: Settings for searching for primer binding sites.

2.15.3 Further reading

A valuable source of information about BLAST can be found at http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=ProgSelectionGuide.

Remember that BLAST is a heuristic method. This means that certain assumptions are made to allow searches to be done in a reasonable amount of time. Thus, you cannot trust BLAST search results to present with an accurate alignment. For more accurate results you should consider using other algorithms, such as Smith-Waterman for a multiple sequence alignment. You can read more in "Bioinformatics explained: BLAST" found here: http://www.clcsupport.com/clcgenomicsworkbench/current/index.php?manual=BE_BLAST.html.

2.16 Folding RNA Molecules

In this tutorial, you will learn how to predict the secondary structure of an RNA molecule. You will also learn how to use the powerful ways of viewing and interacting with graphical displays of the structure.

The sequence to be folded in this tutorial is a tRNA molecule with the characteristic secondary structure as shown in figure 2.139.

The goal for this tutorial is to get a nice-looking graphic result of this structure.

The sequence we are working with is a mitochondrial tRNA molecule from *Drosophila melanogaster*. The name is AB009835, and can be found by searching GenBank:

Download | **Search for Sequences at NCBI** ()

For now, we are not interested in the linear view. Click the **Show Secondary Structure 2D View** () button at the bottom of the view to show the secondary structure. It looks as shown in figure 2.142).

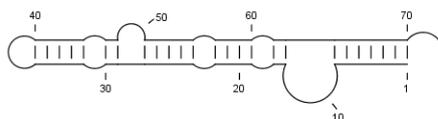


Figure 2.142: The initial 2D view of the secondary structure.

This structure does not look like the one we expected (shown in figure 2.139). We now take a look at some of the other structures (we chose to compute 10 different structures) to see if we can find the classic tRNA structure. First, open a split view of the **Show Secondary Structure Table** ():

Open a split view of the **Show Secondary Structure Table** () by holding Ctrl (or ⌘ on Mac) and the icon for **Show Secondary Structure Table** ()

You will now see a table displaying the ten structures. Selecting a structure in the table will display this structure in the view above. Select the second structure in the table. The views should now look like figure 2.143).

| Name | Created | ΔG | Probability |
|--------------------------------------|---------------------|--------------|-------------|
| $\Delta G = -9,9\text{kcal/mol}$... | 26-11-2007 09:05:59 | -9,9kcal/mol | 6,89E-3 |
| $\Delta G = -9,7\text{kcal/mol}$... | 26-11-2007 09:05:59 | -9,7kcal/mol | 4,98E-3 |
| $\Delta G = -9,4\text{kcal/mol}$... | 26-11-2007 09:05:59 | -9,4kcal/mol | 3,06E-3 |
| $\Delta G = -9,2\text{kcal/mol}$... | 26-11-2007 09:05:59 | -9,2kcal/mol | 2,21E-3 |
| $\Delta G = -9,1\text{kcal/mol}$... | 26-11-2007 09:05:59 | -9,1kcal/mol | 1,88E-3 |
| $\Delta G = -9,1\text{kcal/mol}$... | 26-11-2007 09:05:59 | -9,1kcal/mol | 1,88E-3 |
| $\Delta G = -8,9\text{kcal/mol}$... | 26-11-2007 09:05:59 | -8,9kcal/mol | 1,36E-3 |
| $\Delta G = -8,6\text{kcal/mol}$... | 26-11-2007 09:05:59 | -8,6kcal/mol | 8,35E-4 |
| $\Delta G = -8,4\text{kcal/mol}$... | 26-11-2007 09:05:59 | -8,4kcal/mol | 6,03E-4 |
| $\Delta G = -8,3\text{kcal/mol}$... | 26-11-2007 09:05:59 | -8,3kcal/mol | 5,13E-4 |

Figure 2.143: A split view showing the secondary structure table at the bottom and the Secondary structure 2D view at the top. (You might need to Zoom out to see the structure).

The secondary structure now looks very similar to figure 2.139. By adjusting the layout, we can make it look exactly the same: in the Side Panel of the 2D view, under **Secondary Structure**, choose the **Proportional** layout strategy. You will now see that the appearance of structure changes.

Next, zoom in on the structure to see the residues. This is easiest if you first close () the table view at the bottom.

Zoom in (🔍) | **Click the structure until you see the residues**

If you wish to make some manual corrections of the layout of the structure, first select the **Pan** (🖱️) mode in the Tool bar. 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).

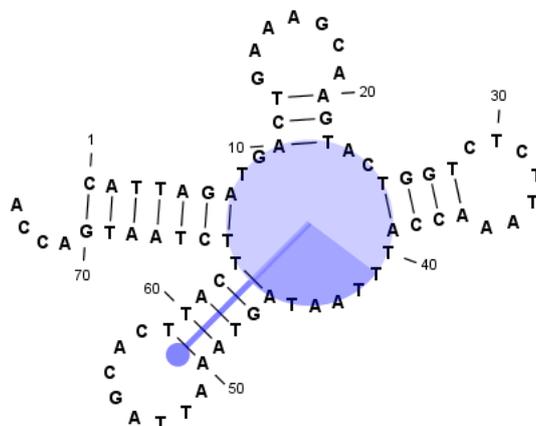


Figure 2.144: The blue circle represents the anchor point for rotating the substructure.

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.

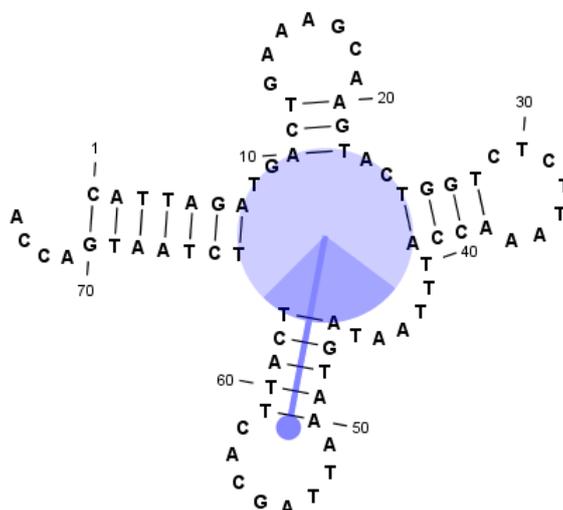


Figure 2.145: The structure has now been rotated.

The view can of course be printed (🖨️) or exported as graphics (📄).

2.17 Align Protein Sequences

This tutorial outlines some of the alignment functionality of the *CLC Main Workbench*. In addition to creating alignments of nucleotide or peptide sequences, the software offers several ways to view alignments. The alignments can then be used for building phylogenetic trees.

Sequences must be available via the **Navigation Area** to be included in an alignment. If you have sequences open in a View that you have not saved, then you just need to select the view tab and press Ctrl + S (or ⌘ + S on Mac) to save them.

In this tutorial six protein sequences from the Example data folder will be aligned. (See figure 2.146).

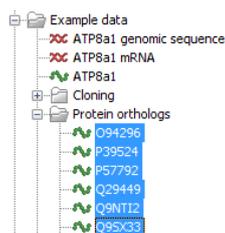


Figure 2.146: Six protein sequences in 'Sequences' from the 'Protein orthologs' folder of the Example data.

To align the sequences:

select and then right click on the sequences from the 'Protein orthologs' folder under 'Example Data' | Toolbox | Alignments and Trees (📁) | Create Alignment (🔍)

2.17.1 The alignment dialog

This opens the dialog shown in figure 2.147.

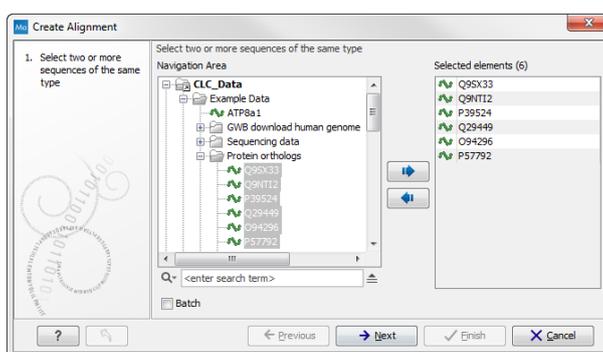


Figure 2.147: The alignment dialog displaying the six protein sequences.

It is possible to add and remove sequences from **Selected Elements** list. Since we had already selected the six proteins, just click **Next** to adjust parameters for the alignment.

Clicking **Next** opens the dialog shown in figure 2.148.

Leave the parameters at their default settings. An explanation of the parameters can be found by clicking the help button (?). Alternatively, a tooltip is displayed by holding the mouse cursor on the parameters.

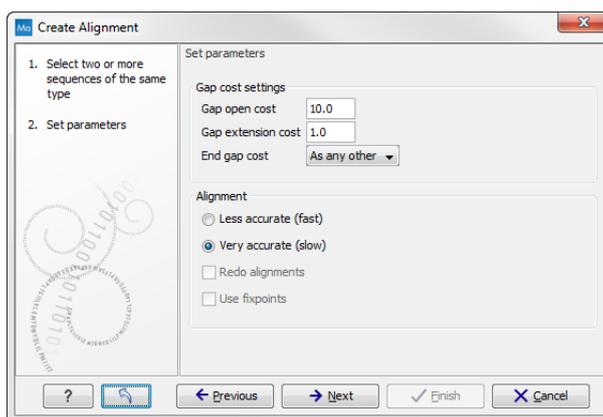


Figure 2.148: The alignment dialog displaying the available parameters which can be adjusted.

Click on the button labeled **Next** and specify where you would like to save the data. Click on the button labeled **Finish** to start the alignment process which is shown in the **Toolbox** under the **Processes** tab. When the program is finished calculating it displays the alignment (see fig. 2.149):

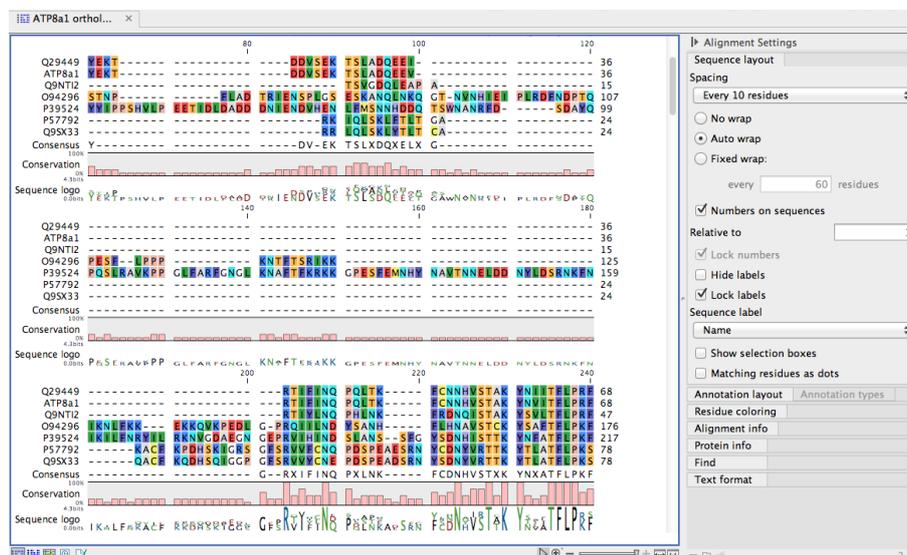


Figure 2.149: The resulting alignment.

Open the generated output from the destination where you choose to save it. Alternatively, the output can be opened by clicking on the small arrow next to the process bar and choosing **Find Results** as shown in figure 2.150):

Installing the Additional Alignments plugin gives you access to two other alignment algorithms: ClustalW (Windows/Mac/Linux) and Muscle (Windows/Mac/Linux). The Additional Alignments Module can be downloaded from <http://www.clcbio.com/plugins>. Note that you will need administrative privileges on your system to install it.

2.18 Find Restriction Sites

This tutorial will show you how to find restriction sites and annotate them on a sequence.

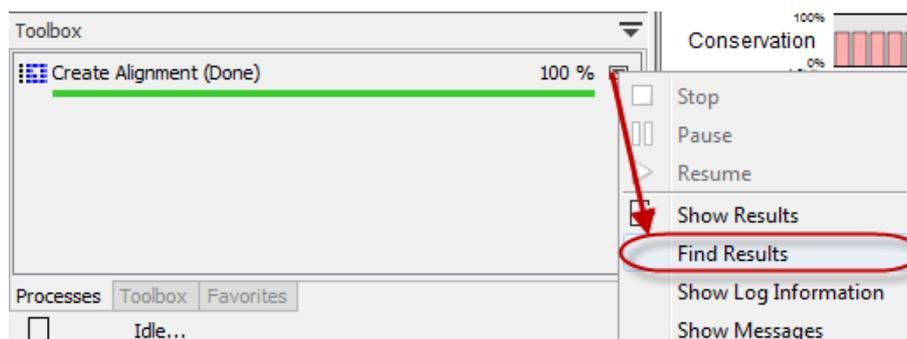


Figure 2.150: Find and open the output that was generated.

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 will be useful, since it is a quick and easy way of showing restriction sites. In the **Toolbox** you will find the other way of doing restriction site analyses. This way provides more control of the analysis and gives you more output options, e.g. a table of restriction sites and a list of restriction enzymes that can be saved for later use. In this tutorial, the first section describes how to use the Side Panel to show restriction sites, whereas the second section describes the restriction map analysis performed from the **Toolbox**.

2.18.1 The Side Panel way of finding restriction sites

When you open a sequence, there is a **Restriction sites** setting in the **Side Panel**. By default, 10 of the most popular restriction enzymes are shown (see figure 2.151).

The restriction sites are shown on the sequence with an indication of cut site and recognition sequence. In the list of enzymes in the **Side Panel**, the number of cut sites is shown in parentheses for each enzyme (e.g. *Sall* cuts three times). If you wish to see the recognition sequence of the enzyme, place your mouse cursor on the enzyme in the list for a short moment, and a tool tip will appear.

You can add or remove enzymes from the list by clicking the **Manage enzymes** button.

2.18.2 The Toolbox way of finding restriction sites

Suppose you are working with sequence 'ATP8a1 mRNA' from the example data, and you wish to know which restriction enzymes will cut this sequence exactly once and create a 3' overhang. Do the following:

select the ATP8a1 mRNA sequence | Toolbox in the Menu Bar | Cloning and Restriction Sites (🔍) | Restriction Site Analysis (✂️)

Click **Next** to set parameters for the restriction map analysis.

In this step first select **Use existing enzyme list** and click the **Browse for enzyme list** button (🔍). Select the 'Popular enzymes' in the Cloning folder under Enzyme lists (figure 2.152).

Then write 3' into the filter below to the left. Select all the enzymes and click the **Add** button (➡️). The result should be like in figure 2.153.

Click on the button labeled **Next**. In this step you specify that you want to show enzymes that cut the sequence only once. This means that you should de-select the **Two restriction sites**

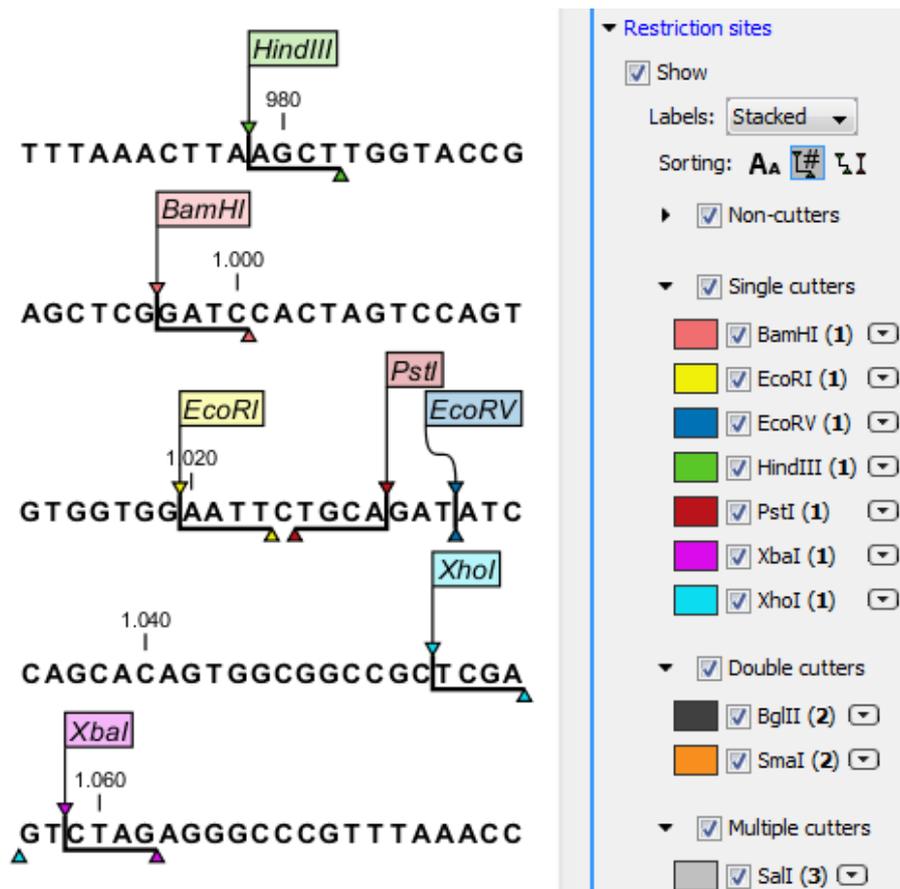


Figure 2.151: Showing restriction sites of ten restriction enzymes.

checkbox (See figure 2.154).

Click on the button labeled **Next** and select that you want to **Add restriction sites as annotations on sequence** and **Create restriction map** (figure 2.155).

Click on the button labeled **Finish** to start the restriction map analysis.

View restriction site

The restriction sites are shown in two views: one view is in a tabular format and the other view displays the sites as annotations on the sequence. The result is shown in figure 2.156.

The restriction map at the bottom can also be shown as a table of fragments produced by cutting the sequence with the enzymes:

Click the Fragments button (📄) **at the bottom of the view**

In a similar way the fragments can be shown on a virtual gel:

Click the Gel button (📄) **at the bottom of the view**

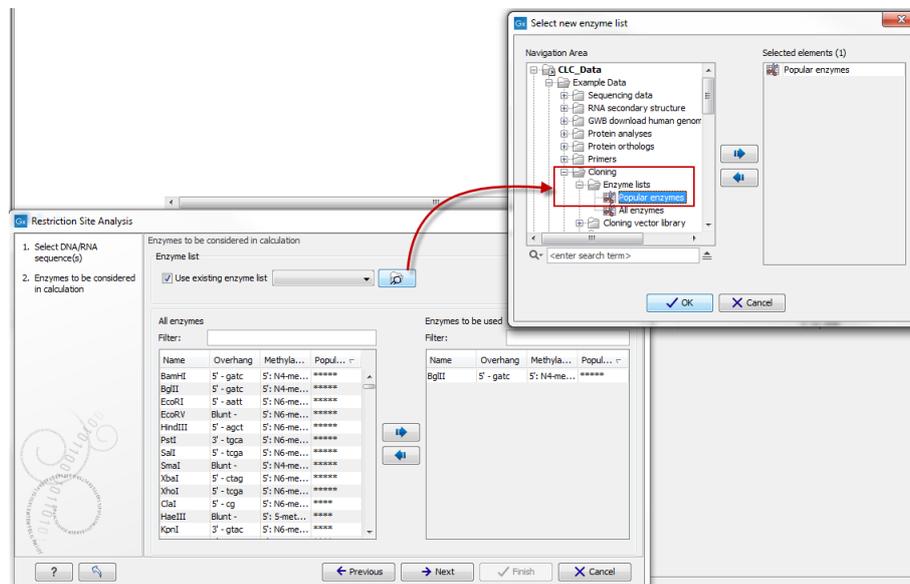


Figure 2.152: Selecting enzyme list.

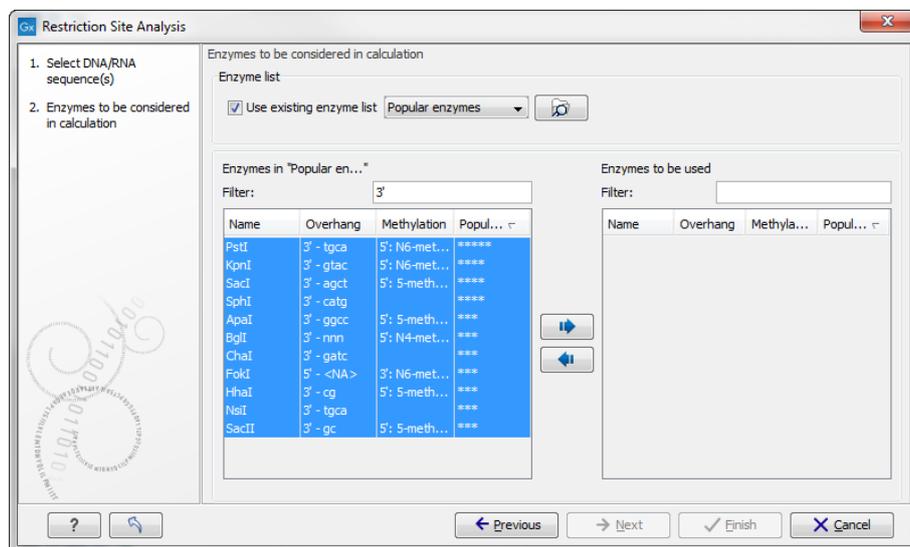


Figure 2.153: Selecting enzymes.

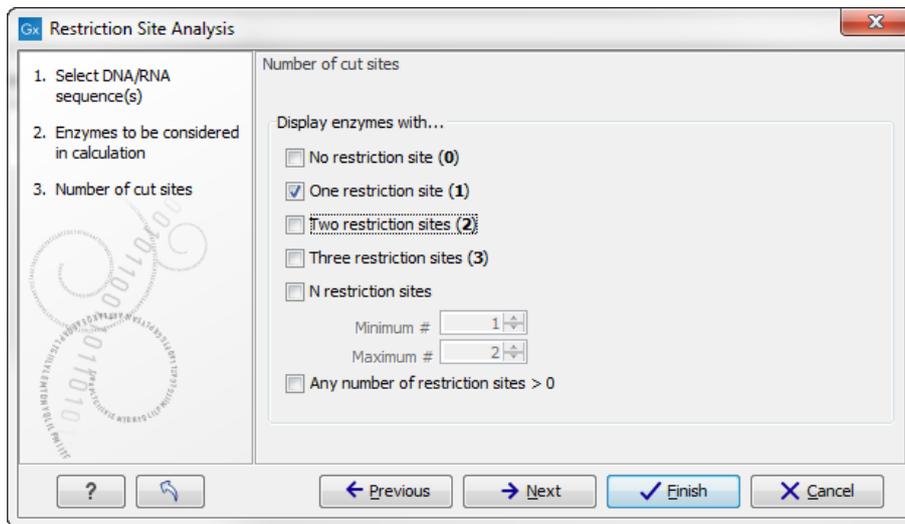


Figure 2.154: Selecting output for restriction map analysis.

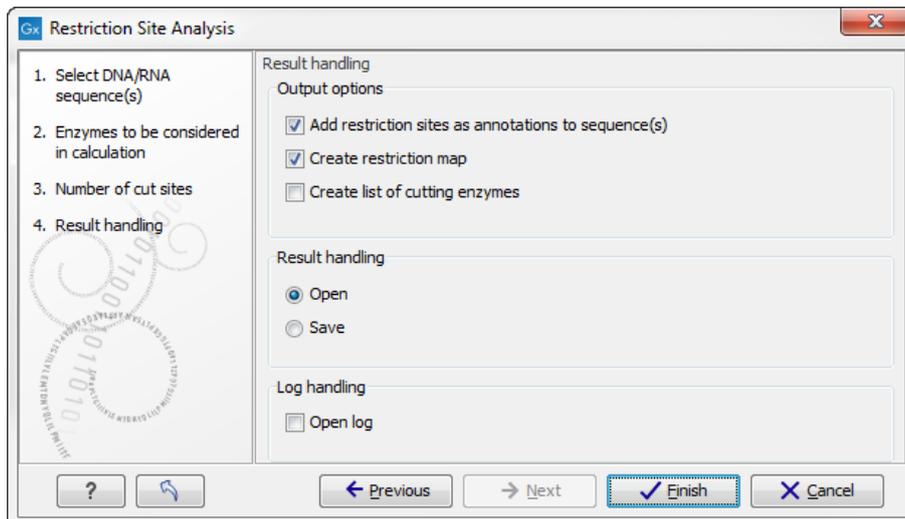


Figure 2.155: Add restriction sites as annotations on sequence and create restriction map.

The screenshot shows a software interface with two main panels. The top panel, titled '* ATP8a1 mRNA (...)', displays a sequence view. A blue bar represents the mRNA sequence, with a red box highlighting a SacII restriction site. The sequence is: CTGGGTGGGAGGCGCGGCCCGCGGCAGCTGAGCCCTCTGCGCGGCGC. The bottom panel, titled '* Restriction m...', shows a table of restriction sites.

| Sequence | Name | Pattern | Length | Overhang | Number of ... | Cut position(s) |
|-------------------------|-------|---------|--------|----------|---------------|-----------------|
| ATP8a1 mRNA (reference) | KpnI | ggtacc | 6 | 3' | 1 | 1208 |
| ATP8a1 mRNA (reference) | NsiI | atgcat | 6 | 3' | 1 | 1363 |
| ATP8a1 mRNA (reference) | SacII | ccgctg | 6 | 3' | 1 | 119 |

Figure 2.156: The result of the restriction map analysis is displayed in a table at the bottom and as annotations on the sequence in the view at the top.

Part II

Core Functionalities

Chapter 3

User interface

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This chapter provides an overview of the different areas in the user interface of *CLC Main Workbench*. As can be seen from figure 3.1 this includes a **Navigation Area**, **View Area**, **Menu Bar**, **Toolbar**, **Status Bar** and **Toolbox**.

A description of the **Navigation Area** is tightly connected to the data organization features of *CLC Main Workbench* and can be found in section 4.1.

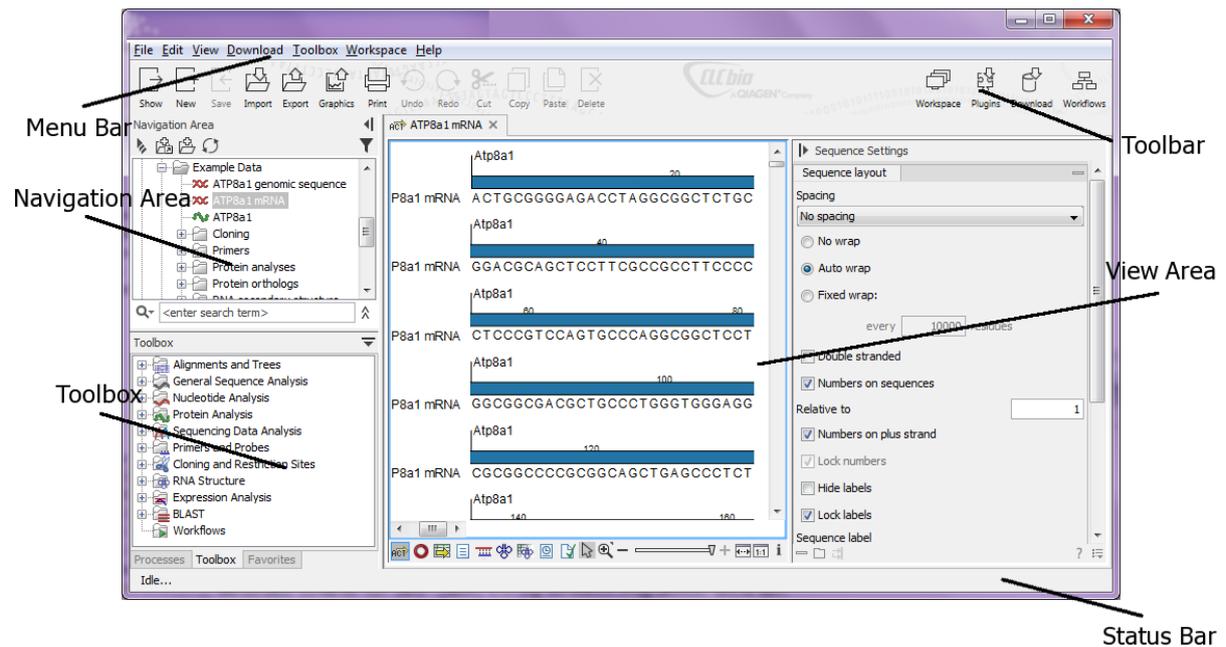


Figure 3.1: The user interface consists of the Menu Bar, Toolbar, Status Bar, Navigation Area, Toolbox, and View Area.

3.1 View Area

The **View Area** is the right-hand part of the screen, displaying your current work. The **View Area** may consist of one or more **Views**, represented by tabs at the top of the **View Area**.

This is illustrated in figure 3.2.

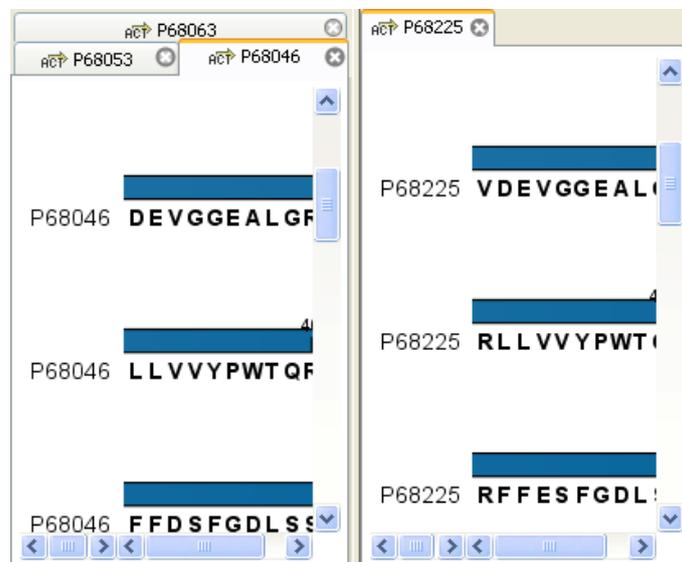


Figure 3.2: A View Area can enclose several views, each view is indicated with a tab (see right view, which shows protein P68225). Furthermore, several views can be shown at the same time (in this example, four views are displayed).

The tab concept is central to working with CLC Main Workbench, because several operations can be performed by dragging the tab of a view, and extended right-click menus can be activated from

the tabs.

This chapter deals with the handling of views inside a **View Area**. Furthermore, it deals with rearranging the views.

Section 3.2 deals with the zooming and selecting functions.

3.1.1 Open view

Opening a view can be done in a number of ways:

double-click an element in the Navigation Area

or **select an element in the Navigation Area | File | Show | Select the desired way to view the element**

or **select an element in the Navigation Area | Ctrl + O (⌘ + B on Mac)**

Opening a view while another view is already open, will show the new view in front of the other view. The view that was already open can be brought to front by clicking its tab.

Note! If you right-click an open tab of any element, click **Show**, and then choose a different view of the same element, this new view is automatically opened in a split-view, allowing you to see both views.

See section 4.1.5 for instructions on how to open a view using drag and drop.

3.1.2 Show element in another view

Each element can be shown in different ways. A sequence, for example, can be shown as linear, circular, text etc.

In the following example, you want to see a sequence in a circular view. If the sequence is already open in a view, you can change the view to a circular view:

Click Show As Circular (○) at the lower left part of the view

The buttons used for switching views are shown in figure 3.3).



Figure 3.3: The buttons shown at the bottom of a view of a nucleotide sequence. You can click the buttons to change the view to e.g. a circular view or a history view.

If the sequence is already open in a linear view (ACT), and you wish to see both a circular and a linear view, you can split the views very easily:

Press Ctrl (⌘ on Mac) while you | Click Show As Circular (○) at the lower left part of the view

This will open a split view with a linear view at the bottom and a circular view at the top (see 12.5).

You can also show a circular view of a sequence without opening the sequence first:

Select the sequence in the Navigation Area | Show (⇨) | As Circular (○)

3.1.3 Close views

When a view is closed, the **View Area** remains open as long as there is at least one open view.

A view is closed by:

right-click the tab of the View | Close

or **select the view | Ctrl + W**

or **hold down the Ctrl-button | Click the tab of the view while the button is pressed**

By right-clicking a tab, the following close options exist. See figure 3.4

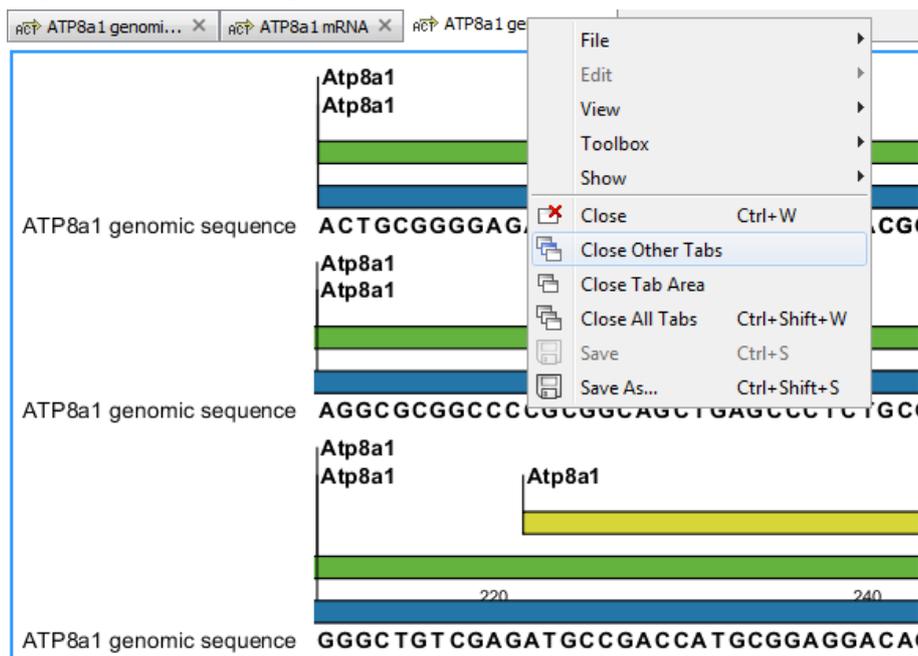


Figure 3.4: By right-clicking a tab, several close options are available.

- **Close.** See above.
- **Close Other Tabs.** Closes all other tabs, in all tab areas, except the one that is selected.
- **Close Tab Area.** Closes all tabs in the tab area.
- **Close All Tabs.** Closes all tabs, in all tab areas. Leaves an empty workspace.

3.1.4 Save changes in a view

When changes to an element are made in a view, the text on the tab appears *bold and italic* (on Mac it is indicated by an * before the name of the tab). This indicates that the changes are not saved. The **Save** function may be activated in two ways:

Click the tab of the view you want to save | Save (⌘) in the toolbar.

or **Click the tab of the view you want to save | Ctrl + S (⌘ + S on Mac)**

If you close a tab of a view containing an element that has been changed since you opened it, you are asked if you want to save.

When saving an element from a new view that has not been opened from the **Navigation Area** (e.g. when opening a sequence from a list of search hits), a save dialog appears (figure 3.5).

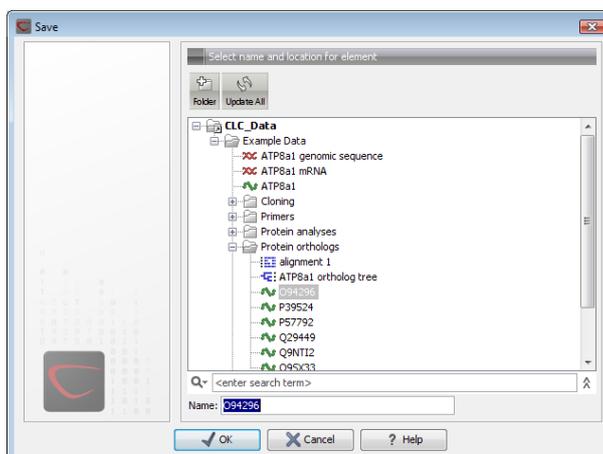


Figure 3.5: Save dialog.

In the dialog you select the folder in which you want to save the element.

After naming the element, press **OK**

3.1.5 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 **Edit | Undo (↶)**

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 **Edit | Redo (↷)**

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 4.1.7).

You can set the number of possible undo actions in the Preferences dialog (see section 5).

3.1.6 Arrange views in View Area

To provide more space for viewing data, you can hide **Navigation Area** and the **Toolbox** by clicking the hide icon (⏏) at the top of the **Navigation Area**.

Views are arranged in the **View Area** by their tabs. The order of the **views** can be changed using drag and drop. E.g. drag the tab of one view onto the tab of another. The tab of the first view is

now placed at the right side of the other tab.

If a tab is dragged into a view, an area of the view is made gray (see fig. 3.6) illustrating that the view will be placed in this part of the **View Area**.

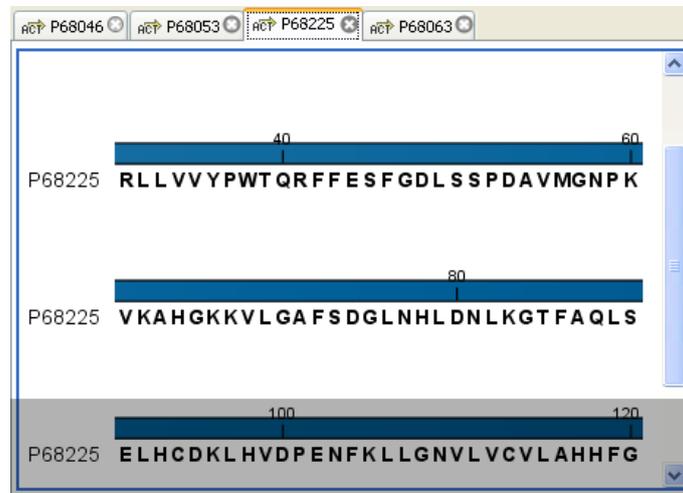


Figure 3.6: When dragging a view, a gray area indicates where the view will be shown.

The results of this action is illustrated in figure 3.7.

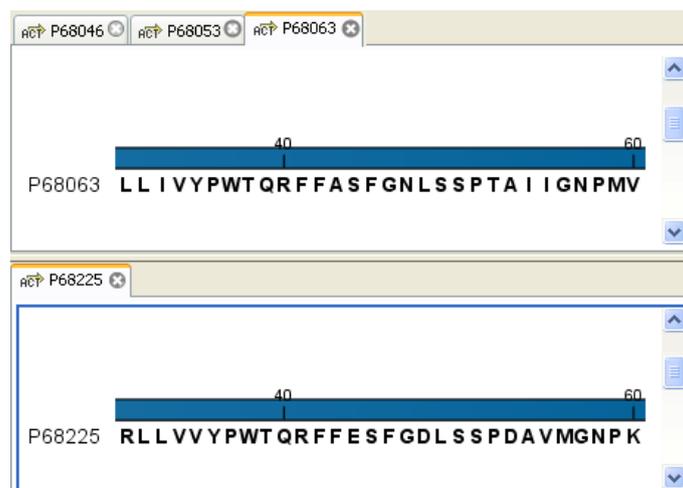


Figure 3.7: A horizontal split-screen. The two views split the View Area.

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. (See figure 3.8). When the split is made vertically, the new view opens to the right of the existing view.

Splitting the **View Area** can be undone by dragging e.g. the tab of the bottom view to the tab of the top view. This is marked by a gray area on the top of the view.



Figure 3.8: A vertical split-screen.

Maximize/Restore size of view

The **Maximize/Restore View** function allows you to see a view in maximized mode, meaning a mode where no other **views** nor the **Navigation Area** is shown.

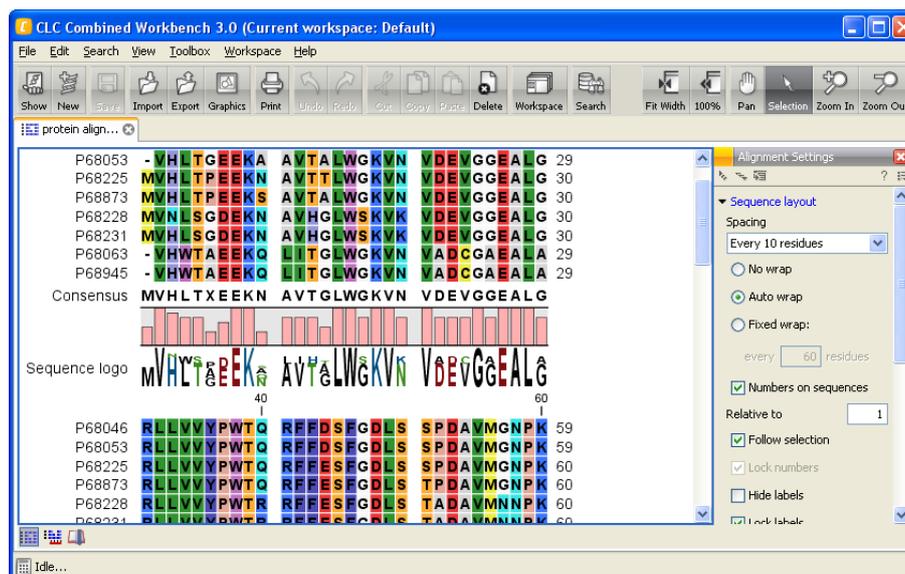


Figure 3.9: A maximized view. The function hides the Navigation Area and the Toolbox.

Maximizing a view can be done in the following ways:

select view | Ctrl + M

or **select view | View | Maximize/restore View** (☐)

or **select view | right-click the tab | View | Maximize/restore View** (☐)

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

The following restores the size of the view:

Ctrl + M

or **View | Maximize/restore View** (☐)

or **double-click title of view**

Please note that you can also hide **Navigation Area** and the **Toolbox** by clicking the hide icon (◀) at the top of the **Navigation Area**

3.1.7 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 3.10, 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.

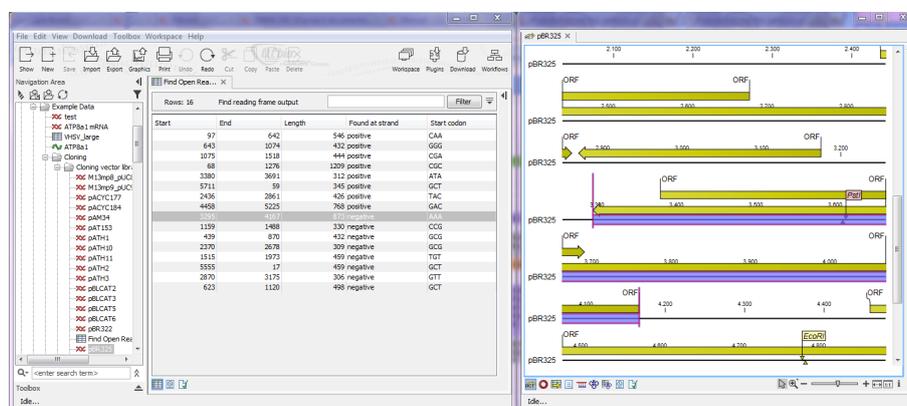


Figure 3.10: Showing the table on one screen while the sequence is displayed on another screen. Clicking the table of open reading frames causes the view on the other screen to follow the selection. Note that the screen resolution in this figure is kept low in order to include it in the manual; in a real scenario, the resolution will be much higher.

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.

You can also split the view area in the detached windows as described in section 3.1.6.

3.1.8 Side Panel

The **Side Panel** allows you to change the way the contents of a view are displayed. The options in the **Side Panel** depend on the kind of data in the view, and they are described in the relevant sections about sequences, alignments, trees etc.

Figure 3.11 shows the default **Side Panel** for a protein sequence. It is organized into *palettes*.

In this example, there is one for Sequence layout, one for Annotation Layout etc. These palettes can be re-organized by dragging the palette name with the mouse and dropping it where you want

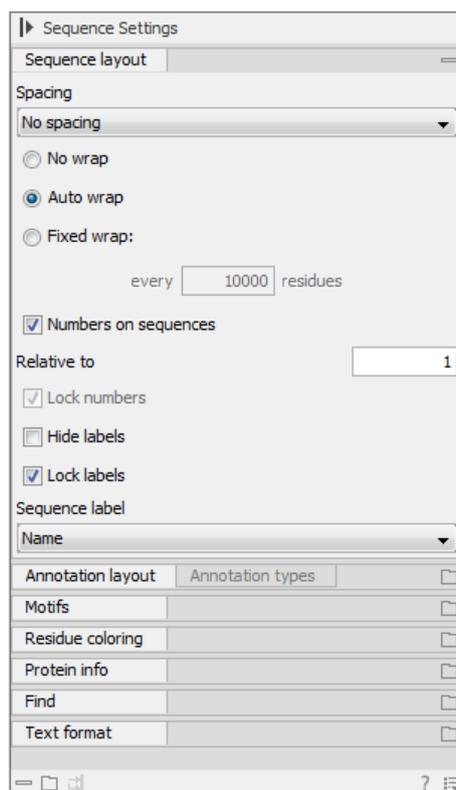


Figure 3.11: The default view of the Side Panel when opening a protein sequence.

it to be. They can either be situated next to each other, so that you can switch between them, or they can be listed on top of each other, so that expanding one of the palettes will push the palettes below further down.

In addition, they can be moved away from the **Side Panel** and placed anywhere on the screen as shown in figure 3.12.

In this example, the **Motifs** palette has been placed on top of the sequence view together with the **Protein info** and the **Residue coloring** palettes. In the **Side Panel** to the right, the **Find** palette has been put on top.

In order to make all palettes dock in the **Side Panel** again, click the **Dock Side Panel** icon (→|).

You can completely hide the **Side Panel** by clicking the **Hide Side Panel** icon (|▶).

At the bottom of the **Side Panel** (see figure 3.13) there are a number of icons used to:

- Expand all settings (□).
- Collapse all settings (≡).
- Dock all palettes (→|)
- Get **Help** for the particular view and settings (?)
- Save the settings of the **Side Panel** or apply already saved settings. Read more in section 5.6

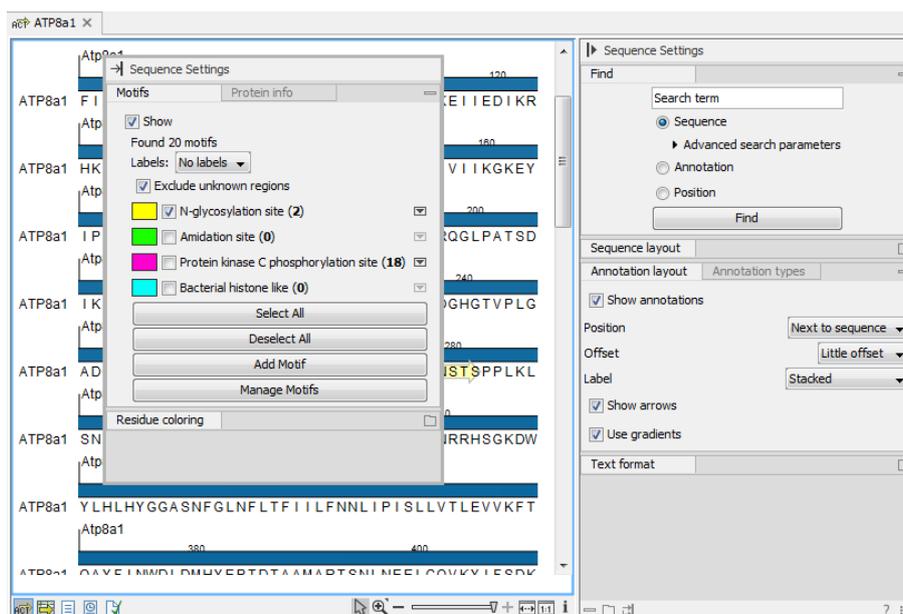


Figure 3.12: Palettes can be organized in the Side Panel as you like or placed anywhere on the screen.

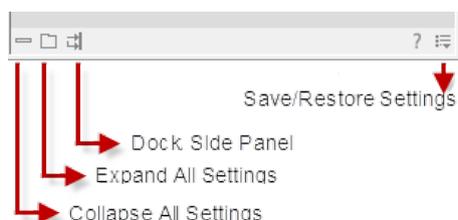


Figure 3.13: Controlling the Side Panel at the bottom

Note! Changes made to the **Side Panel**, including the organization of palettes will not be saved when you save the view. See how to save the changes in section 5.6

3.2 Zoom and selection in View Area

All views except tabular and text views support zooming. Figure 3.14 shows the zoom tools, located at the bottom right corner of the view.

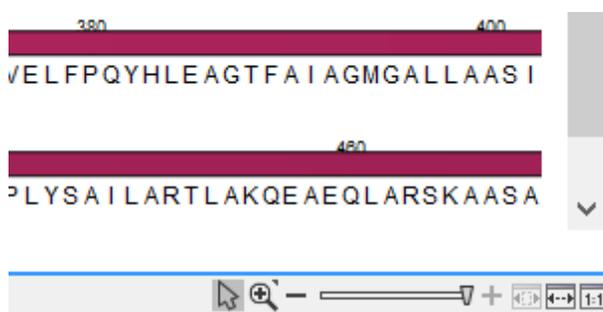


Figure 3.14: The zoom tools are located at the bottom right corner of the view.

The zoom tools consist of some shortcuts for zooming to fit the width of the view () , zoom to 100 % to see details () , zoom to a selection () , a zoom slider, and two mouse mode

buttons () ()

The slider reflects the current zoom level and can be used to quickly adjust this. For more fine-grained control of the zoom level, move the mouse upwards while sliding.

The sections below describes how to use these tools as well as other ways of zooming and navigating data.

Please note that when working with protein 3D structures, there are specific ways of controlling zooming and navigation as explained in section [15.2](#).

3.2.1 Zoom in

There are six ways of **zooming in**:

Click Zoom in mode () in the zoom tools (or press Ctrl+2) | click the location in the view that you want to zoom in on

or **Click Zoom in mode () in the zoom tools | click-and-drag a box around a part of the view | the view now zooms in on the part you selected**

or **Press '+' on your keyboard**

or **Move the zoom slider located in the zoom tools**

or **Click the plus icon in the zoom tools**

The last option for zooming in is only available if you have a mouse with a scroll wheel:

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

Note! You might have to click in the view before you can use the keyboard or the scroll wheel to zoom.

If you press the **Shift** button on your keyboard while in zoom mode, the zoom function is reversed.

If you want to zoom in to 100 % to see all the data, click the **Zoom to base level** () icon.

3.2.2 Zoom out

It is possible to zoom out in different ways:

Click Zoom out mode () in the zoom tools (or press Ctrl+3) | click in the view

or **Press '-' on your keyboard**

or **Move the zoom slider located in the zoom tools**

or **Click the minus icon in the zoom tools**

The last option for zooming out is only available if you have a mouse with a scroll wheel:

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

Note! You might have to click in the view before you can use the keyboard or the scroll wheel to zoom.

If you want to zoom out to see all the data, click the **Zoom to Fit** () icon.

If you press **Shift** while clicking in a **View**, the zoom function is reversed. Hence, clicking on a sequence in this way while the **Zoom out** mode toolbar item is selected, zooms in instead of zooming out.

3.2.3 Selecting, panning and zooming

In the zoom tools, you can control which mouse mode to use. The default is **Selection mode** (☞) which is used for selecting data in a view. Next to the selection mode, you can select the **Zoom in mode** as described in section 3.2.1. If you press and hold this button, two other modes become available as shown in figure 3.15:

- **Panning** (☞) is used for dragging the view with the mouse as a way of scrolling.
- **Zoom out** (☞) is used to change the mouse mode so that whenever you click the view, it zooms out.



Figure 3.15: Additional mouse modes can be found in the zoom tools.

If you hold the mouse over the selection and zoom tools, tooltips will appear that provide further information about how to use the tools.

The mouse modes only apply when the mouse is within the view where they are selected.

The **Selection mode** can also be invoked with the keyboard shortcut Ctrl+1, while the **Panning mode** can be invoked with Ctrl+4.

For some views, if you have made a selection, there is a **Zoom to Selection** (☞) button, which allows you to zoom and scroll directly to fit the view to the selection.

3.3 Toolbox and Status Bar

The **Toolbox** is placed in the left side of the user interface of *CLC Main Workbench* below the **Navigation Area**.

The **Toolbox** shows a **Processes tab**, **Favorites tab** and a **Toolbox tab**.

The **Toolbox** can be hidden, so that the **Navigation Area** is enlarged and thereby displays more elements:

View | Show/Hide Toolbox | Show/Hide Toolbox

You can also click the **Hide Toolbox** (☞) button.

3.3.1 Processes

By clicking the **Processes** tab, the **Toolbox** displays previous and running processes, e.g. an NCBI search or a calculation of an alignment. The running processes can be stopped, paused,

and resumed by clicking the small icon (⏸) next to the process (see figure 3.16).

Running and paused processes are not deleted.

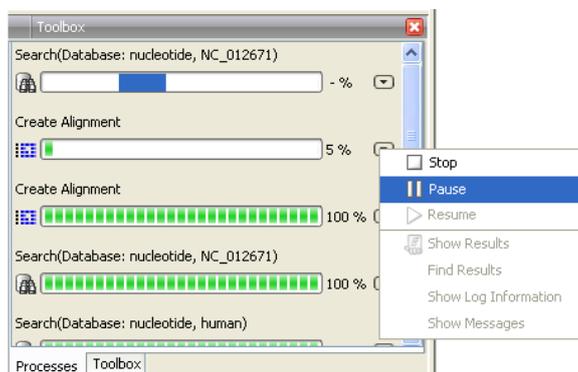


Figure 3.16: A database search and an alignment calculation are running. Clicking the small icon next to the process allow you to stop, pause and resume processes.

Besides the options to stop, pause and resume processes, there are some extra options for a selected number of the tools running from the Toolbox:

- **Show results.** If you have chosen to save the results (see section 9.2), you will be able to open the results directly from the process by clicking this option.
- **Find results.** If you have chosen to save the results (see section 9.2), you will be able to high-light the results in the Navigation Area.
- **Show Log Information.** This will display a log file showing progress of the process. The log file can also be shown by clicking **Show Log** in the "handle results" dialog where you choose between saving and opening the results.
- **Show Messages.** Some analyses will give you a message when processing your data. The messages are the black dialogs shown in the lower left corner of the Workbench that disappear after a few seconds. You can reiterate the messages that have been shown by clicking this option.

The terminated processes can be removed by:

View | Remove Finished Processes (X)

If you close the program while there are running processes, a dialog will ask if you are sure that you want to close the program. Closing the program will stop the process, and it cannot be restarted when you open the program again.

3.3.2 Toolbox

The content of the **Toolbox** tab in the **Toolbox** corresponds to **Toolbox** in the **Menu Bar**.

The tools in the toolbox can be accessed by double-clicking or by dragging elements from the **Navigation Area** to an item in the **Toolbox**.

Quick access to tools

To enable quick launch of tools in *CLC Main Workbench*, press **Ctrl + Shift + T** (**⌘ + Shift + T** on Mac) to show the quick launch dialog (see figure 3.17).

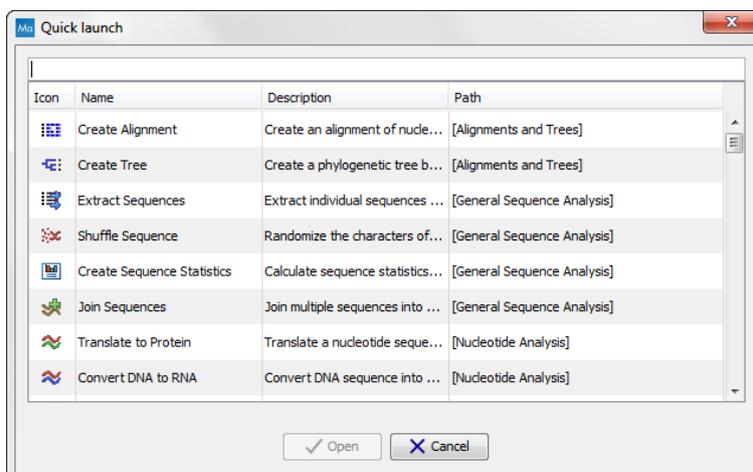


Figure 3.17: Quick access to all tools in **CLC Main Workbench**.

When the dialog is opened, you can start typing search text in the text field at the top. This will bring up the list of tools that match this text either in the name, description or location in the Toolbox. In the example shown in figure 3.18, typing `create` shows a list of tools involving the word "create", and the arrow keys or mouse can be used for selecting and starting a tool.

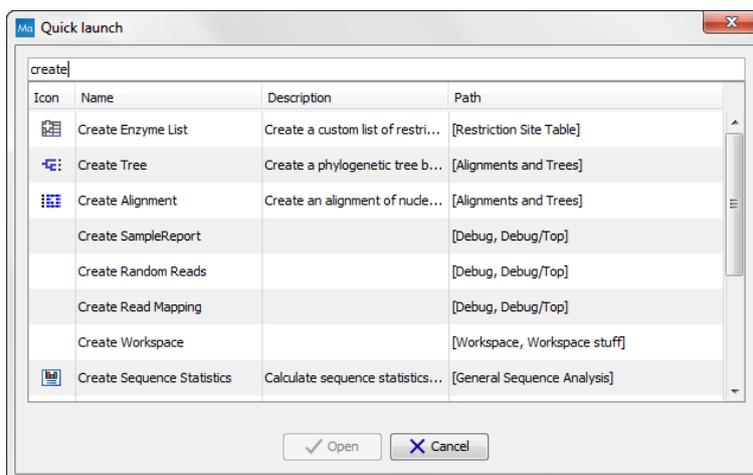


Figure 3.18: Typing in the search field at the top will filter the list of tools to launch.

Favorites toolbox

Next to the **Toolbox** tab, you find the **Favorites** tab. This can be used for organizing and getting quick access to the tools you use the most. It consists of two parts as shown in figure 3.19.

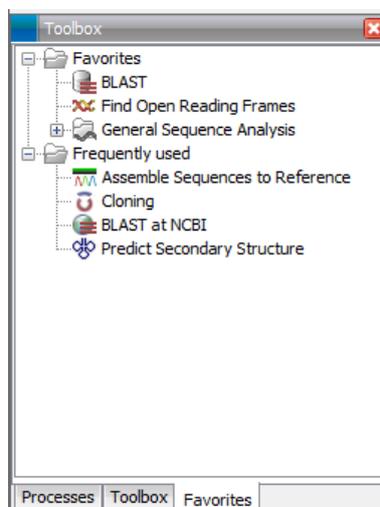


Figure 3.19: Favorites toolbox.

Favorites You can manually add tools to the favorites menu simply by right-clicking the tool in the Toolbox. You can also right-click the Favorites folder itself and select **Add Tool**. To remove a tool, right-click and select **Remove from Favorites**. Note that you can also add complete folders to the favorites.

Frequently used The list of tools in this folder is automatically populated as you use the Workbench. The most frequently used tools are listed at the top.

3.3.3 Status Bar

As can be seen from figure 3.1, the **Status Bar** is located at the bottom of the window. In the left side of the bar is an indication of whether the computer is making calculations or whether it is idle. The right side of the **Status Bar** indicates the range of the selection of a sequence. (See chapter 3.2.3 for more about the Selection mode button.)

3.4 Workspace

If you are working on a project and have arranged the views for this project, you can save this arrangement using **Workspaces**. A Workspace remembers the way you have arranged the views, and you can switch between different workspaces.

The **Navigation Area** always contains the same data across **Workspaces**. It is, however, possible to open different folders in the different **Workspaces**. Consequently, the program allows you to display different clusters of the data in separate **Workspaces**.

All **Workspaces** are automatically saved when closing down *CLC Main Workbench*. The next time you run the program, the **Workspaces** are reopened exactly as you left them.

Note! It is not possible to run more than one version of *CLC Main Workbench* at a time. Use two or more **Workspaces** instead.

3.4.1 Create Workspace

When working with large amounts of data, it might be a good idea to split the work into two or more **Workspaces**. As default the *CLC Main Workbench* opens one **Workspace**. Additional **Workspaces** are created in the following way:

Workspace in the Menu Bar | Create Workspace | enter name of Workspace | OK

When the new **Workspace** is created, the heading of the program frame displays the name of the new **Workspace**. Initially, the selected elements in the **Navigation Area** is collapsed and the **View Area** is empty and ready to work with. (See figure 3.20).

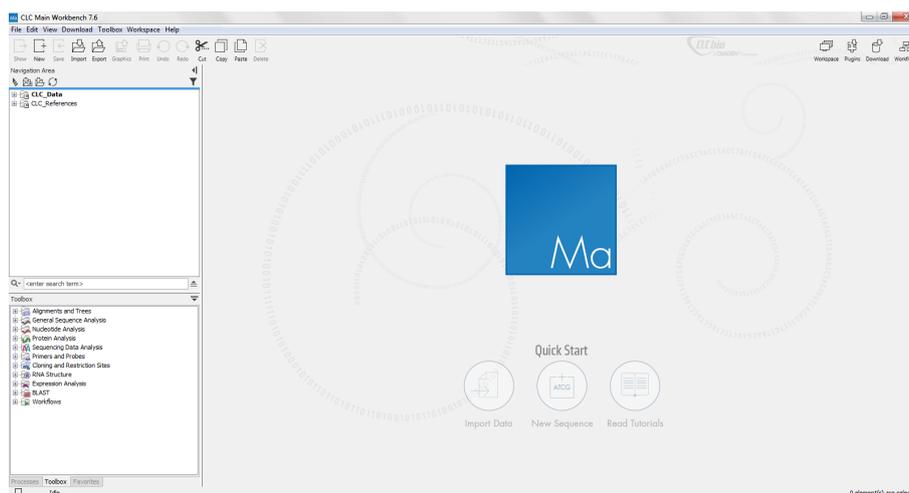


Figure 3.20: An empty Workspace.

3.4.2 Select Workspace

When there is more than one **Workspace** in the *CLC Main Workbench*, there are two ways to switch between them:

Workspace () in the Toolbar | Select the Workspace to activate

or **Workspace in the Menu Bar | Select Workspace () | choose which Workspace to activate | OK**

The name of the selected **Workspace** is shown after "*CLC Main Workbench*" at the top left corner of the main window, in figure 3.20 it says: (default).

3.4.3 Delete Workspace

Deleting a **Workspace** can be done in the following way:

Workspace in the Menu Bar | Delete Workspace | choose which Workspace to delete | OK

Note! Be careful to select the right **Workspace** when deleting. The delete action cannot be undone. (However, no data is lost, because a workspace is only a representation of data.)

It is not possible to delete the default workspace.

3.5 List of shortcuts

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

| Action | Windows/Linux | Mac OS X |
|-------------------------------|----------------------|-------------------------|
| Adjust selection | Shift + arrow keys | Shift + arrow keys |
| Adjust workflow layout | Shift + Alt + L | ⌘ + Shift + Alt + L |
| 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 |
| Maximize/restore size of View | 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 |
| Reverse zoom mode | press and hold Shift | press and hold Shift |
| 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 |
| Sort folder | Ctrl + Shift + R | ⌘ + Shift + R |
| Split Horizontally | Ctrl + T | ⌘ + T |
| Split Vertically | Ctrl + J | ⌘ + J |
| Start Tool Quick Launch | Ctrl + Shift + T | ⌘ + Shift + T |
| Translate to Protein | Ctrl + Shift + P | ⌘ + Shift + P |
| Undo | Ctrl + Z | ⌘ + Z |
| Update folder | F5 | F5 |
| User Preferences | Ctrl + K | ⌘ + ; |

| Action | Windows/Linux | Mac OS X |
|---------------------------------------|---------------------------|------------------------|
| Vertical scroll in read 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 | Alt + Scroll wheel | Alt + Scroll wheel |
| 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 |
| Zoom | Ctrl + Scroll wheel | Ctrl + 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 |

Combinations of keys and mouse movements are listed below.

| Action | Windows/Linux | Mac OS X | 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 that are not grouped together | Ctrl | ⌘ | Click elements |
| Select multiple elements that are grouped together | Shift | Shift | Click elements |

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

Chapter 4

Data management and search

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This chapter explains the 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 through local data.

4.1 Navigation Area

The **Navigation Area** is located in the left side of the screen, under the **Toolbar** (see figure 4.1). It is used for organizing and navigating data. Its behavior is similar to the way files and folders are usually displayed on your computer.

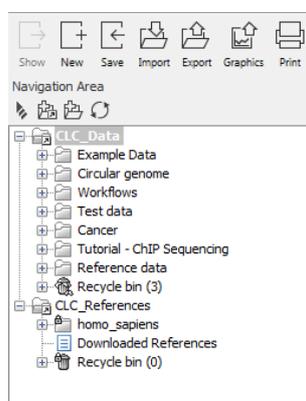


Figure 4.1: The Navigation Area.

To provide more space for viewing data, you can hide **Navigation Area** and the **Toolbox** by clicking the hide icon () at the top.

4.1.1 Data structure

The data in the **Navigation Area** is organized into a number of **Locations**. When the *CLC Main Workbench* is started for the first time, there is one location called *CLC_Data* (unless your computer administrator has configured the installation otherwise).

A location represents a folder on the computer: The data shown under a location in the **Navigation Area** is stored on the computer in the folder which the location points to.

This is explained visually in figure 4.2. The full path to the system folder can be located by mousing over the data location as shown in figure 4.3.

Adding locations

Per default, there is one location in the **Navigation Area** called *CLC_Data*. It points to the following folder:

- On Windows: `C:\Users\\CLC_Data`

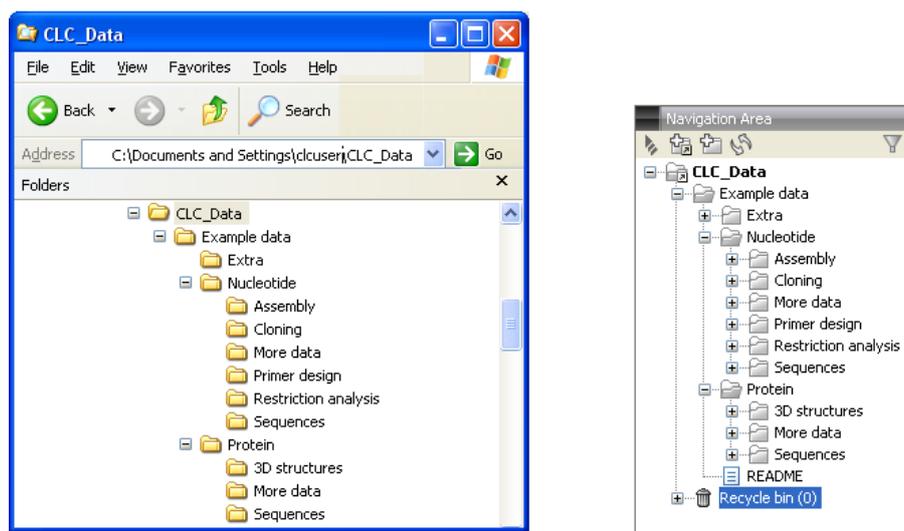


Figure 4.2: In this example the location called 'CLC_Data' points to the folder at C:\Documents and settings\clcuser\CLC_Data.

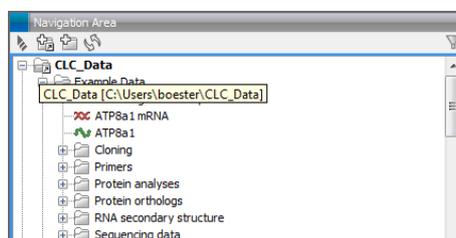


Figure 4.3: Mousing over the location called 'CLC_Data' shows the full path to the system folder, which in this case is C:\Users\boester\CLC_Data.

- On Mac: ~/CLC_Data
- On Linux: /homefolder/CLC_Data

You can easily add more locations to the **Navigation Area**:

File | New | Location (📁)

This will bring up a dialog where you can navigate to the folder you wish to use as your new location (see figure 4.4).

When you click **Open**, the new location is added to the **Navigation Area** as shown in figure 4.5.

The name of the new location will be the name of the folder selected for the location. To see where the folder is located on your computer, place your mouse cursor on the location icon (📁) for a second. This will show the path to the location.

You can use a folder on a network drive or a removable drive as a Data Location. Such a location will appear inactive if the relevant drive is not available when you start up the Workbench. Once the drive is available, click on Update All (Image updates) and the relevant Data Location will become active (note that there might be a few seconds delay from the moment you connect).

Sharing data is possible when a network drive is available to multiple Workbenches. In this case, you can add the same folder as a Data Location on each Workbench. However, it is important to note that data sharing is not actively supported: we do not support concurrent alteration of data

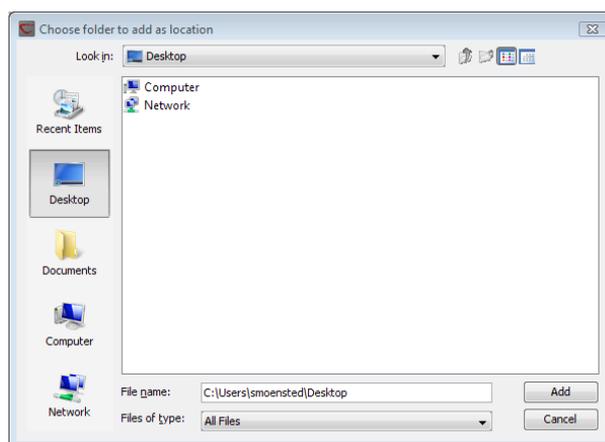


Figure 4.4: Navigating to a folder to use as a new location.

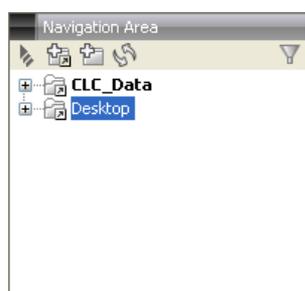


Figure 4.5: The new location has been added.

and 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. In addition, 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 any Workbench that adds a Data Location already used by other Workbenches uses as a Data Location **the exact same folder from the network drive file system hierarchy as the other Workbenches have used**. Indicating a folder higher up or lower down in the hierarchy will cause problems with the indexing of the files, meaning that newly created objects by Workbench A will not be found by Workbench B and vice versa.

Opening data

The elements in the **Navigation Area** are opened by:

Double-clicking on the element

or **Clicking once on the element | Show (☞) in the Toolbar**

or **Clicking once on the element | Right-click on the element | Show (☞)**

or **Clicking once on the element | Right-click on the element | Show (the one without an icon) | Select the desired way to view the element from the menu that appears when mousing over "Show"**

This will open a view in the **View Area**, which is described in section 3.1.

Adding data

Data can be added to the **Navigation Area** in a number of ways. Files can be imported from the file system (see chapter 7). Furthermore, an element can be added by dragging it into the **Navigation Area**. This could be views that are open, elements on lists, e.g. search hits or sequence lists, and files located on your computer. Finally, you can add data by adding a new location (see section 4.1.1).

If a file or another element is dropped on a folder, it is placed at the bottom of the folder. If it is dropped on another element, it will be placed just below that element.

If the element already exists in the **Navigation Area** a copy will be created with the name extension "-1", "-2" etc. if more than one copy exist.

4.1.2 Create new folders

In order to organize your files, they can be placed in 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.

4.1.3 Sorting folders

You can sort the elements in a folder alphabetically:

right-click the folder | Sort Folder

On Windows, subfolders will be placed at the top of the folder, and the rest of the elements will be listed below in alphabetical order. On Mac, both subfolders and other elements are listed together in alphabetical order.

4.1.4 Multiselecting elements

Multiselecting elements means that you select more than one element at the same time. This can be done in the following ways:

- 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.

4.1.5 Moving and copying elements

Elements can be moved and copied in several ways:

- Using **Copy** (⌘), **Cut** (⌘) and **Paste** (⌘) from the **Edit** menu.
- Using Ctrl + C (⌘ + C on Mac), Ctrl + X (⌘ + X on Mac) and Ctrl + V (⌘ + V on Mac).
- Using **Copy** (⌘), **Cut** (⌘) and **Paste** (⌘) in the **Toolbar**.
- Using drag and drop to move elements.
- Using drag and drop while pressing Ctrl / Command to copy elements.

In the following, all of these possibilities for moving and copying elements are described in further detail.

Copy, cut and paste functions

Copies of elements and folders can be made with the copy/paste function which can be applied in a number of ways:

select the files to copy | right-click one of the selected files | Copy (⌘) | right-click the location to insert files into | Paste (⌘)

or **select the files to copy | Ctrl + C (⌘ + C on Mac) | select where to insert files | Ctrl + P (⌘ + P on Mac)**

or **select the files to copy | Edit in the Menu Bar | Copy (⌘) | select where to insert files | Edit in the Menu Bar | Paste (⌘)**

If there is already an element of that name, the pasted element will be renamed by appending a number at the end of the name.

Elements can also be moved instead of copied. This is done with the cut/paste function:

select the files to cut | right-click one of the selected files | Cut (⌘) | right-click the location to insert files into | Paste (⌘)

or **select the files to cut | Ctrl + X (⌘ + X on Mac) | select where to insert files | Ctrl + V (⌘ + V on Mac)**

When you have cut the element, it is "grayed out" until you activate the paste function. If you change your mind, you can revert the cut command by copying another element.

Note that if you move data between locations, the original data is kept. This means that you are essentially doing a copy instead of a move operation.

Move using drag and drop

Using drag and drop in the **Navigation Area**, as well as in general, is a four-step process:

click the element | click on the element again, and hold left mouse button | drag the element to the desired location | let go of mouse button

This allows you to:

- Move elements between different folders in the **Navigation Area**
- Drag from the **Navigation Area** to the **View Area**: A new view is opened in an existing **View Area** if the element is dragged from the **Navigation Area** and dropped next to the tab(s) in that **View Area**.
- Drag from the **View Area** to the **Navigation Area**: The element, e.g. a sequence, alignment, search report etc. is saved where it is dropped. If the element already exists, you are asked whether you want to save a copy. You drag from the **View Area** by dragging the tab of the desired element.

Use of drag and drop is supported throughout the program, also to open and re-arrange views (see section 3.1.6).

Note that if you move data between locations, the original data is kept. This means that you are essentially doing a copy instead of a move operation.

Copy using drag and drop

To copy instead of move using drag and drop, hold the Ctrl (⌘ on Mac) key while dragging:

click the element | click on the element again, and hold left mouse button | drag the element to the desired location | press Ctrl (⌘ on Mac) while you let go of mouse button release the Ctrl/⌘ button

4.1.6 Change element names

This section describes two ways of changing the names of sequences in the **Navigation Area**. In the first part, the sequences themselves are not changed - it's their representation that changes. The second part describes how to change the name of the element.

Change how sequences are displayed

Sequence elements can be displayed in the **Navigation Area** with different types of information:

- 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).

Whether sequences can be displayed with this information depends on their origin. Sequences that you have created yourself or imported might not include this information, and you will only be able to see them represented by their name. However, sequences downloaded from databases like GenBank will include this information. To change how sequences are displayed:

**right-click any element or folder in the Navigation Area | Sequence Representation
| select format**

This will only affect sequence elements, and the display of other types of elements, e.g. alignments, trees and external files, will be not be changed. If a sequence does not have this information, there will be no text next to the sequence icon.

Rename element

Renaming a folder or an element in the **Navigation Area** can be done in two different ways:

select the element | Edit in the Menu Bar | Rename

or **select the element | F2**

When you can rename the element, you can see that the text is selected and you can move the cursor back and forth in the text. When the editing of the name has finished; press **Enter** or select another element in the **Navigation Area**. If you want to discard the changes instead, press the **Esc**-key.

For renaming annotations instead of folders or elements, see section [12.3.3](#).

4.1.7 Delete, restore and remove elements

When one deletes data from a data folder in the Workbench, it is moved to the recycle bin in that data location. Each data location has its own recycle bin. From the recycle bin, data can then be restored, or completely removed. Removal of data from the recycle bin frees disk space.

Deleting a folder or an element from a Workbench data location can be done in two ways:

right-click the element | Delete ()

or **select the element | press Delete key**

This will cause the element to be moved to the **Recycle Bin** () where it is kept until the recycle bin is emptied or until you choose to restore the data object to your data location.

For deleting annotations instead of folders or elements, see section [12.3.4](#).

Items in a recycle bin can be restored in two ways:

Drag the elements with the mouse into the folder where they used to be.

or **select the element | right click and choose the option Restore.**

Once restored, you can continue to work with that data.

All contents of the recycle bin can be removed by choosing to empty the recycle bin:

Edit in the Menu Bar | Empty Recycle Bin ()

This deletes the data and frees up disk space.

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

4.1.8 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 4.6.

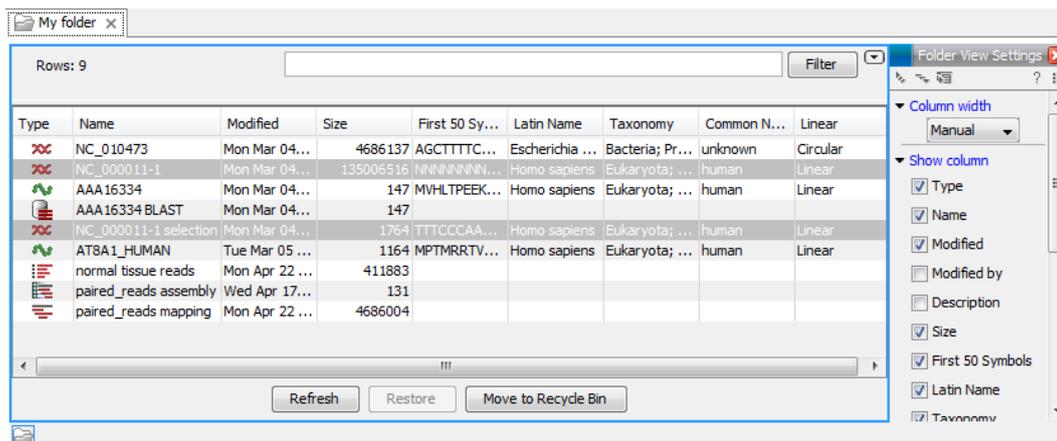


Figure 4.6: Viewing the elements in a folder.

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.

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 4.7 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.

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

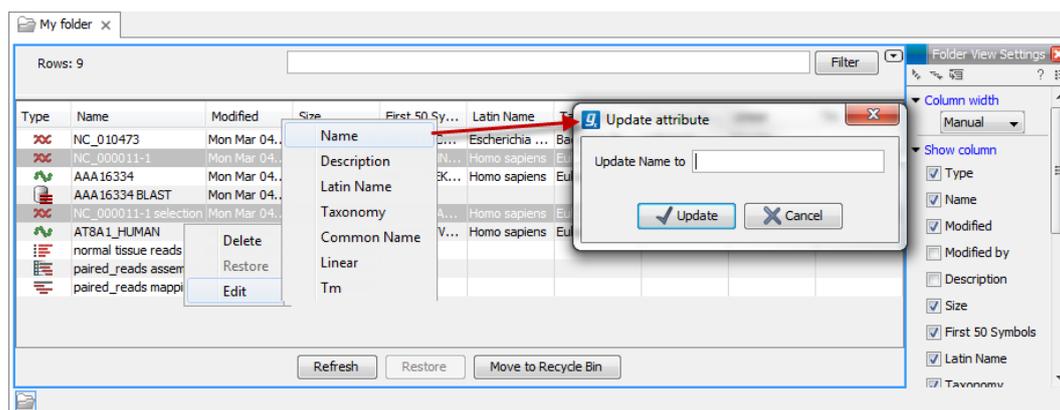


Figure 4.7: Changing the common name of two sequences.

Drag and drop folder elements

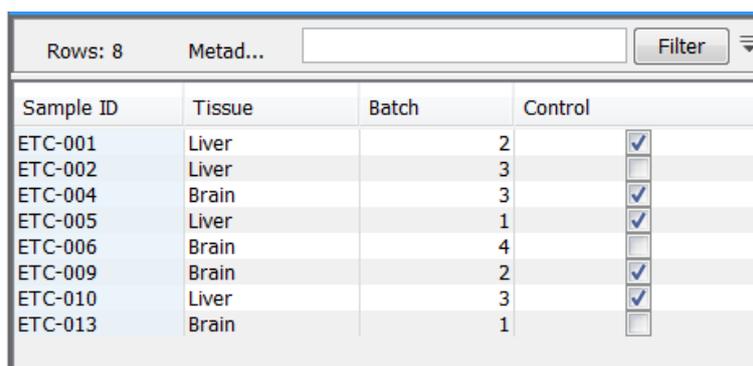
You can drag and drop objects from the folder editor to the **Navigation area**. This will create a copy of the objects at the selected destination. New elements can be included in the folder editor in the view area by dragging and dropping an element from a destination in the **Navigation Area** to 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.

4.2 Metadata

Data elements may be associated with metadata such as information about samples, patients, families, cohortes etc. Metadata is used in a variety of ways in the Workbench, including searching and filtering data elements and annotating phylogenetic trees. The metadata system is intended to help you keep track of your data elements, and how they relate to your samples, without having to resort to elaborate and hard-to-maintain naming conventions or folder structures. For instance, metadata is automatically transferred from input to output elements when an analysis tool is executed.

The Workbench handles metadata in tabular form. A Metadata Table represents a homogeneous collection of external entities, typically samples. Within such a Metadata Table of samples, each row represents a particular sample, and each column represents a property of the samples in the collection. The Workbench does not force any particular interpretation of metadata properties, so you are free to use them to suit your purpose. In the example Metadata Table shown in figure 4.8, the cell containing the value 4 means that the sample with ID ETC-006 has the value 4 for its 'Batch' property. To you, that could e.g. mean that the sample was analyzed with batch 4 chemistry.

Any number of Metadata Tables may be created in the Workbench, each with its own set of columns. A data element may be associated with *at most one* row in each Metadata Table (but you can have references in more metadata tables). Many data elements contain information about a particular sample, and so they may be associated with the same row in a Metadata Table representing samples. For cross-sample analysis results, the association to a sample row would not be unique; such results may instead be associated with a single row in a Metadata Table representing suitable multi-sample entities such as families or cohortes.



| Sample ID | Tissue | Batch | Control |
|-----------|--------|-------|-------------------------------------|
| ETC-001 | Liver | 2 | <input checked="" type="checkbox"/> |
| ETC-002 | Liver | 3 | <input type="checkbox"/> |
| ETC-004 | Brain | 3 | <input checked="" type="checkbox"/> |
| ETC-005 | Liver | 1 | <input checked="" type="checkbox"/> |
| ETC-006 | Brain | 4 | <input type="checkbox"/> |
| ETC-009 | Brain | 2 | <input checked="" type="checkbox"/> |
| ETC-010 | Liver | 3 | <input checked="" type="checkbox"/> |
| ETC-013 | Brain | 1 | <input type="checkbox"/> |

Figure 4.8: A simple Metadata Table.

4.2.1 Setting up Metadata Tables

Metadata may be imported into the Workbench by first setting up an empty Metadata Table with the intended column structure.

File | New | Metadata Table (📄)

This will open a new Metadata Table with no columns and no rows. Columns are added by clicking the **Setup Table** button at the bottom of the view. A dialog is displayed as shown in figure 4.9.

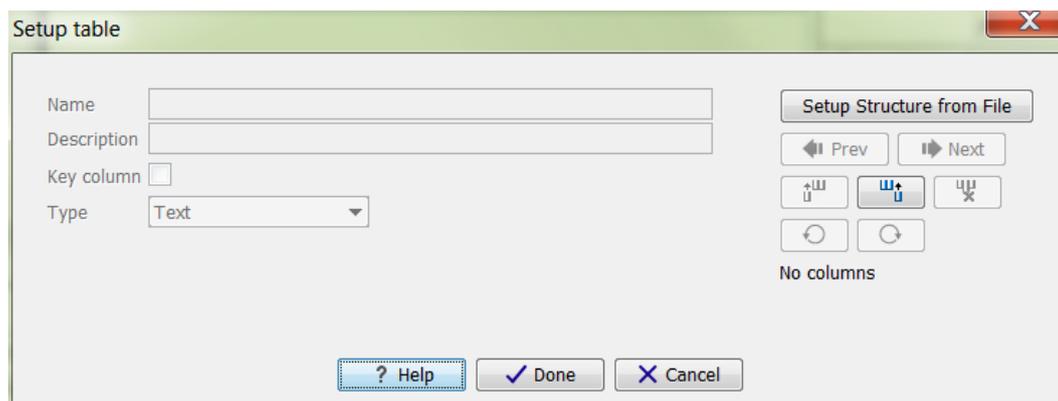


Figure 4.9: Dialog used to add columns to an empty Metadata Table.

You can now add the columns you want by clicking the (📄) button, or you can use an external metadata file as structure for your new table, by clicking the button **Setup Structure from File**. Section 4.2.2 details how you edit the new columns to suit your purpose, and section section 4.2.3 describes how to import the columns. Once a suitable column structure is in place, you can add rows either manually (section 4.2.4) or by importing them from an external file (section 4.2.5).

4.2.2 Editing the metadata structure

Once columns exist, the **Setup table** dialog will contain editable information about them, see figure 4.10.

You may modify the following information for each column:

- **Name.** A mandatory header name or title for the column.

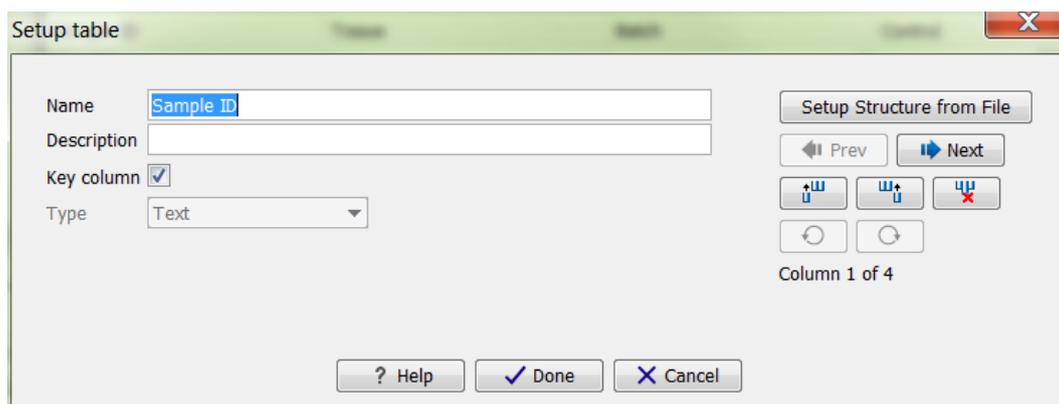


Figure 4.10: Dialog used to edit columns of a Metadata Table.

- **Description.** An optional description of the intended use of the column. The description will appear as a tool tip.
- **Key column.** Select to indicate that the column is a "key" column, meaning that cell values in the column must be present and unique. A typical key column contains identifiers such as sample IDs. At most one key column is allowed in a given Metadata Table.
- **Type.** The type of cell value allowed in the column. The available types are:
 - **Text** Simple text.
 - **Integer** Integral numbers like 42 or -7.
 - **Floating-point number** Decimal values like 3.14 or 1.72e13.
 - **Truth value** True/False or Yes/No.
 - **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.

You navigate between columns by using the (◀) Prev and (▶) Next buttons, or by using left/right arrow keys with Alt held down. Additionally, the Enter key navigates to the next column.

Modifications made to a particular column take effect as you navigate to another column, or if you close the dialog using **Done**. Columns may be deleted using the (✖) button, and new columns may be added using the (⬅) or (➡) buttons which insert new columns before or after the current column, respectively. Deletions and additions take effect immediately, updating the tabular view underneath the edit dialog.

The (↶) and (↷) buttons can be used to undo and redo changes, respectively.

4.2.3 Importing metadata columns

The metadata table structure can be set up to match an existing CSV or Excel file. By clicking the button **Setup Structure from File**, a dialog is displayed as shown in figure 4.11.

You need to specify the following information before importing the columns:

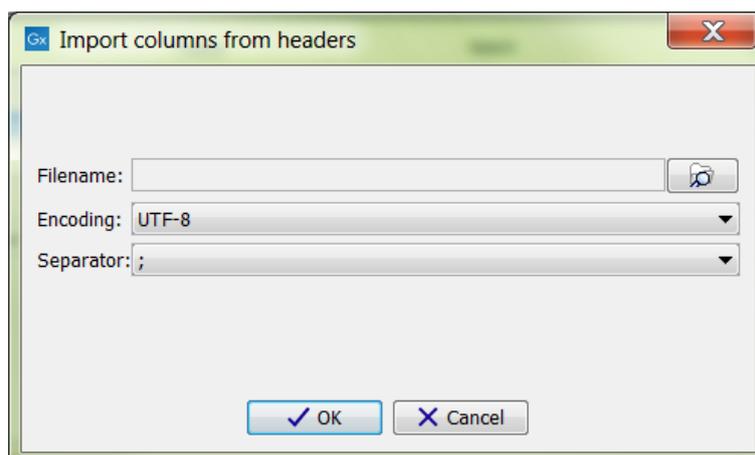


Figure 4.11: Dialog used to import columns into a Metadata Table.

- **Filename.** The file to import. The file should contain the column names in the first row of the file.
- **Encoding.** The encoding used to create the file, default is UTF-8. For excel, this option is not available.
- **Separator.** The separator used to distinguish the separate columns, default is semicolon (;). For excel, this option is not available.

After import, the table should reflect the newly imported columns, and you need to specify the types of the individual columns, as described in the previous section (Section 4.2.2), or delete any columns not needed (for instance formula columns in spreadsheets).

4.2.4 Editing metadata rows

Metadata rows may be edited by clicking the **Manage Data** button at the bottom of the Metadata Table view. Starting from a table with no rows, a dialog appears as shown in figure 4.12.

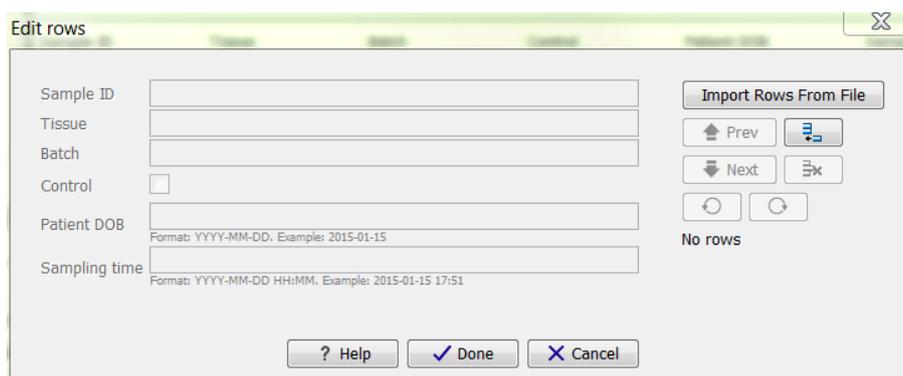


Figure 4.12: Dialog used to add rows to an empty Metadata Table.

You add a new row by clicking the  button, or add them in bulk by clicking the **Import Rows from File** button (see section Section 4.2.5). After adding a new row, you can enter values for each of the columns of your Metadata Table. Once rows exist, the dialog appears as shown in figure 4.13.

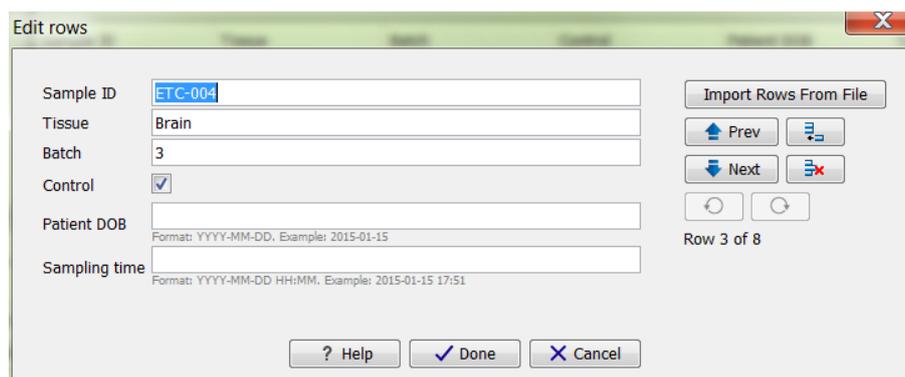


Figure 4.13: Dialog used to edit rows of a Metadata Table.

You navigate between rows by using the (⬆) Prev and (⬇) Next buttons, or by using the keyboard arrow keys or Page Up/Page Down/Home/End keys while holding down the Alt key. (The use of the Alt key is necessary only in circumstances where the key being pressed would otherwise have a different effect, say within a text field, or within the scroll bar that will appear in the dialog if you have many columns). The Enter key navigates to the next row. Row navigation is reflected in the tabular view underneath the edit dialog.

Modifications made to a particular row take effect as you navigate to another row, or if you close the dialog using **Done**. Rows may be deleted using the (✖) button, and new rows may be added using the (+) button. Deletions and additions are immediately visible in the tabular view.

The (↶) and (↷) buttons can be used to undo and redo changes, respectively.

4.2.5 Importing metadata rows

Metadata rows may be imported from an external file by clicking the **Import Rows** button at the bottom of the Metadata Table view. Doing so brings up the dialog shown in figure 4.14.

Row import is parameterized as follows:

- **File.** The file from which to import metadata. Common spreadsheet formats are supported, including .csv, .txt, .xlsx, and .xls.
- **Encoding.** The text encoding of the selected file. Specifying the correct encoding is important to ensure that the character data of the file is correctly interpreted by the Workbench.
- **Separator.** Select the character used to separate two values in the file.
- **Locale.** Select the locale used to format numbers and dates within the file.
- **Date format.** Specifies the date format used in the imported file.
- **Date-time format.** Specifies 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:

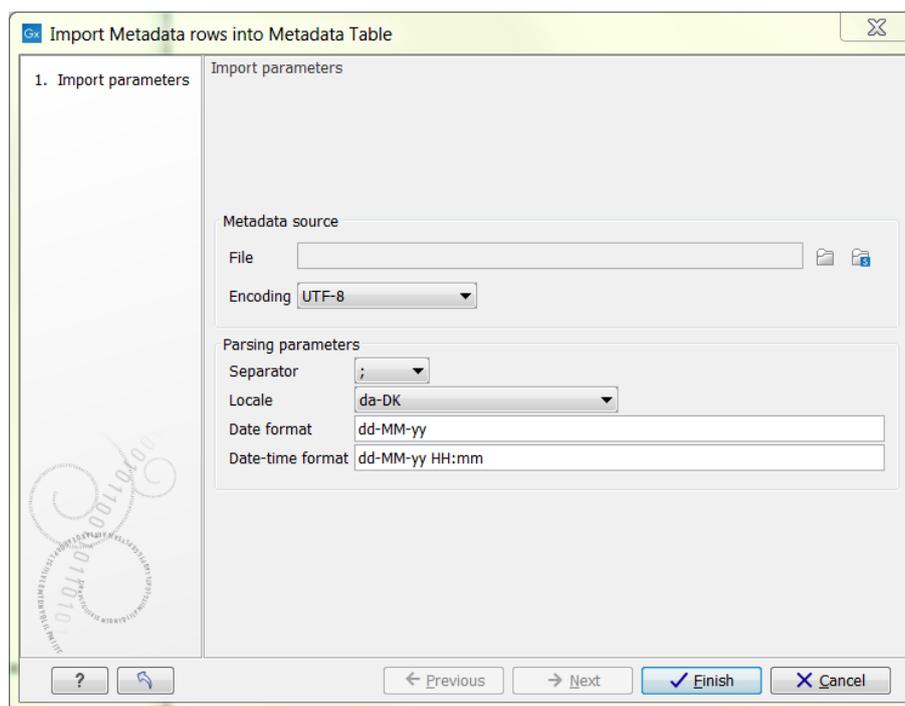


Figure 4.14: Dialog used to import rows into a Metadata Table.

| 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 |

Click the **Finish** button in the dialog when done. The Workbench will then import data from the file, looking for column headers within the file matching those already specified for the columns in the Metadata Table. Only cell values in columns with an exact name match will be imported. This means that if your file contains columns not in your Metadata Table, the values in those columns will be ignored. Conversely, if your Metadata Table contains columns not present in the file, imported rows will have no values for those columns.

The imported rows will appear in the Metadata Table view. The Metadata Table may now be saved.

Importing rows may be done also on a Metadata Table that already contains rows. If a key column is present, the import will overwrite values in rows where the key value matches incoming data. If no existing row matches incoming data in the key column, or if no key column has been specified, incoming data will just be appended to the Metadata Table.

Please note: As importing data can potentially result in a lot of rows being added, a separate process for importing is started. You can see the progress and finally the status of this process in the Process tab of the Toolbox. Any errors resulting from an import that failed can also be seen here, or you may have to consult the 'Advanced' tab in the error dialog shown. Typical examples of errors could be selecting the wrong separator or encoding, or wrong date/time formats.

4.2.6 Associating data elements with metadata

Once a Metadata Table containing a key column has been saved, data elements may be associated with its rows automatically. Typically, this is done on freshly imported data elements. This initial association will then be carried over to results of analyses based on those data elements. This means that you can keep track of e.g. the sample to which a particular analysis result pertains without having to resort to data element naming conventions or an elaborate folder structure.

Each association between a particular data element and a row in your Metadata Table will be qualified by a "role" label that indicates what the role of the data element is with respect to your row. A suitable role for a freshly imported data element may be "Sample data" or "NGS reads". Each analysis tool will provide its own role labels when transferring the metadata association from input to output. As an example, a read mapping tool may assign the role "Un-mapped reads" to the Sequence List it produces, allowing you to keep track of which Sequence List was the original imported NGS reads, and which was the un-mapped ones.

To associate data elements with the rows of a Metadata Table, click the **Associate Data** button at the bottom of the Metadata Table view, and select **Associate Data Automatically**. Note: the button remains disabled until the Metadata Table has been saved (and, in case of the automatic association, has a key column). Clicking the button shows a standard wizard dialog as in figure 4.15.

Select the data elements you wish to associate with the rows of the Metadata Table. You may select both individual elements and folders; folders will be traversed recursively.

Data association is parameterized as follows, cf. figure 4.16.

- **Role of input data.** The role of the data elements with respect to the entities represented by the rows of your Metadata Tables (e.g. 'Sample Data', 'Mapped Reads', 'QC Report').

Click **Next** to specify result handling and to finish the wizard. The selected data elements will then be scanned in a background process, and those elements whose name matches a key in the key column of the Metadata Table (either the whole name or just the beginning of the name) will be modified to include a metadata association to the row in question, and with the role specified in the wizard.

Note: As well as adding new associations to the data elements, any existing associations to this particular metadata table will be *updated* to associate to the matching rows, or *deleted* if no rows match a given data element (e.g. the sample ID was deleted or changed since last automatic

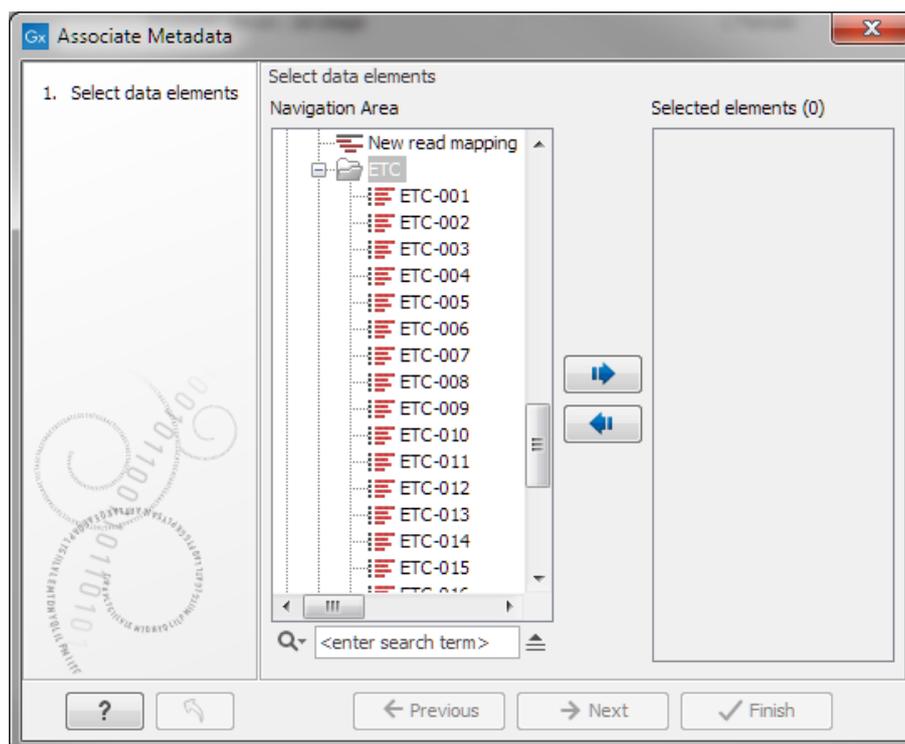


Figure 4.15: Dialog used to associate data elements with rows of a Metadata Table.

association, or the data element was manually associated, see section 4.2.6). If you want to avoid removing existing associations, take special care only to select data elements you want to have automatically associated / dissociated, and not, for instance, a whole folder.

Example of automatically associating data

If we have a metadata table, where the key column contains sample IDs like in figure 4.18) (i.e. ETC-001, ETC-002, . . . , ETC-013), and a folder of files like in figure 4.17.

The automatic association process tries to find the longest key that matches whole parts of the data element name, by splitting up the data element name using word boundaries.¹

If we run the automatic association on the entire folder, the data elements will be associated to keys in the metadata table as follows:

¹A part is considered any length of the data element name that consists of letters (a-z) and / or numbers (0-9), meaning special characters like (,), +, -, *, are boundaries for the search, i.e. 'Sample234-1 (mapped) (trimmed)' has the parts Sample234, -1, (mapped) and (trimmed).

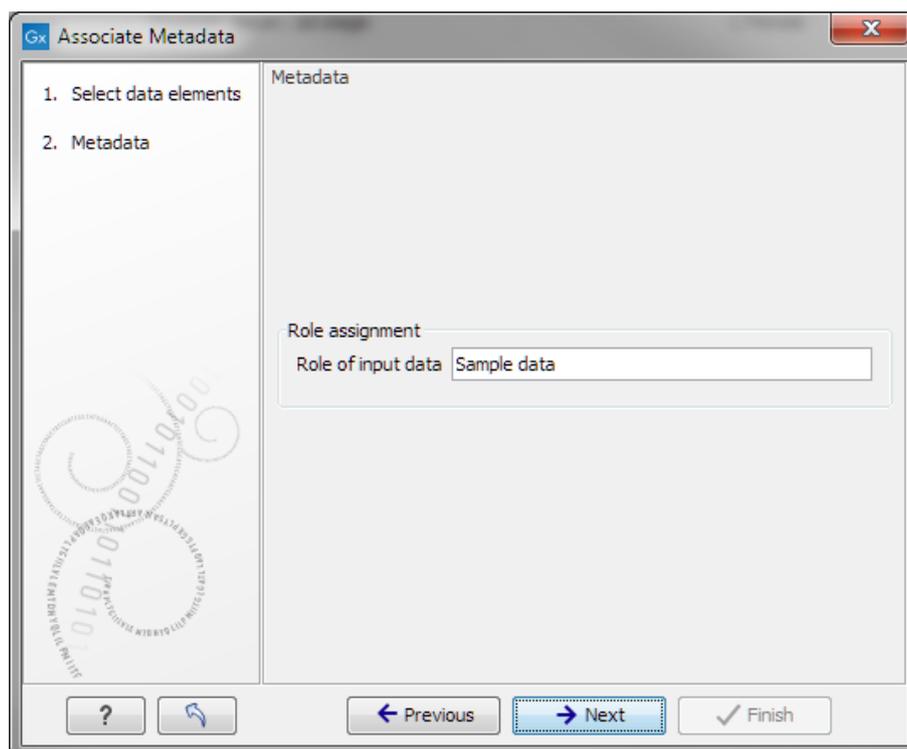


Figure 4.16: Parameters for data association.

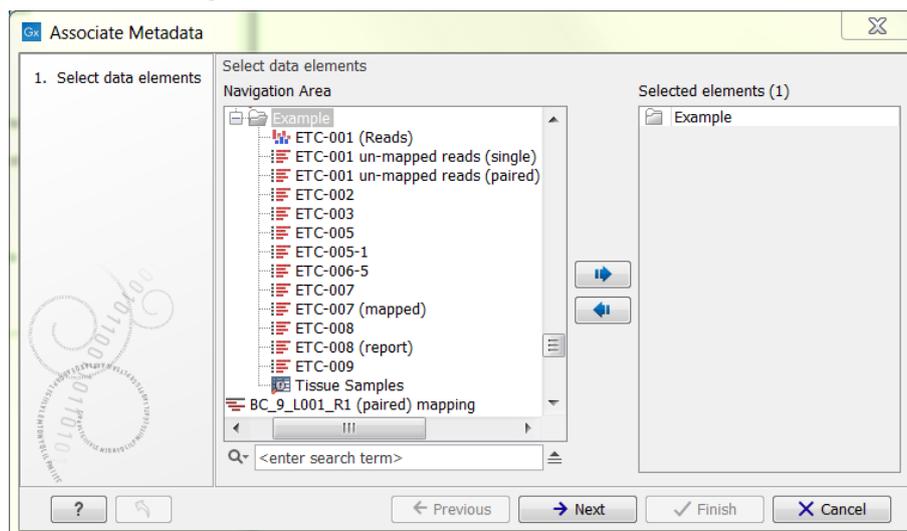


Figure 4.17: Files selected for automatic association.

| 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 |

You can also select individual or groups of elements, and automatically associate them to the metadata table with a special association role, and that way get the original reads mapped with 'Sample data' role, mapped reads with the 'mapped reads' role, etc.

Manually associating a metadata row to data elements

Sometimes, more data elements correspond to a specific metadata row, or the names of the elements do not match the metadata key value to be associated automatically. In these cases, you can manually associate a given metadata role to one or more data elements.

To associate data elements with a row in the Metadata Table, click the row in the editor, and right click to get the **Associate Data with Row** menu (see figure 4.18), or by selecting **Associate Data with row** in the **Associate Data** popup menu at the bottom. The Metadata Table does not need to have a key column for the manual association to work.

This will open up a wizard, where you can select any data elements to be associated with this Metadata row, and supply a role name for the metadata association. (The wizard is identical to the automatic metadata association, shown in figure 4.15 and figure 4.16). After running, any data elements already associated with this Metadata Table, will have this association changed, while associations to other tables will be kept as is.

| 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 4.18: Manual association of data elements to a metadata row.

4.2.7 Finding data elements based on metadata

Using the Metadata Table view you can find data elements associated with rows of the Metadata Table. Select one or more rows of the Metadata Table, then click **Find Associated Data**. A search result table is then displayed below the Metadata Table, see figure 4.19.

The search results table shows the type, name, and navigation area path for each data element found. It also shows the key of the Metadata Table row with which the element is associated, as well as the role of the data element with respect to the entity modelled by that row. In figure 4.19, there are five data elements associated with sample ETC-009. Three are Sequence Lists, two of them containing un-mapped reads resulting from an execution of the Map Reads to Reference tool.

By selecting rows in the search result table, you can open search results of interest by clicking the **Show** button, or you can have them highlighted in the Navigation Area by clicking the **Find in Navigation Area** button. If you invoke a tool or exporter, the selected search results will be pre-selected in the wizard. This functionality can be used together with filtering on the Role column of the search results table to execute analyses on data elements that have a particular role with respect to its associated metadata.

Clicking the **Refresh** button will re-run the search and refresh the search results table. You can close the table by clicking the **Close** button.

Rows: 8 Metadata

| Sample ID | Tissue | Batch | Control | Patient DOB | Sampling time |
|-----------|--------|-------|---------|-------------|---------------|
| 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-010 | Liver | | 3 | | |
| ETC-013 | Brain | | 1 | | |

Setup table... Manage data... Find Associated Data Associate Data

Rows: 6 Metadata Elements

| Sample ID | Role | Type | Name | Path |
|-----------|-----------------|------|--|---------------------|
| ETC-001 | Sample data | | ETC-001 | CLC_Data / Metadata |
| ETC-009 | Sample data | | ETC-009 | CLC_Data / Metadata |
| ETC-009 | Un-mapped reads | | ETC-009 un-mapped reads [no read group] (paired) | CLC_Data / Metadata |
| ETC-009 | Read mapping | | ETC-009 (Reads) | CLC_Data / Metadata |
| ETC-009 | Un-mapped reads | | ETC-009 un-mapped reads [no read group] (single) | CLC_Data / Metadata |
| ETC-009 | Mapping report | | ETC-009 mapping summary report | CLC_Data / Metadata |

Find in Navigation Area Show Refresh Close

Figure 4.19: Metadata Table with search results

4.2.8 Viewing metadata associations

Metadata associations for a data element are shown in the Element Info view (section 12.4), see figure 4.20. To show Element Info,

right-click an element in the Navigation Area | Show | Element Info (📄)

*ETC-001 x

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 4.20: Element Info view with a metadata association

The Element Info view contains the details of each metadata association for the data element. The following operations are available:

- **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.

4.2.9 Exporting metadata

You can use the standard Workbench export functionality to export metadata tables to various formats, including CSV. Your system's default locale will be used for the export, affecting the appearance of numbers and dates etc. in the exported file.

See section 7.2 for more information.

4.3 Customized attributes on data locations

If CLC data is stored in a database then location-specific attributes can be set on all elements stored in that 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.

Note also that for *CLC Genomics Workbench* and *CLC Main Workbench*, a Metadata Import Plugin is available (<http://www.clcbio.com/clc-plugin/metadata-import-plugin/>). The plugin consists of two tools: "Import Sequences in Table Format" and "Associate with Metadata". These tools allow sequences to be imported from a tabular data source and make it possible to add metadata to existing objects.

4.3.1 Configuring which fields should be available

To configure which fields that should be available² go to the Workbench:

right-click the data location | Location | Attribute Manager

This will display the dialog shown in figure 4.21.

Click the **Add Attribute** (+) button to create a new attribute. This will display the dialog shown in figure 4.22.

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.

²If the data location is a server location, you need to be a server administrator to do this

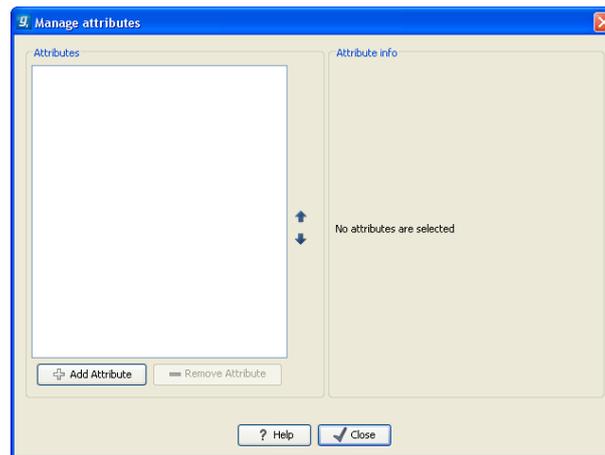


Figure 4.21: Adding attributes.

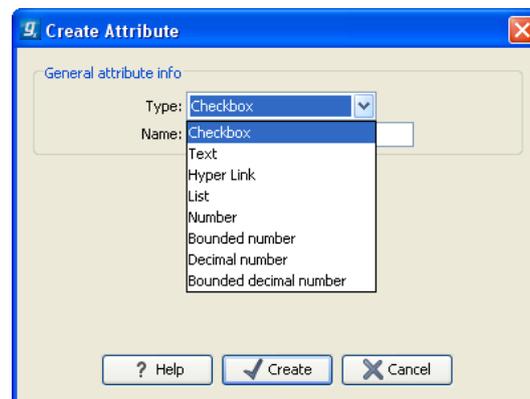


Figure 4.22: The list of attribute types.

- **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 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.

4.3.2 Editing lists

Lists are a little special, since you have to define the items in the list. When you click a list 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 4.23).

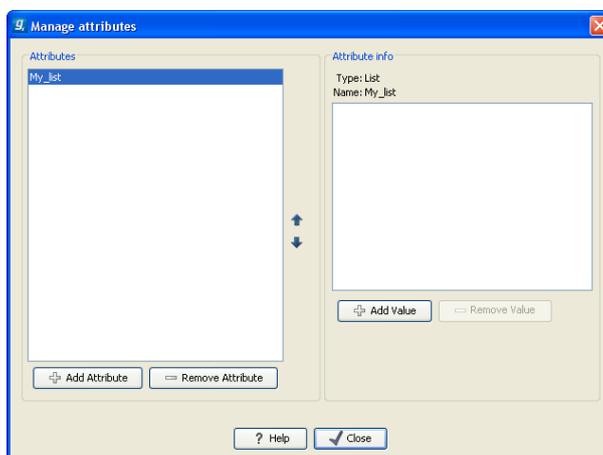


Figure 4.23: Defining items in a list.

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

4.3.3 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.

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.

4.3.4 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.

4.4 Filling in values

When a set of attributes has been created (as shown in figure 4.24), the end users can start filling in information.

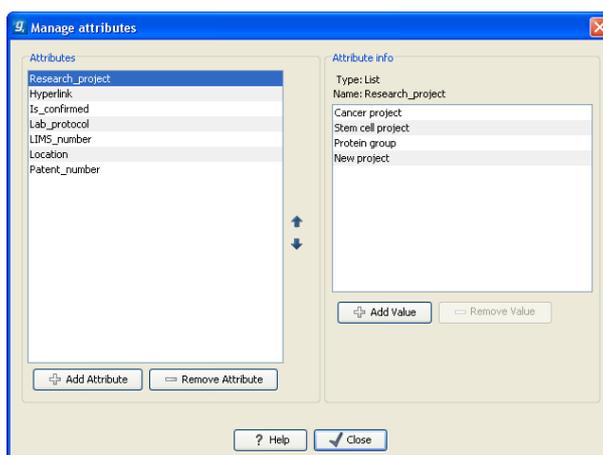


Figure 4.24: A set of attributes defined in the attribute manager.

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 4.25.



Figure 4.25: 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 4.26).



Figure 4.26: 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 4.26, 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 (see section 4.1.8).

4.4.1 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.

4.4.2 Searching

When an attribute has been created, it will automatically be available for searching. This means that in the **Local Search** (🔍), you can select the attribute in the list of search criteria (see figure 4.27).

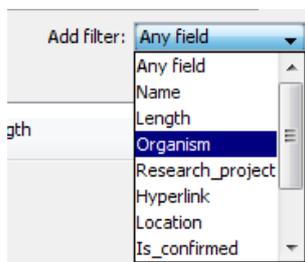


Figure 4.27: The attributes from figure 4.24 are now listed in the search filter.

It will also be available in the **Quick Search** below the **Navigation Area** (press Shift+F1 (Fn+Shift+F1 on Mac) and it will be listed - see figure 4.28).

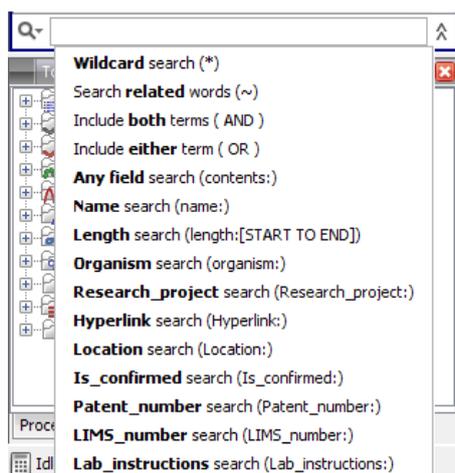


Figure 4.28: The attributes from figure 4.24 are now available in the Quick Search as well.

4.5 Local search

There are two ways of doing text-based searches of your data, as described in this section:

- **Quick-search** directly from the search field in the **Navigation Area**.
- **Advanced search** which makes it easy to make more specific searches.

In most cases, quick-search will find what you need, but if you need to be more specific in your search criteria, the advanced search is preferable.

4.5.1 What kind of information can be searched?

Below is a list of the different kinds of information that you can search for (applies to both quick-search and the advanced search).

- **Name.** The name of a sequence, an alignment or any other kind of element. The name is what is displayed in the **Navigation Area** per default.
- **Length.** The length of the sequence.
- **Organism.** Sequences which contain information about organism can be searched. In this way, you could search for e.g. *Homo sapiens* sequences.
- **Custom attributes.** Read more in section 4.3

Only the first item in the list, **Name**, is available for all kinds of data. The rest is only relevant for sequences.

If you wish to perform a search for sequence similarity, use Local BLAST (see section 26.1.3) instead.

4.5.2 Quick search

At the bottom of the **Navigation Area** there is a text field as shown in figure 4.29).

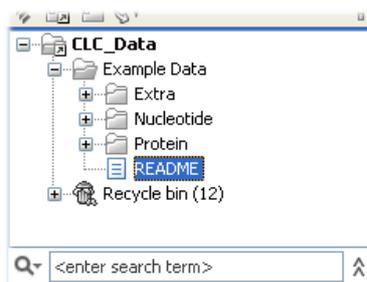


Figure 4.29: Search simply by typing in the text field and press Enter.

To search, simply enter a text to search for and press **Enter**.

Note that the search term supports advanced features known from web search engines, which means that the following list of characters carry special meaning: + - & & || ! () ^ [] " ~ * ? : \ / . To avoid this special interpretation it is suggested to put quotes around the search expression when searching for data containing the special characters, or read the section 4.5.3 on advanced search expressions.

Quick search results

To show the results, the search pane is expanded as shown in figure 4.30).

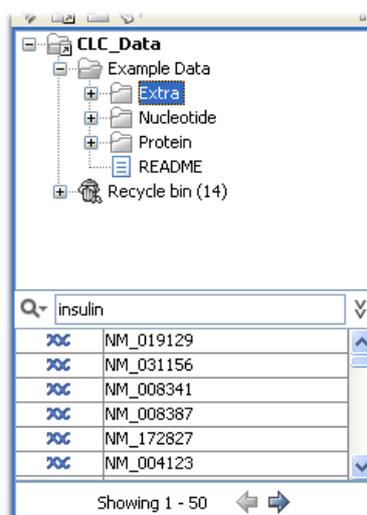


Figure 4.30: Search results.

If there are many hits, only the 50 first hits are immediately shown. At the bottom of the pane you can click **Next** (→) to see the next 50 hits (see figure 4.31).

If a search gives no hits, you will be asked if you wish to search for matches that start with your search term. If you accept this, an asterisk (*) will be appended to the search term.

Pressing the Alt key while you click a search result will high-light the search hit in its folder in the **Navigation Area**.

In the preferences (see Chapter 5), you can specify the number of hits to be shown.

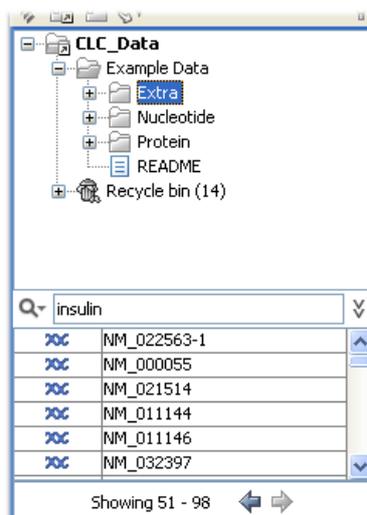


Figure 4.31: Page two of the search results.

Special search expressions

When you write a search term in the search field, you can get help to write a more advanced search expression by pressing **Shift+F1**. This will reveal a list of guides as shown in figure 4.32.

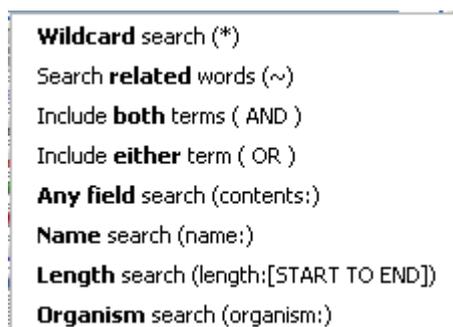


Figure 4.32: Guides to help create advanced search expressions.

You can select any of the guides (using mouse or keyboard arrows), and start typing. If you e.g. wish to search for sequences named BRCA1, select "Name search (name:)", and type "BRCA1". Your search expression will now look like this: "name:BRCA1".

The guides available are these:

- **Wildcard search (*)**. Appending an asterisk * to the search term will find matches starting with the term. E.g. searching for "brca*" will find both *brca1* and *brca2*.
- **Search related words (~)**. If you don't know the exact spelling of a word, you can append a tilde to the search term. E.g. "brac1~" will find sequences with a *brca1* gene.
- **Include both terms (AND)**. If you write two search terms, you can define if your results have to match both search terms by combining them with AND. E.g. search for "brca1 AND human" will find sequences where *both* terms are present.
- **Include either term (OR)**. If you write two search terms, you can define that your results have to match either of the search terms by combining them with OR. E.g. search for "brca1 OR brca2" will find sequences where *either* of the terms is present.

- **Do not include term (NOT)** If you write a term after not, then elements with these terms will not be returned.
- **Name search (name:)**. Search only the name of element.
- **Organism search (organism:)**. For sequences, you can specify the organism to search for. This will look in the "Latin name" field which is seen in the **Sequence Info** view (see section 12.4).
- **Length search (length:[START TO END])**. Search for sequences of a specific length. E.g. search for sequences between 1000 and 2000 residues: "length:1000 TO 2000".

Note! If you have added attributes (see section 4.3), these will also appear on the list when pressing **Shift+F1**.

If you do not use this special syntax, you will automatically search for both name, description, organism, etc., and search terms will be combined as if you had put OR between them.

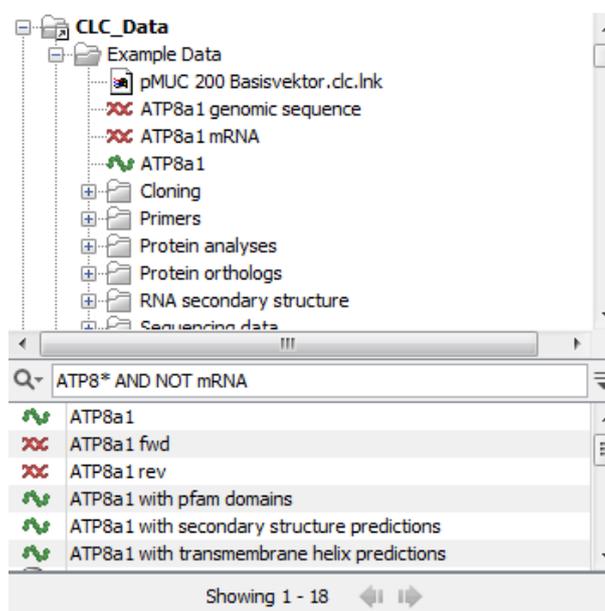


Figure 4.33: An example of searching for elements with the name, description and organism information that includes "ATP8" but do not include the term "mRNA".

Search for data locations

The search function can also be used to search for a specific URL. This can be useful if you work on a server and wish to share a data location with another user. A simple example is shown in figure 4.34. Right click on the object name in the **Navigation Area** (in this case ATP8a1 genomic sequence) and select "Copy". When you use the paste function in a destination outside the Workbench (e.g. in a text editor or in an email), the data location will become visible. The URL can now be used in the search field in the Workbench to locate the object.

Quick search history

You can access the 10 most recent searches by clicking the icon (Q-) next to the search field (see figure 4.35).

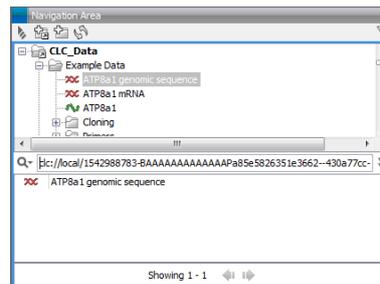


Figure 4.34: The search field can also be used to search for data locations.

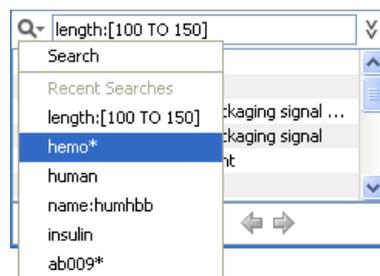


Figure 4.35: Recent searches.

Clicking one of the recent searches will conduct the search again.

4.5.3 Advanced search

As a supplement to the **Quick search** described in the previous section you can use the more advanced search:

Edit | Local Search (📄)

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

This will open the search view as shown in figure 4.36

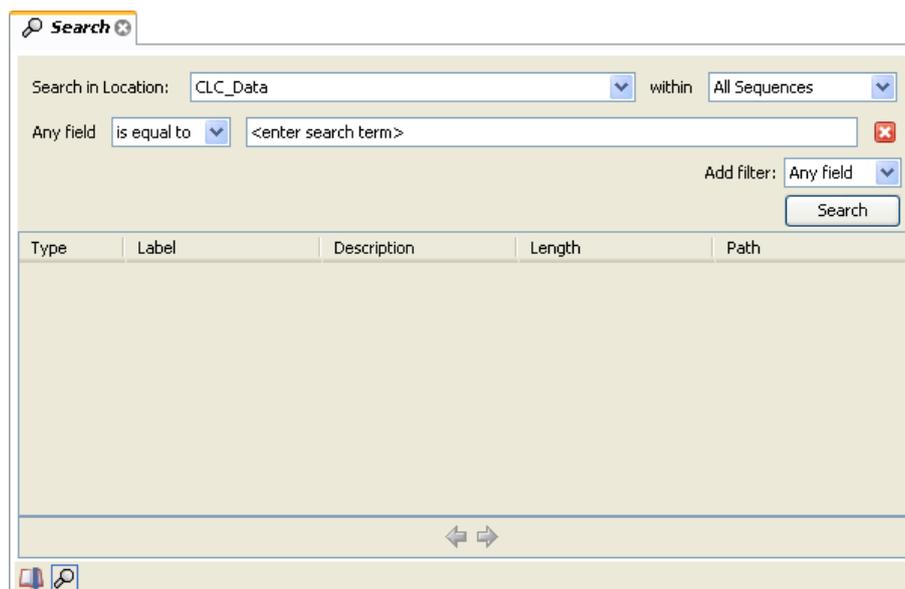


Figure 4.36: Advanced search.

The first thing you can choose is which location should be searched. All the active locations are shown in this list. You can also choose to search all locations. Read more about locations in section [4.1.1](#).

Furthermore, you can specify what kind of elements should be searched:

- All sequences
- Nucleotide sequences
- Protein sequences
- All data

When searching for sequences, you will also get alignments, sequence lists etc as result, if they contain a sequence which match the search criteria.

Below are the search criteria. First, select a relevant search filter in the **Add filter:** list. For sequences you can search for

- Name
- Length
- Organism

See section [4.5.2](#) for more information on individual search terms.

For all other data, you can only search for name.

If you use **Any field**, it will search all of the above plus the following:

- Description
- Keywords
- Common name
- Taxonomy name

To see this information for a sequence, switch to the **Element Info**  view (see section [12.4](#)).

For each search line, you can choose if you want the exact term by selecting "is equal to" or if you only enter the start of the term you wish to find (select "begins with").

An example is shown in figure [4.37](#).

This example will find human nucleotide sequences (organism is *Homo sapiens*), and it will only find sequences shorter than 10,000 nucleotides.

Note that a search can be saved  for later use. You do not save the search results - only the search parameters. This means that you can easily conduct the same search later on when your data has changed.

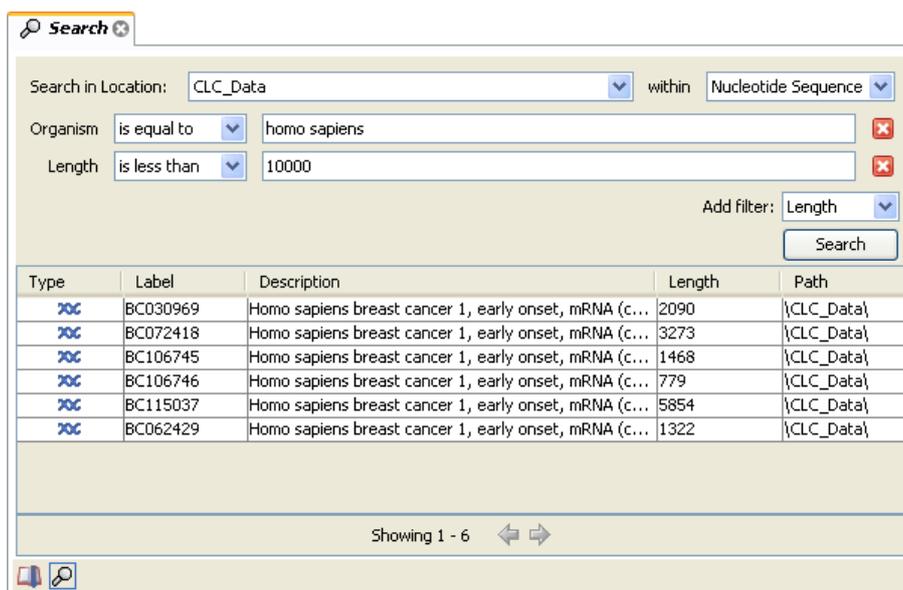


Figure 4.37: Searching for human sequences shorter than 10,000 nucleotides.

4.5.4 Search index

This section has a technical focus and is not relevant if your search works fine.

However, if you experience problems with your search results: if you do not get the hits you expect, it might be because of an index error.

The *CLC Main Workbench* automatically maintains an index of all data in all locations in the **Navigation Area**. If this index becomes out of sync with the data, you will experience problems with strange results. In this case, you can rebuild the index:

Right-click the relevant location | Location | Rebuild Index

This will take a while depending on the size of your data. At any time, the process can be stopped in the process area, see section 3.3.1.

Chapter 5

User preferences and settings

Contents

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The first three sections in this chapter deal with the general preferences that can be set for *CLC Main Workbench* using the **Preferences** dialog. The next section explains how the settings in the **Side Panel** can be saved and applied to other views. Finally, you can learn how to import and export the preferences.

The **Preferences** dialog offers opportunities for changing the default settings for different features of the program.

The **Preferences** dialog is opened in one of the following ways and can be seen in figure 5.1:

Edit | Preferences (⚙️)

or **Ctrl + K** (⌘ + ; on Mac)

5.1 General preferences

The **General** preferences include:

- **Undo Limit.** As default the undo limit is set to 500. By writing a higher number in this field, more actions can be undone. Undo applies to all changes made on molecules, sequences, alignments or trees. See section 3.1.5 for more on this topic.

1. **Toolbar** lets you choose the size of the toolbar icons, and whether to display names below the icons (figure 5.4).

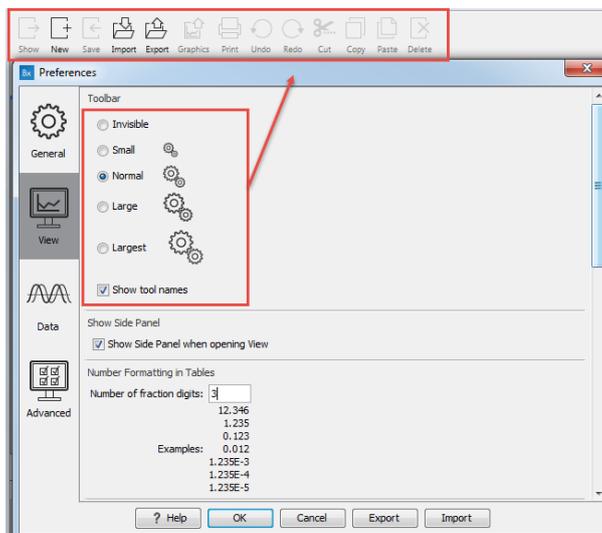


Figure 5.4: Number formatting of tables.

2. **Show Side Panel** allows you to choose whether to display the side panel when opening a new view. Note that 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** specifies how the numbers should be formatted in tables (see figure 5.5). The examples below the text field are updated when you change the value so that you can see the effect. After you have changed the preference, you have to re-open your tables to see the effect.

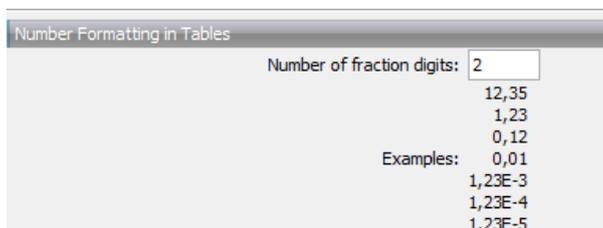


Figure 5.5: Number formatting of tables.

4. **Sequence Representation** allows you to change the way the elements appear in the Navigation Area. The following text can be used to describe the element:

- 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).

5. **User Defined View Settings** gives you an overview of the different Side Panel settings that are saved for each view. See section 5.6 to learn more about how to create and save style sheets. If there are other settings beside CLC Standard Settings, you can use this overview to choose which of the settings should be used per default when you open a view (see an example in figure 5.6).

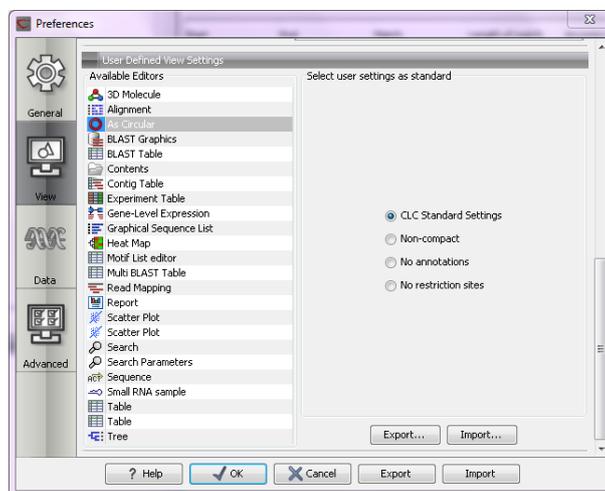


Figure 5.6: Selecting the default view setting. In this example, the CLC Standard Settings is chosen as default.

6. **Molecule Project 3D Editor** gives you the option to turn off the modern OpenGL rendering for **Molecule Projects** (see section 15.2.2).

5.2.1 Import and export Side Panel settings

If you have created a special set of settings in the **Side Panel** that you wish to share with other CLC users, you can export the settings in a file. The other user can then import the settings.

To export the **Side Panel** settings, first select the views that you wish to export settings for. Use Ctrl+click (⌘ + click on Mac) or Shift+click to select multiple views. Next click the **Export...** button. Note that there is also another export button at the very bottom of the dialog, but this will export the other settings of the **Preferences** dialog (see section 5.5).

A dialog will be shown (see figure 5.7) that allows you to select which of the settings you wish to export.

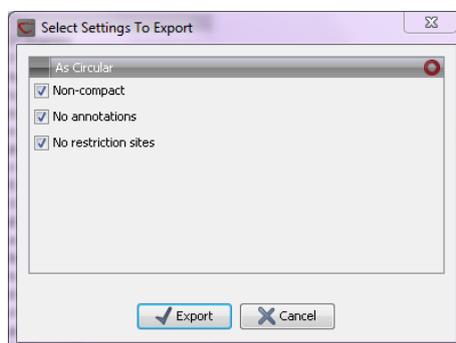


Figure 5.7: Exporting all settings for circular views.

When multiple views are selected for export, all the view settings for the views will be shown in the dialog. Click **Export** and you will now be able to define a save folder and name for the exported file. The settings are saved in a file with a .vsf extension (View Settings File).

To import a **Side Panel** settings file, make sure you are at the bottom of the **View** panel of the **Preferences dialog**, and click the **Import...** button. Note that there is also another import button at the very bottom of the dialog, but this will import the other settings of the **Preferences** dialog (see section 5.5).

The dialog asks if you wish to overwrite existing **Side Panel** settings, or if you wish to merge the imported settings into the existing ones (see figure 5.8).



Figure 5.8: When you import settings, you are asked if you wish to overwrite existing settings or if you wish to merge the new settings into the old ones.

Note! If you choose to overwrite the existing settings, you will lose all the **Side Panel** settings that you have previously saved.

To avoid confusion of the different import and export options, here is an overview:

- Import and export of **bioinformatics data** such as sequences, alignments etc. (described in section 7.1).
- **Graphics** export of the views which creates image files in various formats (described in section 7.3).
- Import and export of **Side Panel Settings** as described above.
- Import and export of all the **Preferences** except the Side Panel settings. This is described in the previous section.

5.3 Data preferences

The data preferences contain preferences related to interpretation of data, e.g. linker sequences:

- Predefined primer additions for Gateway cloning (see section 23.2.1).

5.4 Advanced preferences

The **Advanced** settings include the possibility to set up a proxy server. This is described in section 1.8.

5.4.1 Default data location

The default location is used when you e.g. import a file without selecting a folder or element in the **Navigation Area** first.

The default data location for CLC Workbenches is, by default, a folder called CLC_Data in a user's home area.

This can be changed to a different location for a particular user of the Workbench by going to

Edit | Preferences

and then choosing the **Advanced** tab. This holds a section called **Default Data Location** and here you can choose a default from a drop down list of data locations you have already added.

Note! The default location cannot be removed. You have to select another location as default first.

If the data area you want as your default is not already available in your Workbench, you need to first add it as a new data location (see section 4.1.1).

5.4.2 NCBI BLAST

URL to use for BLAST

It is possible to specify an alternate server URL to use for BLAST searches. The standard URL for the BLAST server at NCBI is: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.

Note! Be careful to specify a valid URL, otherwise BLAST will not work.

5.5 Export/import of preferences

The user preferences of the *CLC Main Workbench* can be exported to other users of the program, allowing other users to display data with the same preferences as yours. You can also use the export/import preferences function to backup your preferences.

To export preferences, open the **Preferences** dialog (Ctrl + K (⌘ + ; on Mac)) and do the following:

Export | Select the relevant preferences | Export | Choose location for the exported file | Enter name of file | Save

Note! The format of exported preferences is .cpf. This notation must be submitted to the name of the exported file in order for the exported file to work.

Before exporting, you are asked about which of the different settings you want to include in the exported file. One of the items in the list is "User Defined View Settings". If you export this, only the information about which of the settings is the default setting for each view is exported. If you wish to export the **Side Panel Settings** themselves, see section 5.2.1.

The process of importing preferences is similar to exporting:

Press Ctrl + K (⌘ + ; on Mac) to open Preferences | Import | Browse to and select the .cpf file | Import and apply preferences

5.5.1 The different options for export and import

To avoid confusion of the different import and export options, you can find an overview here:

- Import and export of **bioinformatics data** such as molecules, sequences, alignments etc. (described in section 7.1).
- **Graphics** export of the views that create image files in various formats (described in section 7.3).
- Import and export of **Side Panel Settings** as described in the next section.
- Import and export of all the **Preferences** except the Side Panel settings. This is described above.

5.6 View settings for the Side Panel

The **Side Panel** is shown to the right of all views that are opened in *CLC Main Workbench* and is described in further detail in section 3.1.8.

When you have adjusted a view of e.g. a sequence, your settings in the **Side Panel** can be saved. When you open other sequences, which you want to display in a similar way, the saved settings can be applied. The options for saving and applying are available at the bottom of the **Side Panel** (see figure 5.9).



Figure 5.9: At the bottom of the Side Panel you save the view settings

5.6.1 Saving, removing and applying saved settings

To save and apply the saved settings, click (☰) seen in figure 5.9. This opens a menu where the following options are available (figure 5.10):

- **Save ... Settings.** (⚙) The settings can be saved in two different ways. When you select either way of saving settings a dialog will open (see figure 5.11) where you can enter a name for your settings.
 - **For ... View in General** (⚙) Will save the currently used settings with all elements of the same type as the one used for adjusting the settings. E.g. if you have selected to save settings "For Track View in General" the settings will be applied each time you open an element of the same type, which in this case means each time one of the saved tracks are opened from the **Navigation Area**. These "general" settings are user specific and will not be saved with or exported with the element.

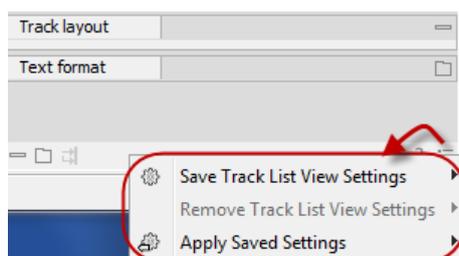


Figure 5.10: When you have adjusted the side panel settings and would like to save these, this can be done with the "Save ... Settings" function, where "..." is the element you are working on - e.g. "Track List View", "Sequence View", "Table View", "Alignment View" etc. Saved settings can be deleted again with "Remove ... Settings" and can be applied to other elements with "Apply Saved Settings".

- **On This Only** (📁) Settings can be saved with the specific element that you are working on in the View area and will not affect any other elements (neither in the View Area or in the **Navigation Area**). E.g. for a track you would get the option to save settings "On This Track Only". The settings are saved with only this element (and will be exported with the element if you later select to export the element to another destination).

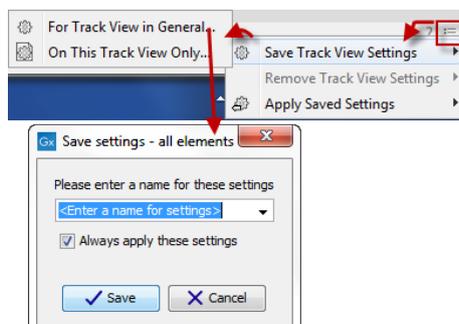


Figure 5.11: The save settings dialog. Two options exist for saving settings. Click on the relevant option to open the dialog shown at the bottom of the figure.

- **Remove ... Settings.** (🗑️) Gives you the option to remove settings specifically for the element that you are working on in the View Area, or on all elements of the same type. When you have selected the relevant option, the dialog shown in figure 5.12 opens and allows you to select which of the saved settings to remove.
 - **From ... View in General** (🗑️) Will remove the currently used settings on all elements of the same type as the one used for adjusting the settings. E.g. if you have selected to remove settings from all alignments using "From Alignment View in General", all alignments in your **Navigation Area** will be opened with the standard settings in stead.
 - **From This ... Only** (📁) When you select this option, the selected settings will only be removed from the particular element that you are working on in the View area and will not affect any other elements (neither in the View Area or in the **Navigation Area**). The settings for this particular element will be replaced with the CLC standard settings (🗑️).
- **Apply Saved Settings.** (📁) This is a submenu containing the settings that you have previously saved (figure 5.13). By clicking one of the settings, they will be applied to the current view. You will also see a number of pre-defined view settings in this submenu. They

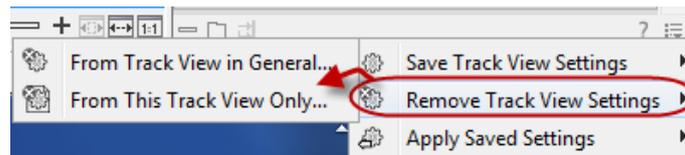


Figure 5.12: The remove settings dialog for a track.

are meant to be examples of how to use the **Side Panel** and provide quick ways of adjusting the view to common usages. At the bottom of the list of settings you will see **CLC Standard Settings** which represent the way the program was set up, when you first launched it. (🗑️)

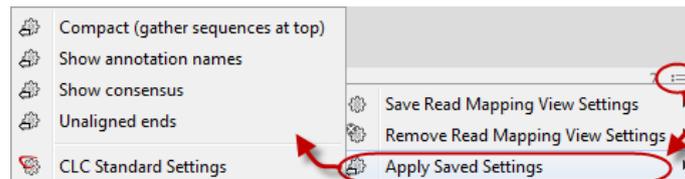


Figure 5.13: Applying saved settings.

The settings are specific to the type of view. Hence, when you save settings of a circular view, they will not be available if you open the sequence in a linear view.

If you wish to export the settings that you have saved, this can be done in the **Preferences** dialog under the **View** tab (see section 5.2.1).

Chapter 6

Printing

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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 7.3) 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 6.1).

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.

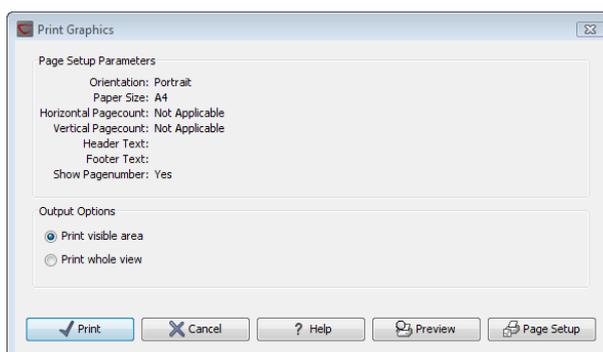


Figure 6.1: The Print dialog.

6.1 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 6.2 is a view of a circular sequence which is zoomed in so that you can only see a part of it.

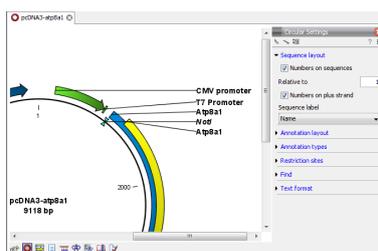


Figure 6.2: A circular sequence as it looks on the screen.

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 6.2 and choosing **Print visible area** can be seen in figure 6.3.

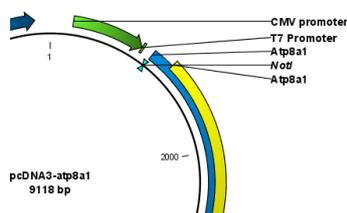


Figure 6.3: A print of the sequence selecting Print visible area.

On the other hand, if you select **Print whole view**, you will get a result that looks like figure 6.4. This means that you also print the part of the sequence which is not visible when you have zoomed in.

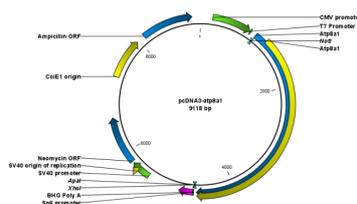


Figure 6.4: A print of the sequence selecting Print whole view. The whole sequence is shown, even though the view is zoomed in on a part of the sequence.

6.2 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 6.5

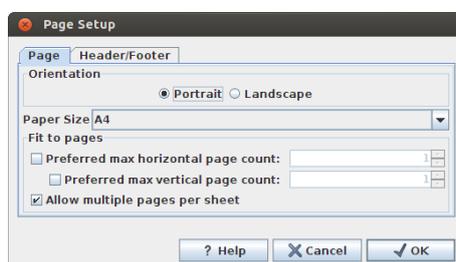


Figure 6.5: Page Setup.

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.
- **Fit to pages.** Can be used to control how the graphics should be split across pages (see figure 6.6 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.

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**.



Figure 6.6: An example where *Fit to pages horizontally* is set to 2, and *Fit to pages vertically* is set to 3.

6.2.1 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.

6.3 Print preview

The preview is shown in figure 6.7.

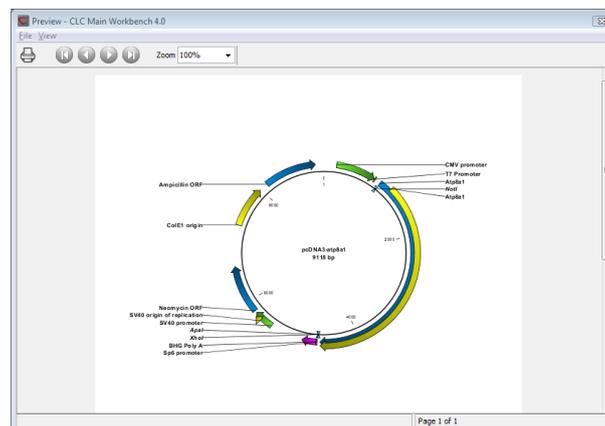


Figure 6.7: *Print preview.*

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 7

Import/export of data and graphics

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CLC Main Workbench handles a large number of different data formats. In order to work with data in the Workbench, it has to be imported (). Data types that are not recognized by the Workbench are imported as "external files" which means that when you open these, they will open in the default application for that file type on your computer (e.g. Word documents will open in Word).

This chapter first deals with importing and exporting data in bioinformatic data formats and as external files. Next comes an explanation of how to export graph data points to a file, and how to export graphics.

7.1 Standard import

CLC Main Workbench has support for a wide range of bioinformatic data such as molecules, sequences, alignments etc. See a full list of the data formats in section [I.1](#).

These data can be imported through the Import dialog, using drag/drop or copy/paste as explained below.

7.1.1 Import using the import dialog

To start the import using the import dialog: **click Import** (📁) **in the Toolbar**

This will show a dialog similar to figure [7.1](#). You can change which kind of file types that should be shown by selecting a file format in the **Files of type** box.

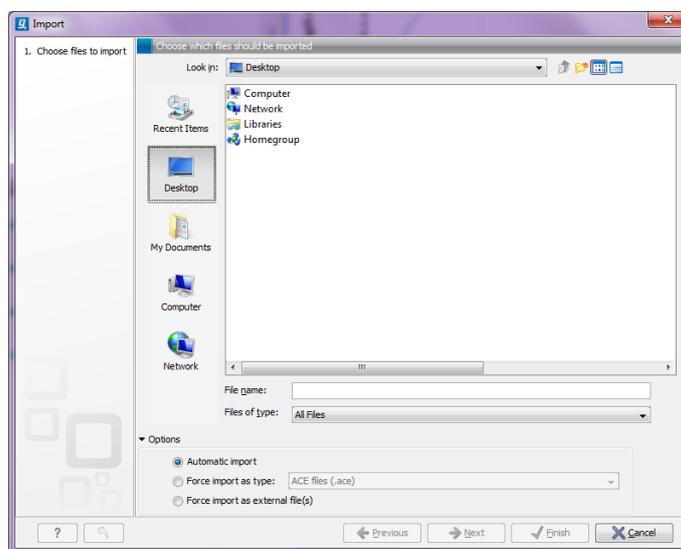


Figure 7.1: The import dialog.

Next, select one or more files or folders to import and click **Next**.

This allows you to select a place for saving the result files.

If you import one or more folders, the contents of the folder is automatically imported and placed in that folder in the **Navigation Area**. If the folder contains subfolders, the whole folder structure is imported.

In the import dialog (figure [7.1](#)), there are three import options:

Automatic import This will import the file and *CLC Main Workbench* will try to determine the format of the file. The format is determined based on the file extension (e.g. SwissProt files have .swp at the end of the file name) in combination with a detection of elements in the file that are specific to the individual file formats. If the file type is not recognized, it will be imported as an external file. In most cases, automatic import will yield a successful result, but if the import goes wrong, the next option can be helpful:

Force import as type This option should be used if *CLC Main Workbench* cannot successfully determine the file format. By forcing the import as a specific type, the automatic determination of the file format is bypassed, and the file is imported as the type specified.

Force import as external file This option should be used if a file is imported as a bioinformatics file when it should just have been external file. It could be an ordinary text file which is imported as a sequence.

7.1.2 Import using drag and drop

It is also possible to drag a file from e.g. the desktop into the **Navigation Area** of *CLC Main Workbench*. This is equivalent to importing the file using the **Automatic import** option described above. If the file type is not recognized, it will be imported as an external file.

7.1.3 Import using copy/paste of text

If you have e.g. a text file or a browser displaying a sequence in one of the formats that can be imported by *CLC Main Workbench*, there is a very easy way to get this sequence into the **Navigation Area**:

Copy the text from the text file or browser | Select a folder in the Navigation Area
| **Paste** ()

This will create a new sequence based on the text copied. This operation is equivalent to saving the text in a text file and importing it into the *CLC Main Workbench*.

If the sequence is not formatted, i.e. if you just have a text like this: "ATGACGAATAGGAGTTC-TAGCTA" you can also paste this into the **Navigation Area**.

Note! Make sure you copy all the relevant text - otherwise *CLC Main Workbench* might not be able to interpret the text.

7.1.4 External files

In order to help you organize your research projects, *CLC Main Workbench* lets you import all kinds of files. E.g. if you have Word, Excel or pdf-files related to your project, you can import them into the **Navigation Area** of *CLC Main Workbench*. Importing an external file creates a copy of the file which is stored at the location you have chosen for import. The file can now be opened by double-clicking the file in the **Navigation Area**. The file is opened using the default application for this file type (e.g. Microsoft Word for .doc-files and Adobe Reader for .pdf).

External files are imported and exported in the same way as bioinformatics files (see section 7.1). Bioinformatics files not recognized by *CLC Main Workbench* are also treated as external files.

7.1.5 Import Vector NTI data

There are several ways of importing your Vector NTI data into the CLC Workbench. The best way to go depends on how your data is currently stored in Vector NTI:

- Your data is stored in the Vector NTI Local Database which can be accessed through Vector NTI Explorer. This is described in the first section below.
- Your data is stored as single files on your computer (just like Word documents etc.). This is described in the second section below.

Import from the Vector NTI Local Database

If your Vector NTI data are stored in a Vector NTI Local Database (as the one shown in figure 7.2), you can import all the data in one step, or you can import selected parts of it.

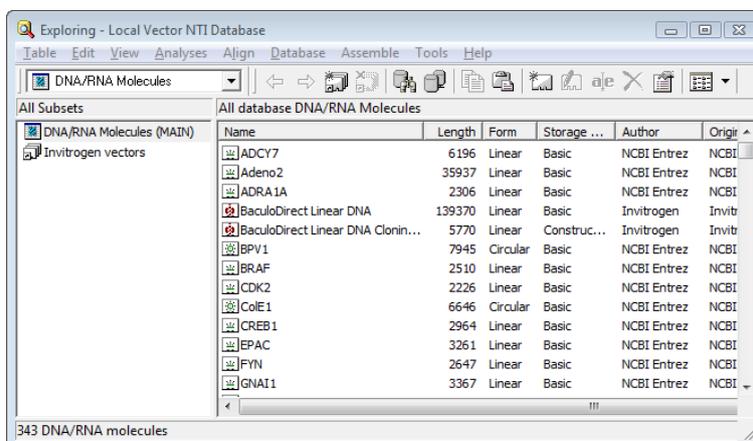


Figure 7.2: Data stored in the Vector NTI Local Database accessed through Vector NTI Explorer.

Importing the entire database in one step

From the Workbench, there is a direct import of the whole database (see figure 7.3):

File | Import Vector NTI Database

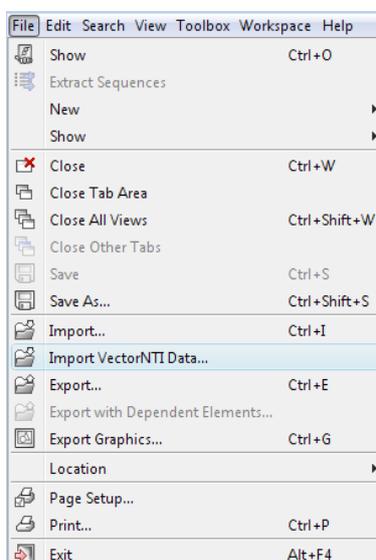


Figure 7.3: Import the whole Vector NTI Database.

This will bring up a dialog letting you choose to import from the default location of the database, or you can specify another location. If the database is installed in the default folder, like e.g. `C:\VNTI Database`, press **Yes**. If not, click **No** and specify the database folder manually.

When the import has finished, the data will be listed in the **Navigation Area** of the Workbench as shown in figure 7.4.

If something goes wrong during the import process, please report the problem to [support-](#)

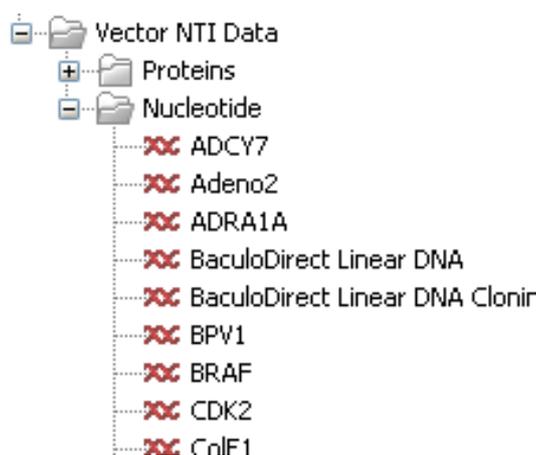


Figure 7.4: The Vector NTI Data folder containing all imported sequences of the Vector NTI Database.

clcbio@qiagen.com. To circumvent the problem, see the following section on how to import parts of the database. It will take a few more steps, but you will most likely be able to import this way.

Importing parts of the database

Instead of importing the whole database automatically, you can export parts of the database from Vector NTI Explorer and subsequently import into the Workbench. First, export a selection of files as an archive as shown in figure 7.5.

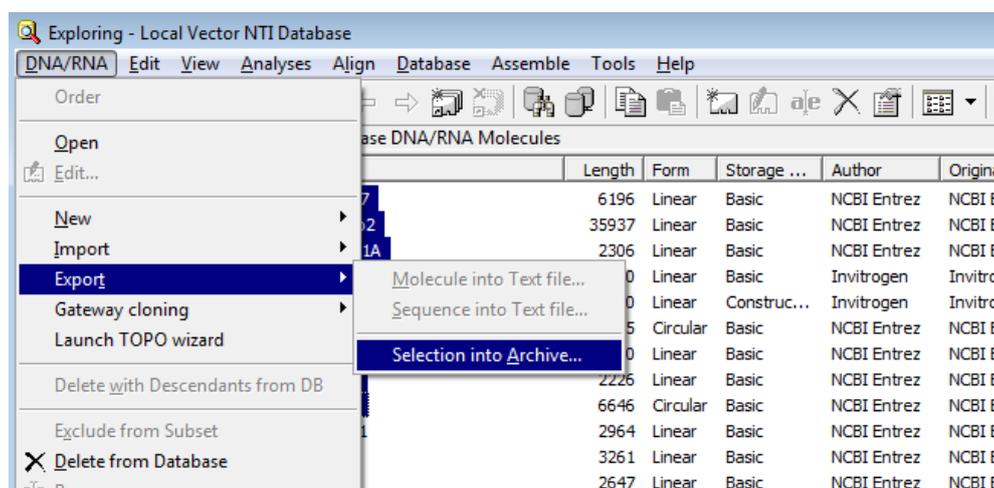


Figure 7.5: Select the relevant files and export them as an archive through the File menu.

This will produce a file with a ma4-, pa4- or oa4-extension. Back in the CLC Workbench, click **Import** (📁) and select the file.

Importing single files

In Vector NTI, you can save a sequence in a file instead of in the database (see figure 7.6).

This will give you file with a .gb extension. This file can be easily imported into the CLC Workbench:

Import (📁) | **select the file** | **Select**

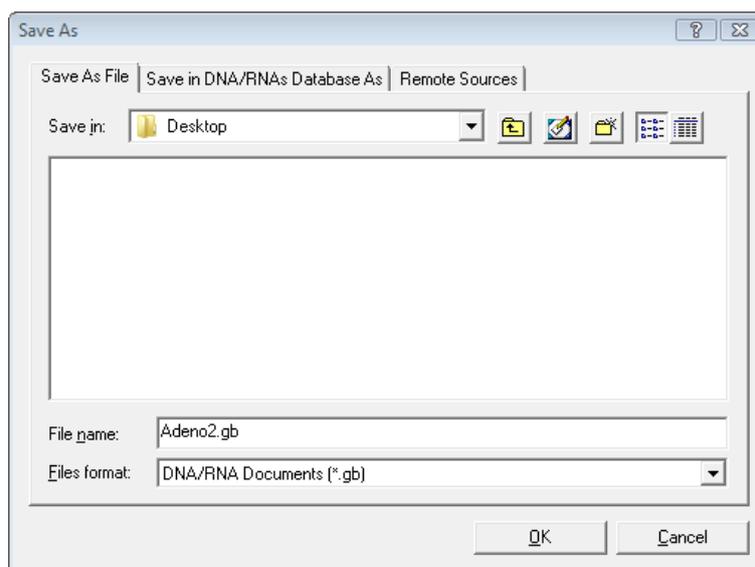


Figure 7.6: Saving a sequence as a file in Vector NTI.

You don't have to import one file at a time. You can simply select a bunch of files or an entire folder, and the CLC Workbench will take care of the rest. Even if the files are in different formats.

You can also simply drag and drop the files into the **Navigation Area** of the CLC Workbench.

The CLC Workbench supports import of several NTI formats, but not all. In case problems are encountered, try exporting NTI files to a more generic file format and then import these.

The Vector NTI import is a plugin which is pre-installed in the Workbench. It can be uninstalled and updated using the plugin manager (see section 1.7).

7.2 Data export

The exporter can be used to:

- Export bioinformatic data in most of the formats that can be imported. There are a few exceptions (see section 1.1).
- Export one or more data elements at a time to a given format. When multiple data elements are selected, each is written out to an individual file, unless compression is turned on, or "Output as single file" is selected.

The standard export functionality can be launched using the Export button on the toolbar, or by going to the menu:

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 7.2.2.

The general steps when configuring a standard export job are:

- (Optional) Select the data to export in the **Navigation Area**.
- Start up the exporter tool via the Export button in the toolbar or using the **Export** option under the File menu.
- Select the format the data should be exported to.
- Select the data to export, or confirm the data to export if it was already selected via the **Navigation Area**.
- Configure the parameters. This includes compression, multiple or single outputs, and naming of the output files, along with other format-specific settings where relevant.
- Select where the data should be exported to.
- Click on the button labeled **Finish**.

Selecting data for export - part I. You can select the data elements to export **before** you run the export tool **or after** the format to export to has been selected. If you are not certain which formats are supported for the data being exported, we recommend selecting the data in the **Navigation Area** before launching the export tool.

Selecting a format to export to. When data is pre-selected in the **Navigation Area** before launching the export tool you will see a column in the export interface called **Supported formats**. Formats that the selected data elements can be exported to are indicated by a "Yes" in this column. Supported formats will appear at the top of the list of formats. See figure 7.7.

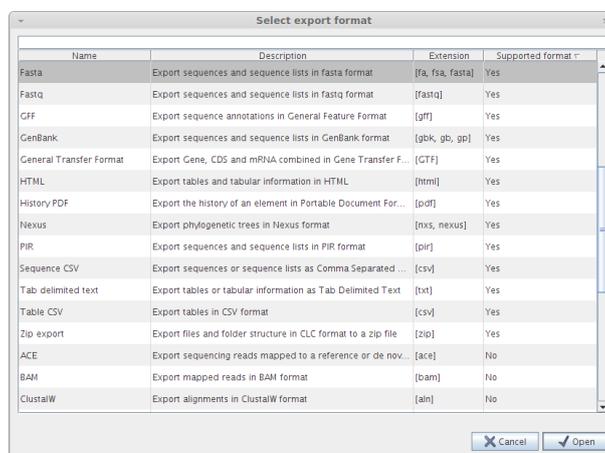


Figure 7.7: The Select exporter dialog where sequence lists were pre-selected in the Navigation Area before launching the export tool. Here, the formats sequence lists can be exported to are listed at the top, with a Yes in the Selected formats column. Other formats are found below, with No in this column.

Formats that cannot be used for export of the selected data have a "No" listed in the **Supported formats** column. If you have selected multiple data elements of different types, then formats which can be used for some of the selected data elements but not all of them are indicated by the text "For some elements" in this column.

Please note that the information in the **Supported formats** column only refers to the data already selected in the **Navigation Area**. If you are going to choose your data later in the export process, then the information in this column will not be pertinent.

Only one export format is available if you select a folder to be exported. This is described in more detail in section 7.2.1.

Finding a particular format in the list. You can quickly find a particular format by using the text box at the top of the exporter window as shown in figure 7.8, where formats that include the term VCF are searched for. This search term will remain in place the next time the Export tool is launched. Just delete the text from the search box if you no longer wish only the formats with that term to be listed.

When the desired export format has been identified, click on the button labeled **Open**.

Selecting data for export - part II. A dialog appears, with a name reflecting the format you have chosen. For example if the "Variant Call Format" (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.

In figure 7.9 we show the selection of a variant track for export to VCF format.

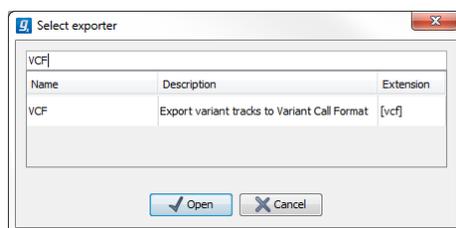


Figure 7.8: The text field has been used to search for VCF format in the Select exporter dialog.

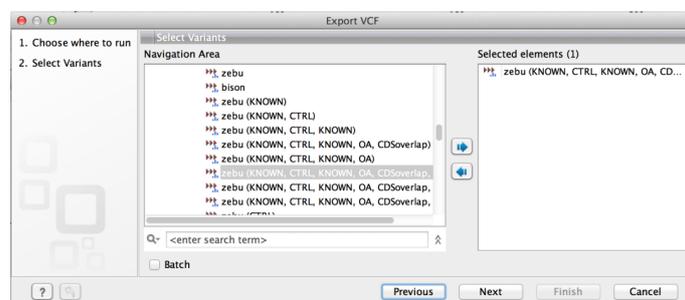


Figure 7.9: The Select exporter dialog. Select the data element(s) to export.

The parameters under **Basic export parameters** and **File name** are offered when exporting to any format.

There may be additional parameters for particular export formats. This is illustrated here with the VCF exporter, where a reference sequence track must be selected (see figure 7.10). To learn more about VCF specific export format, please read section ??.

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.

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

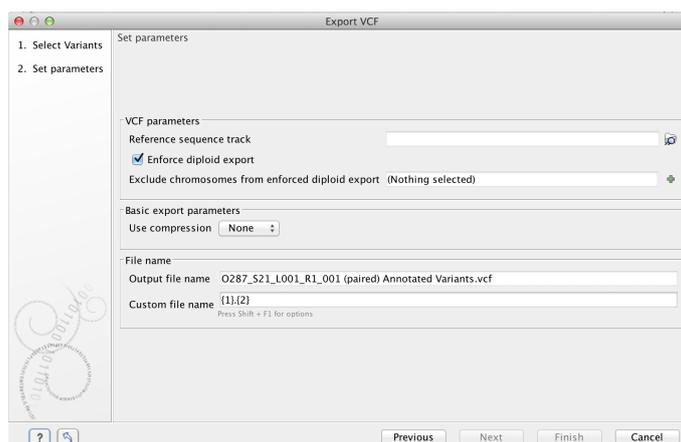


Figure 7.10: Set the export parameters. When exporting in VCF format, a reference sequence track must be selected.

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.

Choosing the exported file name(s) The default setting for the **File name** is to use the original data element name as the basename and the export format as the suffix.

When exporting just one data element, or exporting to a zip file, the desired filename could just be typed in the Custom file name box.

When working with the export of multiple files, using some combination of the terms shown by default in this field and in figure 7.12 are recommended. Clicking in the **Custom file name** field with the mouse and then simultaneously pressing the Shift + F1 keys bring up a list of the available terms that can be included in this field. You can see that "{1}" is the name of the input element and "{2}" is the file name extension. It is possible to change the input file name and the file extension name. We will look at an example to illustrate this:

In this example we would like to change the export file format to .fasta in a situation where .fa was the default format that would be used if you kept the default file extension suggestion ("{2}"). To do this replace "{2}" with ".fasta" in the "Custom file name field". You can see that when changing "{2}" to ".fasta" , the file name extension in the "Output file name" field automatically changes to the new format (see figure 7.11).

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. When working with multiple files, only the name of the first one is shown. Just move the mouse cursor over the name shown in the **Output file name** field to show a listing of the all the filenames.

The last step is to specify the exported data should be saved (figure 7.13).

A note about decimals and Locale settings. When exporting to CSV and tab delimited files, decimal numbers are formatted according to the Locale setting of the Workbench (see section 5.1). If you open the CSV or tab delimited file with spreadsheet software like Excel, you should make sure that both the Workbench and the spreadsheet software are using the same Locale.

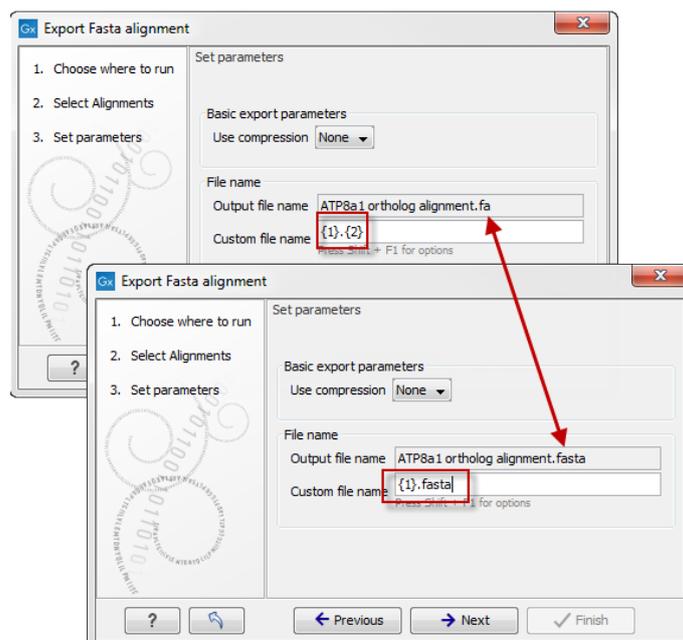


Figure 7.11: The file name extension can be changed by typing in the preferred file name format.

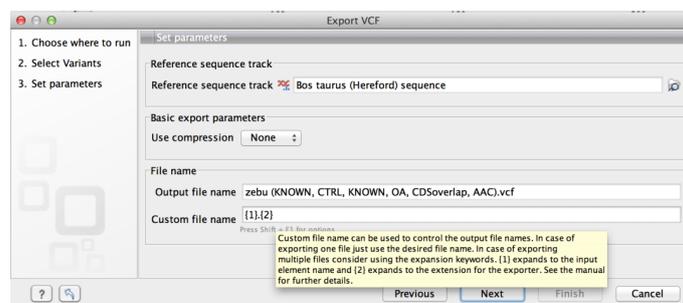


Figure 7.12: Use the custom file name pattern text field to make custom names.

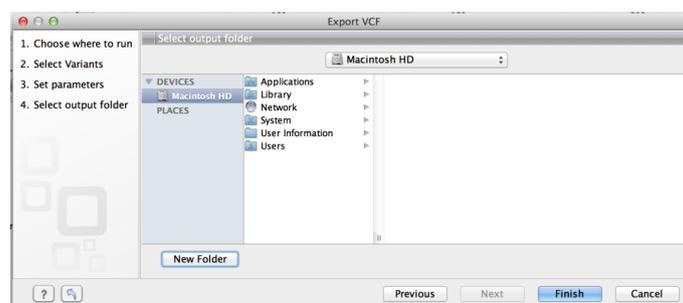


Figure 7.13: Select where to save the exported data.

7.2.1 Export of folders and multiple elements in CLC format

In the list of export formats presented is one called zip format. Choosing this format means that you wish to export the selected data element(s) or folders to a single, compressed CLC format file. This is useful in cases where you wish to exchange data between workbenches or as part of a simple backup procedure.

A zip file generated this way can be imported directly into a CLC Workbench using the Standard Import tool and leaving the import type as Automatic.

Note! When exporting multiple files, the names will be listed in the "Output file name" text field with only the first file name being visible and the rest being substituted by "...", but will appear in a tool tip if you hover the mouse over that field (figure 7.14).

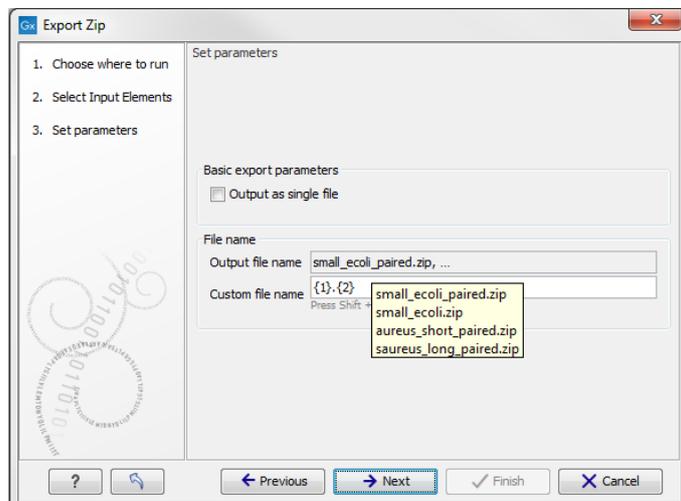


Figure 7.14: The output file names are listed in the "Output file name" text field.

7.2.2 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

In this case, the import type can be left as Automatic.

7.2.3 Export history

Each data element in the Workbench has a history. The history information includes things 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 example, in the case of an alignment, one would see the sequence data used for that alignment listed. You can view this information for each data

element by clicking on the Show History view () at the bottom of the viewing area when a data element is open in the Workbench.

This history information can be exported to a pdf document. To do this:

- (Optional, but preferred) Select the data element (like an alignment) in the **Navigation Area**.
- Start up the exporter tool via the Export button in the toolbar or using the **Export** option under the File menu.
- Select the **History PDF** as the format to export to. See figure 7.15.
- Select the data to export, or confirm the data to export if it was already selected via the **Navigation Area**.
- Edit any parameters of interest, such as the Page Setup details, the output filename(s) and whether or not compression should be applied. See figure 7.16.
- Select where the data should be exported to.
- Click on the button labeled **Finish**.

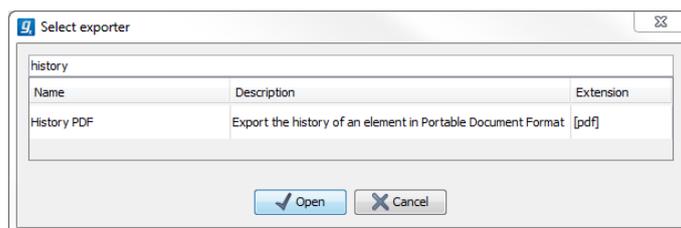


Figure 7.15: Select "History PDF" for exporting the history of an element.

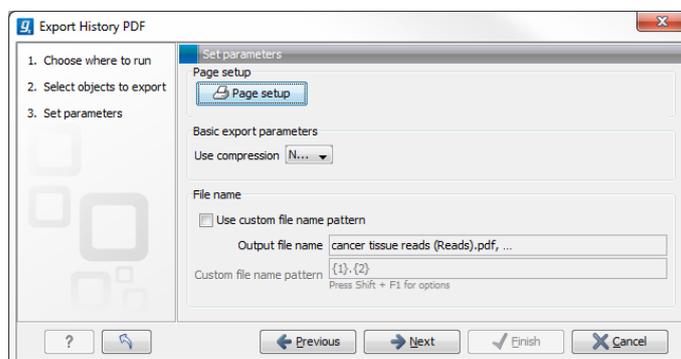


Figure 7.16: When exporting the history in PDF, it is possible to adjust the page setup.

7.2.4 The CLC format

The *CLC Main Workbench* stores bioinformatic data in CLC format. The CLC format contains data, as well as information about that data like history information and comments you may have added.

A given data element in the Workbench can contain different types of data. This is reflected when exporting data, as the choice of different export formats can lead to the extraction of some

parts of that data object rather than others. The part of the data exported reflects the type of data a given format can support. As a simple example, if you export the results of an alignment to Annotation CSV format, you will get just the annotation information. If you exported to Fasta alignment format, you would get the aligned sequences in fasta format, but no annotations.

The CLC format holds all the information for a given data object. Thus if you plan to share the data with colleagues who also have a CLC Workbench or you are communicating with the CLC Support team and you wish to share the data from within the Workbench, exporting to CLC format is usually the best choice as all information associated with that data object in your Workbench will then be available to the other person who imports that data.

If you are planning to share your data with someone who does not have access to a CLC Workbench, then you will wish to export to another data format. Specifically, one they can use with the software they are working with.

7.2.5 Backing up data from the CLC Workbench

Regular backups of your data are advisable.

The data stored in your CLC Workbench is in the areas defined as CLC Data Locations. Whole data locations can be backed up directly (option 1) or, for smaller amounts of data, you could export the selected data elements to a zip file (option 2).

Option 1: Backing up each CLC Data Location

The easiest way for most people to find out where their data is stored is to put the mouse cursor over the top level directories, that is, the ones that have an icon like , in the **Navigation Area** of the Workbench. This brings up a tool tip with the system location for that data location.

To back up all your CLC data, please ensure that all your CLC Data Locations are backed up.

Here, if you needed to recover the data later, you could put add the data folder from backup as a data location in your Workbench. If the original data location is not present, then the data should be usable directly. If the original data location is still present, the Workbench will re-index the (new) data location. For large volumes of data, re-indexing can take some time.

Information about your data locations can also be found in an xml file called model_settings_300.xml. This file is 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 Deployment Manual:

http://www.clcsupport.com/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, certain results files or a whole data location, where that location contains smaller amounts of data. For data that takes up many gigabases of space, this method can be used, but it can be very demanding on space, as well as time.

Select the data items, including any folders, in the Navigation area of your Workbench and choose to export by going to:

File | Export 

and choosing ZIP format.

The zip file created will contain all the data you selected. You can later re-import the zip file into the Workbench by going to:

File | Import (📁)

The only data files associated with the *CLC Main Workbench* not within a specified data location are BLAST databases. It is unusual to back up BLAST databases as they are usually updated relatively frequently and in many cases can be easily re-created from the original files or re-downloaded from public resources. If you do wish to backup your BLAST database files, they can be found in the folders specified in the BLAST Database Manager, which is started by going to:

Toolbox | BLAST | Manage BLAST databases

7.2.6 Export of workflow output

The output from a workflow can be exported by adding one or more workflow export elements (figure 7.17). Multiple elements can be selected by holding down the Ctrl key while clicking on the desired elements.

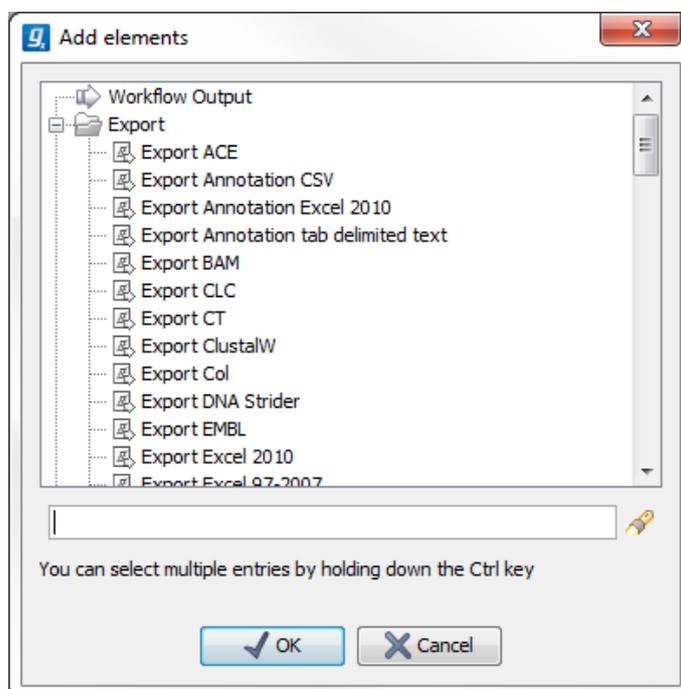


Figure 7.17: Pressing "Add element" enables addition of workflow export elements.

When the workflow has been created, you can set the export parameters and the location to export data to by double clicking on each export element (figure 7.18). Leave fields empty and unlocked if you wish users of the Workflow to enter this information when the Workflow is launched.

7.2.7 Export of tables

Tables can be exported in four different formats; CSV, tab-separated, Excel, or html. When exporting a table in CSV, tab-separated, or Excel format, numbers with many decimals are printed

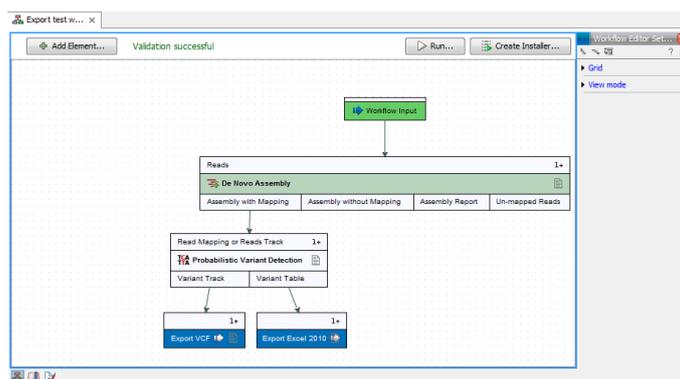


Figure 7.18: A simple workflow with two export elements. The variant track will be exported in VCF format and the variant table in Excel format.

in the exported file with 10 decimals, or in 1.123E-5 format 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 workbench preference settings. When tables are exported in html format from the server or using command line tools, the default number of exported decimals is 3.

The Excel exporters, the CSV and tab delimited exporters, and the HTML exporter have been extended with the ability to export only a sub-set of columns from the object being exported. Uncheck the option "Export all columns" and click next to see a new dialog window in which columns to be exported can be selected (figure 7.19) . The user can choose them one by one or choose a predefined subset:

- All: will select all possible columns.
- None: will clear all preselected column.
- Default: will select the columns preselected by default by the software.
- Last export: will select all windows that were selected during the last export.
- Active editor (only if an active editor is currently open): the columns selected are the same than in the active editor window.

After selecting columns, the user will be directed to the output destination wizard page.

7.3 Export graphics to files

CLC Main Workbench supports export of graphics into a number of formats. This way, the visible output of your work can easily be saved and used in presentations, reports etc. The **Export Graphics** function (📎) is found in the **Toolbar**.

CLC Main Workbench uses a WYSIWYG principle for graphics export: 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 in the program. When you export it, the graphics file will look exactly the same way.

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

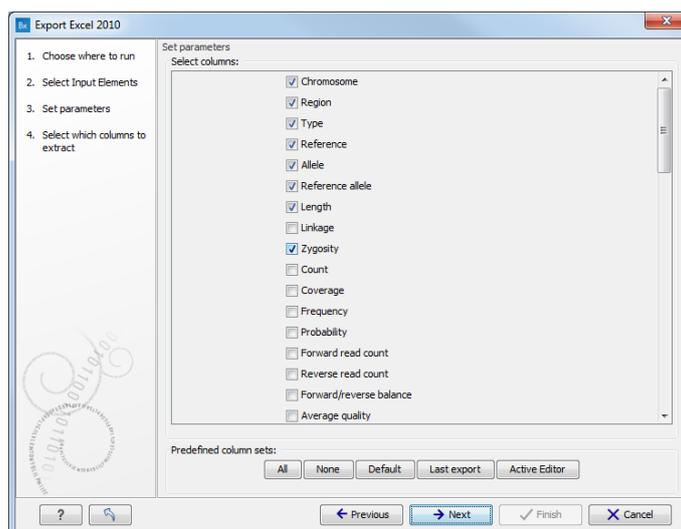


Figure 7.19: Selecting columns to be exported.

select tab of View | Graphics (🖨️) on Toolbar

This will display the dialog shown in figure 7.20.

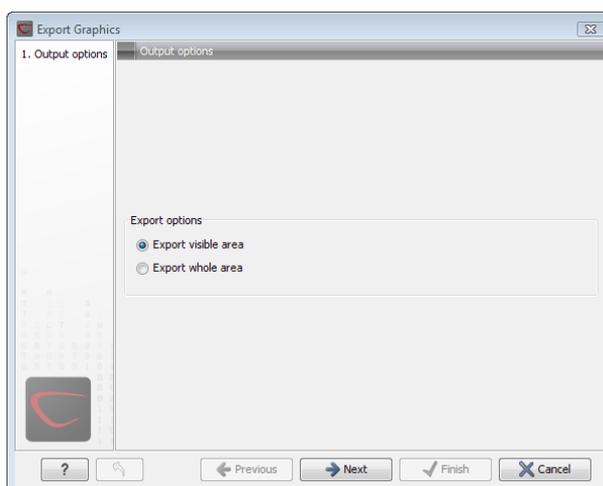


Figure 7.20: Selecting to export whole view or to export only the visible area.

7.3.1 Which part of the view to export

In this dialog you can choose to:

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

These options are available for all views that can be zoomed in and out. In figure 7.21 is a view of a circular sequence which is zoomed in so that you can only see a part of it.

When selecting **Export visible area**, the exported file will only contain the part of the sequence that is *visible* in the view. The result from exporting the view from figure 7.21 and choosing **Export visible area** can be seen in figure 7.22.

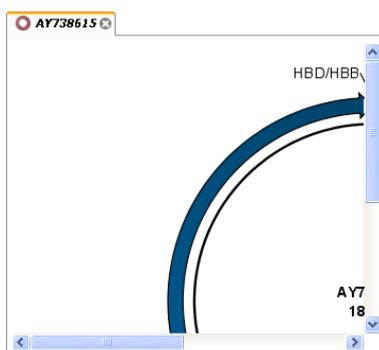


Figure 7.21: A circular sequence as it looks on the screen.

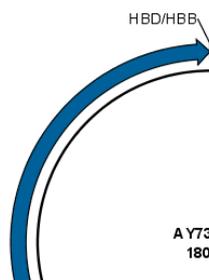


Figure 7.22: The exported graphics file when selecting *Export visible area*.

On the other hand, if you select **Export whole view**, you will get a result that looks like figure 7.23. This means that the graphics file will also include the part of the sequence which is not visible when you have zoomed in.

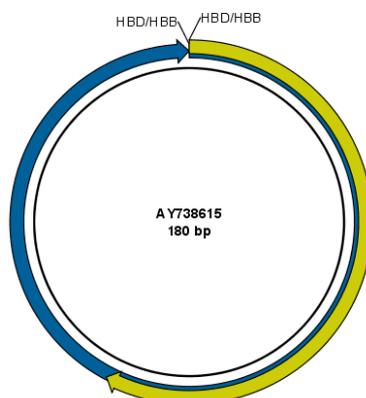


Figure 7.23: The exported graphics file when selecting *Export whole view*. The whole sequence is shown, even though the view is zoomed in on a part of the sequence.

For 3D structures, this first step is omitted and you will always export what is shown in the view (equivalent to selecting **Export visible area**).

Click **Next** when you have chosen which part of the view to export.

7.3.2 Save location and file formats

In this step, you can choose name and save location for the graphics file (see figure 7.24).

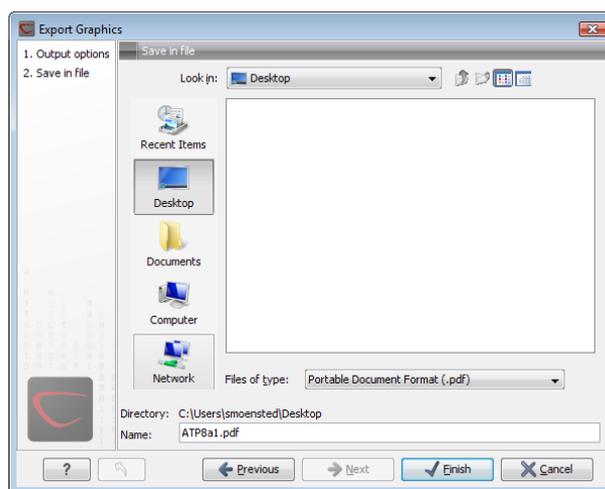


Figure 7.24: Location and name for the graphics file.

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.

Vector graphics

Vector graphic is a collection of shapes. Thus what is stored is e.g. 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 e.g. graphs and reports, but less usable for e.g. 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 like e.g. 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 7.1.4 for more about importing external files into *CLC Main Workbench*.

7.3.3 Graphics export parameters

When you have specified the name and location to save the graphics file, you can either click **Next** or **Finish**. Clicking **Next** allows you to set further parameters for the graphics export, whereas clicking **Finish** will export using the parameters that you have set last time you made a graphics export in that file format (if it is the first time, it will use default parameters).

Parameters for bitmap formats

For bitmap files, clicking **Next** will display the dialog shown in figure 7.25.

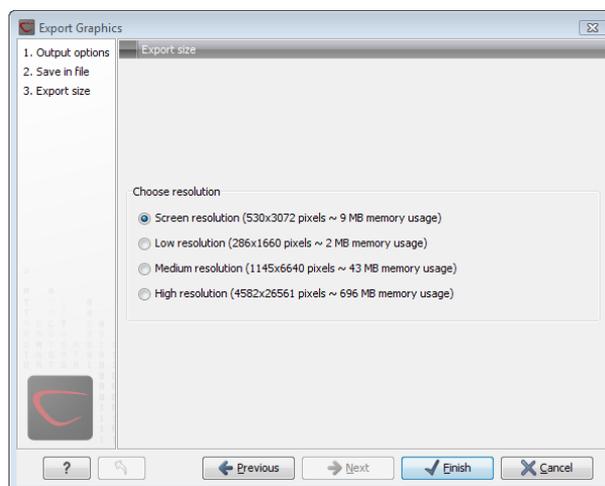


Figure 7.25: Parameters for bitmap formats: size of the graphics file.

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.

Parameters for vector formats

For pdf format, clicking **Next** will display the dialog shown in figure 7.26 (this is only the case if the graphics is using more than one page).

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 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 7.28 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.

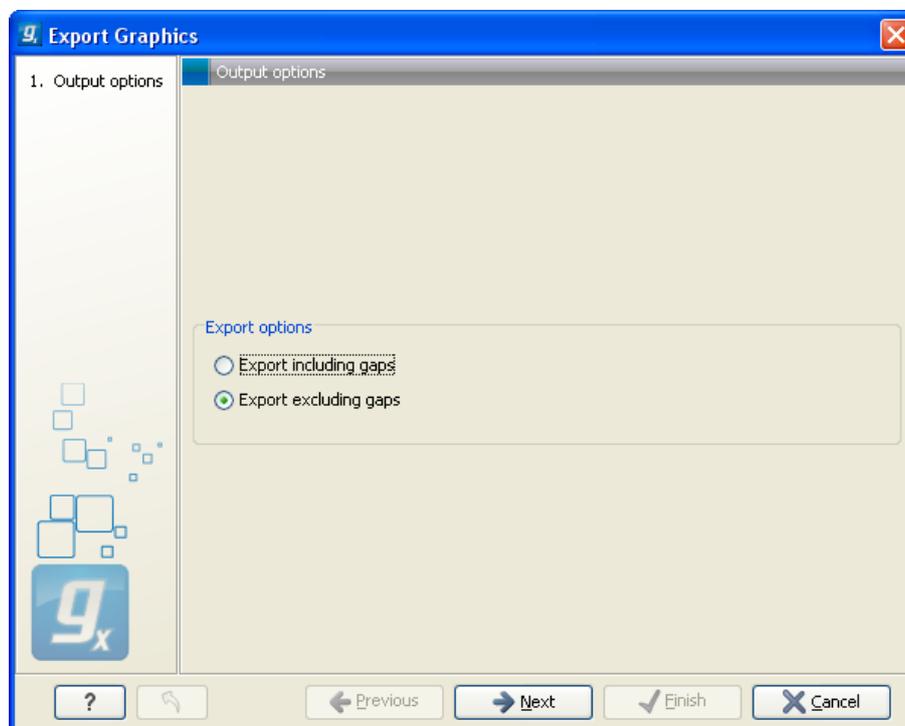


Figure 7.28: Choosing to include data points with gaps

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";  
...
```

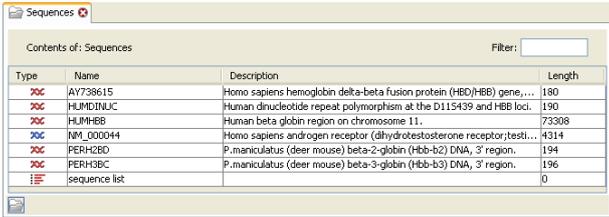
7.5 Copy/paste view output

The content of tables, e.g. in reports, folder lists, and sequence lists can be copy/pasted into different programs, where it can be edited. *CLC Main Workbench* pastes the data in tabulator separated format which is useful if you use programs like Microsoft Word and Excel. There is a huge number of programs in which the copy/paste can be applied. For simplicity, we include one example of the copy/paste function from a **Folder Content** view to Microsoft Excel.

First step is to select the desired elements in the view:

click a line in the Folder Content view | hold Shift-button | press arrow down/up key

See figure 7.29.



| Type | Name | Description | Length |
|------|---------------|--|--------|
| X | AY738615 | Homo sapiens hemoglobin delta-beta fusion protein (HBD/HBB) gene... | 180 |
| X | HLMNDMLC | Human dinucleotide repeat polymorphism at the D11S439 and HBB loci. | 190 |
| X | HLMHBB | Human beta globin region on chromosome 11. | 73308 |
| X | NM_000044 | Homo sapiens androgen receptor (dihydrotestosterone receptor) testi... | 4314 |
| X | PERH2BD | P. maniculatus (deer mouse) beta-2-globin (Hbb-b2) DNA, 3' region. | 194 |
| X | PERH3BC | P. maniculatus (deer mouse) beta-3-globin (Hbb-b3) DNA, 3' region. | 196 |
| | sequence list | | 0 |

Figure 7.29: Selected elements in a Folder Content view.

When the elements are selected, do the following to copy the selected elements:

right-click one of the selected elements | Edit | Copy (📄)

Then:

right-click in the cell A1 | Paste (📄)

The outcome might appear unorganized, but with a few operations the structure of the view in *CLC Main Workbench* can be produced. (Except the icons which are replaced by file references in Excel.)

Note that all tables can also be **Exported** (📄) directly in Excel format.

Chapter 8

History log

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CLC Main Workbench keeps a log of all operations you make in the program. If e.g. you rename a sequence, align sequences, create a phylogenetic tree or translate a sequence, you can always go back and check what you have done. In this way, you are able to document and reproduce previous operations.

This can be useful in several situations: It can be used for documentation purposes, where you can specify exactly how your data has been created and modified. It can also be useful if you return to a project after some time and want to refresh your memory on how the data was created. Also, if you have performed an analysis and you want to reproduce the analysis on another element, you can check the history of the analysis which will give you all parameters you set.

This chapter will describe how to use the **History** functionality of *CLC Main Workbench*.

8.1 Element history

You can view the history of all elements in the **Navigation Area** except files that are opened in other programs (e.g. Word and pdf-files). The history starts when the element appears for the first time in *CLC Main Workbench*. To view the history of an element:

Select the element in the Navigation Area | Show () in the Toolbar | History ()

or **If the element is already open | History () at the bottom left part of the view**

This opens a view that looks like the one in figure 8.1.

When an element's history is opened, the newest change is submitted in the top of the view. The following information is available:

- **Title.** The action that the user performed.
- **Date and time.** Date and time for the operation. The date and time are displayed according

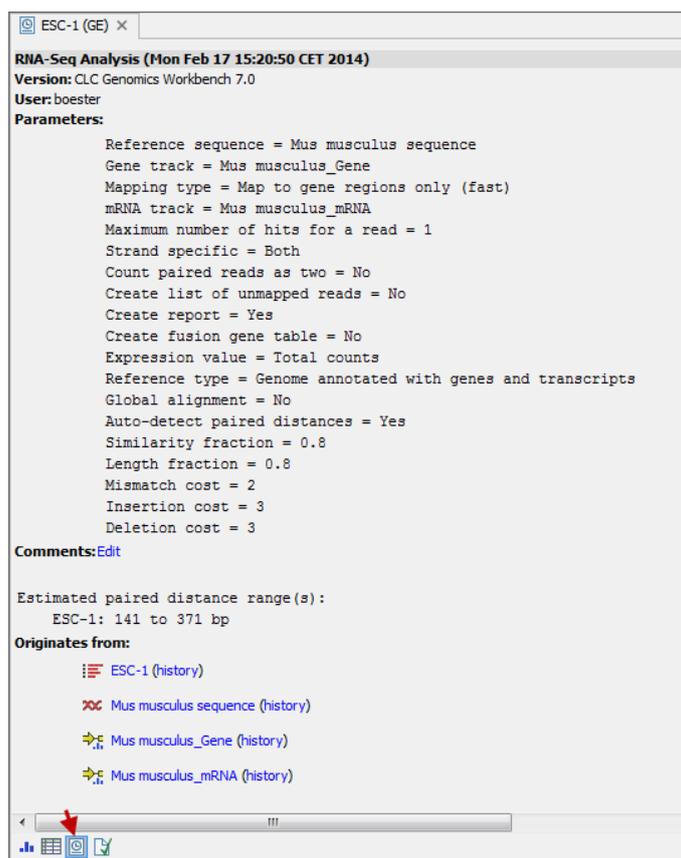


Figure 8.1: An element's history.

to your locale settings (see section 5.1).

- **Version.** The Workbench type and version that has been used.
- **User.** The user who performed the operation. If you import some data created by another person in a CLC Workbench, that person's name will be shown.
- **Parameters.** Details about the action performed. This could be the parameters that were chosen for an analysis.
- **Comments.** By clicking **Edit** you can enter your own comments regarding this entry in the history. These comments are saved.
- **Originates from.** This information is usually shown at the bottom of an element's history. Here, you can see which elements the current element originates from. If you have e.g. created an alignment of three sequences, the three sequences are shown here. Clicking the element selects it in the **Navigation Area**, and clicking the 'history' link opens the element's own history.

8.1.1 Sharing data with history

The history of an element is attached to that element, which means that exporting an element in CLC format (*.clc) will export the history too. In this way, you can share folders and files with others while preserving the history. If an element's history includes source elements (i.e. if

there are elements listed in 'Originates from'), they must also be exported in order to see the full history. Otherwise, the history will have entries named "Element deleted". An easy way to export an element with all its source elements is to use the **Export Dependent Elements** function described in section [7.2.2](#).

The history view can be printed. To do so, click the **Print** icon (). The history can also be exported as a pdf file:

Select the element in the Navigation Area | Export () | in "File of type" choose History PDF | Save

Chapter 9

Batching and result handling

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9.1 Batch processing

Most of the analyses in the **Toolbox** are able to perform the same analysis on several elements in one batch. This means that analyzing large amounts of data is very easily accomplished. As an example, if you use the **Find Binding Sites and Create Fragments**  tool available in *CLC Genomics Workbench* and supply five sequences as shown in figure 9.1, the result table will present an overview of the results for all five sequences.

This is because the input sequences are pooled before running the analysis. If you want individual outputs for each sequence, you would need to run the tool five times, or alternatively use the **Batching mode**.

Batching mode is activated by clicking the **Batch** checkbox in the dialog where the input data is selected. Batching simply means that each data set is run separately, just as if the tool has been run manually for each one. For some analyses, this simply means that each input sequence should be run separately, but in other cases it is desirable to pool sets of files together in one run. This selection of data for a batch run is defined as a **batch unit**.

When batching is selected, the data to be added is the folder containing the data you want to batch. The content of the folder is assigned into batch units based on this concept:

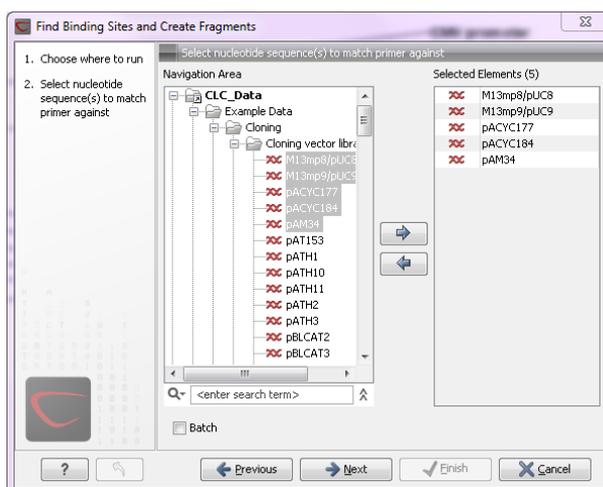


Figure 9.1: Inputting five sequences to Find Binding Sites and Create Fragments.

- All subfolders are treated as individual batch units. This means that if the subfolder contains several input files, they will be pooled as one batch unit. Nested subfolders (i.e. subfolders within the subfolder) are ignored.
- All files that are not in subfolders are treated as individual batch units.

An example of a batch run is shown in figure 9.2.

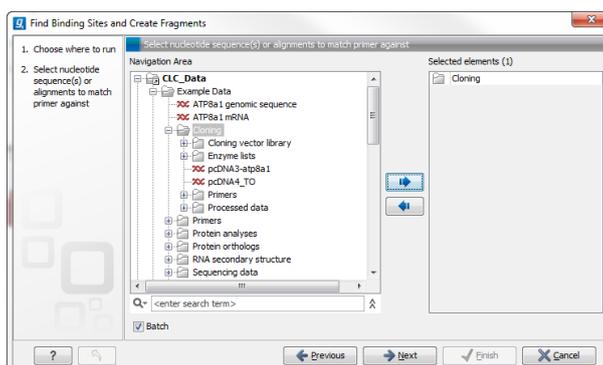


Figure 9.2: The Cloning folder includes both folders and sequences.

The `Cloning` folder that is found in the example data (see section 1.6.2) contains two sequences (X) and four folders (F). If you click **Batch**, only folders can be added to the list of selected elements in the right-hand side of the dialog. To run the contents of the `Cloning` folder in batch, double-click to select it.

When the `Cloning` folder is selected and you click **Next**, a batch overview is shown.

9.1.1 Batch overview

The batch overview lists the batch units to the left and the contents of the selected unit to the right (see figure 9.3).

In this example, the two sequences are defined as separate batch units because they are located at the top level of the `Cloning` folder. There were also four folders in the `Cloning` folder (see

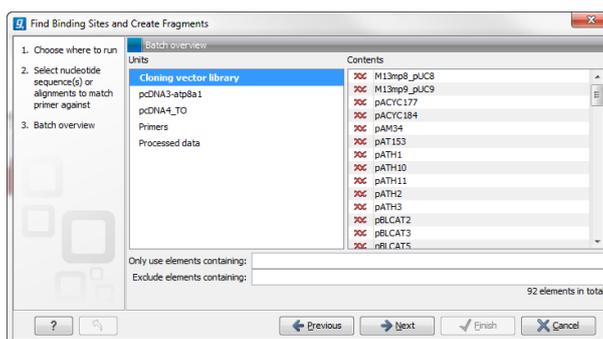


Figure 9.3: Overview of the batch run.

figure 9.2), and three of them are listed as well. This means that the contents of these folders are pooled in one batch run (you can see the contents of the `Cloning vector library` batch run in the panel at the right-hand side of the dialog). The reason why the `Enzyme lists` folder is not listed as a batch unit is that it does not contain any sequences.

In this overview dialog, the Workbench has filtered the data so that only the types of data accepted by the tool is shown (DNA sequences in the example above).

9.1.2 Batch filtering and counting

At the bottom of the dialog shown in figure 9.3, the Workbench counts the number of files that will be run in total (92 in this case). This is counted across all the batch units.

In some situations it is useful to filter the input for the batching based on names. As an example, this could be to include only paired reads for a mapping, by only allowing names where "paired" is part of the name.

This is achieved using the **Only use elements containing** and **Exclude elements containing** text fields. Note that the count is dynamically updated to reflect the number of input files based on the filtering.

If a complete batch unit should be removed, you can select it, right-click and choose **Remove Batch Unit**. You can also remove items from the contents of each batch unit using right-click and **Remove Element**.

9.1.3 Setting parameters for batch runs

For some tools, the subsequent dialogs depend on the input data. In this case, one of the units is specified as parameter prototype and will be used to guide the choices in the dialogs. Per default, this will be the first batch unit (marked in bold), but this can be changed by right-clicking another batch unit and click **Set as Parameter Prototype**.

Note that the Workbench is validating a lot of the input and parameters when running in normal "non-batch" mode. When running in batch, this validation is not performed, and this means that some analyses will fail if combinations of input data and parameters are not right. Therefore batching should only be used when the batch units are very homogenous in terms of the type and size of data.

9.1.4 Running the analysis and organizing the results

At the last dialog before clicking **Finish**, it is only possible to use the **Save** option. When a tool is run in batch mode, the default behavior is to place the result files in the same folder as the input files. In the example shown in figure 9.3, the result of the two single sequences will be placed in the Cloning folder, whereas the results for the `Cloning vector library` and `Processed data` runs will be placed inside these folders.

However, there is an option to save the results in a separate folder structure by checking **Into separate folders**. This will allow you to specify a new save destination, and the *CLC Main Workbench* will create a subfolder for each batch unit where the results are saved.

When the batch run is started, there will be one "master" process representing the overall batch job, and there will then be a separate process for each batch unit. The behavior of this is different between Workbench and Server:

- When running the batch job in the Workbench, only one batch unit is run at a time. So when the first batch unit is done, the second will be started and so on. This is done in order to avoid many parallel analyses that would draw on the same compute resources and slow down the computer.
- When this is run on a CLC Server (see <http://clcbio.com/server>), all the processes are placed in the queue, and the queue is then taking care of distributing the jobs. This means that if the server set-up includes multiple nodes, the jobs can be run in parallel.

If you need to stop the whole batch run, you need to stop the "master" process.

9.2 How to handle results of analyses

This section will explain how results generated from tools in the Toolbox are handled by *CLC Main Workbench*. Note that this also applies to tools not running in batch mode (see above). All the analyses in the **Toolbox** are performed in a step-by-step procedure. First, you select elements for analyses, and then there are a number of steps where you can specify parameters (some of the analyses have no parameters, e.g. when translating DNA to RNA). The final step concerns the handling of the results of the analysis, and it is almost identical for all the analyses so we explain it in this section in general.

In this step, shown in figure 9.4, you have two options:

- **Open**. This will open the result of the analysis in a view. This is the default setting.
- **Save**. This means that the result will not be opened but saved to a folder in the **Navigation Area**. If you select this option, click **Next** and you will see one more step where you can specify where to save the results (see figure 9.5). In this step, you also have the option of creating a new folder or adding a location by clicking the buttons /  at the top of the dialog.

9.2.1 Table outputs

Some analyses also generate a table with results, and for these analyses the last step looks like figure 9.6.

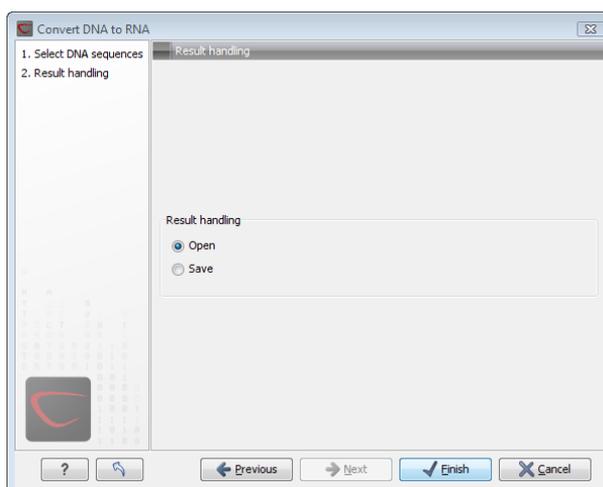


Figure 9.4: The last step of the analyses exemplified by Translate DNA to RNA.

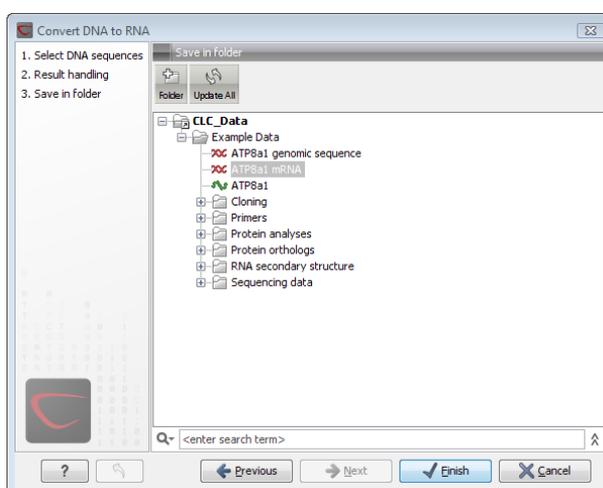


Figure 9.5: Specify a folder for the results of the analysis.

In addition to the **Open** and **Save** options you can also choose whether the result of the analysis should be added as annotations on the sequence or shown on a table. If both options are selected, you will be able to click the results in the table and the corresponding region on the sequence will be selected.

If you choose to add annotations to the sequence, they can be removed afterwards by clicking **Undo** (↶) in the **Toolbar**.

9.2.2 Batch log

For some analyses, there is an extra option in the final step to create a log of the batch process (see e.g. figure 9.6). This log will be created in the beginning of the process and continually updated with information about the results. See an example of a log in figure 9.7. In this example, the log displays information about how many open reading frames were found.

The log will either be saved with the results of the analysis or opened in a view with the results, depending on how you chose to handle the results.

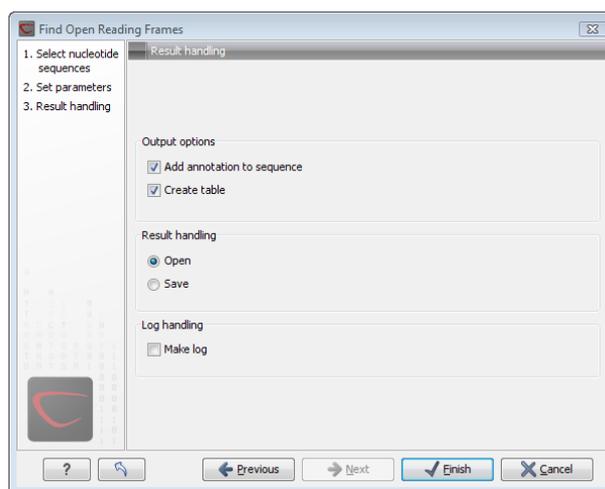


Figure 9.6: Analyses which also generate tables.

| Name | Description | Type | Time |
|----------|-------------------------|------|---------------|
| AY738615 | Found 10 reading frames | | Fri Nov 17... |
| HUMDINUC | Found 5 reading frames | | Fri Nov 17... |
| PERH1BA | Found 5 reading frames | | Fri Nov 17... |
| PERH1BB | Found 7 reading frames | | Fri Nov 17... |
| PERH2BA | Found 4 reading frames | | Fri Nov 17... |
| PERH2BB | Found 7 reading frames | | Fri Nov 17... |
| PERH2BD | Found 8 reading frames | | Fri Nov 17... |
| PERH3BA | Found 3 reading frames | | Fri Nov 17... |
| PERH3BC | Found 7 reading frames | | Fri Nov 17... |

Figure 9.7: An example of a batch log when finding open reading frames.

9.3 Working with tables

Tables are used in a lot of places in the *CLC Main Workbench*. There are some general features for all tables, irrespective of their contents, that are described here.

Figure 9.8 shows an example of a typical table. This is the table result of **Find Open Reading Frames** (🔍). We use this table as an example to illustrate concepts relevant to all kinds of tables.

Table viewing options

Options relevant to the view of the table can be configured in the **Side Panel** on the right.

For example, the columns that can be displayed in the table are listed in the section called **Show column**. The checkboxes allow you to see or hide any of the available columns for that table.

The Column width can be set to **Automatic** or **Manual**. By default, the first time you open a table, it will be set to **Automatic**. The default selected columns are hereby resized to fit the width of the viewing area. When changing to the **Manual** option, column widths will adjust to the actual header size, and each column size can subsequently be adjusted manually. When the table content exceeds the size of the viewing area, a horizontal scroll becomes available for navigation across the columns.

Sorting tables

You can **sort** table according to the values of a particular column by clicking a column header. (Pressing Ctrl - ⌘ on Mac - while you click will refine the existing sorting).

Clicking once will sort in ascending order. A second click will change the order to descending. A

| Sequence | Start | End | Length | Found at strand | Start codon |
|-------------------------|--------|--------|--------|-----------------|-------------|
| ATP8a1 genomic sequence | 18430 | 18747 | 318 | positive | ATG |
| ATP8a1 genomic sequence | 19414 | 19719 | 306 | positive | ATG |
| ATP8a1 genomic sequence | 54871 | 56568 | 1698 | positive | ATG |
| ATP8a1 genomic sequence | 92920 | 93231 | 312 | positive | ATG |
| ATP8a1 genomic sequence | 104521 | 104826 | 306 | positive | ATG |
| ATP8a1 genomic sequence | 136402 | 136773 | 372 | positive | ATG |
| ATP8a1 genomic sequence | 139531 | 139953 | 423 | positive | ATG |
| ATP8a1 genomic sequence | 152548 | 152871 | 324 | positive | ATG |
| ATP8a1 genomic sequence | 186019 | 186384 | 366 | positive | ATG |
| ATP8a1 genomic sequence | 7226 | 7582 | 357 | positive | ATG |
| ATP8a1 genomic sequence | 32537 | 32857 | 321 | positive | ATG |
| ATP8a1 genomic sequence | 54902 | 56518 | 1617 | positive | ATG |
| ATP8a1 genomic sequence | 76304 | 76642 | 339 | positive | ATG |
| ATP8a1 genomic sequence | 102089 | 102427 | 339 | positive | ATG |
| ATP8a1 genomic sequence | 169274 | 169849 | 576 | positive | ATG |
| ATP8a1 genomic sequence | 186452 | 186766 | 315 | positive | ATG |
| ATP8a1 genomic sequence | 54861 | 56594 | 1734 | positive | ATG |
| ATP8a1 genomic sequence | 95214 | 95522 | 309 | positive | ATG |
| ATP8a1 genomic sequence | 125520 | 125828 | 309 | positive | ATG |
| ATP8a1 genomic sequence | 132096 | 132647 | 552 | positive | ATG |
| ATP8a1 genomic sequence | 206397 | 206735 | 339 | positive | ATG |
| ATP8a1 genomic sequence | 222615 | 222920 | 306 | positive | ATG |
| ATP8a1 genomic sequence | 135831 | 136946 | 1116 | negative | ATG |
| ATP8a1 genomic sequence | 56598 | 57182 | 585 | negative | ATG |
| ATP8a1 genomic sequence | 31281 | 31619 | 339 | negative | ATG |
| ATP8a1 genomic sequence | 187208 | 187516 | 309 | negative | ATG |
| ATP8a1 genomic sequence | 132515 | 135790 | 3276 | negative | ATG |
| ATP8a1 genomic sequence | 131945 | 132511 | 567 | negative | ATG |
| ATP8a1 genomic sequence | 46934 | 47242 | 309 | negative | ATG |
| ATP8a1 genomic sequence | 178993 | 179358 | 366 | negative | ATG |
| ATP8a1 genomic sequence | 166075 | 166452 | 378 | negative | ATG |
| ATP8a1 genomic sequence | 160519 | 160878 | 360 | negative | ATG |
| ATP8a1 genomic sequence | 140920 | 141243 | 324 | negative | ATG |
| ATP8a1 genomic sequence | 127864 | 128187 | 324 | negative | ATG |

Figure 9.8: A table showing the results of an open reading frames analysis.

third click will set the order back its original order.

9.3.1 Filtering tables

The final concept to introduce is **Filtering**. The table filter as an advanced and a simple mode. The simple mode is the default and is applied simply by typing text or numbers (see an example in figure 9.9).¹

| 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 9.9: Typing "neg" in the filter in simple mode.

Typing "neg" in the filter will only show the rows where "neg" is part of the text in any of the columns (also the ones that are not shown). The text does not have to be in the beginning, thus "ega" would give the same result. This simple filter works fine for fast, textual and non-complicated filtering and searching.

¹Note that for tables with more than 10000 rows, you have to actually click the **Filter** button for the table to take effect.

However, if you wish to make use of numerical information or make more complex filters, you can switch to the advanced mode by clicking the **Advanced filter** (☑) button. The advanced filter is structure in a different way: First of all, you can have more than one criterion in the filter. Criteria can be added or removed by clicking the **Add** (+) or **Remove** (✖) buttons. At the top, you can choose whether all the criteria should be fulfilled (**Match all**), or if just one of the needs to be fulfilled (**Match any**).

For each filter criterion, you first have to select which column it should apply to. Next, you choose an operator. For numbers, you can choose between:

- = (equal to)
- < (smaller than)
- > (greater than)
- <> (not equal to)
- **abs. value <** (absolute value smaller than. This is useful if it doesn't matter whether the number is negative or positive)
- **abs. value >** (absolute value greater than. This is useful if it doesn't matter whether the number is negative or positive)

Note, that the number of digits displayed is a formatting option which can be set in the View Preferences. The true number may well be (slightly) larger. This behaviour can lead to problems when filtering on exact matches using the = (equal to) operator on numbers. Instead, users are advised to use two filters of inequalities (< (smaller than) and > (greater than)) delimiting a (small) interval around the target value.

For text-based columns, you can choose between:

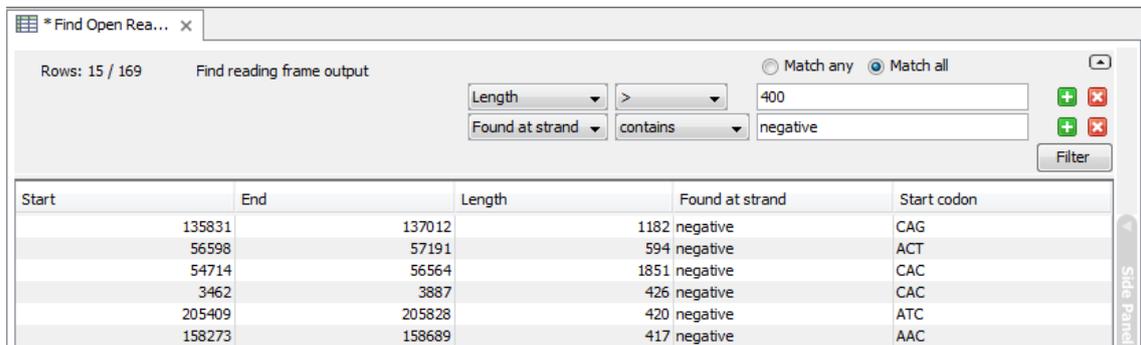
- **starts with** (the text starts with your search term)
- **contains** (the text does not have to be in the beginning)
- **doesn't contain**
- = (the whole text in the table cell has to match, also lower/upper case)
- ≠ (the text in the table cell has to not match)
- **is in list** (The text in the table cell has to match one of the items of the list. Items are separated by comma, semicolon, or space. This filter is case-insensitive)

Once you have chosen an operator, you can enter the text or numerical value to use.

If you wish to reset the filter, simply remove (✖) all the search criteria. Note that the last one will not disappear - it will be reset and allow you to start over.

Figure 9.10 shows an example of an advanced filter which displays the open reading frames larger than 400 that are placed on the negative strand.

Both for the simple and the advanced filter, there is a counter at the upper left corner which tells you the number of rows that pass the filter (91 in figure 9.9 and 15 in figure 9.10).



Rows: 15 / 169 Find reading frame output

Match any Match all

Length > 400

Found at strand contains negative

Filter

| Start | End | Length | Found at strand | Start codon |
|--------|--------|--------|-----------------|-------------|
| 135831 | 137012 | 1182 | negative | CAG |
| 56598 | 57191 | 594 | negative | ACT |
| 54714 | 56564 | 1851 | negative | CAC |
| 3462 | 3887 | 426 | negative | CAC |
| 205409 | 205828 | 420 | negative | ATC |
| 158273 | 158689 | 417 | negative | AAC |

Figure 9.10: The advanced filter showing open reading frames larger than 400 that are placed on the negative strand.

Chapter 10

Workflows

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The *CLC Main Workbench* provides a framework for creating, distributing, installing and running workflows. A workflow consists of a series of connected tools where the output of one tool is used as input for another tool. Once the workflow is set up, it can be installed (either in your own Workbench or it can be shared with colleagues and installed on a server). In that way it becomes possible to analyze lots of samples using the same standard pipeline, the same reference data and the same parameters.

This chapter will first explain how to create a new workflow, and next go into details about the installation and execution of a workflow.

Note that the examples below are using tools from the *CLC Genomics Workbench* that are not necessarily available in the *CLC Main Workbench*. But the principles and workflow framework can be used in the same way with tools from *CLC Main Workbench*.

10.1 Creating a workflow

A workflow can be created by pressing the "Workflows" button (🔗) in the toolbar and then selecting "New Workflow..." (🔗).

Alternatively, a workflow can be created via the menu bar:

File | New | Workflow (🔗)

This will open a new view with a blank screen where a new workflow can be created.

10.1.1 Adding workflow elements

First, click the **Add Element** (+) button at the bottom (or use the shortcut Shift + Alt + E). This will bring up a dialog that lists the elements and tools, which can be added to a workflow (see figure 10.1).

Alternatively elements can be dragged directly from the **Toolbox** into the workflow. Not all elements are workflow enabled. This means that only workflow enabled elements can be dropped in the workflow.

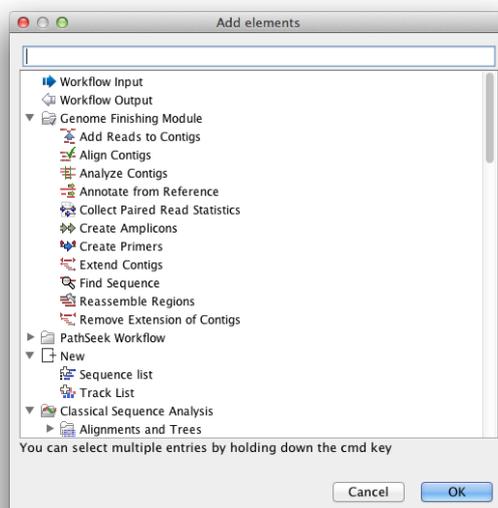


Figure 10.1: Adding elements in the workflow.

Elements that can be selected in the dialog are mostly tools from the Toolbox. However, there are two special elements on the list; the elements that are used for input and output. These two elements are explained in section 10.1.5.

You can select more than one element in the dialog by pressing Ctrl (⌘ on Mac) while selecting. Click OK when you have selected the relevant tools (you can always add more later on).

You will now see the selected elements in the editor (see figure 10.2).

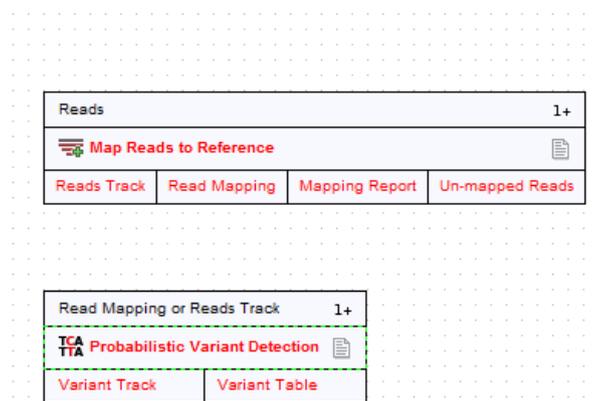


Figure 10.2: Read mapping and variant calling added to the workflow.

Once added, you can move and re-arrange the elements by dragging with the mouse (grab the box with the name of the element).

10.1.2 Configuring workflow elements

Each of the tools can be configured by right-clicking the name of the tool as shown in figure 10.3.

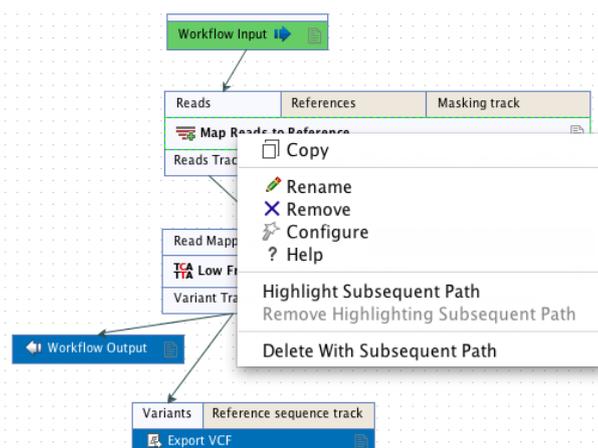


Figure 10.3: Configuring a tool.

The first option you are presented with is the option to **Rename** the element. This is for example useful when you wish to discriminate several copies of the same tool in a workflow. The name of the element is also visible as part of the process description when the workflow is executed. Right click on the tool in the workflow and select "Rename" or click on the tool in the workflow and use the F2 key as a shortcut.

With the **Remove** option, elements can be removed from the workflow. The shortcut Alt + Shift + R removes all elements from the workflow.

You can also **Configure** the tool from the right click menu or alternatively it can be done by double-clicking the element. This will open a dialog with options for setting parameters, selecting reference data, export destination of specified columns, etc. An example is shown in figure 10.4.

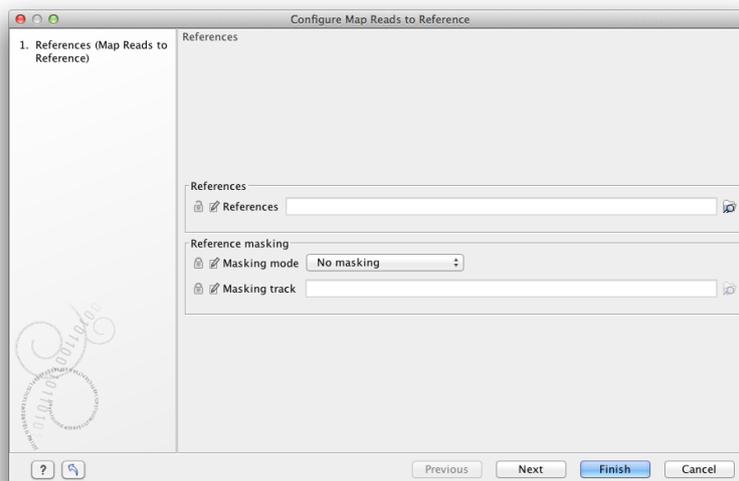


Figure 10.4: Configuring read mapper parameters.

Click through the dialogs using **Next** and press **Finish** when you are done. This will save the parameter settings that will then be applied when the workflow is executed.

You can also change the name of the parameter into something that fits the vocabulary of the users that are intended to execute the workflow. This is done by clicking the edit icon (✎) and entering a new name.

Note that reference data are a bit special. In the example with the read mapper in figure 10.3, you have to define a reference genome. This is done by pointing to data in the **Navigation Area**. If you distribute the workflow and install it in a different setting where this data is not accessible, the installation procedure will involve defining the new reference data to use (e.g. the reference genome sequence for read mapping). This is explained in more detail in section 10.2.

The lock icons in the dialog are used for specifying whether the parameter should be locked and unlocked as described in the next section. Hereby it is possible to lock so the workflow runs with the same parameters like references(s) every time.

Once an element has been configured, the workflow element gets a darker color to make it easy to see which elements have been configured.

With **Highlight Subsequent Path** the path from Name of the tool that was clicked on and further downstream will be highlighted whereas all other elements will be grayed out (figure 10.5). The **Remove Highlighting Subsequent Path** reverts the highlighting to the normal workflow layout.

In some workflows, many elements use the same reference data, and there is a quick way of configuring all these: right-click the empty space and choose **Configure All References**. This will show a dialog listing all reference data needed by the workflow. When you click on the button labeled Finish, only the elements where the 'active' column has been checked will be configured.

Similarly, instead of configuring the various tools individually, the **Configuration Editor** enable specification of all settings, references, masking parameters etc. through a single wizard window (figure 10.6). The Editor is accessed through the (⚙️) icon located lower left corner.

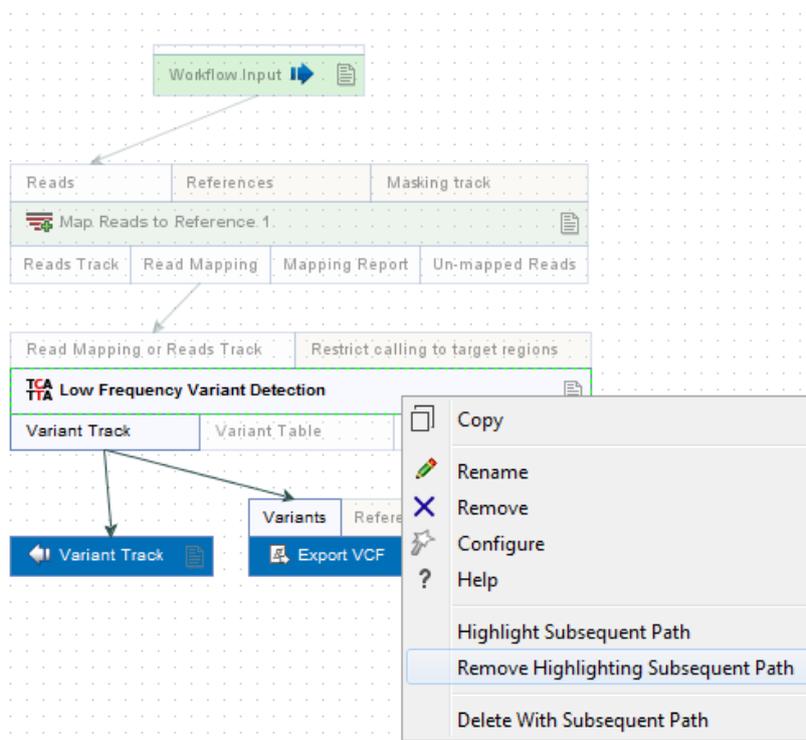


Figure 10.5: Highlight path from the selected tool and downstream.

10.1.3 Locking and unlocking parameters

Figure 10.7 shows the different stages in a workflow.

At the top, the workflow creation is illustrated. Workflow creation is explained above. Next, the workflow can be installed in a Workbench or Server (explained in section 10.2). Subsequently, the workflow can be executed as any other tool in the **Toolbox**.

At the creation step, the workflow creator can specify which parameters should be locked or unlocked. If a parameter is locked, it means that it cannot be changed neither in the installation nor the execution step. The lock icons shown in figure 10.4 specifies whether the parameter should be open or locked.

If the parameter is left open, it is possible to adjust it as part of the installation (see section 10.2). Furthermore, it can also be locked at this stage.

Parameters that are left open both from the workflow creation and installation, will be available for adjustment when the workflow is executed.

Please note that data parameters per default are marked as unlocked. When installing the workflow somewhere else, the connection to the data needs to be re-established, and this is only possible when the parameter is unlocked. Data parameters should only be locked if they should not be set, or if the workflow will only be installed in a setting where there is access to the same data.

10.1.4 Connecting workflow elements

Figure 10.8 explains the different parts of a workflow element.

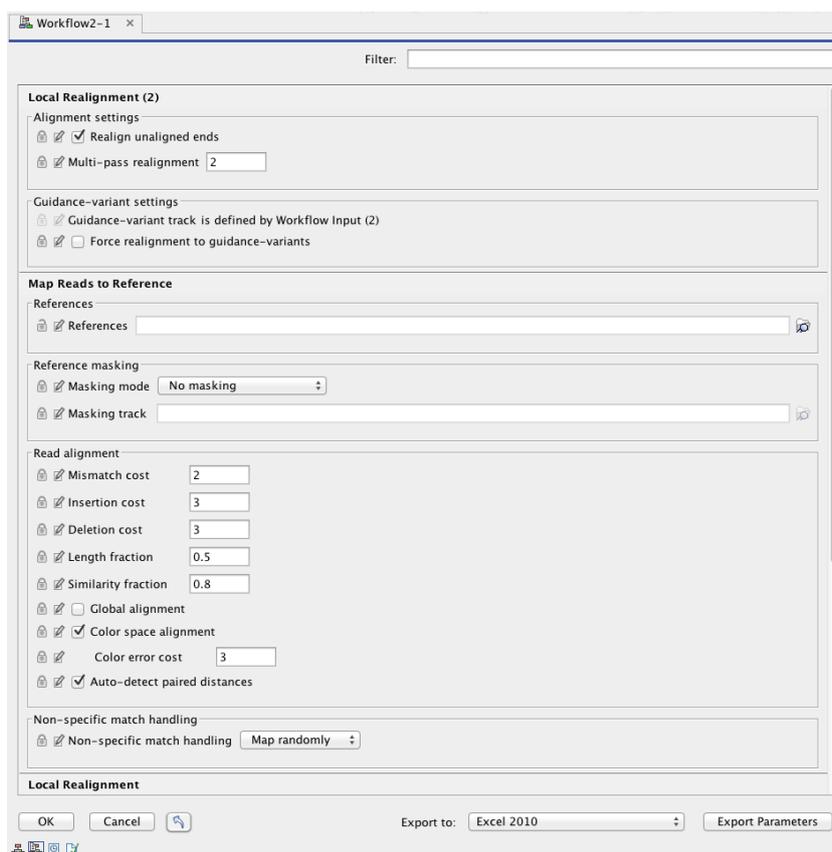


Figure 10.6: The Configuration Editor enable setting of all configurable tools through a single window.

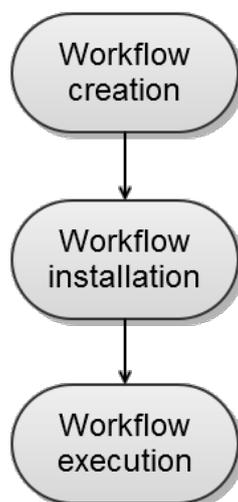


Figure 10.7: The life cycle of a workflow.

At the top of each element a description of the required type of input is found. In the right-hand side, a symbol specifies whether the element accepts multiple incoming connections, e.g. +1 means that more than one output can be connected, and no symbol means that only one can be connected. At the bottom of each element there are a number of small boxes that represent the different kinds of output that is produced. In the example with the read mapper shown in

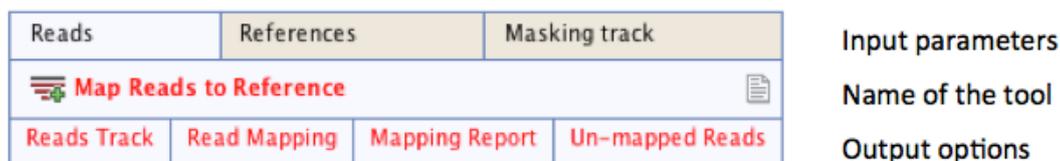


Figure 10.8: A workflow element consists of three parts: input, name of the tool, and output.

figure 10.2, the read mapper is able to produce a reads track, a report etc.

Each of the output boxes can be connected to further analysis in three ways:

- By dragging with the mouse from the output into the input box of the next element. This is shown in figure 10.9. A green border around the box will tell you when the mouse button can be released, and an arrow will connect the two elements (see figure 10.10).
- Right-clicking the output box will display a list of the possible elements that this output could be connected to. You can also right-click the input box of an element and connect this to a matching output of another element.
- Alternatively, if the element to connect to is not already added, you can right-click the output and choose **Add Element to be Connected**. This will bring up the dialog from figure 10.1, but only showing the tools that accepts this particular output. Selecting a tool will both add it to the workflow and connect with the output you selected. You can also add an upstream element of workflow in the same way by right-clicking the input box.



Figure 10.9: Dragging the reads track output with the mouse.

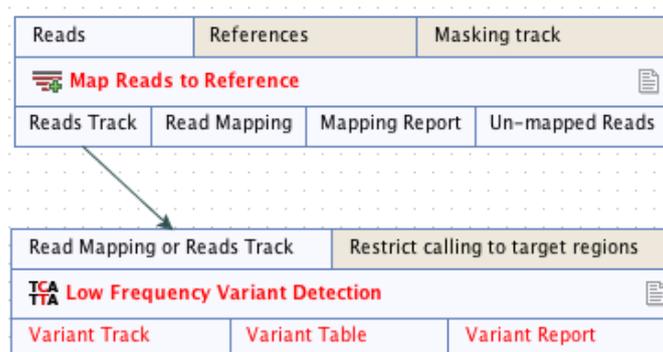


Figure 10.10: The reads track is now used for variant calling.

All the logic of combining output and input is based on matching the type of input. So the read mapper creates a reads track and a report as output. The variant caller accepts reads tracks as input but not mapping reports. This means that you will not be able to connect the mapping report to the variant caller.

Figure 10.11 demonstrates how one tool can receive input from two different sources; 1) a reads track that is the input that hold the data that is to be analyzed (in this case reads that is to be locally realigned), and 2) a parameter that can have different functions depending on the tool that it is connected to (in this case the InDel track is used as a guidance track for the local realignment. In other situations the parameter track could be used for e.g. annotation or could provide a reference sequence).

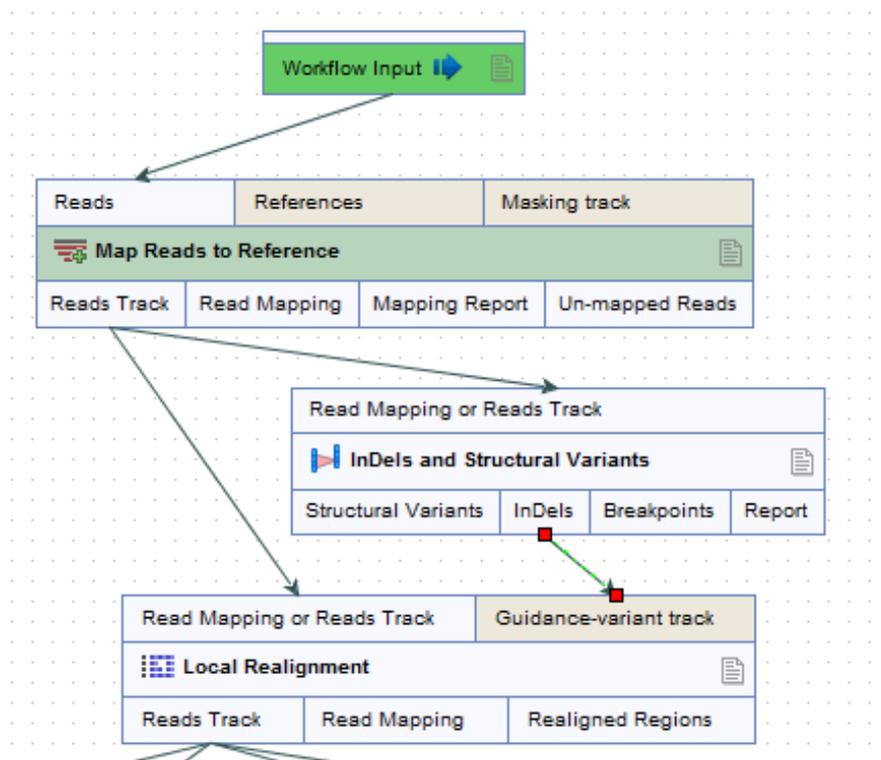


Figure 10.11: A tool can receive input from both the generated output from another tool (in this example a reads track) and from a parameter (in this case InDels detected with the InDels and Structural Variants tool).

10.1.5 Input and output

Besides connecting the elements together, you have to decide what the input and the output of the workflow should be. We will first look at specification of the output, which is done by right-clicking the output box of any tool and selecting **Use as Workflow Output** as shown in figure 10.12.

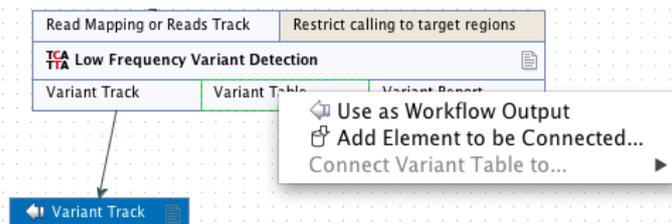


Figure 10.12: Selecting a workflow output.

You can mark several outputs this way throughout the workflow. Note that no intermediate results are saved unless they are marked as workflow output¹.

By double-clicking the output box, you can specify how the result should be named as shown in figure 10.13.

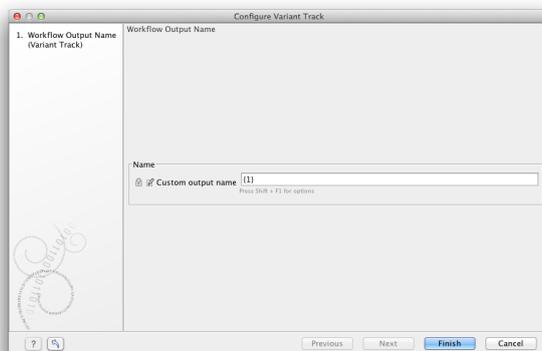


Figure 10.13: Specifying naming of a workflow output.

In this dialog you can enter a name for the output result, and you can make use of two dynamic placeholders for creating this name (press Shift + F1 to get assistance):

- {1} Represents the default name of the result. When running the tool outside of a workflow, this is the name given to the result.
- {2} Represents the name of the workflow input (not the input to this particular tool but the input to the entire workflow).

An example of a meaningful name to a variant track could be {2} variant track as shown in figure 10.14. If your workflow input is named Sample 1, the result would be Sample 1 variant track.

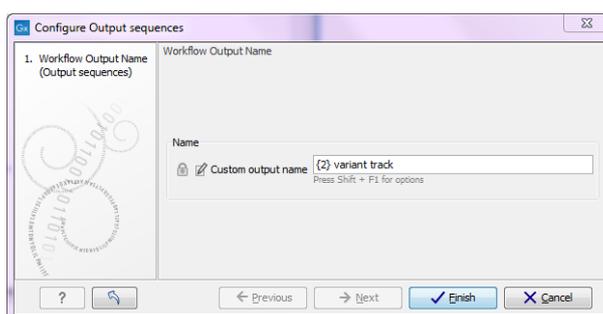


Figure 10.14: Providing a custom name for the result.

In addition to output, you also have to specify where the data should go into the workflow by adding an element called **Workflow Input**. This can be done by:

¹When the workflow is executed, all the intermediate results are indeed saved temporarily but they are automatically deleted when the workflow is completed. If a part of the workflow fails, the intermediate results are not deleted.

- Right-clicking the input box of the first tool and choosing **Connect to Workflow Input**. By dragging from the workflow input box to other input boxes several tools can use the input data directly.
- Pressing the button labeled **Add Element** (or right-click somewhere in the workflow background area and select **Add Element** from the menu that appears). The input box must then be connected to the relevant tool(s) in the workflow by dragging from the Workflow Input box to the "input description" part of the relevant tool(s) in the workflow.

At this point you have only prepared the workflow for receiving input data, but not specified which data to use as input. To be able to do this you must first save the workflow. When this has been done, the button labeled **Run** is enabled which allows you to start executing the workflow. When you click on the button labeled **Run** you will be asked to provide the input data.

Multiple input files can be used when:

- Data is generated within the Workflow
- Data is held within the Workbench
- Data is a combination of the two situations above

Please note that once the multiple input feature is used in a workflow, it is not possible to run the workflow in batch mode. Please also note that the input elements can be renamed, which is useful if you want to be able to discriminate the elements in the process description shown during workflow execution.

You can also choose the order in which inputs will be processed by right clicking on the input parameter box at the top of the element. The feature 'order inputs' is enabled as soon as 2 or more inputs have been connected to the element (see figure 10.15). It will open a small window in which you can move the different inputs up and down (see figure 10.16). From now on there the order of the inputs will be displayed on the branches connecting inputs to elements.

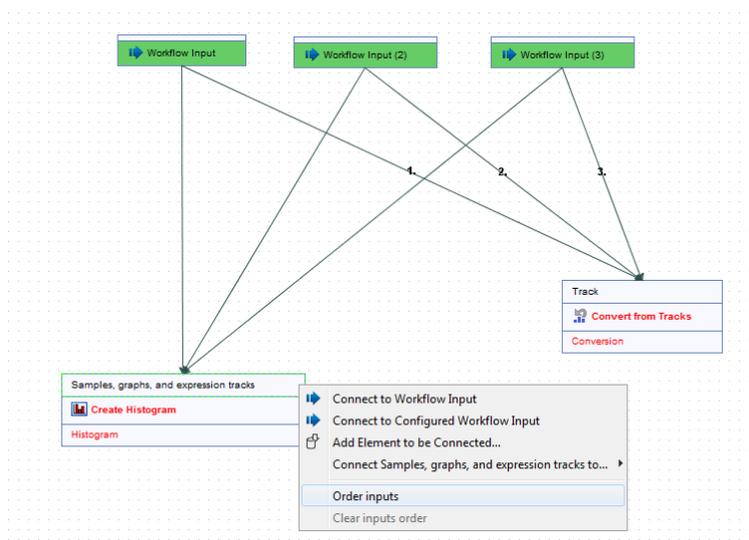


Figure 10.15: Right-click on the input parameter box to see the 'order inputs' function.

The example in figure 10.17 shows how to generate a track list in a workflow. Any track based on a compatible genome can be added to the same track list. This includes reference tracks as

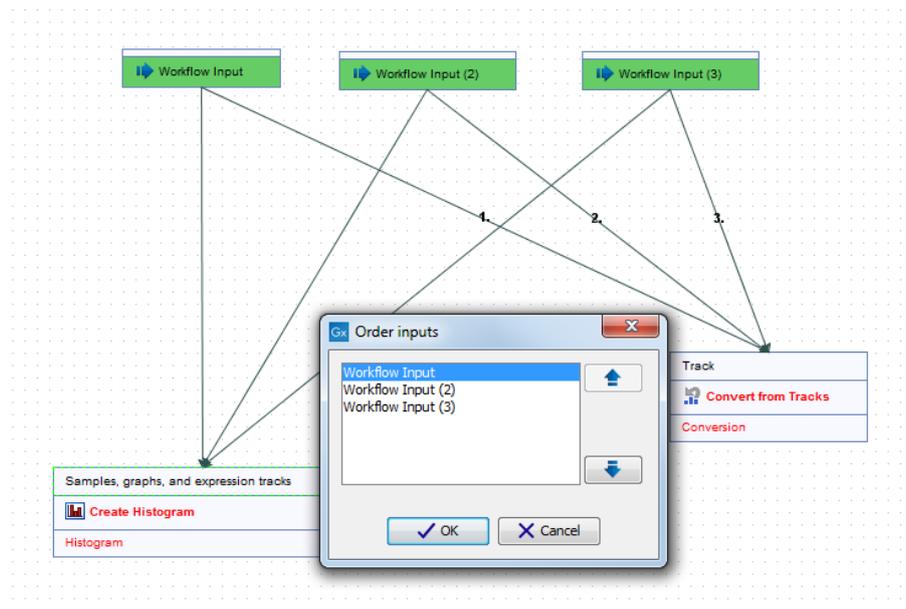


Figure 10.16: Define the inputs order.

well as track results generated by elements of that workflow. In the latter case, only those for which a workflow output element has been configured can be included in a track list.

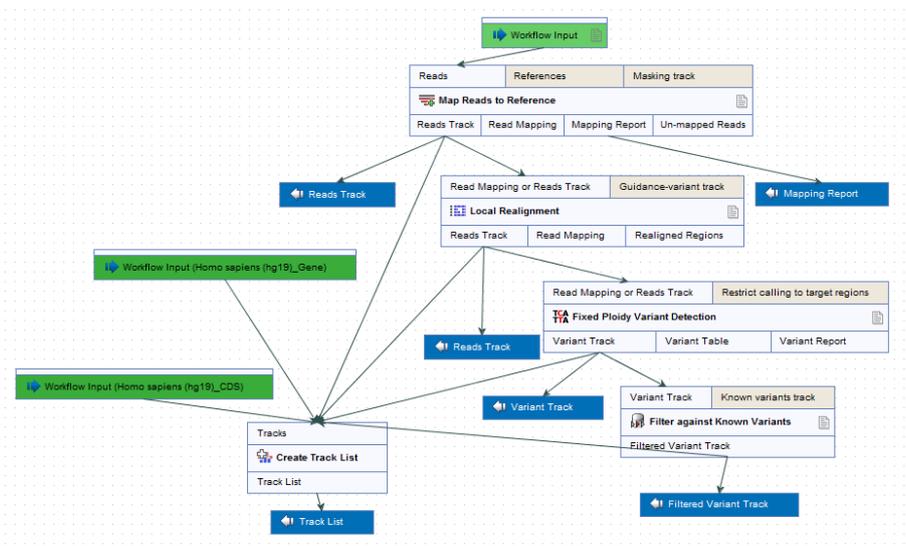


Figure 10.17: Generation of a track list including data generated within the Workflow, as well as data held in the Workbench.

10.1.6 Layout

The workflow layout can be adjusted automatically. Right clicking in the workflow editor will bring up a pop-up menu with the option "Layout". Click on "Layout" to adjust the layout of the selected elements (Figure 10.18). Only elements that have been connected will be adjusted.

Note! The layout can also be adjusted with the quick command Shift + Alt + L.

Note! It is very easy to make an image of the workflow. Simply select the elements in the

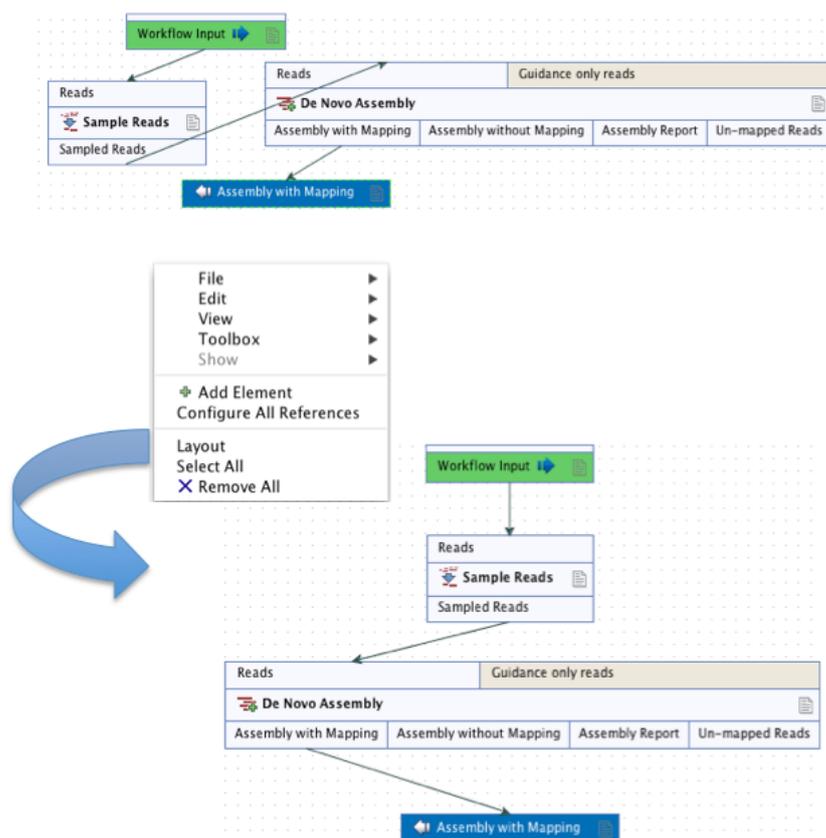


Figure 10.18: A workflow layout can be adjusted automatically with the "Layout" function.

workflow (this can be done pressing Ctrl + A, by dragging the mouse around the workflow while holding down the left mouse button, or by right clicking in the editor and then selecting "Select All"), then press the Copy button in the toolbar (□) or CTRL + C. Press Ctrl + V to paste the image into the wanted destination e.g. an email or a text or presentation program.

10.1.7 Input modifying tools

An input modifying tool is a tool that manipulates its input objects (e.g. adds annotations) without producing a new object. This behavior differs from the rest of the tools and requires special handling in the workflow.

In the workflow an input modifying tool is marked with the symbol (M) (figure 10.19).



Figure 10.19: Input modifying tools are marked with the letter M.

Restrictions apply to workflows that contain input modifying tools. For example, branches are not allowed where one of the elements is a modifying tool (see figure 10.20), as it cannot be

guaranteed which workflow branch will be executed first, which in turn means that different runs can result in production of different objects. Hence, if a workflow is constructed with a branch where one of the succeeding elements is a modifying tool, a message in red letters will appear saying "Branching before a modifying tool can lead to non-deterministic behavior". In such a situation the "Run" and "Create Installer" buttons will be disabled (figure 10.20).

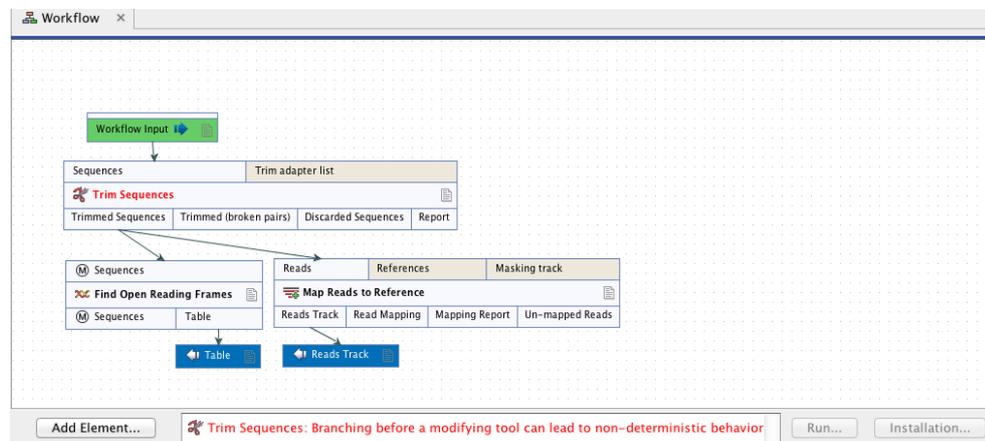


Figure 10.20: A branch containing an input modifying tool is not allowed in a workflow.

The problem can be solved by resolving the branch by putting the elements in the right order (with respect to order of execution). This is shown in figure 10.21 that also shows that the "Run" and "Create Installer" buttons are now enabled. In addition, a message in green letters has appeared saying "Validation successful".

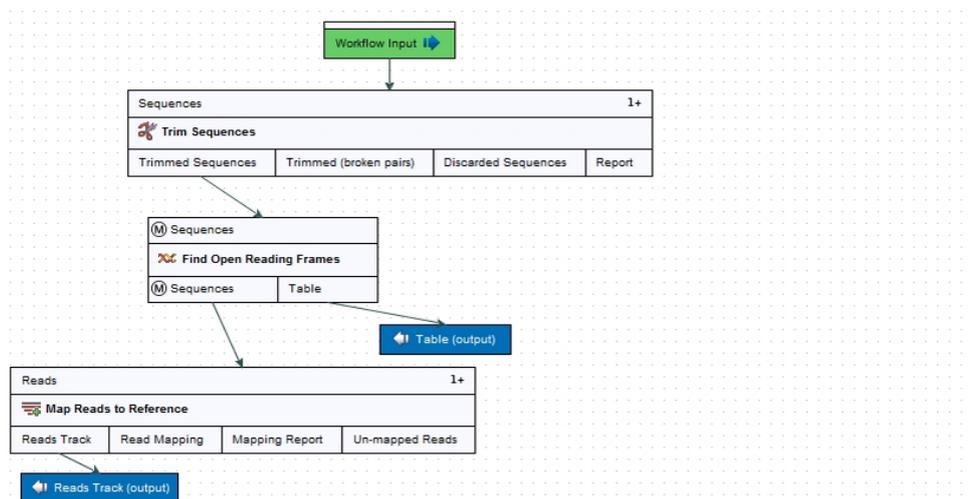


Figure 10.21: A branch containing an input modifying tool has been resolved and the workflow can now be run or installed.

As input modifying tools only modify existing objects without producing a new object, it is not possible to add a workflow output element directly after an input modifying tool (figure 10.22).

A workflow output element can only be added when other tools than input modifying tools are included in the workflow.

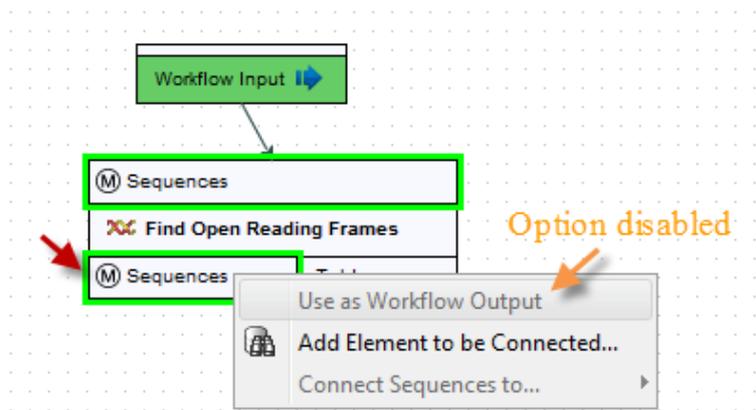


Figure 10.22: A workflow output element cannot be added if the workflow only contains an input modifying tool.

If the situation occur where more input modifying tools are used succeedingly, a copy of the object will be created in addition to using the modified object as input at the next step of the chain (see figure 10.23). In order to see this output you must right click on the output option (marked with a red arrow in figure 10.23) and select "Use as Workflow Output".

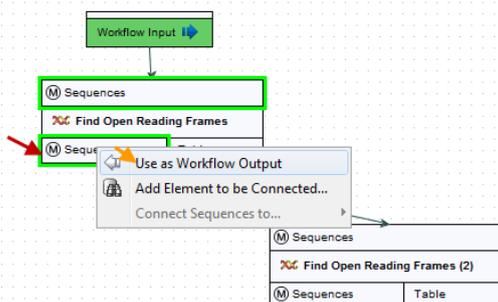


Figure 10.23: A workflow output element can be added when more than one input modifying tool is used succeedingly (despite that the workflow only contains input modifying tools). Select "Use as Workflow Output" to make a copy of the output.

When running a workflow where a workflow output has been added after the first input modifying tool in the chain (see figure 10.24) the output arrow is marked with "copy" to indicate that this is a copy of the result that is used as input at the next level in the chain. When running this workflow you will be able to see the copy of the output from the first input modifying tool in the **Navigation Area** (at the destination that you selected when running the workflow).

10.1.8 Workflow validation

At the bottom of the view, there is a text with a status of the workflow (see figure 10.25). It will inform about the actions you need to take to finalize the workflow.

The validation may contain several lines of text. Scroll the list to see more lines. If one of the errors pertain to a specific element in the workflow, clicking the error will highlight this element.

The following needs to be in place before a workflow can be executed:

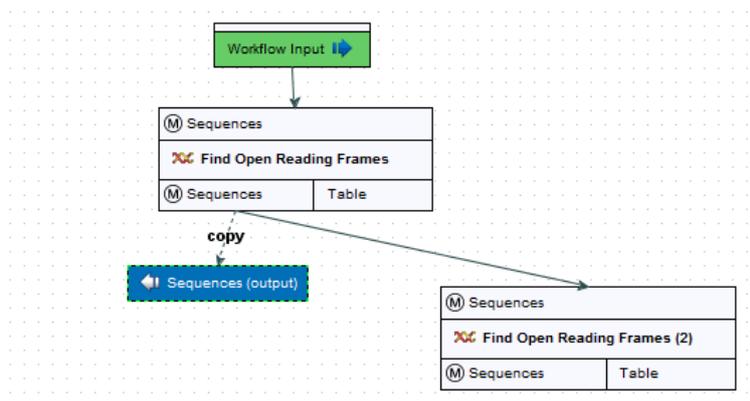


Figure 10.24: A workflow output element can be added when more than one input modifying tool is used succeedingly (despite that the workflow only contains input modifying tools). Note that this output is marked with "copy" to indicate that this is a copy of the result that is used as input at the next level in the chain.



Figure 10.25: A workflow is constantly validated at the bottom of the view.

- All input boxes need to be connected either to the workflow input or to the output of other tools.
- At least one output box from each tool needs to be connected to either a workflow output or to the input box of another tool.
- Additional checks that the workflow is consistent.

Once these conditions are fulfilled, the status will be "Validation successful", the **Run** button is enabled. Clicking this button will enable you to try running a data set through the workflow to test that it produces the expected results. If reference data has not been configured (see section 10.1.2), there will be a dialog asking for this as part of the test run.

10.1.9 Workflow creation helper tools

In the workflow editor **Side Panel**, you will find the following workflow display settings that can be useful to know (figure 10.26):

Grid

- Enable grid You can display a grid and control the spacing and color of the grid. Per default, the grid is shown, and the workflow elements snap to the grid when they are moved around.

View mode

- Collapsed The elements of the workflow can be collapsed to allow a cleaner view and especially for large workflows this can be useful.

- Highlight used elements Ticking **Highlight used elements** (or using the shortcut Alt + Shift + U) will show all elements that are used in the workflow whereas unused elements are grayed out.
- Rulers Vertical and horizontal rules can be visualised
- Auto Layout Ticking **Auto Layout** will ensure rearrangement of elements once new elements are added.
- Connections to background Connecting arrows are shown behind elements. This may ease reading of element names and accessible parameters.

Design

- Round elements Enable rounding of the element boxes.
- Show shadow Shadows of element boxes can be added.
- Configured elements Background color can be customized.
- Input elements Background color can be customized.
- Edges Color of connecting arrows can be customized.

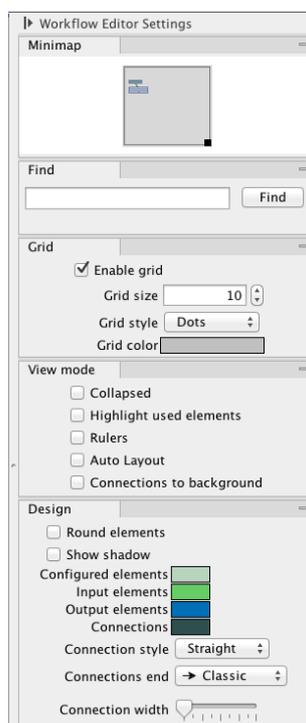


Figure 10.26: The Side Panel of the workflow editor.

10.1.10 Adding to workflows

Additional elements can be added to an already existing workflow by dragging it from the navigation area into the workflow editor and joining more elements as necessary. The new workflow must be saved and validated before it can be executed. Two or more workflows can be joined by dragging and dropping one from the Navigation Area, into another that is already open in the main viewing area. The output of one must be connected to the input of the next to allow the whole workflow to run in one go.

Workflows do not need to be valid to be dragged in to the workflow editor, but they must have been migrated to the current version of the workbench.

10.1.11 Snippets in workflows

When creating a new workflow, you will often have a number of connected elements that are shared between workflows. Instead of building workflows from scratch it is possible to reuse components of an existing workflow. These components are called snippets and can exist of e.g. a read mapper and a variant caller.

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 10.27.

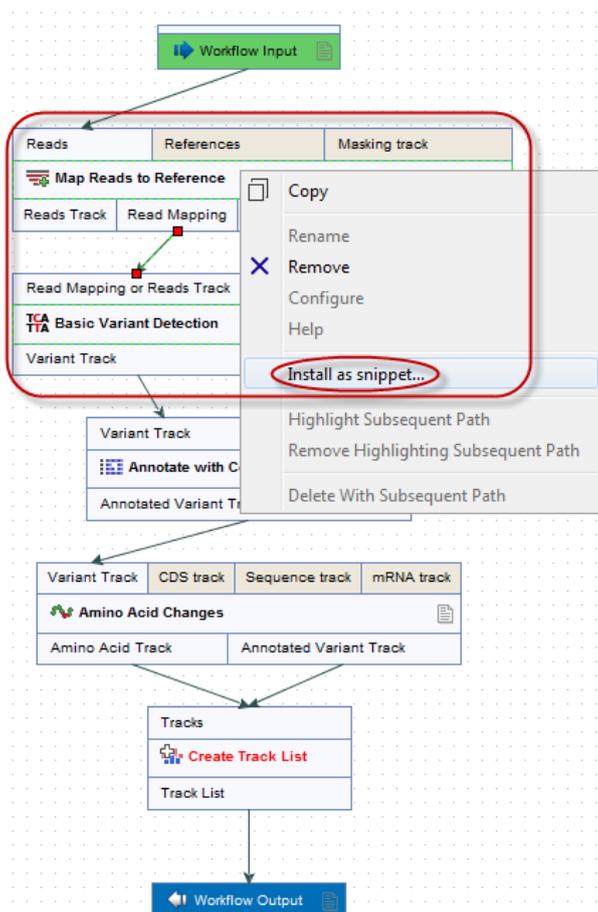


Figure 10.27: The selected elements are highlighted with a red box in this figure. Select "Install as snippet".

When you have clicked on "Install as snippet" the dialog shown in figure 10.28 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 on the button labeled **OK**. This will install your snippet and the installed snippet will now appear in the **Side Panel** under the "Snippets" tab (see figure 10.29)

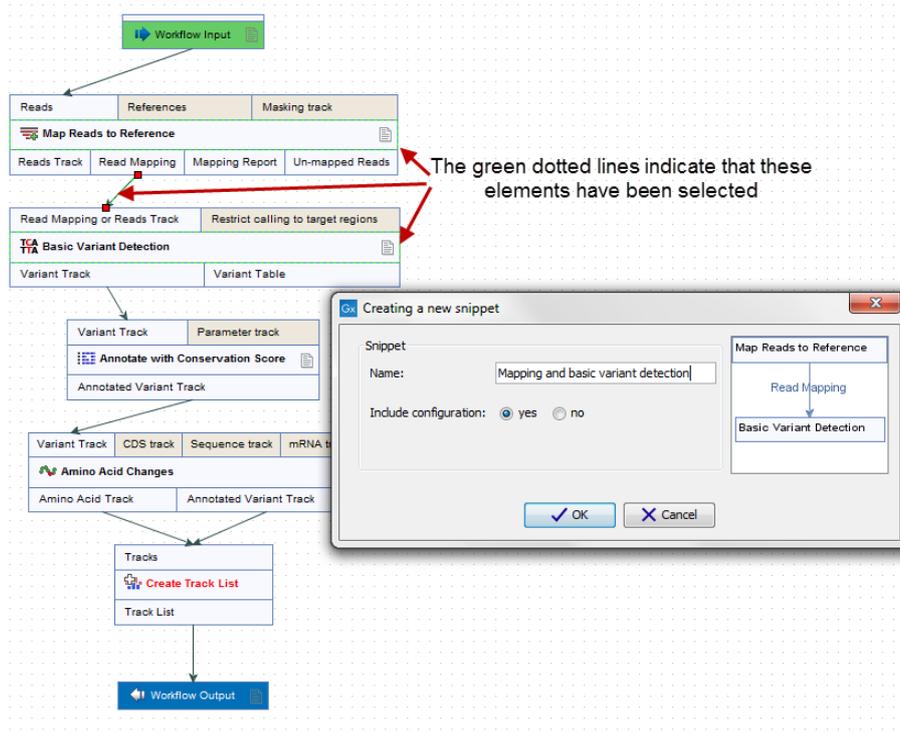


Figure 10.28: In the "Create a new snippet" dialog you can name the snippet and select whether or not you would like to include the configuration. In the right-hand side of the dialog you can see the elements that are included in the snippet.

Right-clicking on the installed snippet in the **Side Panel** will bring up the following options (figure 10.30):

- **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** Migrates the snippet (if migration is required).

If you right-click on the top-level folder you get the options shown in figure 10.31:

- **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)

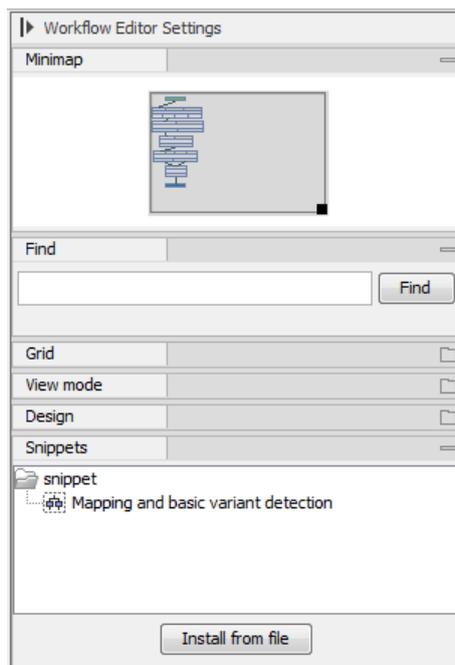


Figure 10.29: When a snippet is installed, it appears in the Side Panel under the "Snippets" tab.

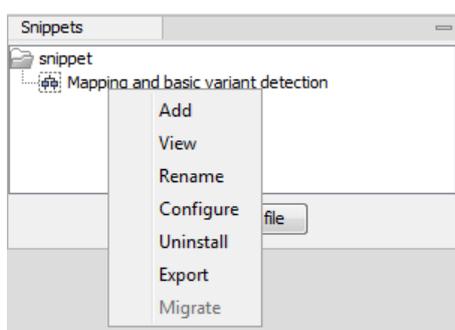


Figure 10.30: Right-clicking on an installed snippet brings up a range of different options.

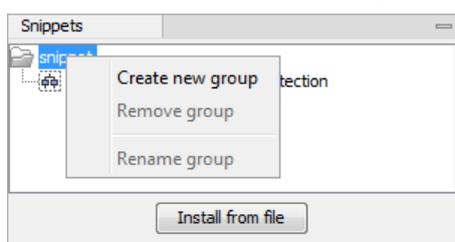


Figure 10.31: Right-clicking on the snippet top-level folder makes it possible to manipulate the groups.

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.

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 elements" option that is shown in figure 10.32.

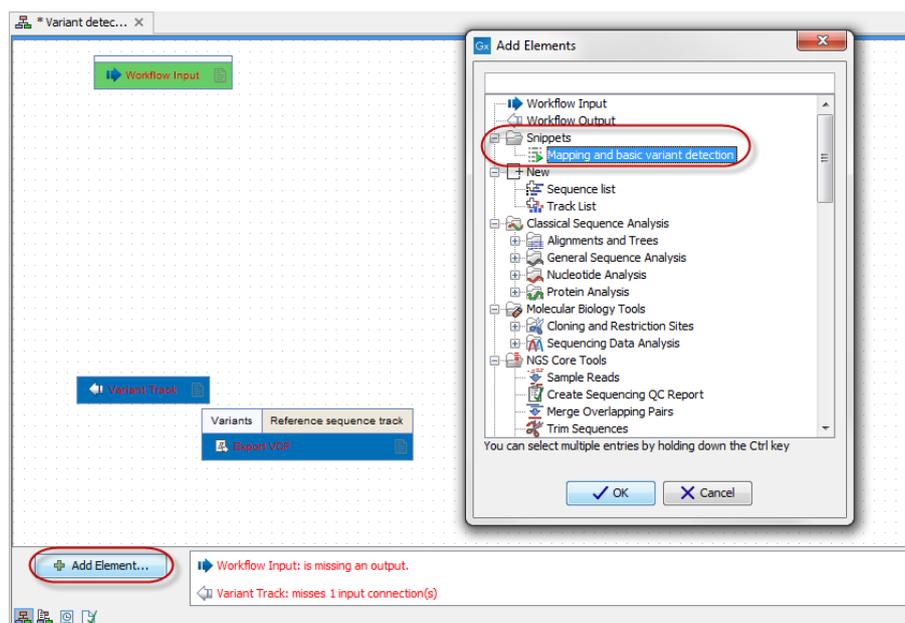


Figure 10.32: Snippets can be added to a workflow in the workflow editor using the 'Add Elements' button found in the lower left corner.

10.1.12 Supported data flows

The current version of the workflow framework supports single-sample workflows. This means processing one sample through various analysis steps. When it comes to comparative analysis, this has to be done outside the workflow.

A typical example that would explain how this works is a trio analysis study where you want to compare variants found in a child with those from the mother and father. For this, you would create a workflow including mapping, variant detection, variant annotation and maybe some quality control. All three samples would be processed through this workflow in batch mode (see section 10.3). At the end, you can manually create a track list with all the relevant tracks (reads and variants) and run the trio analysis tool manually.

Since all the comparative tools are relatively quick, the bulk of the computation work can usually be incorporated into the workflow which can take care of the more tedious parts of the manual work involved.

10.2 Distributing and installing workflows

Once the workflow has been configured, you can use the **Run** button (see section 10.1.8) to process data through the workflow, but the real power of the workflow is its ability to be distributed and installed in the **Toolbox** alongside the other tools that come with the *CLC Main Workbench*, as well as the ability to install the same workflow on a *CLC Genomics Server*. The mechanism for distributing the workflow is a workflow installer file which can be created from the workflow editor and distributed and installed in any Workbench or Server.

10.2.1 Creating a workflow installation file

At the bottom of the workflow editor, click the **Create Installer** button (or use the shortcut Shift + Alt + I) to bring up a dialog where you provide information about the workflow to be distributed (see an example with information from a CLC bio workflow in figure 10.33).

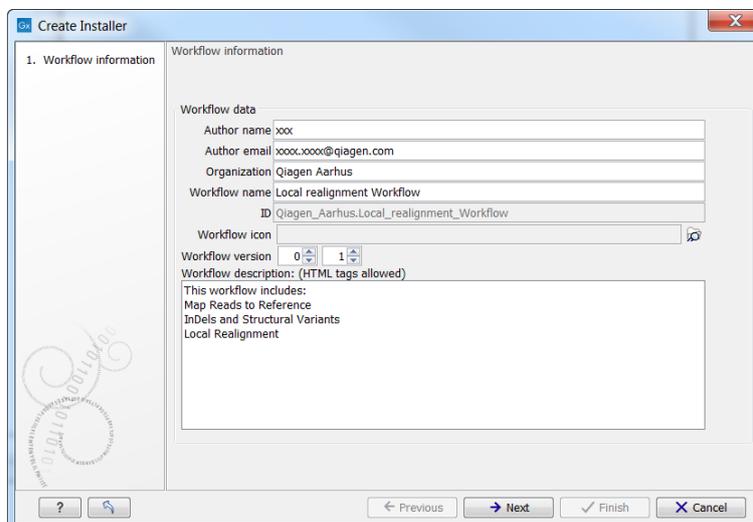
The image shows a 'Create Installer' dialog box with a sidebar on the left containing a circular logo and a search icon. The main area is titled 'Workflow information' and contains several input fields: 'Author name' (xxx), 'Author email' (xxxx.xxxx@qiagen.com), 'Organization' (Qiagen Aarhus), 'Workflow name' (Local realignment Workflow), and 'ID' (Qiagen_Aarhus.Local_realignment_Workflow). There is a 'Workflow icon' field with a file selection icon. Below these is a 'Workflow version' spinner set to 0.1. A 'Workflow description' field contains the text: 'This workflow includes: Map Reads to Reference, InDels and Structural Variants, Local Realignment'. At the bottom are buttons for '?', a search icon, 'Previous', 'Next', 'Finish', and 'Cancel'.

Figure 10.33: Workflow information for the installer.

Author information Providing name, email and organization of the author of the workflow. This will be visible for users installing the workflow and will enable them to look up the source of the workflow any time. The organization name is important because it is part of the workflow id (see more in section 10.2.4)

Workflow name The workflow name is based on the name used when saving the workflow in the **Navigation Area**. The workflow name is essential because it is used as part of the workflow id (see more in section 10.2.4). The workflow name can be changed during the installation of the workflow. This is useful whenever you have a workflow that you would like to use e.g. with small variations. The original workflow name will remain the same in the **Navigation Area** - only the installed workflow will receive the customized name.

ID The final id of the workflow.

Workflow icon An icon can be provided. This will show up in the installation overview and in the **Toolbox** once the workflow is installed. The icon should be a 16 x 16 pixels gif or png file. If the icon is larger, it will automatically be resized to fit 16 x 16 pixels.

Workflow version A major and minor version can be provided.

Include original workflow file This will include the design file to be included with the installer. Once the workflow is installed in a workbench, you can extract the original workflow file and modify it.

Workflow description Provide a textual description of the workflow. This information will be displayed when a user mouses-over the name of the installed Workflow in the Workbench Toolbox, and is also presented in the Description tab for that Workflow in the Manage Workflows tool, described in section 10.2.3. Simple HTML tags are allowed (should be HTML 3.1 compatible, see <http://www.w3.org/TR/REC-html32>).

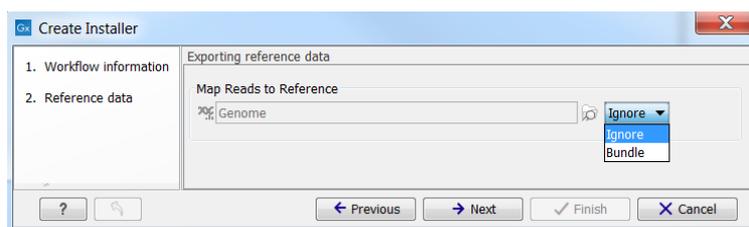


Figure 10.34: Bundling data with the workflow installer.

If you configured any of the workflow elements with data, clicking **Next** will give you the following options (see figure 10.34):

- **Ignore** This will exclude these reference data from the workflow.
- **Bundle** Includes data in the workflow by bundling the reference data with the workflow.
Note! Bundling data should only be used to bundle small data sets with the workflow installer.

Click **Next** and you will be asked to specify where to install the workflow (figure 10.35). You can install your workflow directly on your local computer. If you are logged on a server and are the administrator, the option "Install the workflow on the current server" will be enabled. Finally, you can select to save the workflow as a .cpw file that can be installed on another computer. Click **Finish**. This will install the workflow directly on the selected destination. If you have selected to save the workflow for installation on another computer, you will be asked where to save the file after clicking **Finish**. If you chose to bundle data with your workflow installation, you will be asked for a location to put the bundled data on the workbench. Installing a workflow with bundled data on a server, the data will be put in a folder created in the first writeable persistence location. Should this location not suit your needs, you can always move it afterwards, using the normal persistence operations.

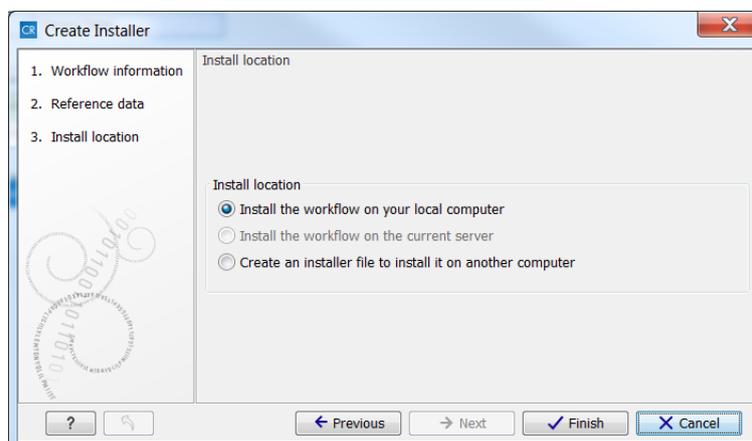


Figure 10.35: Select whether the workflow should be installed on your local computer or on the current server. A third option is to create an installer file (.cpw) that can be installed on another computer.

In cases where an existing workflow, that has already been installed, is modified, the workflow must be reinstalled. This can be done by first saving the workflow after it has been modified and then pressing the **Create Installer** button. Click through the wizard and select whether you wish to install the modified workflow on your local computer or on a server. Press **Finish**. This will

open a pop-up dialog "Workflow is already installed" (figure 10.36) with the option that you can force the installation. This will uninstall the existing workflow and install the modified version of the workflow. **Note!** When forcing installation of the modified workflow, the configuration of the original workflow will be lost.

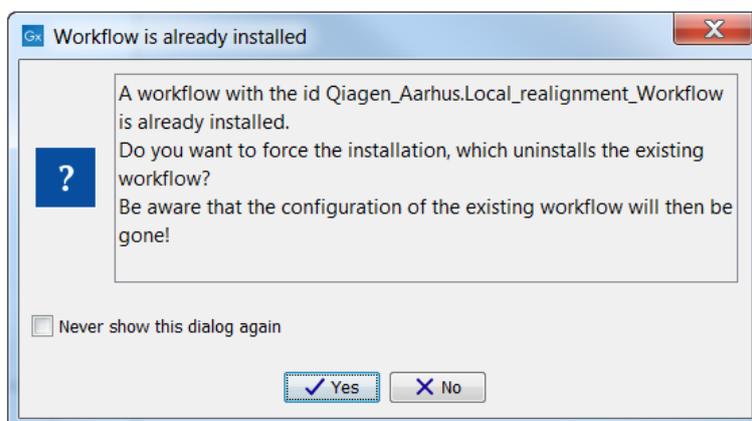


Figure 10.36: Select whether you wish to force the installation of the workflow or keep the original workflow.

10.2.2 Installing a workflow

Workflows are installed in the workflow manager (for information about installing a workflow on the *CLC Genomics Server*, please see the user manual at <http://www.clcbio.com/usermanuals>):

Help | Manage Workflows (⚙️)

or press the "Workflows" button (🔧) in the toolbar and then select "Manage Workflow..." (⚙️).

This will display a dialog listing the installed workflows. To install an existing workflow, click **Install from File** and select a workflow .cpw file .

Once installed, it will appear in the workflow manager as shown in figure 10.37.

If the workflow was bundled with data, installing it on the workbench will ask you for a location to put the bundled data. Installing a workflow with bundled data on a server, the data will be put in a folder created in the first writeable persistence location.

10.2.3 Managing workflows

Workflows can be managed from the workflow manager:

Help | Manage Workflows (⚙️)

or using the "Workflows" button (🔧) in the toolbar and then select "Manage Workflow..." (⚙️).

The workflow manager lists Custom workflows and Ready-to-Use workflows, but the functionalities described below (Configure, Rename, and Uninstall) are only available to custom workflows. You can always create a copy of a Ready-to-Use workflow (by opening the Ready-to-Use workflow and saving a copy in your Navigation Area) to enable the options described below.

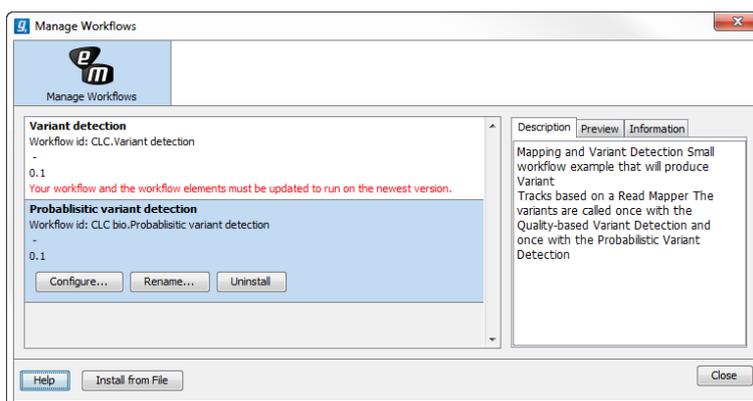


Figure 10.37: Workflows available in the workflow manager. Note the alert on the "Variant detection" workflow, that means that this workflow needs to be updated.

Configure Select the workflow of interest and click on the button labeled Configure. You will be presented with a dialog listing all the reference data that need to be selected. An example is shown in figure 10.38.

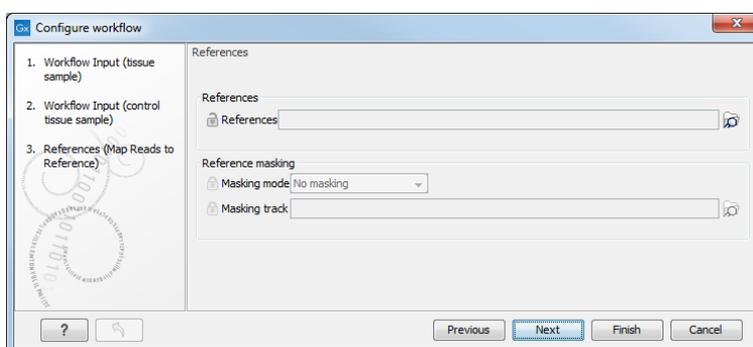


Figure 10.38: Configuring parameters for the workflow.

This dialog also allows you to lock parameters of the workflow (see more about locking in section 10.1.3).

Note that if the workflow is intended to be executed on a server, it is important to select reference data that is located on the server.

Rename In addition to the configuration option, it is also possible to rename the workflow. This will change the name of the workflow in the **Toolbox**. The workflow id (see below) remains the same. To rename an element right click on the element name in the Navigation Area and select "Rename" or click on the F2 button.

Uninstall Use this button to install a workflow.

Description, Preview and Information In the right side of the window, you will find three tabs. **Description** contains the description that was entered when creating the workflow installer (see figure 10.33), the **Preview** shows a graphical representation of the workflow (figure 10.39), and finally you can get **Information** about the workflow (figure 10.40).

The "Information" field (figure 10.40) contains the following:

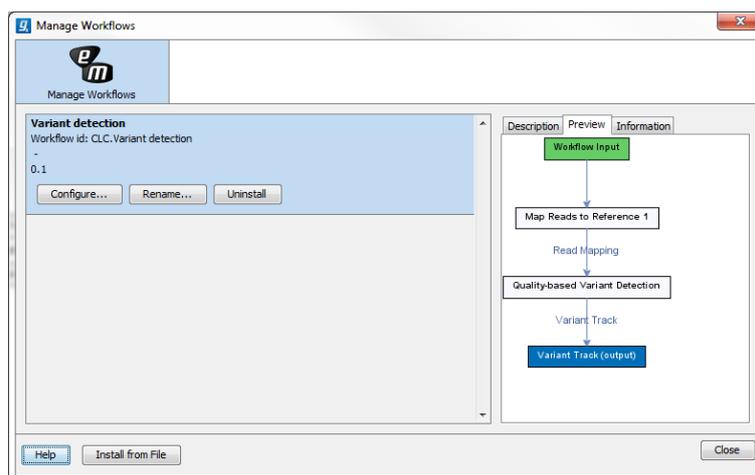


Figure 10.39: Preview of the workflow.

Build id The date followed by the time

Download href The name of the workflow .cpw file

Id The unique id of a workflow, by which the workflow is identified

Major version The major version of the workflow

Minor version The minor version of the workflow

Name Name of workflow

Rev version Revision version. The functionality is activated but currently not in use

Vendor id ID of vendor that has created the workflow

Version <Major version>.<Minor version>

Workbench api version Workbench version

Workflow api version Workflow version (a technical number that can be used for troubleshooting)

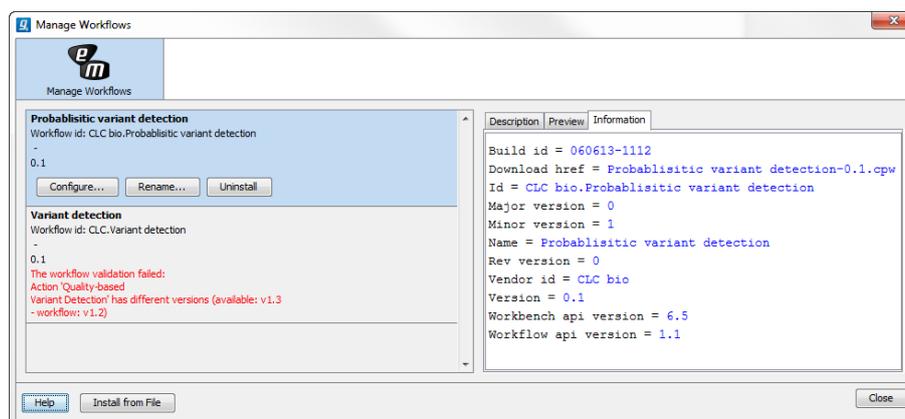


Figure 10.40: With "Manage Workflows" it is possible to configure, rename and uninstall workflows.

10.2.4 Workflow identification and versioning

A workflow has a version. The version is used to make it easy to distribute an improved version of the same workflow. To do this, create a new installer with an incremented version number. In order to install a new and updated version, the old one has to be uninstalled.

The way the *CLC Main Workbench* checks whether a workflow already exists in a previous version is by looking at the workflow id. The id is a combination of the organization name and the name of the workflow itself as it is shown in the dialog shown in figure 10.33. Once installed this information is also available in the workflow manager (in figure 10.37 this is `CLC bio.Simple variant detection and annotation-1.2`).

If you create two different workflows with the same name and using the same organization name when creating the installer, they cannot both be installed.

10.2.5 Automatic update of workflow elements

When new versions of the *CLC Main Workbench* are released, some of the tools that are part of a workflow may change. When this happens, the workflow may no longer be valid. This will happen both to the workflow configurations saved in the **Navigation Area** and the installed workflows.

When a workflow is opened from the **Navigation Area**, an editor will appear, if tools used in the workflow have been updated (see figure 10.41).

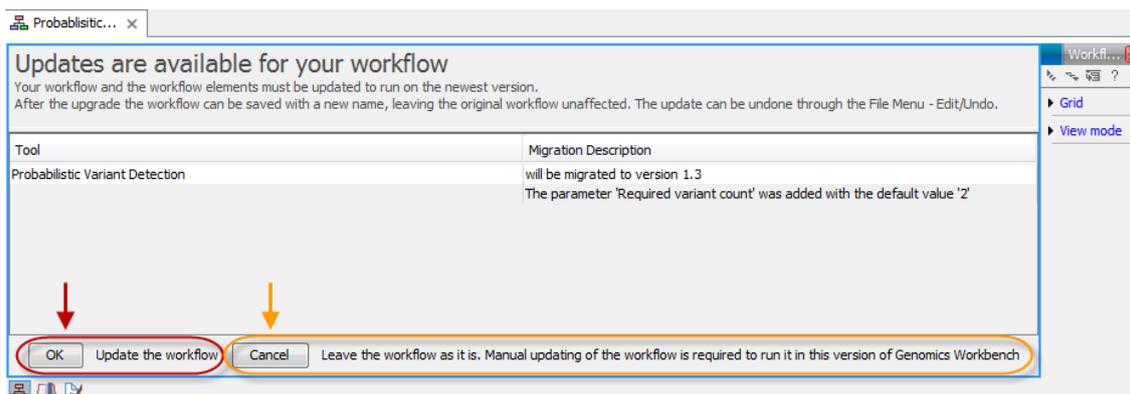


Figure 10.41: When updates are available an editor appears with information about which tools should be updated. Press "OK" to update the workflow. The workflow must be updated to be able to run the workflow on the newest version of the Workbench.

Updating a workflow means that the tools in your workflow is updated with the most recent version of these particular tools. To update your workflow, press the **OK** button at the bottom of the page.

There may be situations where it is important for you to keep the workflow in its original form. This could be the case if you have used a workflow to generate results for a publication. In such cases it may be necessary for you to be able to go back to the original workflow to e.g. repeat an analysis.

You have two options to keep the old workflow:

- If you do not wish to update the workflow at all, press the **Cancel** button. This will keep the workflow unchanged. However, the next time you open the workflow, you will again be

asked whether you wish to update the workflow. Please note that only updated workflows can run on the newest versions of the Workbench.

- Another option is to update the workflow and save the updated workflow with a new name. This will ensure that the old workflow is kept rather than being overwritten.

Note! In cases where new parameters have been added, these will be used with their default settings.

If you have used the toolbar "Workflow" button () and "Manage Workflow..." () to access a specific workflow in order to e.g. change the workflow configuration or are going to use the "Install from File" function, a button labeled "Update..." will appear whenever tools have been changed and the workflow needs to be updated (figure 10.42). When you click the button labeled "Update...", your workflow will be updated and the existing workflow will be overwritten.

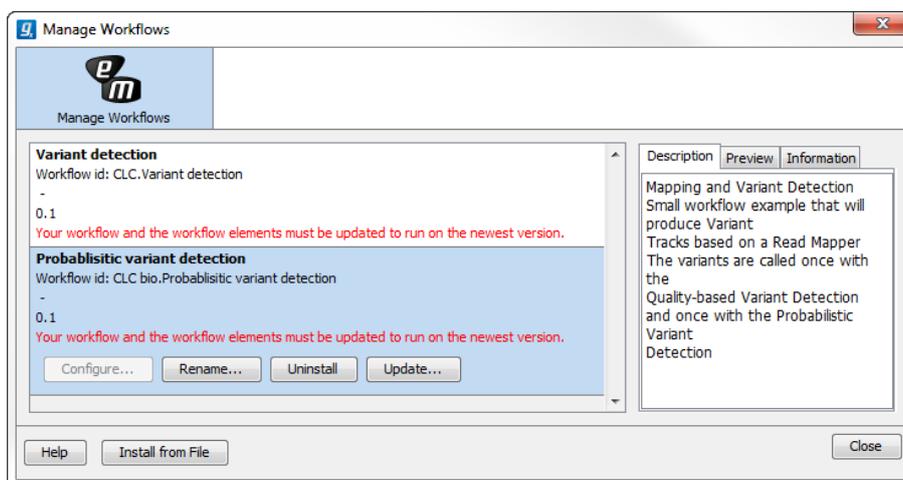


Figure 10.42: Workflow migration.

10.3 Executing a workflow

Once installed and configured, a workflow will appear in the **Toolbox** under **Workflows** (). If an icon was provided with the workflow installer this will also be shown (see figure 10.43).

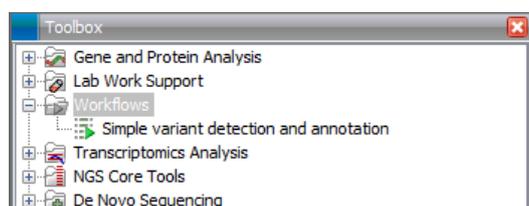


Figure 10.43: A workflow is installed and ready to be used.

The workflow is executed just as any other tool in the **Toolbox** by double-clicking or selecting it in the menu (or with the shortcut Ctrl + Enter). This will open a dialog where you provide input data and with options to run the workflow in batch mode (see section 9.1). In the last page of the dialog, you can preview all the parameters of the workflow, as well as the input data, before clicking "Next" to choose where to save the output, and then "Finish" to execute the workflow.

If you are connected to a *CLC Genomics Server*, you will be presented with the option to run the workflow locally on the Workbench or on the Server. When you are selecting where to run the workflow, you should also see a message should there be any missing configurations. There are more details about running Workflows on the Server in the Server manual (<http://www.clcsupport.com/clcgenomicsserver/current/admin/index.php?manual=Workflows.html>).

When the workflow is started, you can see the log file with detailed information from each step in the process.

If the workflow is not properly configured, you will see that in the dialog when the workflow is started ².

10.4 Open copy of installed workflow

A copy of an installed and configured workflow found in the **Toolbox** under **Workflows** (📁) can be opened in the View Area by clicking once and then right-clicking on the name of the installed workflow in the toolbox (figure 10.44).

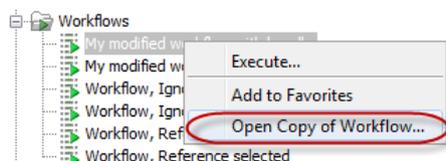


Figure 10.44: A copy of an installed workflow can be opened from the Toolbox. The copied workflow will open in the View Area.

An example of a copy of a workflow that has been opened in the **View Area** is shown in figure 10.45.

²If the workflow uses a tool that is part of a plugin, a missing plugin can also be the reason why the workflow is not enabled. A workflow can also become outdated because the underlying tools have changed since the workflow was created (see section 10.2.4)

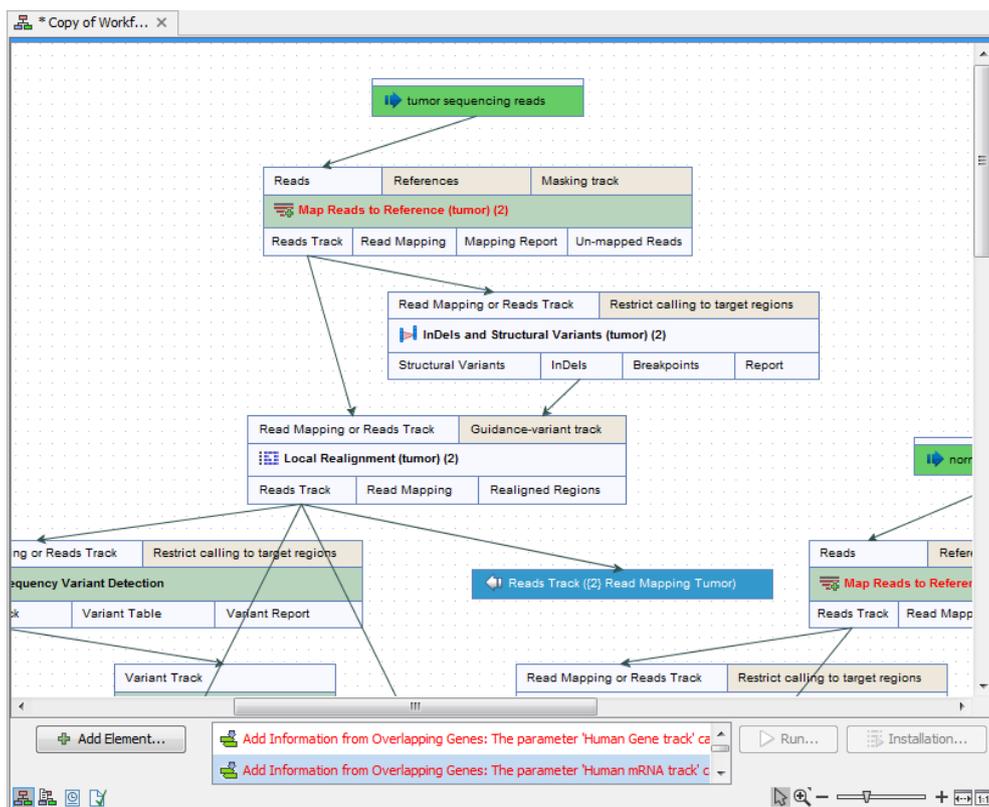


Figure 10.45: A copy of an installed workflow after it has been opened in the View Area.

Chapter 11

Other data types

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11.1 Tracks

The *CLC Main Workbench* supports viewing of data in track format, which is the preferred data format used to visualize and analyze data in the CLC Genomics Workbench and the Biomedical Genomics Workbench. For a description of the track format we refer to <http://www.clcsupport.com/clcgenomicsworkbench/current/index.php?manual=Tracks.html>.

Part III

Bioinformatics

Chapter 12

Viewing and editing sequences

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CLC Main Workbench offers five different ways of viewing and editing single sequences as described in the first five sections of this chapter. Furthermore, this chapter also explains how to create a new sequence and how to gather several sequences in a sequence list.

12.1 View sequence

When you double-click a sequence in the **Navigation Area**, the sequence will open automatically, and you will see the nucleotides or amino acids. The zoom options described in section 3.2 allow

you to e.g. zoom out in order to see more of the sequence in one view. There are a number of options for viewing and editing the sequence which are all described in this section.

All the options described in this section also apply to alignments (further described in section 16.2).

12.1.1 Sequence settings in Side Panel

Each view of a sequence has a **Side Panel** located at the right side of the view (see figure 12.1).

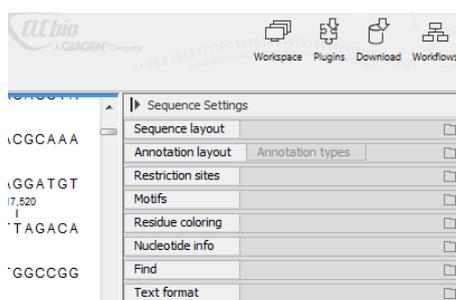


Figure 12.1: Overview of the Side Panel which is always shown to the right of a view.

When you make changes in the **Side Panel** the view of the sequence is instantly updated. To show or hide the **Side Panel**:

select the View | Ctrl + U

or **Click the (▶) at the top right corner of the Side Panel to hide | Click the (◀) to the right to show**

Below, each group of settings will be explained. Some of the preferences are not the same for nucleotide and protein sequences, but the differences will be explained for each group of settings.

Note! When you make changes to the settings in the **Side Panel**, they are not automatically saved when you save the sequence. Click **Save/restore Settings** (⚙) to save the settings (see section 5.6 for more information).

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 [12.3.1](#).

Restriction sites

See section [12.1.2](#).

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 only apply to nucleotide sequences.

- **Color space encoding.** Lets you define a few settings for how the colors should appear.
 - Infer encoding** This is used if you want to display the colors for non-color space sequence (e.g. a reference sequence). The colors are then simply inferred from the sequence.
 - Show corrections** This is only relevant for mapping results - it will show where the mapping process has detected color errors. An example of a color error is shown in figure **??**.

Hide unaligned This option determines whether color for the unaligned ends of reads should be displayed. It also controls whether colors should be shown for gaps. The idea behind this is that these color dots will interfere with the color alignment, so it is possible to turn them off.

- **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 [12.1.3](#).
 - * **+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 [19.5](#).
 - **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.
- **Trace data.** See section [21.1](#).
- **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 score is displayed on a graph (Learn how to export the data behind the graph in section [7.4](#)).
 - * **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.
- **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 level is displayed on a graph (Learn how to export the data behind the graph in section 7.4).
 - * **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.
- **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 20.5.2.

- **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.** Searches 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. Below this option you can choose to search for translations as well. Sequences annotated with coding regions often have the translation specified which can lead to undesired results.
- **Position search.** Finds 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 and end number (see section 12.3.2). 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.** Searches for sequence names. This is useful for searching sequence lists, mapping results and BLAST results.

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.** Five different sizes.
- **Font.** Shows a list of Fonts available on your computer.
- **Bold residues.** Makes the residues bold.

12.1.2 Restriction sites in the Side Panel

Please see section 23.3.1.

12.1.3 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 several parts at the same time (multiselect)

You can select several 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 [12.1.2.](#))

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 | Toolbox | 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.

12.1.4 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 23.1.4.

Note When editing annotated nucleotide sequences, the annotation content is not updated automatically (but its position is). Please refer to section 12.3.3 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.

12.1.5 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 12.2 is an example of three regions with separate colors.

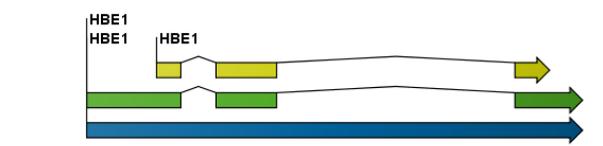


Figure 12.2: Three regions on a human beta globin DNA sequence (HUMHBB).

Figure 12.3 shows an artificial sequence with all the different kinds of regions.

12.2 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" ()

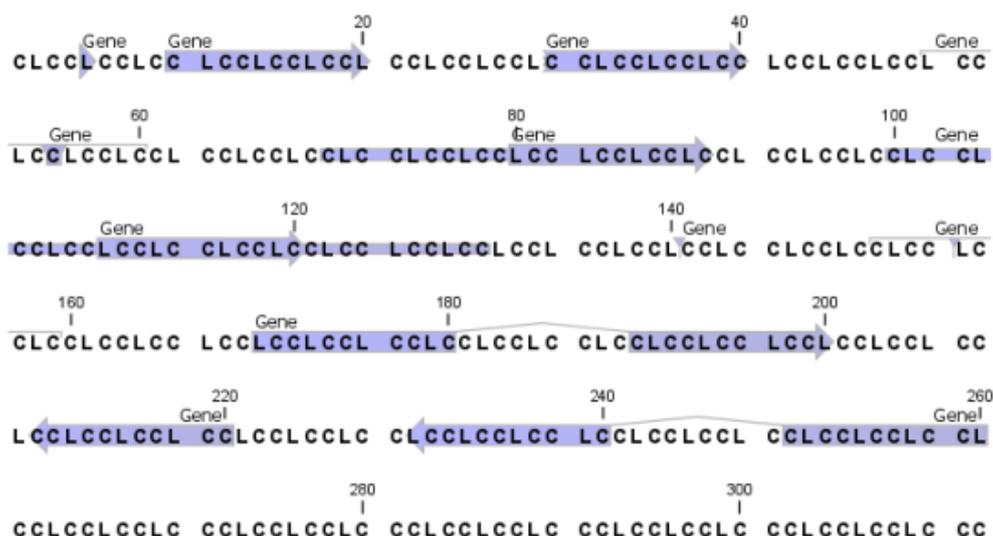


Figure 12.3: *Region #1: A single residue, Region #2: A range of residues including both endpoints, Region #3: A range of residues starting somewhere before 30 and continuing up to and including 40, Region #4: A single residue somewhere between 50 and 60 inclusive, Region #5: A range of residues beginning 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.*

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 12.4.

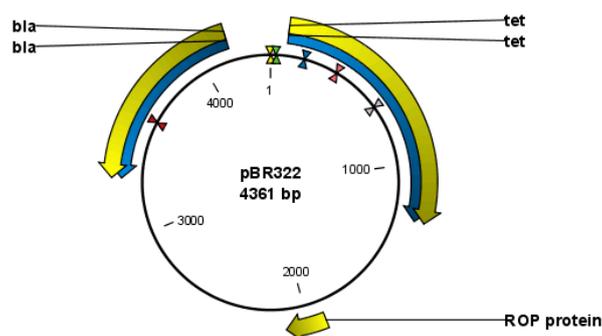


Figure 12.4: *A molecule shown in a circular view.*

This view of the sequence shares some of the properties of the linear view of sequences as described in section 12.1, 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.

- **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.

12.2.1 Using split views to see details of the circular molecule

In order 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 (AC) 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 12.5.

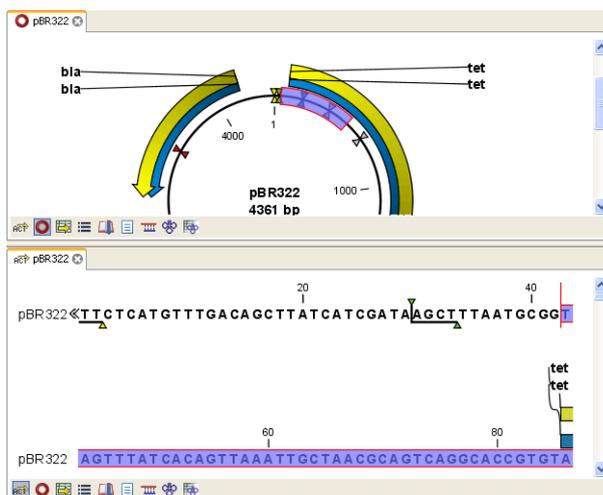


Figure 12.5: Two views showing the same sequence. The bottom view is zoomed in.

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.

12.2.2 Mark molecule as circular and specify starting point

You can mark a DNA molecule as circular by right-clicking its name in either the sequence view or the circular view. In the right-click menu you can also make a circular molecule linear. A circular molecule displayed in the normal sequence view, will have the sequence ends marked with a $\frac{1}{2}$.

The starting point of a circular sequence can be changed by:

make a selection starting at the position that you want to be the new starting point | right-click the selection | Move Starting Point to Selection Start

Note! This can only be done for sequence that have been marked as circular.

12.3 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 15.5.2), and atom groups defined on the structure transferred to sequence annotations or vica versa (section 15.5.3).
- You can manually add annotations to a sequence (described in the section 12.3.2).

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 18.1.

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.

12.3.1 Viewing annotations

Annotations can be viewed in a number of different ways:

- As arrows or boxes in all views displaying sequences (sequence lists, alignments etc)
- In the table of annotations (.
- In the text view of sequences (.

In the following sections, these view options will be described in more detail. In all the views except the text view () , annotations can be added, modified and deleted. This is described in the following sections.

View Annotations in sequence views

Figure 12.6 shows an annotation displayed on a sequence.

The various sequence views listed in section 12.3.1 have different default settings for showing annotations. However, they all have two groups in the **Side Panel** in common:

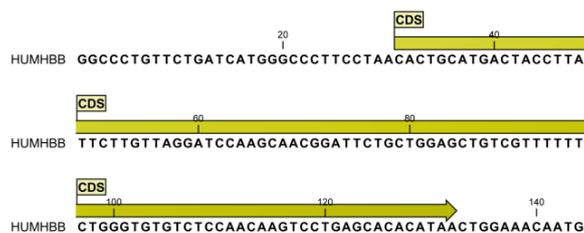


Figure 12.6: An annotation showing a coding region on a genomic dna sequence.

- **Annotation Layout**
- **Annotation Types**

The two groups are shown in figure 12.7.

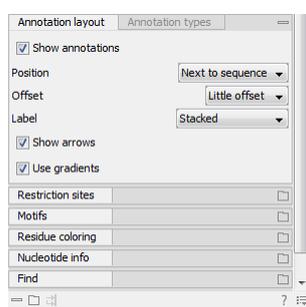


Figure 12.7: The annotation layout in the Side Panel. The annotation types can be shown by clicking on the "Annotation types" tab.

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 12.8).



Figure 12.8: Browsing the gene annotations on a sequence.

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 12.9) 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.

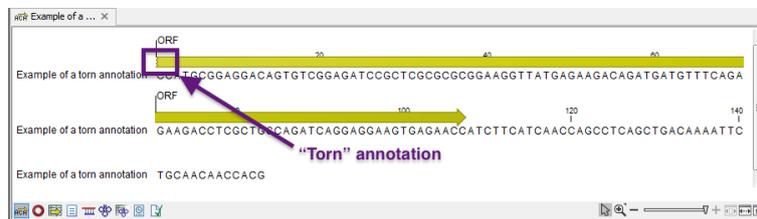


Figure 12.9: Example of a torn annotation on a sequence.

View Annotations in a table

Annotations can also be viewed in a table:

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 12.10).

| 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... |

Figure 12.10: A table showing annotations on the sequence.

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.**
- **Qualifiers.**

The Name, Type and Region for each annotation can be edited simply by double-clicking, typing the change directly, and pressing **Enter**.

This information corresponds to the information in the dialog when you edit and add annotations (see section 12.3.2).

You can benefit from this table in several ways:

- It provides an intelligible overview of all the annotations on the sequence.
- You can use the filter at the top to search the annotations. Type e.g. "UCP" into the filter and you will find all annotations which have "UCP" in either the name, the type, the region or the qualifiers. Combined with showing or hiding the annotation types in the **Side Panel**, this makes it easy to find annotations or a subset of annotations.
- You can copy and paste annotations, e.g. from one sequence to another.
- If you wish to edit many annotations consecutively, the double-click editing makes this very fast (see section 12.3.2).

12.3.2 Adding annotations

Adding annotations to a sequence can be done in two ways:

Open the sequence in a sequence view (double-click in the Navigation Area) | make a selection covering the part of the sequence you want to annotate¹ | right-click the selection | Add Annotation (➡)

or **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 (📄) | right click anywhere in the annotation table | select Add Annotation (➡)**

This will display a dialog like the one in figure 12.11.

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 ².

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. (Whether the name is actually shown depends on the **Annotation Layout** preferences, see section 12.3.1).

²Note 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, your own annotation type will be preserved

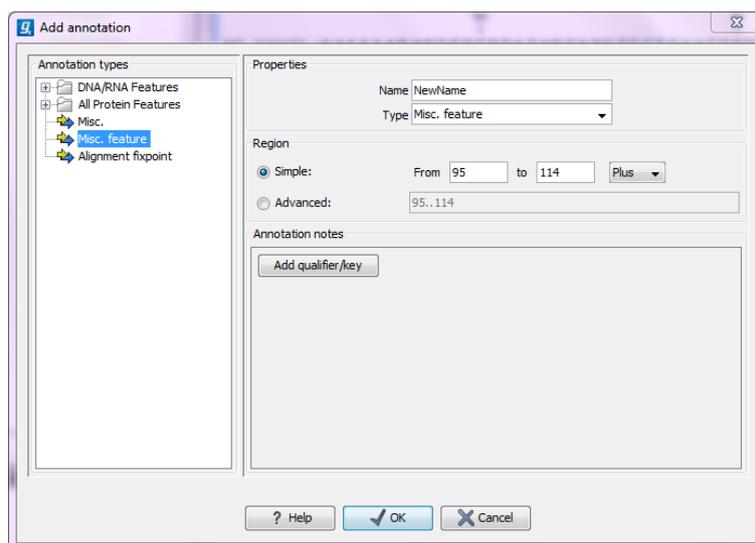


Figure 12.11: *The Add Annotation dialog.*

- **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 <http://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.
 - **123^124.** 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 12.3.1) 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. "www.clcbio.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.

12.3.3 Edit annotations

To edit an existing annotation from within a sequence view:

right-click the annotation | Edit Annotation (✎)

This will show the same dialog as in figure 12.11, with the exception that some of the fields are filled out depending on how much information the annotation contains.

There is another way of quickly editing annotations which is particularly useful when you wish to edit several annotations.

To edit the information, simply double-click and you will be able to edit e.g. the name or the annotation type. If you wish to edit the qualifiers and double-click in this column, you will see the dialog for editing annotations.

Advanced editing of annotations

Sometimes you end up with annotations which do not have a meaningful name. In that case there is an advanced batch rename functionality:

Open the Annotation Table (📄) | **select the annotations that you want to rename | right-click the selection | Advanced Rename**

This will bring up the dialog shown in figure 12.12.

In this dialog, you have two options:

- **Use this qualifier.** Use one of the qualifiers as name. A list of all qualifiers of all the selected annotations is shown. Note that if one of the annotations do not have the qualifier you have chosen, it will not be renamed. If an annotation has multiple qualifiers of the same type, the first is used for naming.

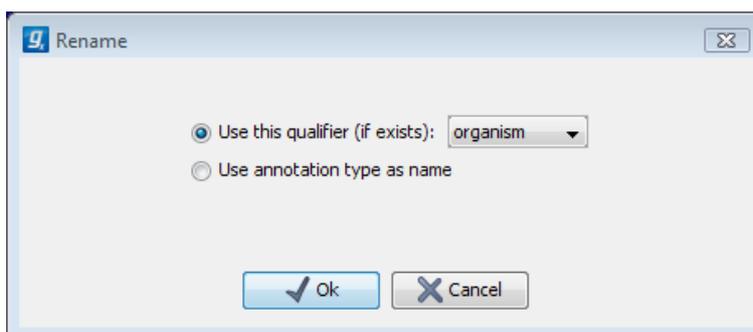


Figure 12.12: The Advanced Rename dialog.

- **Use annotation type as name.** The annotation's type will be used as name (e.g. if you have an annotation of type "Promoter", it will get "Promoter" as its name by using this option).

A similar functionality for batch re-typing annotations is available in the right-click menu as well, in case your annotations are not typed correctly:

Open the Annotation Table (📄) | select the annotations that you want to retype | right-click the selection | Advanced Retype

This will bring up the dialog shown in figure 12.13.

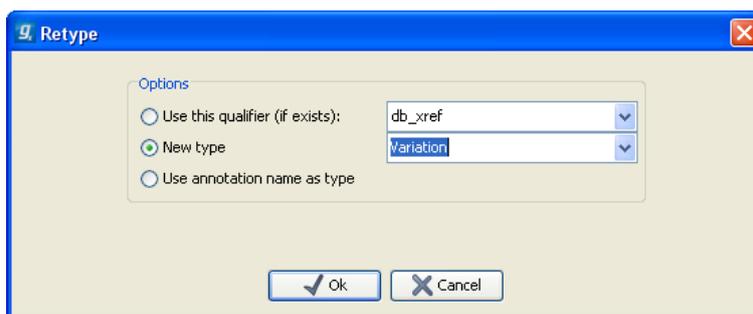


Figure 12.13: The Advanced Retype dialog.

In this dialog, you have two options:

- **Use this qualifier.** Use one of the qualifiers as type. A list of all qualifiers of all the selected annotations is shown. Note that if one of the annotations do not have the qualifier you have chosen, it will not be retyped. If an annotation has multiple qualifiers of the same type, the first is used for the new type.
- **New type.** You can select from a list of all the pre-defined types as well as enter your own annotation type. All the selected annotations will then get this type.
- **Use annotation name as type.** The annotation's name will be used as type (e.g. if you have an annotation named "Promoter", it will get "Promoter" as its type by using this option).

12.3.4 Removing annotations

Annotations can be hidden using the **Annotation Types** preferences in the **Side Panel** to the right of the view (see section 12.3.1). 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**

12.4 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 12.14.



Figure 12.14: 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.

- **Name.** The name of the sequence which is also shown in sequence views and in the **Navigation Area**.
- **Description.** A description of 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 (section 8) 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.

The information available depends on the origin of the sequence. Sequences downloaded from database like NCBI and UniProt (see section 13) have this information. On the other hand, some sequence formats like fasta format do not contain this information.

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.

Note that for other kinds of data, the **Element info** will only have **Name** and **Description**.

12.5 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 12.4.)

In the **Side Panel**, you find a search field for searching the text in the view.

12.6 Sequence Lists

The **Sequence List** shows a number of sequences in a tabular format or it can show the sequences together in a normal sequence view.

Having sequences in a sequence list can help organizing sequence data.

Sequence lists are generated automatically when you import files containing more than one sequence. Sequence lists may also be created as the output from particular Workbench tool including database searches.

See (chapter 13.1).

Sequence List can also be created from single sequences or by merging already existing sequence lists with the Workbench. To do this:

select two or more sequences or sequence lists | right-click the elements | New | Sequence List (☰)

Alternatively, you can launch this tool via the menu system:

File | New | Sequence List (☰)

This opens the **Sequence List Wizard**:

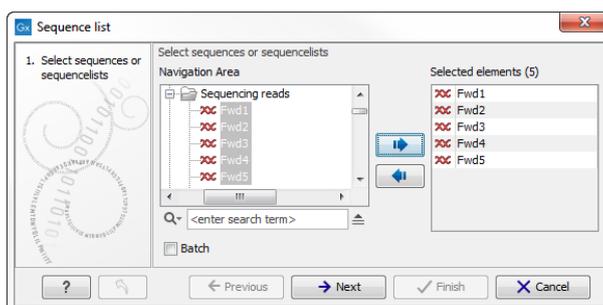


Figure 12.15: A Sequence List dialog.

The dialog allows you to select more sequences to include in the list, or to remove already chosen sequences from the list.

Clicking **Finish** opens the sequence list. It can be saved by clicking **Save** (☒) or by dragging the tab of the view into the **Navigation Area**.

Opening a Sequence list is done by:

right-click the sequence list in the Navigation Area | Show (☒) | Graphical Sequence List (☰) OR Table (☒)

The two different views of the same sequence list are shown in split screen in figure 12.16.

12.6.1 Graphical view of sequence lists

The graphical view of sequence lists is almost identical to the view of single sequences (see section 12.1). The main difference is that you now can see more than one sequence in the same view.

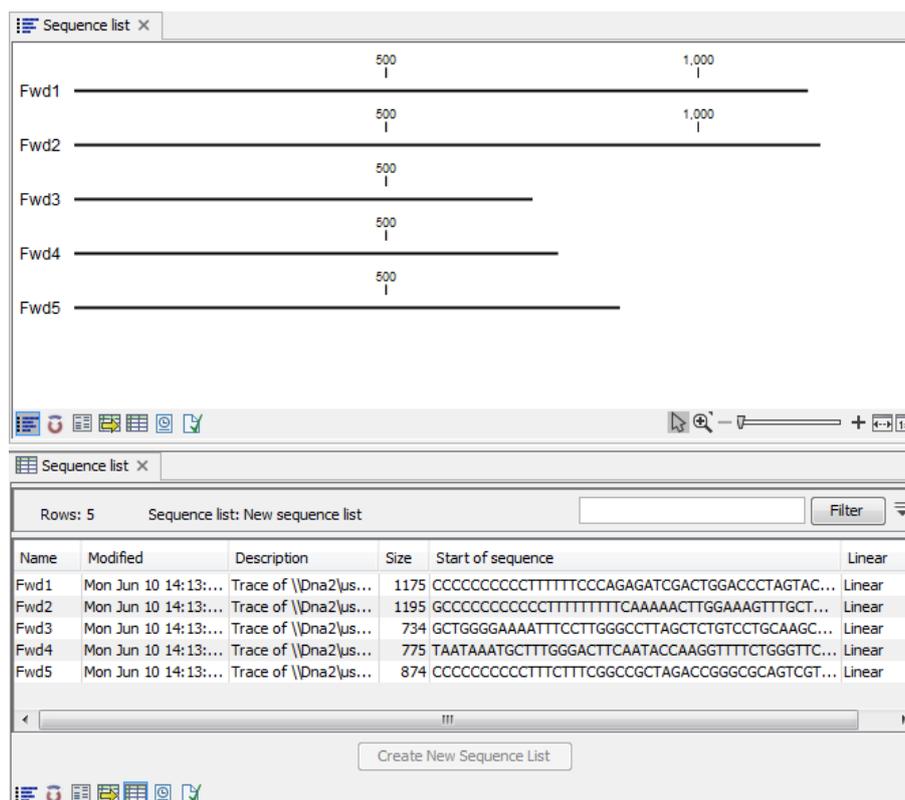


Figure 12.16: A sequence list containing multiple sequences can be viewed in either a table or in a graphical sequence list. The graphical view is useful for viewing annotations and the sequence itself, while the table view provides other information like sequence lengths, and the number of sequences in the list (number of Rows reported).

However, you also have a few extra options for sorting, deleting and adding sequences:

- To add extra sequences to the list, right-click an empty (white) space in the view, and select **Add Sequences**.
- To delete a sequence from the list, right-click the sequence's name and select **Delete Sequence**.
- To sort the sequences in the list, right-click the name of one of the sequences and select **Sort Sequence List by Name** or **Sort Sequence List by Length**.
- To rename a sequence, right-click the name of the sequence and select **Rename Sequence**.

12.6.2 Sequence list table

Each sequence in the table sequence list is displayed with:

- Name.
- Accession.
- Description.

- Modification date.
- Length.
- First 50 residues.

The number of sequences in the list is reported as the number of Rows at the top of the table view.

Adding and removing sequences from the list is easy: adding is done by dragging the sequence from another list or from the **Navigation Area** and drop it in the table. To delete sequences, simply select them and press **Delete** ().

You can also create a subset of the sequence list:

select the relevant sequences | right-click | Create New Sequence List

This will create a new sequence list, which only includes the selected sequences.

Learn more about tables in Appendix [9.3](#).

12.6.3 Extract sequences from sequence list

Sequences can be extracted from a sequence list when the sequence list is opened in tabular view. One or more sequences can be dragged (with the mouse) directly from the table into the **Navigation Area**. This allows you to extract specific sequences from the entire list. Another option is to extract all sequences found in the list. This can be done with the **Extract Sequences** tool:

Toolbox | General Sequence Analysis () | **Extract Sequences** ()

A description of how to use the **Extract Sequences** tool can be found in section [18.2](#).

Click **Next** if you wish to adjust how to handle the results (see section [9.2](#)). If not, click **Finish**.

Chapter 13

Data download

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CLC Main Workbench offers different ways of searching and downloading online data. You must be online when initiating and performing the following searches:

13.1 GenBank search

This section describes searches for sequences in GenBank - the **NCBI Entrez** database. The NCBI search view is opened in this way (figure 13.1):

Download | Search for Sequences at NCBI ()

or **Ctrl + B** (**⌘ + B** on Mac)

This opens the following view:

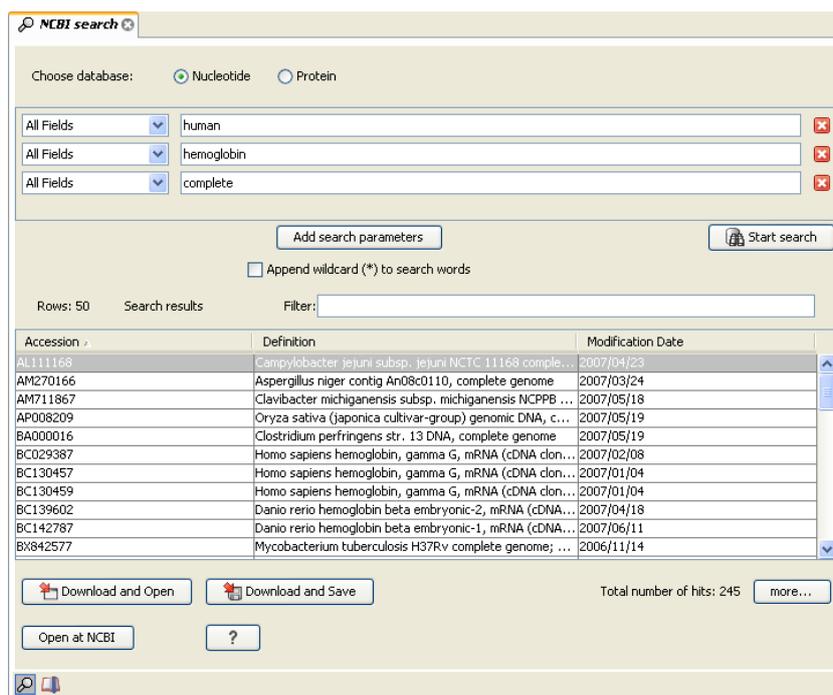


Figure 13.1: The GenBank search view.

13.1.1 GenBank search options

Conducting a search in the **NCBI Database** from *CLC Main Workbench* corresponds to conducting the search on NCBI's website. When conducting the search from *CLC Main Workbench*, the results are available and ready to work with straight away.

You can choose whether you want to search for nucleotide sequences or protein sequences.

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 checking the checkbox at the bottom. This means that you only have to enter the first part of the search text, e.g. searching for "genom" will find both "genomic" and "genome".

The following parameters can be added to the search:

- **All fields.** Text, searches in all parameters in the NCBI database at the same time.
- **Organism.** Text.
- **Description.** Text.
- **Modified Since.** Between 30 days and 10 years.
- **Gene Location.** Genomic DNA/RNA, Mitochondrion, or Chloroplast.
- **Molecule.** Genomic DNA/RNA, mRNA or rRNA.
- **Sequence Length.** Number for maximum or minimum length of the sequence.

- **Gene Name.** Text.

The search parameters are the most recently used. The **All fields** allows searches in all parameters in the NCBI 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. You can also write e.g. `CD9 NOT homo sapiens` in **All fields**.

Note! The 'Feature Key' option is only available in GenBank when searching for nucleotide sequences. For more information about how to use this syntax, see <http://www.ncbi.nlm.nih.gov/books/NBK3837/>

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.

13.1.2 Handling of GenBank 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. This can be changed in the **Preferences** (see chapter 5). More hits can be displayed by clicking the **More...** button at the bottom right of the **View**.

Each sequence hit is represented by text in three columns:

- Accession.
- Description.
- Modification date.
- Length.

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 5.6.

Several sequences can be selected, and by clicking the buttons in the bottom of the search view, you can do the following:

- Download and open, doesn't save the sequence.
- Download and save, lets you choose location for saving sequence.
- Open at NCBI, searches the sequence at NCBI's web page.

Double-clicking a hit will download and open the sequence. 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.

Even if you don't save the search, the next time you open the search view, it will remember the parameters from the last time you did a search.

13.2 UniProt (Swiss-Prot/TrEMBL) search

This section describes searches in UniProt and the handling of search results. UniProt is a global database of protein sequences.

The UniProt search view (figure 13.3) is opened in this way:

Download | Search for Sequences in UniProt (🔍)

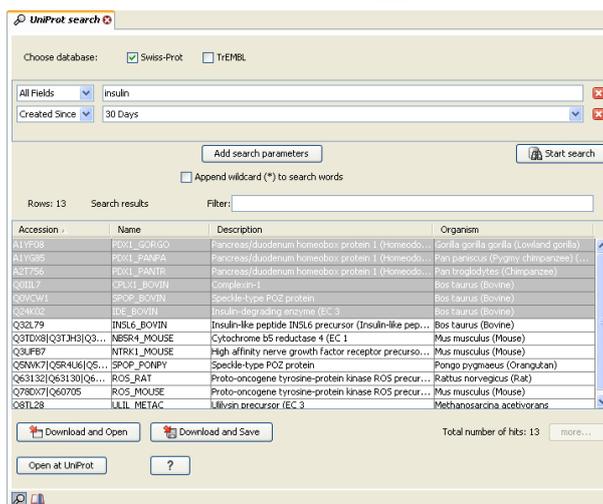


Figure 13.3: The UniProt search view.

13.2.1 UniProt search options

Conducting a search in **UniProt** from *CLC Main Workbench* corresponds to conducting the search on UniProt's website. When conducting the search from *CLC Main Workbench*, the results are available and ready to work with straight away.

Above the search fields, you can choose which database to search:

- **Swiss-Prot** This is believed to be the most accurate and best quality protein database available. All entries in the database has been curated manually and data are entered according to the original research paper.
- **TrEMBL** This database contain computer annotated protein sequences, thus the quality of the annotations is not as good as the Swiss-Prot database.

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 checking the checkbox at the bottom. This means that you only have to enter the first part of the search text, e.g. searching for "genom" will find both "genomic" and "genome".

The following parameters can be added to the search:

- **All fields.** Text, searches in all parameters in the UniProt database at the same time.
- **Organism.** Text.
- **Description.** Text.
- **Created Since.** Between 30 days and 10 years.
- **Feature.** Text.

The search parameters listed in the dialog are the most recently used. The **All fields** allows searches in all parameters in the UniProt database at the same time.

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 UniProt database. This ensures a much faster search.

13.2.2 Handling of UniProt search results

The search result is presented as a list of links to the files in the UniProt database. The **View** displays 50 hits at a time (can be changed in the **Preferences** (see chapter 5)). More hits can be displayed by clicking the **More...** button at the bottom right of the **View**. More hits can be displayed by clicking the **More...** button at the bottom left of the **View**.

Each sequence hit is represented by text in three columns:

- Accession
- Name
- Description
- Organism
- Length.

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 5.6.

Several sequences can be selected, and by clicking the buttons in the bottom of the search view, you can do the following:

- Download and open, does not save the sequence.
- Download and save, lets you choose location for saving sequence.
- Open at UniProt, searches the sequence at UniProt's web page.

Double-clicking a hit will download and open the sequence. 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 UniProt search results

The sequences from the search results can be opened by dragging them into a position in the **View Area**.

Note! A sequence is not saved until the **View** displaying the sequence is closed. When that happens, a dialog opens: Save changes of sequence x? (Yes or No).

The sequence can also be saved by dragging it into the **Navigation Area**. It is possible to select more sequences and drag all of them into the **Navigation Area** at the same time.

Download UniProt search results using right-click menu

You may also select one or more sequences from the list and download using the right-click menu (see figure 13.2). Choosing **Download and Save** lets you select a folder or location where the sequences are saved when they are downloaded. Choosing **Download and Open** opens a new view for each of the selected sequences.

Copy/paste from UniProt search results

When using copy/paste to bring the search results into the **Navigation Area**, the actual files are downloaded from UniProt.

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 **Toolbox** under the **Processes** tab) it is possible to continue other tasks in the program. Like the search process, the download process can be stopped, paused, and resumed.

13.2.3 Save UniProt search parameters

The search view can be saved either using dragging the search tab and dropping it in the **Navigation Area** or by clicking **Save** (). When saving the search, only the parameters are saved - not the results of the search. This is useful if you have a special search that you perform from time to time.

Even if you don't save the search, the next time you open the search view, it will remember the parameters from the last time you did a search.

13.3 Search for structures at NCBI

This section describes searches for three dimensional structures from the NCBI structure database <http://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 structures at NCBI ()

or **Ctrl + B** (⌘ + B on Mac)

This opens the view shown in figure 13.4:

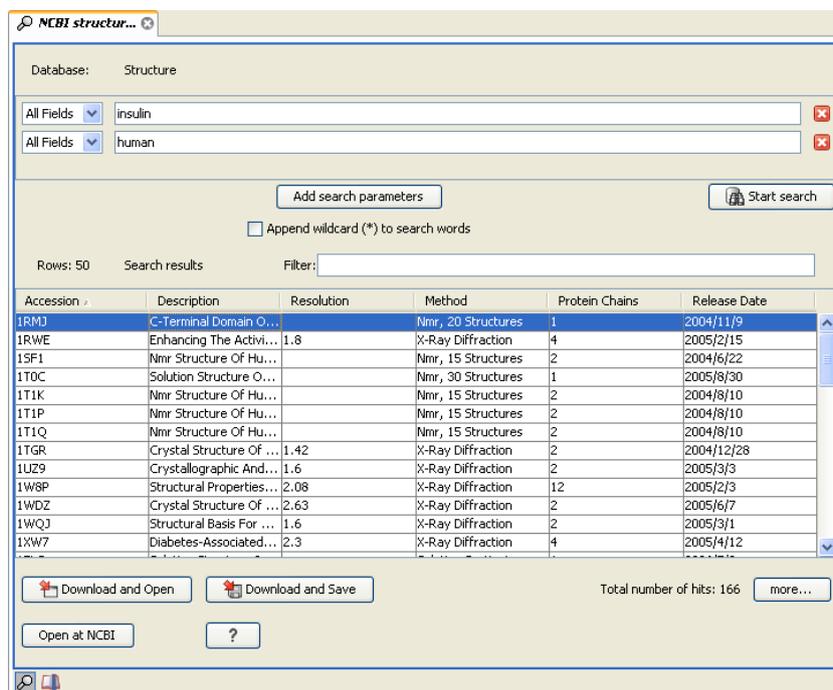


Figure 13.4: The structure search view.

13.3.1 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.

13.3.2 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 5)). 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 5.6.

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 13.5). 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.

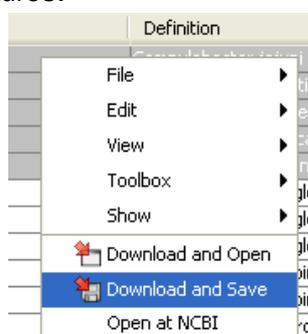


Figure 13.5: By right-clicking a search result, it is possible to choose how to handle the relevant structure.

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 **Toolbox** in the **Processes** tab.

13.3.3 Save structure search parameters

The search view can be saved either using dragging the search tab and dropping it in the **Navigation Area** or by clicking **Save** (↵). When saving the search, only the parameters are saved

- not the results of the search. This is useful if you have a special search that you perform from time to time.

Even if you don't save the search, the next time you open the search view, it will remember the parameters from the last time you did a search.

13.4 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 12.5). 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 13.6):

Open a sequence or a sequence list | Right-click the name of the sequence | Web Info (🌐) | select the desired search function

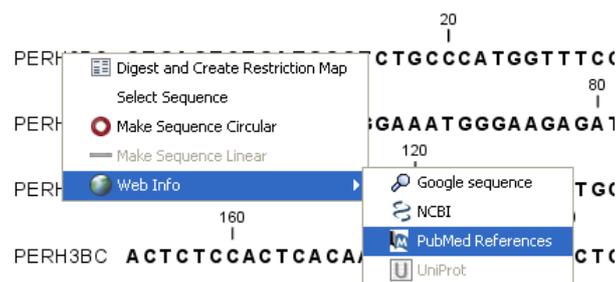


Figure 13.6: Open webpages with information about this sequence.

This will open your computer's default browser searching for the sequence that you selected.

13.4.1 Google sequence

The Google search function uses the accession number of the sequence which is used as search term on <http://www.google.com>. The resulting web page is equivalent to typing the accession number of the sequence into the search field on <http://www.google.com>.

13.4.2 NCBI

The NCBI search function searches in GenBank at NCBI (<http://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.

13.4.3 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 see a dialog and the browser will not open.

13.4.4 UniProt

The UniProt search function searches in the UniProt database (<http://www.ebi.uniprot.org>) using the accession number. Furthermore, it checks whether the sequence was indeed downloaded from UniProt.

13.4.5 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 14

3D Molecule Viewer

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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) <http://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.

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

14.1 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 (15.1.1)**
- **From your own file system (15.1.2)**
- **Using BLAST search against the PDB database (15.1.3)**

14.1.1 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.

Select the molecule structure of interest and click on the button labeled "Download and Open" (see figure 15.1) 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.

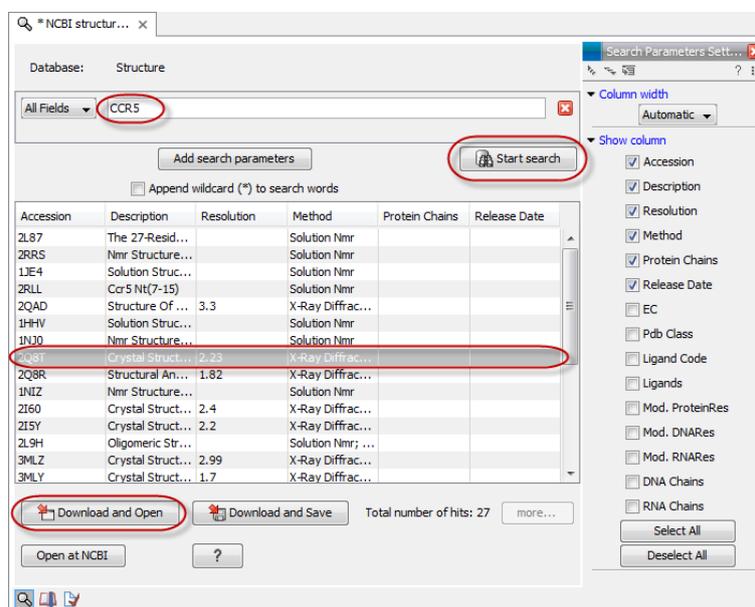


Figure 14.1: Download protein structure from the Protein Data Bank. It is possible to open a structure file directly from the output of the search by clicking the "Download and Open" button or by double clicking directly on the relevant row.

14.1.2 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**.

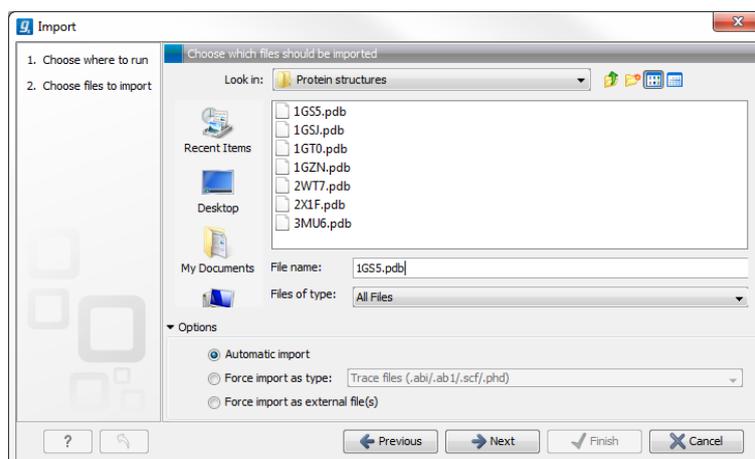


Figure 14.2: A PDB file can be imported using the "Standard Import" function.

14.1.3 BLAST search against the PDB database

It is also possible to make a BLAST search against the PDB database, by going to:

Toolbox | 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).

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.

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

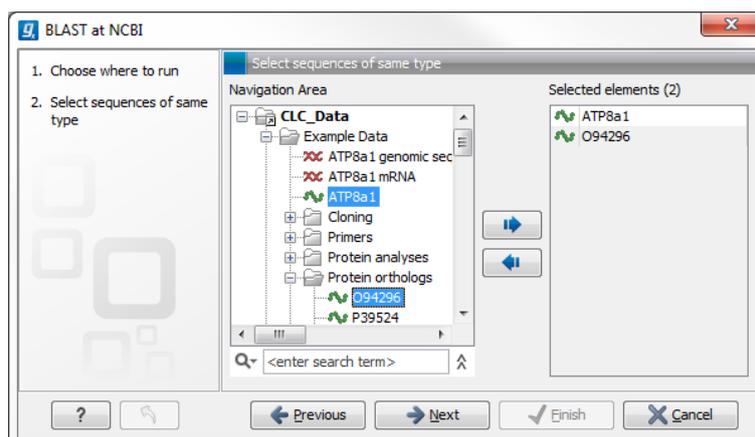


Figure 14.3: Select the input sequence of interest. In this example a protein sequence for ATPase class I type 8A member 1 and an ATPase ortholog from *S. pombe* have been selected.

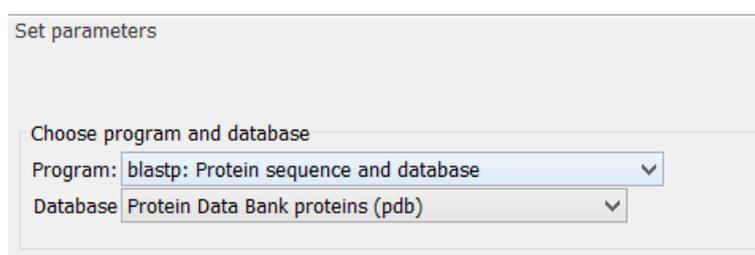


Figure 14.4: Select database and program.

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**.

14.1.4 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

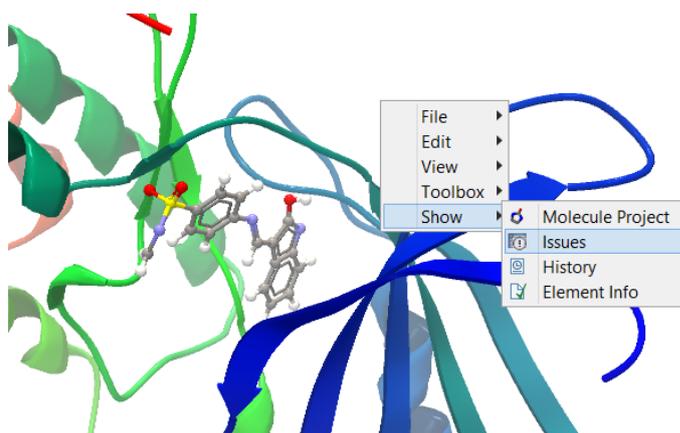


Figure 14.6: At the bottom of the Molecule Project it is possible to switch to the "Show Issues" view by clicking on the "table-with-exclamation-mark" icon.

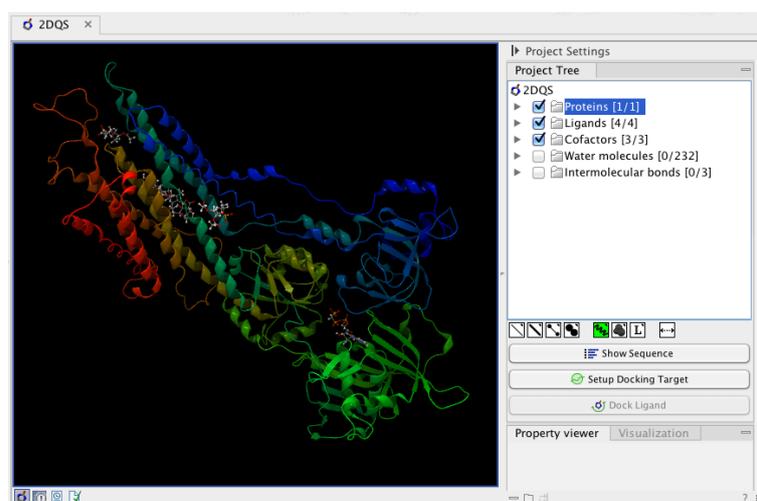


Figure 14.7: 3D view of a calcium ATPase. All molecules in the PDB file are shown in the Molecule Project. The Project Tree in the right side of the window lists the involved molecules.

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-checking 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 (\leftrightarrow) at the bottom of the **Project Tree** view.

14.2.2 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.

14.2.3 Updating old structure files

A completely redesign of the 3D Molecule Viewer was released in August 2013. It is therefore necessary to update older structure files. To update existing structure files, double click on the name in the **Navigation Area**. This will bring up the dialog shown in figure 15.8, which via the "Download from PDB..." button gives access to downloading the specific structure in PDB format.

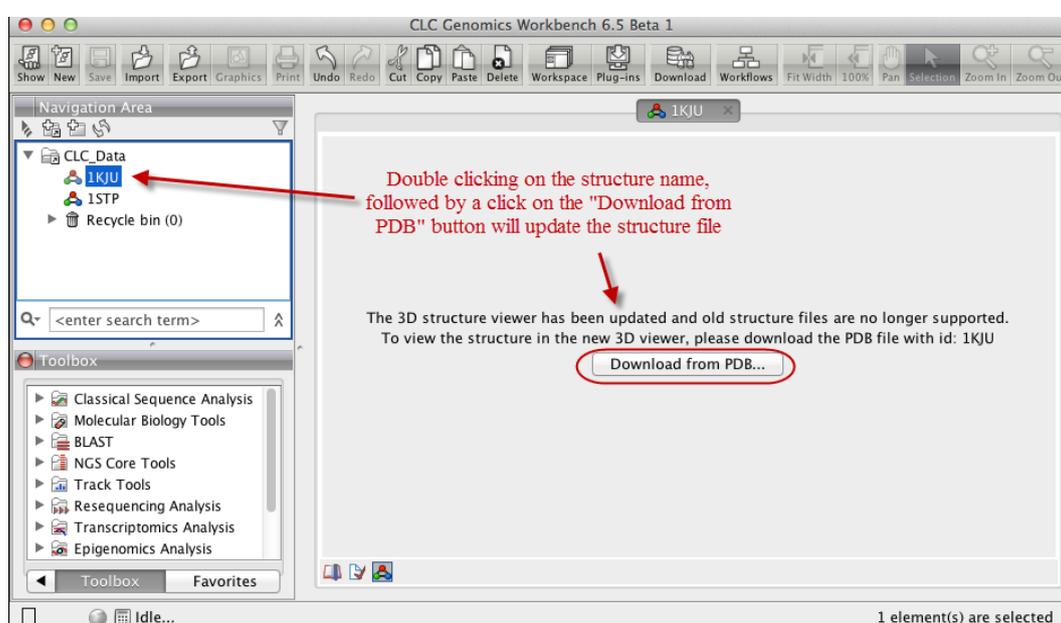


Figure 14.8: Old structure files are not supported by the new 3D Molecule Viewer and must be updated.

14.3 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.

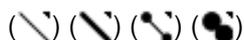
The six leftmost quick-style buttons below the **Project Tree** view give access to the molecule visualization styles, while context menus on the buttons (accessible via right-click or left-click-hold) give access to the color schemes available for the visualization styles. Visualization styles and color 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.

14.3.1 Visualization styles and colors

Wireframe, Stick, Ball and stick, Space-filling/CPK



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



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 α 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.

Notice that visual artifacts may appear when rotating a transparent surface. These artifacts disappear as soon as the mouse is released.

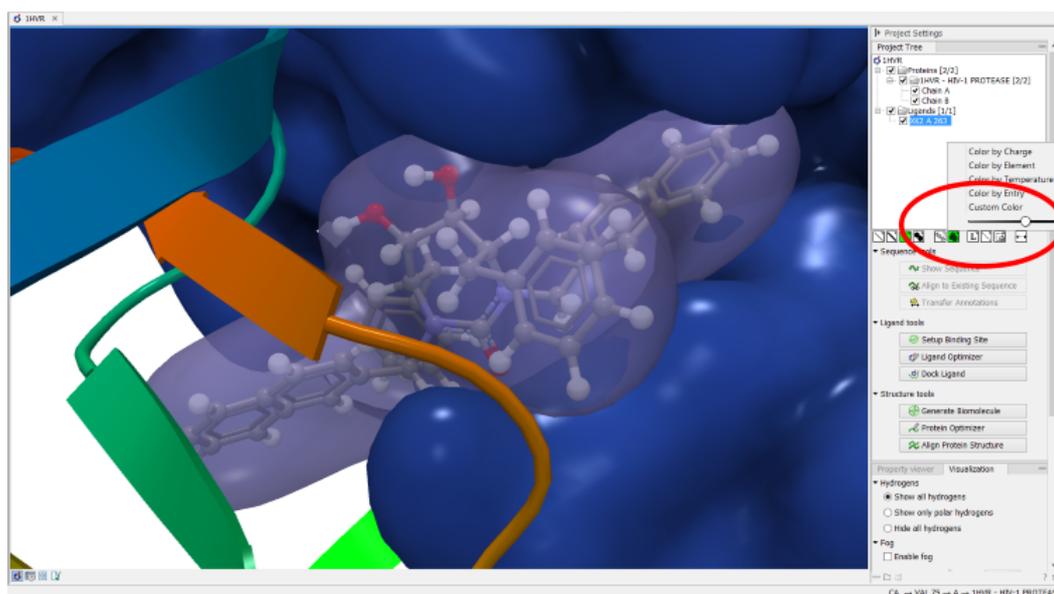


Figure 14.9: Transparent surfaces

Labels

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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 15.10).

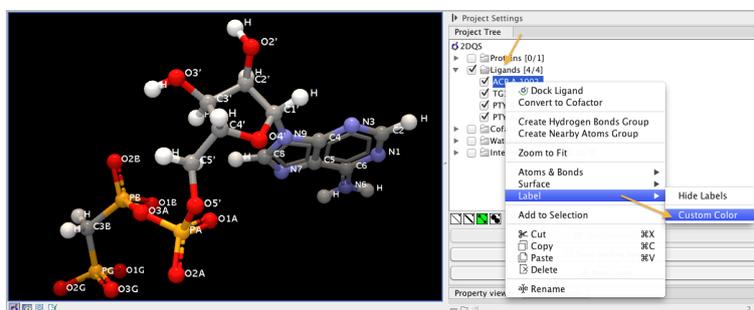


Figure 14.10: The color of the labels can be adjusted in two different ways. Either directly using the label button by right clicking the button, or by right clicking on the molecule or category of interest in the Project Tree.

- For proteins and nucleic acids, each residue is labelled with the PDB name and number.
- For ligands, each atom is labelled 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 labelled with the PDB name and number.
- For atom groups not including protein atoms, each atom is labelled with the atom name as given in the input.

Labels can be removed again by clicking on the label button.

Hydrogen bonds



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.

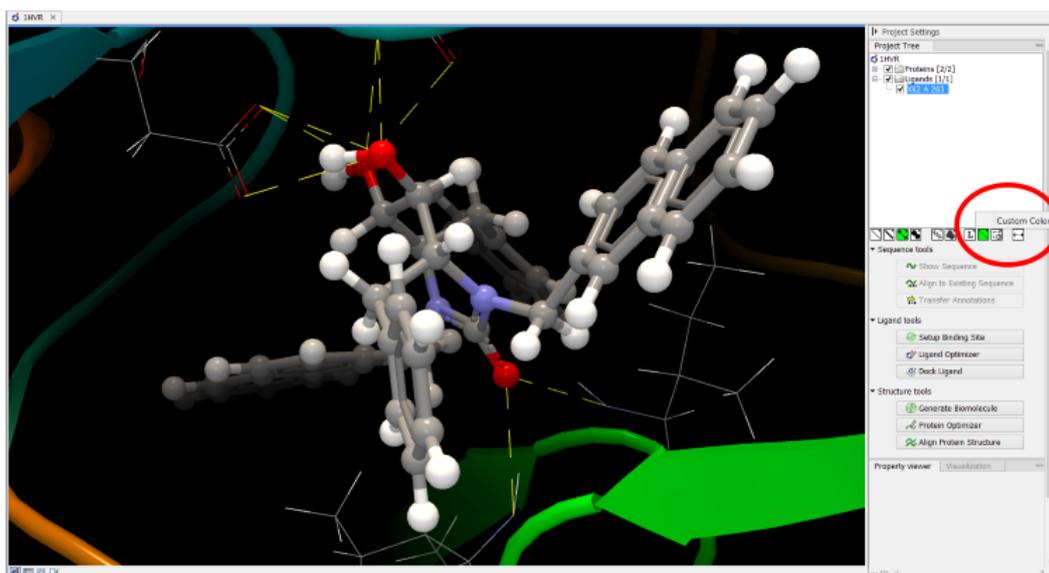


Figure 14.11: The hydrogen bond visualization setting, with custom bond color

Create atom group



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.

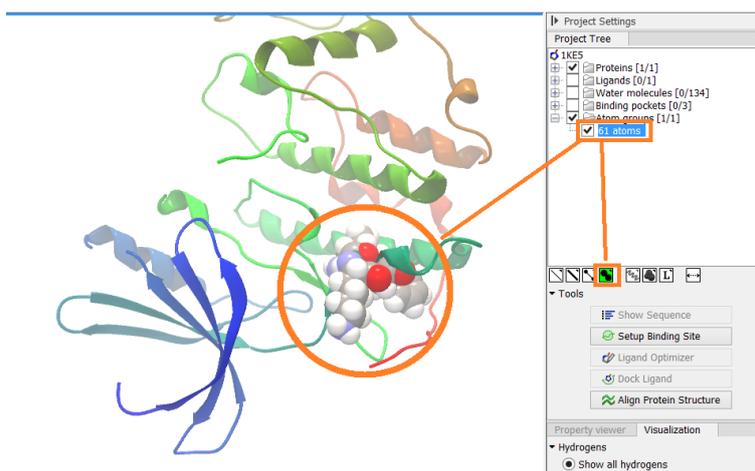


Figure 14.12: An atom group that has been highlighted by adding a unique visualization style.

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 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 (section 15.5.1). A split-view will appear with a sequence editor for each of the sequence data types (Protein, DNA, RNA) (figure 15.13). 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 (section 15.5.2). 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.

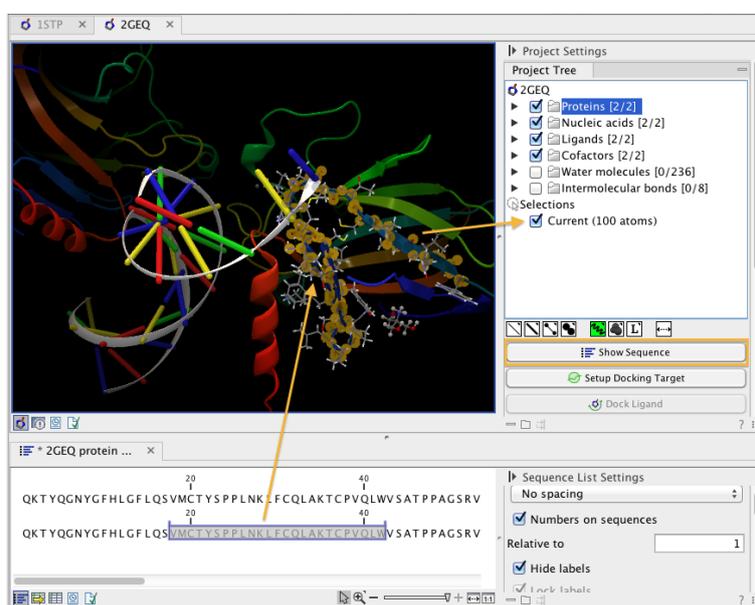


Figure 14.13: The protein sequence in the split view is linked with the protein structure. This means that when a part of the protein sequence is selected, the same region in the protein structure will be selected.

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. This option is also available on binding pocket entries (binding pockets can only be created in *CLC Drug Discovery Workbench*).
- **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 can only be created using *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

(\leftrightarrow)

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 (\leftrightarrow) at the bottom of the Project Tree view (figure 15.14). Double-clicking an entry in the Project Tree will have the same effect.

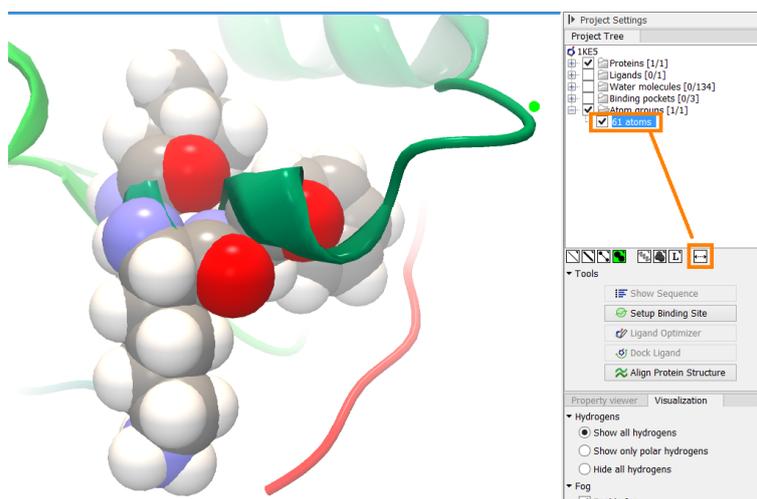


Figure 14.14: The "Fit to screen" button can be used to bring a particular molecule or category of molecules in focus.

14.3.2 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** (\equiv). This is described in detail in section 5.6.

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 [15.5.1](#).

- **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 [15.5.2](#).
- **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 [15.5.3](#).
- **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 [15.6](#).

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:

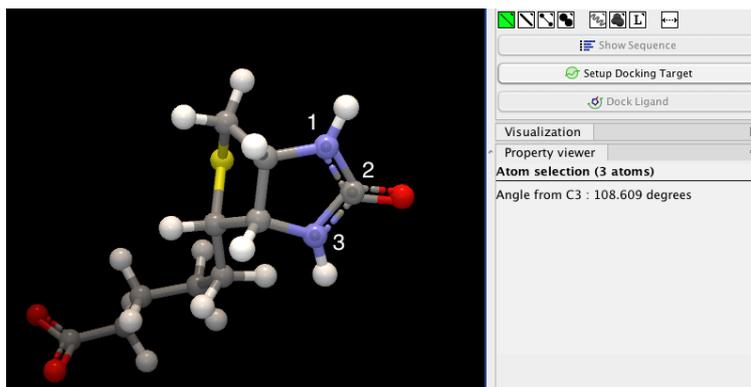


Figure 14.15: Selecting two, three, or four atoms will display the distance, angle, or dihedral angle, respectively.

- **Atoms** Number of atoms in the molecule.
- **Weight** The weight of the molecule in Daltons.

Visualization settings

Under "Visualization" five options exist:

- **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.

14.4 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 5.6).

14.5 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 15.5.3).

14.5.1 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 15.16). 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 15.5.2).

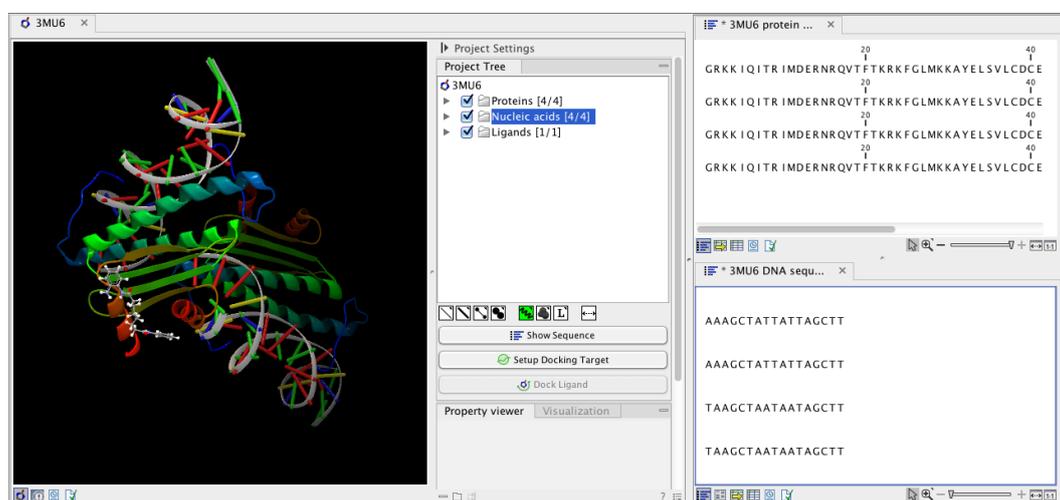


Figure 14.16: Protein chain sequences and DNA sequences are shown in separate views.

14.5.2 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 15.5.1) or Align to Existing Sequence.

Select a single protein chain in the project tree (see figure 15.17). 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 - section 15.5.1), and a sequence alignment is created between this sequence and the sequences or alignments selected from the Navigation Area. The

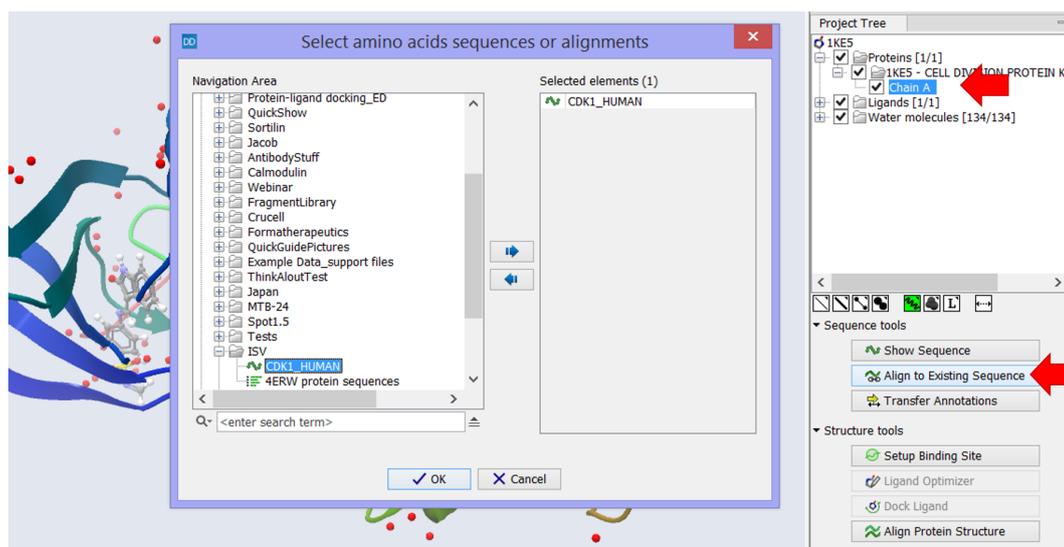


Figure 14.17: Select a single protein chain in the Project Tree and invoke "Align to Existing Sequence".

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

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 15.5.3). Notice, 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 15.20 and section 16.3.4).

14.5.3 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 12.3 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 15.5.1) or by mapping to an existing sequence or sequence alignment using the 'Align to Existing Sequence' button (see section 15.5.2).

Invoke the Transfer Annotations dialog by selecting a linked protein chain in the Project Tree and press 'Transfer Annotations' (see figure 15.18).

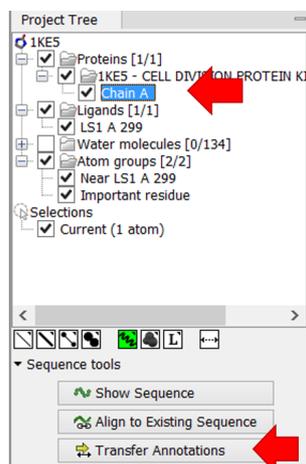


Figure 14.18: Select a single protein chain in the Project Tree and invoke "Transfer Annotations".

The dialog contains two tables (see figure 15.19). 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, changes to the visualization styles are allowed.

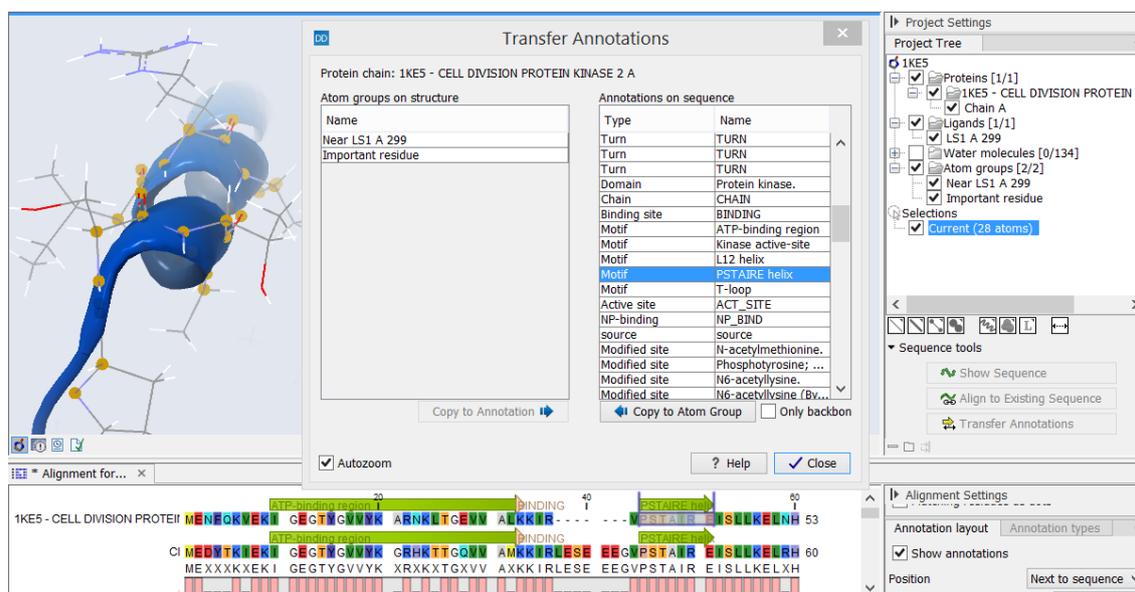


Figure 14.19: The Transfer Annotations dialog allow you to select annotations listed in the two tables, and copy them from structure to sequence or vice versa.

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 15.20 and section 16.3.4).

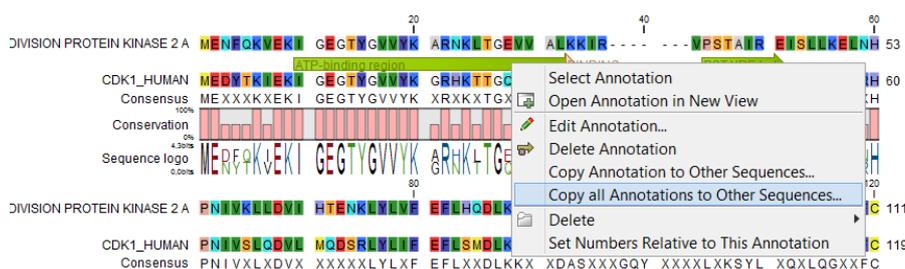


Figure 14.20: Copy annotations from sequences in the alignment to the sequence linked to the 3D view.

14.6 Protein structure alignment

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 15.21). 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.

14.6.1 The Align Protein Structure dialog box

The dialog box contains three fields:

- **Select reference (protein chain or atom group)** This drop-down menu shows all the protein 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,

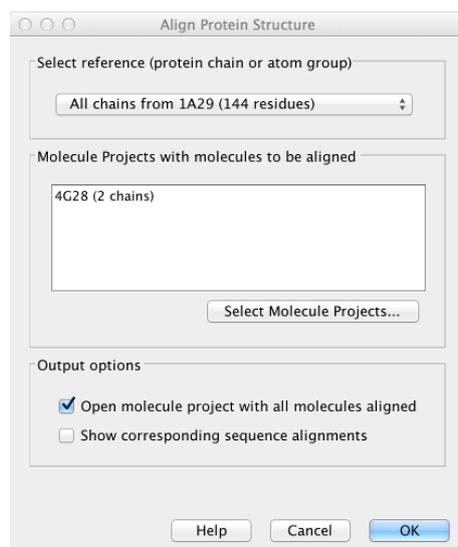


Figure 14.21: *The Align Protein Structure dialog box.*

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.

14.6.2 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 15.21. 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 15.22. 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).

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 15.23). Using the new atom group as the reference in the alignment dialog leads to the alignment shown in figure 15.24. 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.

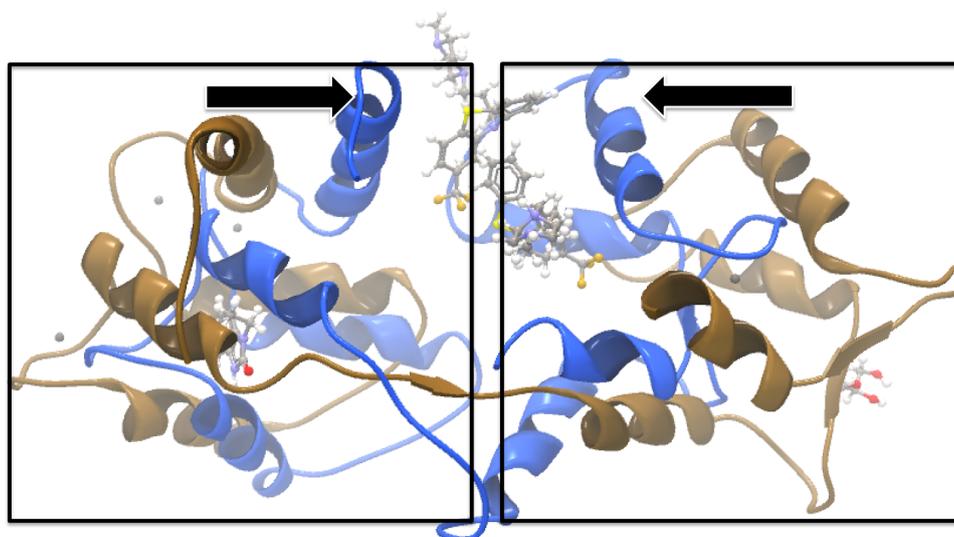


Figure 14.22: Global alignment of two calmodulin structures (blue and brown). The two domains of calmodulin (shown within black boxes) can undergo large changes in relative orientation. In this case, the different orientation of the domains in the blue and brown structures makes a good global alignment 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.

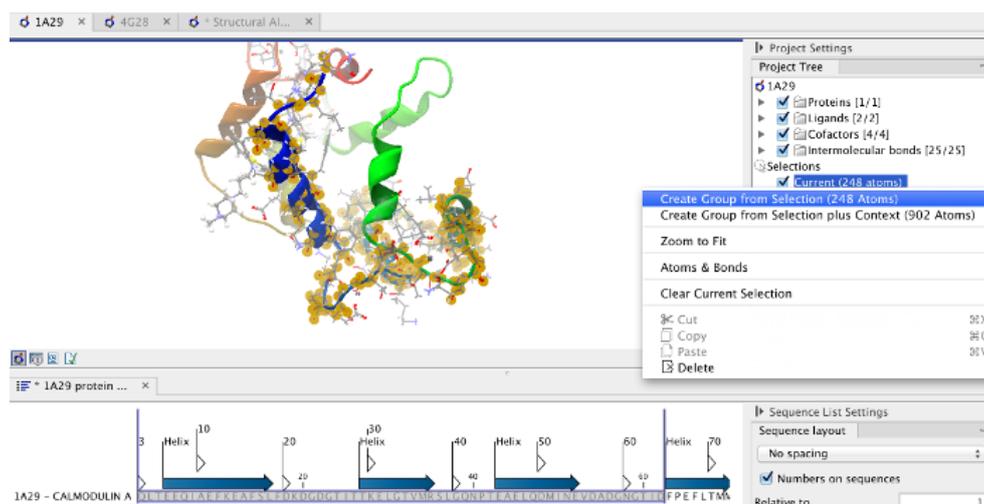


Figure 14.23: Creation of an atom group containing the N-terminal domain of calmodulin.

Aligning a binding site Two bound calcium atoms, one from each calmodulin structure, are shown in the black box of figure 15.24. 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 15.25.

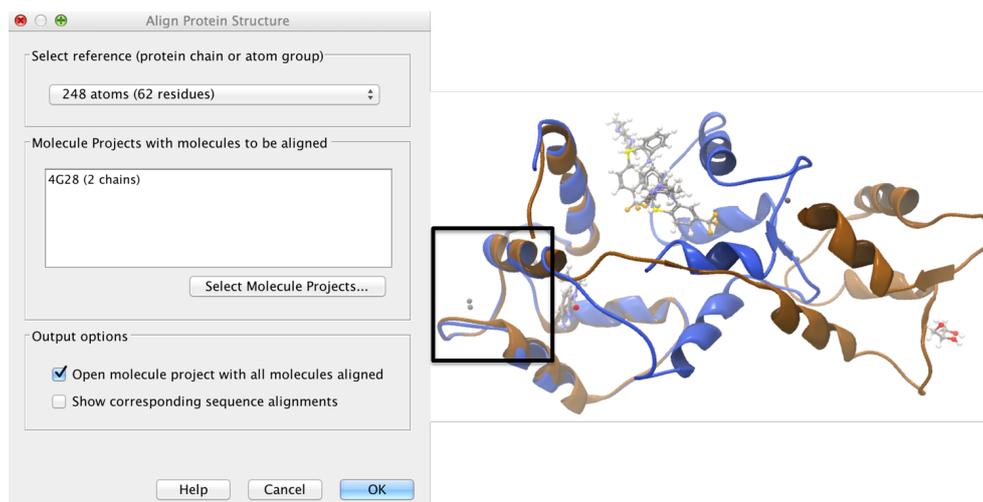


Figure 14.24: Alignment of the same two calmodulin proteins as in figure 15.22, but this time with a focus on the N-terminal domain. The blue and brown structures are now well-superimposed in the N-terminal region. The black box encloses two calcium atoms that are bound to the structures.

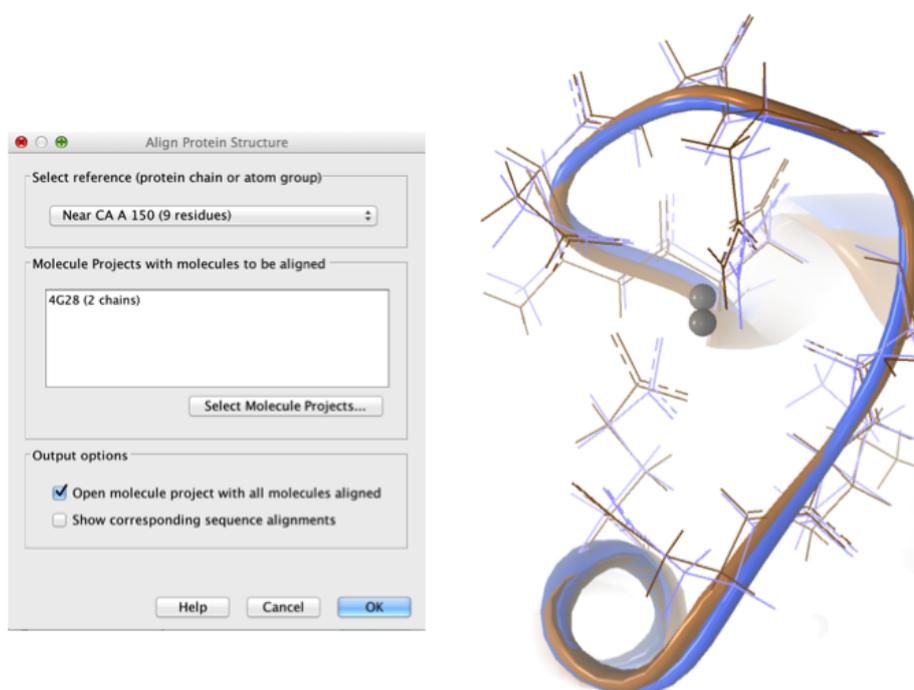


Figure 14.25: Alignment of the same two calmodulin domains as in figure 15.22, but this time with a focus on the calcium atom within the black box of figure 15.24. The calcium atoms are less than 1 Å apart – compatible with thermal motion encoded in the atoms' temperature factors.

14.6.3 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).

Chapter 15

Protein viewer

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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) <http://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.

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 (15.1.1)**
- **From your own file system (15.1.2)**
- **Using BLAST search against the PDB database (15.1.3)**

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.

Select the molecule structure of interest and click on the button labeled "Download and Open" (see figure 15.1) 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.

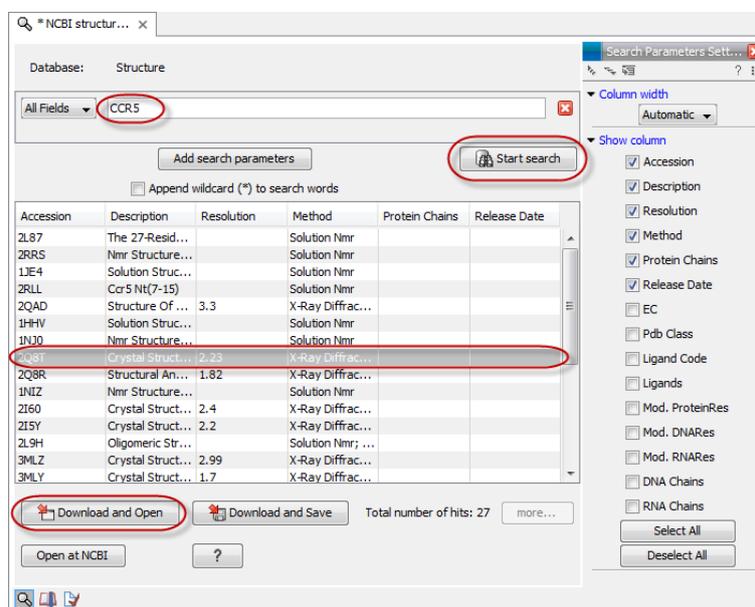


Figure 15.1: Download protein structure from the Protein Data Bank. It is possible to open a structure file directly from the output of the search by clicking the "Download and Open" button or by double clicking directly on the relevant row.

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**.

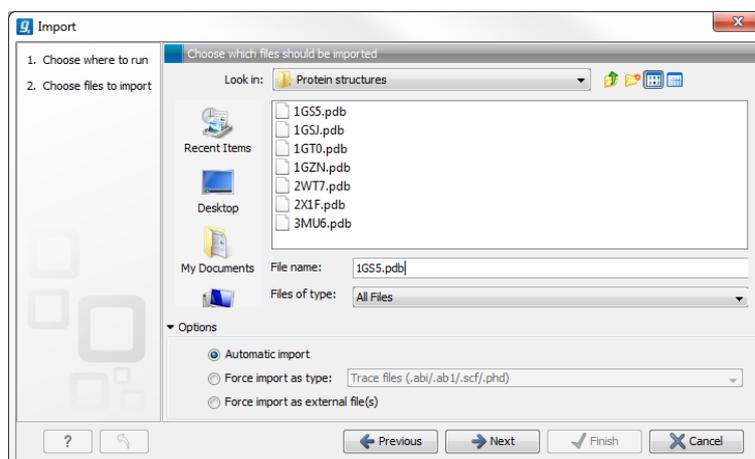


Figure 15.2: A PDB file can be imported using the "Standard Import" function.

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:

Toolbox | 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).

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.

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

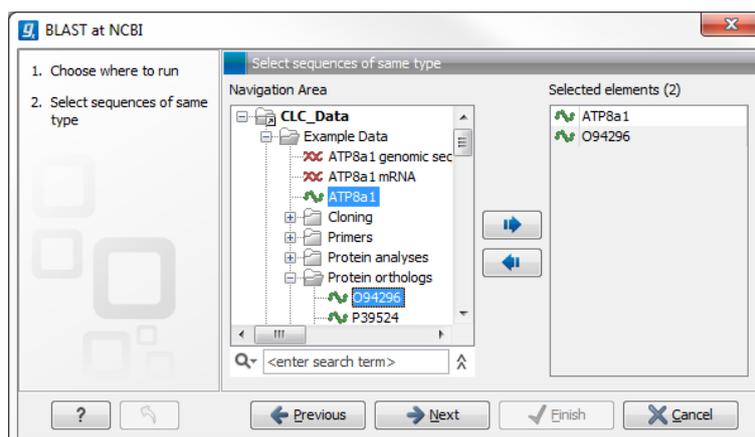


Figure 15.3: Select the input sequence of interest. In this example a protein sequence for ATPase class I type 8A member 1 and an ATPase ortholog from *S. pombe* have been selected.

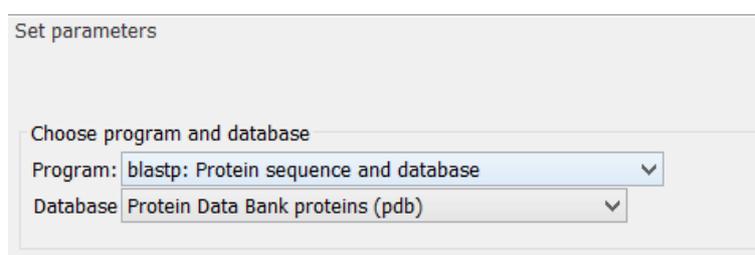


Figure 15.4: Select database and program.

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

The screenshot displays the Protein Viewer interface. The top window shows the BLAST search results for the query 'ATP8a1'. The sequence 'AMARTSNLNEELGQVQYIFSDKTGTLTCNVMQFKKC-TIAGVAY' is highlighted. Below it, several protein sequences are listed with their accession numbers and descriptions. The bottom window shows a table of hits from the query. The table has columns for Hit, Description, E-value, Score, and %Gaps. The row for '2DQ5_A' is selected, and the buttons 'Download and Open', 'Download and Save', 'Open at NCBI', and 'Open Structure' are visible below the table. The 'BLAST Table Settings' panel on the right shows various options for displaying the table.

| Hit | Description | E-value | Score | %Gaps |
|--------|--|---------|--------|-------|
| 3TLM_A | Chain A, Crystal Structure Of Endoplasmic Reticulum Ca2+-ATPase (Serca) From Bovine Musc... | 1.20E-8 | 143.00 | 20.00 |
| 1KJU_A | Chain A, Ca2+-ATPase In The E2 State >g 25200158 1IWO A Chain A, Crystal Structure ... | 3.03E-8 | 139.00 | 23.00 |
| 2DQ5_A | Chain A, Crystal Structure Of The Calcium Pump With Ampcp In The Absence Of Calcium >g 3... | 3.03E-8 | 139.00 | 23.00 |
| 3W5B_A | Chain A, Crystal Structure Of The Recombinant Serca 1a (calcium Pump Of Fast Twitch Skeletal ... | 3.03E-8 | 139.00 | 23.00 |
| 3IXZ_A | Chain A, Pig Gastric H+K+-ATPase Complexed With Aluminium Fluoride >g 320089708 pdb 2ZK... | 2.21E-7 | 132.00 | 16.00 |
| 3IXZ_A | Chain A, Pig Gastric H+K+-ATPase Complexed With Aluminium Fluoride >g 320089708 pdb 2ZK... | 0.17 | 82.00 | 6.00 |
| 3BA6_A | Chain A, Structure Of The Ca2e Ip Phosphoenzyme Intermediate Of The Serca Ca2+-ATPase | 2.46E-7 | 132.00 | 23.00 |
| 3B8E_A | Chain A, Crystal Structure Of The Sodium-Potassium Pump >g 163311039 pdb 3B8E C Chain C... | 2.31E-4 | 106.00 | 6.00 |
| 3B8E_A | Chain A, Crystal Structure Of The Sodium-Potassium Pump >g 163311039 pdb 3B8E C Chain C... | 0.15 | 82.00 | 10.00 |
| 3N23_A | Chain A, Crystal Structure Of The High Affinity Complex Between Ouabain And The E2p Form O... | 2.38E-4 | 106.00 | 6.00 |
| 3N23_A | Chain A, Crystal Structure Of The High Affinity Complex Between Ouabain And The E2p Form O... | 0.17 | 82.00 | 10.00 |
| 22XE_A | Chain A, Crystal Structure Of The Sodium - Potassium Pump In The E2.2k+.Pi State >g 257471... | 1.61E-3 | 99.00 | 14.00 |
| 22XE_A | Chain A, Crystal Structure Of The Sodium - Potassium Pump In The E2.2k+.Pi State >g 257471... | 3.76E-3 | 96.00 | 6.00 |
| 2HCB_A | Chain A, Structure Of The A. Fulgidus Copa A-Domain >g 238537685 pdb 2VOY F Chain F, Cr... | 0.01 | 85.00 | 8.00 |
| 3J08_A | Chain A, High Resolution Helical Reconstruction Of The Bacterial P-Type AtPase Copper Transp... | 0.02 | 90.00 | 8.00 |
| 3J08_A | Chain A, High Resolution Helical Reconstruction Of The Bacterial P-Type AtPase Copper Transp... | 3.06 | 71.00 | 4.00 |
| 3J09_A | Chain A, High Resolution Helical Reconstruction Of The Bacterial P-Type AtPase Copper Transp... | 0.02 | 90.00 | 8.00 |
| 3J09_A | Chain A, High Resolution Helical Reconstruction Of The Bacterial P-Type AtPase Copper Transp... | 3.44 | 71.00 | 4.00 |
| 3RFU_A | Chain A, Crystal Structure Of A Copper-Transporting Pib-Type AtPase >g 340708460 pdb 3RF... | 0.06 | 86.00 | 16.00 |
| 1MHS_A | Chain A, Model Of Neurospora Crassa Proton AtPase >g 24159071 pdb 1MHS B Chain B, Mod... | 0.39 | 79.00 | 29.00 |
| 2W0M_A | Chain A, Crystal Structure Of Sso2452 From Sulfolobus Solfataricus P2 | 0.46 | 76.00 | 3.00 |
| 3P96_A | Chain A, Crystal Structure Of Phosphoserine Phosphatase Serb From Mycobacterium Avium, Na... | 0.51 | 77.00 | 6.00 |
| 2RAR_A | Chain A, X-Ray Crystallographic Structures Show Conservation Of A Trigonal-Bipyramidal Inter... | 0.56 | 76.00 | 7.00 |
| 3M1Y_A | Chain A, Crystal Structure Of A Phosphoserine Phosphatase (Serb) From Helicobacter Pylori >g ... | 0.76 | 74.00 | 0.00 |

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.

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 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.

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.

15.2.1 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.

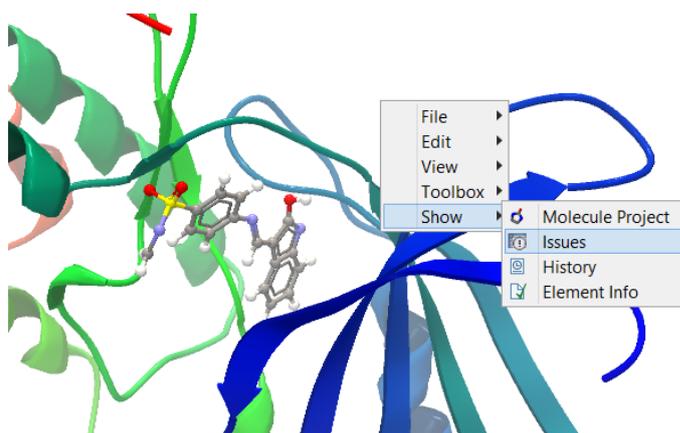


Figure 15.6: At the bottom of the Molecule Project it is possible to switch to the "Show Issues" view by clicking on the "table-with-exclamation-mark" icon.

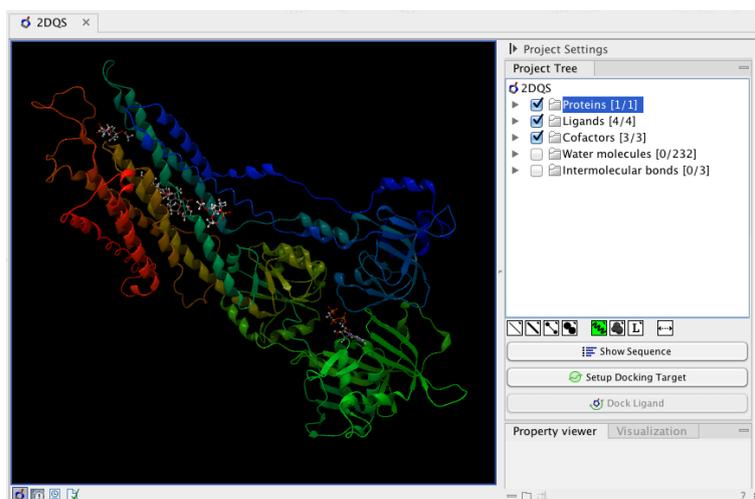


Figure 15.7: 3D view of a calcium ATPase. All molecules in the PDB file are shown in the Molecule Project. The Project Tree in the right side of the window lists the involved molecules.

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-checking 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.

15.2.2 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.

15.2.3 Updating old structure files

A completely redesign of the 3D Molecule Viewer was released in August 2013. It is therefore necessary to update older structure files. To update existing structure files, double click on the name in the **Navigation Area**. This will bring up the dialog shown in figure 15.8, which via the "Download from PDB..." button gives access to downloading the specific structure in PDB format.

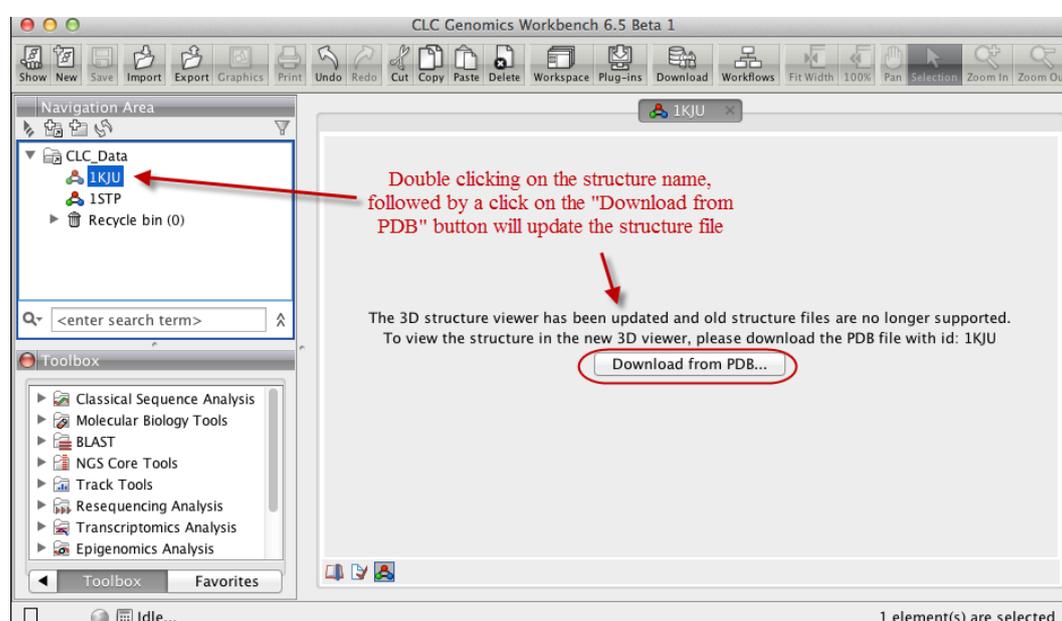


Figure 15.8: Old structure files are not supported by the new 3D Molecule Viewer and must be updated.

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.

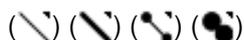
The six leftmost quick-style buttons below the **Project Tree** view give access to the molecule visualization styles, while context menus on the buttons (accessible via right-click or left-click-hold) give access to the color schemes available for the visualization styles. Visualization styles and color 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



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



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 α 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.

Notice that visual artifacts may appear when rotating a transparent surface. These artifacts disappear as soon as the mouse is released.

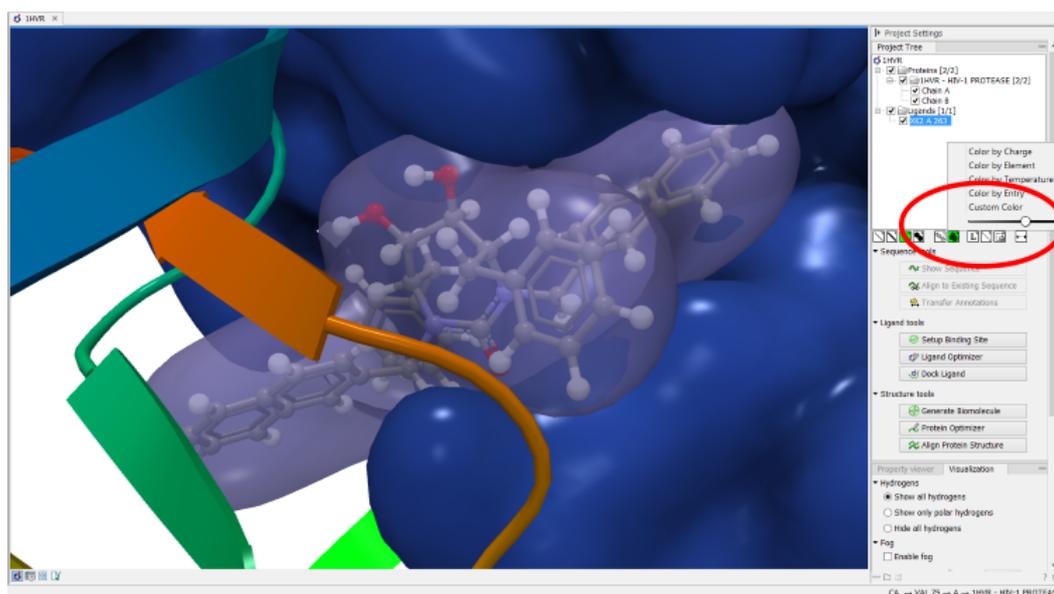


Figure 15.9: Transparent surfaces

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 15.10).

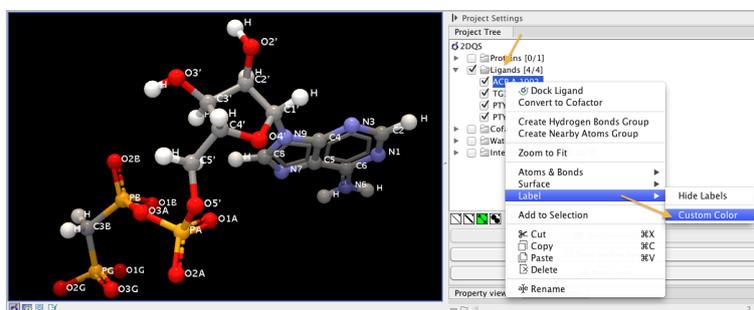


Figure 15.10: The color of the labels can be adjusted in two different ways. Either directly using the label button by right clicking the button, or by right clicking on the molecule or category of interest in the Project Tree.

- For proteins and nucleic acids, each residue is labelled with the PDB name and number.
- For ligands, each atom is labelled 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 labelled with the PDB name and number.
- For atom groups not including protein atoms, each atom is labelled with the atom name as given in the input.

Labels can be removed again by clicking on the label button.

Hydrogen bonds



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.

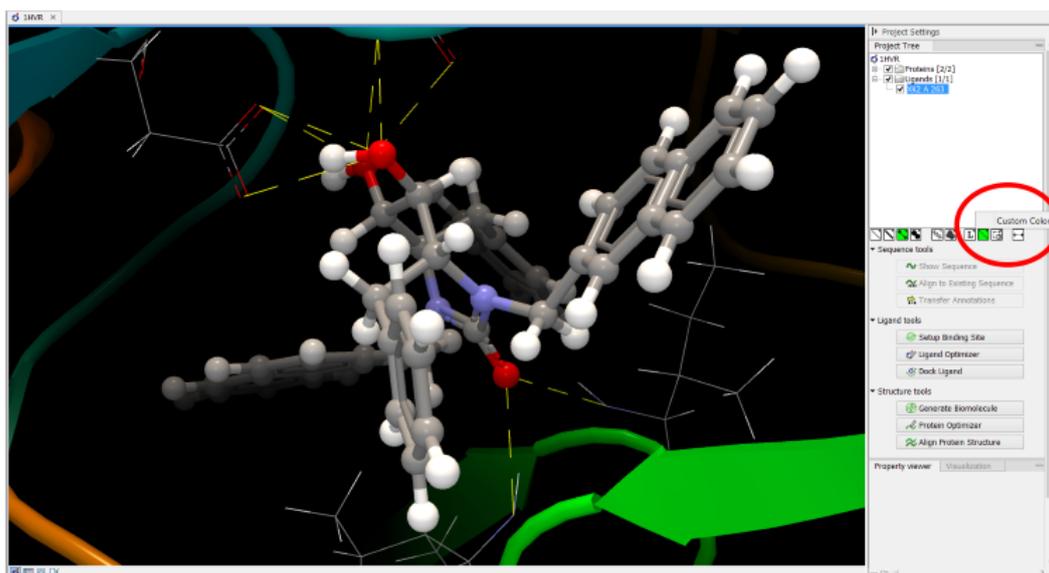


Figure 15.11: The hydrogen bond visualization setting, with custom bond color

Create atom group



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.

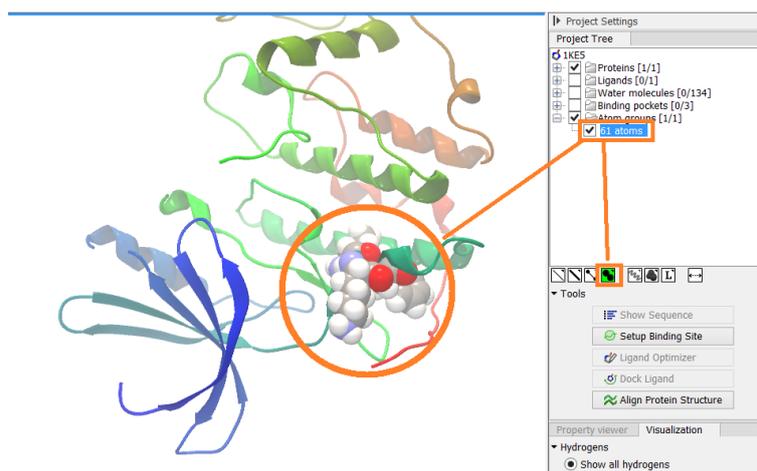


Figure 15.12: An atom group that has been highlighted by adding a unique visualization style.

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 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 (section 15.5.1). A split-view will appear with a sequence editor for each of the sequence data types (Protein, DNA, RNA) (figure 15.13). 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 (section 15.5.2). 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.

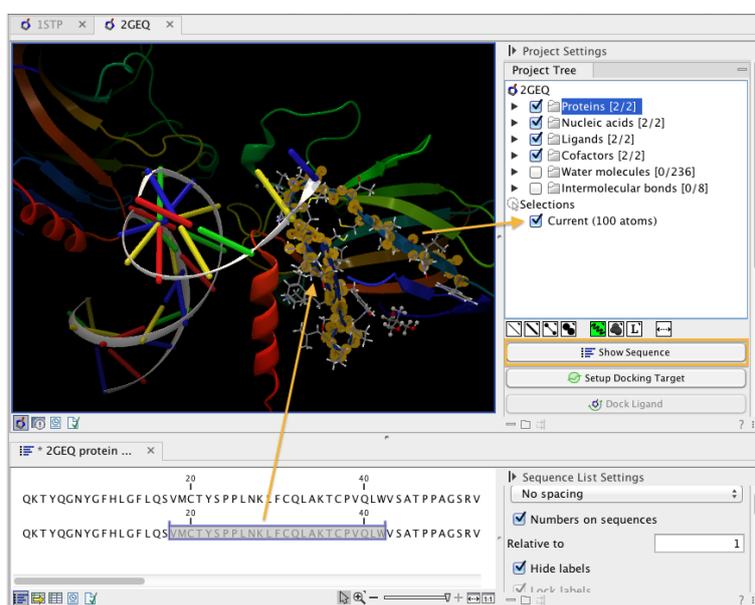


Figure 15.13: The protein sequence in the split view is linked with the protein structure. This means that when a part of the protein sequence is selected, the same region in the protein structure will be selected.

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. This option is also available on binding pocket entries (binding pockets can only be created in *CLC Drug Discovery Workbench*).
- **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 can only be created using *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

(\longleftrightarrow)

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 (\longleftrightarrow) at the bottom of the Project Tree view (figure 15.14). Double-clicking an entry in the Project Tree will have the same effect.

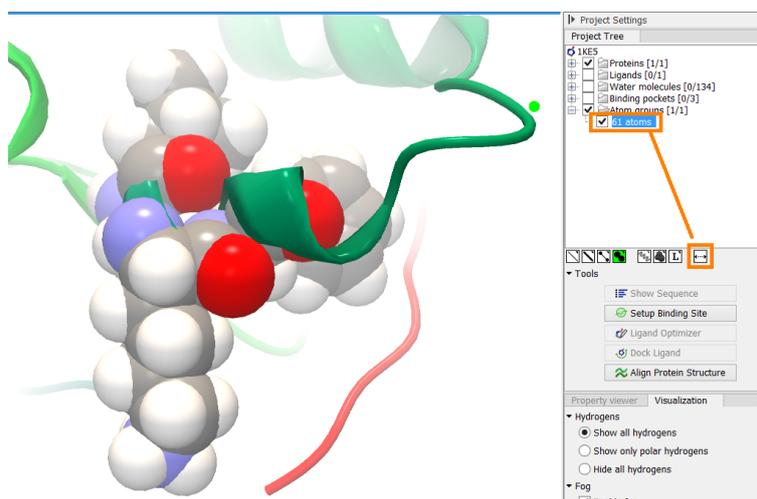


Figure 15.14: The "Fit to screen" button can be used to bring a particular molecule or category of molecules in focus.

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** (\equiv). This is described in detail in section 5.6.

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 [15.5.1](#).

- **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 [15.5.2](#).
- **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 [15.5.3](#).
- **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 [15.6](#).

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:

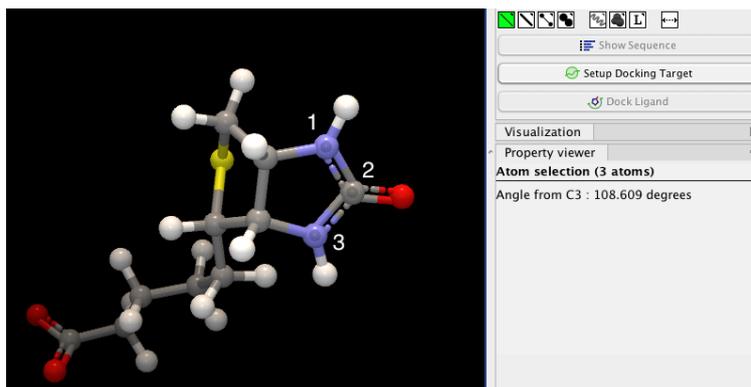


Figure 15.15: Selecting two, three, or four atoms will display the distance, angle, or dihedral angle, respectively.

- **Atoms** Number of atoms in the molecule.
- **Weight** The weight of the molecule in Daltons.

Visualization settings

Under "Visualization" five options exist:

- **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.

15.4 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 5.6).

15.5 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 15.5.3).

15.5.1 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 15.16). 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 15.5.2).

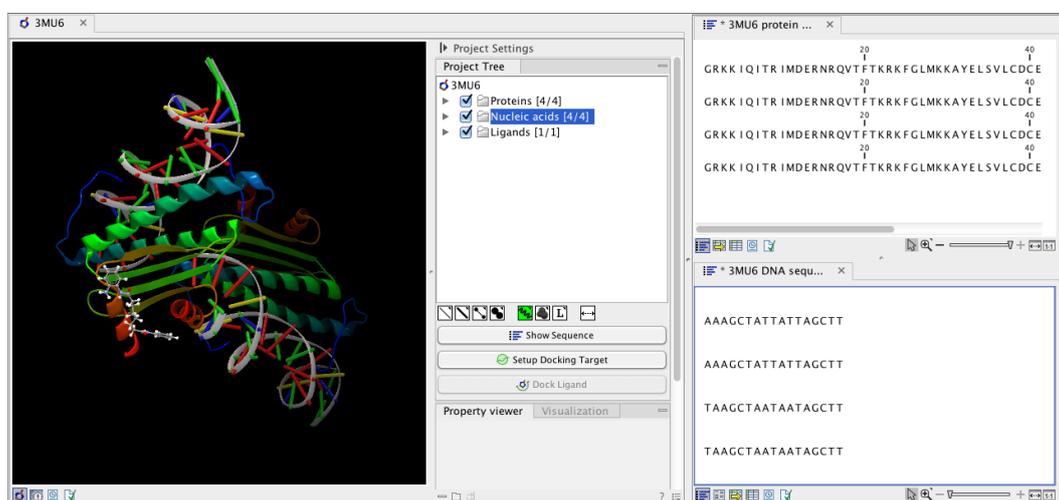


Figure 15.16: Protein chain sequences and DNA sequences are shown in separate views.

15.5.2 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 15.5.1) or Align to Existing Sequence.

Select a single protein chain in the project tree (see figure 15.17). 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 - section 15.5.1), and a sequence alignment is created between this sequence and the sequences or alignments selected from the Navigation Area. The

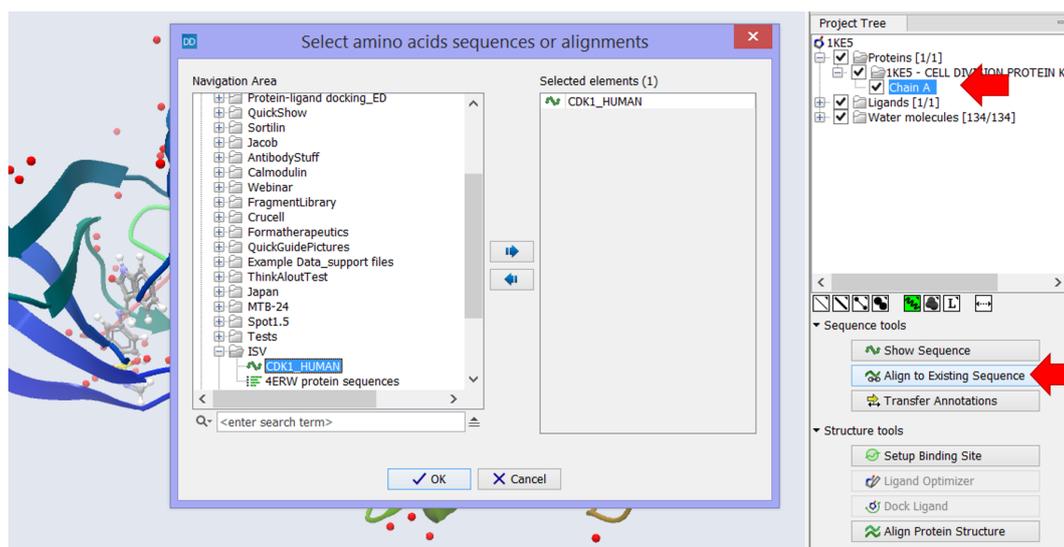


Figure 15.17: Select a single protein chain in the Project Tree and invoke "Align to Existing Sequence".

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

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 15.5.3). Notice, 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 15.20 and section 16.3.4).

15.5.3 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 12.3 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 15.5.1) or by mapping to an existing sequence or sequence alignment using the 'Align to Existing Sequence' button (see section 15.5.2).

Invoke the Transfer Annotations dialog by selecting a linked protein chain in the Project Tree and press 'Transfer Annotations' (see figure 15.18).

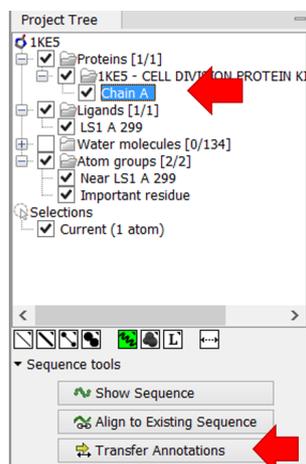


Figure 15.18: Select a single protein chain in the Project Tree and invoke "Transfer Annotations".

The dialog contains two tables (see figure 15.19). 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, changes to the visualization styles are allowed.

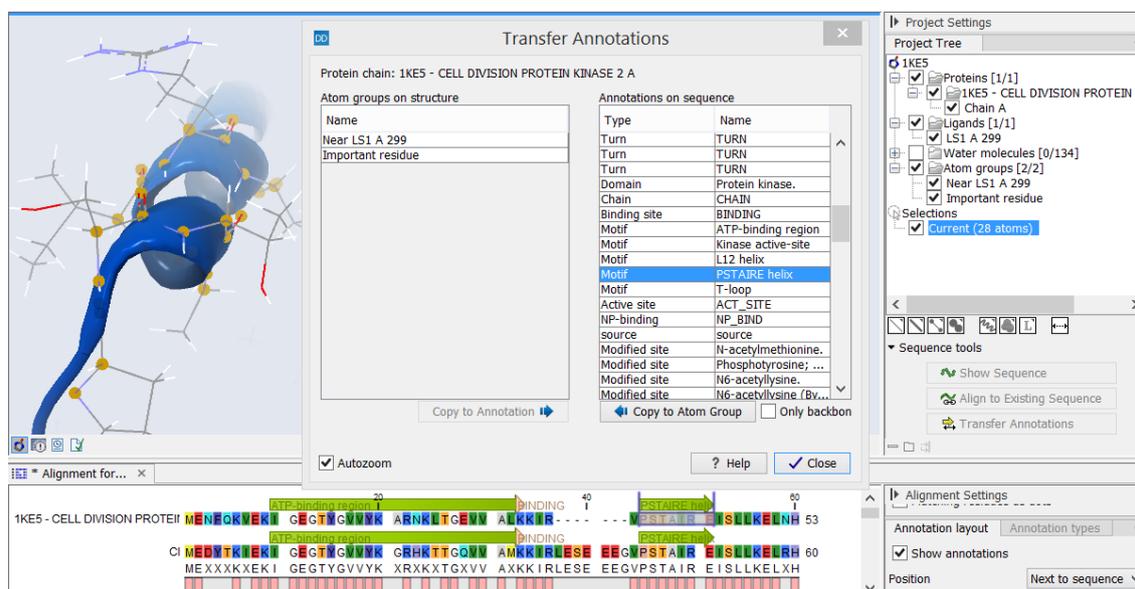


Figure 15.19: The Transfer Annotations dialog allow you to select annotations listed in the two tables, and copy them from structure to sequence or vice versa.

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 15.20 and section 16.3.4).

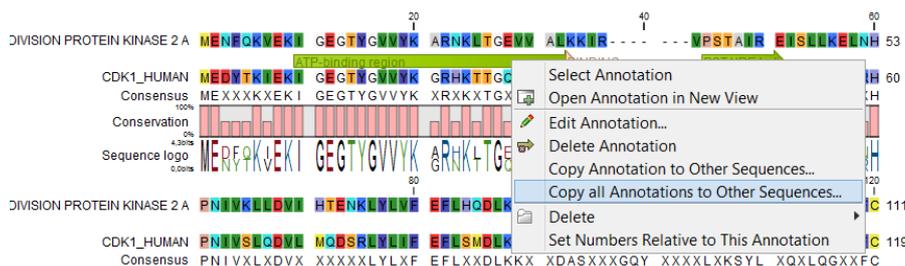


Figure 15.20: Copy annotations from sequences in the alignment to the sequence linked to the 3D view.

15.6 Protein structure alignment

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 15.21). 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.

15.6.1 The Align Protein Structure dialog box

The dialog box contains three fields:

- **Select reference (protein chain or atom group)** This drop-down menu shows all the protein 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,

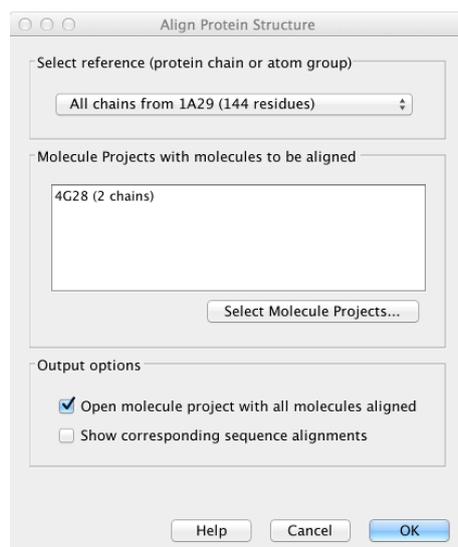


Figure 15.21: *The Align Protein Structure dialog box.*

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.

15.6.2 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 15.21. 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 15.22. 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).

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 15.23). Using the new atom group as the reference in the alignment dialog leads to the alignment shown in figure 15.24. 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.

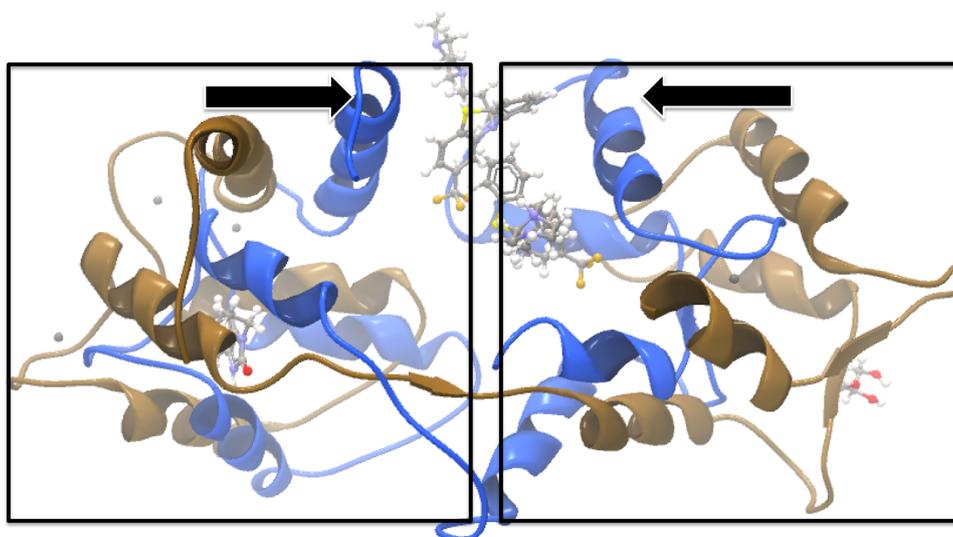


Figure 15.22: Global alignment of two calmodulin structures (blue and brown). The two domains of calmodulin (shown within black boxes) can undergo large changes in relative orientation. In this case, the different orientation of the domains in the blue and brown structures makes a good global alignment 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.

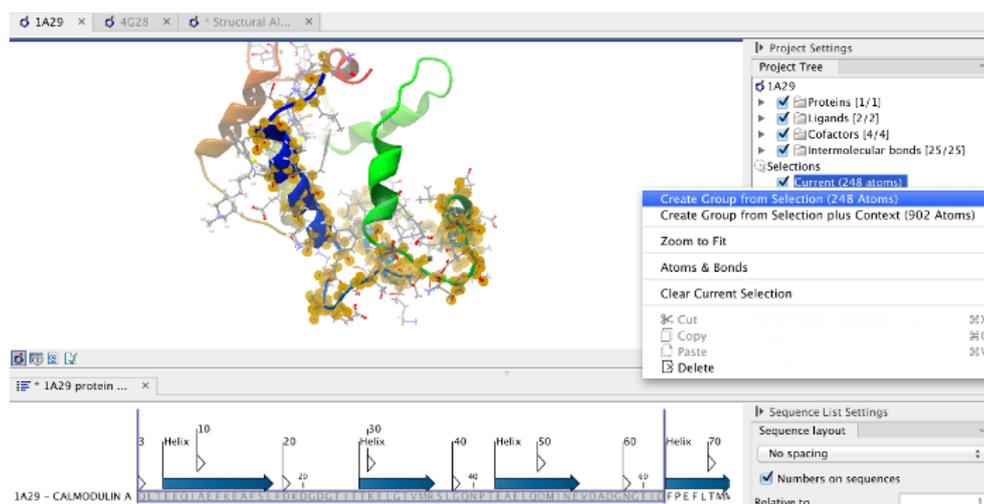


Figure 15.23: Creation of an atom group containing the N-terminal domain of calmodulin.

Aligning a binding site Two bound calcium atoms, one from each calmodulin structure, are shown in the black box of figure 15.24. 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 15.25.

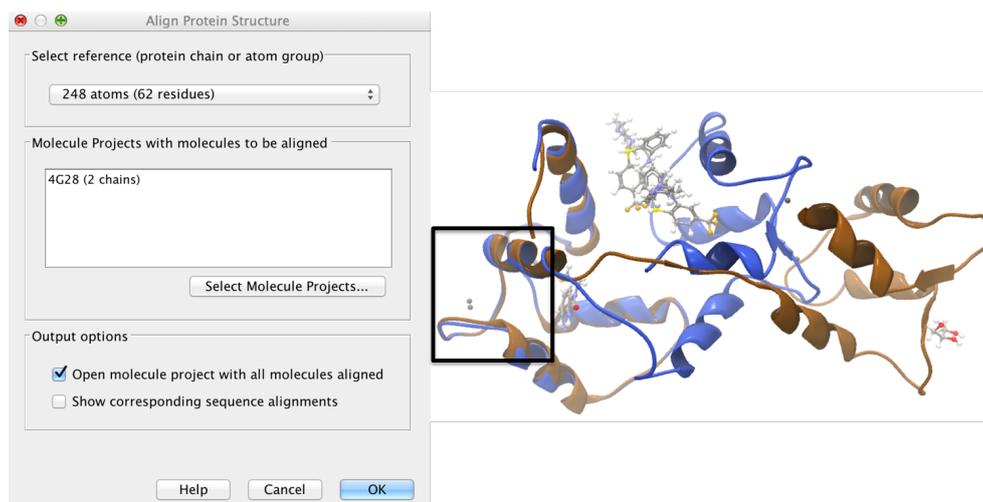


Figure 15.24: Alignment of the same two calmodulin proteins as in figure 15.22, but this time with a focus on the N-terminal domain. The blue and brown structures are now well-superimposed in the N-terminal region. The black box encloses two calcium atoms that are bound to the structures.

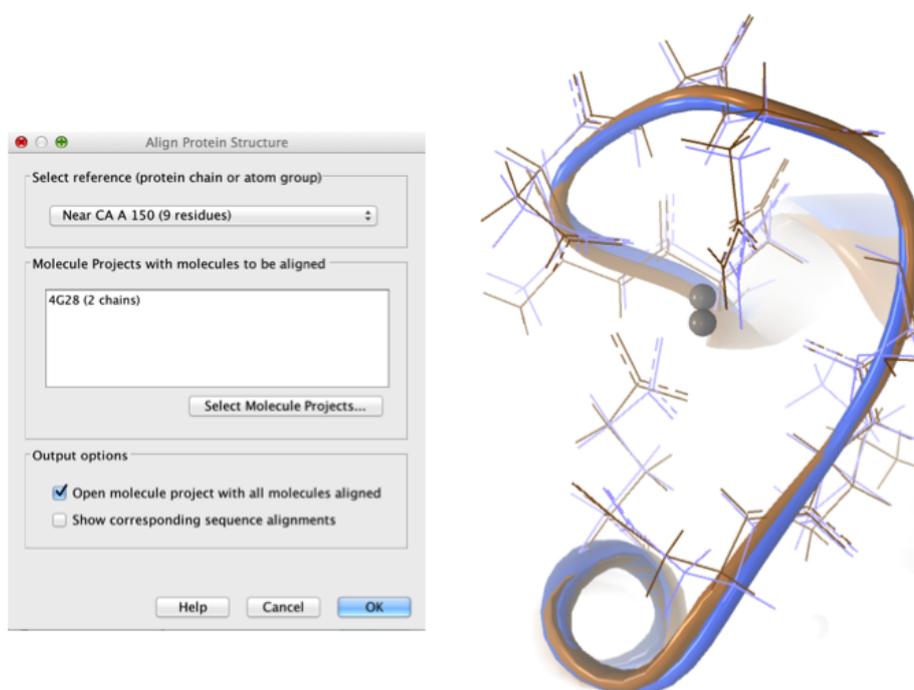


Figure 15.25: Alignment of the same two calmodulin domains as in figure 15.22, but this time with a focus on the calcium atom within the black box of figure 15.24. The calcium atoms are less than 1 Å apart – compatible with thermal motion encoded in the atoms' temperature factors.

15.6.3 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).

Chapter 16

Sequence alignment

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CLC Main Workbench can align nucleotides and proteins using a *progressive alignment* algorithm (see section 16.6 or read the White paper on alignments in the **Science** section of <http://www.clcbio.com>).

This chapter describes how to use the program to align sequences. The chapter also describes alignment algorithms in more general terms.

16.1 Create an alignment

Alignments can be created from sequences, sequence lists (see section 12.6), existing alignments and from any combination of the three.

To create an alignment in *CLC Main Workbench*:

Toolbox | Alignments and Trees (📁) | Create Alignment (🔍)

This opens the dialog shown in figure 16.1.

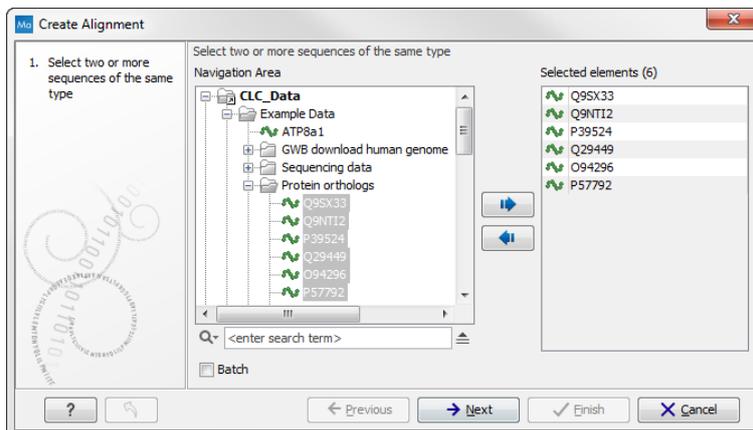


Figure 16.1: Creating an alignment.

If you have selected some elements before choosing the Toolbox action, they are now listed in the **Selected Elements** window of the dialog. Use the arrows to add or remove sequences, sequence lists or alignments from the selected elements. Click **Next** to adjust alignment algorithm parameters. Clicking **Next** opens the dialog shown in figure 16.2.

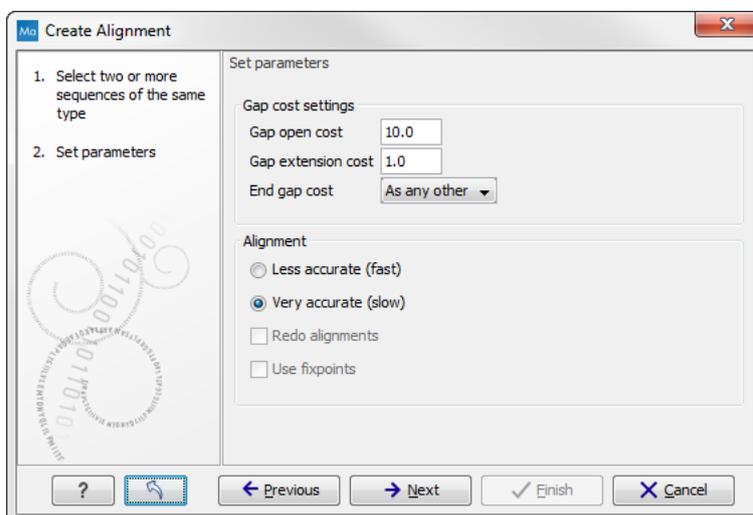


Figure 16.2: Adjusting alignment algorithm parameters.

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.

16.1.2 Fast or accurate alignment algorithm

CLC Main Workbench has two algorithms for calculating alignments:

- **Fast (less accurate).** This allows for use of an optimized alignment algorithm which is very fast. The fast option is particularly useful for data sets with very long sequences.
- **Slow (very accurate).** This is the recommended choice unless you find the processing time too long.

Both algorithms use progressive alignment. The faster algorithm builds the initial tree by doing more approximate pairwise alignments than the slower option.

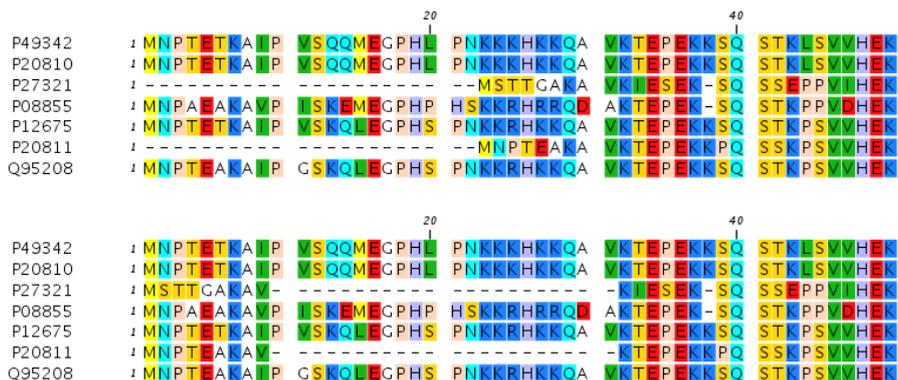


Figure 16.3: The first 50 positions of two different alignments of seven calpastatin sequences. The top alignment is made with cheap end gaps, while the bottom alignment is made with end gaps having the same price as any other gaps. In this case it seems that the latter scoring scheme gives the best result.

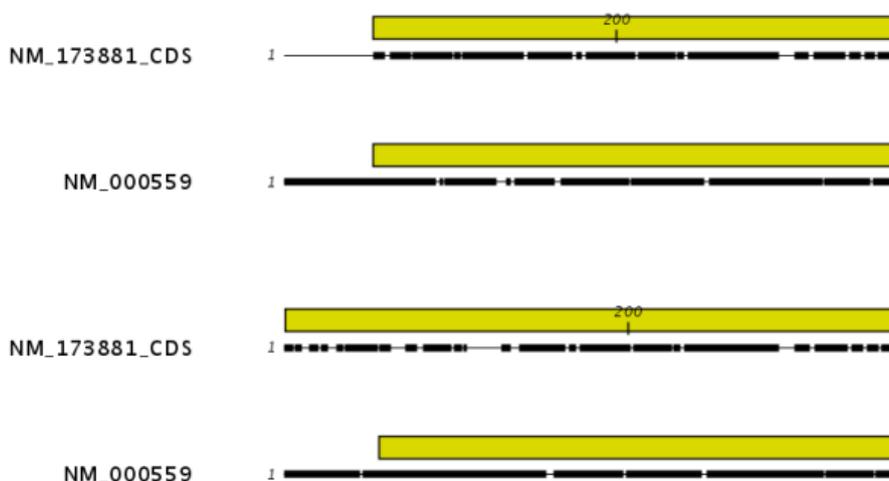


Figure 16.4: The alignment of the coding sequence of bovine myoglobin with the full mRNA of human gamma globin. The top alignment is made with free end gaps, while the bottom alignment is made with end gaps treated as any other. The yellow annotation is the coding sequence in both sequences. It is evident 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

If you have selected an existing alignment in the first step (16.1), you have to decide how this alignment should be treated.

- **Redo alignment.** The original alignment will be realigned if this checkbox is checked. Otherwise, the original alignment is kept in its original form except for possible extra equally sized gaps in all sequences of the original alignment. This is visualized in figure 16.5.

This feature is useful if you wish to add extra sequences to an existing alignment, in which case you just select the alignment and the extra sequences and choose not to redo the alignment.

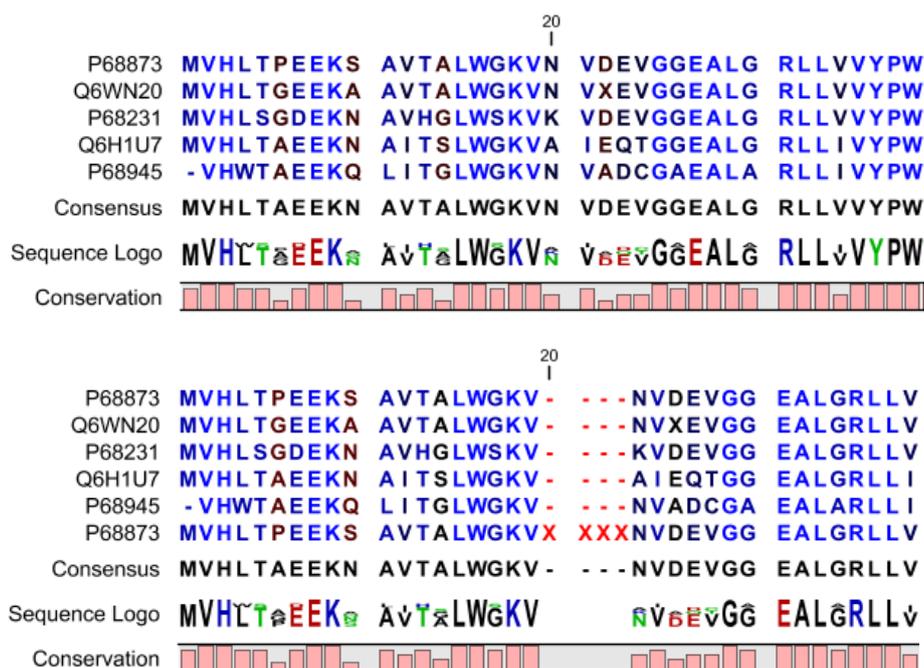


Figure 16.5: The top figures shows the original alignment. In the bottom panel a single sequence with four inserted X's are aligned to the original alignment. This introduces gaps in all sequences of the original alignment. All other positions in the original alignment are fixed.

It is also useful if you have created an alignment where the gaps are not placed correctly. In this case, you can realign the alignment with different gap cost parameters.

16.1.4 Fixpoints

With fixpoints, you can get full control over the alignment algorithm. The fixpoints are points on the sequences that are forced to align to each other.

Fixpoints are added to sequences or alignments before clicking "Create alignment". To add a fixpoint, open the sequence or alignment and:

Select the region you want to use as a fixpoint | right-click the selection | Set alignment fixpoint here

This will add an annotation labeled "Fixpoint" to the sequence (see figure 16.6). Use this procedure to add fixpoints to the other sequence(s) that should be forced to align to each other.

When you click "Create alignment" and go to **Step 2**, check **Use fixpoints** in order to force the alignment algorithm to align the fixpoints in the selected sequences to each other.

In figure 16.7 the result of an alignment using fixpoints is illustrated.

You can add multiple fixpoints, e.g. adding two fixpoints to the sequences that are aligned will force their first fixpoints to be aligned to each other, and their second fixpoints will also be aligned to each other.

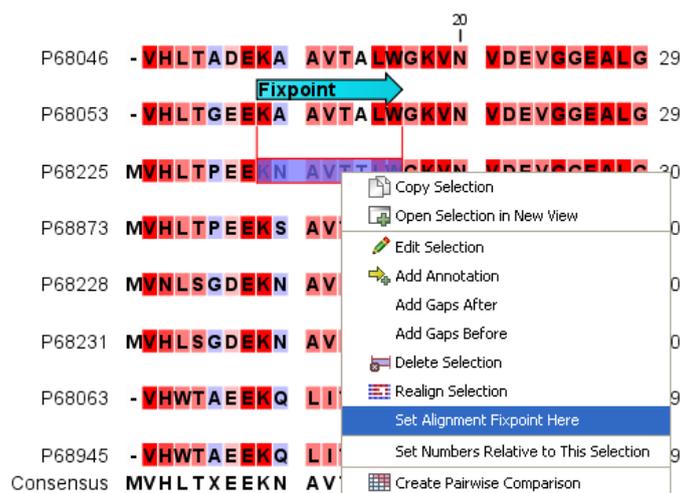


Figure 16.6: Adding a fixpoint to a sequence in an existing alignment. At the top you can see a fixpoint that has already been added.

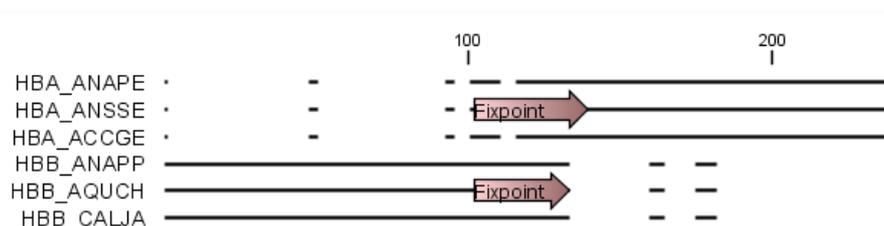
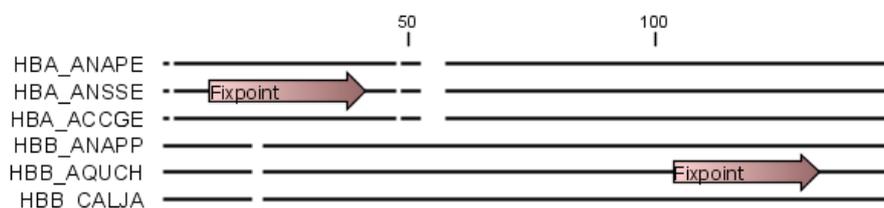


Figure 16.7: Realigning using fixpoints. In the top view, fixpoints have been added to two of the sequences. In the view below, the alignment has been realigned using the fixpoints. The three top sequences are very similar, and therefore they follow the one sequence (number two from the top) that has a fixpoint.

Advanced use of fixpoints

Fixpoints with the same names will be aligned to each other, which gives the opportunity for great control over the alignment process. It is only necessary to change any fixpoint names in very special cases.

One example would be three sequences A, B and C where sequences A and B has one copy of a domain while sequence C has two copies of the domain. You can now force sequence A to align to the first copy and sequence B to align to the second copy of the domains in sequence C. This is done by inserting fixpoints in sequence C for each domain, and naming them 'fp1' and 'fp2' (for example). Now, you can insert a fixpoint in each of sequences A and B, naming them 'fp1' and 'fp2', respectively. Now, when aligning the three sequences using fixpoints, sequence A will

align to the first copy of the domain in sequence C, while sequence B would align to the second copy of the domain in sequence C.

You can name fixpoints by:

right-click the Fixpoint annotation | Edit Annotation (👉) | type the name in the 'Name' field

16.2 View alignments

Since an alignment is a display of several sequences arranged in rows, the basic options for viewing alignments are the same as for viewing sequences. Therefore we refer to section [12.1](#) for an explanation of these basic options.

However, there are a number of alignment-specific view options in the **Alignment info** and the **Nucleotide info** in the **Side Panel** to the right of the view. Below is more information on these view options.

Under **Translation** in the **Nucleotide info**, 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.

The options in the **Alignment info** relate to each column in the alignment:

- **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. E.g. 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 [H](#) and [G](#).) 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. Learn how to export the data behind the graph in section 7.4.
 - * **Height.** Specifies the height of the graph.
 - * **Type.** The type of the graph.
 - **Line plot.** Displays the graph as a line plot.
 - **Bar plot.** Displays the graph as a bar plot.
 - **Colors.** Displays the graph 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 (Learn how to export the data behind the graph in section 7.4).
 - * **Height.** Specifies the height of the graph.
 - * **Type.** The type of the graph.
 - **Line plot.** Displays the graph as a line plot.
 - **Bar plot.** Displays the graph as a line plot.
 - **Colors.** Displays the graph 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.

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 researchers 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 16.8). 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 16.8). 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 16.8 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.

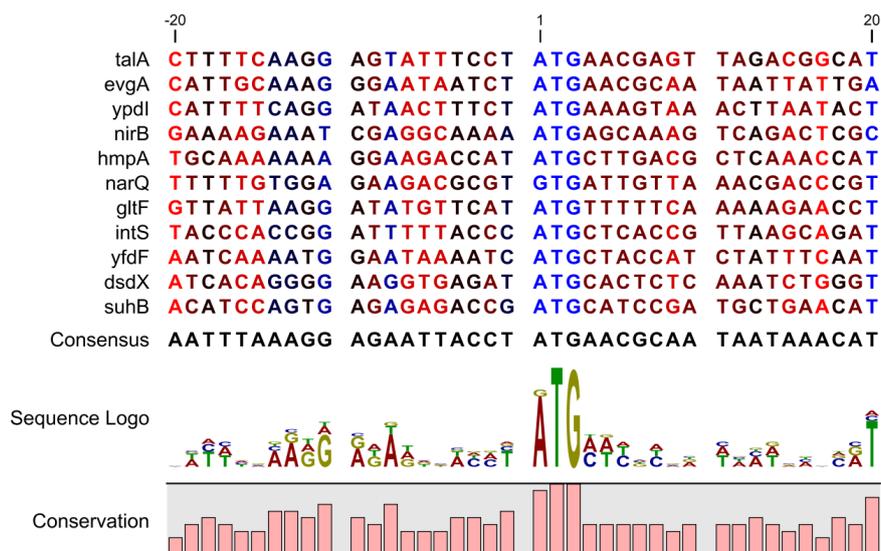


Figure 16.8: Ungapped sequence alignment of eleven *E. coli* sequences defining a start codon. The start codons start at position 1. Below the alignment is shown the corresponding sequence logo. As seen, a GTG start codon and the usual ATG start codons are present in the alignment. This can also be visualized in the logo at position 1.

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}),

$$R_{seq} = S_{max} - S_{obs} = \log_2 N - \left(- \sum_{n=1}^N p_n \log_2 p_n \right)$$

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 $\log_2 4 = 2 \text{ bits}$ for DNA/RNA and $\log_2 20 \approx 4.32 \text{ bits}$ 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 height 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

16.3.1 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 16.9).

Note! Residues can only be moved when they are next to a gap.

```

AGG GAGTCAT      AGG GAGTCAT
AGG GAGTCAT      AGG GAGTCAT
AGG GAGCAGT      AGG GAGCAGT
- - - - -        - - - - -
AGG GTACAGT      AGG GTACAGT
- - - GAGTAGC    - GA G - - TAGC
- - - GAGTAGC    - GA G - - TAGC
- - - GAGTAGC    - GA G - - TAGG
ATG GTGCACC      ATG GTGCACC
ATG GTGCATC      ATG GTGCATC
  
```

Figure 16.9: *Moving a part of an alignment. Notice the change of mouse pointer to a horizontal arrow.*

16.3.2 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 e.g. five residues, 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.

16.3.3 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'

16.3.4 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. That is, 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, not the new sequence, which may differ.

16.3.5 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

16.3.6 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

This can be undone by clicking **Undo** () in the Toolbar.

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.

16.3.7 Realign selection

If you have created an alignment, it is possible to realign a part of it, leaving the rest of the alignment unchanged:

select a part of the alignment to realign | right-click the selection | Realign selection

This will open **Step 2** in the "Create alignment" dialog, allowing you to set the parameters for the realignment (see section 16.1).

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.

Realigning a selection is a very powerful tool for editing alignments in several situations:

- **Removing changes.** If you change the alignment in a specific region by hand, you may end up being unhappy with the result. In this case you may of course undo your edits, but another option is to select the region and realign it.
- **Adjusting the number of gaps.** If you have a region in an alignment which has too many gaps in your opinion, you can select the region and realign it. By choosing a relatively high gap cost you will be able to reduce the number of gaps.
- **Combine with fixpoints.** If you have an alignment where two residues are not aligned, but you know that they should have been. You can now set an alignment fixpoint on each of the two residues, select the region and realign it using the fixpoints. Now, the two residues are aligned with each other and everything in the selected region around them is adjusted to accommodate this change.

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 by:

Toolbox | Alignments and Trees (📁) | Join Alignments (🔗)

This opens the dialog shown in figure 16.10.

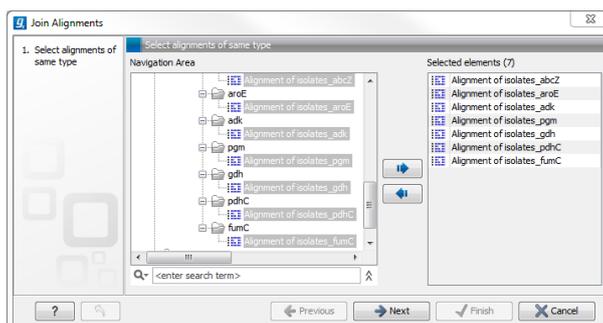


Figure 16.10: Selecting two alignments to be joined.

If you have selected some alignments before choosing the Toolbox action, they are now listed 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 16.11.

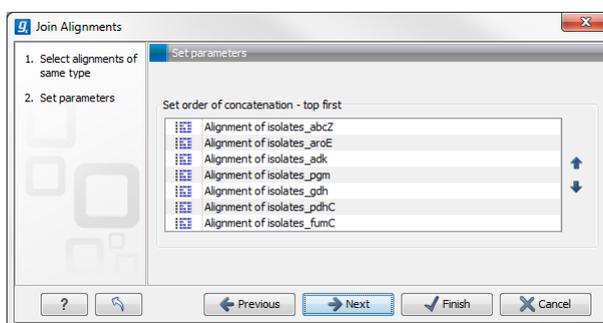


Figure 16.11: Selecting order of concatenation.

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 16.12.

16.4.1 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 shown in figure 16.12

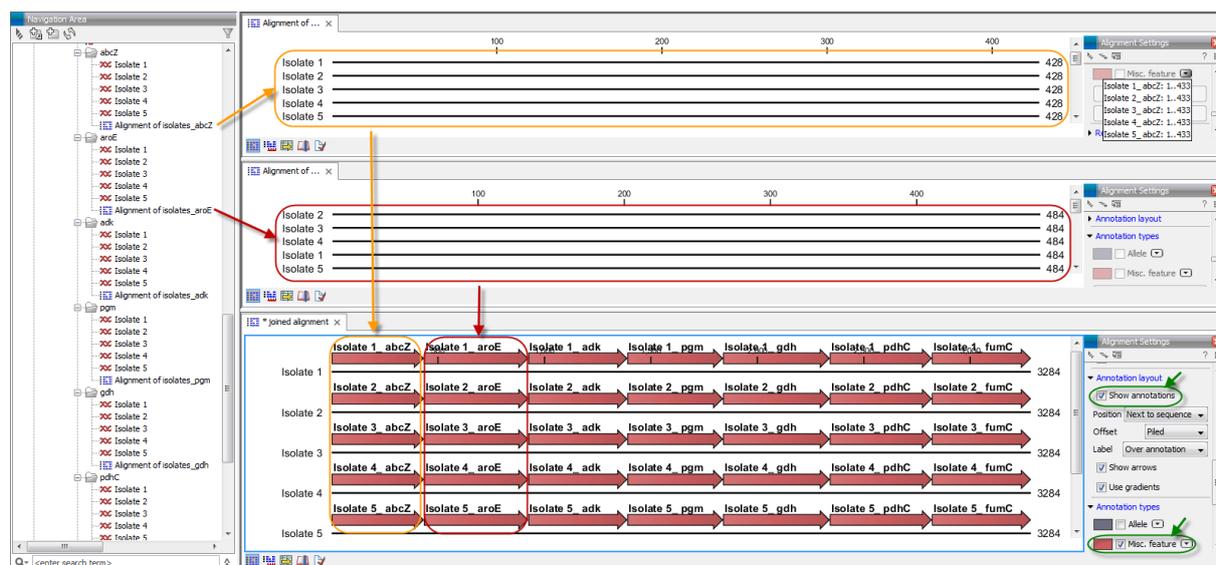


Figure 16.12: The upper part of the figure shows two of the seven alignments for the genes "abcZ" and "aroE" respectively. Each alignment consists of sequences from one gene from five different isolates. The lower part of the figure shows the result of "Join Alignments". Seven genes have been joined 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.

"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:

Toolbox | Alignments and Trees (📄) | Create Pairwise Comparison (📊)

This opens the dialog displayed in figure 16.13:

If an alignment was selected before choosing the Toolbox action, this alignment is now 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.

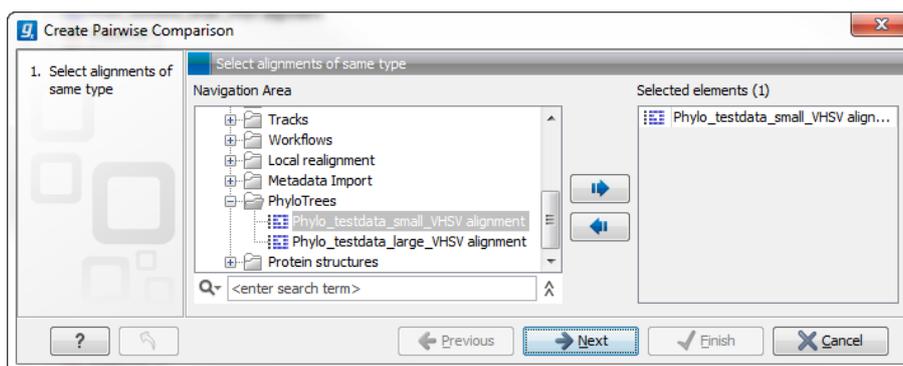


Figure 16.13: Creating a pairwise comparison table.

16.5.1 Pairwise comparison on alignment selection

A pairwise comparison can also be performed for a selected part of an alignment:

right-click on an alignment selection | Pairwise Comparison ()

This leads directly to the dialog described in the next section.

16.5.2 Pairwise comparison parameters

There are five kinds of comparison that can be made between the sequences in the alignment, as shown in figure 16.14.

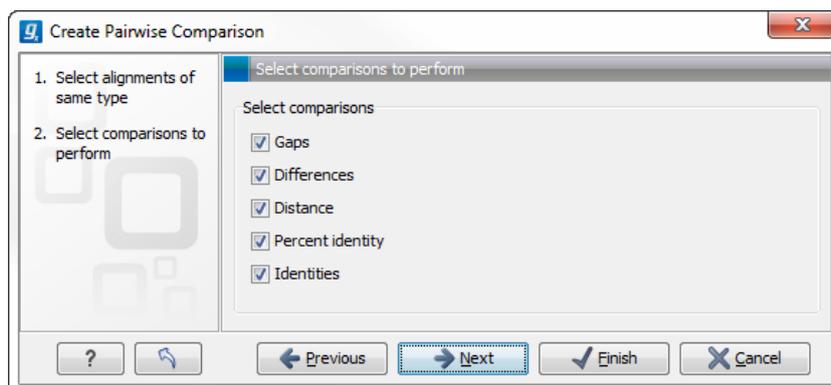


Figure 16.14: Adjusting parameters for pairwise comparison.

- **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.

16.5.3 The pairwise comparison table

The table shows the results of selected comparisons (see an example in figure 16.15). 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.

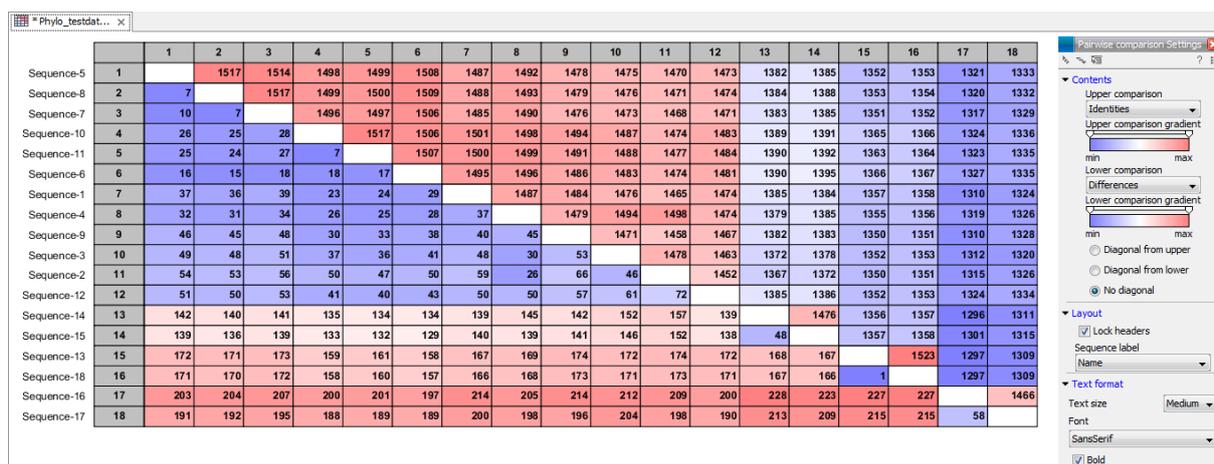


Figure 16.15: A pairwise comparison table.

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.

16.6 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 16.16) 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.

16.6.1 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.

16.6.2 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

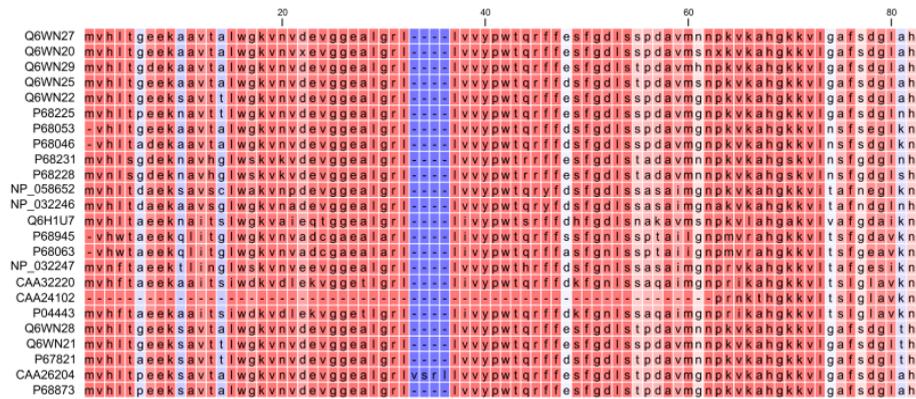


Figure 16.16: The tabular format of a multiple alignment of 24 Hemoglobin protein sequences. Sequence names appear at the beginning of each row and the residue position is indicated by the numbers at the top of the alignment columns. The level of sequence conservation is shown on a color scale with blue residues being the least conserved and red residues being the most conserved.

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.

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

Presently, the most exciting development in multiple alignment methodology is the construction of *statistical alignment* algorithms [Hein, 2001], [Hein et al., 2000]. These algorithms employ a scoring function which incorporates the underlying phylogeny and use an explicit stochastic model of molecular evolution which makes it possible to compare different solutions in a statistically rigorous way. The optimization step, however, still relies on dynamic programming and practical use of these algorithms thus awaits further developments.

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Chapter 17

Phylogenetic trees

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17.1 Phylogenetic tree features

Phylogenetics describes the taxonomical 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 [17.2.5](#) 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.

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- Coloring and labeling of subtrees.
- Curved edges.
- Editable node sizes and line width.
- Intelligent visualization of overlapping labels and nodes.

17.2 Create Trees

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 using one of two distance based methods (see "Bioinformatics explained" in section 17.2.5).

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 17.2.5). 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 17.2.5). 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 related, 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 17.2.4 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 17.2.3) in order to identify the best suitable models for creating a tree.
- **Create Tree** (🌲): Is a tool that uses distance estimates computed from multiple alignments to create trees. 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 17.2.5).

17.2.1 K-mer Based Tree Construction

The "K-mer Based Tree Construction" 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:

Toolbox | Alignments and Trees (📁) | K-mer Based Tree Construction (🌲)

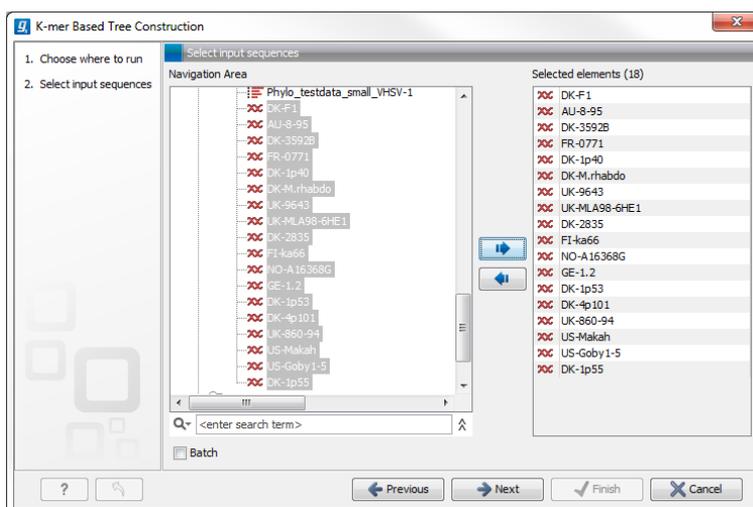


Figure 17.1: Creating a tree with K-mer based tree construction. Select sequences.

Select sequences or a sequence list (figure 17.1):

Next, select the construction method, specify the k-mer length and select a distance measure for tree construction (figure 17.2):

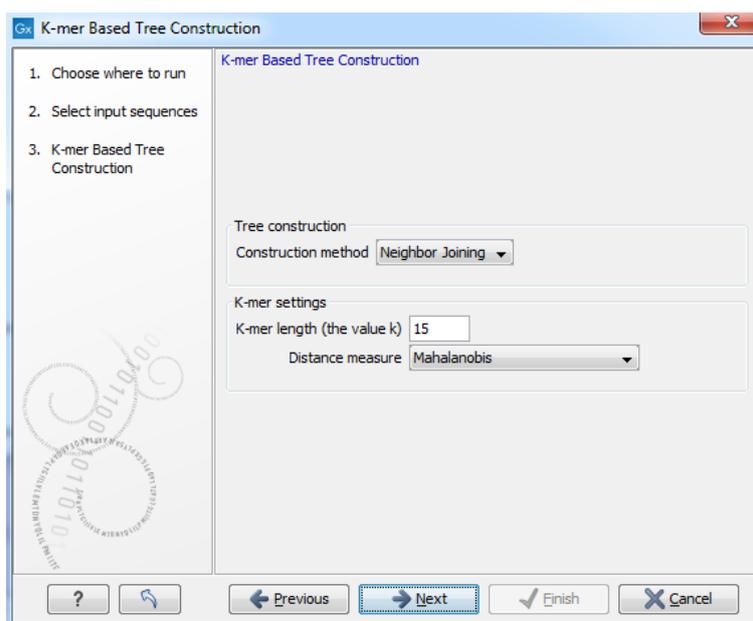


Figure 17.2: Creating a tree with K-mer based tree construction. Select construction method, specify the k-mer length and select a distance measure.

- **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 17.2.5):

- * 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 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 17.2.5 for further details.

17.2.2 Create tree

The "Create tree" tool can be used to generate a distance-based phylogenetic tree with multiple alignments as input:

Toolbox | Alignments and Trees (📁) | Create Tree (🌳)

This will open the dialog displayed in figure 17.3:

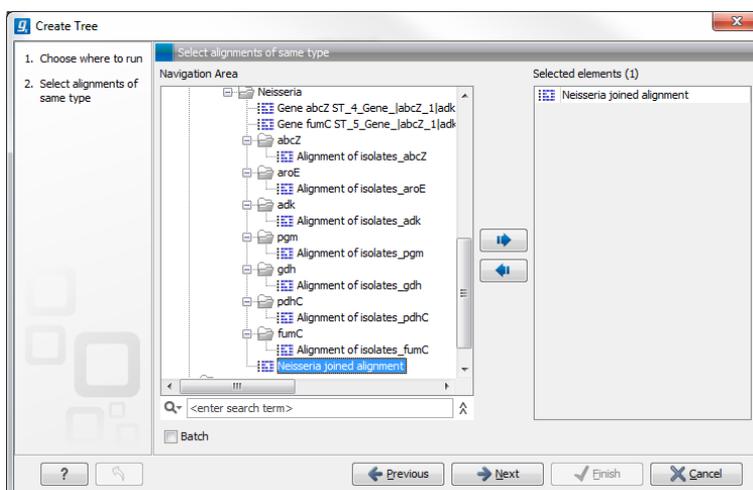


Figure 17.3: Creating a tree.

If an alignment was selected before choosing the Toolbox action, this alignment is now 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.

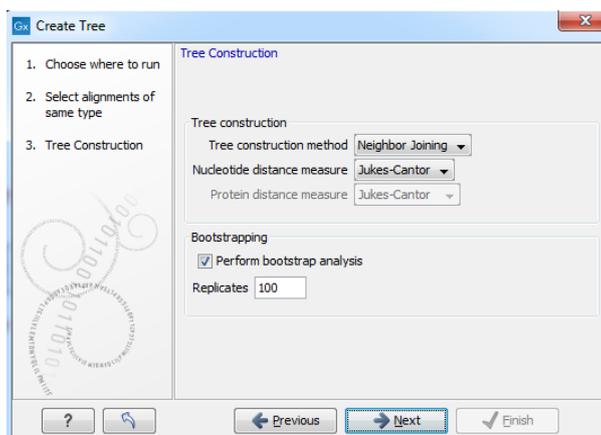


Figure 17.4: Adjusting parameters for distance-based methods.

Figure 17.4 shows the parameters that can be set for this distance-based tree creation:

- Tree construction (see section [17.2.5](#))
 - Tree construction method
 - * The **UPGMA** method. Assumes constant rate of evolution.
 - * The **Neighbor Joining** method. Well suited for trees with varying rates of evolution.
 - 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 [17.2.5](#)). 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 [17.2.5](#).

17.2.3 Model Testing

As the "Model Testing" tool can help identify the best substitution model ([17.2.5](#)) to be used for "Maximum Likelihood Phylogeny" tree construction, it is recommended to do "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:

Toolbox | Alignments and Trees (📁) | Model Testing (🔧)

Select the alignment that you wish to use for the tree construction (figure 17.5):

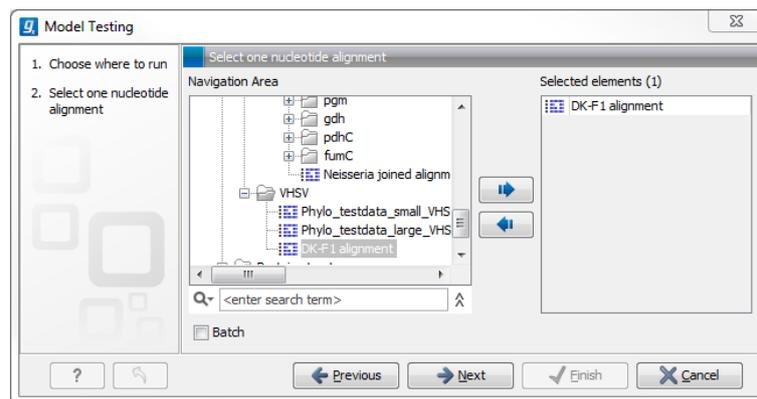


Figure 17.5: Select alignment for model testing.

Specify the parameters to be used for model testing (figure 17.6):

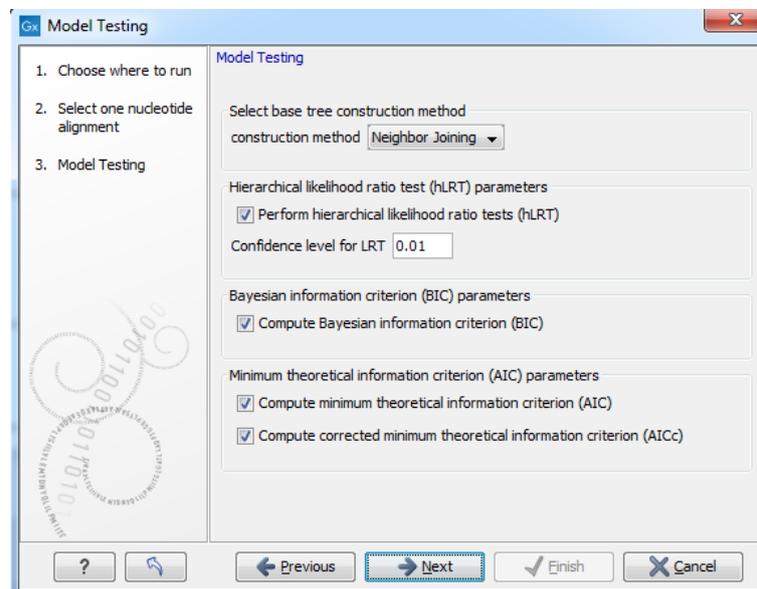


Figure 17.6: Specify parameters for model testing.

- **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 $BIC = -2\ln(L) + K\ln(n)$, 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 $AIC = -2\ln(L) + 2K$, 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 (AICc)** Rank substitution models based on minimum corrected theoretical information criterion (AICc). Formula used is $AICc = -2\ln(L) + 2K + 2K(K+1)/(n-K-1)$, 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.

17.2.4 Maximum Likelihood Phylogeny

To generate a maximum likelihood based phylogenetic tree:

Toolbox | Alignments and Trees (📁) | Maximum Likelihood Phylogeny (🔍)

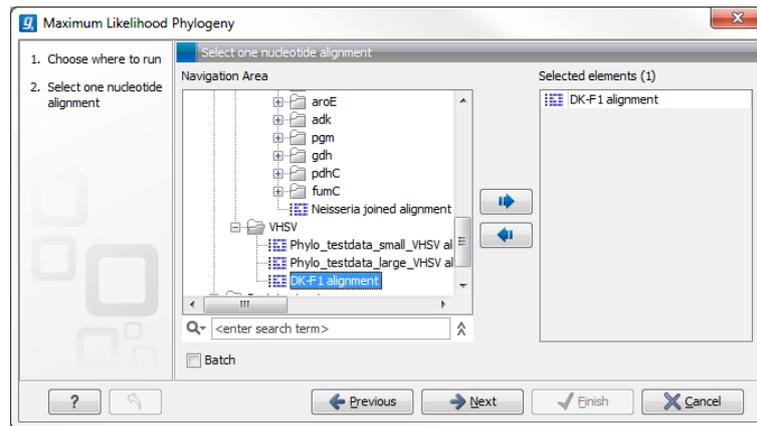


Figure 17.7: Select the alignment for tree construction

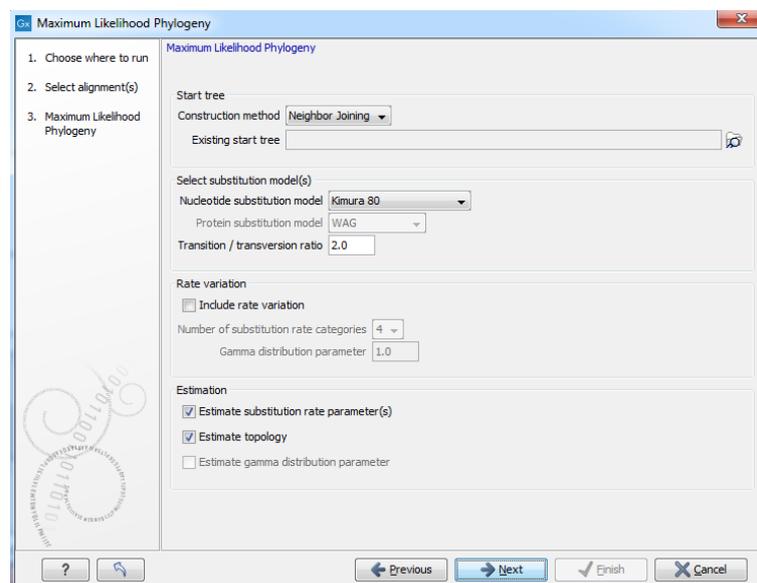


Figure 17.8: Adjusting parameters for maximum likelihood phylogeny

The following parameters can be set for the maximum likelihood based phylogenetic tree (see figure 17.8):

- **Starting tree**

- **Construction method** Specify the tree construction method which should be used to create the initial tree. There are two possibilities:
 - * Neighbor Joining
 - * 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 use the browser function to identify the desired starting tree.

- **Select substitution model**

- **Nucleotide 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 transition/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 17.2.5.

- **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).

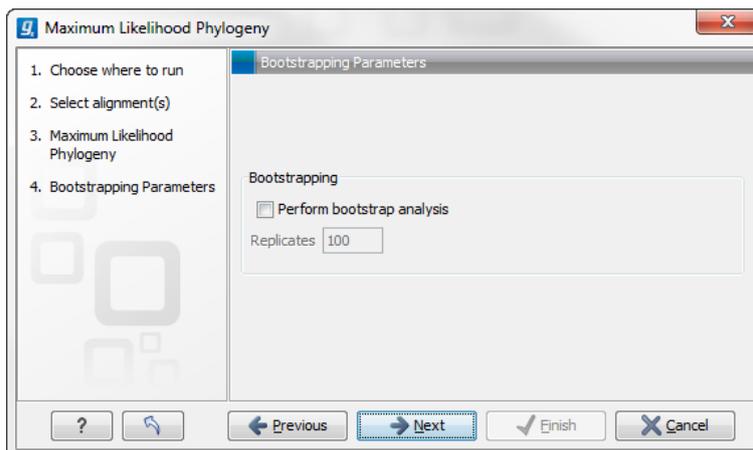


Figure 17.9: Adjusting parameters for ML phylogeny

- **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 17.2.5). 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.

17.2.5 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 taxonomical units. In this case, the terminal nodes (also called leaves or tips of the tree) represent extant species of *Hominidae* and are the *operational taxonomical units* (OTUs). The internal nodes, which here represent extinct common ancestors of the great apes, are termed *hypothetical taxonomical units* since they are not directly observable.

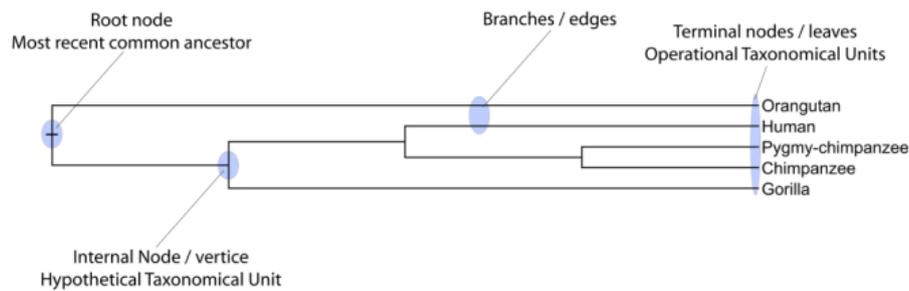


Figure 17.10: A proposed phylogeny of the great apes (*Hominidae*). Different components of the tree are marked, see text for description.

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.

Modern usage of phylogenies

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 were 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) occurring 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 17.2.5).

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 lengths. 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 $P(\text{tree}|\text{data})$ 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 $P(\text{tree}|\text{data})$ so instead ML based methods have to compute the probability of the data given a tree, i.e. $P(\text{data}|\text{tree})$. 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 and to help users choose the correct model for a data, set the "Model Testing" tool (see section 17.2.3) can be used to test a range of different models for nucleotide input 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 resampling 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.

17.3 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 Tree Settings Side Panel found in the left side of the view area can be used to adjust the tree layout.

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.

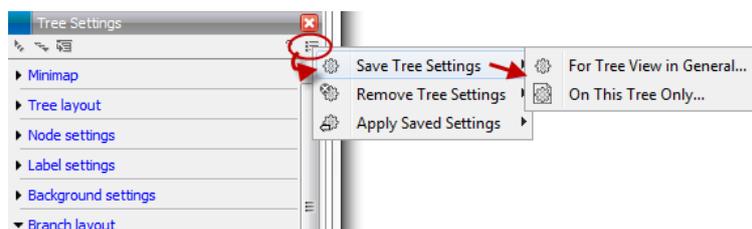


Figure 17.11: Save, remove or apply preferred layout settings.

Tree Settings contains the following categories:

- Minimap
- Tree layout
- Node settings
- Label settings
- Background settings
- Branch layout
- Bootstrap settings
- Metadata

17.3.1 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.

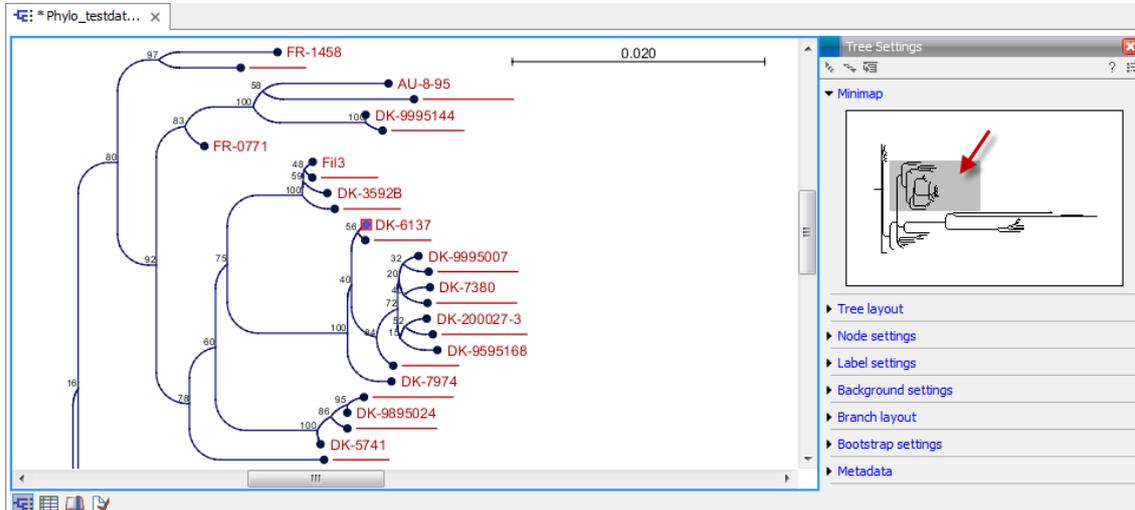


Figure 17.12: Visualization of a phylogenetic tree. The grey square in the Minimap shows the part of the tree that is shown in the View Area.

17.3.2 Tree layout

The **Tree Layout** can be adjusted in the Side Panel (figure 17.13).

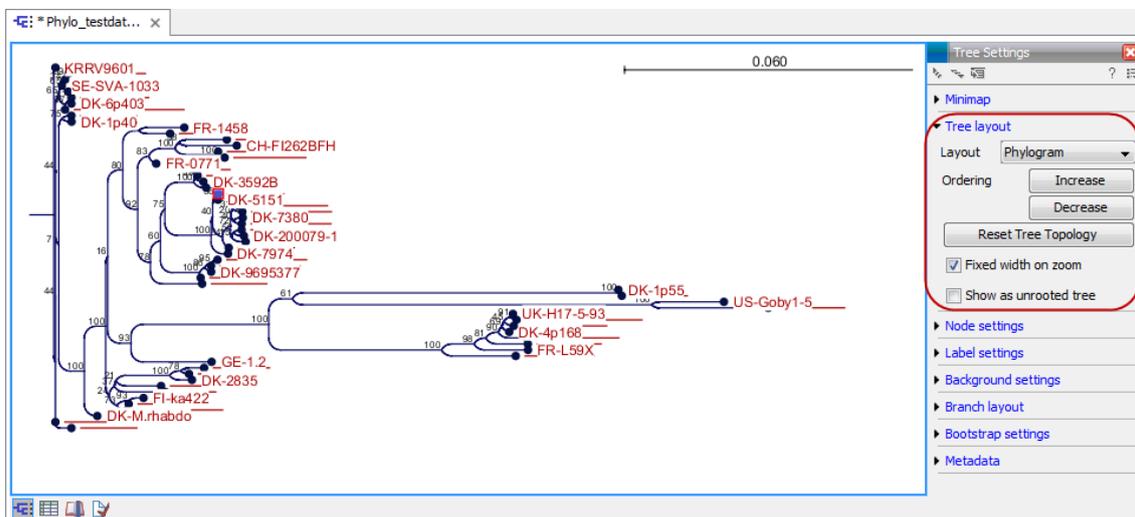


Figure 17.13: The tree layout can be adjusted in the Side Panel. Five different layouts can be selected and the node order can be changed to increasing or decreasing. The tree topology and node order can be reverted to the original view with the button labeled "Reset Tree Topology".

- **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 17.15) or **Decreasing**.
 - **Reset Tree Topology** Resets to the default tree topology and node order (see figure 17.15).
 - **Ordering** The nodes can be ordered after the branch length; either **Increasing** (shown in figure 17.15) or **Decreasing**.
 - **Reset Tree Topology** Resets to the default tree topology and node order (see figure 17.15).
 - **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.

17.3.3 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 17.16.

17.3.4 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.



Figure 17.14: 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.

- **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 17.18).
- **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 17.3.9).
- **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.

When working with big trees there is typically not enough space to show all labels. As illustrated in figure 17.18, only some of the labels are shown. The hidden labels are illustrated with thin horizontal lines (figure 17.19).

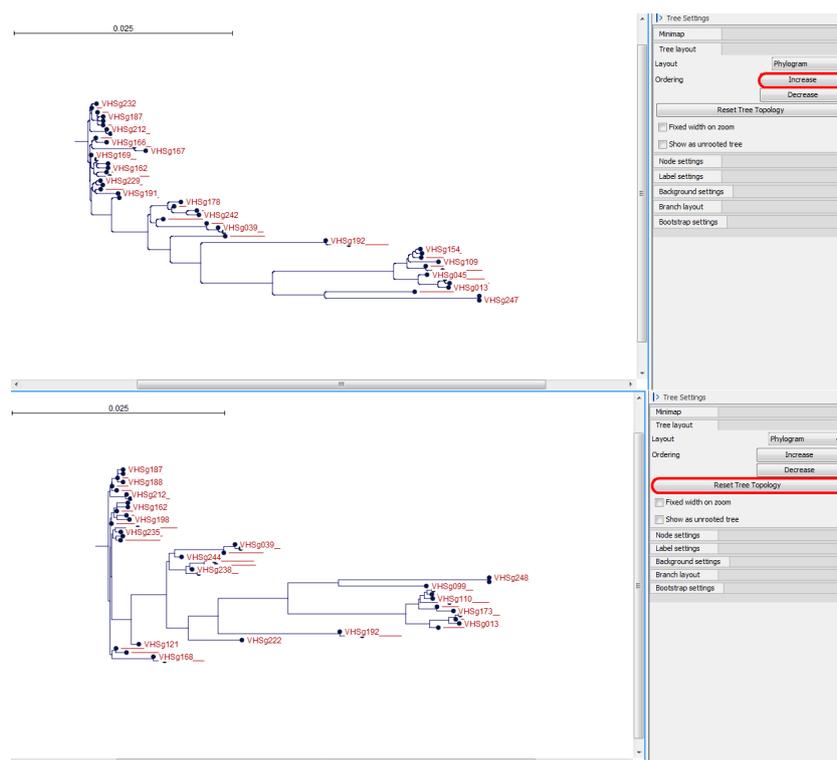


Figure 17.15: The tree layout can be adjusted in the Side Panel. The top part of the figure shows a tree with increasing node order. In the bottom part of the figure the tree has been reverted to the original tree topology.

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 17.19 and figure 17.20). 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 17.3.5).

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.

17.3.5 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.
- **Show label background** Show a background color for each label. Once ticked, it is possible to specify a background color.

17.3.6 Branch layout

- **Branch length font settings** Specify/adjust font type, size and typography (Bold, Italic or normal).

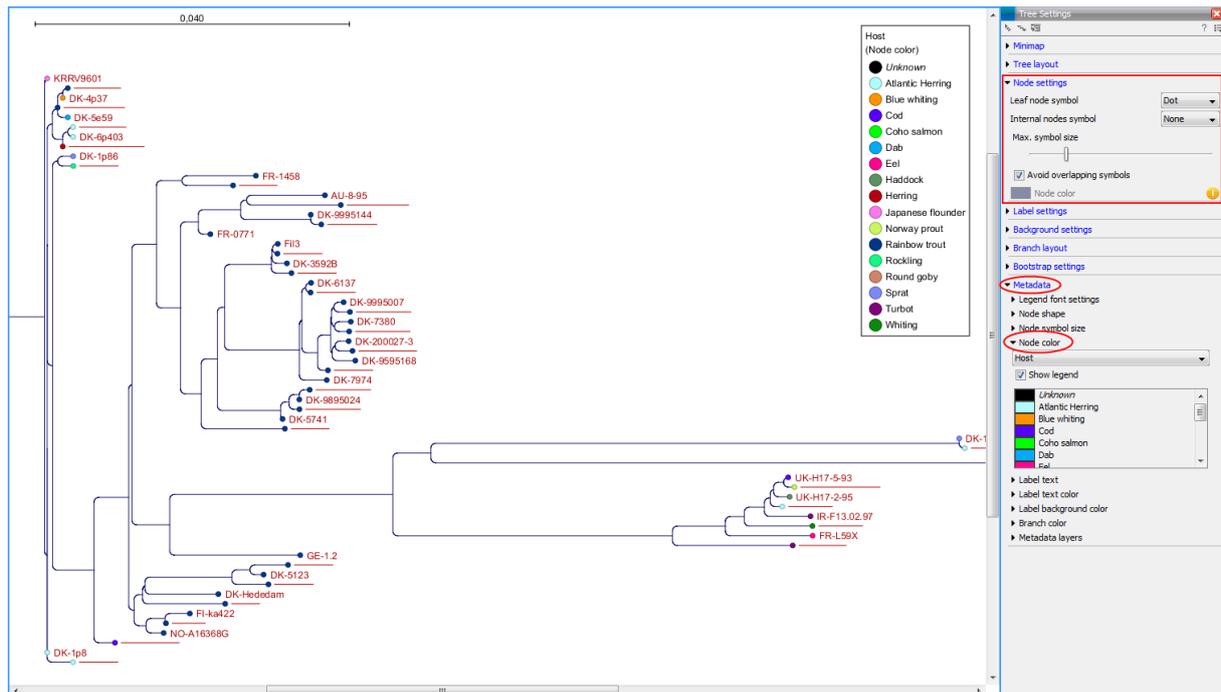


Figure 17.16: The Node Layout settings. Node color is specified by metadata and is therefore inactive in this example.

- **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 17.22.

17.3.7 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

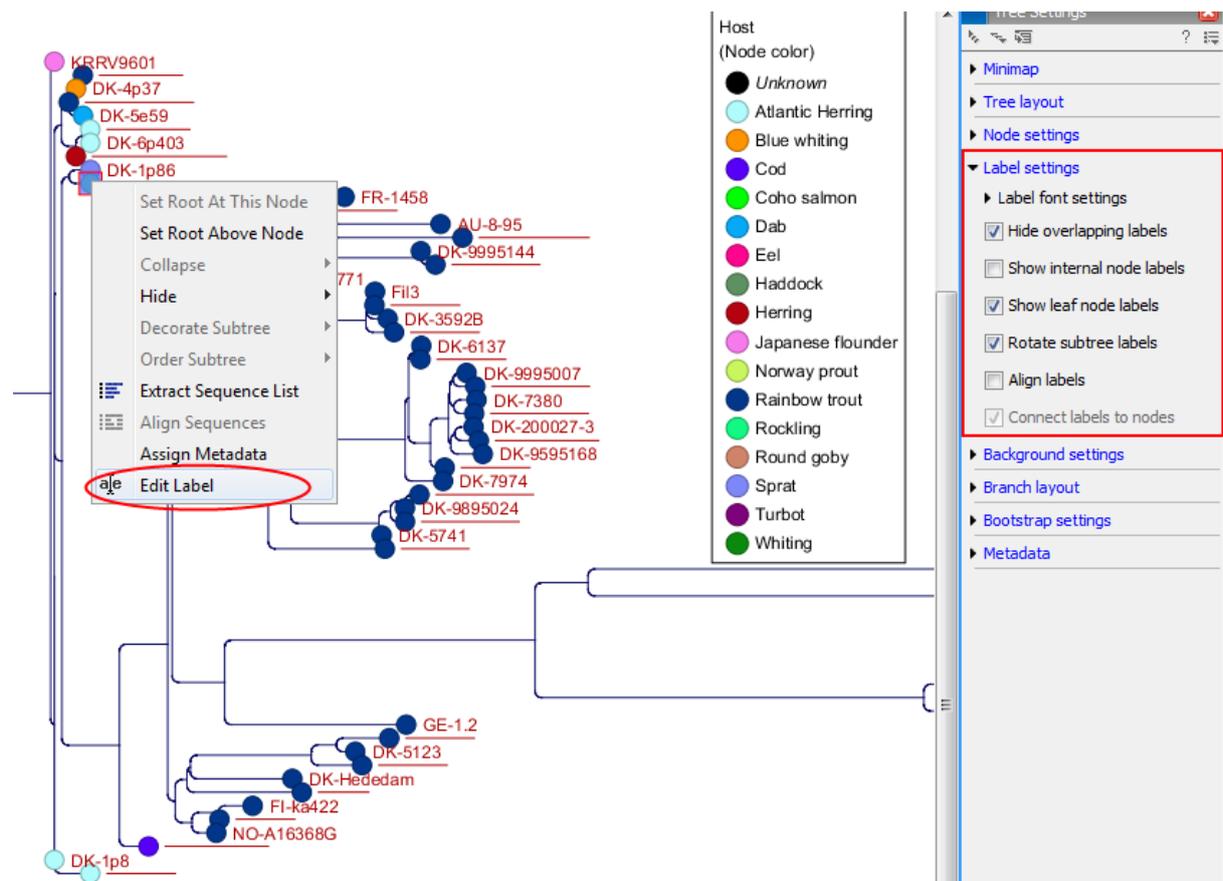


Figure 17.17: "Edit label" in the right click menu can be used to customize the label text. The way node labels are displayed can be controlled through the labels settings in the right side panel.

- **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.

17.3.8 Visualizing metadata

Metadata associated with a phylogenetic tree (described in detail in section 17.4) 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.

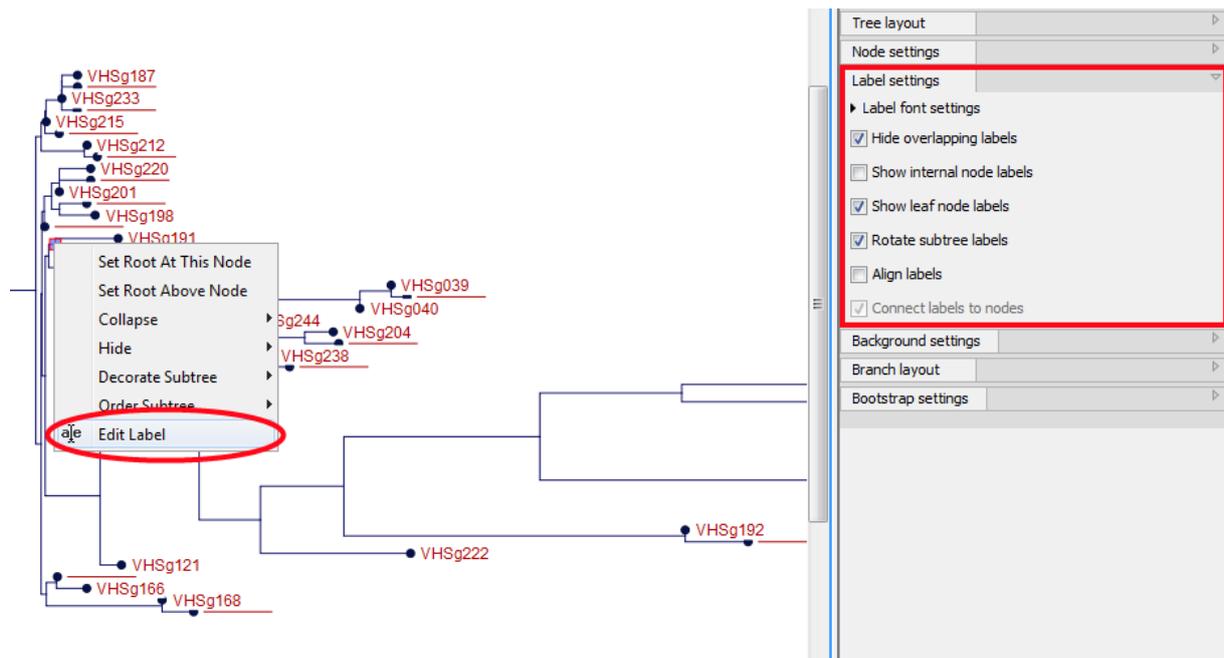


Figure 17.18: "Edit label" in the right click menu can be used to customize the label text. The way node labels are displayed can be controlled through the labels settings in the right side panel.

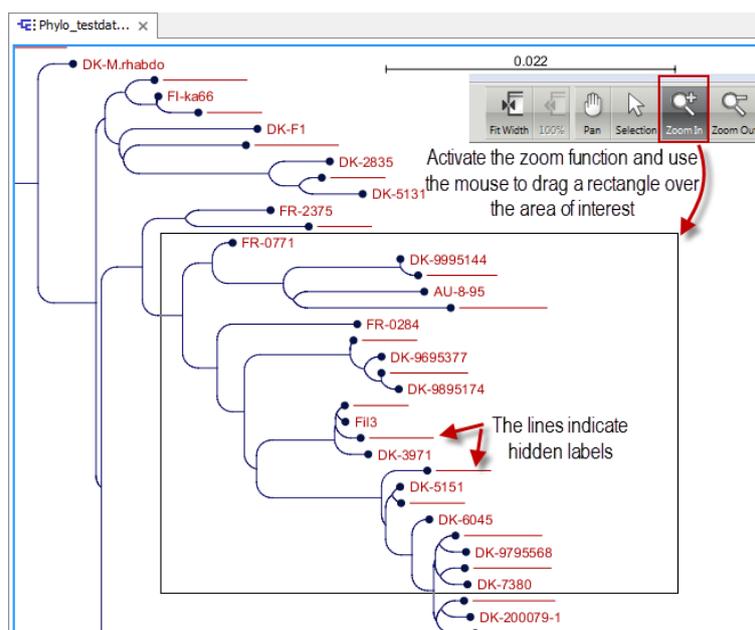


Figure 17.19: The zoom function in the upper right corner of CLC Genomics Workbench can be used to zoom in on a particular region of the tree. When the zoom function has been activated, use the mouse to drag a rectangle over the area that you wish to zoom in at.

- **Node color** Change the node color to visualize metadata.
- **Label text** The metadata can be shown directly as text labels as shown in figure 17.23.
- **Label text color** The label text can be colored and used to visualize metadata (see figure 17.23).

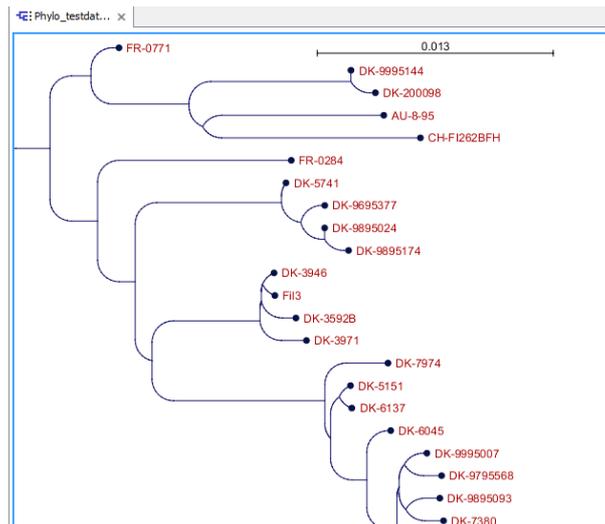


Figure 17.20: After zooming in on a region of interest more labels become visible. In this example all labels are now visible.

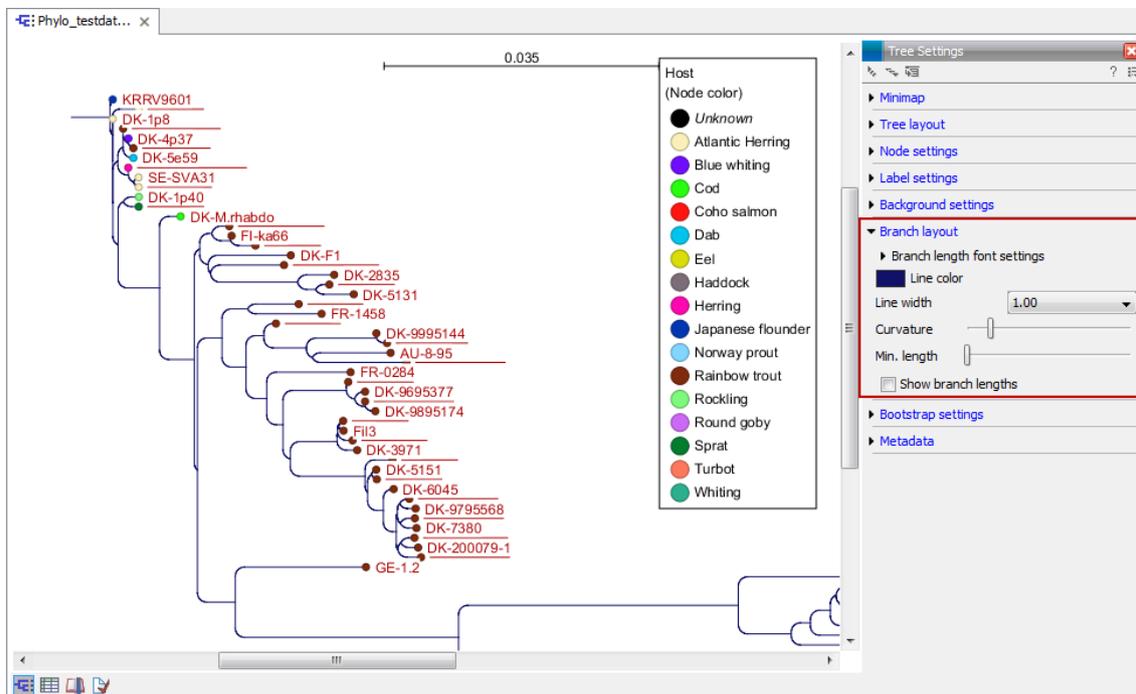


Figure 17.21: Branch Layout settings.

- **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 17.16).

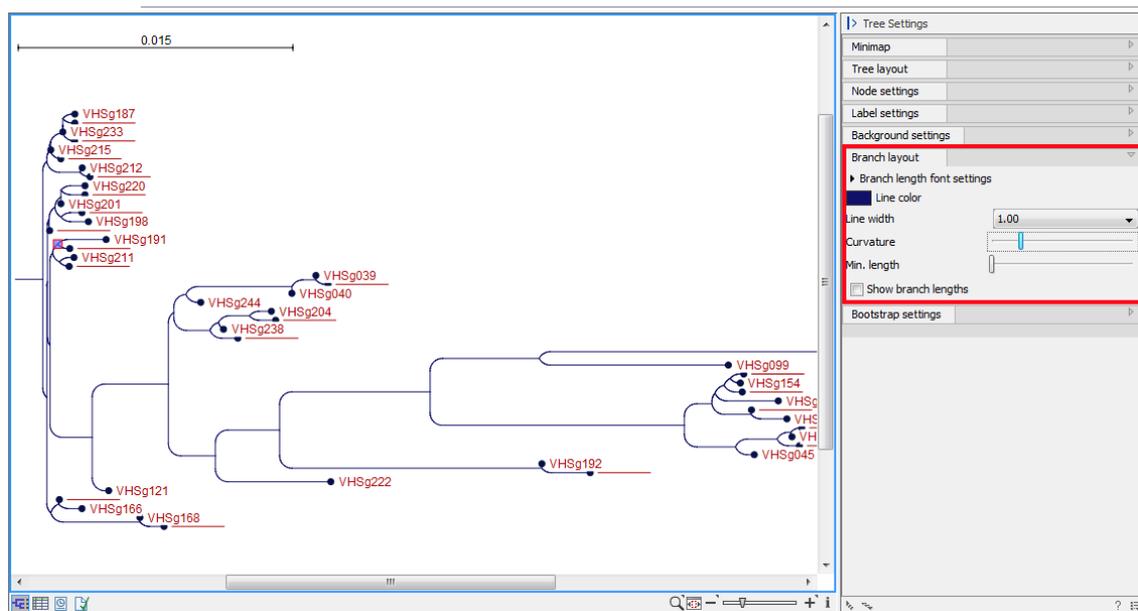


Figure 17.22: Branch Layout settings.

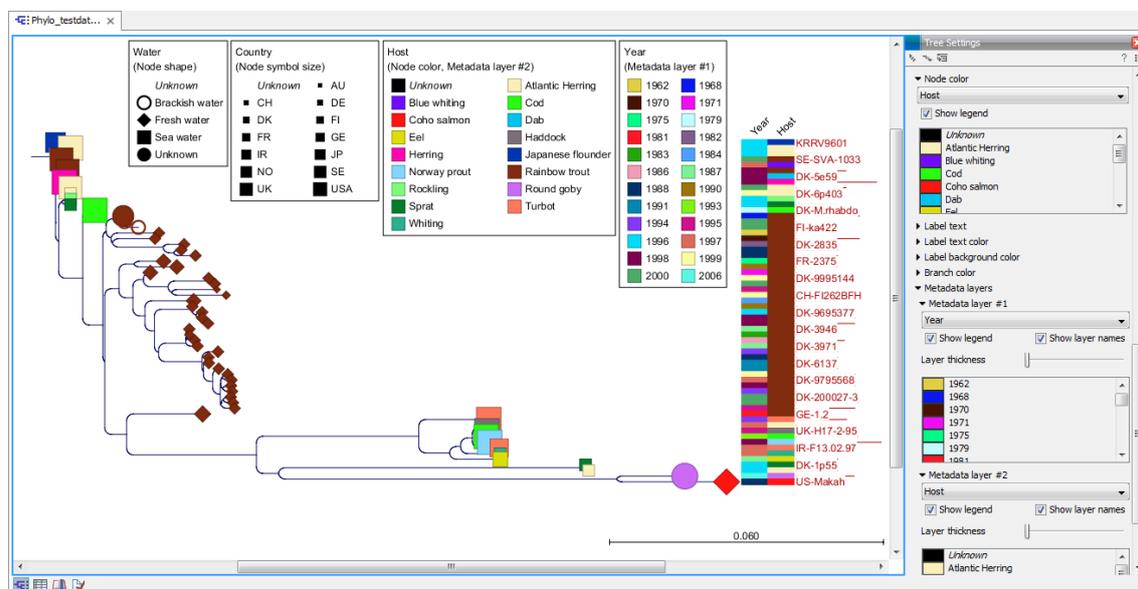


Figure 17.23: Different types of metadata can be visualized by adjusting node size, shape, and color. Two color-code metadata layers (Year and Host) are shown in the right side of the tree.

17.3.9 Node right click menu

Additional options for layout and extraction of subtree data are available when right clicking the nodes (figure 17.18):

- **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.
- **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.

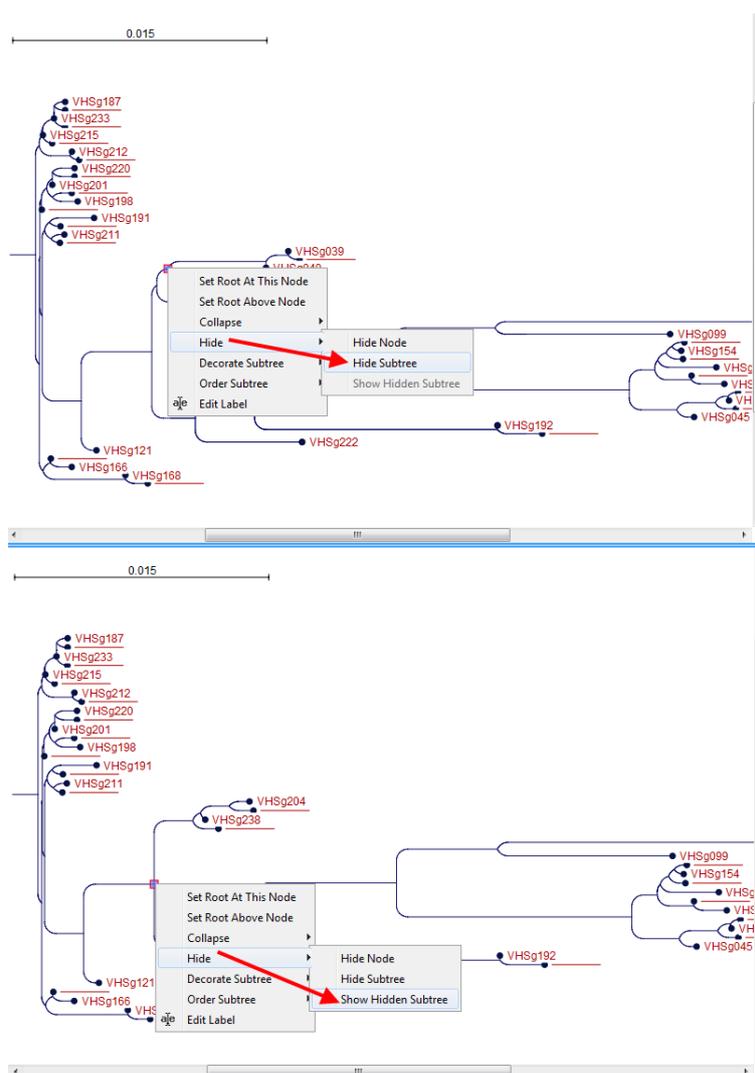


Figure 17.24: A subtree can be hidden by selecting "Hide Subtree" and is shown again when selecting "Show Hidden Subtree" on a parent node.

- 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.
- 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 17.25). When hiding nodes, a new button appears labeled "Show X hidden nodes" in the Side Panel under "Tree Layout" (figure 17.26). 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.

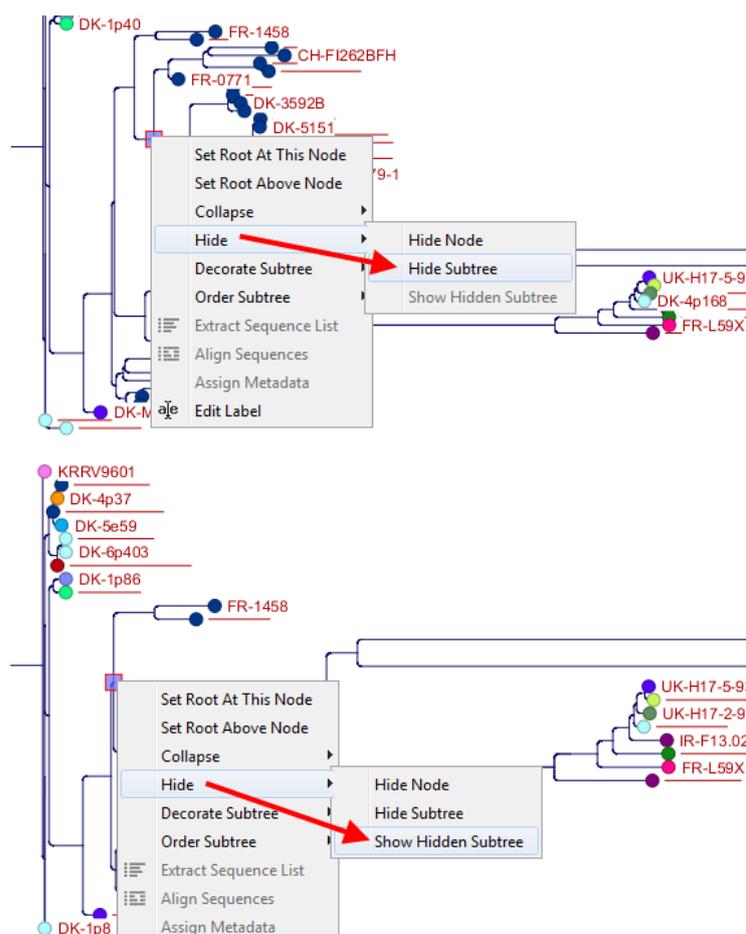


Figure 17.25: A subtree can be hidden by selecting "Hide Subtree" and is shown again when selecting "Show Hidden Subtree" on a parent node.

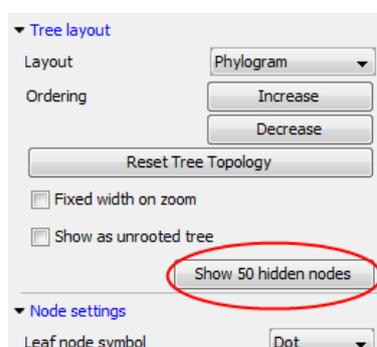


Figure 17.26: When hiding nodes, a new button labeled "Show X hidden nodes" appears in the Side Panel under "Tree Layout". When pressing this button, all hidden nodes are brought back.

- **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.

- **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**

17.4 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 17.27).

Additional metadata can be associated with a tree by clicking the **Import Metadata** button. This will open up the dialog shown in figure 17.28.

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 17.28. 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.

17.4.1 Table Settings and Filtering

How to use the metadata table (see figure 17.29):

- **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.

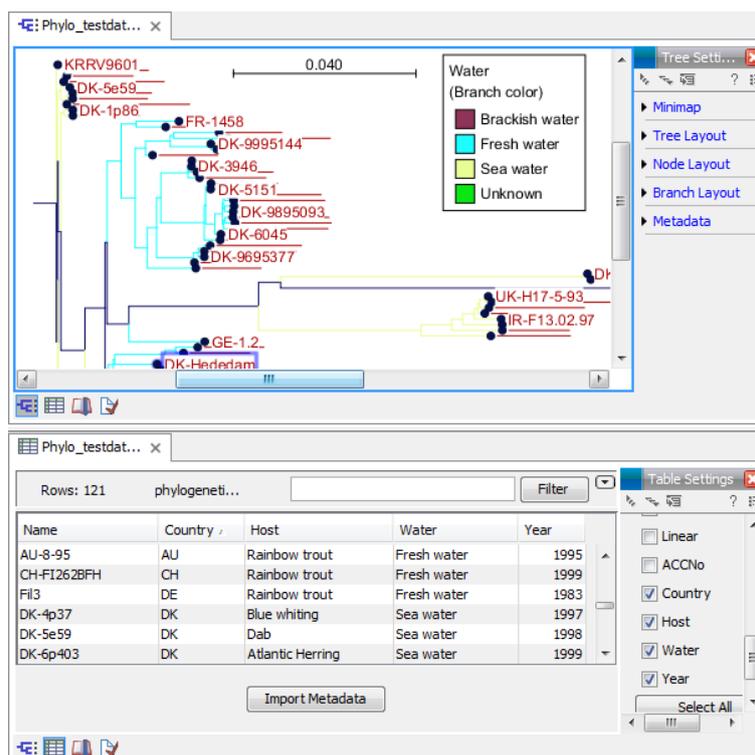


Figure 17.27: Tabular metadata that is associated with an existing tree shown in a split view.

- **Filtering Metadata information** Metadata information in a table can be filtered by a simple or advanced mode (this is described in the CLC Genomics Workbench manual, Appendix D, Filtering tables).

17.4.2 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 17.30):

- **Assign Metadata** The right click option "Assign Metadata" can be used for four purposes.
 - 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.

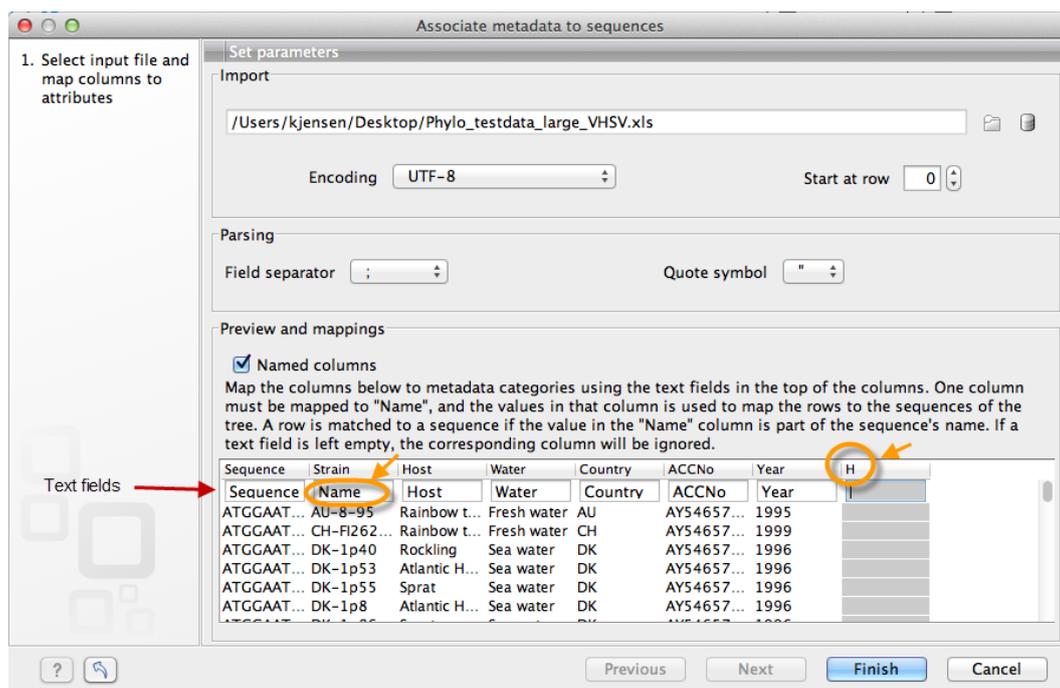


Figure 17.28: Import of metadata for a tree. The second column named "Strain" is chosen as the common denominator by entering "Name" in the text field of the column. The column labeled "H" is ignored by not assigning a column heading to this column.

- **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" (see an example in figure 17.31). 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". This will change values in all selected rows in the column that was clicked.

17.4.3 Undefined metadata values on a tree

When visualizing a metadata category where one or more nodes in the tree have undefined values, these nodes will be visualized using a *default* value. This value will always be shown in italics in the top of the legend (see figure 17.32). To remove this entry in the legend, all nodes must have a value assigned in the corresponding metadata category.

17.4.4 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 17.33).

Rows: 121 phylogenetic tree: DK-4p37 alignment_tree

| Name | Leaf | Size | ACCNo | Country | Host | Water | Year |
|---------------|------|------|------------|---------|-------------------|-------------|------|
| KRRV9601 | Leaf | 1524 | AB672614.1 | JP | Japanese flounder | Sea water | 1996 |
| SE-SVA14 | Leaf | 1524 | AY546622.1 | SE | Rainbow trout | Sea water | 1998 |
| DK-4p37 | Leaf | 1524 | FJ460590.1 | DK | Blue whiting | Sea water | 1997 |
| SE-SVA-1033 | Leaf | 1524 | FJ460591.1 | SE | Rainbow trout | Sea water | 2000 |
| DK-5e59 | Leaf | 1524 | AY546583.1 | DK | Dab | Sea water | 1998 |
| SE-SVA31 | Leaf | 1524 | AY546626.1 | SE | Atlantic Herring | Sea water | 2000 |
| DK-6p403 | Leaf | 1524 | AY546584.1 | DK | Atlantic Herring | Sea water | 1999 |
| UK-MLA98-6HE1 | Leaf | 1524 | AY546631.1 | UK | Herring | Sea water | 1998 |
| DK-1p86 | Leaf | 1524 | AY546579.1 | DK | Sprat | Sea water | 1996 |
| DK-1p40 | Leaf | 1524 | AY546575.1 | DK | Rockling | Sea water | 1996 |
| FR-1458 | Leaf | 1524 | AF143863 | FR | Rainbow trout | Fresh water | 1990 |
| FR-2375 | Leaf | 1524 | AY546617.1 | FR | Rainbow trout | Fresh water | 1975 |
| AU-8-95 | Leaf | 1524 | AY546570.1 | AU | Rainbow trout | Fresh water | 1995 |
| CH-FI2628FH | Leaf | 1524 | AY546571.1 | CH | Rainbow trout | Fresh water | 1999 |
| DK-9995144 | Leaf | 1524 | AY546602.1 | DK | Rainbow trout | Fresh water | 1999 |
| DK-200098 | Leaf | 1524 | AY546605.1 | DK | Rainbow trout | Fresh water | 2000 |
| FR-0771 | Leaf | 1524 | AY546616.1 | FR | Rainbow trout | Fresh water | 1971 |
| FI3 | Leaf | 1524 | Y18263.1 | DE | Rainbow trout | Fresh water | 1983 |
| DK-3946 | Leaf | 1524 | AY546586.1 | DK | Rainbow trout | Fresh water | 1987 |
| DK-3592B | Leaf | 1524 | X66134 | DK | Rainbow trout | Fresh water | 1986 |
| DK-3971 | Leaf | 1524 | AY546587.1 | DK | Rainbow trout | Fresh water | 1987 |
| DK-6137 | Leaf | 1524 | AY546593.1 | DK | Rainbow trout | Fresh water | 1991 |
| DK-5151 | Leaf | 1524 | AF345859.1 | DK | Rainbow trout | Fresh water | 1988 |
| DK-9995007 | Leaf | 1524 | AY546601.1 | DK | Rainbow trout | Fresh water | 1999 |
| DK-9795568 | Leaf | 1524 | AY546598.1 | DK | Rainbow trout | Fresh water | 1997 |
| DK-7380 | Leaf | 1524 | AY546594.1 | DK | Rainbow trout | Fresh water | 1994 |
| DK-9895093 | Leaf | 1524 | AY546600.1 | DK | Rainbow trout | Fresh water | 1998 |
| DK-200079-1 | Leaf | 1524 | AY546613.1 | DK | Rainbow trout | Fresh water | 2000 |

Figure 17.29: 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 (this is described in the CLC Genomics Workbench manual, Appendix D, Filtering tables).

| Water | Country | ACCNo | Year | Host |
|----------------|---------|------------|------|---------------|
| Unknown | NO | AY546621.1 | 1968 | Rainbow trout |
| Brackish water | | | 2000 | Rainbow trout |
| Brackish water | | | 2000 | Rainbow trout |
| Fresh water | | | 1962 | Rainbow trout |
| Fresh water | | | 1970 | Rainbow trout |

Figure 17.30: Right click options in the metadata table.

- **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

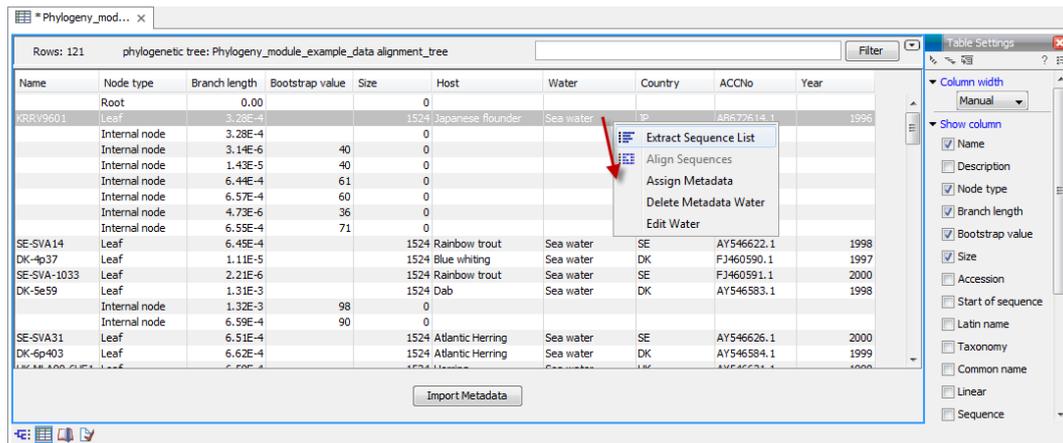


Figure 17.31: To include an extra metadata column, use the right click option "Assign Metadata", provide "Name" (the column header) and "Value". To modify existing metadata, click on the specific field, select "Edit column header" and provide new value.

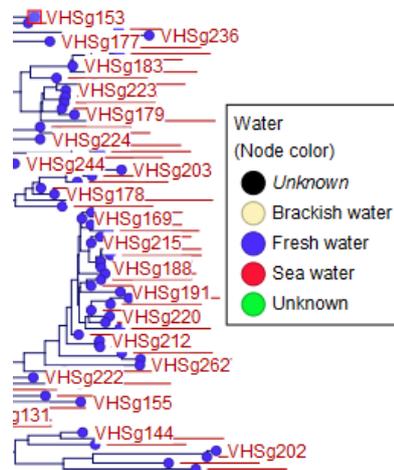


Figure 17.32: A legend for a metadata category where one or more values are undefined.

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 16.6). Next, change relevant option in the multiple alignment wizard that pops up and click **Finish**. The multiple alignment will now be generated.

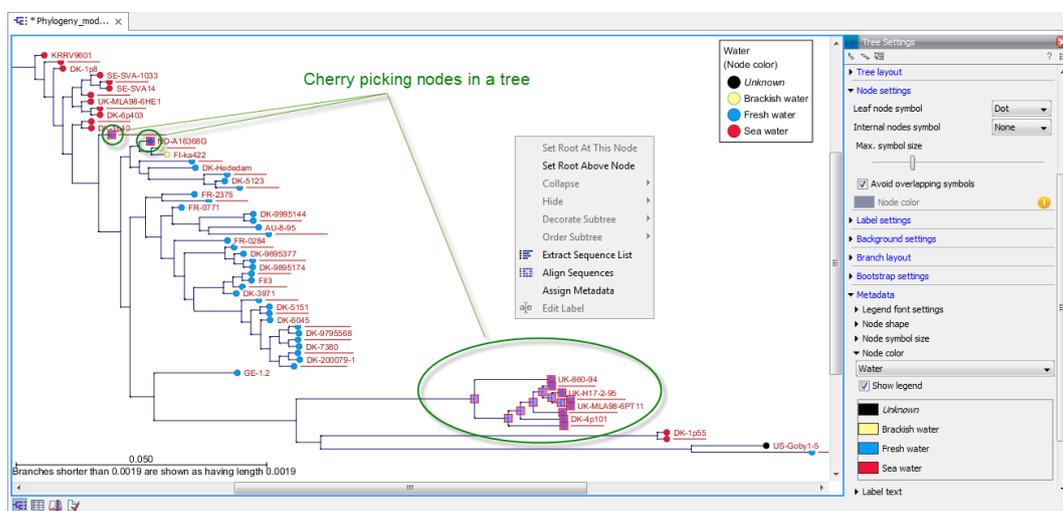


Figure 17.33: *Cherry picking nodes in a tree.* The selected leaf sequences can be extracted by right clicking on one of the selected nodes and selecting "Extract Sequence List". It is also possible to Align Sequences directly by right clicking on the nodes or leaves.

Chapter 18

General sequence analyses

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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 Extract Annotations

The **Extract annotations** tool makes it very easy to extract parts of a sequence (or several sequences) based on its annotations. Using a few steps it is possible to:

- extract e.g. all tRNA genes from the *E. coli* genome.

- automatically add flanking regions to the annotated sequences.
- search for specific words in all available annotations.

The output is a sequence list that contains sequences carrying the annotation specified (including the flanking regions, if this option was selected).

To extract annotations from a sequence, go to:

Toolbox | General Sequence Analysis (🔧) | Extract Annotations (👉)

This opens the dialog shown in figure 18.1 that allows specification of which sequence to extract annotations from.

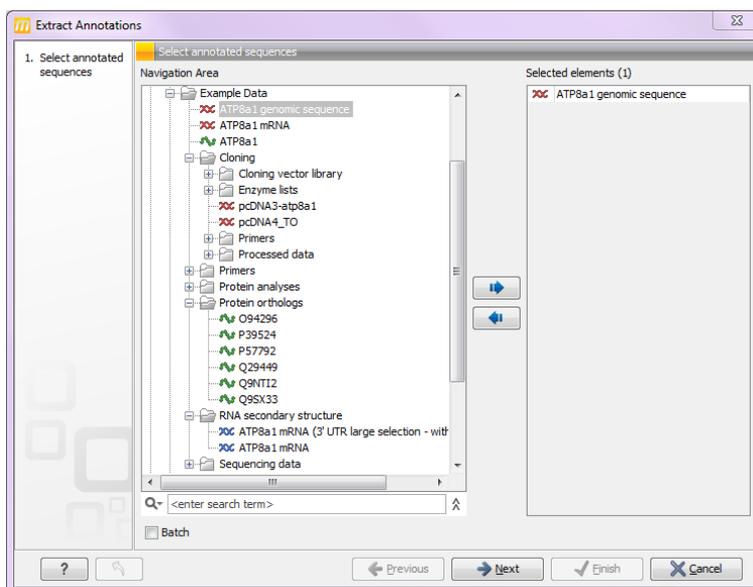


Figure 18.1: Select one or more sequences to extract annotations from.

Click **Next**. At the top of the dialog shown in figure 18.2 you can specify which annotations to use:

- **Search term.** 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. If you e.g. 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, e.g. "MLH1, Human" will find annotations including both "MLH1" and "Human".
- **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.

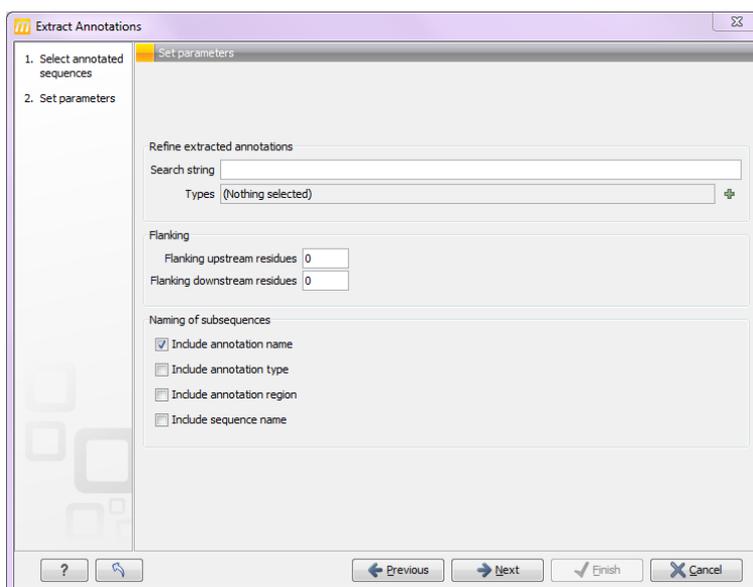


Figure 18.2: Adjusting parameters for extract annotations.

- **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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

18.2 Extract sequences

This tool allows the extraction of sequences from other types of data in the Workbench, such as sequence lists or alignments. The data types you can extract sequences from are:

- Alignments (📄)
- BLAST result (📄)
- BLAST overview tables (📄)

- sequence lists (📄)
- Contigs and read mappings (📄)
- Read mapping tables (📄)
- Read mapping tracks (📄)
- RNA-Seq mapping results (📄)

Note! When the Extract Sequences tool is run via the Workbench toolbox on an entire file of one of the above types, **all** sequences are extracted from the data used as input. If only a **subset** of the sequences is desired, for example, the reads from just a small area of a mapping, or the sequences for only a few blast results, then a data set containing just this subsection or subset should be created and the Extract Sequences tool should be run on that.

For extracting a subset of a mapping, please see section 21.7.6 that describes the function "Extract from Selection" that also can be selected from the right click menu (see figure 18.3).

For extracting a subset of a sequence list, you can highlight the sequences of interest in the table view of the sequence list, right click on the selection and launch the Extract Sequences tool.

The Extract Sequences tool can be launched via the Toolbox menu, by going to:

Toolbox | General Sequence Analysis (📄) | Extract Sequences (📄)

Alternatively, on all the data types listed above except sequence lists, the option to run this tool appears by right clicking in the relevant area; a row in a table or in the read area of mapping data. An example is shown in figure 18.3.

Please note that for mappings, only the read sequences are extracted. Reference and consensus sequences are not extracted using this tool. Similarly, when extracting sequences from BLAST results, the sequence hits are extracted, not the original query sequence or a consensus sequence.

"Note also, that paired reads will be extracted in accordance with the read group settings, which is specified during the original import of the reads. If the orientation has since been changed (e.g. 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."

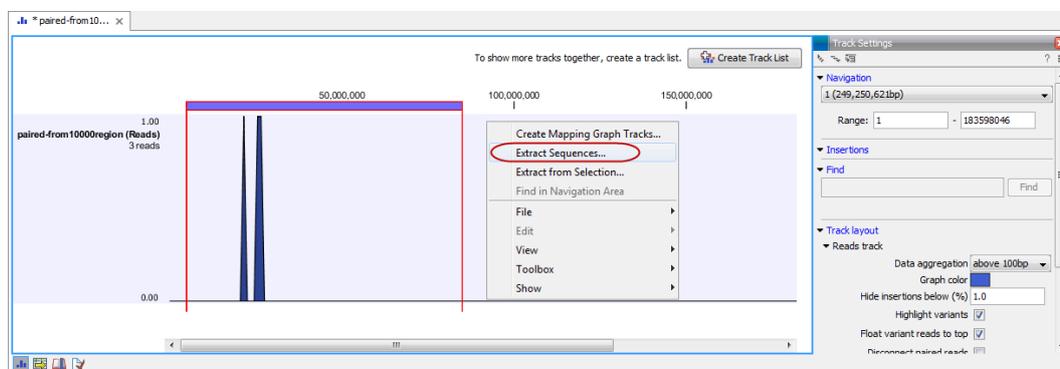


Figure 18.3: Right click somewhere in the reads track area and select "Extract Sequences".

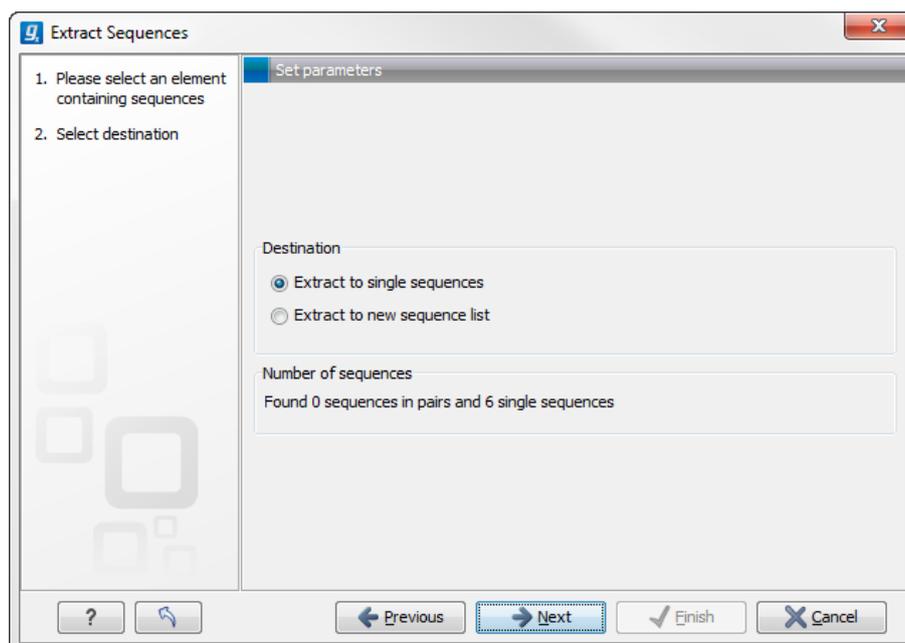


Figure 18.4: Choosing whether the extracted sequences should be placed in a new list or as single sequences.

The dialog allows you to select the **Destination**. Here you can choose whether the extracted sequences should be extracted as single sequences or placed in a new sequence list. For most data types, it will make most sense to choose to extract the sequences into a sequence list. The exception to this is when working with a sequence list, where choosing to extract to a sequence list would create a copy of the same sequence list. In this case, the other option would generally be chosen. This would then result in the generation of individual sequence objects for each sequence in the sequence list.

Below these options, in the dialog, you can see the number of sequences that will be extracted.

18.3 Shuffle sequence

In some cases, it is beneficial to shuffle a sequence. This is an option in the **Toolbox** menu under **General Sequence Analyses**. It is normally used for statistical analyses, e.g. 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 tool, go to:

Toolbox | General Sequence Analysis (📄) | Shuffle Sequence (🔀)

This opens the dialog displayed in figure 18.5:

If a sequence was selected before choosing the Toolbox action, 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** to determine how the shuffling should be performed.

In this step, shown in figure 18.6:

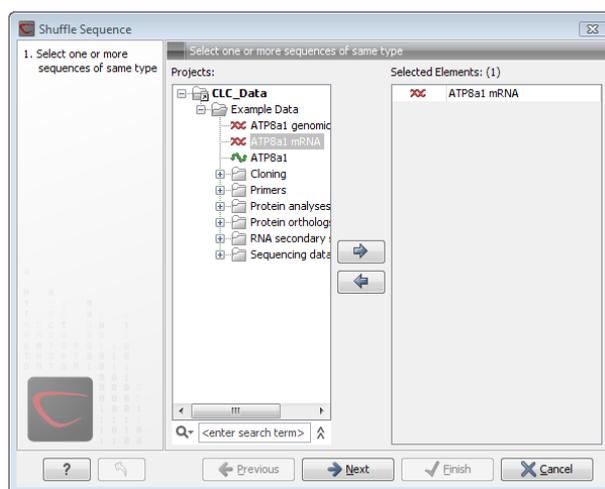


Figure 18.5: Choosing sequence for shuffling.

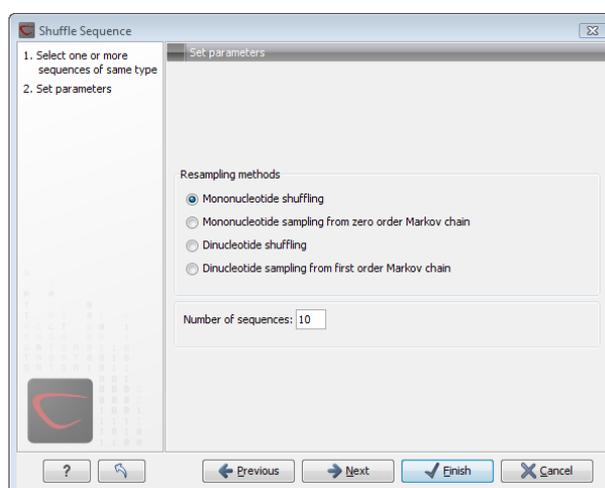


Figure 18.6: Parameters for shuffling.

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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. This chapter first describes how to create and second how to

This section describes how to adjust the view of the plot.

18.4.1 Create dot plots

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.

To create a dot plot, go to:

Toolbox | General Sequence Analysis (🔧) | Create Dot Plot (📊)

This opens the dialog shown in figure 18.7.

If a sequence was selected before choosing the **Toolbox** action, this sequence is now listed in the **Selected Elements** window of the dialog. Use the arrows to add or remove elements from the selected elements. Click **Next** to adjust dot plot parameters. Clicking **Next** opens the dialog shown in figure 18.8.

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

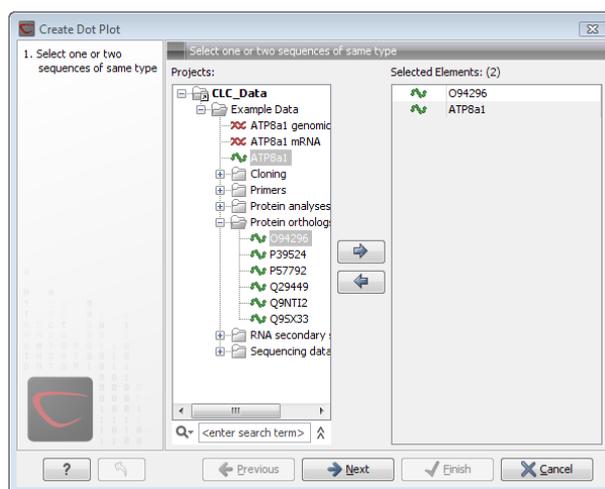


Figure 18.7: Selecting sequences for the dot plot.

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.

Adjust 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'.

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

18.4.2 View dot plots

A view of a dot plot can be seen in figure 18.9. 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.

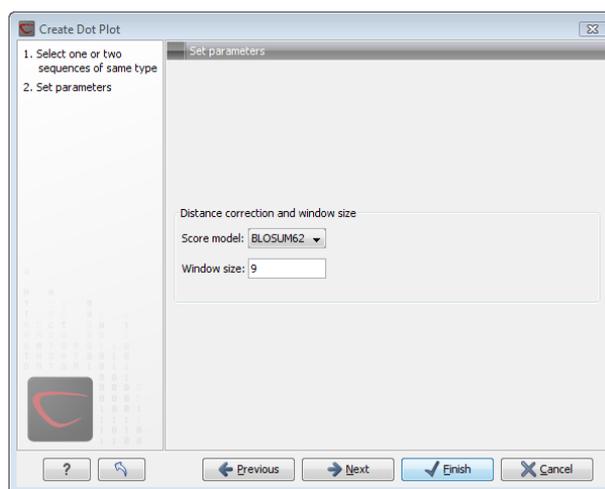


Figure 18.8: Setting the dot plot parameters.

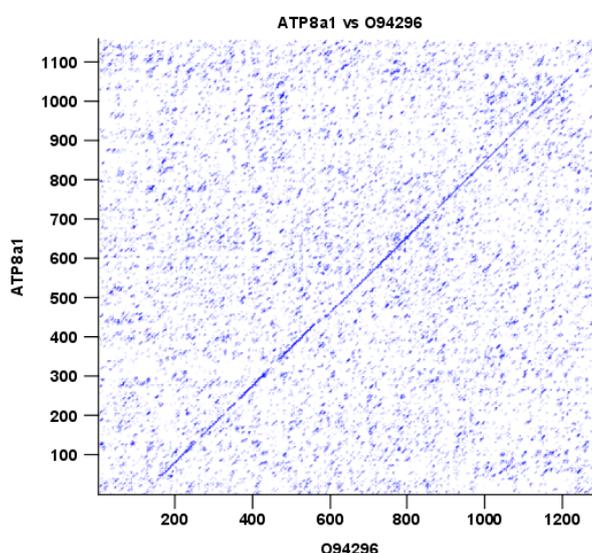


Figure 18.9: A view is opened showing the dot plot.

Adjusting the sliders above the gradient box is also practical, when producing an output for printing. (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. (If you choose a color gradient, which includes white). See figure 18.9.

18.4.3 Bioinformatics explained: Dot plots

Realization of 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.

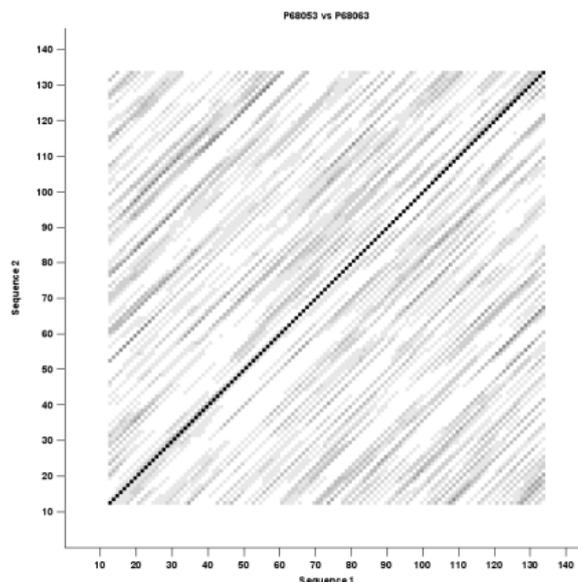


Figure 18.10: Dot plot with inverted colors, practical for printing.

- 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 18.11 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>.

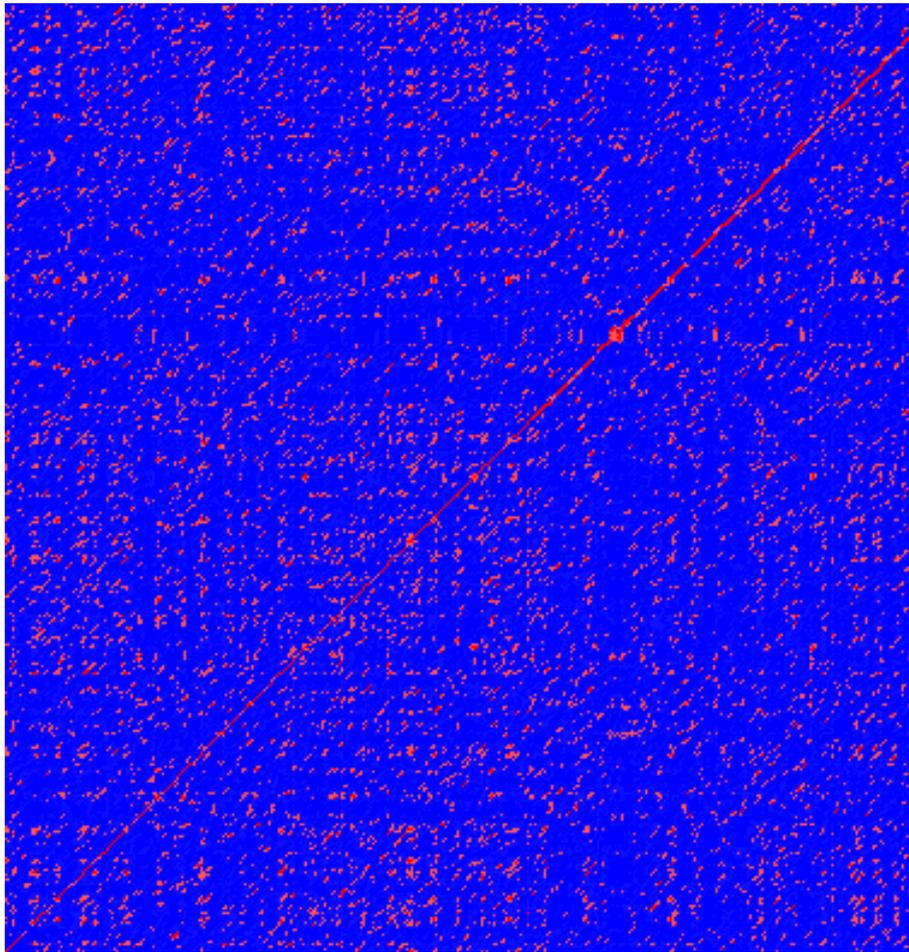


Figure 18.11: Dot plot of DQ232610 vs. DQ023146 (Influenza A virus nucleoproteins) showing and overall similarity

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.



Figure 18.12: 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 18.13 you can see a sequence with repeats.

Frame shifts

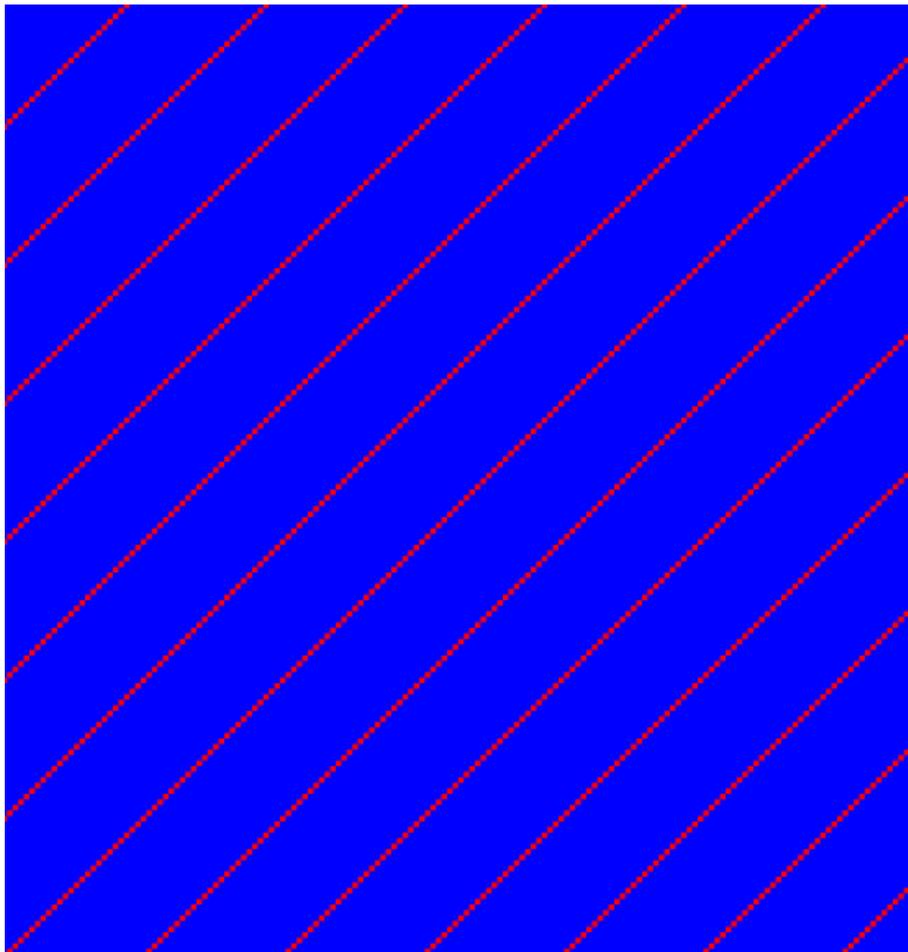


Figure 18.13: The dot plot of a sequence showing repeated elements. See also figure 18.12.

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 18.14. 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)

Sequence inversions

In dot plots you can see an inversion of sequence as contrary diagonal to the diagonal showing similarity. In figure 18.15 you can see a dot plot (window length is 3) with an inversion.

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 18.16 is a square shows the low-complexity region of this sequence.

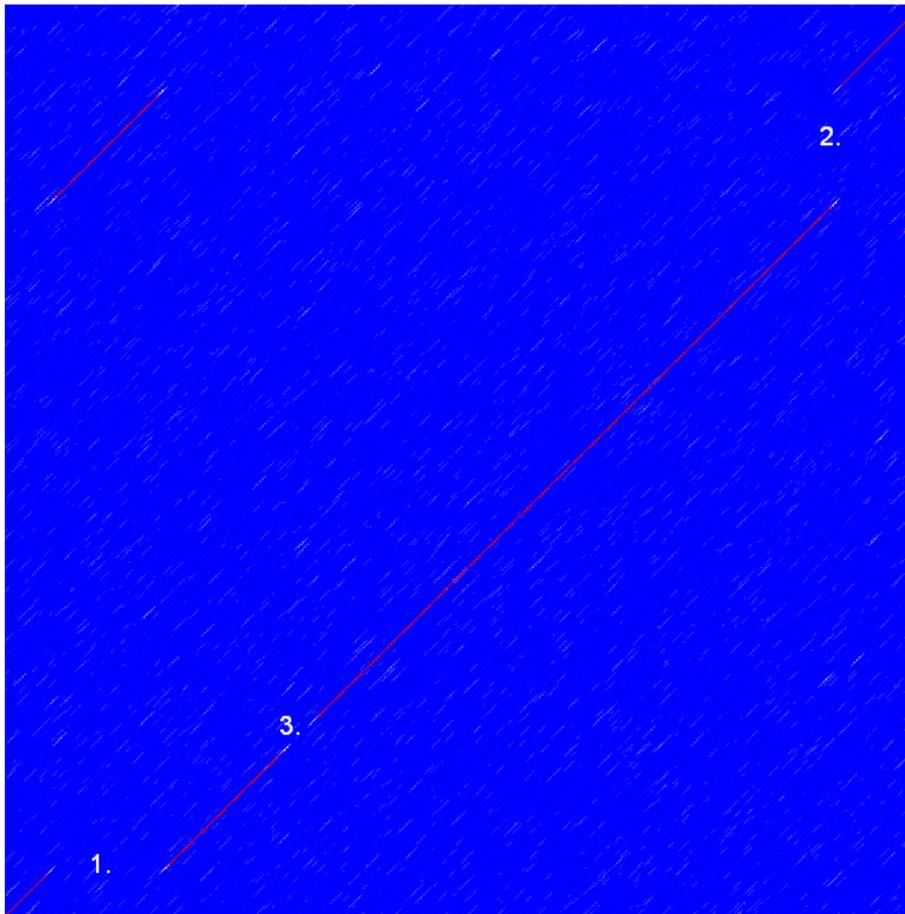


Figure 18.14: This dot plot show various frame shifts in the sequence. See text for details.

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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).

Based on evolution of proteins it became apparent that these changes or substitutions of amino

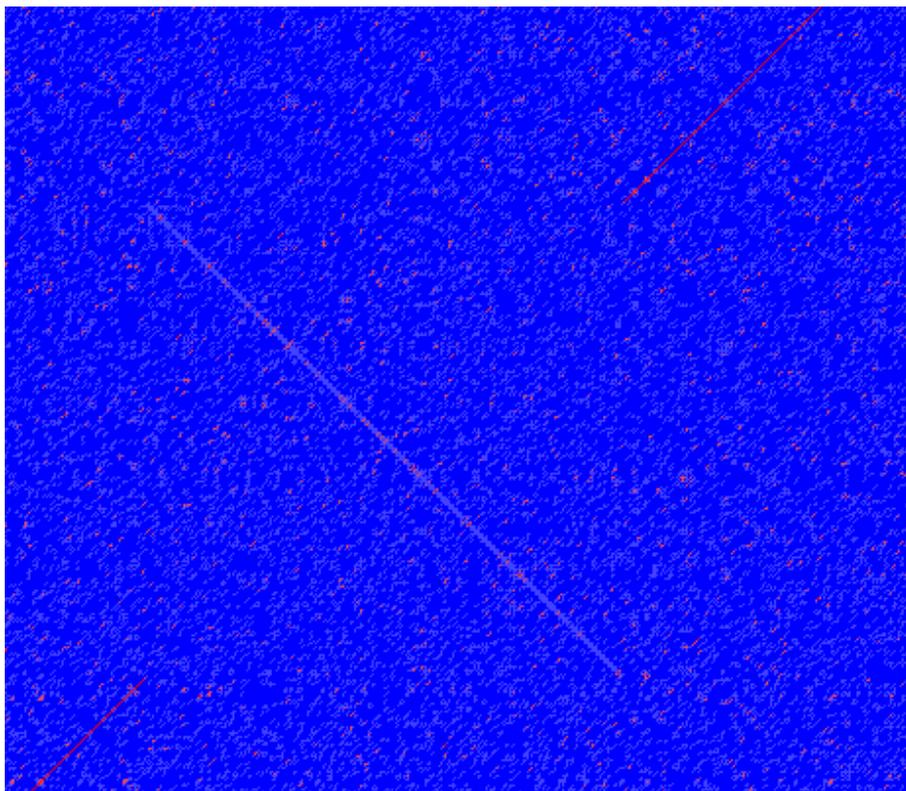


Figure 18.15: The dot plot showing an inversion in a sequence. See also figure 18.12.

acids can be modeled by a scoring matrix also referred 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 18.17).

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

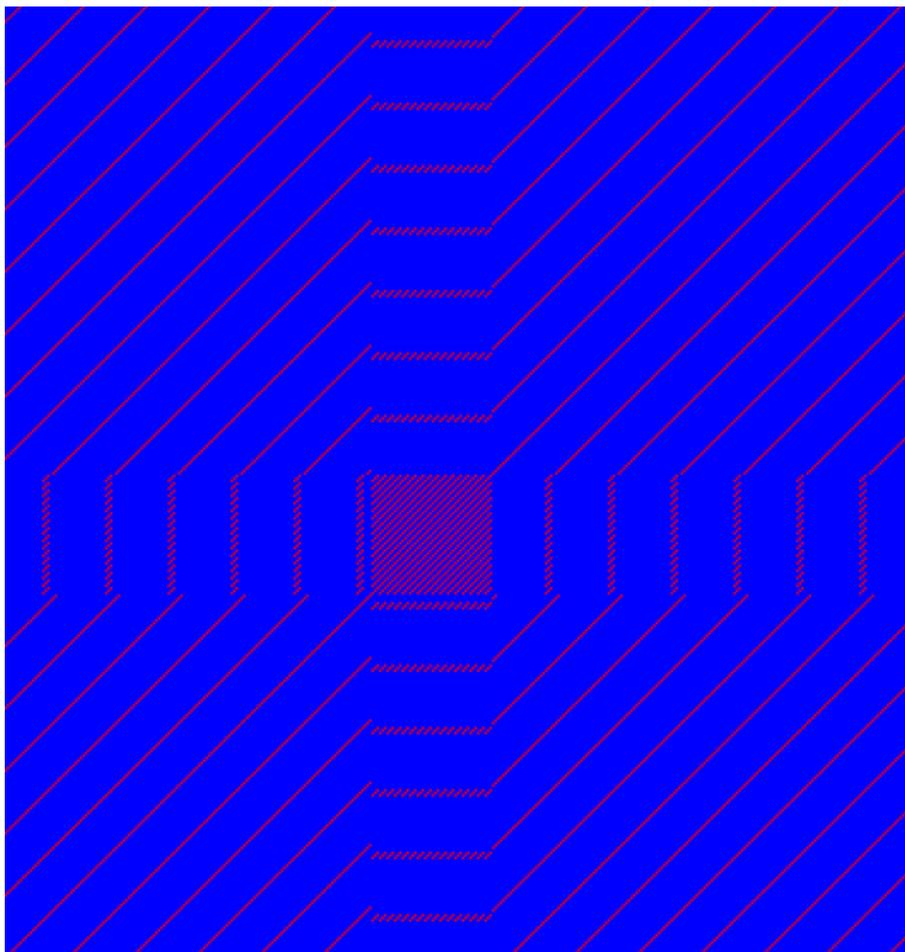


Figure 18.16: The dot plot showing a low-complexity region in the sequence. The sequence is artificial and low complexity regions do not always show as a square.

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 $\lambda_{\frac{1}{2}}$ 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 to 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 probably strongly influence on the outcome of the analysis. In general a few rules apply to the selection of scoring matrices.

| | 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].

- 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 18.17) 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.

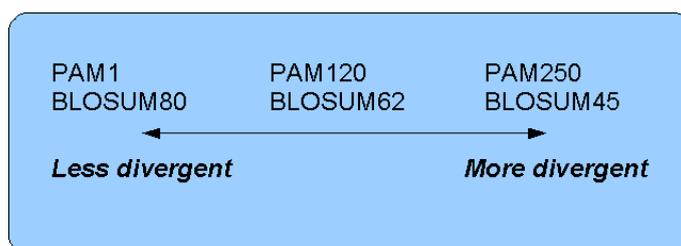


Figure 18.17: *Relationship between scoring matrices. The BLOSUM62 has become a de facto standard scoring matrix for a wide range of alignment programs. It is the default matrix in BLAST.*

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

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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:

Toolbox | 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 18.18 shows an example of a local complexity plot.

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. The values of the complexity plot approaches 1.0 as the distribution of amino acids become more complex.

See section B 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:

Toolbox | General Sequence Analysis (📁) | Create Sequence Statistics (📊)

This opens a dialog where you can alter your choice of sequences. If you had already selected sequences in the Navigation Area, these will be shown in the **Selected Elements** window. However you can remove these, or add others, by using the arrows to move sequences in or out of the **Selected Elements** window. You can also add sequence lists.

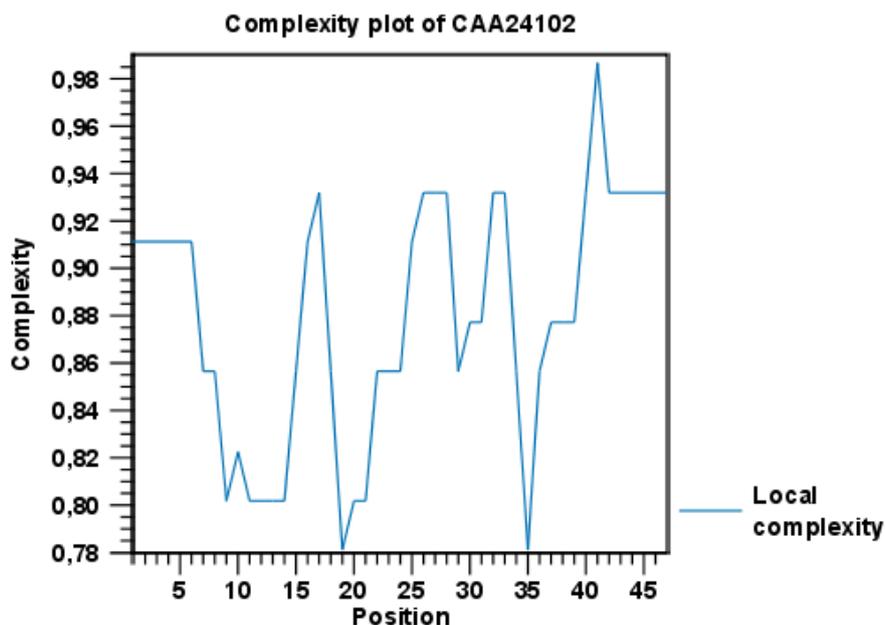


Figure 18.18: An example of a local complexity plot.

Note! You cannot create statistics for DNA and protein sequences at the same time; they must be run separately.

When the sequences are selected, click **Next**.

This opens the dialog displayed in figure 18.19.

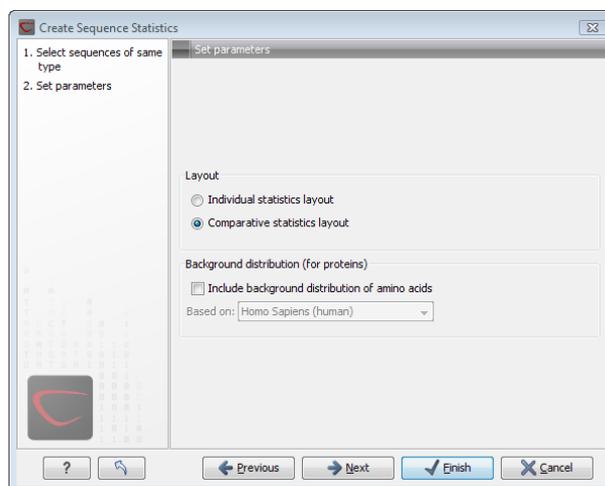


Figure 18.19: Setting parameters for the sequence statistics.

The dialog offers to adjust the following parameters:

- **Individual statistics layout.** If more sequences were selected in **Step 1**, this function generates separate statistics for each sequence.
- **Comparative statistics layout.** If more sequences were selected in **Step 1**, this function generates statistics with comparisons between the sequences.

You can also 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.)

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. An example of protein sequence statistics is shown in figure 18.20.

1 Protein statistics

1.1 Sequence information

| | |
|-------------------|---|
| Sequence type | Protein |
| Length | 147 |
| Organism | Mus musculus |
| Name | CAA32220 |
| Description | haemoglobin beta-h0 chain [Mus musculus]. |
| Modification Date | 18-APR-2005 |
| Weight | 16,412 kDa |

1.2 Half-life

| N-terminal aa | Half-life mammals | Half-life yeast | Half-life E.Coli |
|---------------|-------------------|-----------------|------------------|
|---------------|-------------------|-----------------|------------------|

Figure 18.20: Example of protein sequence statistics.

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 comparative protein sequence statistics include:

- Sequence information:
 - Sequence type
 - Length
 - Organism
 - Name
 - Description
 - Modification Date
 - Weight. This is calculated like this: $sum_{unitsinsequence}(weight(unit)) - links * weight(H_2O)$ 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
- Half-life
- Extinction coefficient
- Counts of Atoms

- Frequency of Atoms
- Count of hydrophobic and hydrophilic residues
- Frequencies of hydrophobic and hydrophilic residues
- Count of charged residues
- Frequencies of charged residues
- Amino acid distribution
- Histogram of amino acid distribution
- Annotation table
- Counts of di-peptides
- Frequency of di-peptides

- Sequence Information:
 - Sequence type
 - Length
 - Organism
 - Name
 - Description
 - Modification Date
 - Weight
 - Isoelectric point
 - Aliphatic index

- Amino acid distribution
- Annotation table

The output of nucleotide sequence statistics include:

- General statistics:
 - Sequence type
 - Length
 - Organism
 - Name
 - Description
 - Modification Date

- Weight. This is calculated like this: $sum_{unitsinsequence}(weight(unit)) - links * weight(H_2O)$ where `links` is the sequence length minus one for linear sequences and sequence length for circular molecules. The `units` are monophosphates. Both the weight for single- and double stranded molecules are included. The atomic composition is defined the same way.
- Atomic composition
- Nucleotide distribution table
- Nucleotide distribution histogram
- Annotation table
- Counts of di-nucleotides
- Frequency of di-nucleotides
- General statistics:
 - Sequence type
 - Length
 - Organism
 - Name
 - Description
 - Modification Date
 - Weight (calculated as single-stranded DNA)
- Nucleotide distribution table
- Annotation table

If nucleotide sequences are used as input, and these are annotated with CDS, a section on Codon statistics for Coding Regions is included.

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 posttranslational 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.

$$\text{Aliphatic index} = X(\text{Ala}) + a * X(\text{Val}) + b * X(\text{Leu}) + b * X(\text{Ile})$$

$X(\text{Ala})$, $X(\text{Val})$, $X(\text{Ile})$ and $X(\text{Leu})$ 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 using the following equation:

$$\text{Ext}(\text{Protein}) = \text{count}(\text{Cysteine}) * \text{Ext}(\text{Cysteine}) + \text{count}(\text{Tyr}) * \text{Ext}(\text{Tyr}) + \text{count}(\text{Trp}) * \text{Ext}(\text{Trp})$$

where Ext is the extinction coefficient of amino acid in question. At 280nm the extinction

| 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*.

coefficients are: Cys=120, Tyr=1280 and Trp=5690.

This equation is only valid under the following conditions:

- pH 6.5
- 6.0 M guanidium hydrochloride
- 0.02 M phosphate buffer

The extinction coefficient values of the three important amino acids at different wavelengths are found in [Gill and von Hippel, 1989].

Knowing the extinction coefficient, the absorbance (optical density) can be calculated using the following formula:

$$\text{Absorbance}(\text{Protein}) = \frac{\text{Ext}(\text{Protein})}{\text{Molecular weight}}$$

Two values are reported. The first value is computed assuming that all cysteine residues appear as half cystines, meaning they form di-sulfide bridges to other cysteines. The second number assumes that no di-sulfide bonds are formed.

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.

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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 by:

Toolbox | General Sequence Analyses | Join sequences (🟢)

This opens the dialog shown in figure 18.21.

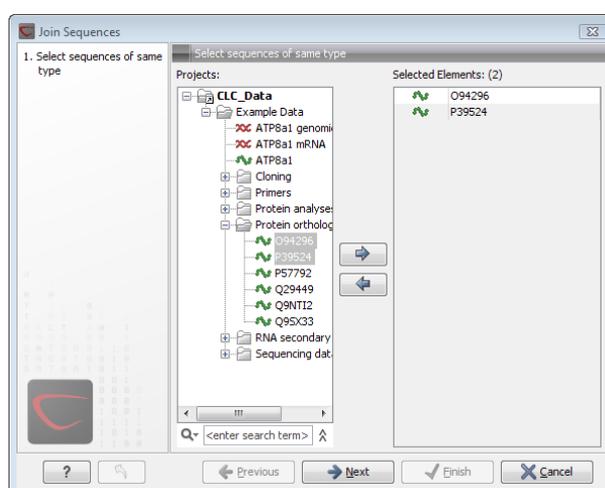


Figure 18.21: Selecting two sequences to be joined.

If you have selected some sequences before choosing the Toolbox action, they are now listed in the **Selected Elements** window of the dialog. Use the arrows to add or remove sequences from the selected elements. Click **Next** opens the dialog shown in figure 18.22.

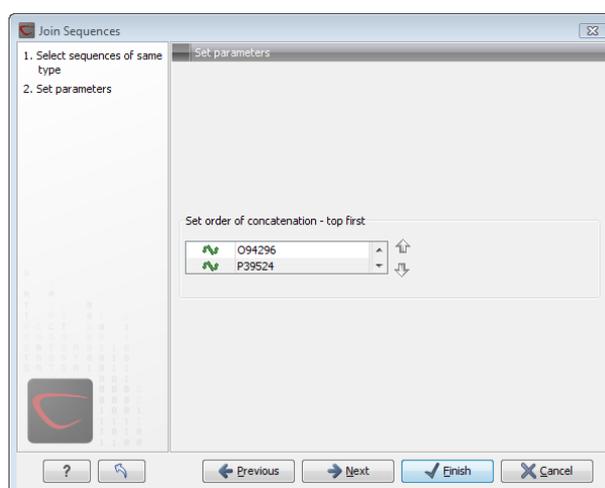


Figure 18.22: Setting the order in which sequences are joined.

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

The result is shown in figure 18.23.

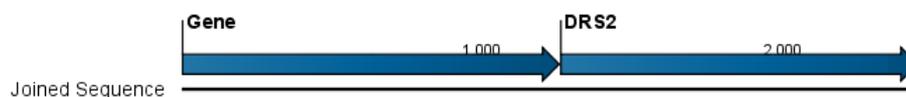


Figure 18.23: The result of joining sequences is a new sequence containing the annotations of the joined sequences (they each had a HBB annotation).

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:

Toolbox | General Sequence Analysis (📁) | Pattern Discovery (🔍)

If a sequence was selected before choosing the Toolbox action, the 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.

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 18.24).

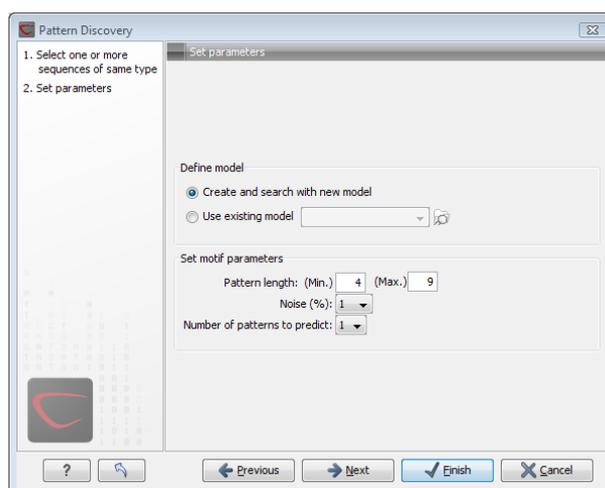


Figure 18.24: Setting parameters for the pattern discovery. See text for details.

In order to search unknown sequences with an already existing model:

Select to use an already existing model which is seen in figure 18.24. 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 18.24.

- **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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. This will open a view showing the patterns found as annotations on the original sequence (see figure 18.25). If you have selected several sequences, a corresponding number of views will be opened.



```
      Pattern1      Pattern1
      ┌──────────┐  ┌──────────┐
      │VCNKNQTA  │  │QTARSSGE  │
      └──────────┘  └──────────┘
3VCNKNQTA EDLAWSYGFP ECARFLTMIK CMQTARSSGE
```

Figure 18.25: 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

CLC Main Workbench offers advanced and versatile options to search for known motifs represented either by a simple sequence or a more advanced regular expression. These advanced search capabilities are available for use in both DNA and protein sequences.

There are two ways to access this functionality:

- When viewing sequences, it is possible to have motifs calculated and shown on the sequence in a similar way as restriction sites (see section 23.3.1). This approach is called *Dynamic motifs* and is an easy way to spot known sequence motifs when working with sequences for cloning etc.
- A more refined and systematic search for motifs can be performed through the **Toolbox**. This will generate a table and optionally add annotations to the sequences.

The two approaches are described below.

18.9.1 Dynamic motifs

In the **Side Panel** of sequence views, there is a group called **Motifs** (see figure 18.26).



Figure 18.26: *Dynamic motifs in the Side Panel.*

The Workbench will look for the listed motifs in the sequence that is open and by clicking the check box next to the motif it will be shown in the view as illustrated in figure 18.27.

```

      380                400                420
      |                |                |
      CCCATTGACGTCAATGGGAGTTTGTGTTTGGCACCAAATCAACGGGACTTTCC
      |                |                |
      440                460
      |                |
      AAAATGTCGTAACAACCTCCGCCCCATTGACGCAAAATGGGCGGTAGGCGTGTAC
      |                |                |
      480                500                520
      |                |                |
      GGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAAGATCGCCTGG
  
```

Figure 18.27: Showing dynamic motifs on the sequence.

This case shows the CMV promoter primer sequence which is one of the pre-defined motifs in *CLC Main Workbench*. The motif is per default shown as a faded arrow with no text. The direction of the arrow indicates the strand of the motif.

Placing the mouse cursor on the arrow will display additional information about the motif as illustrated in figure 18.28.

```

      3CCCCATTGACGCAAAATGGGCGGTAGGCGTGTACGGTGGGAGG
      |
      |motif=CGCAAATGGGCGGTAGGCGTG, list index: 5 (CMV):
      /type=Simple
      /description=CMV promoter primer
  
```

Figure 18.28: Showing dynamic motifs on the sequence.

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. 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 18.26 is a pre-defined list that is included with the *CLC Main Workbench*. You can define your own set of motifs to use instead. In order to do this, you can either click on the **Add Motif** button in the side panel (see figure 18.26) and directly define and add motifs of choice as illustrated in figure 18.32. Alternatively, you can create and save a **Motif list** (📄) (see section 18.10). Subsequently, in the sequence view click the **Manage Motifs** button in the side panel which will bring up the dialog shown in figure 18.29.

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**.

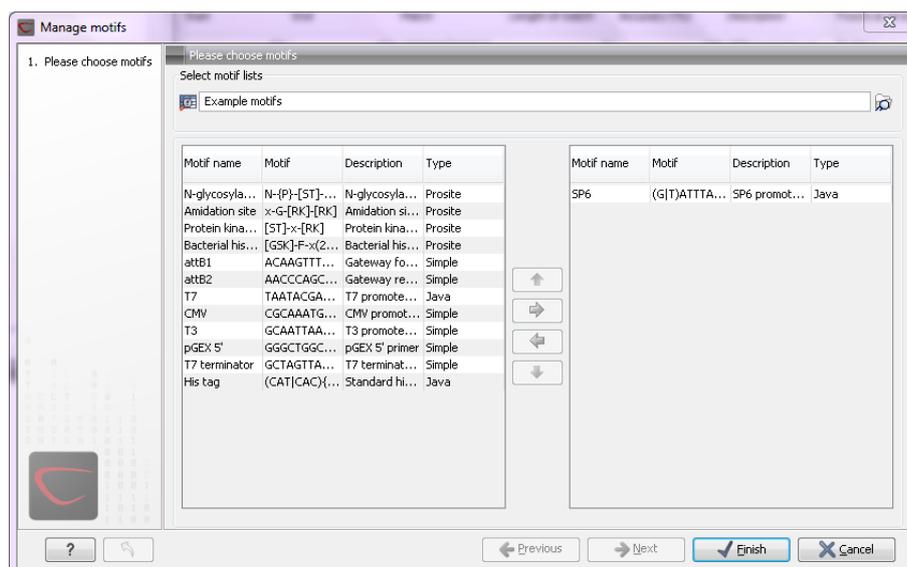


Figure 18.29: Managing the motifs to be shown.

18.9.2 Motif search from the Toolbox

The dynamic motifs described in section 18.9.1 provide a quick way of routinely scanning a sequence for commonly used motifs, but in some cases a more systematic approach is needed. The motif search in the **Toolbox** provides an option to search for motifs with a user-specified similarity to the target sequence, and furthermore the motifs found can be displayed in an overview table. This is particularly useful when searching for motifs on many sequences.

To start the Toolbox motif search, go to:

Toolbox | General Sequence Analysis (📁) | Motif Search (🔍)

A dialog window will be launched. Use the arrows to add or remove sequences or sequence lists between the Navigation Area and the selected elements.

You can perform the analysis on several DNA or several protein sequences at a time. In this case, the method will search for patterns in the sequences and create an overview table of the motifs found in all sequences.

Click **Next** to adjust parameters (see figure 18.30).

The options for the motif search 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 <http://www.expasy.org/cgi-bin/prosite-list.pl>).

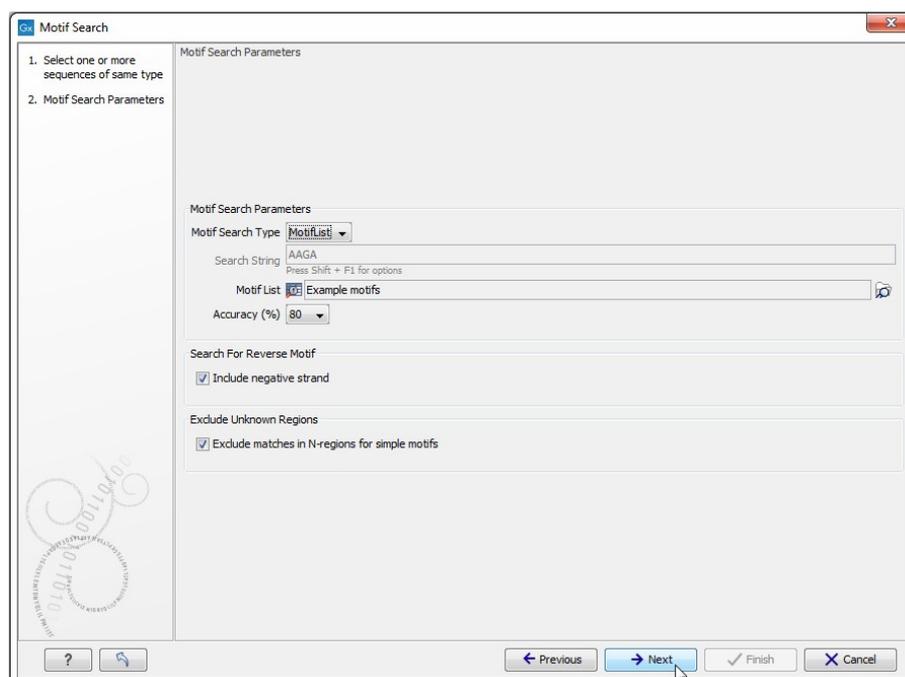


Figure 18.30: Setting parameters for the motif search.

- 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 two types of results that can be produced:

- **Add annotations.** This will add an annotation to the sequence when a motif is found (an example is shown in figure 18.31).
- **Create table.** This will create an overview table of all the motifs found for all the input sequences.

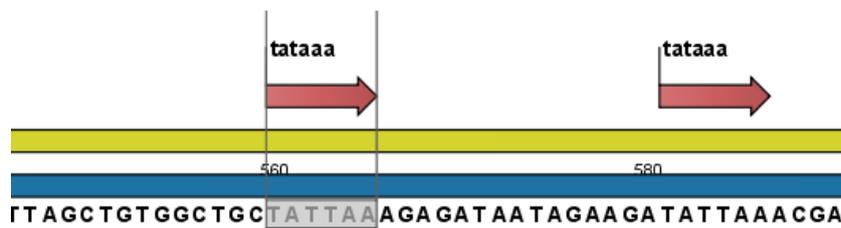


Figure 18.31: Sequence view displaying the pattern found. The search string was 'tataaa'.

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 <http://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.

`[^A-M]` will match any character except those between A and M (Excluding). You can also put single characters between the brackets: The expression `[^AG]` matches any character except A and G.

`[A-Z&&[^M-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&&[^CG]]` 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 \wedge restricts the search to the beginning of your sequence. For example, if you search through a sequence with the regular expression $\wedge AC$, 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 $[ACG][\wedge AC]G\{2\}$ 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 $G.[\wedge A]\$$ 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 one of two ways:

Toolbox | General Sequence Analysis (📁) | Create Motif List (🛠️)

File | New | Motif List (📄)

Add (+) button at the bottom of the view. This will open a dialog shown in figure 18.32.

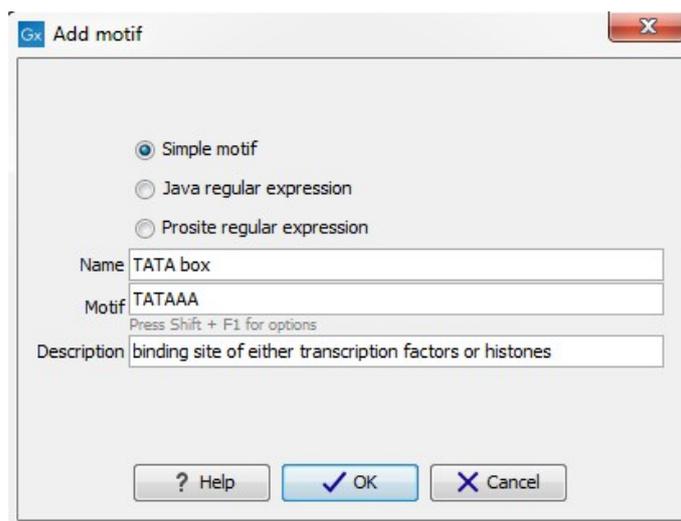


Figure 18.32: Entering a new motif in the list.

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".

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

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CLC Main Workbench offers different kinds of sequence analyses, which only apply to DNA and RNA.

19.1 Convert DNA to RNA

CLC Main Workbench lets you convert a DNA sequence into RNA, substituting the T residues (Thymine) for U residues (Urasil):

Toolbox | Nucleotide Analysis  | **Convert DNA to RNA** 

This opens the dialog displayed in figure 19.1:

If a sequence was selected before choosing the Toolbox action, 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** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Note! You can select multiple DNA sequences and sequence lists at a time. If the sequence list contains RNA sequences as well, they will not be converted.

19.2 Convert RNA to DNA

CLC Main Workbench lets you convert an RNA sequence into DNA, substituting the U residues

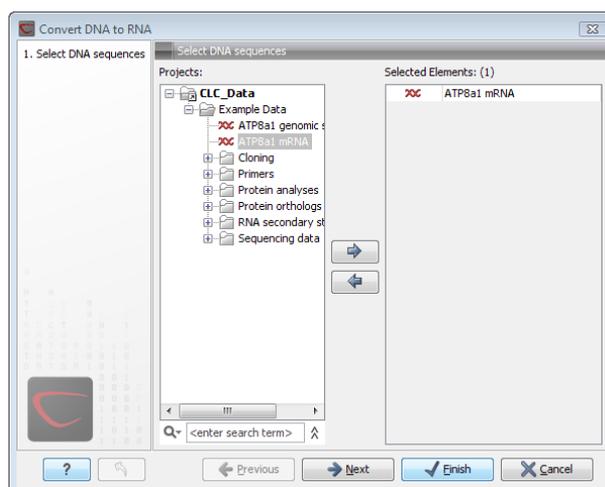


Figure 19.1: Translating DNA to RNA.

(Urasil) for T residues (Thymine):

Toolbox | Nucleotide Analysis (🗑️) | Convert RNA to DNA (🔄)

This opens the dialog displayed in figure 19.2:

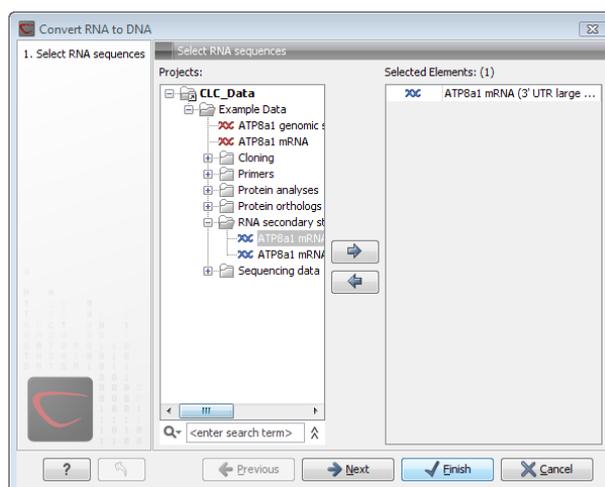


Figure 19.2: Translating RNA to DNA.

If a sequence was selected before choosing the Toolbox action, 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** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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.

Note! You can select multiple RNA sequences and sequence lists at a time. If the sequence list contains DNA sequences as well, they will not be converted.

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:

Toolbox | Nucleotide Analysis  | **Reverse Complement Sequence** 

This opens the dialog displayed in figure 19.3:

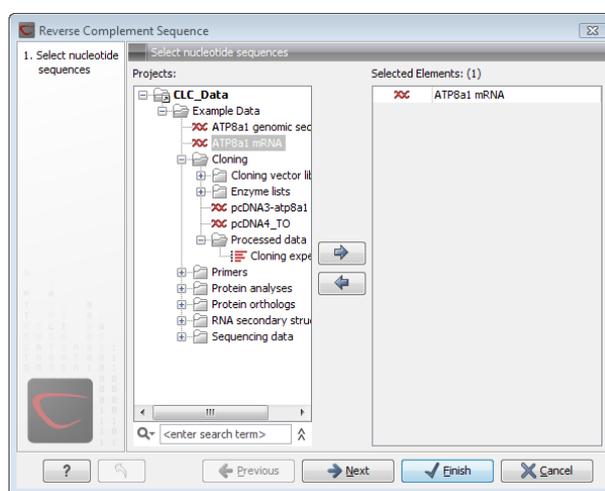


Figure 19.3: Creating a reverse complement sequence.

If a sequence was selected before choosing the Toolbox action, the 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** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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 Reverse sequence

CLC Main Workbench is able to create the reverse of a nucleotide sequence.

Note! This is not the same as a reverse complement. If you wish to create the reverse complement, please refer to section 19.3.

To run the tool, go to:

Toolbox | Nucleotide Analysis (📄) | Reverse Sequence (↔)

This opens the dialog displayed in figure 19.4:

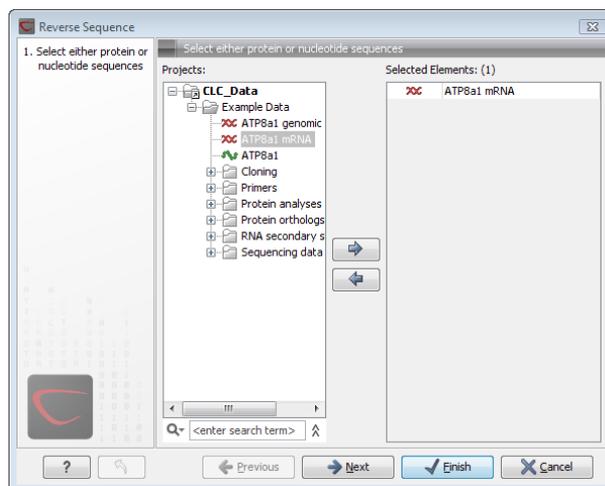


Figure 19.4: Reversing a sequence.

If a sequence was selected before choosing the Toolbox action, the 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** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Note! This is not the same as a reverse complement. If you wish to create the reverse complement, please refer to section 19.3.

19.5 Translation of DNA or RNA to protein

In *CLC Main Workbench* you can translate a nucleotide sequence into a protein sequence using the **Toolbox** tools. Usually, you use the +1 reading frame which means that the translation starts from the first nucleotide. Stop codons result in an asterisk being inserted in the protein sequence at the corresponding position. It is possible to translate in any combination of the six reading frames in one analysis. To translate, go to:

Toolbox | Nucleotide Analysis (📄) | Translate to Protein (📄)

This opens the dialog displayed in figure 19.5:

If a sequence was selected before choosing the Toolbox action, the 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.

Clicking **Next** generates the dialog seen in figure 19.6:

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.

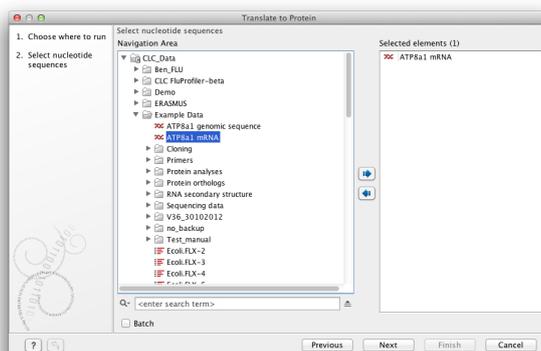


Figure 19.5: Choosing sequences for translation.

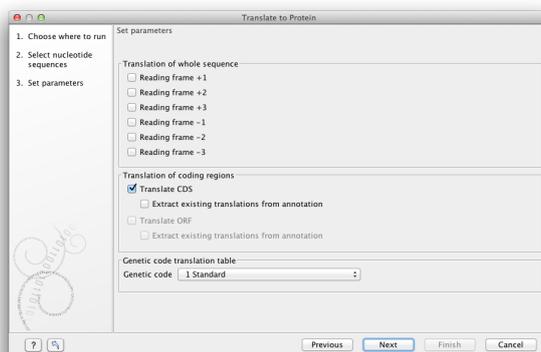


Figure 19.6: Choosing translation of CDS's using standard translation table.

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 translation table Lets you specify the genetic code for the translation. The translation tables are occasionally updated from NCBI. The tables are not available in this printable version of the user manual. Instead, the tables are included in the **Help**-menu in the **Menu Bar** (in the appendix).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. 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.

19.5.1 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 and ORF" in the translation dialog (see figure 19.6).

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 (👉) | choose a translation table | OK

If the annotation contains information about the translation, this information will be used, and you do not have to specify a translation table.

The CDS and ORF annotations are colored yellow as default.

19.6 Find open reading frames

The *CLC Main Workbench* **Find Open Reading Frames** function can be used to find all open reading frames (ORF) in a sequence, or, by choosing particular start codons to use, it can be used as a rudimentary gene finder. ORFs identified will be shown as annotations on the sequence. You have the option of choosing a translation table, the start codons to use, minimum ORF length as well as a few other parameters. These choices are explained in this section.

To find open reading frames:

Toolbox | Nucleotide Analysis (📁) | Find Open Reading Frames (🔍)

This opens the dialog displayed in figure 19.7:

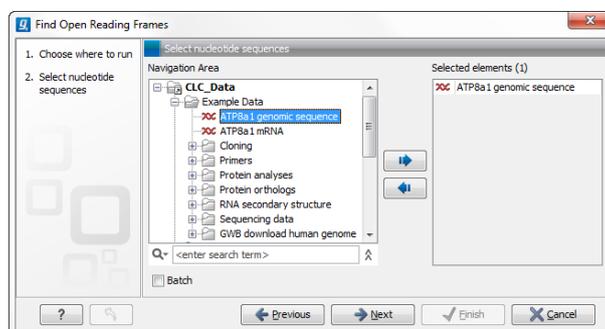


Figure 19.7: Create Reading Frame dialog.

If a sequence was selected before choosing the Toolbox action, the 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.

The **Find Open Reading Frames** tool simply looks for start and stop codons and reports any open reading frames that satisfy the parameters. If you want to adjust the parameters for finding open reading frames click **Next**.

19.6.1 Open reading frame parameters

This opens the dialog displayed in figure 19.8:

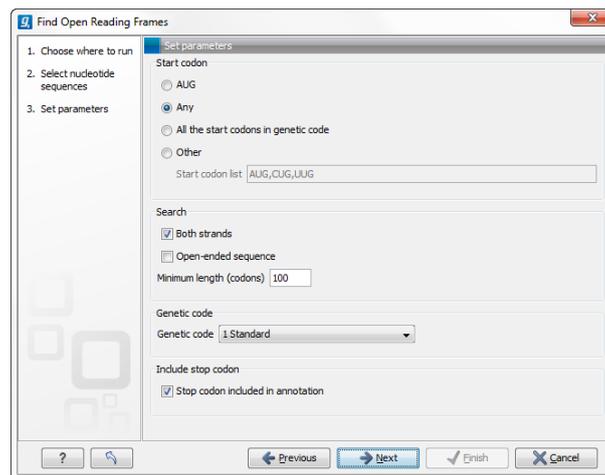


Figure 19.8: Create Reading Frame dialog.

The adjustable parameters for the search are:

- **Start codon:**
 - **AUG.** Most commonly used start codon.
 - **Any.** Find all open reading frames of specified length. Any combination of three bases that is not a stop-codon is interpreted as a start codon, and translated according to the specified genetic code.
 - **All start codons in genetic code.**
 - **Other.** Here you can specify a number of start codons separated by commas.
- **Both strands.** Finds reading frames on both strands.
- **Open-ended Sequence.** Allows the ORF to start or end outside the sequence. If the sequence studied is a part of a larger sequence, it may be advantageous to allow the ORF to start or end outside the sequence.
- **Genetic code translation table.**
- **Include stop codon in result** The ORFs will be shown as annotations which can include the stop codon if this option is checked. The translation tables are occasionally updated from NCBI. The tables are not available in this printable version of the user manual. Instead, the tables are included in the **Help**-menu in the **Menu Bar** (in the appendix).
- **Minimum Length.** Specifies the minimum length for the ORFs to be found. The length is specified as number of codons.

Using open reading frames for gene finding 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).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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.

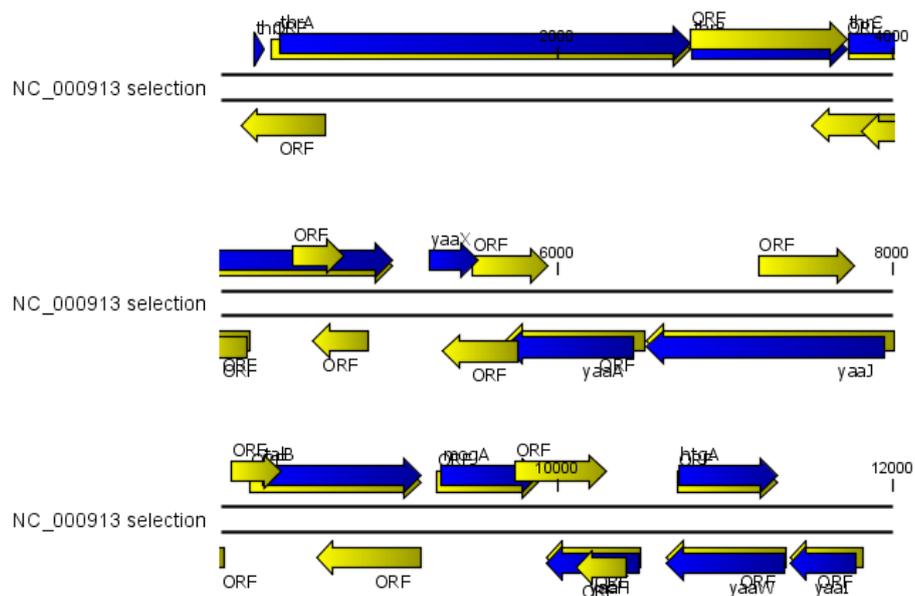


Figure 19.9: The first 12,000 positions of the *E. coli* sequence NC_000913 downloaded from GenBank. The blue (dark) annotations are the genes while the yellow (brighter) annotations are the ORFs with a length of at least 100 amino acids. On the positive strand around position 11,000, a gene starts before 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.

Chapter 20

Protein analyses

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CLC Main Workbench offers a number of analyses of proteins as described in this chapter.

20.1 Signal peptide prediction

Signal peptides target proteins to the extracellular environment either through direct plasmamembrane translocation in prokaryotes or is routed through the Endoplasmatic Reticulum in eukaryotic cells. The signal peptide is removed from the resulting mature protein during translocation across the membrane. For prediction of signal peptides, we query SignalP [Nielsen et al., 1997, Bendtsen et al., 2004b] located at <http://www.cbs.dtu.dk/services/SignalP/>. Thus an active internet connection is required to run the signal peptide prediction. Additional information on SignalP and Center for Biological Sequence analysis (CBS) can be found at <http://www.cbs.dtu.dk> and in the original research papers [Nielsen et al., 1997, Bendtsen et al., 2004b].

In order to predict potential signal peptides of proteins, the D-score from the SignalP output is used for discrimination of signal peptide versus non-signal peptide (see section 20.1.3). This score has been shown to be the most accurate [Klee and Ellis, 2005] in an evaluation study of signal peptide predictors.

In order to use SignalP, you need to download the *SignalP plugin* using the plugin manager, see section 1.7.1.

When the plugin is downloaded and installed, you can use it to predict signal peptides:

Toolbox | Protein Analysis (📁) | Signal Peptide Prediction (🌿)

If a sequence was selected before choosing the Toolbox action, 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. The SignalP service is limited to 2,000 sequences and 200,000 amino acids for one submission. Each sequence may be no longer than 6,000 amino acids.

Click **Next** to set parameters for the SignalP analysis.

20.1.1 Signal peptide prediction parameter settings

You should select which organism group the input sequences belong to. the default is eukaryote (see figure 20.1).

- Eukaryote (default)
- Gram-negative bacteria
- Gram-positive bacteria

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 if a signal peptide is found. If no signal peptide is found in the sequence, a dialog box will be shown.

The predictions obtained can either be shown as annotations on the sequence, listed in a table or be shown as the detailed and full text output from the SignalP method. This can be used to interpret borderline predictions:

- Add annotations to sequence

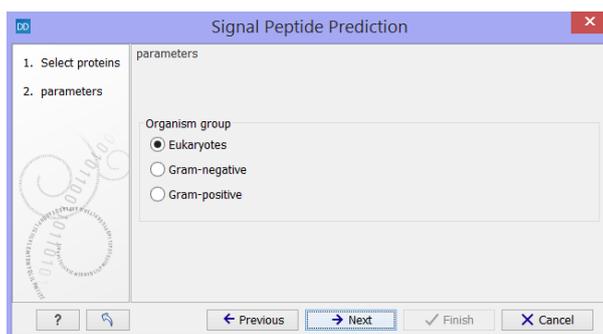


Figure 20.1: Setting the parameters for signal peptide prediction.

- Create table
- Text

Click **Next** to adjust how to handle the results, then click **Finish**.

20.1.2 Signal peptide prediction output

After running the prediction as described above, the protein sequence will show predicted signal peptide as annotations on the original sequence (see figure 20.2). Make sure the Side Panel settings of the sequence is so that 'Show annotations' is checked in the 'Annotation layout' palette, and that the annotation type 'Signal peptide' is checked in the 'Annotation types' palette.

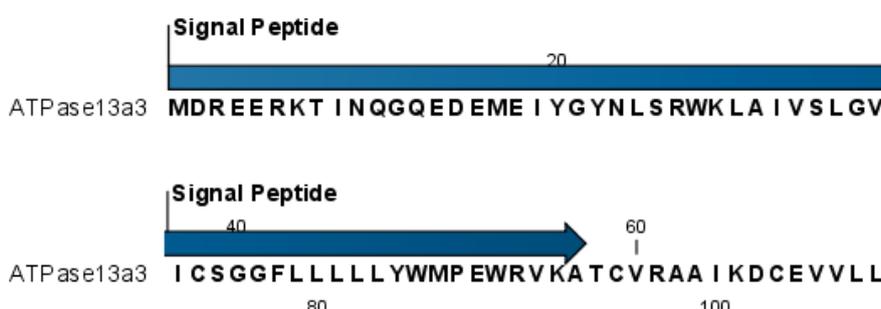


Figure 20.2: N-terminal signal peptide shown as annotation on the sequence.

Additional notes can be added through the **Edit annotation** (👉) right-click mouse menu. See section 12.3.2.

Undesired annotations can be removed through the **Delete Annotation** (🗑️) right-click mouse menu. See section 12.3.4.

20.1.3 Bioinformatics explained: Prediction of signal peptides

Why the interest in signal peptides?

The importance of signal peptides was shown in 1999 when Günter Blobel received the Nobel Prize in physiology or medicine for his discovery that "proteins have intrinsic signals that govern their transport and localization in the cell" [Blobel, 2000]. He pointed out the importance of defined peptide motifs for targeting proteins to their site of function.

Performing a query to PubMed¹ reveals that thousands of papers have been published, regarding signal peptides, secretion and subcellular localization, including knowledge of using signal peptides as vehicles for chimeric proteins for biomedical and pharmaceutical industry. Many papers describe statistical or machine learning methods for prediction of signal peptides and prediction of subcellular localization in general. After the first published method for signal peptide prediction [von Heijne, 1986], more and more methods have surfaced, although not all methods have been made available publicly.

Different types of signal peptides

Soon after Günter Blobel's initial discovery of signal peptides, more targeting signals were found. Most cell types and organisms employ several ways of targeting proteins to the extracellular environment or subcellular locations. Most of the proteins targeted for the extracellular space or subcellular locations carry specific sequence motifs (signal peptides) characterizing the type of secretion/targeting it undergoes.

Several new different signal peptides or targeting signals have been found during the later years, and papers often describe a small amino acid motif required for secretion of that particular protein. In most of the latter cases, the identified sequence motif is only found in this particular protein and as such cannot be described as a new group of signal peptides.

Describing the various types of signal peptides is beyond the scope of this text but several review papers on this topic can be found on PubMed. Targeting motifs can either be removed from, or retained in the mature protein after the protein has reached the correct and final destination. Some of the best characterized signal peptides are depicted in figure 20.3.

Numerous methods for prediction of protein targeting and signal peptides have been developed; some of them are mentioned and cited in the introduction of the SignalP research paper [Bendtsen et al., 2004b]. However, no prediction method will be able to cover all the different types of signal peptides. Most methods predicts classical signal peptides targeting to the general secretory pathway in bacteria or classical secretory pathway in eukaryotes. Furthermore, a few methods for prediction of non-classically secreted proteins have emerged [Bendtsen et al., 2004a, Bendtsen et al., 2005].

Prediction of signal peptides and subcellular localization

In the search for accurate prediction of signal peptides, many approaches have been investigated. Almost 20 years ago, the first method for prediction of classical signal peptides was published [von Heijne, 1986]. Nowadays, more sophisticated machine learning methods, such as neural networks, support vector machines, and hidden Markov models have arrived along with the increasing computational power and they all perform superior to the old weight matrix based methods [Menne et al., 2000]. Also, many other "classical" statistical approaches have been carried out, often in conjunction with machine learning methods. In the following sections, a wide range of different signal peptide and subcellular prediction methods will be described.

Most signal peptide prediction methods require the presence of the correct N-terminal end of the preprotein for correct classification. As large scale genome sequencing projects sometimes assign the 5'-end of genes incorrectly, many proteins are annotated without the correct N-terminal [Reinhardt and Hubbard, 1998] leading to incorrect prediction of subcellular localization.

¹<http://www.ncbi.nlm.nih.gov/entrez/>

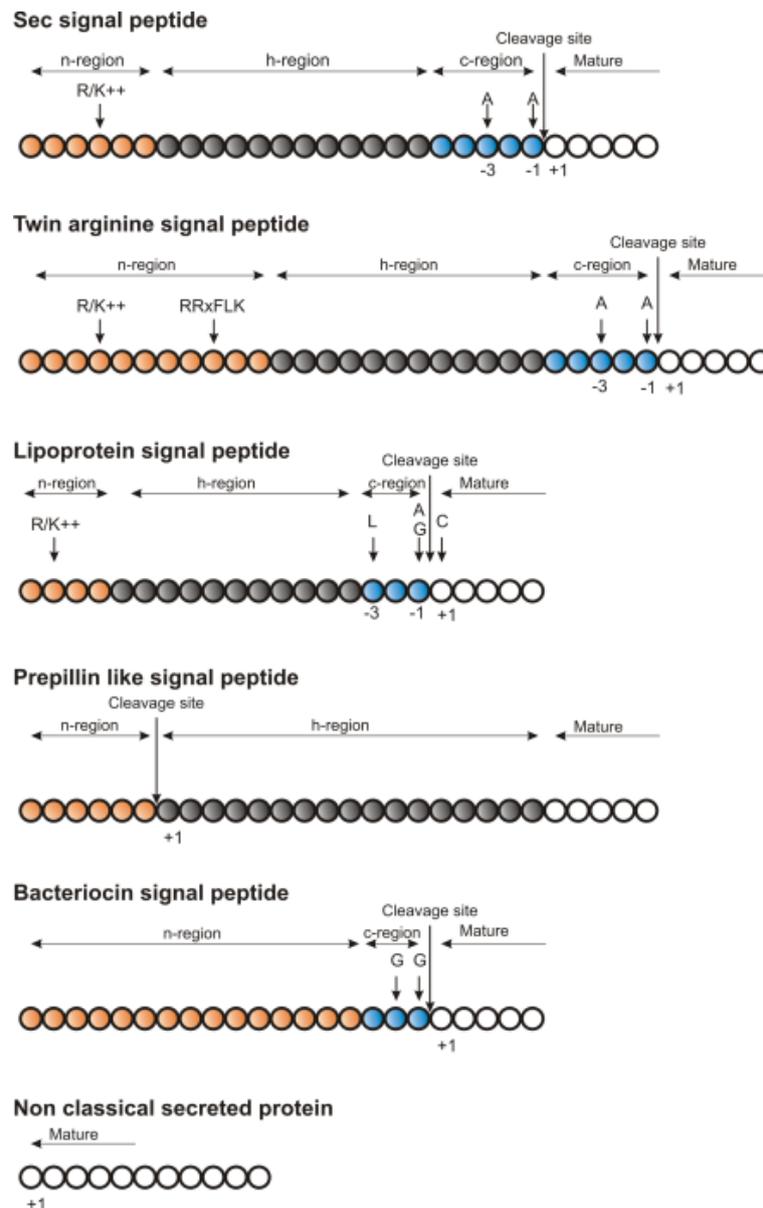


Figure 20.3: Schematic representation of various signal peptides. Red color indicates n-region, gray color indicates h-region, cyan indicates c-region. All white circles are part of the mature protein. +1 indicates the first position of the mature protein. The length of the signal peptides is not drawn to scale.

These erroneous predictions can be ascribed directly to poor gene finding. Other methods for prediction of subcellular localization use information within the mature protein and therefore they are more robust to N-terminal truncation and gene finding errors.

The SignalP method

One of the most cited and best methods for prediction of classical signal peptides is the SignalP method [Nielsen et al., 1997, Bendtsen et al., 2004b]. In contrast to other methods, SignalP also predicts the actual cleavage site; thus the peptide which is cleaved off during translocation over the membrane. Recently, an independent research paper has rated SignalP version 3.0

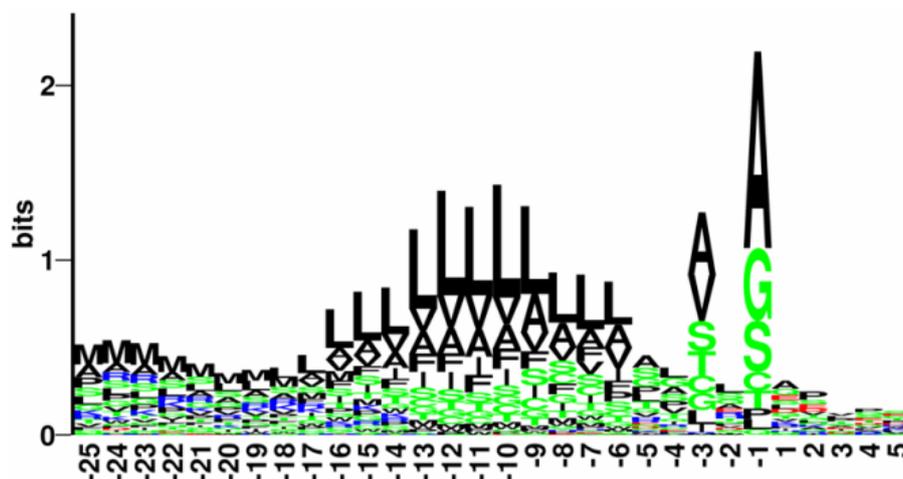


Figure 20.4: Sequence logo of eukaryotic signal peptides, showing conservation of amino acids in bits [Schneider and Stephens, 1990]. Polar and hydrophobic residues are shown in green and black, respectively, while blue indicates positively charged residues and red negatively charged residues. The logo is based on an ungapped sequence alignment fixed at the -1 position of the signal peptides.

to be the best standalone tool for signal peptide prediction. It was shown that the D-score which is reported by the SignalP method is the best measure for discriminating secretory from non-secretory proteins [Klee and Ellis, 2005].

SignalP is located at <http://www.cbs.dtu.dk/services/SignalP/>

What do the SignalP scores mean?

Many bioinformatics approaches or prediction tools do not give a yes/no answer. Often the user is facing an interpretation of the output, which can be either numerical or graphical. Why is that? In clear-cut examples there are no doubt; yes: this is a signal peptide! But, in borderline cases it is often convenient to have more information than just a yes/no answer. Here a graphical output can aid to interpret the correct answer. An example is shown in figure 20.5.

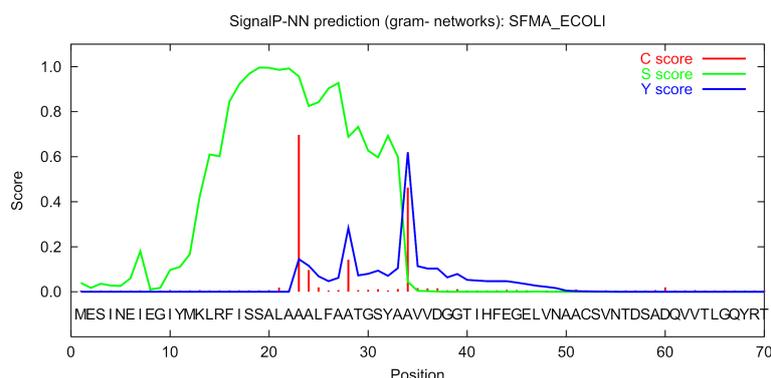


Figure 20.5: Graphical output from the SignalP method of Swiss-Prot entry *SFMA_ECOLI*. Initially this seemed like a borderline prediction, but closer inspection of the sequence revealed an internal methionine at position 12, which could indicate a erroneously annotated start of the protein. Later this protein was re-annotated by Swiss-Prot to start at the M in position 12. See the text for description of the scores.

The graphical output from SignalP (neural network) comprises three different scores, C, S and Y. Two additional scores are reported in the SignalP3-NN output, namely the *S-mean* and the *D-score*, but these are only reported as numerical values.

For each organism class in SignalP; Eukaryote, Gram-negative and Gram-positive, two different neural networks are used, one for predicting the actual signal peptide and one for predicting the position of the signal peptidase I (SPase I) cleavage site. The *S-score* for the signal peptide prediction is reported for every single amino acid position in the submitted sequence, with high scores indicating that the corresponding amino acid is part of a signal peptide, and low scores indicating that the amino acid is part of a mature protein.

The *C-score* is the "cleavage site" score. For each position in the submitted sequence, a *C-score* is reported, which should only be significantly high at the cleavage site. Confusion is often seen with the position numbering of the cleavage site. When a cleavage site position is referred to by a single number, the number indicates the first residue in the mature protein. This means that a reported cleavage site between amino acid 26-27 corresponds to the mature protein starting at (and include) position 27.

Y-max is a derivative of the *C-score* combined with the *S-score* resulting in a better cleavage site prediction than the raw *C-score* alone. This is due to the fact that multiple high-peaking *C-scores* can be found in one sequence, where only one is the true cleavage site. The cleavage site is assigned from the *Y-score* where the slope of the *S-score* is steep and a significant *C-score* is found.

The *S-mean* is the average of the *S-score*, ranging from the N-terminal amino acid to the amino acid assigned with the highest *Y-max* score, thus the *S-mean* score is calculated for the length of the predicted signal peptide. The *S-mean* score was in SignalP version 2.0 used as the criteria for discrimination of secretory and non-secretory proteins.

The *D-score* is introduced in SignalP version 3.0 and is a simple average of the *S-mean* and *Y-max* score. The score shows superior discrimination performance of secretory and non-secretory proteins to that of the *S-mean* score which was used in SignalP version 1 and 2.

For non-secretory proteins all the scores represented in the SignalP3-NN output should ideally be very low.

The hidden Markov model calculates the probability of whether the submitted sequence contains a signal peptide or not. The eukaryotic HMM model also reports the probability of a signal anchor, previously named uncleaved signal peptides. Furthermore, the cleavage site is assigned by a probability score together with scores for the n-region, h-region, and c-region of the signal peptide, if it is found.

Other useful resources

<http://www.cbs.dtu.dk/services/SignalP>

Pubmed entries for some of the original papers.

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=9051728&query_hl=1&itool=pubmed_docsum

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=15223320&dopt=Citation

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20.2 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:

Toolbox | Protein Analysis (🌿) | Create Protein Charge Plot (📊)

This opens the dialog displayed in figure 20.6:

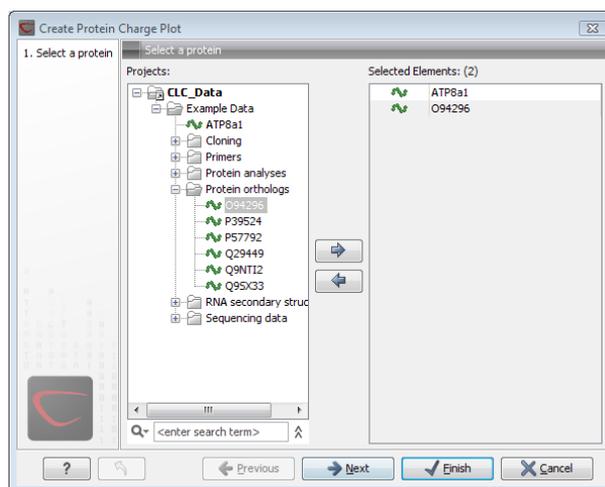


Figure 20.6: Choosing protein sequences to calculate protein charge.

If a sequence was selected before choosing the Toolbox action, the 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.

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

20.2.1 Modifying the layout

Figure 20.7 shows the electrical charges for three proteins. In the **Side Panel** to the right, you can modify the layout of the graph.

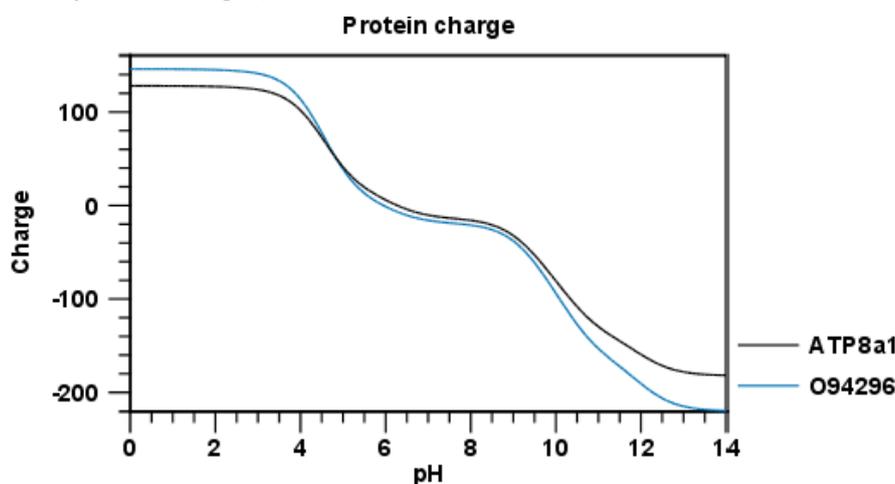


Figure 20.7: View of the protein charge.

See section B in the appendix for information about the graph view.

20.3 Transmembrane helix prediction

Many proteins are integral membrane proteins. Most membrane proteins have hydrophobic regions which span the hydrophobic core of the membrane bi-layer and hydrophilic regions located on the outside or the inside of the membrane. Many receptor proteins have several transmembrane helices spanning the cellular membrane.

For prediction of transmembrane helices, *CLC Main Workbench* uses TMHMM version 2.0 [Krogh et al., 2001] located at <http://www.cbs.dtu.dk/services/TMHMM/>, thus an active internet connection is required to run the transmembrane helix prediction. Additional information on THMHH and Center for Biological Sequence analysis (CBS) can be found at <http://www.cbs.dtu.dk> and in the original research paper [Krogh et al., 2001].

In order to use the transmembrane helix prediction, you need to download the plugin using the plugin manager (see section 1.7.1).

When the plugin is downloaded and installed, you can use it to predict transmembrane helices:

Toolbox | Protein Analysis (📁) | Transmembrane Helix Prediction (🔍)

If a sequence was selected before choosing the Toolbox action, 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.

The predictions obtained can either be shown as annotations on the sequence, in a table or as the detailed and text output from the TMHMM method.

- Add annotations to sequence
- Create table
- Text

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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 if a transmembrane helix is found. If a transmembrane helix is not found a dialog box will be presented.

After running the prediction as described above, the protein sequence will show predicted transmembrane helices as annotations on the original sequence (see figure 20.8). Moreover, annotations showing the topology will be shown. That is, which part the proteins is located on the inside or on the outside.

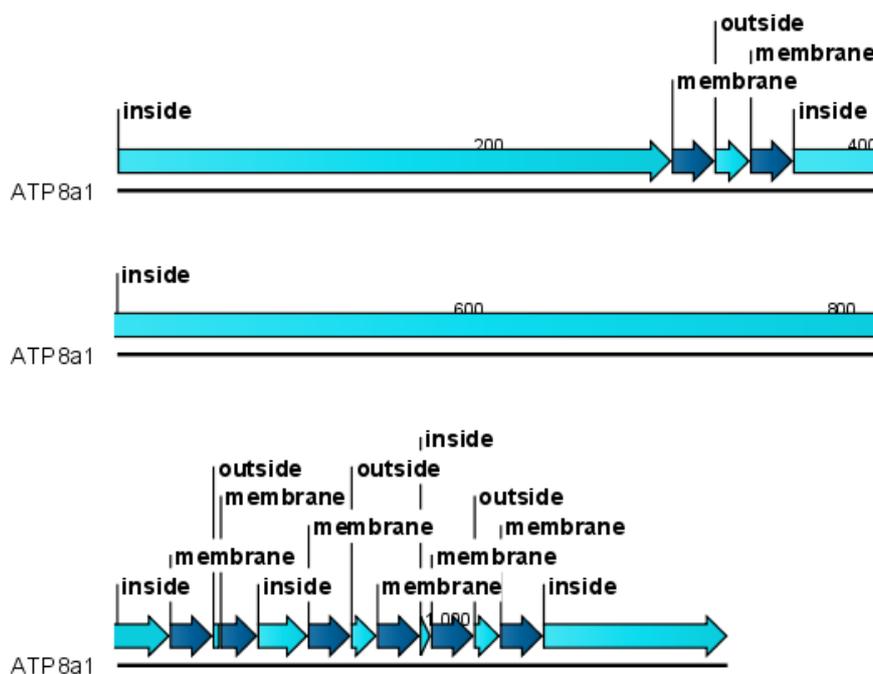


Figure 20.8: Transmembrane segments shown as annotation on the sequence and the topology.

Each annotation will carry a tooltip note saying that the corresponding annotation is predicted with TMHMM version 2.0. Additional notes can be added through the **Edit annotation** (👉) right-click mouse menu. See section 12.3.2.

Undesired annotations can be removed through the **Delete Annotation** (🗑️) right-click mouse menu. See section 12.3.4.

20.4 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 method can not always be expected as the two methods are based on different training sets.

20.4.1 Plot of antigenicity

Displaying the antigenicity for a protein sequence in a plot is done in the following way:

Toolbox | 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 Toolbox action, these are shown in the **Selected Elements**. Clicking **Next** takes you through to **Step 2**, which is displayed in figure 20.9.

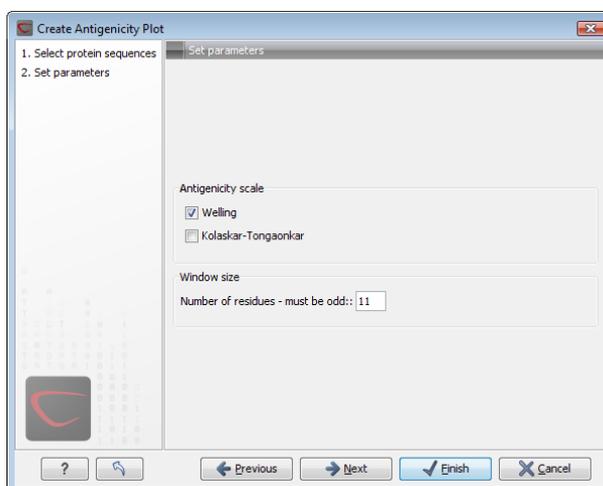


Figure 20.9: Step two in the Antigenicity Plot allows you to choose different antigenicity scales and the window size.

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. The result can be seen in figure 20.10.

See section B in the appendix for information about the graph view.

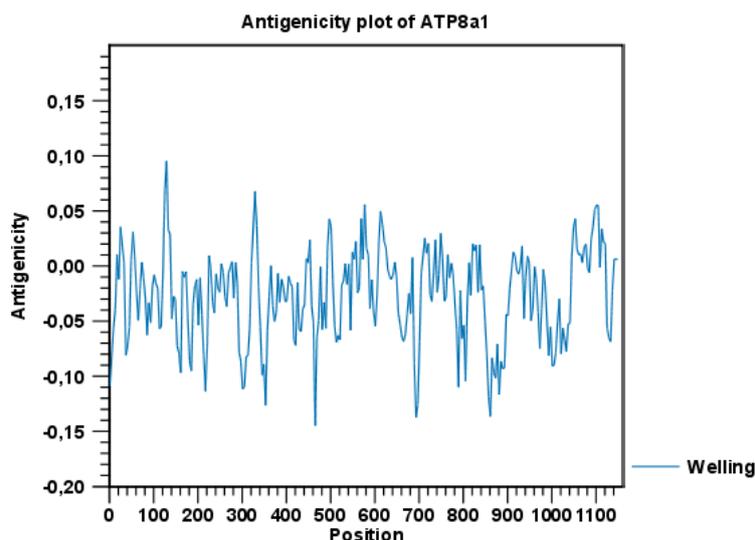


Figure 20.10: The result of the antigenicity plot calculation and the associated Side Panel.

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.

20.4.2 Antigenicity graphs along sequence

Antigenicity graphs along the sequence can be displayed using the **Side Panel**. The functionality is similar to hydrophobicity (see section 20.5.2).

20.5 Hydrophobicity

CLC Main Workbench can calculate the hydrophobicity of protein sequences in different ways, using different algorithms. (See section 20.5.3). 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.

20.5.1 Hydrophobicity plot

Displaying the hydrophobicity for a protein sequence in a plot is done in the following way:

Toolbox | **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 20.11.

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 choose from a number of hydrophobicity scales

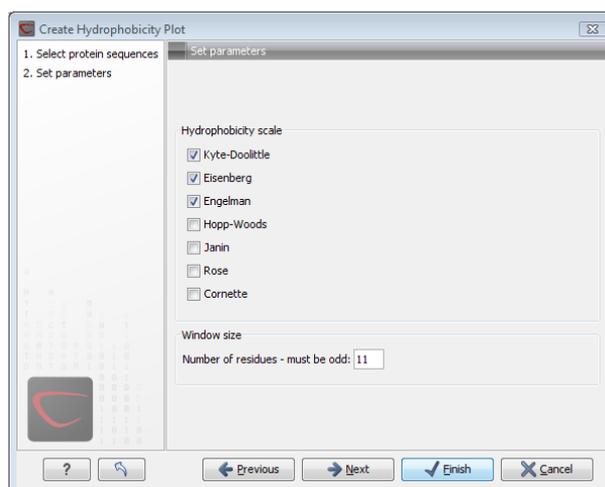


Figure 20.11: Step two in the Hydrophobicity Plot allows you to choose hydrophobicity scale and the window size.

which are further explained in section 20.5.3 Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. The result can be seen in figure 20.12.

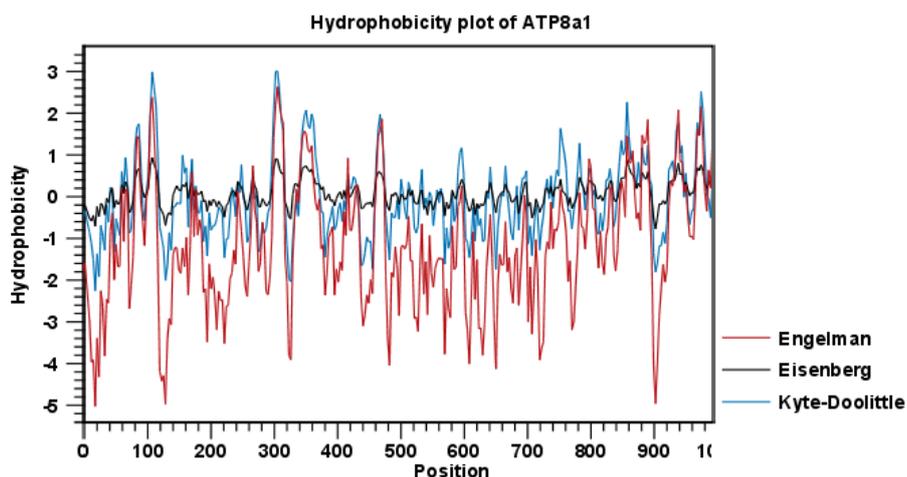


Figure 20.12: The result of the hydrophobicity plot calculation and the associated Side Panel.

See section B in the appendix for information about the graph view.

20.5.2 Hydrophobicity graphs along sequence

Hydrophobicity graphs along sequence can be displayed easily by activating the calculations from the **Side Panel** for a sequence.

right-click protein sequence in Navigation Area | Show | Sequence | open Protein info in Side Panel

or **double-click protein sequence in Navigation Area | Show | Sequence | open Protein info in Side Panel**

These actions result in the view displayed in figure 20.13.

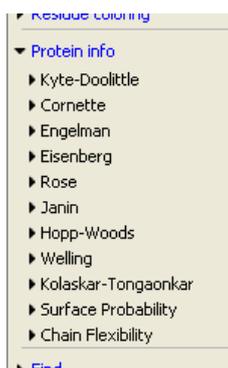


Figure 20.13: The different available scales in Protein info in **CLC Main Workbench**.

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 20.5.3).

In the following we will focus on the different ways that *CLC Main 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 hydrophobicity scores. You can choose one, two or all three options by selecting the boxes. (See figure 20.14).



Figure 20.14: 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 20.14. Notice that you can choose the height of the graphs underneath the sequence.

20.5.3 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.

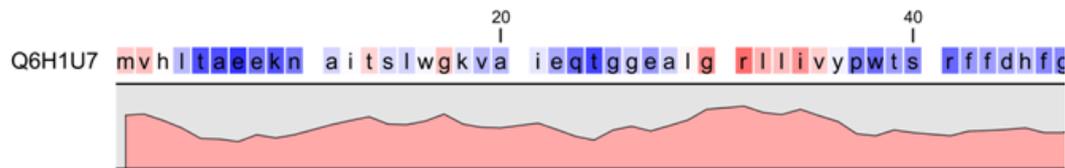


Figure 20.15: Plot of hydrophobicity along the amino acid sequence. Hydrophobic regions on the sequence have higher numbers according to the graph below the sequence, furthermore hydrophobic regions are colored on the sequence. Red indicates regions with high hydrophobicity and blue indicates regions 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 20.15).

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

| aa | aa | Kyte-Doolittle | Hopp-Woods | Cornette | Eisenberg | Rose | Janin | Engelman (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.*

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].

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>

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20.6 Pfam domain search

With *CLC Main Workbench* you can perform a search for domains in protein sequences using the Pfam database. The Pfam database [Bateman et al., 2004] at <http://pfam.sanger.ac.uk/> was initially developed to aid the annotation of the *C. elegans* genome. The database is a large collection of multiple sequence alignments that cover 14831 protein domains and protein families as of March 2014. The database contains profile hidden Markov models (HMMs) for individual domain alignments, which can be used to quickly identify domains in protein sequences.

Many proteins have a unique combination of domains, which can be responsible for e.g. 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.

Using the **Pfam Domain Search** tool in *CLC Main Workbench*, you can search for domains in sequence data which otherwise do not carry any annotation information. The domain search is performed using the `hmmsearch` tool from the HMMER3 package version 3.1b1 (<http://hmmer.janelia.org/>). The Pfam search tool annotates protein sequences with all domains in the Pfam database that have a significant match. It is possible to lower the significance cutoff thresholds in the `hmmsearch` algorithm, which will reduce the number of domain annotations. Individual domain annotations can be removed manually as described in section 12.3.4.

20.6.1 Download of Pfam database

To be able to run the **Pfam Domain Search** tool you must first download the Pfam database. The Pfam database can be downloaded using:

Toolbox | Protein Analysis (📁) | Download Pfam Database (🔗)

Specify where you would like to save the downloaded Pfam database. The output of the **Download Pfam Database** tool is a database object, which can be selected as a parameter for the Pfam Domain Search tool. It doesn't really make sense to try to open the database object directly from the **Navigation Area** as all you can see directly is the element history (which version of the Workbench that has been used and the name of the downloaded files) and the element info, which in this case only provides information about the database name.

20.6.2 Running Pfam Domain Search

When you have downloaded the Pfam database you are ready to perform a Pfam domain search. To do this start the Pfam search tool:

Toolbox | Protein Analysis (📁) | Pfam Domain Search (↔)

If a sequence was selected before choosing the Toolbox action, 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.

You can perform the analysis on several protein sequences at a time. This will add annotations to all the sequences. Click **Next** to adjust parameters (see figure 20.16).

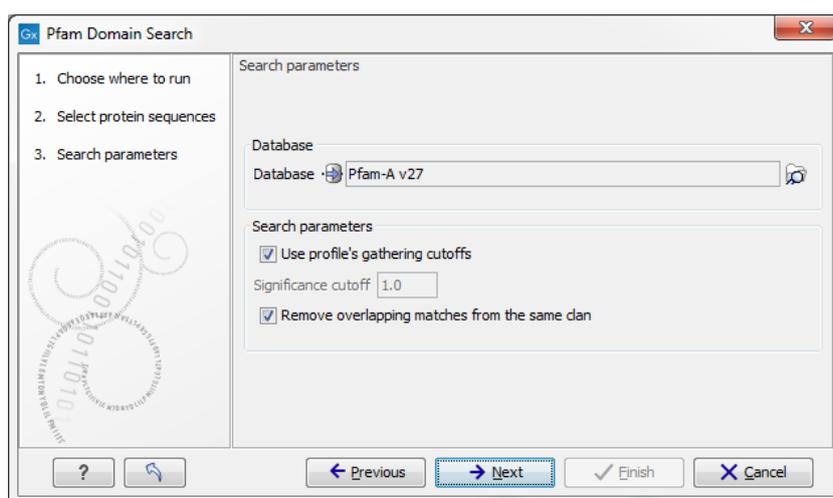


Figure 20.16: Setting parameters for Pfam Domain Search.

- **Database.** Choose which database to use when searching for Pfam domains. For information on how to download a Pfam database see section 20.6.1
- 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 compared to 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.

Click **Next** to adjust the output of the tool. The Pfam search tool can produce two types of output. It can add annotations on the input sequences that show the domains found (see figure 20.17) and it can output a table with all the domains found.

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

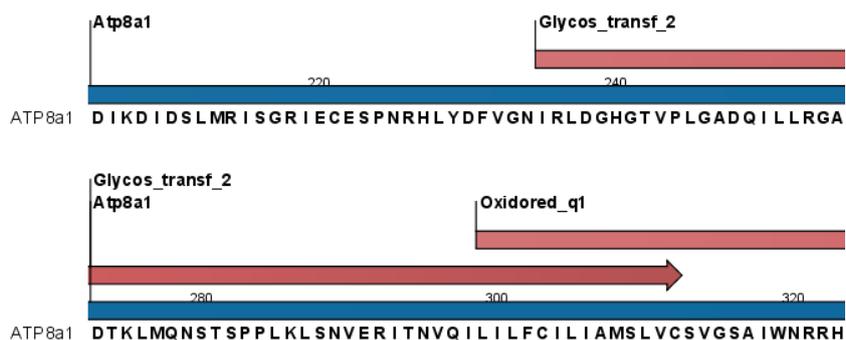


Figure 20.17: Annotations (in red) that were added by the Pfam search tool.

Domain annotations added by the Pfam search tool have the type **Region**. If the annotations are not visible they have to be enabled in the side panel. Detailed information for each domain annotation, such as the bit score which is the basis for the prediction of domains, is available through the annotation tool tip.

A more detailed description of the scores provided in the annotation tool tips can be found here: <http://pfam.sanger.ac.uk/help#tabview=tab5>.

20.7 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 databank (<http://www.rcsb.org/pdb/>) 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:

Toolbox | Protein Analysis (📁) | **Predict secondary structure** (🌀)

This opens the dialog displayed in figure 20.18:

If a sequence was selected before choosing the Toolbox action, 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.

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.

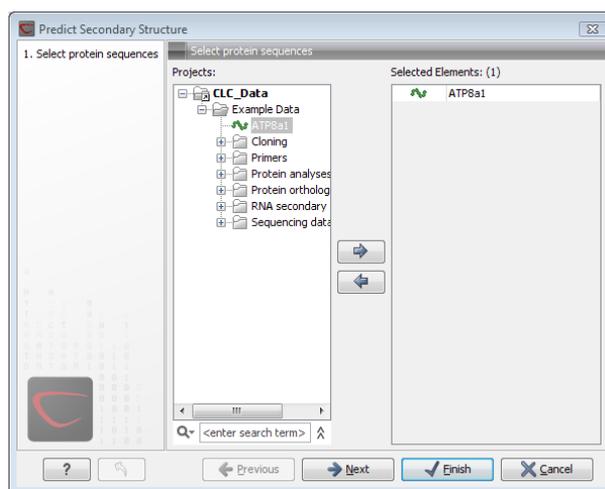


Figure 20.18: Choosing one or more protein sequences for secondary structure prediction.

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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 20.19).

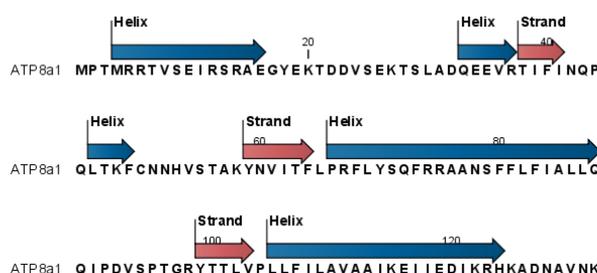


Figure 20.19: Alpha-helices and beta-strands shown as annotations on the sequence.

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 12.3.2.

Undesired alpha-helices or beta-sheets can be removed through the **Delete Annotation** (🗑️) right-click mouse menu. See section 12.3.4.

20.8 Protein report

CLC Main Workbench is able to produce protein reports, that allow you to easily generate different kinds of information regarding a protein.

Actually a protein report is a collection of some of the protein analyses which are described elsewhere in this manual.

To create a protein report do the following:

Toolbox | **Protein Analysis** (📁) | **Create Protein Report** (📄)

This opens dialog **Step 1**, 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 Toolbox action, this will

be shown in the **Selected Elements**. However, you can use the arrows to change this. When the correct one is chosen, click **Next**.

In dialog **Step 2** 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.** See section 18.6 for more about this topic.
- **Plot of charge as function of pH.** See section 20.2 for more about this topic.
- **Plot of hydrophobicity.** See section 20.5 for more about this topic.
- **Plot of local complexity.** See section 18.5 for more about this topic.
- **Dot plot against self.** See section 18.4 for more about this topic.
- **Secondary structure prediction.** See section 20.7 for more about this topic.
- **Pfam domain search.** See section 20.6 for more about this topic.
- **Local BLAST.** See section 26.1.3 for more about this topic.
- **NCBI BLAST.** See section 26.1.1 for more about this topic.

When you have selected the relevant analyses, click **Next. Step 3 to Step 7** (if you select all the analyses in **Step 2**) are adjustments of parameters for the different analyses. The parameters are mentioned briefly in relation to the following steps, and you can turn to the relevant chapters or sections (mentioned above) to learn more about the significance of the parameters.

In **Step 3** you can adjust parameters 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.

In **Step 4** you can adjust parameters for hydrophobicity plots:

- **Window size.** Width of window on sequence (odd number).
- **Hydrophobicity scales.** Lets you choose between different scales.

In **Step 5** you can adjust a parameter for complexity plots:

- **Window size.** Width of window on sequence (must be odd).

In **Step 6** you can adjust parameters for dot plots:

- **Score model.** Different scoring matrices.

- **Window size.** Width of window on sequence.

In **Step 7** you can adjust parameters for Pfam domain search:

- **Database and search type.** Lets you choose different databases and specify the search for full domains or fragments. See section 20.6.2 for more info about this topic.
- **Significance cutoff.** Lets you set your E-value. See section 20.6.2 for more info about this topic.

In **Step 8** you can adjust parameters for BLAST search:

- **Program.** Lets you choose between different BLAST programs.
- **Database.** Lets you limit your search to a particular database.

20.8.1 Protein report output

An example of Protein report can be seen in figure 20.20.

1 Protein statistics

1.1 Sequence information

| | |
|-------------------|-------------------------------|
| Sequence type | Protein |
| Length | 47 nuc |
| Organism | Mus musculus (house mouse) |
| Name | CAA24102 |
| Description | beta-globin HO [Mus musculus] |
| Modification Date | 18-APR-2005 |
| Weight | 5.326,084 Da |

1.2 Half-life

| N-terminal aa | Half-life mammals | Half-life yeast | Half-life E.Coli |
|---------------|-------------------|-----------------|------------------|
| Proline | >20 hours | >20 hours | Unknown |

1.3 Extinction coefficient

| Conditions | Extinction coefficient at | Absorption at 280nm 0.1% |
|------------|---------------------------|--------------------------|
| | | |

Figure 20.20: 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. To do so:

Select content of table | Right-click the selection | Copy

You can also **Export** (📄) the report in Excel format.

20.9 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 20.9.2.

In order to make a reverse translation:

Toolbox | Protein Analysis (📁) | Reverse Translate (🔄)

This opens the dialog displayed in figure 20.21:

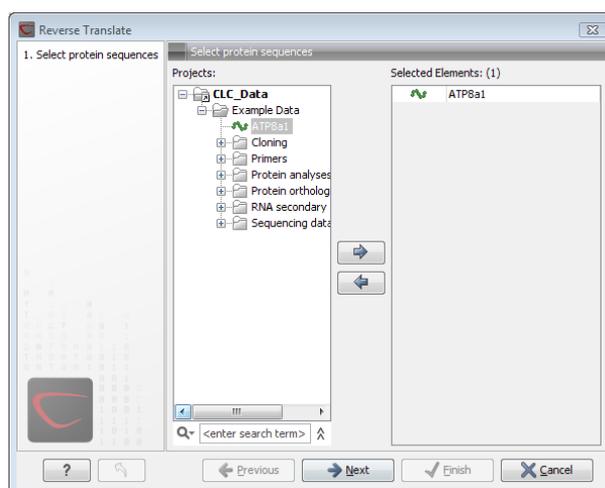


Figure 20.21: Choosing a protein sequence for reverse translation.

If a sequence was selected before choosing the Toolbox action, the 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. You can translate several protein sequences at a time.

Click **Next** to adjust the parameters for the translation.

20.9.1 Reverse translation parameters

Figure 20.22 shows the choices for making the 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

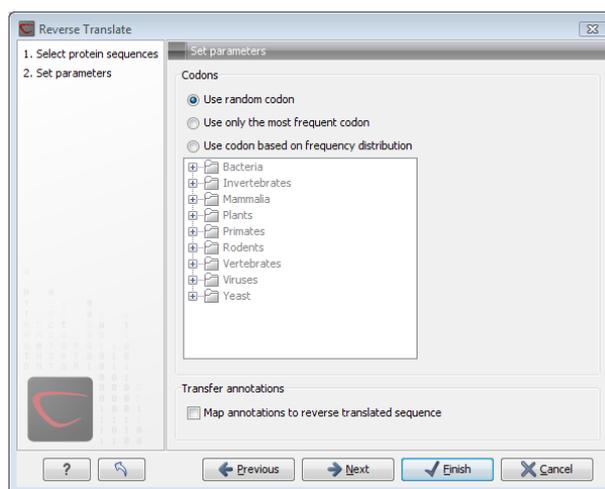


Figure 20.22: Choosing parameters for the reverse translation.

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 <http://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 K.

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. 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. 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 show the save dialog.

20.9.2 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 21st 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

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20.10 Proteolytic cleavage detection

CLC Main Workbench offers to analyze protein sequences with respect to cleavage by a selection of proteolytic enzymes. This section explains how to adjust the detection parameters and offers basic information on proteolytic cleavage in general.

20.10.1 Proteolytic cleavage parameters

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 and in textual format in a table below the sequence view.

Detection of proteolytic cleavage sites is initiated by:

Toolbox | Protein Analysis (📁) | Proteolytic Cleavage, (✂️)

This opens the dialog shown in figure 20.23:

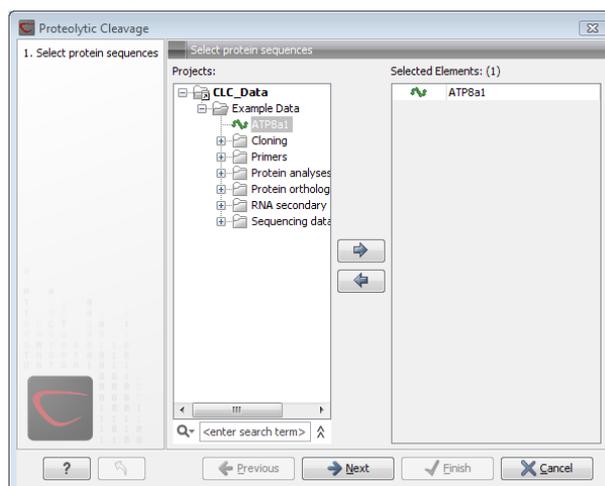


Figure 20.23: Choosing sequence CAA32220 for proteolytic cleavage.

CLC Main Workbench allows you to detect proteolytic cleavages for several sequences at a time. Correct the list of sequences by selecting a sequence and clicking the arrows pointing left and right. Then click **Next** to go to **Step 2**.

In **Step 2** you can select proteolytic cleavage enzymes. The list of available enzymes will be expanded continuously. Presently, the list contains the enzymes shown in figure 20.24. The full list of enzymes and their cleavage patterns can be seen in Appendix, section D.

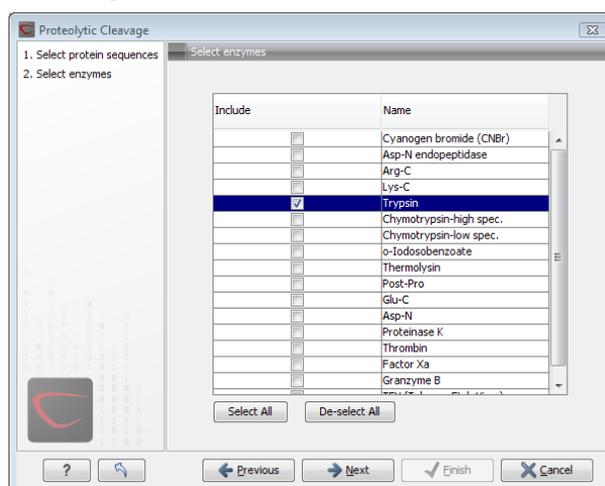


Figure 20.24: Setting parameters for proteolytic cleavage detection.

Select the enzymes you want to use for detection. When the relevant enzymes are chosen, click

Next.

In **Step 3** you can set parameters for the detection. This limits the number of detected cleavages. Figure 20.25 shows an example of how parameters can be set.

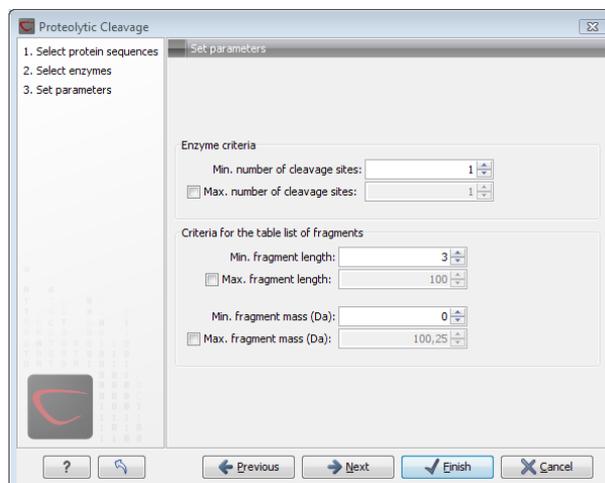


Figure 20.25: Setting parameters for proteolytic cleavage detection.

- **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.
- **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.

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

The result of the detection is displayed in figure 20.26.

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 these.

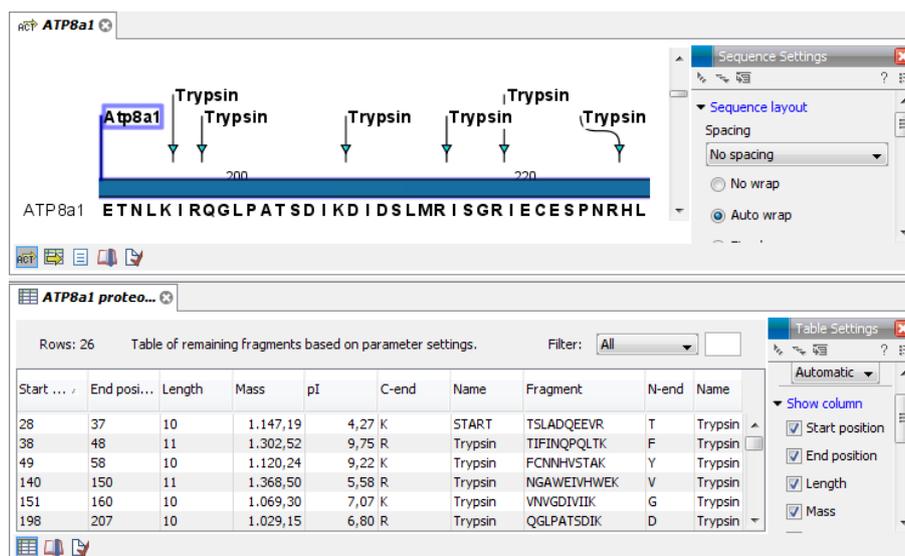


Figure 20.26: The result of the proteolytic cleavage detection.

20.10.2 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 20.27.

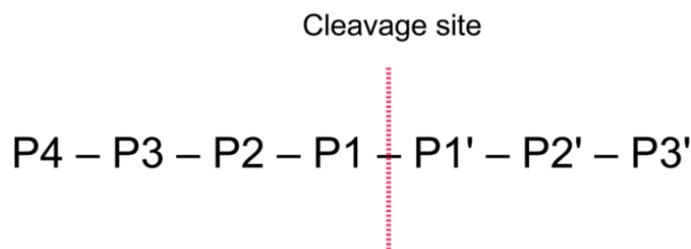


Figure 20.27: Nomenclature of the peptide substrate. The substrate is cleaved between position P1-P1'.

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 20.28).

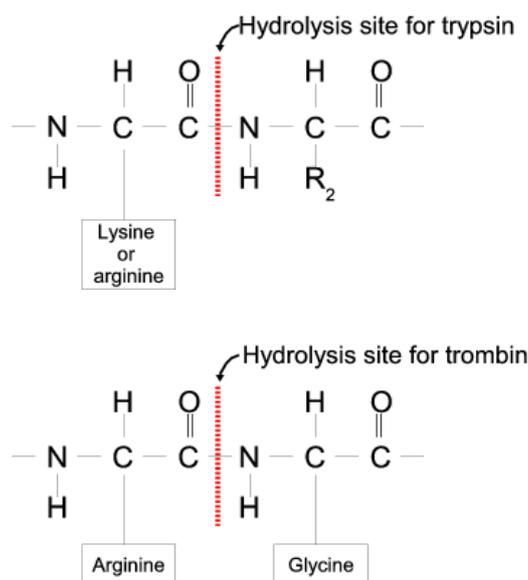


Figure 20.28: Hydrolysis of the peptide bond between two amino acids. Trypsin cleaves unspecifically at lysine or arginine residues whereas trombin cleaves at arginines if aspartate or glutamate is absent.

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/>

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Chapter 21

Sequencing data analyses and Assembly

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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, including e.g. BLAST and open reading frame prediction.

You can see additional information about the quality of the traces by holding the mouse cursor on the imported sequence. This will display a tooltip as shown in figure 21.1.

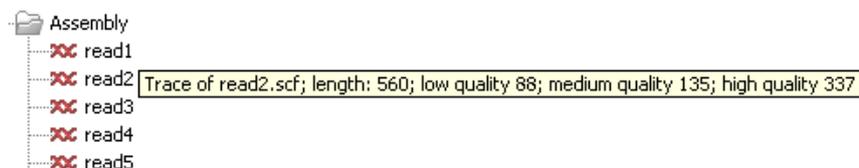


Figure 21.1: A tooltip displaying information about the quality of the chromatogram.

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 (ACT).

21.1.1 Scaling traces

The traces can be scaled by dragging the trace vertically as shown in 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 in section 21.1.2.



Figure 21.2: Grab the traces to scale.

21.1.2 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 21.1.1.



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. Please see section 21.7.1.

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 section of the Workbench toolbox, 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 12.3.2).

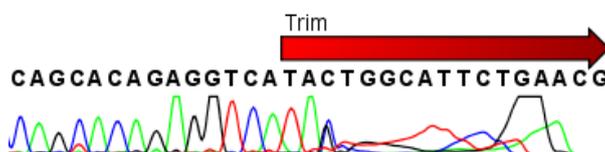


Figure 21.4: Trimming creates annotations on the regions that will be ignored in the assembly process.

When exporting sequences in fasta format, there is an option to remove the parts of the sequence covered by trim annotations.

21.2.1 Trimming using the Trim 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 tool in the Workbench, go to the menu option:

Toolbox | Sequencing Data Analysis (A) | Trim Sequences (T)

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.

When the sequences are selected, click **Next**.

This opens the dialog displayed in figure 21.5.

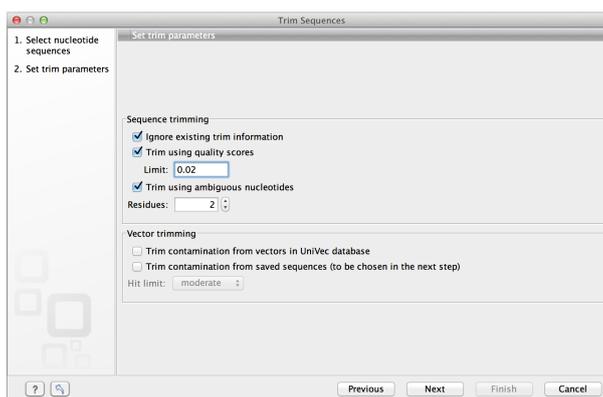


Figure 21.5: Setting parameters for trimming.

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: $Q = -10\log_{10}(P)$, 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 <http://www.clcbio.com/files/usermanuals/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 H).
 - **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 database is included when you install the *CLC Main Workbench*). A list of all the vectors in the UniVec database can be found at <http://www.ncbi.nlm.nih.gov/VecScreen/replist.html>.
 - **Hit limit.** Specifies how strictly vector contamination is trimmed. 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 (<http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html>):
 - * **Weak.** 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.** 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.** Expect 1 random match in 1,000,000 queries of length 350 kb
 - Terminal match with Score ≥ 24 .
 - Internal match with Score ≥ 30 .
- Note that selecting e.g. **Weak** will also include matches in the **Moderate** and **Strong** categories.
- **Trim contamination from saved sequences.** This option lets you select your own vector sequences that you have imported into the Workbench. If you select this option, you will be able to select one or more sequences when you click **Next**.

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. 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 Workbench Toolbox as the Trim 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 21.5). To perform the assembly:

Toolbox | Sequencing Data 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.

Note! You can assemble a maximum of 2000 sequences at a time.

To assemble more sequences, you need the *CLC Genomics Workbench* (see <http://www.clcbio.com/genomics>).

When the sequences are selected, click **Next**. This will show the dialog in figure 21.6

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 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. Three stringency levels can be set:

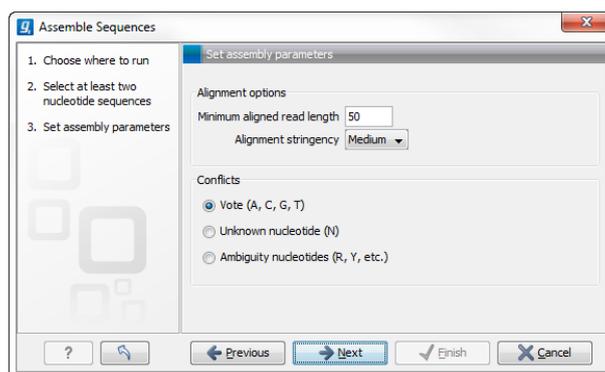


Figure 21.6: Setting assembly parameters.

- **Low.**
- **Medium.**
- **High.**
- **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 H.

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.

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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 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:

Toolbox | Molecular Biology Tools  | **Sequencing Data 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 21.7.

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. 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 21.7.

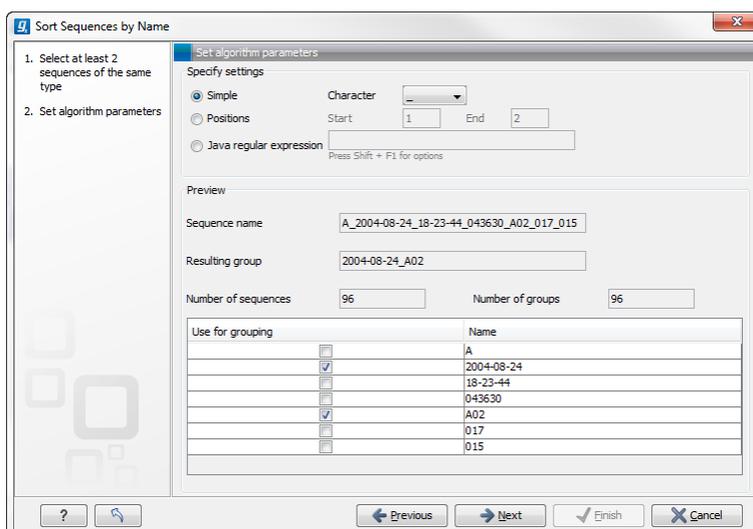


Figure 21.7: Splitting up the name at every underscore (`_`) and using the date and analysis position for grouping.

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 21.7 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 21.8.

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.

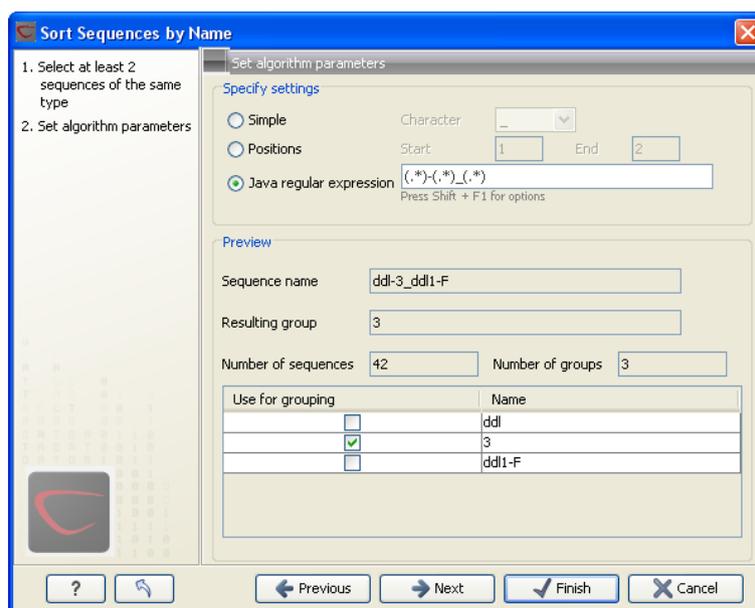


Figure 21.8: Dividing the sequence into three groups based on the number in the middle of the name.

21.5 Assemble sequences to reference

This section describes how to assemble a number of sequence reads into a contig using a reference sequence. A reference sequence can be particularly helpful when the objective is to characterize SNP variation in the data.

To start the assembly:

Toolbox | Sequencing Data Analysis (AA) | Assemble Sequences to Reference (MW)

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.

Note! You can assemble a maximum of 2000 sequences at a time.

To assemble more sequences, you need the *CLC Genomics Workbench* (see <http://www.clcbio.com/genomics>).

When the sequences are selected, click **Next**, and you will see the dialog shown in figure 21.9

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

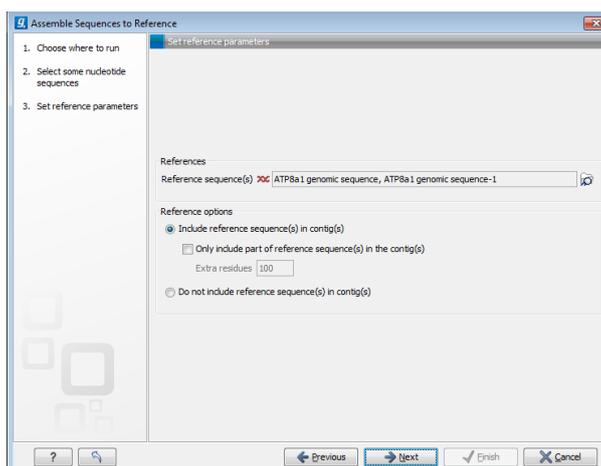


Figure 21.9: Parameters for how the reference should be handled when assembling sequences to a reference sequence.

only cover a small part of a reference sequence, it may not be desirable to include the 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 21.10

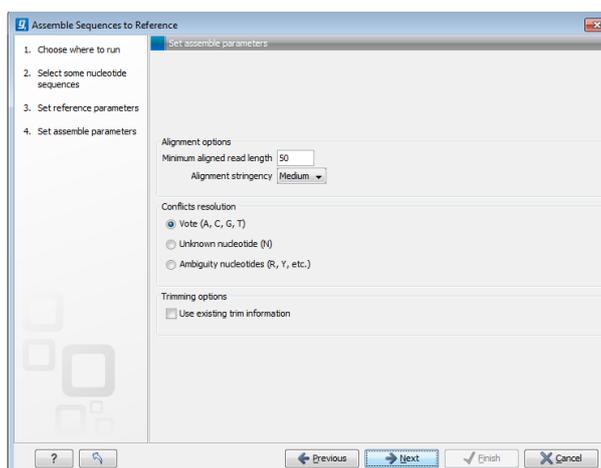


Figure 21.10: Options for how the input sequences should be aligned and how nucleotide conflicts should be handled.

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 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. Three stringency levels can be set:
 - **Low.**
 - **Medium.**
 - **High.**

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 H.
 - **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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. This will start the assembly process. See section 21.7 on how to use the resulting contigs.

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 start the assembly:

Toolbox in the Menu Bar | Sequencing Data Analysis (🔧) | Add Sequences to Contig (📁)

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 21.5).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. This will start the assembly process. See section 21.7 on how to use the resulting 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

The result of the assembly process is one or more contigs where the sequence reads have been aligned (see figure 21.12).

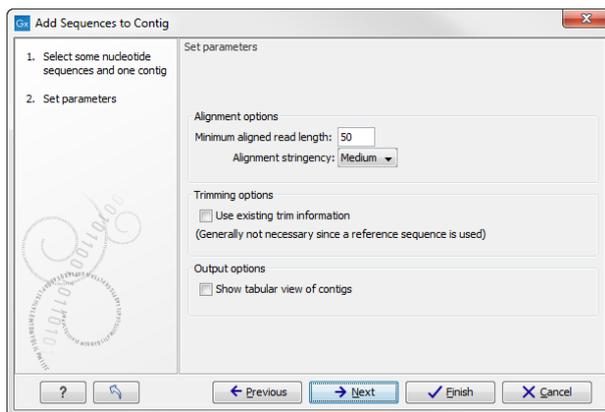


Figure 21.11: Setting assembly parameters when assembling to an existing contig.

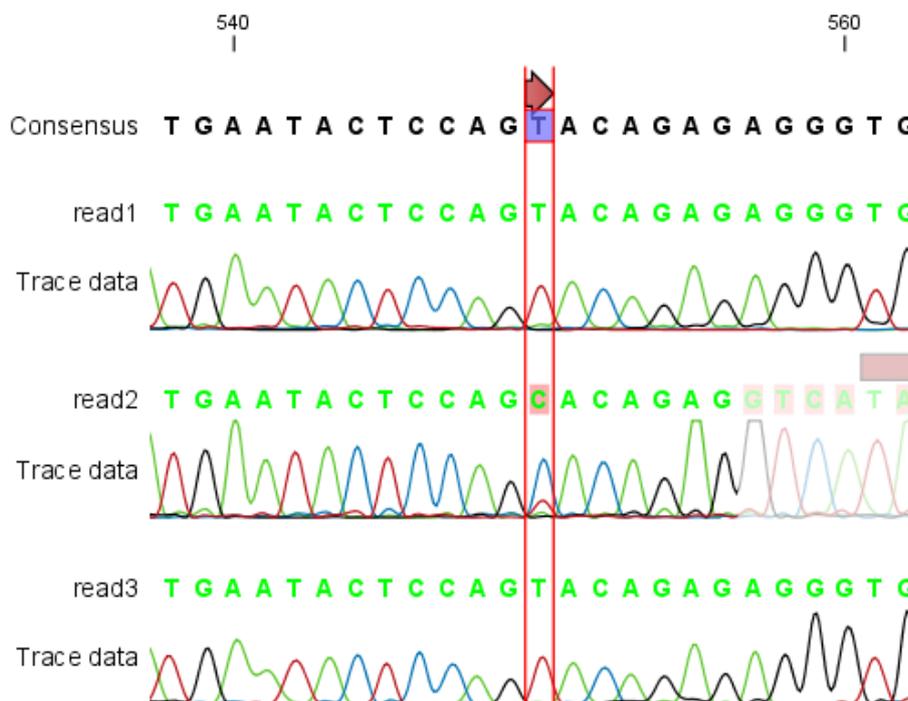


Figure 21.12: The view of a contig. Notice that you can zoom to a very detailed level in contigs.

You can see that color of the residues and trace at the end of one of the reads has been faded. This indicates, that this region has not contributed to the contig. This may be due to trimming before or during the assembly or due to misalignment to the other reads.

You can easily adjust the trimmed area to include more of the read in the contig: simply drag the edge of the faded area as shown in figure 21.13.



Figure 21.13: Dragging the edge of the faded area.

Note! This is only possible when you can see the residues on the reads. This means that you need to have zoomed in to 100% or more and chosen **Compactness** levels "Not compact", "Low" or "Packed". Otherwise the handles for dragging are not available (this is done in order to make

the visual overview more simple).

If reads have been reversed, this is indicated by red. Otherwise, the residues are colored green. The colors can be changed in the **Side Panel** as described in section 21.7.1

If you find out that the reversed reads should have been the forward reads and vice versa, you can reverse complement the whole contig (imagine flipping the whole contig):

right-click in the empty white area of the contig | Reverse Complement Sequence

21.7.1 View settings in the Side Panel

Apart from this the view resembles that of alignments (see section 16.2) but has some extra preferences in the **Side Panel**:

Apart from this the view resembles that of alignments but has some extra preferences in the **Side Panel**:

- **Read layout.** This section appears at the top of the **Side Panel** when viewing a stand alone read mapping:
 - **Compactness.** The compactness setting options let you control the level of detail to be displayed. This setting affects many of the other settings in the **Side Panel** as well as the general behavior of the view. For example: if the compactness is set to **Compact**, you will not be able to see quality scores or annotations on the reads, even if these are turned on via the Nucleotide info section of the Side Panel. You can change the Compactness setting in the Side Panel directly, or you can use the shortcut: press and hold the Alt key while you scroll 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 this is relevant. To view such information, additional viewing options under the **Nucleotide info** view settings must also be selected. For further details on these, please see section 21.1.2 and section 12.1.
 - * **Low.** Hides trace data, quality scores and puts the reads' annotations on the sequence.
 - * **Medium.** The labels of the reads and their annotations are hidden, and the residues of the reads cannot be seen.
 - * **Compact.** Even less space between the reads.
 - * **Packed.** All the other compactness settings will stack the reads on top of each other, but the packed setting will use all space available for displaying the reads. When zoomed in to 100%, you can see the residues but when zoomed out the reads will be represented as lines just as with the Compact setting. The packed mode is very useful when viewing large amounts of data. However certain functionality possible with other views are not available in packed view. For example, no editing of the read mapping or selections of it can be done and color coding changes are not possible. An example of the packed setting is shown in figure 21.14.
 - **Gather sequences at top.** Enabling this option affects the view that is shown when scrolling horizontally. If selected, the sequence reads which did not contribute to the

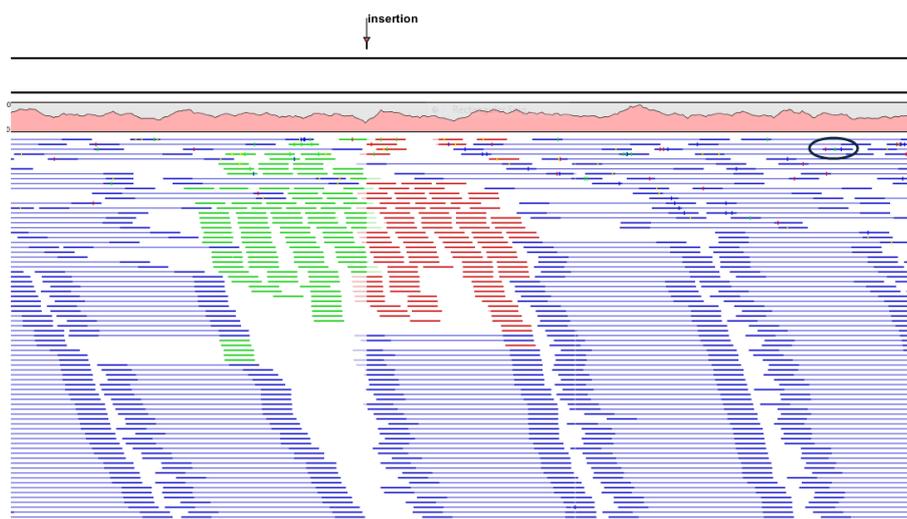


Figure 21.14: An example of the packed compactness setting. We highlighted in black an example of 3 narrow vertical lines representing mismatching residues.

visible part of the mapping will be omitted whereas the contributing sequence reads will automatically be placed right below the reference. This setting is not relevant when the compactness is packed.

- **Show sequence ends.** Regions that have been trimmed are shown with faded traces and residues. This illustrates that these regions have been ignored during the assembly.
- **Show mismatches.** When the compactness is packed, you can highlight mismatches which will get a color according to the Rasmol color scheme. A mismatch is whenever the base is different from the reference sequence at this position. This setting also causes the reads that have mismatches to be floated at the top of the view.
- **Disconnect paired reads.** This option will break up the paired reads in the display (they are still marked as pairs - this just affects the visualization). The reads are marked with colors for the direction (default red and green) instead of the color for pairs (default blue). This is particularly useful when investigating overlapping pairs in packed view and when the strand / read orientation is important.
- **Packed read height.** When the compactness is set to "packed", you can choose the height of the visible reads. When there are more reads than the height specified, an overflow graph will be displayed below the reads. The overflow graph is shown in the same colors as the sequences, and mismatches in reads are shown as narrow vertical lines (see figure 21.14). The colors of the small lines represent the mismatching residue. The color codes for the horizontal lines correspond to the color used for highlighting mismatches in the sequences (red = A, blue = C, yellow = G, and green = T), meaning that a red line with half the height of the blue part of the overflow graph will represent a mismatching "A" in half of the paired reads at this particular position.
- **Find Conflict.** Clicking this button selects the next position where there is a conflict between the sequence reads. Residues that are different from the reference are colored (as default), providing an overview of the conflicts. Since the next conflict is automatically selected it is easy to make changes. You can also use the Space key to find the next conflict.
- **Low coverage threshold.** All regions with coverage up to and including this value are

considered low coverage. When clicking the 'Find low coverage' button the next region in the read mapping with low coverage will be selected.

- **Alignment info.** There is one additional parameter:
 - **Coverage:** Shows how many sequence reads that are contributing information to a given position in the contig. The level of coverage is relative to the overall number of sequence reads.
 - * **Foreground color.** Colors the letters 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 letters using a gradient, where the left side color is used for low coverage and the right side is used for maximum coverage
 - * **Graph.** The coverage is displayed as a graph (Learn how to export the data behind the graph in section 7.4).
 - **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.
- **Residue coloring.** There is one additional parameter:
 - **Sequence colors.** This option lets you use different colors for the reads.
 - * **Main.** The color of the consensus and reference sequence. Black per default.
 - * **Forward.** The color of forward reads (single reads). Green per default.
 - * **Reverse.** The color of reverse reads (single reads). Red per default.
 - * **Paired.** The color of paired reads. Blue per default. Note that reads from **broken pairs** are colored according to their Forward/Reverse orientation or as a Non-specific match, but with a darker nuance than ordinary single reads.
 - * **Non-specific matches.** When a read would have matched equally well another place in the mapping, it is considered a non-specific match. This color will "overrule" the other colors. Note that if you are mapping with several reference sequences, a read is considered a double match when it matches more than once *across all the contigs/references*. A non-specific match is yellow per default.
- **Sequence layout.** At the top of the **Side Panel**:
 - **Matching residues as dots** Matching residues will be presented as dots. Only the top sequence will be preserved in its original format.

There are many other viewing options available, both general and aimed as specific elements of a mapping, which can be adjusted in the View settings. Those covered here were the key ones relevant for standard review of mapping results.

21.7.2 Editing the contig

When editing contigs, you are typically interested in confirming or changing single bases, and this can be done simply by:

selecting the base | typing the right base

Some users prefer to use lower-case letters in order to be able to see which bases were altered when they use the results later on. In *CLC Main Workbench* all changes are recorded in the history log (see section 8) allowing the user to quickly reconstruct the actions performed in the editing session.

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 view, you can use **Zoom in** () to zoom to a greater level of detail than in other views (see figure 21.12). This is useful for discerning the trace curves.

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 ()

This will show a warning dialog, but you can choose never to see this dialog again by clicking the checkbox at the bottom of the dialog.

Note that for contigs with more than 1000 reads, you can only do single-residue replacements (you can't delete or edit a selection). When the compactness is **Packed**, you cannot edit any of the reads.

21.7.3 Sorting reads

If you wish 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

When the contig is created, 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 have a different residue*.

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 contig

Due to the integrated nature of *CLC Main Workbench* it is easy to use the consensus sequences as input for additional analyses.

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 contig and will be saved together with the contig. It also means that if you add annotations to the sequence, they will be shown in the contig view as well. This can be very convenient e.g. for Primer design .

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 Extract parts of a contig

Sometimes it is useful to extract part of a contig for in-depth analysis. This could be the case if you have performed an analysis of a whole genome data set and have found a region that you are particularly interested in analyzing further. Rather than running all further analysis on your full data, you may prefer to run only on a subset of the data. You can extract a subset of your contig data by running the **Extract from Selection** tool on a selected region in your contig. The result of running this tool is a new contig which contains only the reads (and optionally only those that are of a particular type) in your selected region.

To select a region, use the **Selection mode**  (see Section 3.2.3 for a detailed description of the different modes) and select your region of interest in your contig, then right-click. You are now presented with the dialog shown in Figure 21.15.

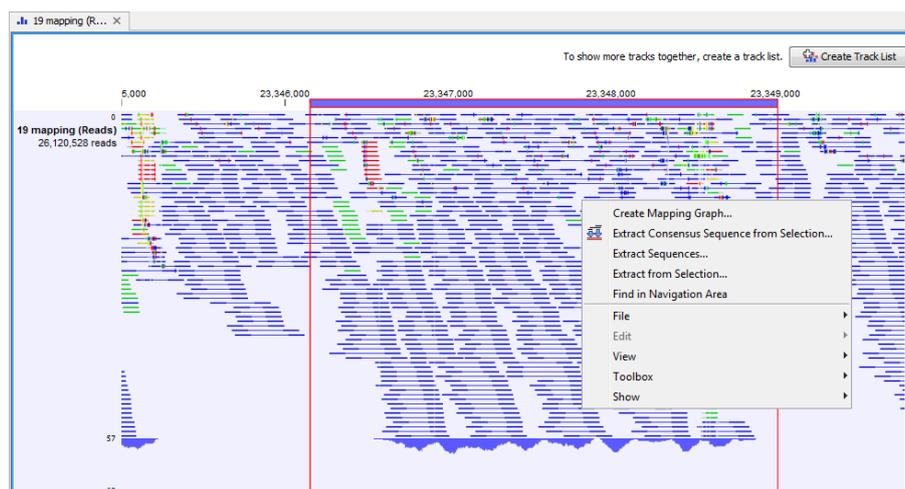


Figure 21.15: Extracting parts of a contig.

When you choose the **Extract from Selection** option you are presented by the dialog shown in figure 21.16.

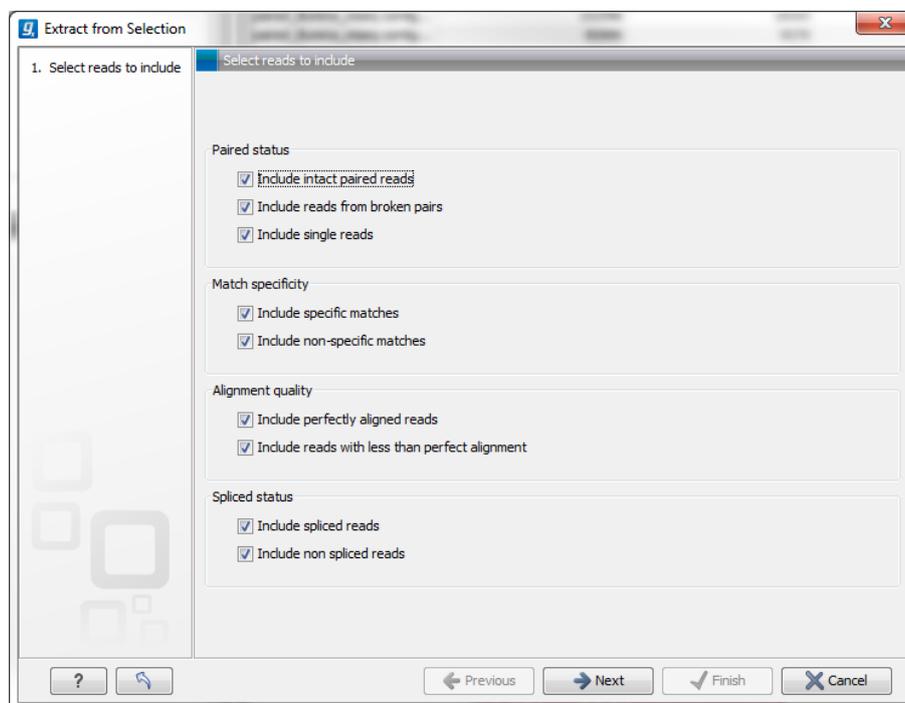


Figure 21.16: Selecting the reads to include.

The purpose of this dialog is to let you specify what kind of reads you want to include. Per default all reads are included. The options are:

Paired status Include intact paired reads When paired reads are placed within the paired distance specified, they will fall into this category. Per default, these reads are colored in blue.

Include paired reads from broken pairs When a pair is broken, either because only one read in the pair matches, or because the distance or relative orientation is wrong, the reads are placed and colored as single reads, but you can still extract them by

checking this box.

Include single reads This will include reads that are marked as single reads (as opposed to paired reads). Note that paired reads that have been broken during assembly are not included in this category. Single reads that come from trimming paired sequence lists are included in this category.

Match specificity Include specific matches Reads that only are mapped to one position.

Include non-specific matches Reads that have multiple equally good alignments to the reference. These reads are colored yellow per default.

Alignment quality Include perfectly aligned reads Reads where *the full read* is perfectly aligned to the reference sequence (or consensus sequence for de novo assemblies). Note that at the end of the contig, reads may extend beyond the contig (this is not visible unless you make a selection on the read and observe the position numbering in the status bar). Such reads are not considered perfectly aligned reads because they don't align in their entire length.

Include reads with less than perfect alignment Reads with mismatches, insertions or deletions, or with unaligned nucleotides at the ends (the faded part of a read).

Spliced status Include spliced reads Reads that are across an intron.

Include non spliced reads Reads that are not across an intron.

Note that only reads that are completely covered by the selection will be part of the new contig.

One of the benefits of this is that you can actually use this tool to extract subset of reads from a contig. An example work flow could look like this:

1. Select the whole reference sequence
2. Right-click and **Extract from Selection**
3. Choose to include only paired matches
4. Extract the reads from the new file (see section 18.2)

You will now have all paired reads from the original mapping in a list.

21.7.7 Variance table

In addition to the standard graphical display of a contig 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 21.17.

The table has the following columns:

- **Reference position.** The position of the conflict measured from the starting point of the reference sequence.

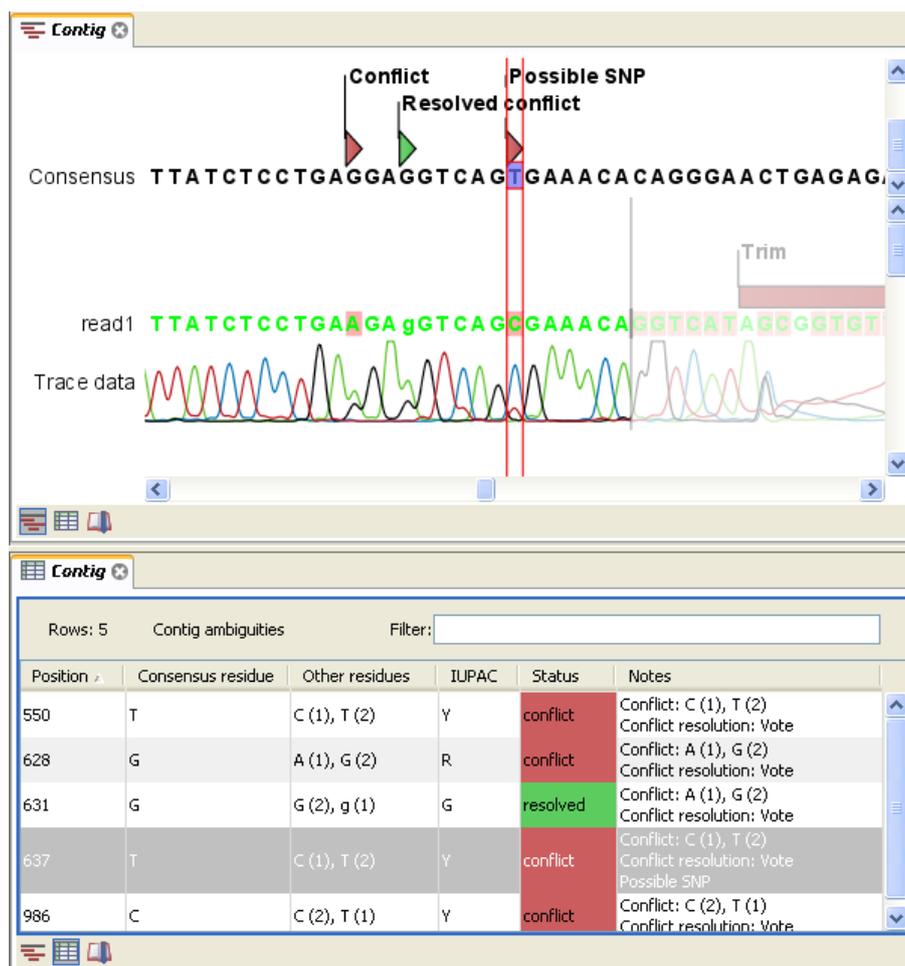


Figure 21.17: The graphical view is displayed at the top. At the bottom the conflicts are shown in a table. At the conflict at position 637, the user has entered a comment in the table. This comment is now also reflected on the tooltip of the conflict annotation in the graphical view above.

- **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 21.17, you 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 H.)
- **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 21.17). 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, apart from 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:

Toolbox | Sequencing Data Analysis (AA) | Reassemble Contig (R) | select the contig from Navigation Area, move to 'Selected Elements' and click Next

or **right-click in the empty white area of the contig | Reassemble contig (R)**

This opens a dialog as shown in figure 21.18

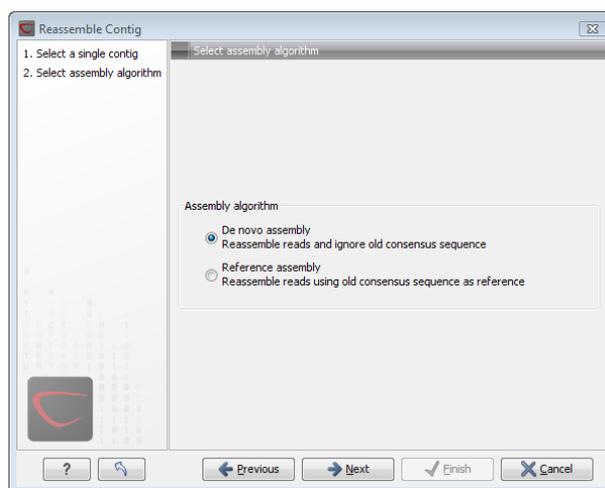


Figure 21.18: Re-assembling a contig.

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 21.5.

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 change 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:

Toolbox | Sequencing Data Analysis (A) | Call Secondary Peaks (A)

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 21.19.

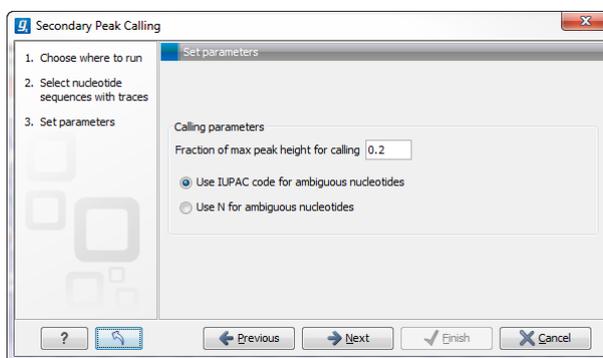


Figure 21.19: Setting parameters secondary peak calling.

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.
- **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 H).

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. 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.

Chapter 22

Primers and probes

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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:

Toolbox | Primers and Probes () | Design Primers () | OK

or **right-click sequence in Navigation Area | Show | Primer ()**

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 12.1 for an explanation of these options.

Note! This means that annotations such as e.g. known SNPs or exons, can be displayed on the template sequence to guide the choice of primer regions. Also, traces in sequencing reads can be shown along with the structure to guide e.g. the re-sequencing of poorly resolved regions.

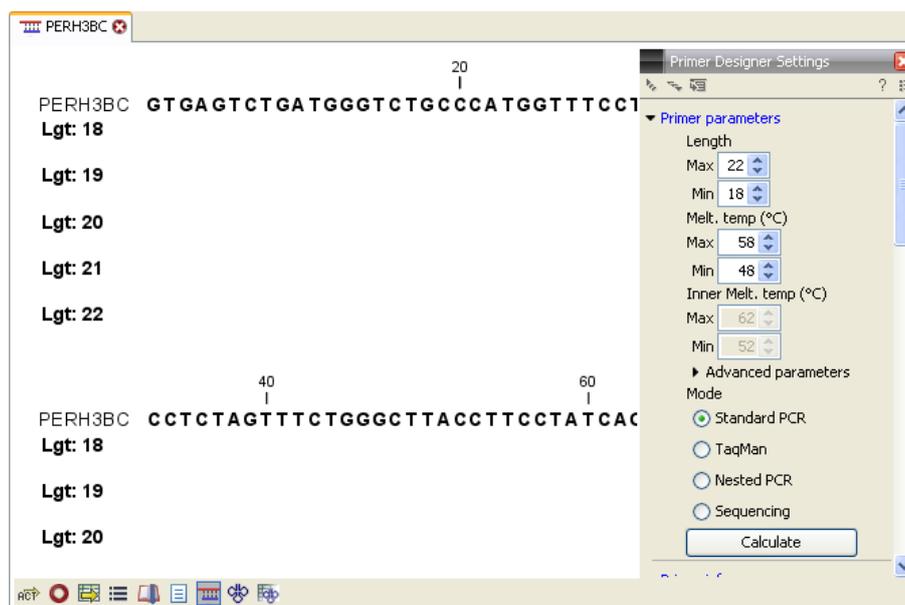


Figure 22.1: The initial view of the sequence used for primer design.

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 possible 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).

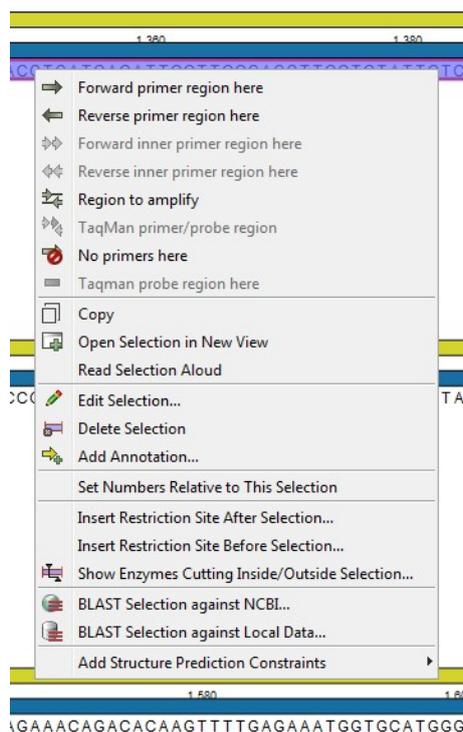


Figure 22.2: Right-click menu allowing you to specify regions for the primer design

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 fulfills 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 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).

The image shows two side panels from a software interface. The left panel, titled 'Primer parameters', contains several settings: 'Length' with 'Max' at 22 and 'Min' at 18; 'Melt. temp (°C)' with 'Max' at 58 and 'Min' at 48; 'Inner Melt. temp (°C)' with 'Max' at 62 and 'Min' at 52. Below these are 'Advanced parameters' and 'Mode' options: 'Standard PCR' (selected), 'TaqMan', 'Nested PCR', and 'Sequencing'. A 'Calculate' button is at the bottom. The right panel, titled 'Primer information', has a 'Show' checkbox checked, followed by radio buttons for 'Compact' (selected) and 'Detailed'. Below are several unchecked checkboxes: 'G/C content(G/C)', 'Melting temp.(Tm)', 'Self annealing(SA)', 'Self end annealing(SEA)', 'Secondary structure(SS)', '3' end G/C', and '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 GC 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).

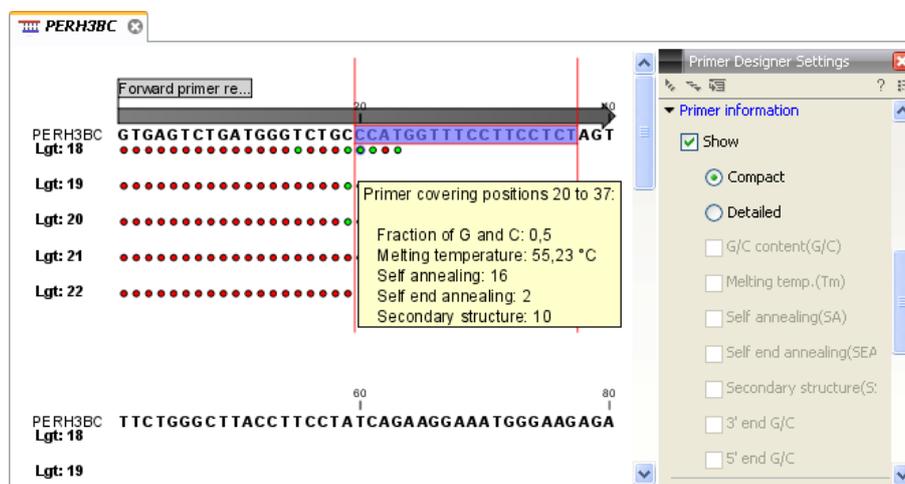


Figure 22.4: Compact information mode

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 fulfills 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, red 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).

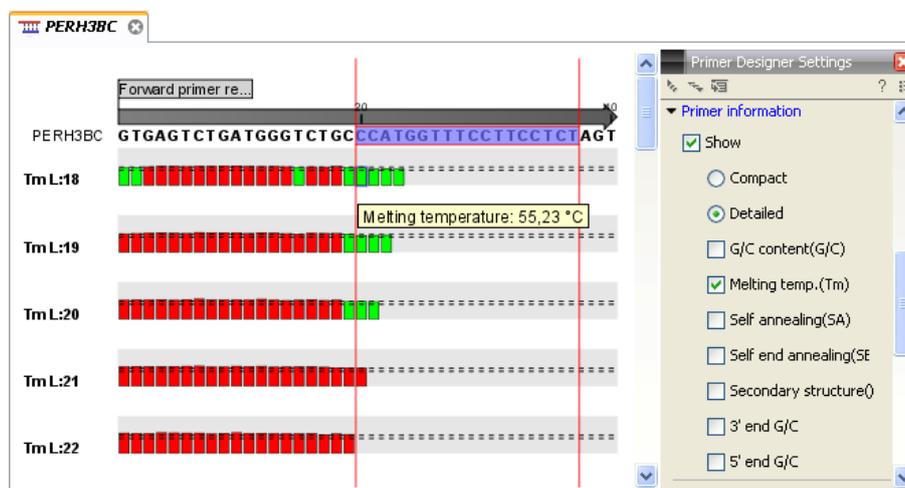


Figure 22.5: Detailed information mode

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
- Self end annealing score
- Secondary structure score

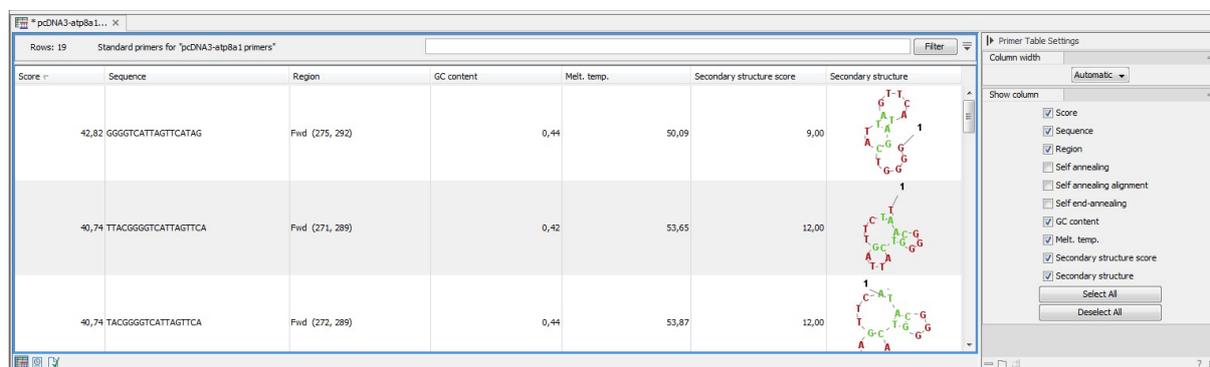
Properties with Yes - No values. If a primer meets the set requirement a green circle will be shown at its starting position and if it fails to meet the requirement a red dot is shown at its starting position:

- 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).



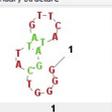
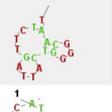
| Score | Sequence | Region | GC content | Melt. temp. | Secondary structure score | Secondary structure |
|-------|--------------------|----------------|------------|-------------|---------------------------|---|
| 42,82 | GGGGTCATTAGTTCATAG | Fwd (275, 292) | 0,44 | 50,09 | 9,00 |  |
| 40,74 | TTACGGGGTCATTAGTTC | Fwd (271, 289) | 0,42 | 53,65 | 12,00 |  |
| 40,74 | TACGGGGTCATTAGTTC | Fwd (272, 289) | 0,44 | 53,87 | 12,00 |  |

Figure 22.6: Proposed primers

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.

22.4.1 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, including BLAST. Furthermore, the primers can be edited using the standard sequence view to introduce e.g. mutations and restriction sites.

22.4.2 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.

22.4.3 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.

22.5.1 User input

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.

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).

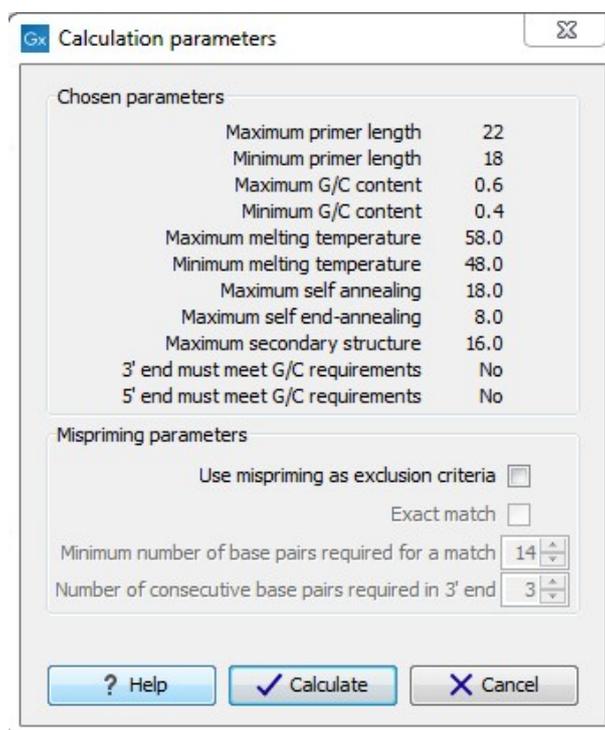


Figure 22.7: Calculation dialog for PCR primers when only a single primer region has been defined.

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.

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).

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.

| 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 | <input type="checkbox"/> |
| Exact match | <input type="checkbox"/> |
| 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.

22.5.2 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).

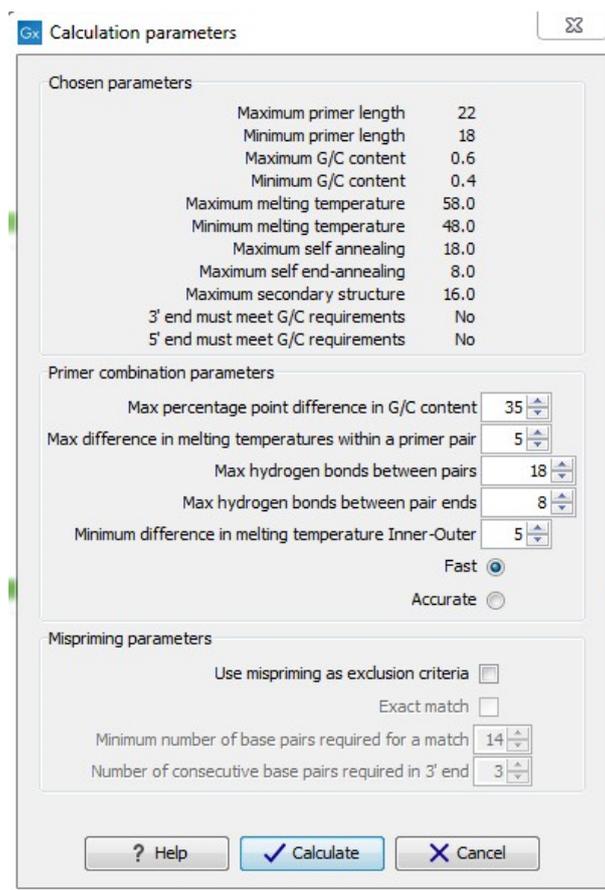


Figure 22.9: Calculation dialog

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.

22.6.1 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 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).

| 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 | <input type="checkbox"/> |
| Exact match | <input type="checkbox"/> |
| 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

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.

22.7.1 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 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 the section 22.5 for a description.

22.8.1 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.

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 in two ways:

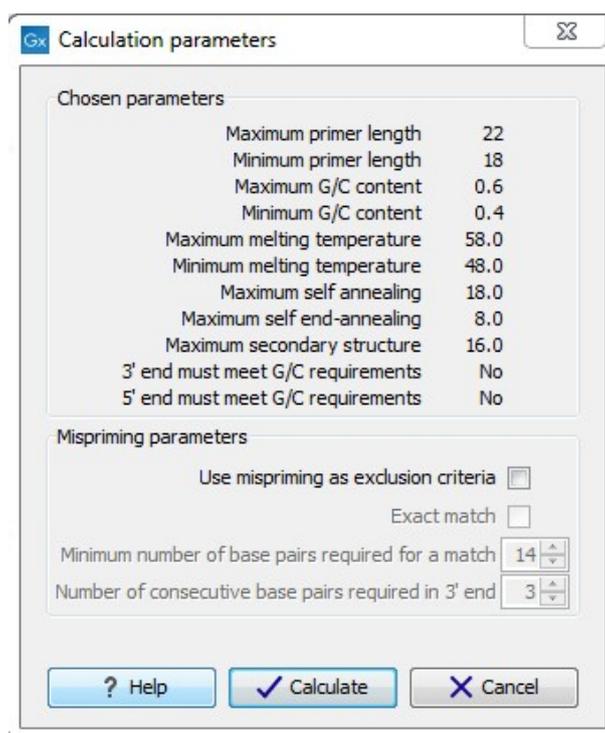


Figure 22.11: Calculation dialog for sequencing primers

Toolbox | 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.

Note! This means that annotations such as e.g. known SNPs or exons, can be displayed on the template sequence to guide the choice of primer regions. Since the definition of groups of sequences is essential to the primer design, the selection boxes of the standard view are shown as default in the alignment primer view.

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. This is elaborated below. The **Primer Parameters** group in the **Side Panel** has the same options for specifying primer requirements, but differs by the following (see figure 22.12):

- In the **Mode** submenu which specifies the reaction types the following options are found:
 - **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.



Figure 22.12: The initial view of an alignment used for primer design.

- The **Primer solution** submenu is used to 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 work flow when designing alignment based primers and probes is as follows:

- 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 22.13.

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.

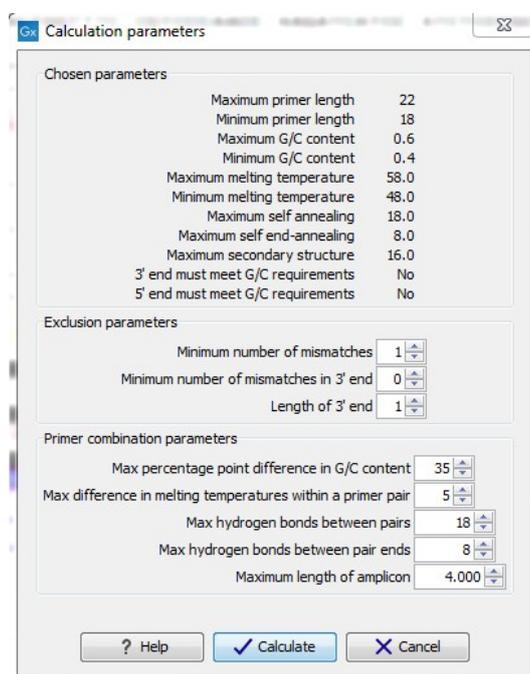


Figure 22.13: Calculation dialog shown when designing alignment based PCR primers.

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 22.14 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.

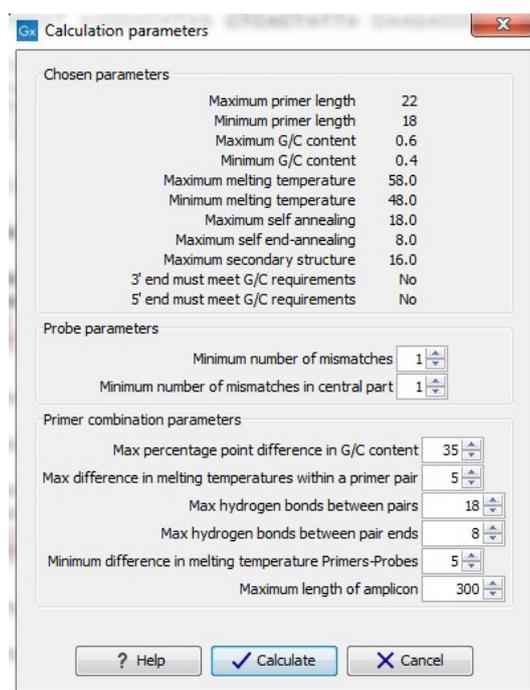


Figure 22.14: Calculation dialog shown when designing alignment based TaqMan probes.

22.10 Analyze primer properties

CLC Main Workbench can calculate and display the properties of predefined primers and probes:

Toolbox | Primers and Probes (📁) | Analyze Primer Properties (🔍)

If a sequence was selected before choosing the Toolbox action, this sequence is now 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 22.15:

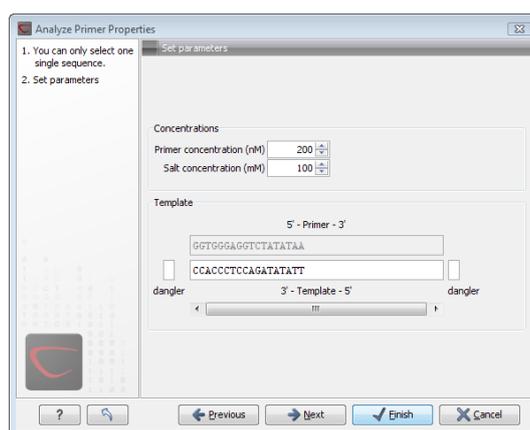


Figure 22.15: The parameters for analyzing primer properties.

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**. The result is shown in figure 22.16:

| Sequence | Melt. temp. | Self annealing alignment | Secondary structure |
|-------------------|-------------|--|---------------------|
| GGTGGGAGGCTATATAA | 50,49 | GGTGGGAGGCTATATAA AATATATCTGGAGGGTGG | |

Figure 22.16: Properties of a primer from the Example Data.

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 22.5.2.

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 e.g. 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:

Toolbox | 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 22.17:

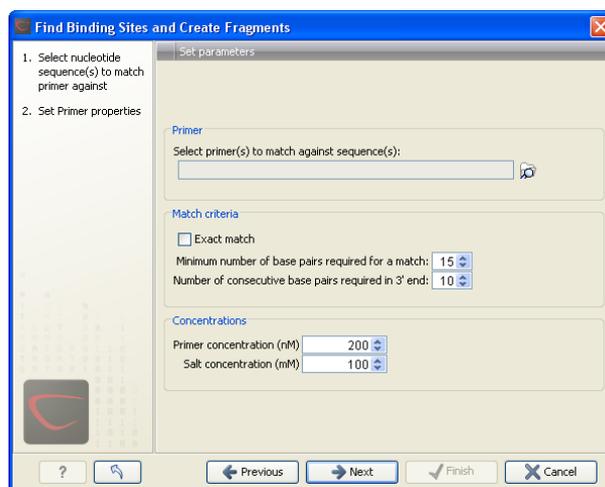


Figure 22.17: Search parameters for finding primer binding sites.

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

Click **Next** to specify the output options as shown in figure 22.18:

The output options are:

An example of the **primer binding site table** is shown in figure 22.20.

| 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 | GGAAGTGAATAGAGGA | rev | complement(1373..1390) | 0 | 0 |
| Primer 6 | AGCAGGCAATAAGAGAA | rev | complement(1412..1430) | 0 | 0 |

Figure 22.20: 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 22.5.2. You can use this table to browse the binding sites. If you make a split view of the table and the sequence (see section 3.1.6), 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 22.21.

| 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 |

Figure 22.21: 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 3.1.6), 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 22.22.

| Rows: 7 | | Fragments | | Filter: | |
|-----------------|--------------------|-----------------|------------|---------|--|
| Fwd | Rev | Fragment length | Region | Other f | |
| primer-3 | primer-2 | 1488 | 1575..3062 | | |
| primer-6 | primer-1 | 51 | 151..401 | | |
| primer-6 | primer-5 - HindIII | 65 | 151..1615 | | |
| primer-6 | primer-5 | 51 | 151..1601 | | |
| primer-6- EcoRV | primer-1 | 269 | 133..401 | | |
| primer-6- EcoRV | primer-5 - HindIII | 1483 | 133..1615 | | |

Figure 22.22: 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 22.22, 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 23.1).

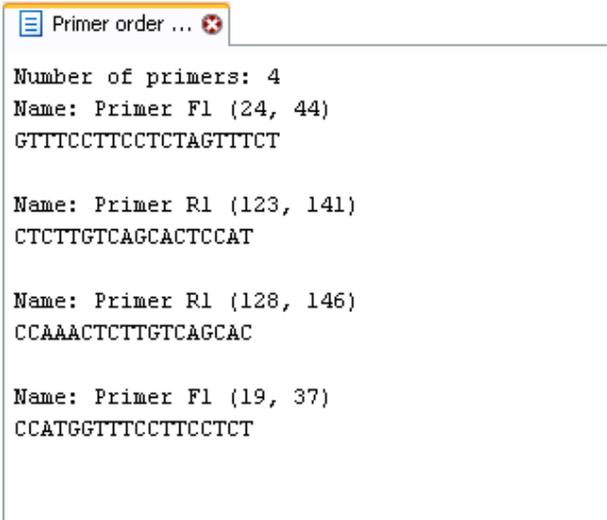
22.12 Order primers

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:

Toolbox | Primers and Probes (📁) | Order Primers (📄)

This opens a dialog where you can choose additional primers. Clicking **OK** opens a textual representation of the primers (see figure 22.23). 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. The created object can also be saved and exported as a text file.

See figure 22.23



```
Primer order ...  
Number of primers: 4  
Name: Primer F1 (24, 44)  
GTTTCCTTCCTCTAGTTTCT  
  
Name: Primer R1 (123, 141)  
CTCTTGTCAGCACTCCAT  
  
Name: Primer R1 (128, 146)  
CCAAACTCTTGTCAGCAC  
  
Name: Primer F1 (19, 37)  
CCATGGTTTCCTTCCTCT
```

Figure 22.23: A primer order for 4 primers.

Chapter 23

Cloning and restriction sites

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CLC Main Workbench offers graphically advanced *in silico* cloning and design of vectors for various purposes together with restriction enzyme analysis and functionalities for managing lists of restriction enzymes.

First, after a brief introduction, restriction cloning and general vector design is explained. Next, we describe how to do Gateway Cloning ¹. Finally, the general restriction site analyses are described.

¹Gateway is a registered trademark of Invitrogen Corporation

23.1 Molecular cloning

Molecular cloning is a very important tool in the quest to understand gene function and regulation. Through molecular cloning it is possible to study individual genes in a controlled environment. Using molecular cloning it is possible to build complete libraries of fragments of DNA inserted into appropriate cloning vectors.

The *in silico* cloning process in *CLC Main Workbench* begins with the selection of sequences to be used:

Toolbox | Cloning and Restriction Sites (🔗) | Cloning (🔗)

This will open a dialog where you can select the sequences containing the fragments you want to clone as well as sequences to be used as vector (figure 23.1).

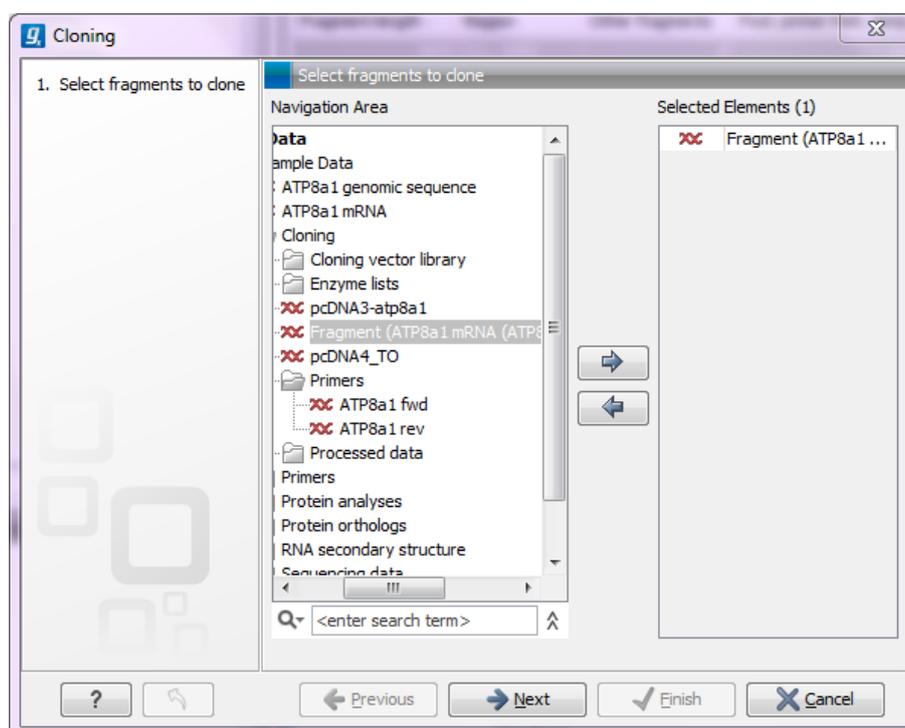


Figure 23.1: Selecting one or more sequences containing the fragments you want to clone.

The *CLC Main Workbench* will now create a sequence list of the selected fragments and vector sequences (if you have selected both fragments and vectors) and open it in the cloning editor as shown in figure 23.2.

When you save the cloning experiment, it is saved as a **Sequence list**. See section 12.6 for more information about sequence lists. If you need to open the list later for cloning work, simply switch to the **Cloning** (🔗) editor at the bottom of the view.

If you later in the process need additional sequences, you can easily add more sequences to the view. Just:

right-click anywhere on the empty white area | Add Sequences

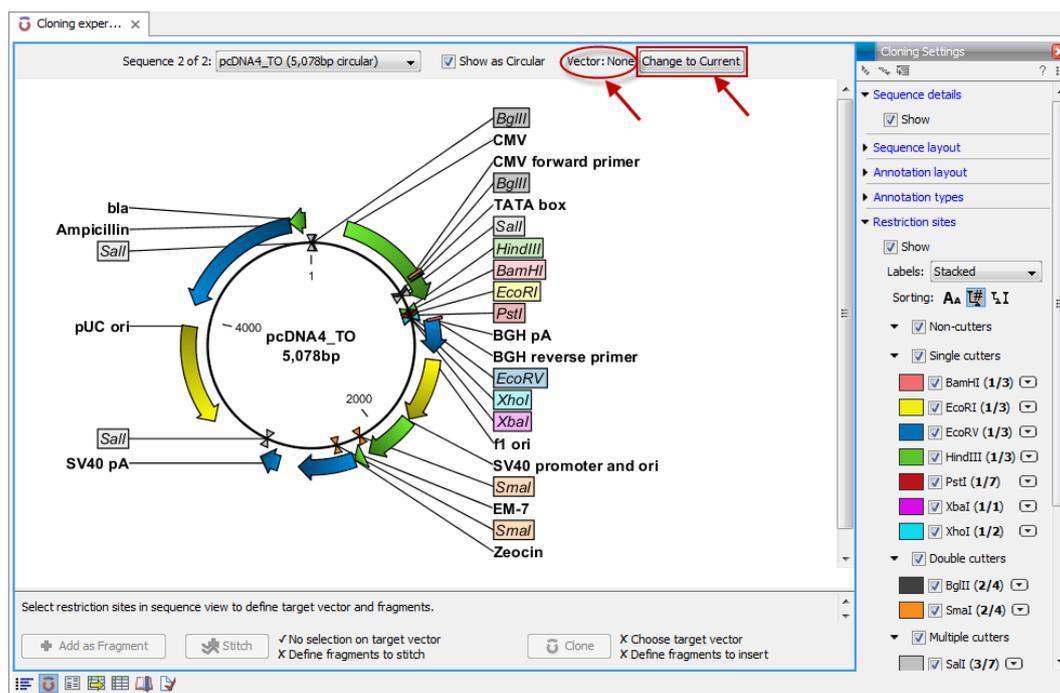


Figure 23.2: Cloning editor.

23.1.1 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. See section 12.1 for an explanation of these options. This means that e.g. known SNP's, exons and other 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. At the right-hand side, you can select whether or not to select a vector. When no vector has been selected a button **Change to Current** is enabled. This button can be used 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. This is explained in details below.
- At the bottom, there is a panel where the selection of fragments and target vector is performed (see elaboration below).

There are essentially three ways of performing cloning in the *CLC Main Workbench*. The *first* is the most straight-forward approach, which is based on a simple model of selecting restriction sites for cutting out one or more fragments and defining how to open the vector to insert the fragments. This is described as *the cloning workflow* below. The *second* approach is unguided and more flexible and allows you to manually cut, copy, insert and replace parts of the sequences. This approach is described under *manual cloning* below. Finally, the *CLC Main Workbench* also supports *Gateway cloning* (see section 23.2).

The cloning editor

The cloning editor can be activated in different ways. One way is to click on the **Cloning Editor** icon () in the view area when a sequence list has been opened in the sequence list editor. Another way is to create a new cloning experiment (the actual data object will still be a sequence list) using the **Cloning** () action from the toolbox. Using this action the user collects a set of existing sequences and creates a new sequence list.

The cloning editor can be used in two different ways:

1. **The cloning mode** is utilized when the user has selected one of the sequences as 'Vector'. In the cloning mode, the user opens up the vector by applying 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 user can switch the order of the inserted fragments and rotate them prior to adjusting the overhangs to match the cloning conditions.
2. **The stitch mode** is utilized when the user deselects or has not selected a sequence as 'Vector'. In stitch mode, the user can select a number of fragments (either full sequences or cuttings) from the cloning experiment. These fragments can then be stitched together into one single new and longer sequence. In the stitching adapter dialog, the user can switch order and rotate the fragments prior to adjusting the overhangs to match the stitch conditions.

23.1.2 The cloning workflow

The *cloning workflow* is designed to support restriction cloning workflows through the following steps:

1. Define one or more fragments
2. Define how the vector should be opened
3. Specify orientation and order of the fragment

Defining 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 23.3.1). 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.

When this is done, the panel below will update to reflect the selections (see figure 23.3).

In this example you can see that there are now two options listed in the panel below the view. This is because there are now two options for selecting the fragment that should be used for cloning. 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 the **Add Current Sequence as Fragment** ().

The screenshot shows a cloning software interface. The main window displays a DNA sequence with various restriction sites and features like promoters and origins of replication. The 'Cloning Settings' panel on the right is open, showing 'Restriction sites' checked and several enzymes selected. A fragment selection window at the bottom shows two options for cutting out a fragment between HindIII and XhoI sites, with the second option selected. A red arrow points to the 'Fragments to insert: 1' field in the bottom right.

Figure 23.3: *HindIII* and *XhoI* cut sites selected to cut out fragment.

At any time, the selection of cut sites can be cleared by clicking the **Remove** (✖) icon to the right of the fragment selections. 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**.

Defining target vector

When selecting among the sequences in the panel at the top, the vector sequence has "vector" appended to its name. If you wish to use one of the other sequences as vector, select this sequence in the list and click **Change to Current**.

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. 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) (figure 23.4).

Just as when cutting out the fragment, there is a lot of choices regarding which sequence should be used as the vector.

At any time, the selection of cut sites can be cleared by clicking the **Remove** (✖) icon to the right of the target vector selections. 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**.

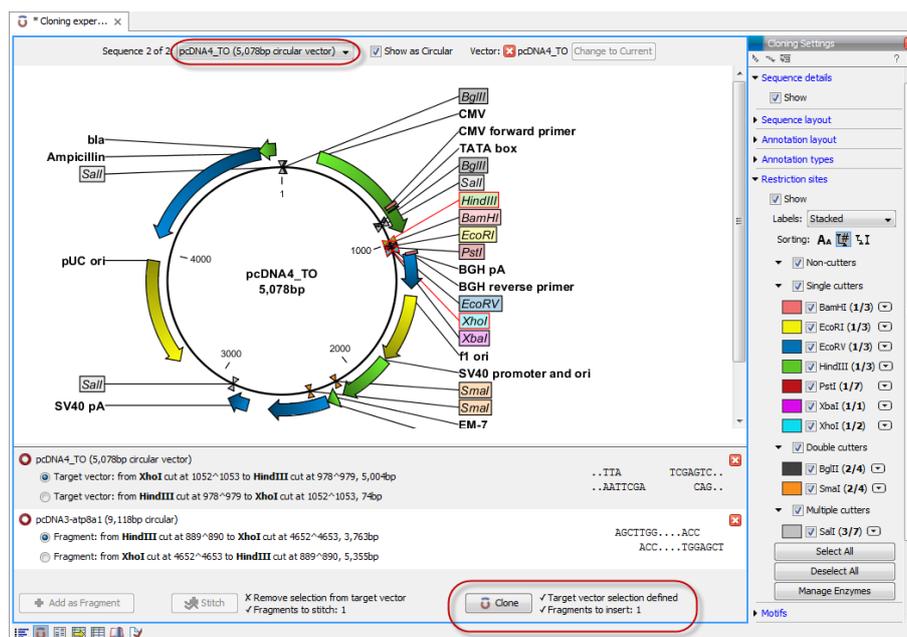


Figure 23.4: *HindIII* and *XhoI* sites used to open the vector. Note that the "Cloning" button has now been enabled as both criteria ("Target vector selection defined" and "Fragments to insert:...") have been defined.

When the right target vector is selected, you are ready to **Perform Cloning** (🔗), see below.

Perform cloning

Once selections have been made for both fragments and vector, click **Cloning** (🔗). This will display a dialog to adapt overhangs and change orientation as shown in figure 23.5)

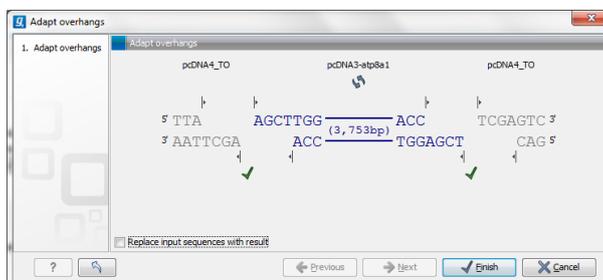


Figure 23.5: Showing the insertion point of the vector.

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 can blunt end or fill in the overhangs using the **drag handles** (⊥). Click and drag with the mouse to adjust the overhangs.

Whenever you drag the handles, the status of the insertion point is indicated below:

- The overhangs match (✓).
- The overhangs do not match (⊘). In this case, you will not be able to click **Finish**. Drag the handles to make 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 (➡)/ (⬅).

There is an option for the result of the cloning: **Replace input sequences with result**. Per default, the construct will be opened in a new view and can be saved separately. By selecting this option, the construct will also be added to the input sequence list and the original fragment and vector sequences will be deleted.

When you click **Finish** the final construct will be shown (see figure 23.6).

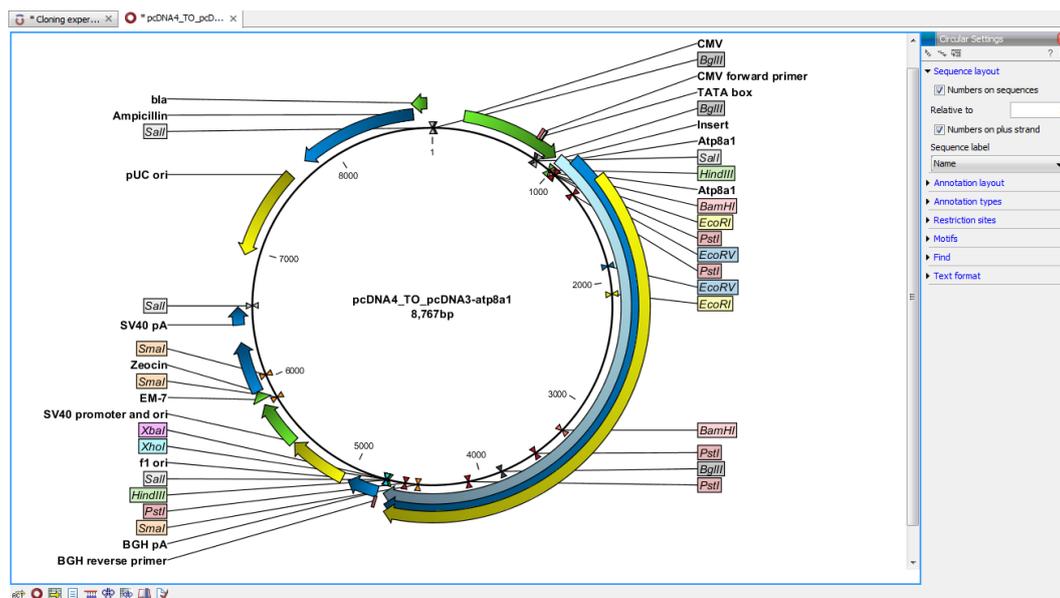


Figure 23.6: The final construct.

You can now **Save** (↵) this sequence for later use. 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.

23.1.3 Manual cloning

If you wish to use the manual way of cloning (as opposed to using the cloning workflow explained above in section 23.1.2), you can disregard the panel at the bottom. The manual cloning approach is based on a number of ways that you can manipulate the sequences. All manipulations of sequences are done manually, giving you full control over how the final construct is made. Manipulations are performed through right-click menus, which have three different appearances depending on where you click, as visualized in figure 23.7.

- **Right-click the sequence name (to the left) to manipulate the whole sequence.**
- **Right-click a selection to manipulate the selection.**

The two menus are described in the following:

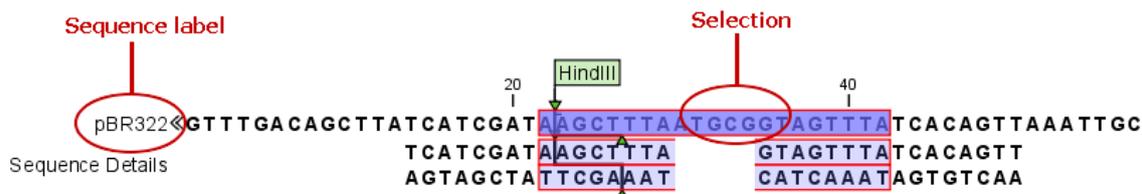


Figure 23.7: The red circles mark the two places you can use for manipulating the sequences.

Manipulate the whole sequence

Right-clicking the sequence name at the left side of the view reveals several options on sorting, opening and editing the sequences in the view (see figure 23.8).

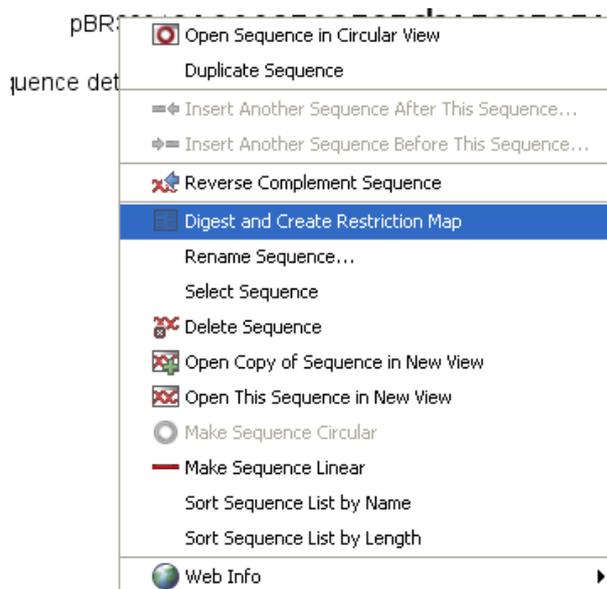


Figure 23.8: Right click on the sequence in the cloning view.

- Open sequence in circular view** (☐)

Opens the sequence in a new circular view. If the sequence is not circular, you will be asked if you wish to make it circular or not. (This will not forge ends with matching overhangs together - use "Make Sequence Circular" (○) instead.)
- Duplicate sequence**

Adds a duplicate of the selected sequence. The new sequence will be added to the list of sequences shown on the screen.
- Insert sequence after this sequence** (⇨⇩)

Insert another sequence after this sequence. The sequence to be inserted can be selected from a list which contains the sequences present in the cloning editor. 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. Otherwise a warning is displayed.
- Insert sequence before this sequence** (⇩⇨)

Insert another sequence before this sequence. The sequence to be inserted can be selected from a list which contains the sequences present in the cloning editor. 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. Otherwise a warning is displayed.

- **Reverse sequence**
Reverse 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* (see the following item in the list).
- **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 Sequence with Selected Enzymes and Run on Gel** (📄) 
See section [23.4.1](#)
- **Rename sequence**
Renames the sequence.
- **Select sequence**
This will select the entire sequence.
- **Delete sequence** (🗑️) 
This deletes the given sequence from the cloning editor.
- **Open sequence** (📄) 
This will open the selected sequence in a normal sequence view.
- **Make sequence circular** (🔄) 
This will convert 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** (—) 
This will convert a sequence from a circular to a linear form, removing the << and >> at the ends.

Manipulate parts of the sequence

Right-clicking a selection reveals several options on manipulating the selection (see figure [23.9](#)).

- **Duplicate Selection.** If a selection on the sequence is duplicated, the selected region will be added as a new sequence to the cloning editor with a new sequence name representing the length of the fragment. When a sequence region between two restriction sites are double-clicked the entire region will automatically be selected. This makes it very easy to make a new sequence from a fragment created by cutting with two restriction sites (right-click the selection and choose **Duplicate selection**).
- **Replace Selection with sequence.** This will replace the selected region with a sequence. The sequence to be inserted can be selected from a list containing all sequences in the cloning editor.

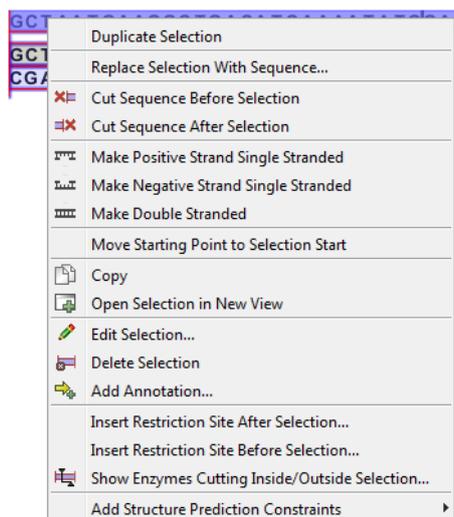


Figure 23.9: Right click on a sequence selection in the cloning view.

- **Cut Sequence Before Selection** (✂). This will cleave the sequence before the selection and will result in two smaller fragments.
- **Cut Sequence After Selection** (✂). This will cleave the sequence after the selection and will result in two smaller fragments.
- **Make Positive Strand Single Stranded** (☰). This will make the positive strand of the selected region single stranded.
- **Make Negative Strand Single Stranded** (☷). This will make 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** (📄). This will copy the selected region to the clipboard, which will enable it for use in other programs.
- **Open Selection in New View** (📄+). This will open the selected region in the normal sequence view.
- **Edit Selection** (✎). This will open a dialog box, in which is it possible to edit the selected residues.
- **Delete Selection** (🗑). This will delete the selected region of the sequence.
- **Add Annotation** (➕). This will open the **Add annotation** dialog box.
- **Insert Restriction Sites After/Before Selection**. This will show a dialog where you can choose from a list restriction enzymes (see section 23.1.4).
- **Show Enzymes Cutting Inside/Outside Selection** (✂). This will add enzymes cutting this selection to the Side Panel.
- **Add Structure Prediction Constraints**. This is relevant for RNA secondary structure prediction (see section 24.1.4).

Insert one sequence into another

Sequences can be inserted into each other in several ways as described in the lists above. When you chose to insert one sequence into another you will be presented with a dialog where all sequences in the view are present (see figure 23.10).

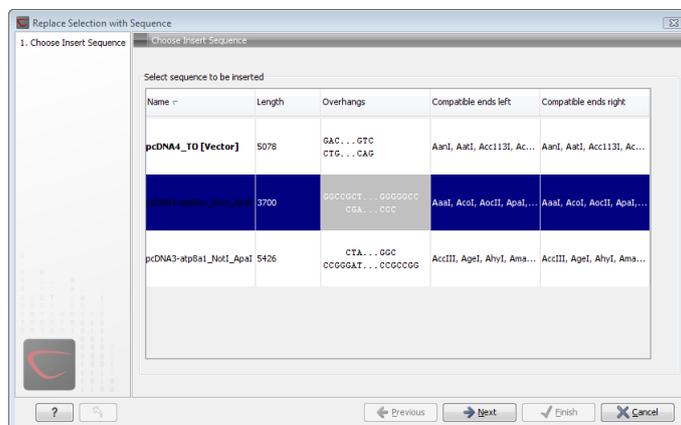


Figure 23.10: Select a sequence for insertion.

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 23.1.2.

The list furthermore 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.

Select the sequence you wish to insert and click **Next**.

This will show the dialog in figure 23.11).

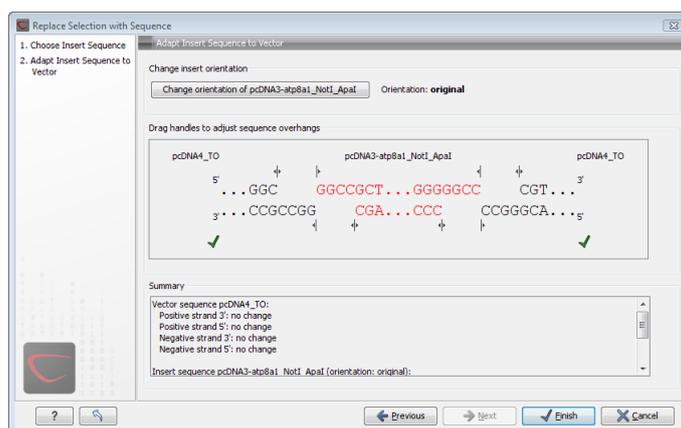


Figure 23.11: Drag the handles to adjust overhangs.

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** (↕).

Whenever you drag the handles, the status of the insertion point is indicated below:

- The overhangs match (✓).
- The overhangs do not match (⊘). In this case, you will not be able to click **Finish**. Drag the handles to make the overhangs match.

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 (📄) when you click **Finish**.

When you click **Finish** and the sequence is inserted, it will be marked with a selection.

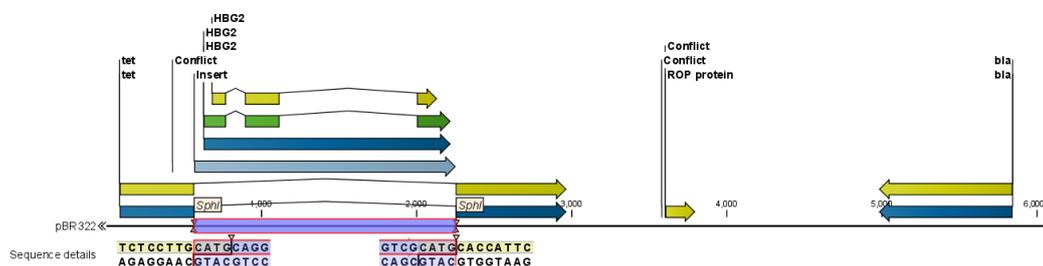


Figure 23.12: One sequence is now inserted into the cloning vector. The sequence inserted is automatically selected.

23.1.4 Insert restriction site

If you make a selection on the sequence, right-click, 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 23.13

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 etc., this can be typed into the text fields adjacent to the recognition sequence. .

Click **OK** will insert the sequence 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. Furthermore, a restriction site annotation is added.

23.2 Gateway cloning

CLC Main Workbench offers tools to perform *in silico* Gateway cloning², including Multi-site Gateway cloning.

The three tools for doing Gateway cloning in the CLC Main Workbench mimic the procedure followed in the lab:

²Gateway is a registered trademark of Invitrogen Corporation

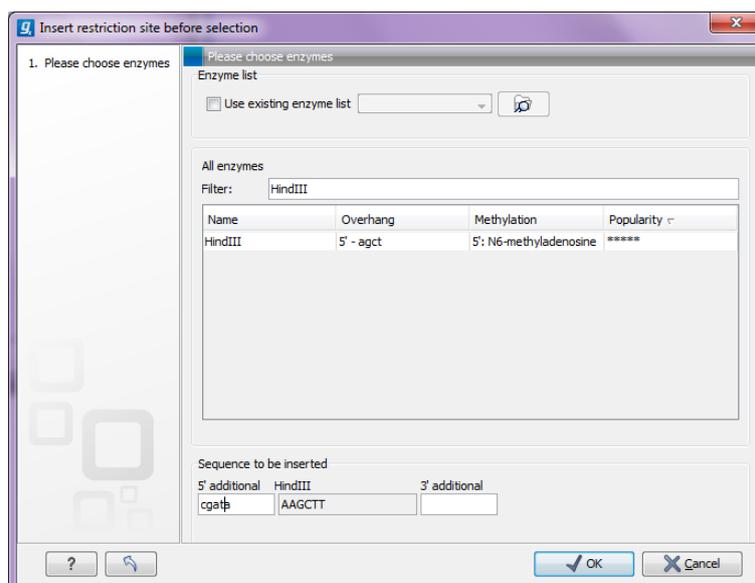


Figure 23.13: Inserting the HindIII recognition sequence.

- 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 <http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning.html>

To perform these analyses in the *CLC Main Workbench*, you need to import donor and expression vectors. These can be downloaded from Invitrogen's web site and directly imported into the Workbench: <http://tools.invitrogen.com/downloads/Gateway%20vectors.ma4>

23.2.1 Add attB sites

The first step in the Gateway cloning process is to amplify the target sequence with primers including so-called attB sites. In the *CLC Main Workbench*, you can add attB sites to a sequence fragment in this way:

Toolbox | Cloning and Restriction Sites (🔧) | Gateway Cloning (📁) | Add attB Sites (🔗)

This will open a dialog where you can select one or more sequences. Note that if your fragment is part of a longer sequence, you will need to extract it first. This can be done in two ways:

- If the fragment is covered by an annotation (if you want to use e.g. a CDS), simply right-click the annotation and **Open Annotation in New View**

- Otherwise you can simply make a selection on the sequence, right-click and **Open Selection in New View**

In both cases, the selected part of the sequence will be copied and opened as a new sequence which can be **Saved** (⇧⌘S).

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 23.14.

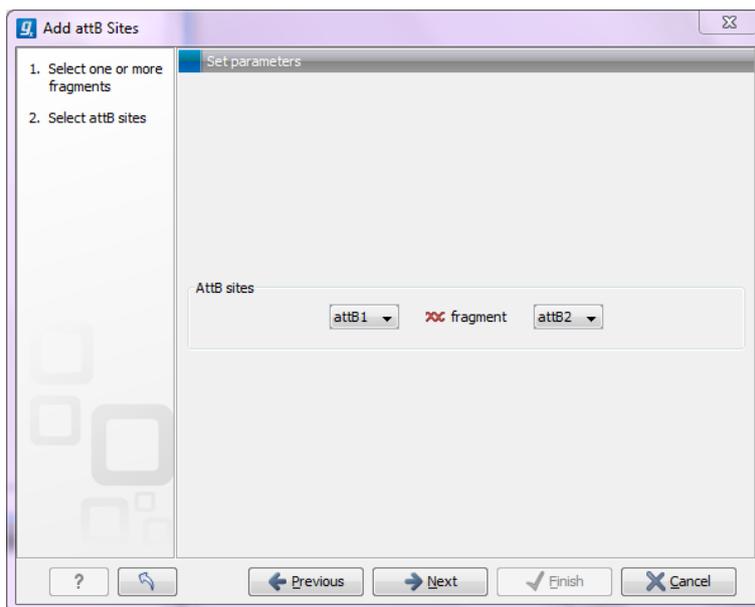


Figure 23.14: Selecting which attB sites to add.

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.

Click **Next** will give you options 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). See an example of this in figure 23.20 where a Shine-Dalgarno site has been added between the attB site and the gene of interest.

At the top of the dialog (see figure 23.15), you can specify primer additions such as a Shine-Dalgarno site, start codon etc. Click in the text field and press **Shift + F1 (Shift + Fn + F1 on Mac)** to show some of the most common additions (see figure 23.16).

Use the up and down arrow keys to select a tag and press **Enter**. This will insert the selected sequence as shown in figure 23.17.

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 (i.e. the sequence you selected as input). 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 (like the green Shine-Dalgarno site in figure 23.17).

This default list of primer additions can be modified, see section 23.2.1.

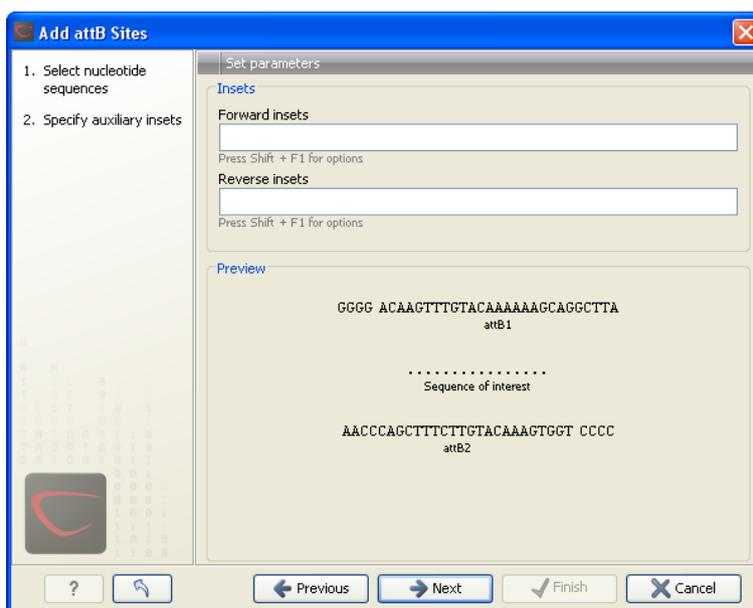


Figure 23.15: Primer additions 5' of the template-specific part of the primer.

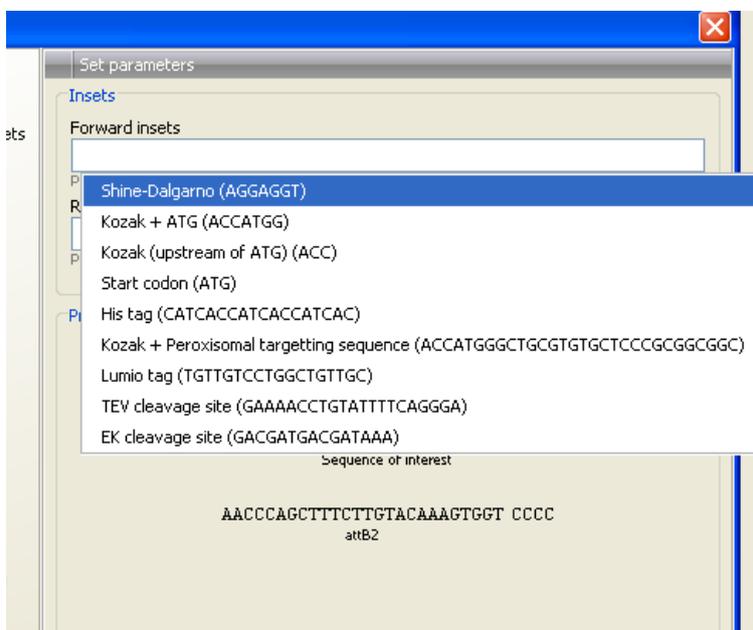


Figure 23.16: Pressing **Shift + F1** shows some of the common additions. This default list can be modified, see section [23.2.1](#).

You can also manually type a sequence with the keyboard or paste in a sequence from the clipboard by pressing **Ctrl + v** (**⌘ + v** on Mac).

Clicking **Next** allows you to specify the length of the template-specific part of the primers as shown in figure [23.18](#).

The *CLC Main 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 and you can also get a list of primers as you can see when clicking **Next** (see figure [23.19](#)).

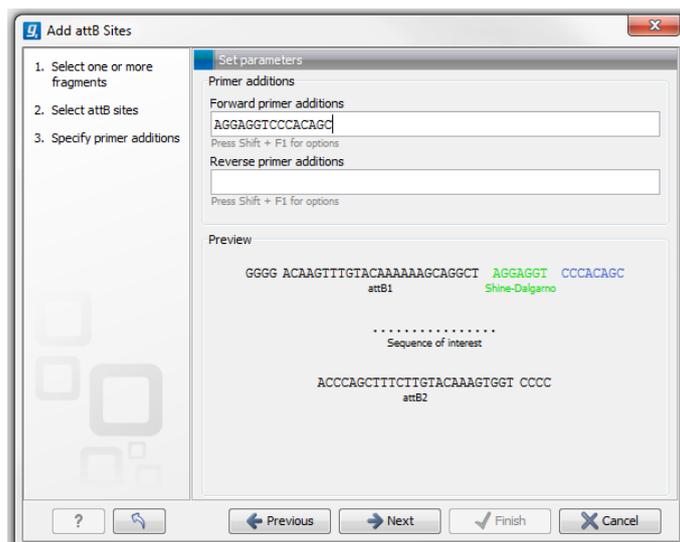


Figure 23.17: A Shine-Dalgarno sequence has been inserted.

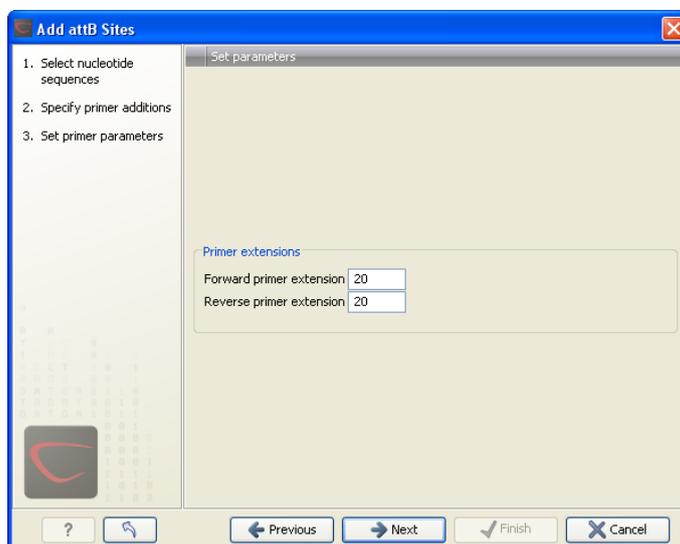


Figure 23.18: Specifying the length of the template-specific part of the primers.

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. Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

The attB sites, the primer additions and the primer regions are annotated in the final result as shown in figure 23.20.

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 work flow (see section 23.2.2). When you open the sequence again, you may need to switch on the relevant annotation types to show the sites and primer additions as illustrated in figure 23.20.

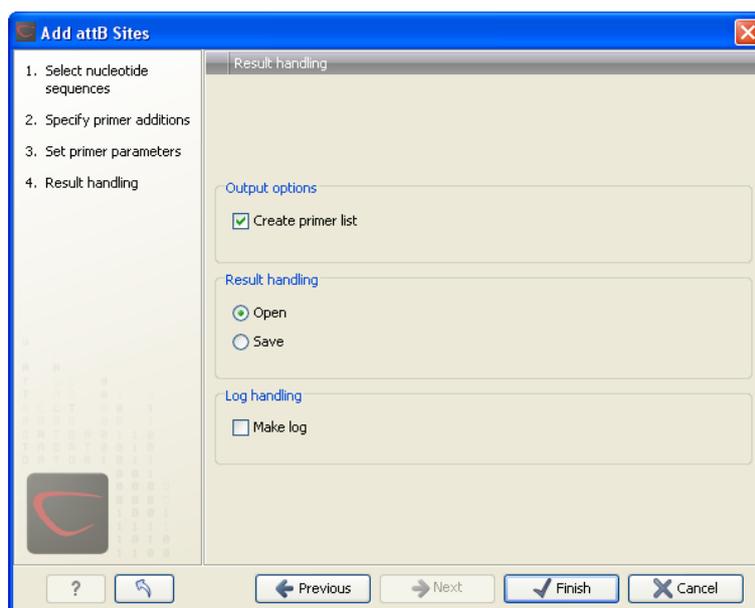


Figure 23.19: Besides the main output which is a copy of the input sequence(s) now including attB sites and primer additions, you can get a list of primers as output.

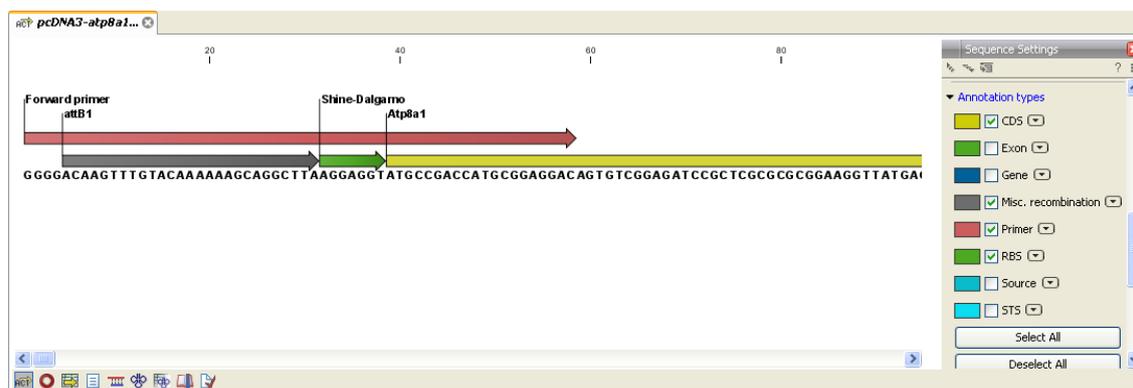


Figure 23.20: the attB site plus the Shine-Dalgarno primer addition is annotated.

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 23.15 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**:

Edit | Preferences | Advanced

In the advanced preferences dialog, scroll to the part called **Gateway cloning primer additions** (see figure 23.21).

Each element in the list has the following information:

Name The name of the sequence. 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. The sequence is always defined on the sense strand (although the reverse primer would be reverse complement).

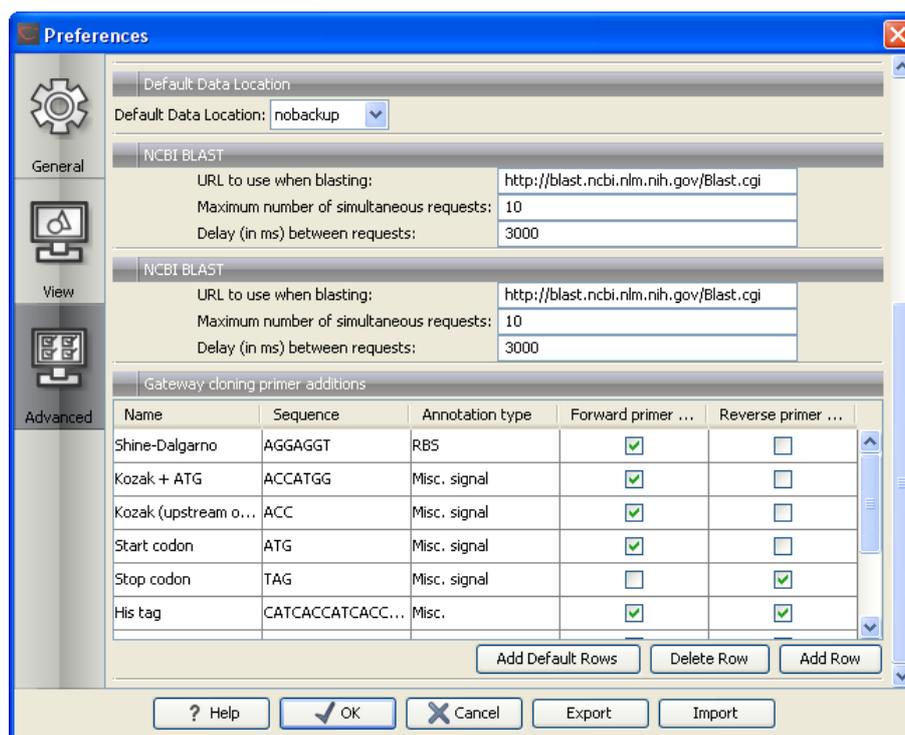


Figure 23.21: Configuring the list of primer additions available when adding attB sites.

Annotation type The annotation type used for the annotation 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.

You can either change 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.

23.2.2 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, the so-called BP reaction:

Toolbox | Cloning and Restriction Sites (🔧) | Gateway Cloning (📁) | Create Entry Clone (🔄)

This will open a dialog where you can select one or more sequences that will be the sequence of interest to be recombined into your donor vector. Note that the sequences you select should be flanked with attB sites (see section 23.2.1). You can select more than one sequence as input, and the corresponding number of entry clones will be created.

When you have selected your sequence(s), click **Next**.

This will display the dialog shown in figure 23.22.

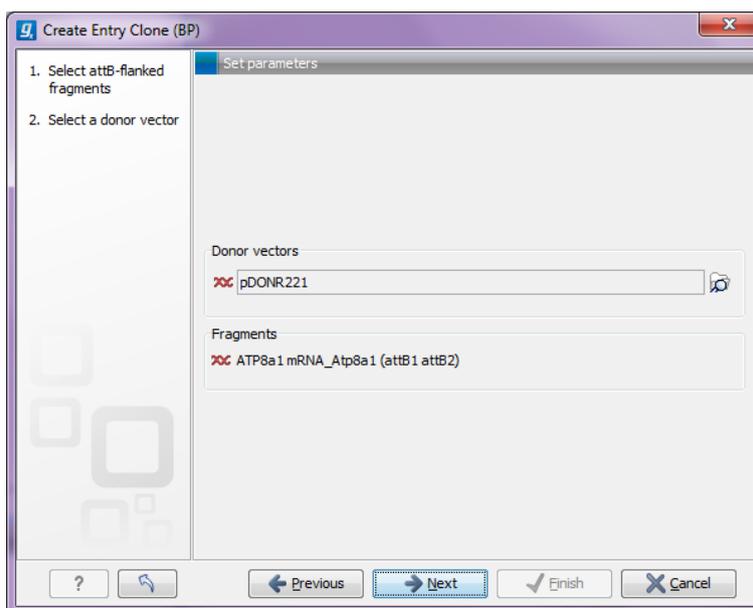


Figure 23.22: Selecting one or more donor vectors.

Clicking the **Browse** (🔍) button opens a dialog where you can select a donor vector. You can download donor vectors from Invitrogen's web site: <http://tools.invitrogen.com/downloads/Gateway%20vectors.ma4> and import into the *CLC Main Workbench*. Note that the *Workbench* looks for the specific sequences of the attP sites in the sequences that you select in this dialog (see how to change the definition of sites in appendix F). Note that the *CLC Main Workbench* only checks that valid attP sites are found - 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.

Below there is a preview of the fragments selected and the attB sites that they contain. 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.

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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 23.23.

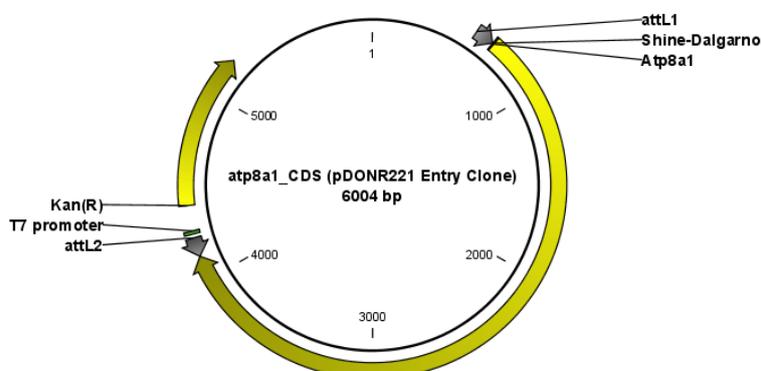


Figure 23.23: The resulting entry vector opened in a circular view.

Note that the bi-product of the recombination is not part of the output.

23.2.3 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, the so-called LR reaction:

Toolbox | Cloning and Restriction Sites (🔧) | Gateway Cloning (📁) | Create Expression Clone (🔄)

This will open a dialog where you can select one or more entry clones (see how to create an entry clone in section 23.2.2). If you wish to perform separate LR reactions with multiple entry clones, you should run the **Create Expression Clone** in batch mode (see section 9.1).

When you have selected your entry clone(s), click **Next**.

This will display the dialog shown in figure 23.24.

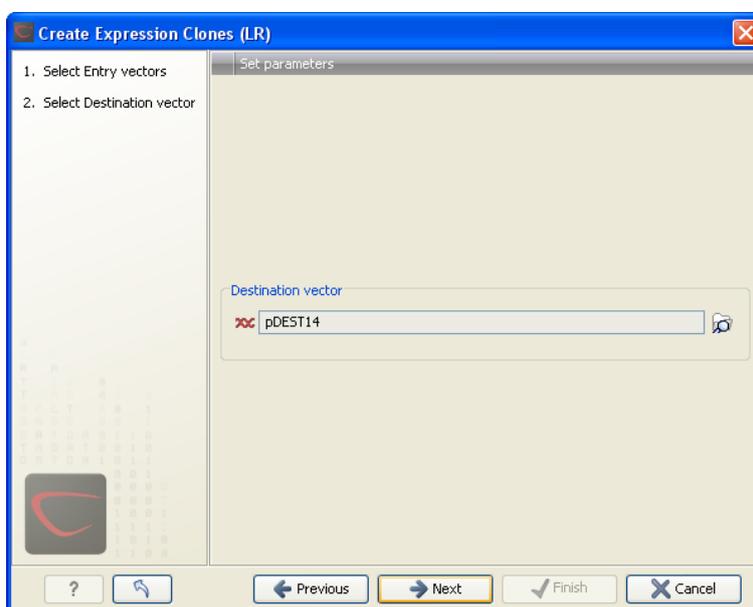


Figure 23.24: Selecting one or more destination vectors.

Clicking the **Browse** (🔍) button opens a dialog where you can select a destination vector. You can download donor vectors from Invitrogen's web site: <http://tools.invitrogen.com/downloads/Gateway%20vectors.ma4> and import into the *CLC Main Workbench*. 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 F). Note that the *CLC Main Workbench* only checks that valid attR sites are found - it does not check that they correspond to the attL sites of the selected fragments at this step. If the right combination of attL and attR sites is not found, no entry clones will be produced.

When performing multi-site gateway cloning, the *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 <http://tools.invitrogen.com/downloads/gateway-multisite-seminar.html>

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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 23.25.

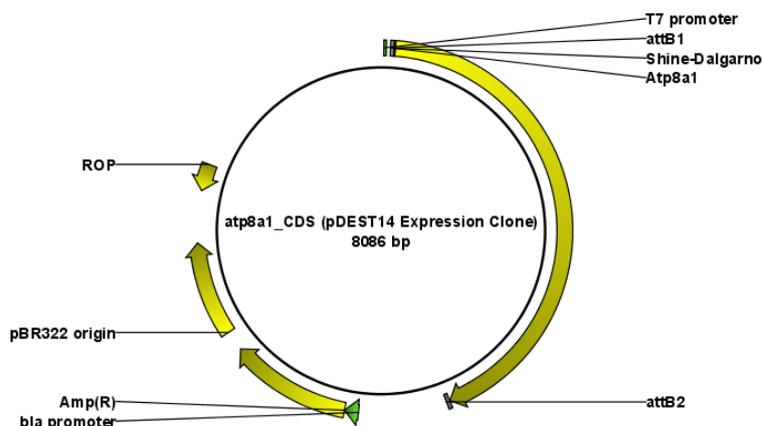


Figure 23.25: The resulting expression clone opened in a circular view.

You can choose to create a sequence list with the bi-products as well.

For a destination vector to be recognized, apart from the appropriate att sites, it must contain the *ccdB* gene. This must be present either as a 'ccdB' annotation, or as the exact sequence:

```
ATGCAGTTTAAGGTTTACACCTATAAAAGAGAGAGCCGTTATCGTCTGTTTGTGGATGTACAGAGTGATATT
ATTGACACGCCCGGGCGACGGATGGTATCCCCCTGGCCAGTGCACGTCTGCTGTCAGATAAAGTCTCC
CGTGAACCTTACCCGGTGGTGCATATCGGGGATGAAAGCTGGCGCATGATGACCACCGATATGGCCAGT
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 the relevant part of the sequence, right-click and choose 'Add Annotation'. Name the annotation 'ccdB'.

23.3 Restriction site analysis

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 will be useful, since it is a quick and easy way of showing restriction sites.
- In the **Toolbox** you will find the other way of doing restriction site analyses. This way provides more control of the analysis and gives you more output options, e.g. a table of restriction sites and you can perform the same restriction map analysis on several sequences in one step.

This chapter first describes the dynamic restriction sites, followed by a description of how to do restriction site analyses via the toolbox (you can run more extensive analysis via the restriction analysis tools available in the toolbox). This section also includes an explanation of how to

simulate a gel with the selected enzymes. The final section in this chapter focuses on enzyme lists which represent an easy way of managing restriction enzymes.

23.3.1 Dynamic restriction sites

If you open a sequence, a sequence list etc, you will find the **Restriction Sites** group in the **Side Panel**.

As shown in figure 23.26 you can display restriction sites as colored triangles and lines on the sequence. The **Restriction sites** group in the side panel shows a list of enzymes, represented by different colors corresponding to the colors of the triangles on the sequence. By selecting or deselecting the enzymes in the list, you can specify which enzymes' restriction sites should be displayed.

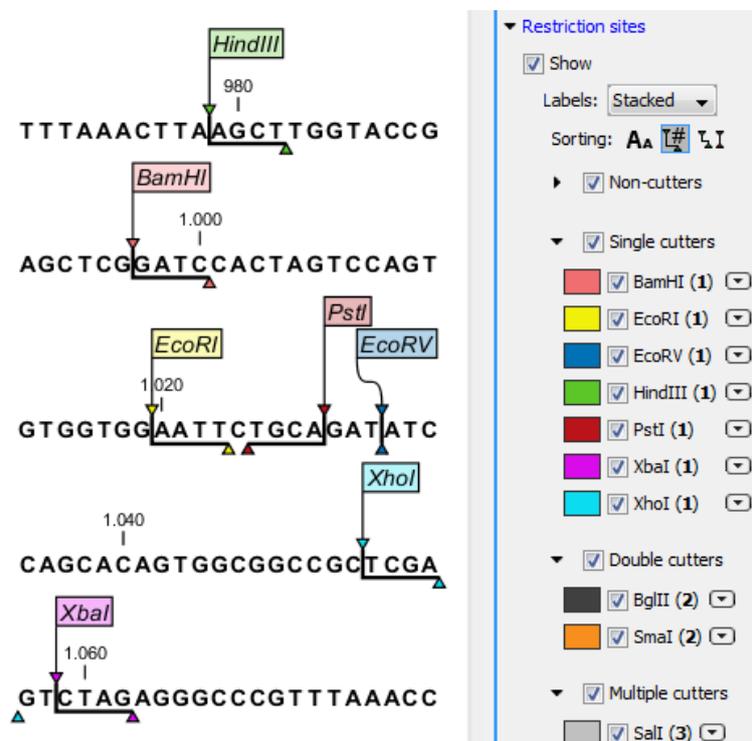


Figure 23.26: Showing restriction sites of ten restriction enzymes.

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 name flags** above the list of restriction enzymes.

There is also an option to specify how the **Labels** shown 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 23.27). 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:

- **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 23.29).
- **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 23.28).



Figure 23.27: Restriction site labels shown as flags.

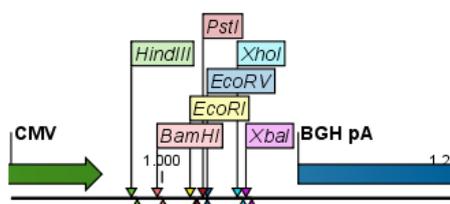


Figure 23.28: Restriction site labels stacked.

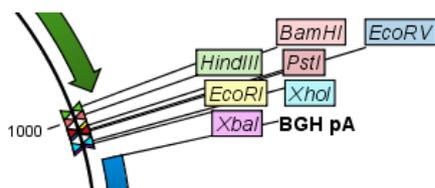


Figure 23.29: Restriction site labels in radial layout.

Note that in a circular view, the **Stacked** and **Radial** options also affect the layout of annotations.

Sort enzymes

Just above the list of enzymes there are three buttons to be used for sorting the list (see figure 23.30):

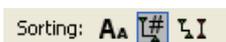


Figure 23.30: Buttons to sort restriction enzymes.

- **Sort enzymes alphabetically (AA).** Clicking this button will sort the list of enzymes alphabetically.
- **Sort enzymes by number of restriction sites (T#).** 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** (T I). 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 20 of the most popular enzymes, but you can easily modify this list and add more enzymes by clicking the **Manage enzymes button**. This will display the dialog shown in figure 23.31.

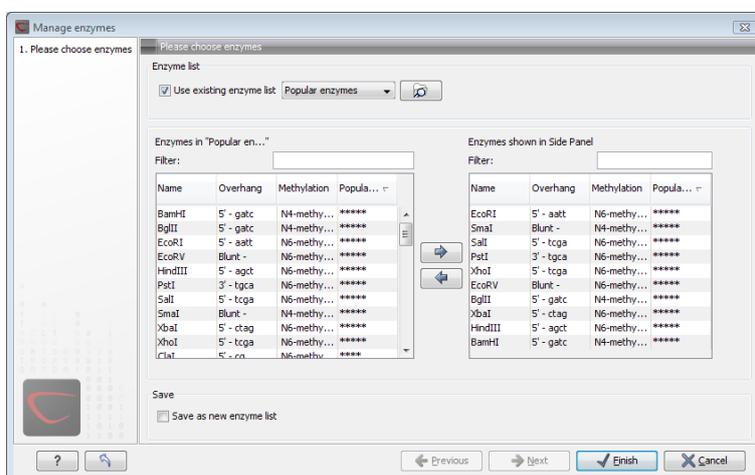


Figure 23.31: Adding or removing enzymes from the Side Panel.

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**. See section 23.5 for more about creating and modifying enzyme lists.

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 an existing enzyme list, this panel shows all the enzymes available³.
- 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 (➡). If you e.g. wish to use EcoRV and BamHI, select these two enzymes and add them to the right side panel.

³The CLC Main Workbench comes with a standard set of enzymes based on <http://rebase.neb.com/rebase/rebase.html>. You can customize the enzyme database for your installation, see section E

If you wish to use all the enzymes in the list:

Click in the panel to the left | press Ctrl + A (⌘ + A on Mac) | Add (➡)

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 e.g. a 3' overhang. In this case, you can sort the list by clicking the Overhang column heading, and all the enzymes producing 3' overhangs will be listed together for easy selection.

When looking for a specific enzyme, it is easier to use the Filter. If you wish to find e.g. HindIII sites, simply type HindIII into the filter, and the list of enzymes will shrink automatically to only include the HindIII enzyme. This can also be used to only show enzymes producing e.g. a 3' overhang as shown in figure 23.50.

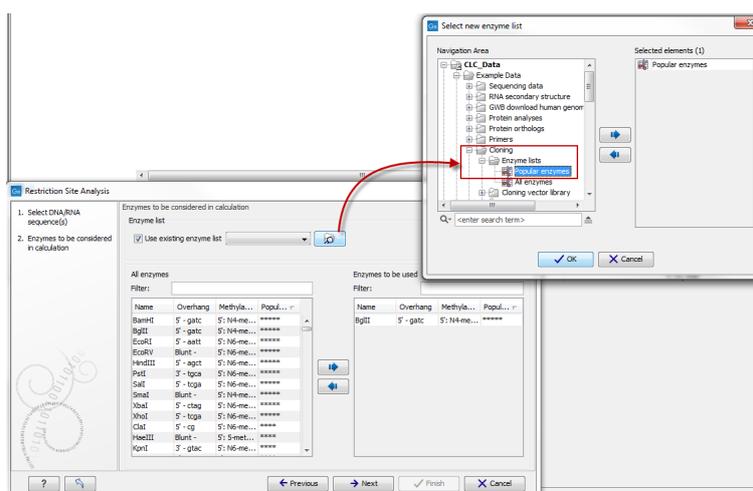


Figure 23.32: Selecting enzymes.

If you need more detailed information and filtering of the enzymes, either place your mouse cursor on an enzyme for one second to display additional information (see figure 23.51), or use the view of enzyme lists (see 23.5).

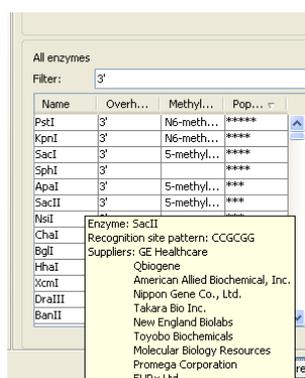


Figure 23.33: Showing additional information about an enzyme like recognition sequence or a list of commercial vendors.

At the bottom of the dialog, you can select to save this list of enzymes as a new file. In this way, you can save the selection of enzymes for later use.

When you click **Finish**, the enzymes are added to the Side Panel and the cut sites are shown on the sequence.

If you have specified a set of enzymes which you always use, it will probably be a good idea to save the settings in the Side Panel (see section 5.6) for future use.

Show enzymes cutting inside/outside selection

Section 23.3.1 describes how to add more enzymes to the list in the Side Panel based on the name of the enzyme, overhang, methylation sensitivity etc. However, you will often find yourself in a situation where you need a more sophisticated and explorative approach.

An illustrative example: you have a selection on a sequence, and you wish to find enzymes cutting within the selection, but not outside. This problem often arises during design of cloning experiments. In this case, you do not know the name of the enzyme, so you want the Workbench to find the enzymes for you:

right-click the selection | Show Enzymes Cutting Inside/Outside Selection (🔍)

This will display the dialog shown in figure 23.34 where you can specify which enzymes should initially be considered.

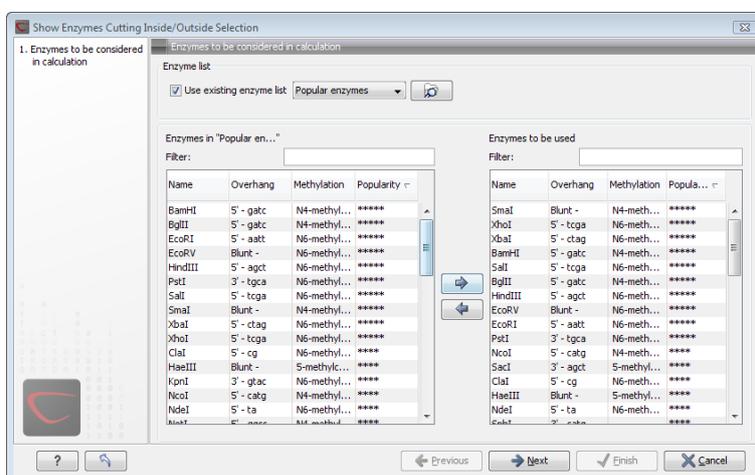


Figure 23.34: Choosing enzymes to be considered.

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**. See section 23.5 for more about creating and modifying enzyme lists.

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 an existing enzyme list, this panel shows all the enzymes available ⁴.
- 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 (➡). If you e.g. wish to use EcoRV and BamHI, select these two enzymes and add them to the right side panel.

⁴The CLC Main Workbench comes with a standard set of enzymes based on <http://rebase.neb.com/rebase/rebase.html>. You can customize the enzyme database for your installation, see section E

If you wish to use all the enzymes in the list:

Click in the panel to the left | press Ctrl + A (⌘ + A on Mac) | Add (➡)

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 e.g. a 3' overhang. In this case, you can sort the list by clicking the Overhang column heading, and all the enzymes producing 3' overhangs will be listed together for easy selection.

When looking for a specific enzyme, it is easier to use the Filter. If you wish to find e.g. HindIII sites, simply type HindIII into the filter, and the list of enzymes will shrink automatically to only include the HindIII enzyme. This can also be used to only show enzymes producing e.g. a 3' overhang as shown in figure 23.50.

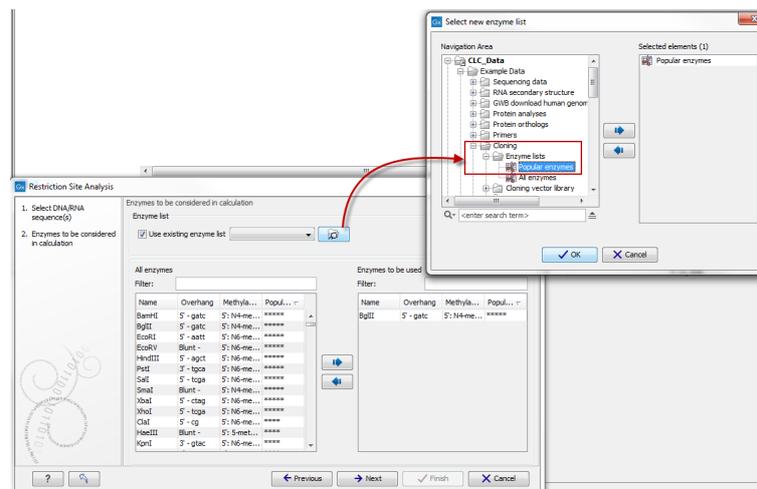


Figure 23.35: Selecting enzymes.

If you need more detailed information and filtering of the enzymes, either place your mouse cursor on an enzyme for one second to display additional information (see figure 23.51), or use the view of enzyme lists (see 23.5).

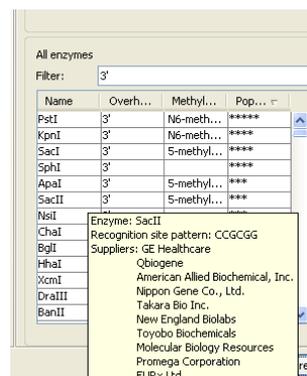


Figure 23.36: Showing additional information about an enzyme like recognition sequence or a list of commercial vendors.

Clicking **Next** will show the dialog in figure 23.37.

At the top of the dialog, you see the selected region, and below are two panels:

- **Inside selection.** Specify how many times you wish the enzyme to cut inside the selection.

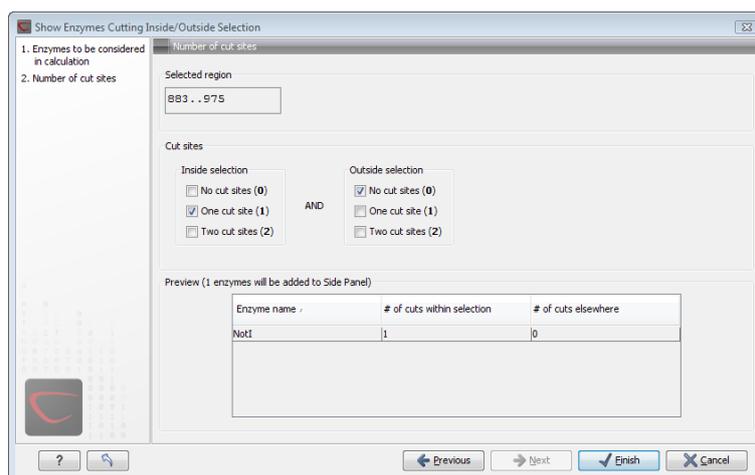


Figure 23.37: Deciding number of cut sites inside and outside the selection.

In the example described above, "One cut site (1)" should be selected to only show enzymes cutting once in the selection.

- **Outside selection.** Specify how many times you wish the enzyme to cut outside the selection (i.e. the rest of the sequence). In the example above, "No cut sites (0)" should be selected.

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

Besides what is described above, there is a third way of adding enzymes to the Side Panel and thereby displaying them on the sequence. It is based on the overhang produced by cutting with an enzyme and will find enzymes producing a compatible overhang:

right-click the restriction site | Show Enzymes with Compatible Ends (⌘ I)

This will display the dialog shown in figure 23.38.

At the top you can choose whether the enzymes considered should have an exact match or not. 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.

We advice trying **Exact match** first, and use **All matches** as an alternative if a satisfactory result cannot be achieved.

At the bottom of the dialog, the list of enzymes producing compatible overhangs is shown. Use the arrows to add enzymes which will be displayed on the sequence which you press **Finish**.

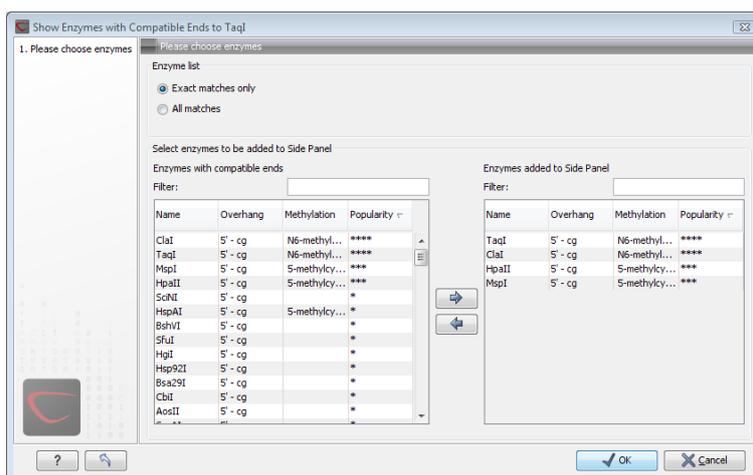


Figure 23.38: *Enzymes with compatible ends.*

When you have added the relevant enzymes, click **Finish**, and the enzymes will be added to the Side Panel and their cut sites displayed on the sequence.

23.3.2 Restriction site analysis from the Toolbox

Besides the dynamic restriction sites, you can do a more elaborate restriction map analysis with more output format using the Toolbox:

Toolbox | Cloning and Restriction Sites (🔧) | Restriction Site Analysis (✂️)

This will display the dialog shown in figure 23.39.

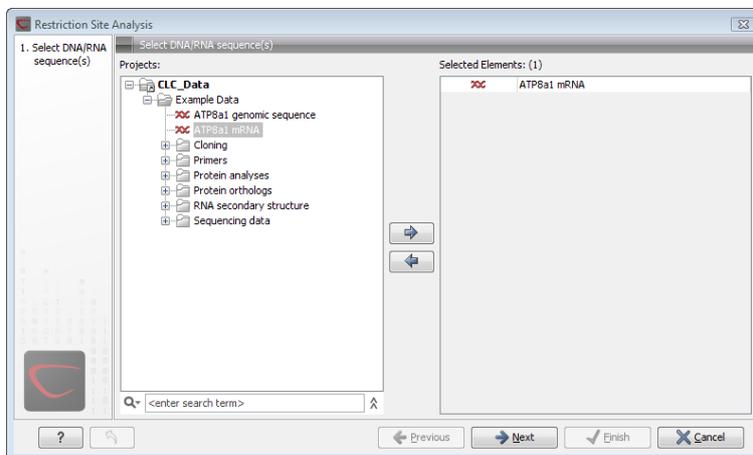


Figure 23.39: *Choosing sequence ATP8a1 mRNA for restriction map analysis.*

If a sequence was selected before choosing the Toolbox action, 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.

Selecting, sorting and filtering enzymes

Clicking **Next** lets you define which enzymes to use as basis for finding restriction sites on the sequence. 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**. See section 23.5 for more about creating and modifying enzyme lists.

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 an existing enzyme list, this panel shows all the enzymes available ⁵.
- 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 (➡). If you e.g. wish to use EcoRV and BamHI, select these two enzymes and add them to the right side panel.

If you wish to use all the enzymes in the list:

Click in the panel to the left | press Ctrl + A (⌘ + A on Mac) | Add (➡)

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 e.g. a 3' overhang. In this case, you can sort the list by clicking the Overhang column heading, and all the enzymes producing 3' overhangs will be listed together for easy selection.

When looking for a specific enzyme, it is easier to use the Filter. If you wish to find e.g. HindIII sites, simply type HindIII into the filter, and the list of enzymes will shrink automatically to only include the HindIII enzyme. This can also be used to only show enzymes producing e.g. a 3' overhang as shown in figure 23.50.

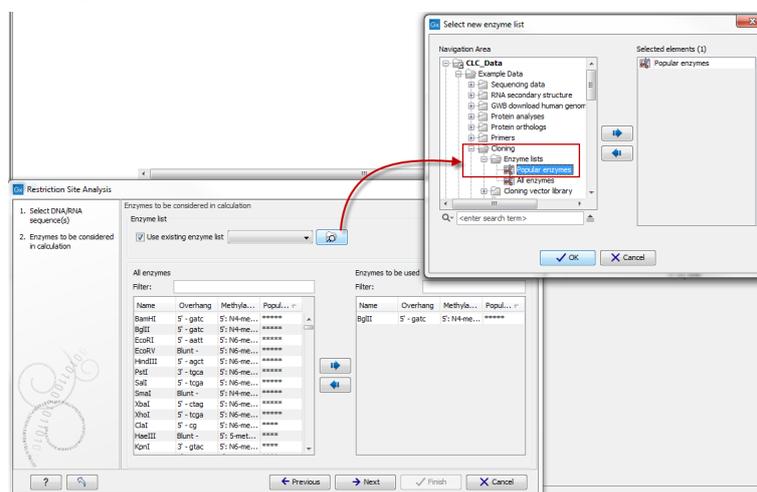


Figure 23.40: Selecting enzymes.

If you need more detailed information and filtering of the enzymes, either place your mouse cursor on an enzyme for one second to display additional information (see figure 23.51), or use the view of enzyme lists (see 23.5).

⁵The CLC Main Workbench comes with a standard set of enzymes based on <http://rebase.neb.com/rebase/rebase.html>. You can customize the enzyme database for your installation, see section E

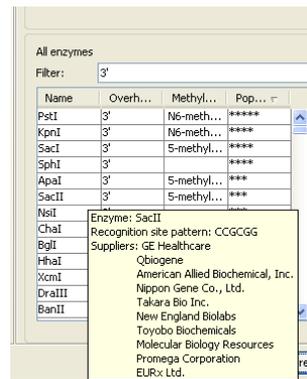


Figure 23.41: Showing additional information about an enzyme like recognition sequence or a list of commercial vendors.

Number of cut sites

Clicking **Next** confirms the list of enzymes which will be included in the analysis, and takes you to the dialog shown in figure 23.42.

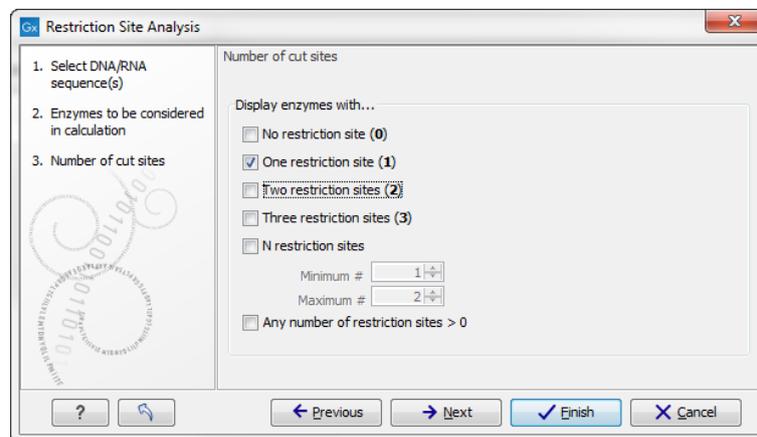


Figure 23.42: Selecting number of cut sites.

If you wish the output of the restriction map analysis only to include restriction enzymes which cut the sequence a specific number of times, use the checkboxes in this dialog:

- No restriction site (**0**)
- One restriction site (**1**)
- Two restriction sites (**2**)
- Three restriction site (**3**)
- N restriction sites
 - Minimum
 - Maximum
- Any number of restriction sites > 0

The default setting is to include the enzymes which cut the sequence one or two times.

You can use the checkboxes to perform very specific searches for restriction sites: e.g. if you wish to find enzymes which do not cut the sequence, or enzymes cutting exactly twice.

Output of restriction map analysis

Clicking next shows the dialog in figure 23.43.

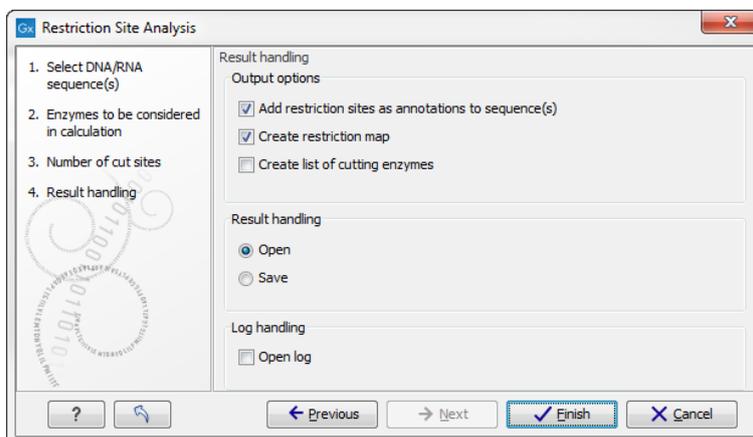


Figure 23.43: Choosing to add restriction sites as annotations or creating a restriction map.

This dialog lets you specify how the result of the restriction map analysis should be presented:

- **Add restriction sites as annotations to sequence(s).** This option makes it possible to see the restriction sites on the sequence (see figure 23.44) and save the annotations for later use.
- **Create restriction map.** The restriction map is a table of restriction sites as shown in figure 23.45. 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 (or more).
- **Add restriction sites as annotations to sequence(s).** This option makes it possible to see the restriction sites on the sequence (see figure 23.44) and save the annotations for later use.
- **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 23.45. 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.
 - As a **table of fragments** which shows the sequence fragments that would be the result of cutting the sequence with the selected enzymes (see figure 23.46).
 - 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 23.4.

The following sections will describe these output formats in more detail.

In order to complete the analysis click **Finish** (see section 9.2 for information about the Save and Open options).

Restriction sites as annotation on the sequence

If you chose to add the restriction sites as annotation to the sequence, the result will be similar to the sequence shown in figure 23.44. See section 12.3 for more information about viewing



Figure 23.44: The result of the restriction analysis shown as annotations.

annotations.

Table of restriction sites

The restriction map can be shown as a table of restriction sites (see figure 23.45).

| Sequ... | Name | Pattern | Overhang | Number ... | Cut position(s) |
|---------|----------|-------------|----------|------------|-----------------|
| PERH3BC | CjePI | ccannnnnntc | 3' | 1 | (151, 184) |
| PERH3BC | MboII | gaaga | 3' | 1 | 86 |
| PERH3BC | NcuI | gaaga | 3' | 1 | 86 |
| PERH3BC | TsoI | tarcca | 3' | 1 | [134] |
| PERH3BC | Tth111II | caarca | 3' | 1 | [101] |

Figure 23.45: The result of the restriction analysis shown as annotations.

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.
- **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 cuts more than once, the positions are separated by commas.
 - [] If the enzyme's recognition sequence is on the negative strand, the cut position is put in brackets (as the enzyme TsoI in figure 23.45 whose cut position is [134]).

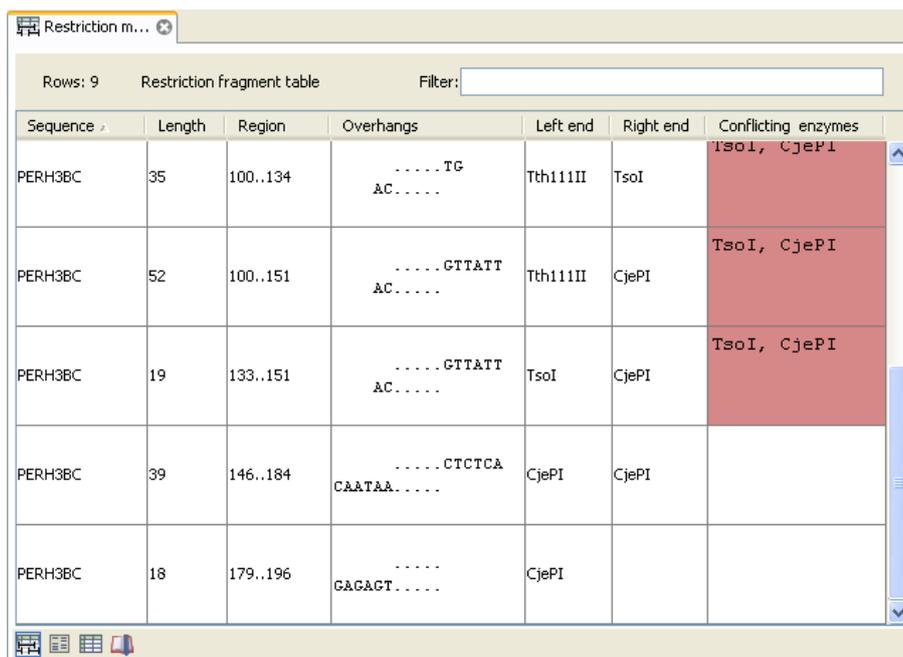
- () Some enzymes cut the sequence twice for each recognition site, and in this case the two cut positions are surrounded by parentheses.

Table of restriction fragments

The restriction map can be shown as a table of fragments produced by cutting the sequence with the enzymes:

Click the Fragments button () at the bottom of the view

The table is shown in see figure 23.46.



| Sequence | Length | Region | Overhangs | Left end | Right end | Conflicting enzymes |
|----------|--------|----------|----------------------------|----------|-----------|---------------------|
| PERH3BC | 35 | 100..134 |TC AC..... | Tth111II | TsoI | TsoI, CjePI |
| PERH3BC | 52 | 100..151 |GTTATT AC..... | Tth111II | CjePI | TsoI, CjePI |
| PERH3BC | 19 | 133..151 |GTTATT AC..... | TsoI | CjePI | TsoI, CjePI |
| PERH3BC | 39 | 146..184 |CTCTCA CAATAA..... | CjePI | CjePI | |
| PERH3BC | 18 | 179..196 | GAGACT..... | CjePI | | |

Figure 23.46: The result of the restriction analysis shown as annotations.

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 ⁶. 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.** 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.

⁶Furthermore, if this is the case, you will see the names of the other enzymes in the **Conflicting Enzymes** column

- **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.

Gel

The restriction map can also be shown as a gel. This is described in section [23.4.1](#).

23.4 Gel electrophoresis

CLC Main Workbench enables the user to simulate the separation of nucleotide sequences on a gel. This feature is useful when e.g. 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 two main ways to simulate gel separation of nucleotide sequences:

- One or more sequences can be digested with restriction enzymes and the resulting fragments can be separated on a gel.
- A number of existing sequences can be separated on a gel.

There are several ways to apply these functionalities as described below.

23.4.1 Separate fragments of sequences on gel

This section explains how to simulate a gel electrophoresis of one or more sequences which are digested with restriction enzymes. There are two ways to do this:

- When performing the **Restriction Site Analysis** from the **Toolbox**, you can choose to create a restriction map which can be shown as a gel.
This is explained in section [23.3.2](#).
- From all the graphical views of sequences, you can right-click the name of the sequence and choose: **Digest Sequence with Selected Enzymes and Run on Gel** (). The views where this option is available are listed below:
 - Circular view (see section [12.2](#)).
 - Ordinary sequence view (see section [12.1](#)).

- Graphical view of sequence lists (see section 12.6).
- Cloning editor (see section 23.1).
- Primer designer (see section 22.3).

Furthermore, you can also right-click an empty part of the view of the graphical view of sequence lists and the cloning editor and choose **Digest All Sequences with Selected Enzymes and Run on Gel**.

Note! When using the right-click options, the sequence will be digested with the enzymes that are selected in the **Side Panel**. This is explained in section 12.1.2.

The view of the gel is explained in section 23.4.3

23.4.2 Separate sequences on gel

To separate sequences without restriction enzyme digestion, first create a sequence list of the sequences in question (see section 12.6). Then click the **Gel** button () at the bottom of the view of the sequence list.

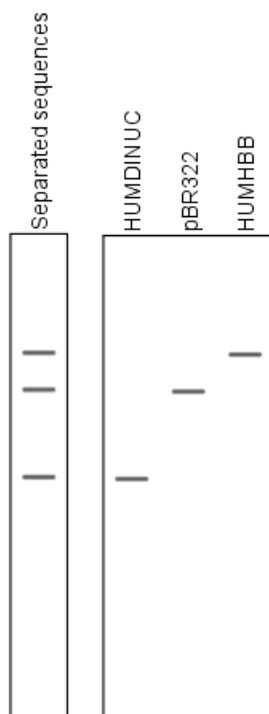


Figure 23.47: A sequence list shown as a gel.

For more information about the view of the gel, see the next section.

23.4.3 Gel view

In figure 23.48 you can see a simulation of a gel with its **Side Panel** to the right. This view will be explained in this section.

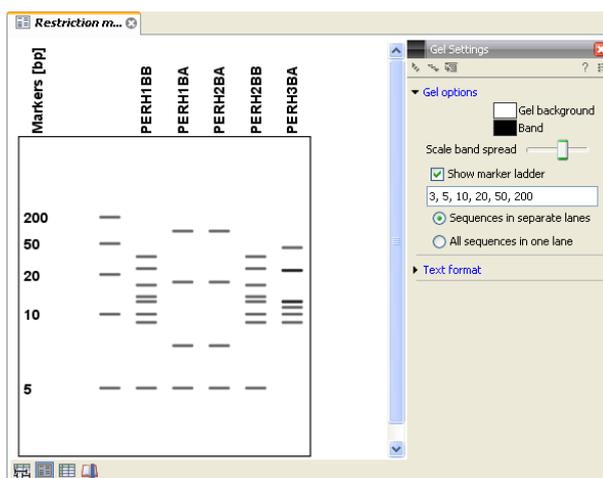


Figure 23.48: Five lanes showing fragments of five sequences cut with restriction enzymes.

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:

- 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**.

23.5 Restriction enzyme lists

CLC Main Workbench includes all the restriction enzymes available in the **REBASE** database⁷. 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 e.g. all enzymes available in the laboratory freezer, all enzymes used to create a given restriction map or all enzymes that are available from the preferred vendor.

In the example data (see section 1.6.2) 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*.

This section describes how you can create an enzyme list, and how you can modify it.

23.5.1 Create enzyme list

CLC Main Workbench uses enzymes from the **REBASE** restriction enzyme database at <http://rebase.neb.com>⁸.

To create an enzyme list of a subset of these enzymes:

File | New | Enzyme list (📄)

This opens the dialog shown in figure 23.49

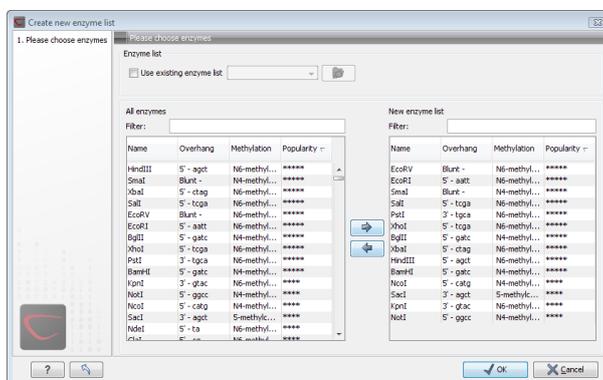


Figure 23.49: Choosing enzymes for the new enzyme list.

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**. See section 23.5 for more about creating and modifying enzyme lists.

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

⁷You can customize the enzyme database for your installation, see section E

⁸You can customize the enzyme database for your installation, see section E

chosen to use an existing enzyme list, this panel shows all the enzymes available ⁹.

- 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 (➡). If you e.g. wish to use EcoRV and BamHI, select these two enzymes and add them to the right side panel.

If you wish to use all the enzymes in the list:

Click in the panel to the left | press Ctrl + A (⌘ + A on Mac) | Add (➡)

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 e.g. a 3' overhang. In this case, you can sort the list by clicking the Overhang column heading, and all the enzymes producing 3' overhangs will be listed together for easy selection.

When looking for a specific enzyme, it is easier to use the Filter. If you wish to find e.g. HindIII sites, simply type HindIII into the filter, and the list of enzymes will shrink automatically to only include the HindIII enzyme. This can also be used to only show enzymes producing e.g. a 3' overhang as shown in figure 23.50.

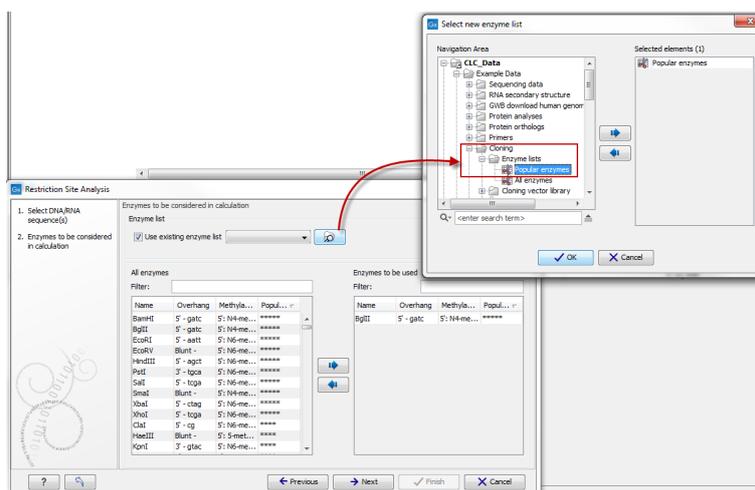


Figure 23.50: Selecting enzymes.

If you need more detailed information and filtering of the enzymes, either place your mouse cursor on an enzyme for one second to display additional information (see figure 23.51), or use the view of enzyme lists (see 23.5).

Click **Finish** to open the enzyme list.

23.5.2 View and modify enzyme list

An enzyme list is shown in figure 23.52. The list 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.

⁹The CLC Main Workbench comes with a standard set of enzymes based on <http://rebase.neb.com/rebase/rebase.html>. You can customize the enzyme database for your installation, see section E

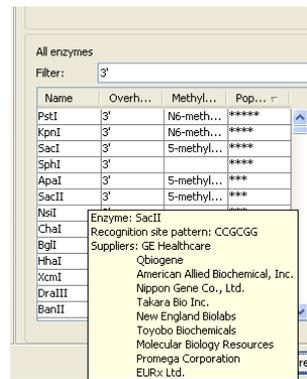


Figure 23.51: Showing additional information about an enzyme like recognition sequence or a list of commercial vendors.

| Name | Recognition sequence | Length | Overhang | Suppliers |
|--------|----------------------|--------|----------|---|
| PstI | gaatnctc | 6 | 3' | GE Healthcare; Takara Bio Inc.; New England Biolabs |
| KpnI | gtaccacg | 6 | 3' | GE Healthcare; Invitrogen Corporation; American Allied Biochemical, Inc.; Takara Bio Inc.; Roche Applied Sciences |
| SacI | gaatcgtc | 6 | Blunt | New England Biolabs |
| SphI | catatg | 6 | 3' | GE Healthcare; Fermentas International Inc.; Qbiogene; Takara Bio Inc.; Roche Applied Sciences; Takara Bio Inc. |
| Apal | gtacacac | 6 | 3' | GE Healthcare; Fermentas International Inc.; Qbiogene; Takara Bio Inc.; Roche Applied Sciences; Takara Bio Inc. |
| SacII | ccgctg | 6 | 3' | GE Healthcare; Fermentas International Inc.; Qbiogene; Takara Bio Inc.; Roche Applied Sciences; Takara Bio Inc. |
| NsiI | catatg | 6 | 3' | GE Healthcare; Fermentas International Inc.; Qbiogene; Takara Bio Inc.; Roche Applied Sciences; Takara Bio Inc. |
| Chai | ccgctg | 6 | 3' | GE Healthcare; Fermentas International Inc.; Qbiogene; Takara Bio Inc.; Roche Applied Sciences; Takara Bio Inc. |
| BglI | atctg | 6 | Blunt | GE Healthcare; Invitrogen Corporation; American Allied Biochemical, Inc.; Takara Bio Inc.; Roche Applied Sciences |
| HhaI | gacgtc | 6 | Blunt | GE Healthcare; Invitrogen Corporation; American Allied Biochemical, Inc.; Takara Bio Inc.; Roche Applied Sciences |
| XcmI | ccgctg | 6 | 3' | GE Healthcare; Fermentas International Inc.; Qbiogene; Takara Bio Inc.; Roche Applied Sciences; Takara Bio Inc. |
| DraIII | catatg | 6 | 3' | GE Healthcare; Fermentas International Inc.; Qbiogene; Takara Bio Inc.; Roche Applied Sciences; Takara Bio Inc. |
| BanII | ggtacc | 6 | Blunt | GE Healthcare; Invitrogen Corporation; American Allied Biochemical, Inc.; Takara Bio Inc.; Roche Applied Sciences |

Figure 23.52: An enzyme list.

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 23.49 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 | 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. E.g. if you wish to create a list of enzymes sold by a particular distributor, type the name of the distributor into the filter, and select and create a new enzyme list from the selection.

Chapter 24

RNA structure

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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 known 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 [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, see <http://mfold.rna.albany.edu/?q=mfold/mfold-references>.

24.1.1 Selecting sequences for prediction

Secondary structure prediction can be accessed in the **Toolbox**:

Toolbox | RNA Structure () | Predict Secondary Structure ()

This opens the dialog shown in figure [24.1](#).

If you have selected sequences before choosing the Toolbox action, they are now listed in the **Selected Elements** window of the dialog. Use the arrows to add or remove sequences or

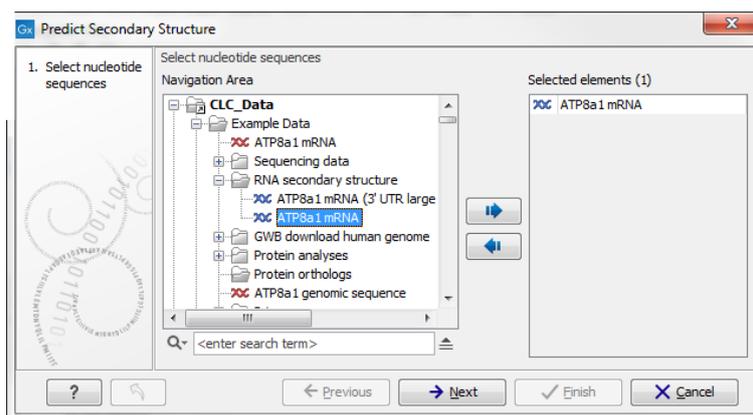


Figure 24.1: Selecting RNA or DNA sequences for structure prediction (DNA is folded as if it was RNA).

sequence lists from the selected elements. You can use both DNA and RNA sequences - DNA will be folded as if it were RNA. Click **Next** to adjust secondary structure prediction parameters. Clicking **Next** opens the dialog shown in figure 24.2.

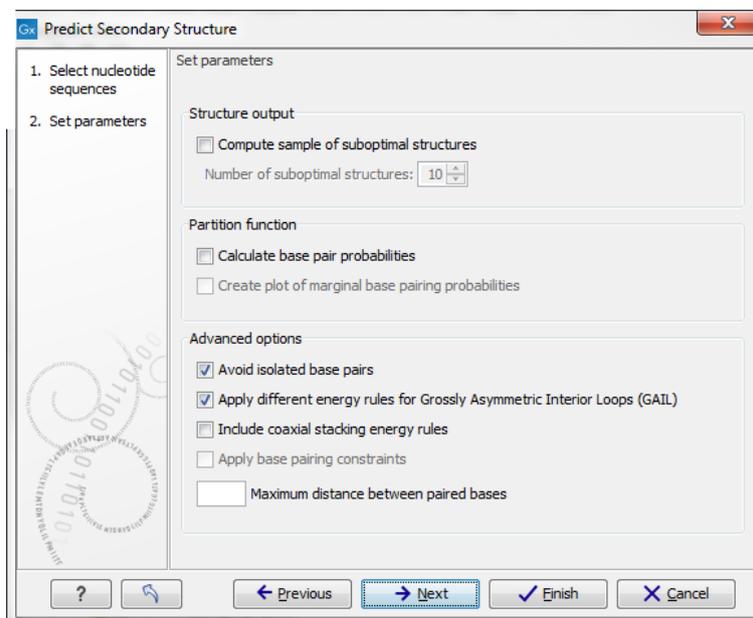


Figure 24.2: Adjusting parameters for secondary structure prediction.

24.1.2 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 labeled **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].

24.1.3 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.

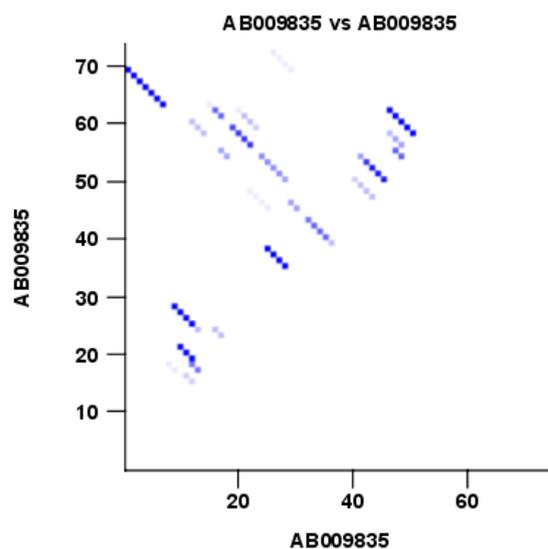


Figure 24.3: *The marginal base pair probability of all possible base pairs.*

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

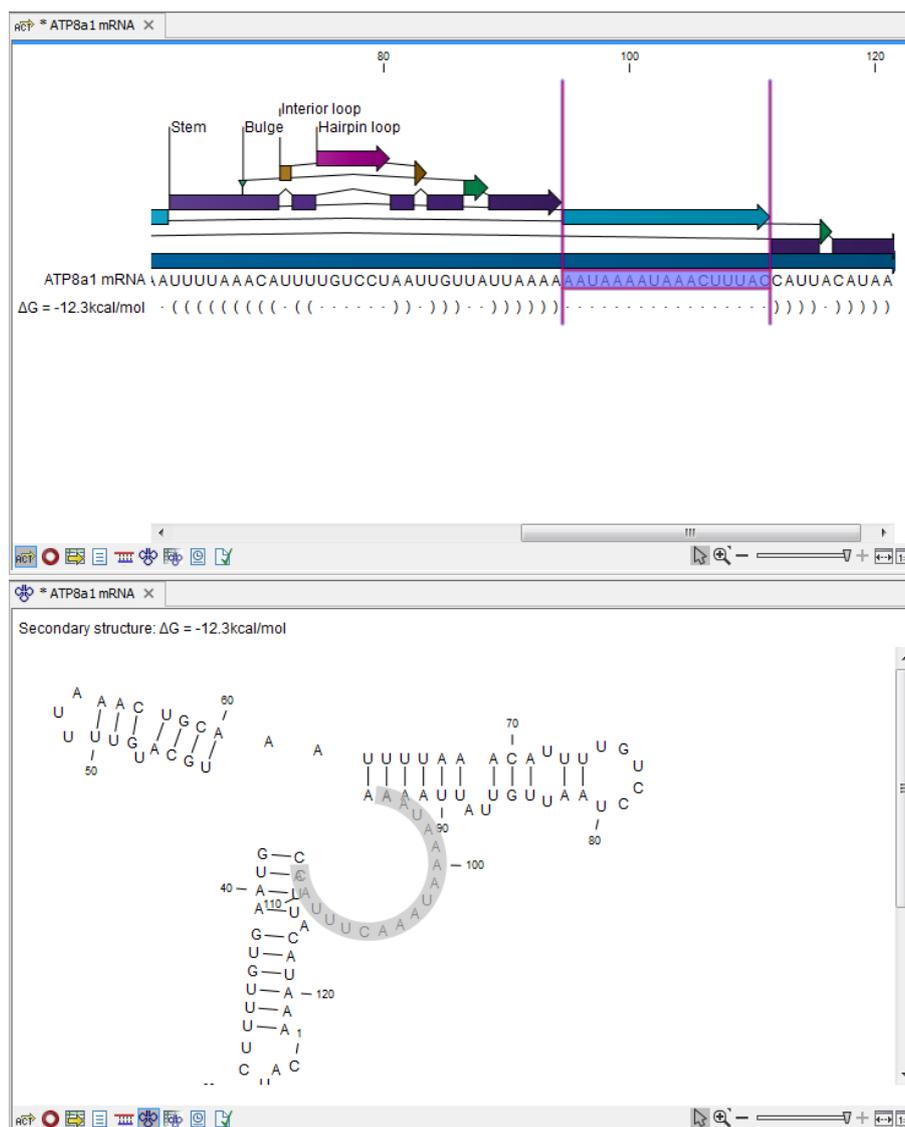


Figure 24.4: Marginal probability of base pairs shown in linear view (top) and marginal probability of being unpaired shown in the secondary structure 2D view (bottom).

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.

24.1.4 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://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].
- **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 24.1.3).

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).

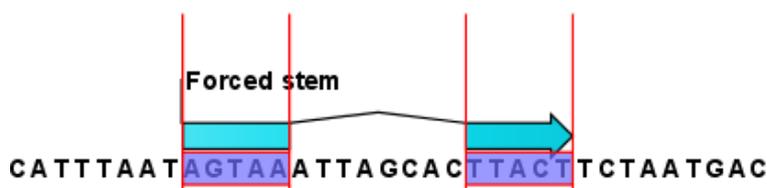


Figure 24.5: Force a stem of the selected bases.

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).



Figure 24.6: Prohibit the selected bases from forming a stem.

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:

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.

24.1.5 Structure as annotation

You can choose to add the elements of the best structure as annotations (see figure 24.8).

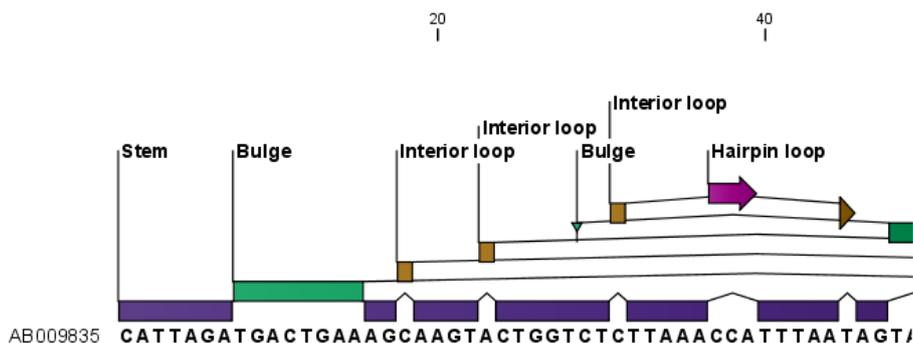


Figure 24.8: Annotations added for each structure element.

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.

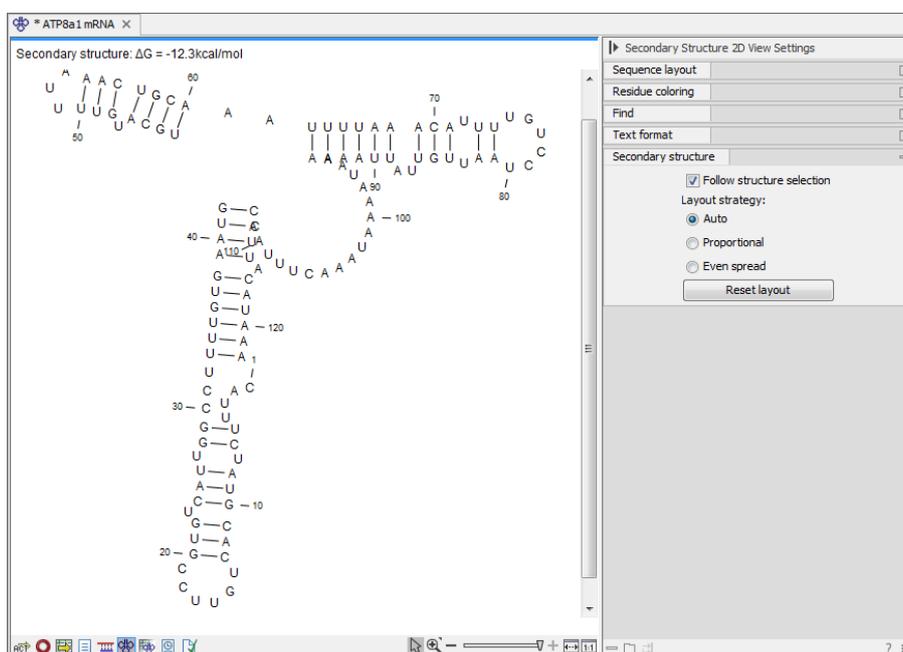


Figure 24.9: The secondary structure view of an RNA sequence zoomed in.

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 12.1.1. 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.

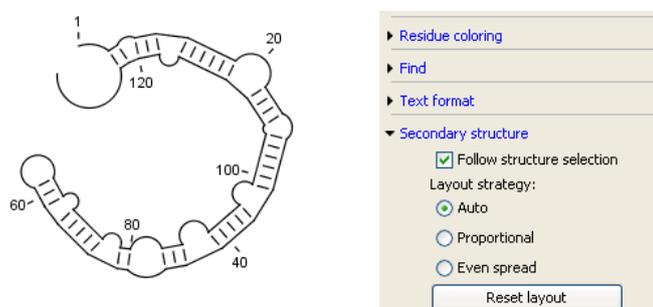


Figure 24.10: *Auto layout. Overlaps are minimized.*

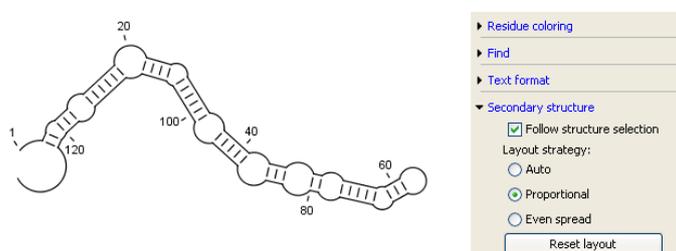


Figure 24.11: *Proportional layout. Length of the arc is proportional to the number of residues in the arc.*

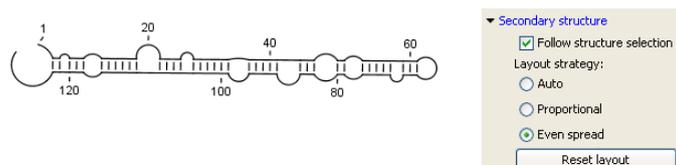


Figure 24.12: *Even spread*. Stems are spread evenly around loops.

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).

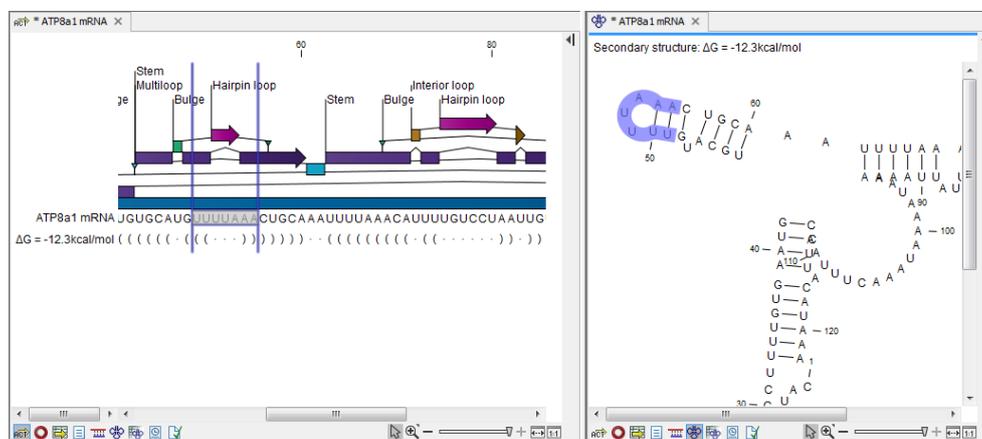


Figure 24.13: A *split view* of the secondary structure view and a linear sequence view.

If you make a selection in another sequence view, this will 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. 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).

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.

Press **Reset layout** in the **Side Panel** to reset the layout to the way it looked when the structure

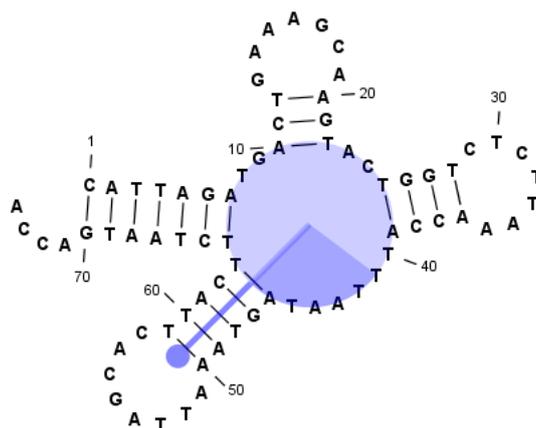


Figure 24.14: The blue circle represents the anchor point for rotating the substructure.

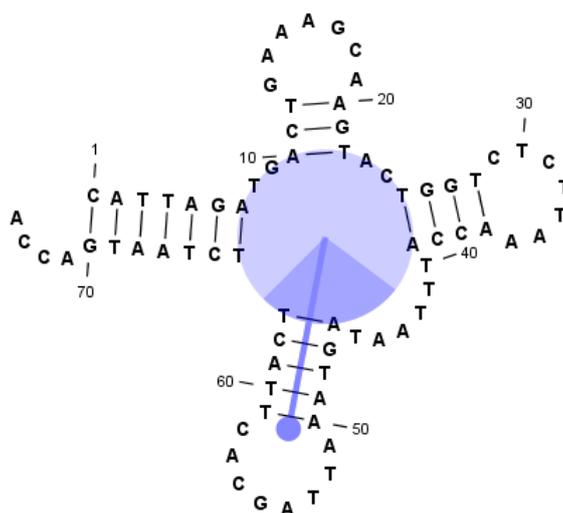


Figure 24.15: The structure has now been rotated.

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.

The screenshot shows a window titled 'ATP8a1 mRNA (...)' with a table of 11 rows. The table has columns for Name, Created, ΔG, and Probability. To the right of the table is a list of substructures for the selected structure, each with a description and its ΔG value.

| 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.

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 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

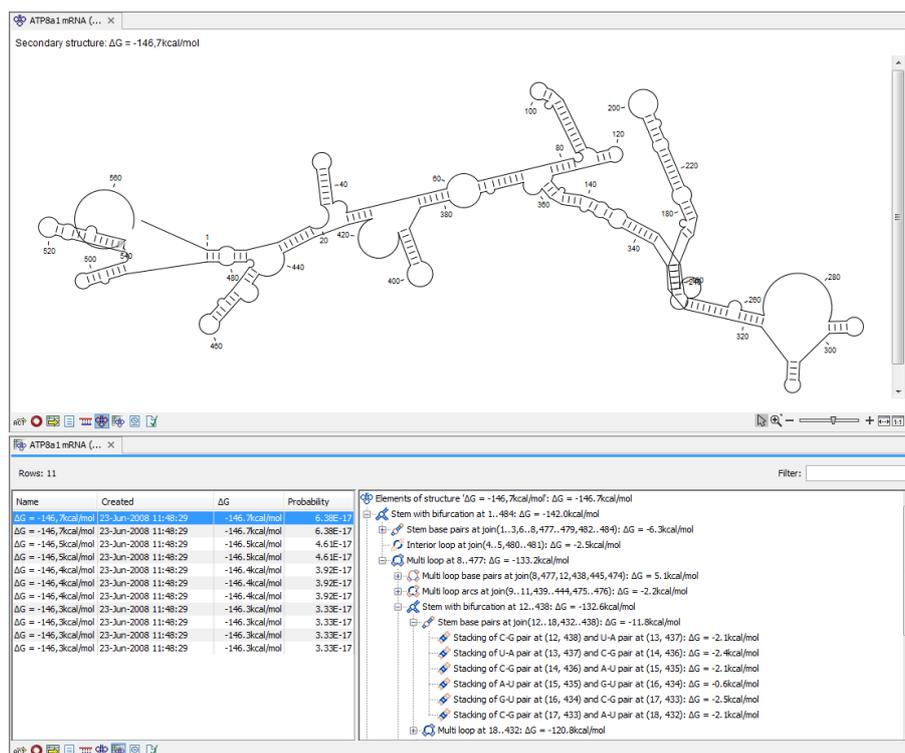


Figure 24.17: A split view showing a structure table to the right and the secondary structure 2D view to the left.

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.

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.

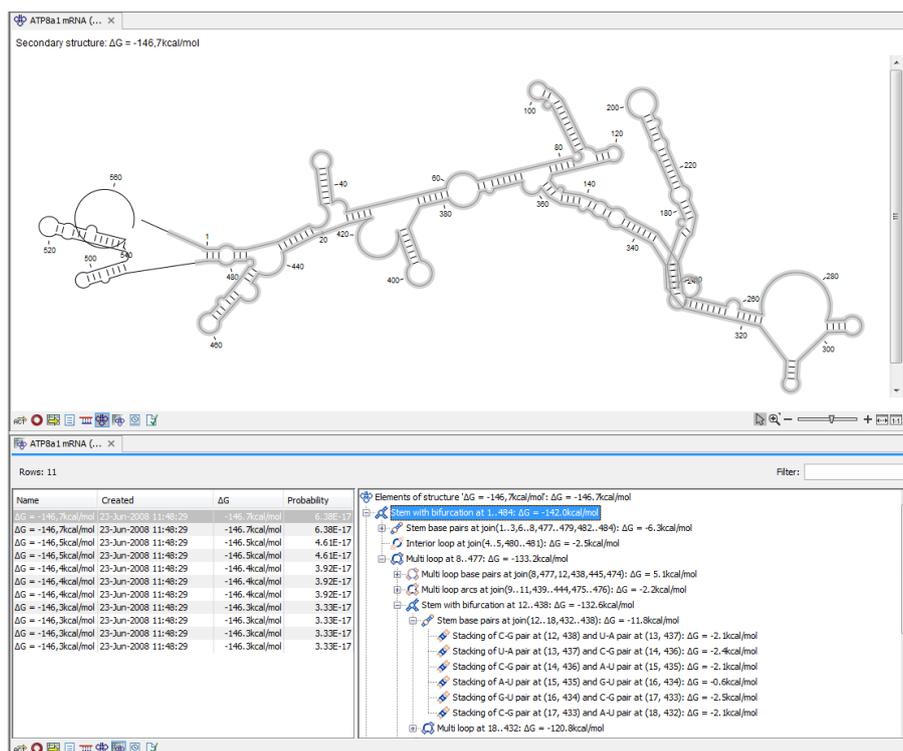


Figure 24.18: Now the "Stem with bifurcation" node has been selected in the table and a corresponding selection has been made in the view of the secondary structure to the left.

- **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.

24.2.3 Symbolic representation in sequence view

In the **Side Panel** of normal sequence views (RCP), 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).

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 .

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%** (☐) 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 24.1.4. 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:

$$P(H) = \frac{\sum_{s_H \in S_H} P(s_H)}{\sum_{s \in S} P(s)} = \frac{PF_H}{PF_{\text{full}}},$$

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.

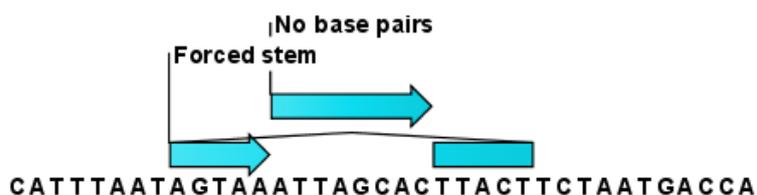


Figure 24.21: Two constraints defining a structural hypothesis.

24.3.1 Selecting sequences for evaluation

The evaluation is started from the **Toolbox**:

Toolbox | RNA Structure (☞) | Evaluate Structure Hypothesis (☞)

This opens the dialog shown in figure 24.22.

If you have selected sequences before choosing the Toolbox action, they are now listed in the **Selected Elements** window of the dialog. Use the arrows to add or remove sequences or

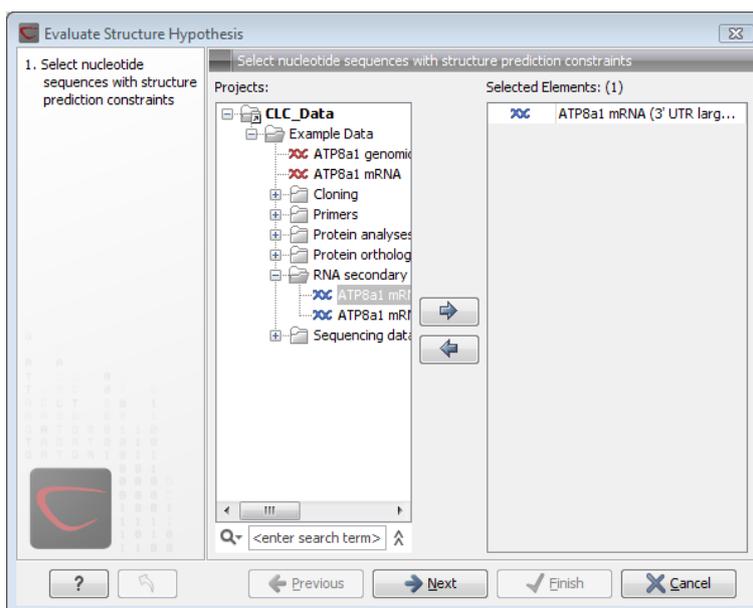


Figure 24.22: Selecting RNA or DNA sequences for evaluating structure hypothesis.

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).
- **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).

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

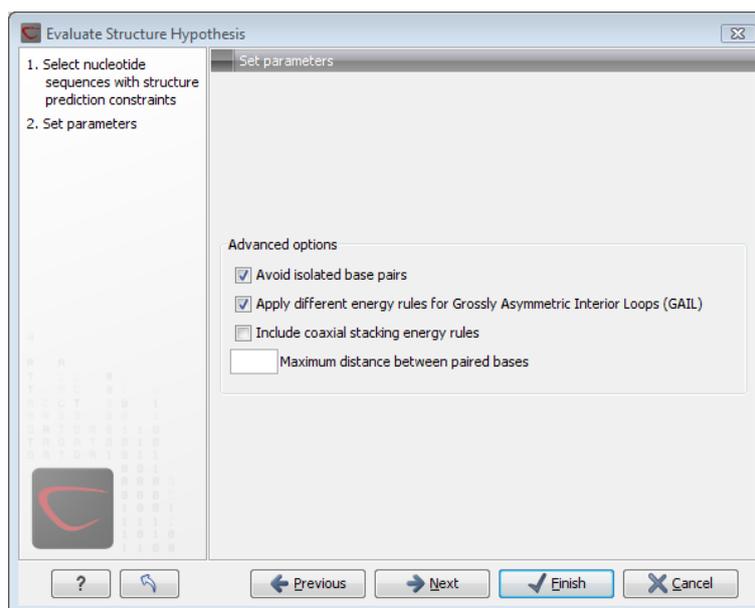


Figure 24.23: Adjusting parameters for hypothesis evaluation.

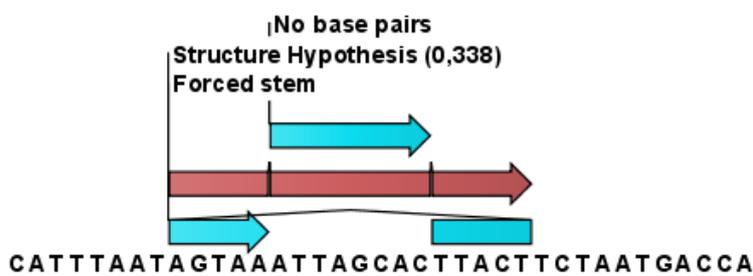


Figure 24.24: This hypothesis has a probability of 0.338 as shown in the annotation.

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

The scanning is started from the **Toolbox**:

Toolbox | RNA Structure (🔧) | Evaluate Structure Hypothesis (📊)

This opens the dialog shown in figure 24.25.

If you have selected sequences before choosing the Toolbox action, they are now listed in the **Selected Elements** window of the dialog. Use the arrows to add or remove sequences or

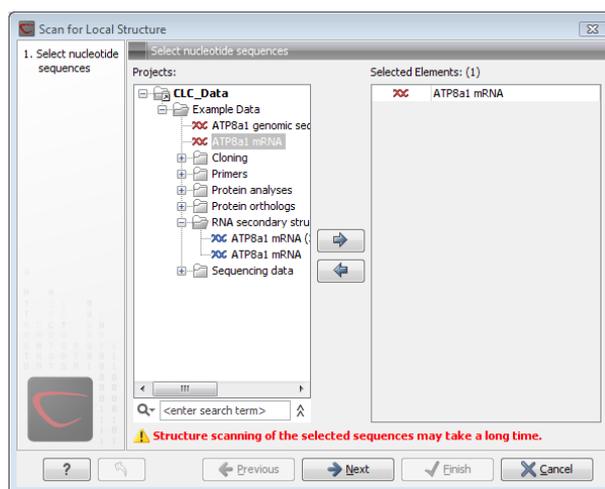


Figure 24.25: Selecting RNA or DNA sequences for structure scanning.

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.
- **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.

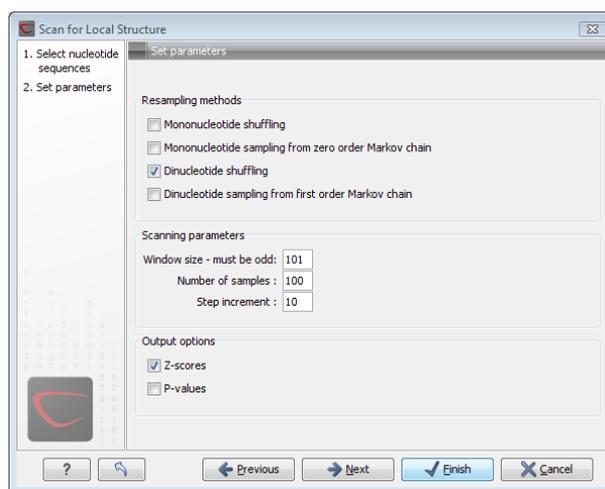


Figure 24.26: Adjusting parameters for structure scanning.

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).

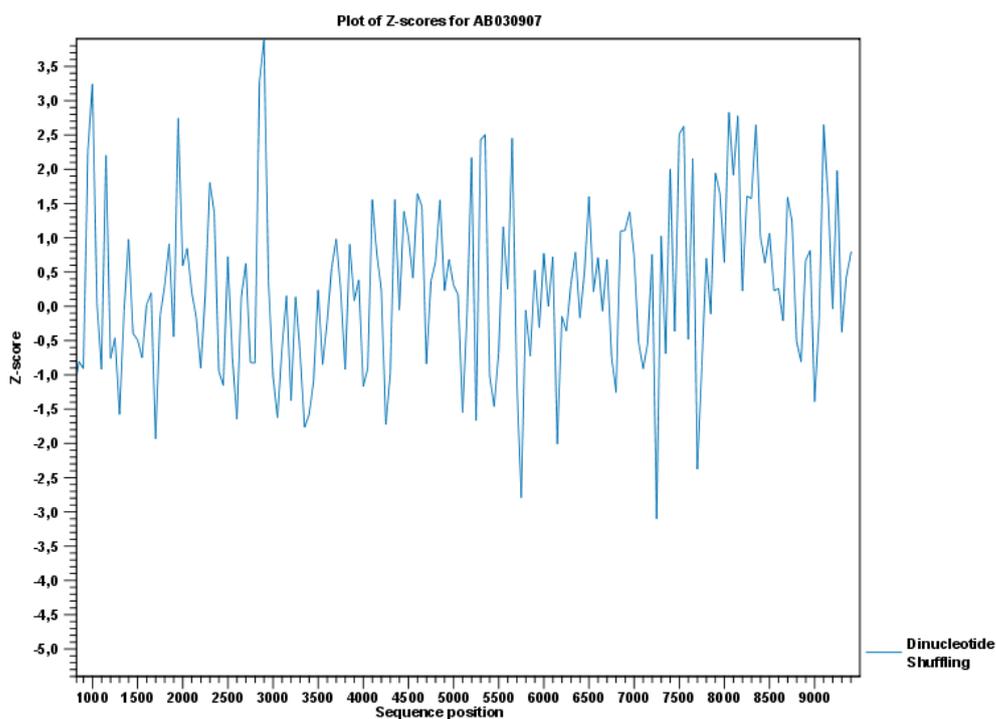


Figure 24.27: A plot of the Z -scores produced by sliding a window along a sequence.

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.

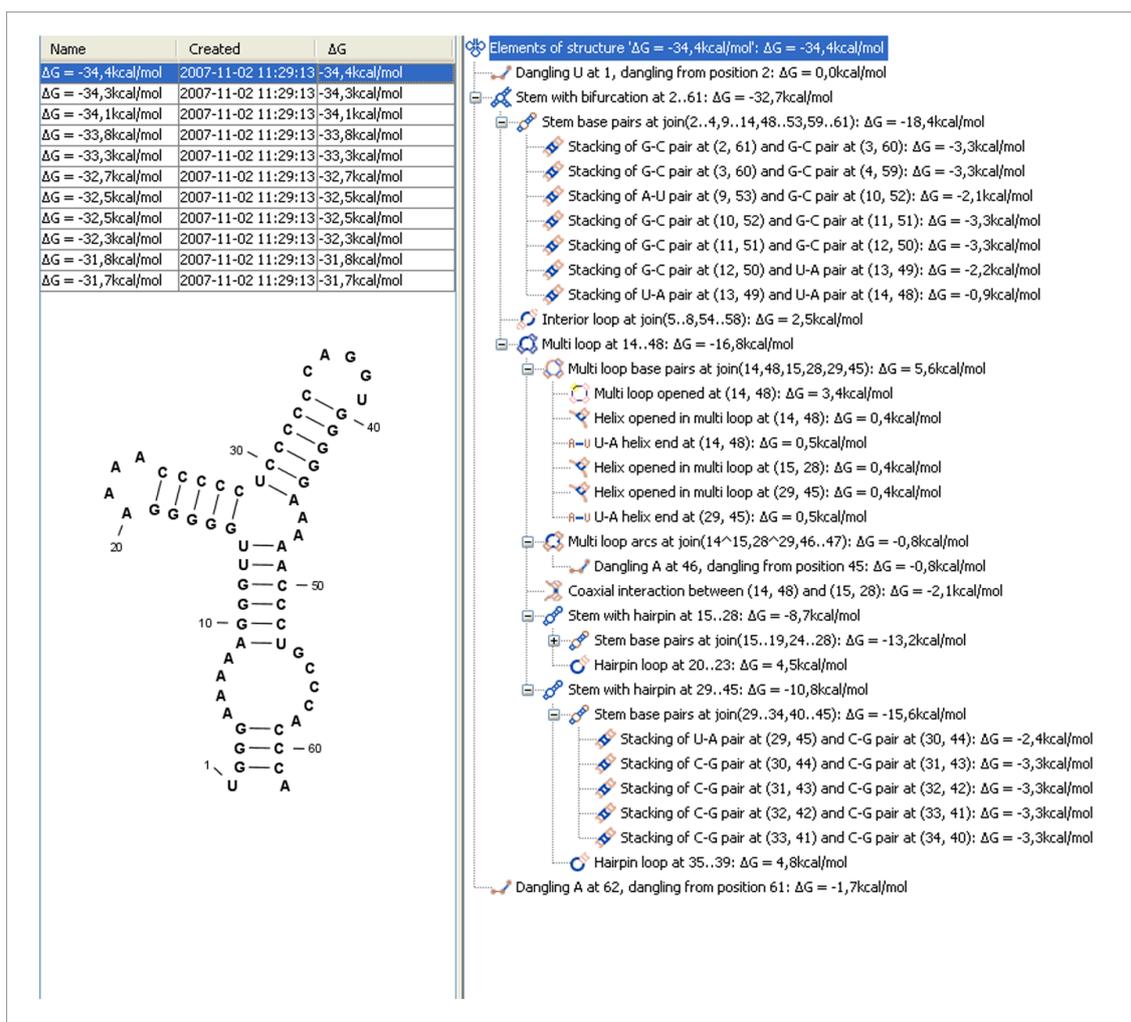


Figure 24.28: A number of suboptimal structures have been predicted using **CLC Main Workbench** and are listed at the top left. At the right hand side, the structural components of the selected structure are listed in a hierarchical structure and on the left hand side the structure is displayed.

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.

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 base pairs, we can define the following distinct structure

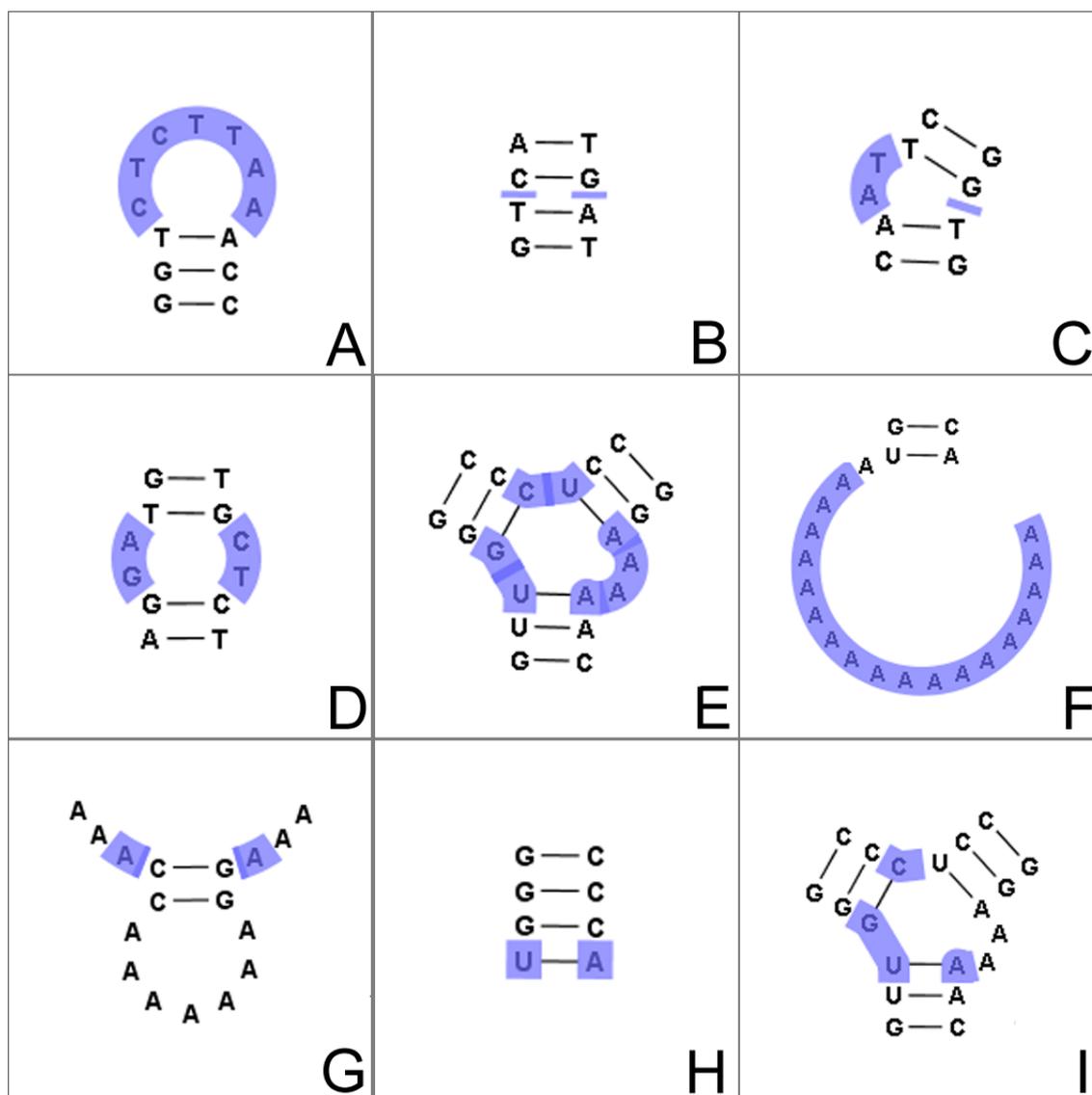


Figure 24.29: The different structure elements of RNA secondary structures predicted with the free energy minimization algorithm in **CLC Main Workbench**. See text for a detailed description.

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** (). 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].

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Figure 24.30: *Known structural features can be added as constraints to the secondary structure prediction algorithm in **CLC Main Workbench**.*

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Chapter 25

Expression analysis

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The *CLC Main Workbench* is able to analyze expression data produced on microarray platforms and high-throughput sequencing platforms (also known as Next-Generation Sequencing platforms). Note that the *CLC Main Workbench* is not able to calculate expression levels based on the raw sequence data. This analysis has to be performed with the *CLC Genomics Workbench*. The result of this analysis can be imported and further analyzed in the *CLC Main Workbench*.

The *CLC Main Workbench* provides 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 used to aid the interpretation of the results.

25.1 Experimental design

In order to make full use of the various tools for interpreting expression data, you need to know the central concepts behind the way the data is organized in the *CLC Main Workbench*.

The first piece of data you are faced with is the **sample**. In the Workbench, a sample contains the expression values from either one array or from sequencing data of one sample. Note that the *CLC Main Workbench* is not able to calculate expression levels based on the raw sequence data. This analysis has to be performed with the *CLC Genomics Workbench*. The result of this analysis can be imported and further analyzed in the *CLC Main Workbench*.

See more below on how to get your expression data into the Workbench as samples in section J.

In a sample, there is a number of **features**, usually genes, and their associated expression levels.

To analyze differential expression, you need to tell the workbench how the samples are related. This is done by setting up an **experiment**. An experiment is essentially a set of samples which are grouped. By creating an experiment defining the relationship between the samples, it becomes possible to do statistical analysis to investigate differential expression between the groups. The **Experiment** is also used to accumulate calculations like t-tests and clustering because this information is closely related to the grouping of the samples.

25.1.1 Setting up an experiment

To set up an experiment:

Toolbox | Transcriptomics Analysis  | **Set Up Experiment** 

Select the samples that you wish to use by double-clicking or selecting and pressing the **Add**  button (see figure 25.1).

Note that we use "samples" as the general term for both microarray-based sets of expression values and sequencing-based sets of expression values (e.g. an expression track from RNA-Seq).

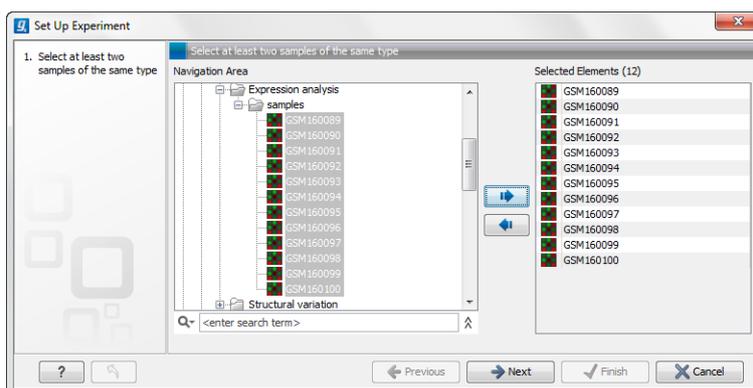


Figure 25.1: Select the samples to use for setting up the experiment.

Clicking **Next** shows the dialog in figure 25.2.

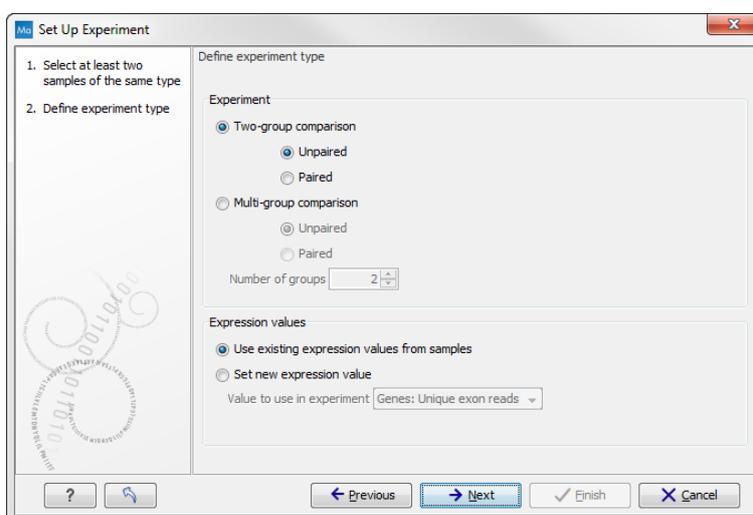


Figure 25.2: Defining the number of groups and expression value type.

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 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.** For RNA-Seq experiments, you can also choose which expression value to be used when setting up the experiment. This value will then be used for all subsequent analyses. 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 – 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]: $RPKM = \frac{\text{total exon reads}}{\text{mapped reads (millions)} \times \text{exon length (KB)}}$. 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. See more in section ??.
- **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 (see the description of the "Maximum number of hits for a read" option, ??). 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.

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.

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.

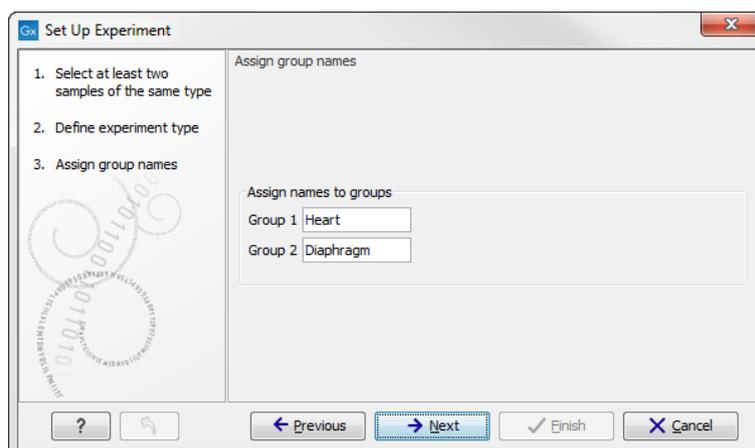


Figure 25.3: Naming the groups.

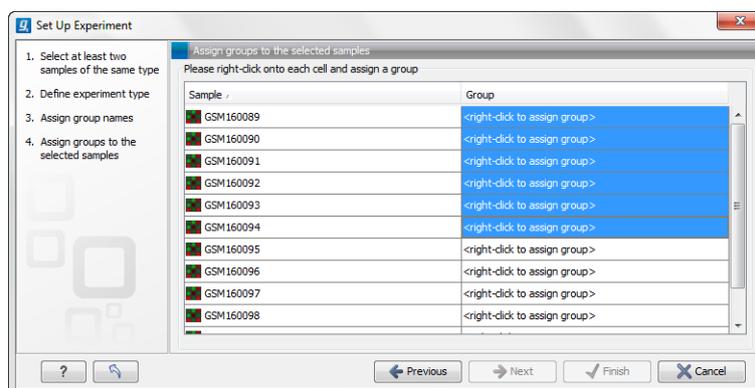


Figure 25.4: Putting the samples into groups.

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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 9.3.

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 5.6).

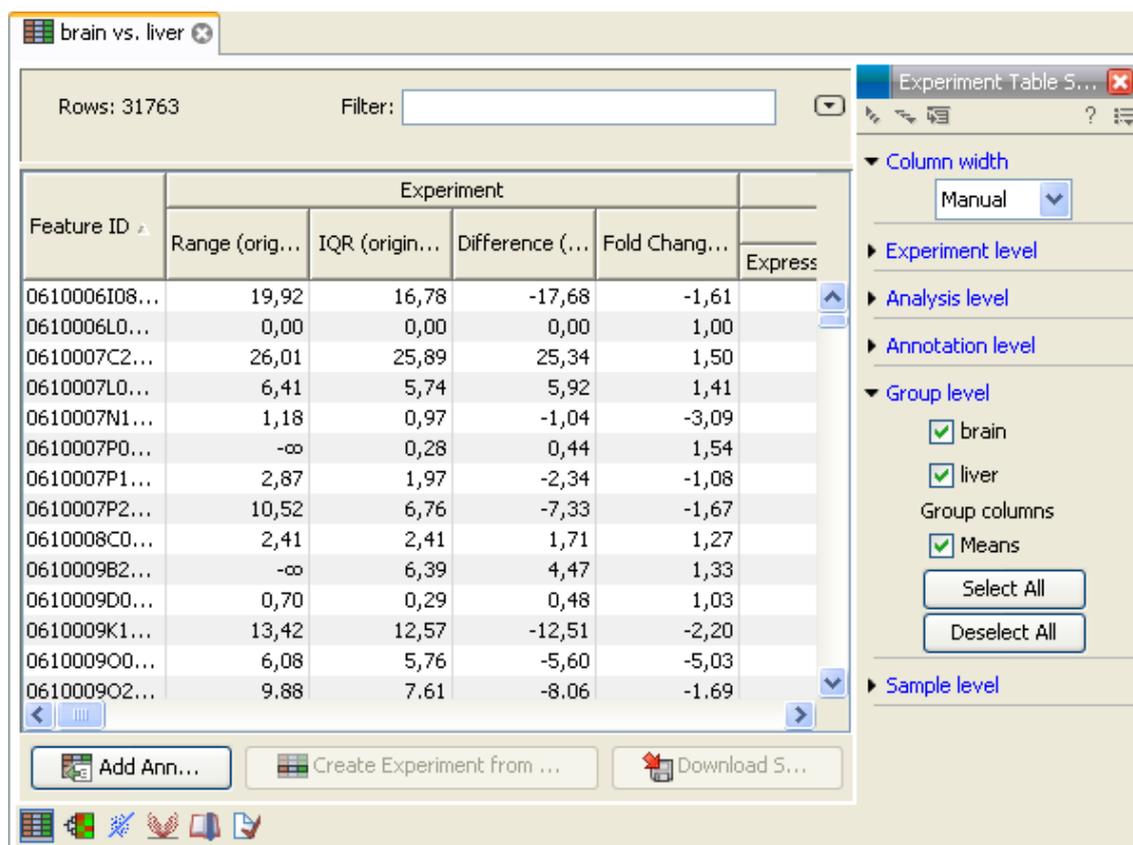


Figure 25.5: Opening the experiment.

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.

For more information on visualizing RNA-Seq read tracks from the experiment, see section ??.

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).

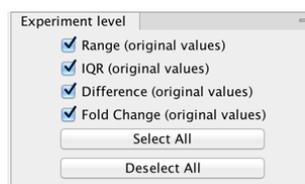


Figure 25.6: The initial view of the experiment level for a two-group experiment.

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 25.3.3 and 25.3.2, 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 25.5.5. 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.

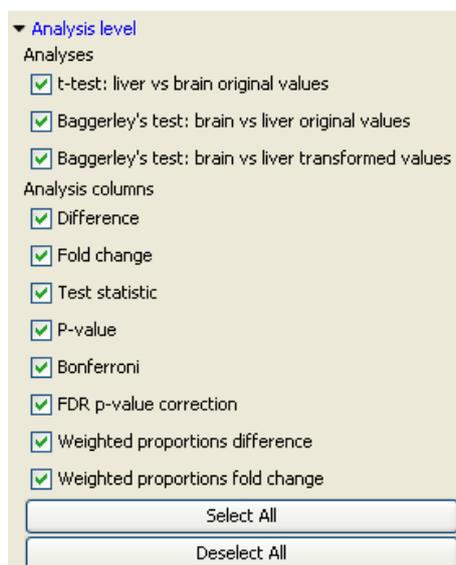


Figure 25.7: An example of columns available under the Analysis level section.

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.

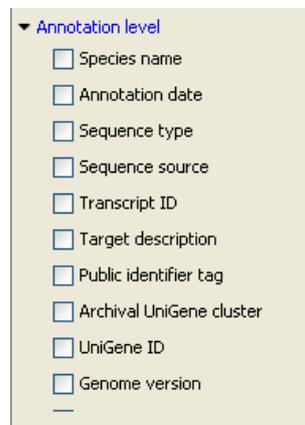


Figure 25.8: An annotated experiment.

In order to avoid too much detail and cluttering the table, only a few of the columns are shown 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 5.6).

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 25.3.3 and 25.3.2, respectively), the means of the normalized and transformed values will also appear.

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 25.3.3 and 25.3.2, respectively), the normalized and transformed values will also appear.

An example is shown in figure 25.9.

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 9.3).

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**

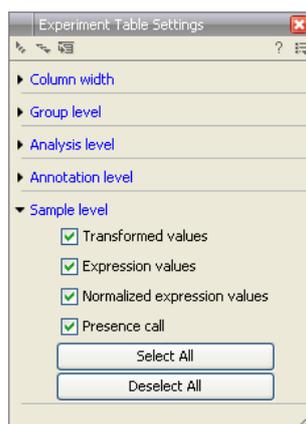


Figure 25.9: Sample level when transformation and normalization has been performed.

Experiment from Selection () button at the bottom of the table (see figure 25.10).

| | | | | | |
|---|------|------|------|------|------|
| 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 |

Buttons: Add Annotations, Create Experiment from Selection, Download Sequence

Figure 25.10: 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 25.11).

| | | | | | |
|---|------|------|------|------|------|
| 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 |

Buttons: Add Annotations, Create Experiment from Selection, Download Sequence

Figure 25.11: Select sequences and press the download button.

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 12. You can now use the downloaded sequences for further analysis in the Workbench, e.g. performing BLAST searches and designing primers for QPCR experiments.

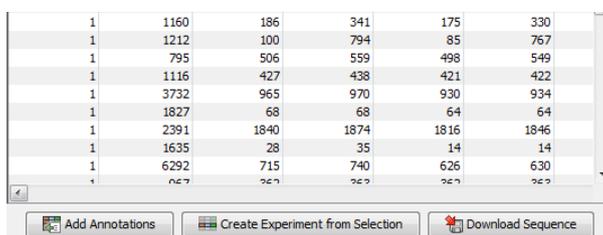
25.1.3 Adding annotations to an experiment

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 J 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 J. 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:

Toolbox | Transcriptomics 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 25.12).

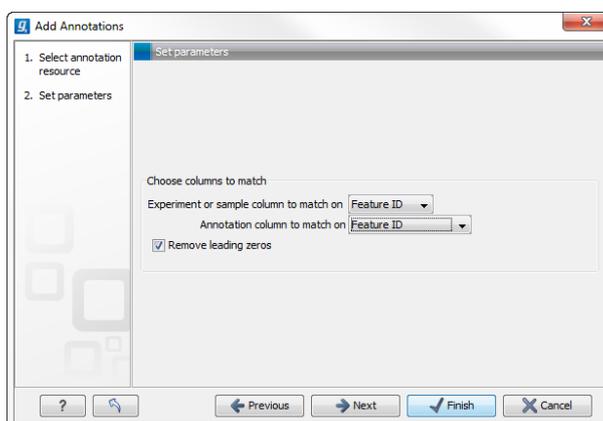


| | | | | | |
|---|------|------|------|------|------|
| 1 | 1160 | 186 | 341 | 175 | 330 |
| 1 | 1212 | 100 | 794 | 85 | 767 |
| 1 | 795 | 506 | 559 | 498 | 549 |
| 1 | 1116 | 427 | 438 | 421 | 422 |
| 1 | 3732 | 965 | 970 | 930 | 934 |
| 1 | 1827 | 68 | 68 | 64 | 64 |
| 1 | 2391 | 1840 | 1874 | 1816 | 1846 |
| 1 | 1635 | 28 | 35 | 14 | 14 |
| 1 | 6292 | 715 | 740 | 626 | 630 |
| 1 | 647 | 667 | 669 | 667 | 667 |

Buttons: Add Annotations, Create Experiment from Selection, Download Sequence

Figure 25.12: 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 25.13).



Add Annotations

1. Select annotation resource
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

Buttons: Previous, Next, Finish, Cancel

Figure 25.13: 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 25.14).



Figure 25.14: 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 25.15.

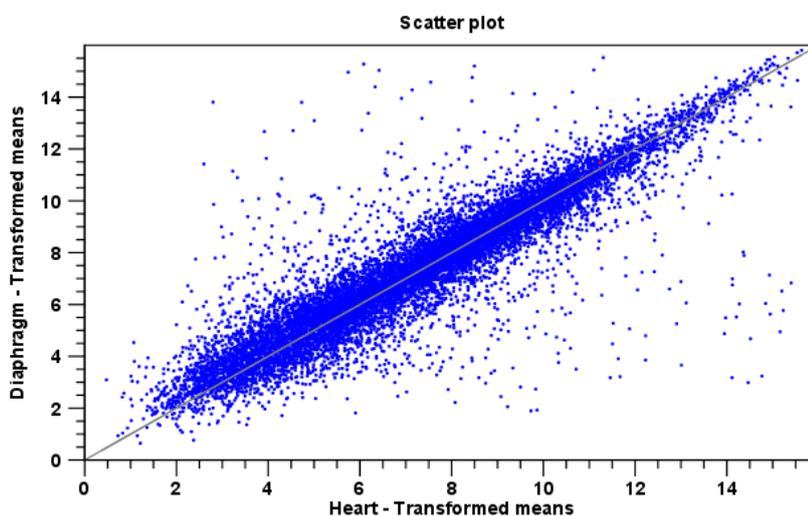


Figure 25.15: A scatter plot of group means for two groups (transformed expression values).

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.
 - Outside
 - Inside

- **Tick lines at.** Choosing Major ticks will show a grid behind the graph.
 - None
 - Major ticks
- **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
 - Wide
- **Line type**
 - None
 - Line
 - Long dash
 - Short dash
- **Line color.** Allows you to choose between many different colors. 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**
 - None
 - Cross
 - Plus
 - Square
 - Diamond
 - Circle
 - Triangle
 - Reverse triangle

– Dot

- **Dot color.** Allows you to choose between many different colors. 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 5.6).

25.1.5 Cross-view selections

There are a number of different ways of looking at an experiment as shown in figure 25.16).



Figure 25.16: 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 25.16, 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 25.17.

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 Working with tracks and experiments

The *CLC Main Workbench* provides several tools for the analysis, organization, and visualization of expression data. In this section, we describe how Tracks and Experiments complement each other, and how they can be used together for the analysis of transcriptomics data using the tools found in the **Transcriptomics** toolbox.

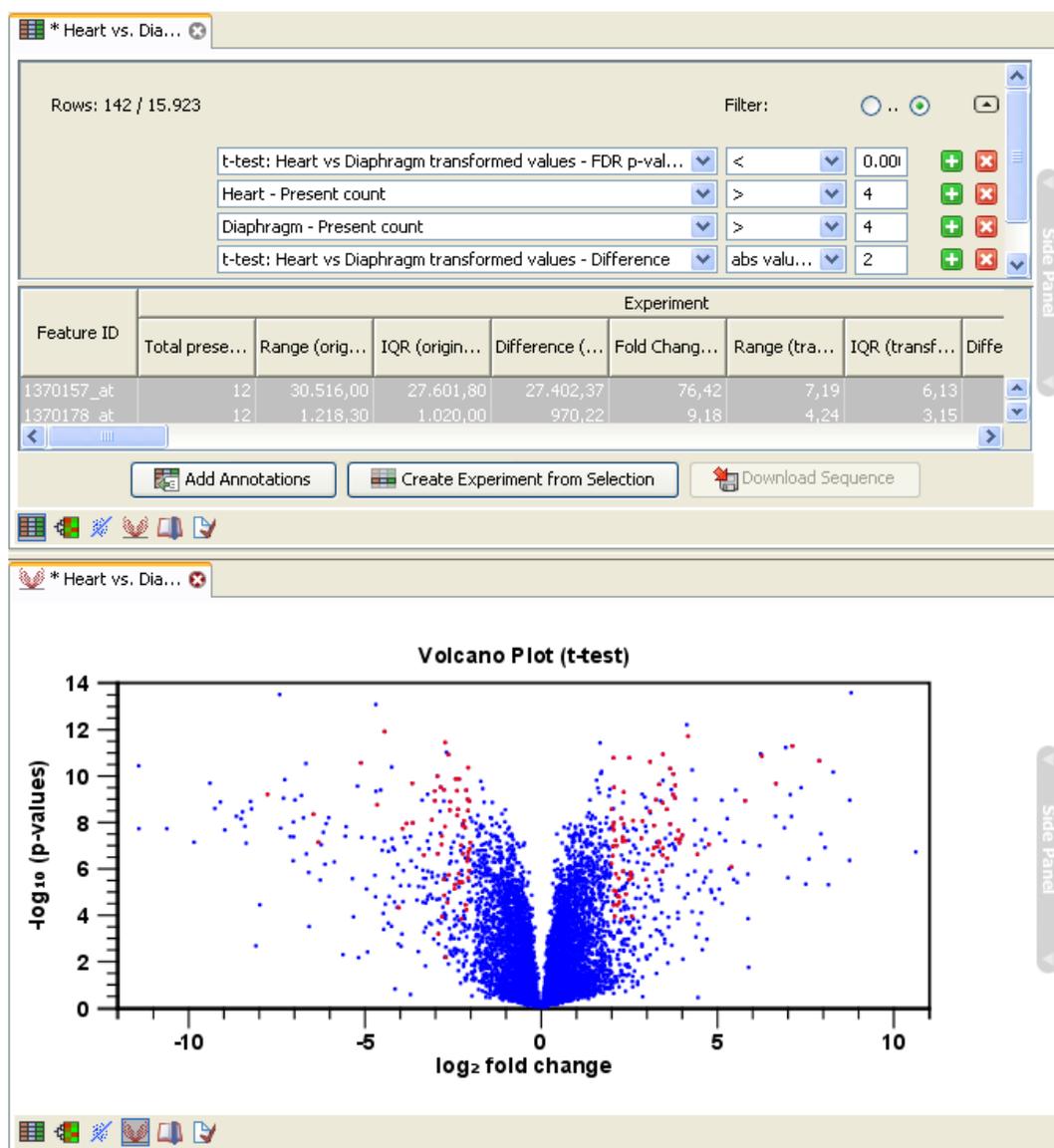


Figure 25.17: A split view showing an experiment table at the top and a volcano plot at the bottom (note that you need to perform statistical analysis to show a volcano plot, see section 25.5).

25.2.1 Data structures for transcriptomics

The two main data structures used for transcriptomics data analysis in the *CLC Main Workbench* are tracks and experiments.

The track format can be used to visualize and analyze data in the *CLC Main Workbench*. All information is tied to genomic positions, and a central coordinate-system is provided by a reference genome. This allows different types of data or results for different samples to be seen and analyzed together.

Experiments (see section 25.1), on the other hand, are used to represent complex relationships between expression samples, and to carry out statistical analysis (see section 25.5) of differential expression.

Tracks and experiments are intimately related, and it is possible in most cases to convert from

one type to the other.

From Tracks to Experiments

The starting point is a set of reads from a sequencing study that can be analyzed with the RNA-Seq Analysis tool in the CLC Genomics Workbench or the Biomedical Genomics Workbench and imported to the *CLC Main Workbench*. As part of the RNA-Seq Analysis tool, these reads are mapped onto a reference genome. The RNA-Seq tool produces expression tracks, which are compatible with the reference genome, and can be visualized together with the genome in the **Track List View**

Once expression tracks have been obtained from the RNA-Seq Analysis tool, they can be used as sequencing-based sets of expression values in setting up an experiment. This can be done using the **Set up Experiment** tool, also found in the **Transcriptomics** toolbox. This is described in more detail in section 25.1.

An experiment set up in this manner from expression tracks is intimately coupled to the tracks it originated from. To see this coupling in action, perform the following steps:

1. Use the **Set up Experiment** tool on two or more expression tracks to set up an experiment, as described in section 25.1.
2. Save and open the resulting experiment, by double-clicking its name in the **Navigation Area**.
3. Use the **Create Track List** tool to create a track list from the expression tracks you used to set up the experiment.
4. Save and open the resulting track list by double-clicking its name in the **Navigation Area**.
5. Drag the experiment tab downwards, until you see the blue shadow indicating the resulting placement (figure 25.18), and drop it in place. You should now have a divided view, with the experiment in the bottom half (figure 25.19).
6. Clicking on any line in the experiment will now automatically jump to the corresponding genomic location in the upper view. Use the **Zoom to Selection**  button to zoom in to the desired genomic region.

25.2.2 From Experiments to Tracks

Experiments can be used to carry out statistical analysis on the expression values obtained from RNA-seq analysis as described in section 25.5. The results of the statistical analysis are annotated on the experiment as additional columns.

It can be advantageous to visualize the results of the statistical analysis as tracks. The **Create Track from Experiment** tool in the *CLC Main Workbench* enables the conversion of experiments to tracks.

25.2.3 Running the Create Track from Experiment tool

You can find the **Create Track from Experiment** tool here:

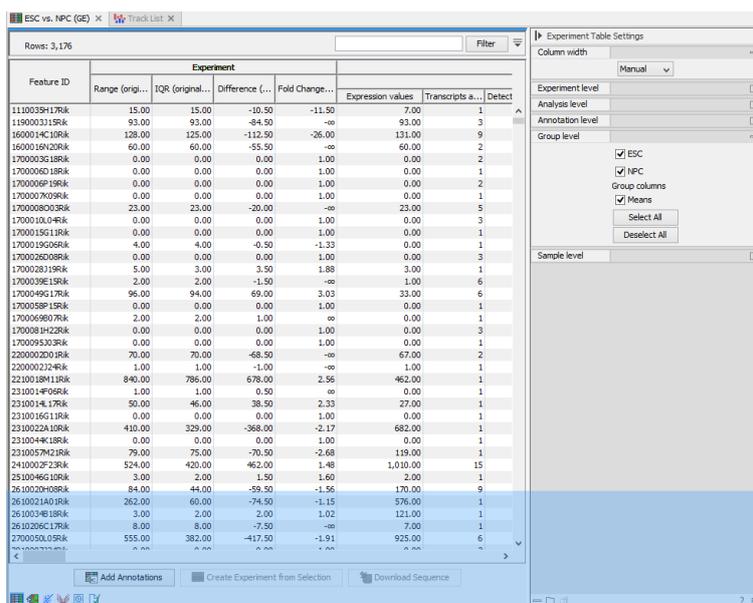


Figure 25.18: Dragging a tab to the lower half of the view area.

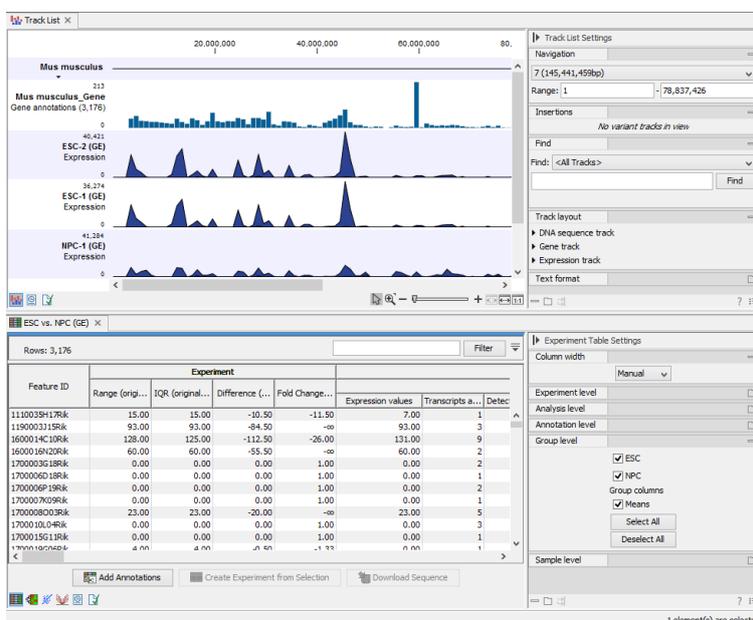


Figure 25.19: After dropping a tab to the lower half of the view area.

Toolbox | Transcriptomics Analysis | Create Track from Experiment

After you start the tool, you are presented with a wizard where you can choose the experiment that you would like to create a track of. The **Create Track from Experiment** tool can be run on two types of experiments:

1. Experiments with associated genomic information, such as those created using expression tracks from the **RNA-Seq Analysis** tool.
2. Experiments without associated genomic information, such as those created using samples from the legacy RNA-Seq Analysis tool.

In the case where the experiment has associated genomic information, the **Create Track from Experiment** tool will automatically infer these and the wizard will jump directly to the filtering step, as shown in figure 25.21.

In the case where the experiment does not have associated genomic information, you will first need to specify how the genomic information should be obtained in the parameters step of the **Create Track from Experiment** tool (figure 25.20).

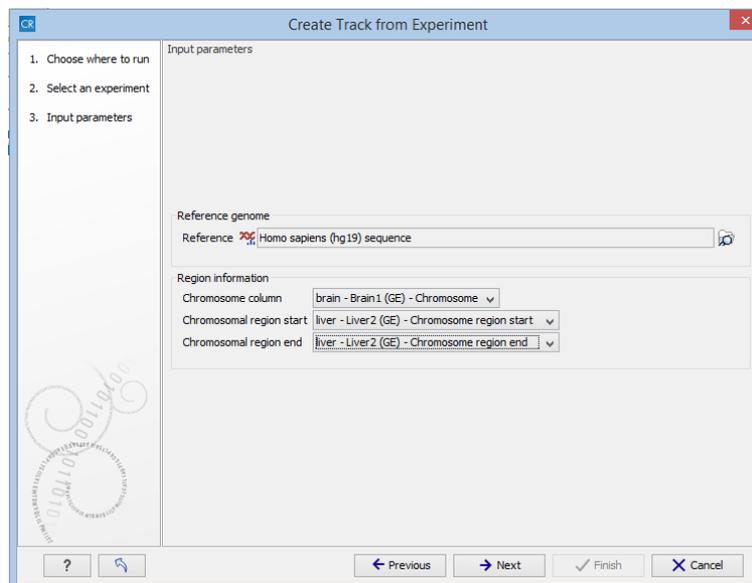


Figure 25.20: The "Input parameters" step in the Create Track from Experiment tool.

In the Input parameters step, you must specify the following parameters:

- **Reference genome.** The chosen genome will be used as the reference genome for the resulting track.
- **Chromosome column.** The column containing the chromosome names must be chosen from the drop-down menu.
- **Chromosomal region start.** The column containing the start of the genomic regions must be chosen from the drop-down menu.
- **Chromosomal region end.** The column containing the end of the genomic regions must be chosen from the drop-down menu.

Note! The drop-down menus will only contain the columns that potentially represent the information required by the given parameter. If the experiment does not contain any columns that potentially represent the required genomic information, the drop-down menus may appear empty. In this case, it is not possible to convert the given experiment to a track.

In the filtering step (figure 25.21), you have the following options:

- **Filter based on statistical analysis results** This allows to filter which annotations are transferred to the track on the basis of the statistical analysis. To enable filtering, check the **Filter based on statistical analysis results** checkbox. The filtering option is only available if a statistical analysis has previously been carried out on the experiment, and

the drop-down menu will only contain the statistical analyses that are present on the experiment.

- **Statistical analysis** Allows you to choose statistical analysis from the drop-down list. The selection of available statistical analyses depends on which tests have been used when you set up the experiment that you are about to convert to track format.
- **Type of p-value** This drop-down menu allows you to select between raw and corrected p-values (see section 25.5.4). Only the types of p-values available for the given statistical analysis will be present in the drop-down menu.
- **Maximum p-value** In this input field, you can enter the maximum allowed p-value, as a number between 0 and 1. If you do not want any filtering based on p-value, enter 1.
- **Minimum fold-change value** You can also specify the minimum allowed fold-change value as a number greater than zero. If you do not want any filtering based on fold-change, enter 0.

You can then select in the drop-down menu which analysis you want to use for filtering.

The fold change values are stored as different columns in the experiment, depending on the statistical analysis performed. The Create Track from Experiment tool will automatically use the fold-change column appropriate for the different statistical analyses:

- Kal's Z-test (see section 25.5.2): Proportions fold change.
- Baggerley's test(see section 25.5.2): Weighted proportions fold change.
- T-test (see section 25.5.3): Fold change.
- ANOVA (see section 25.5.3): Max fold change.
- Empirical analysis of DGE (see section 25.5.1): Fold change.

The resulting track will contain only differentially expressed genes whose p-value is lower than the specified threshold and whose fold-enrichment is above the specified threshold.

If the chosen statistical analysis was performed on several pairs of groups, there will be an output track for each tested pair of groups. For example, if the same statistical analysis has been carried out on 'group 1 vs. group 2' and 'group 1 vs. group 3', then the output will contain two tracks, where one is filtered according to the 'group 1 vs. group 2' analysis results and the other one is filtered according to the 'group 1 vs. group 3' analysis results.

When running the **Create Track from Experiment** tool as part of a workflow, there are a few differences in how the parameters are set (see figure 25.23).

- The **Source of genomic information** parameter determines the behavior of the algorithm if the incoming experiment is *not* coupled to a genome. If the value of this parameter is set to **Require genomic information in experiment**, then the algorithm will expect the incoming experiment to be coupled to a genome, and will fail with an error alerting the user in case the experiment does not fulfill this criterion. If the value of the parameter is set to **Automatic: use genomic information if available**, then the algorithm will still use

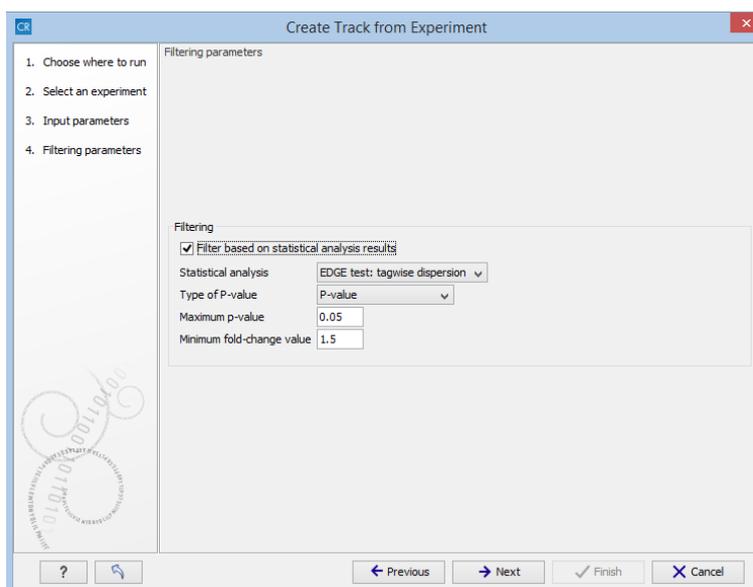


Figure 25.21: The filtering step in the Create Track from Experiment tool.

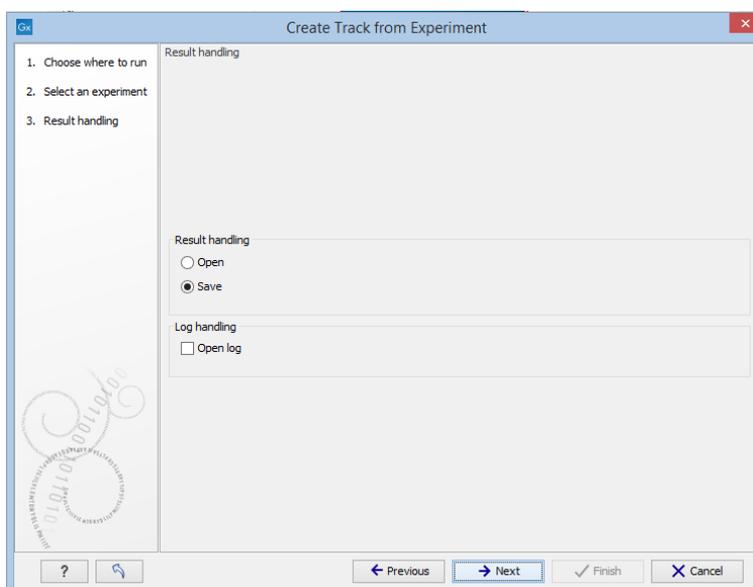


Figure 25.22: The result handling step in the Create Track from Experiment tool.

the genomic information in a genome-coupled experiment. But if this information is not available, the algorithm will attempt to use the information specified by the user in the workflow parameters. *Note:* If the incoming experiment is coupled to a genome (as will usually be the case), the value of this parameter makes no difference.

- In a workflow setting, the column titles for the chromosome, region end and region start fields can be specified as texts. These fields may be left empty, if the incoming experiment contains the genomic information. If filling out these fields, note that the format for this text is very strict, and must exactly match the text appearing in the drop-down menu when running the tool from the toolbox. For example, if 'Chromosome' is a sample-specific column, for a sample called 'Liver (GE)' in the 'liver' group in the experiment, then the column name text will be: 'liver - Liver (GE) - Chromosome'.

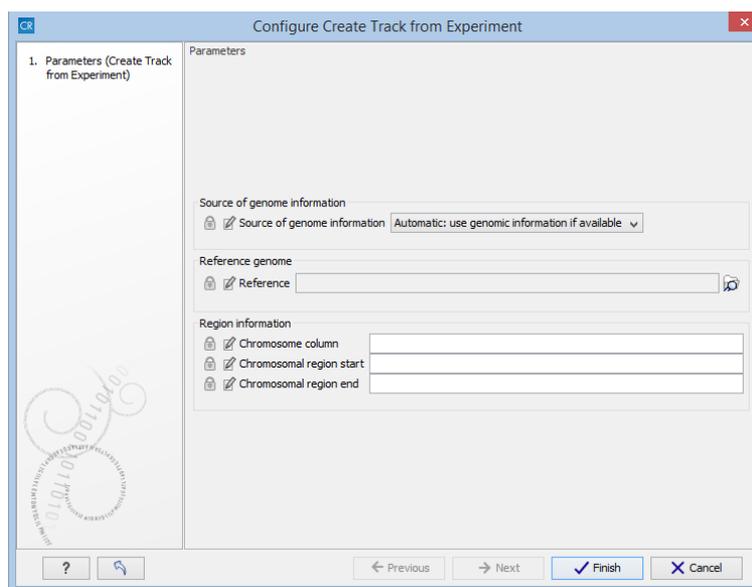


Figure 25.23: Setting the parameters for the Create Track from Experiment tool in a workflow

25.2.4 Interpreting the results of the Create Track from Experiment tool

The **Create Track from Experiment** tool will produce a track or several tracks, if filtering based on analysis results was chosen. The track(s) will contain the following annotations:

- All experiment-specific columns from the experiment
- All user-defined annotations added to the experiment
- All analysis-specific columns from the experiment
- All group-specific columns from the experiment
- Those of the following sample-specific columns when present in the experiment (for each sample): Expression values, Total exon reads, and RPKM.

Two different view options exist: the Track List and the Table View. When opening the annotated output result, the default view is the Track List. It is possible to open both views in split view by holding down the Ctrl key while clicking on the table icon in the lower left corner of the View Area. The two different views are linked together. This means that when you click once on an entry in the table, the Track List will jump the selected region. With the **Zoom to Selection** () button it is possible to jump to and zoom in on the selected region (figure 25.24).

The results of any statistical test executed on the experiment, including fold-changes and p-values, can be seen in the tooltip when hovering over each region in the annotation track shown in the Track List (figure 25.25).

25.3 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

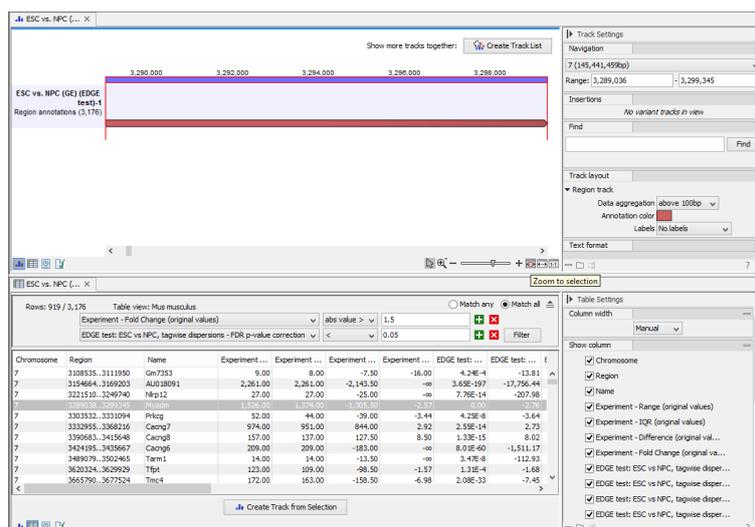


Figure 25.24: Viewing the track produced by the Create Track from Experiment Tool

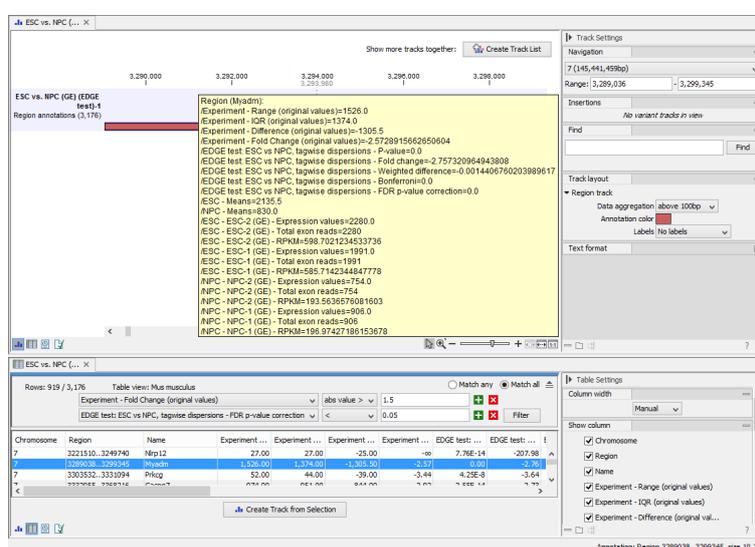


Figure 25.25: The annotations on the track produced by the Create Track from Experiment Tool

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).

25.3.1 Selecting transformed and normalized values for analysis

A number of the tools in the **Expression Analysis** (📁) folder use expression levels. All of these tools let you choose between *Original*, *Transformed* and *Normalized* expression values as shown in figure 25.26.



Figure 25.26: 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.

25.3.2 Transformation

The *CLC Main Workbench* lets you transform expression values based on logarithm and adding a constant:

Toolbox | Transcriptomics 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 25.27.

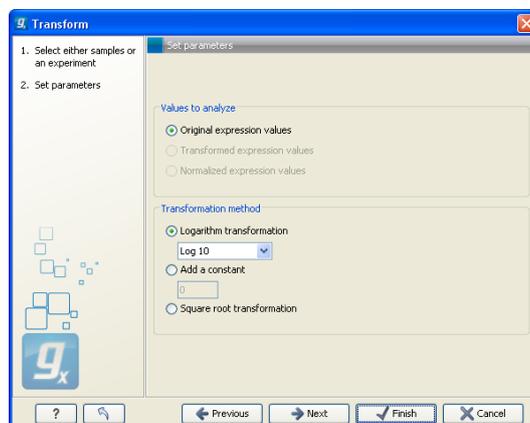


Figure 25.27: Transforming expression values.

At the top, you can select which values to transform (see section 25.3.1).

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

25.3.3 Normalization

The *CLC Main Workbench* lets you normalize expression values.

To start the normalization:

Toolbox | Transcriptomics 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 25.28.

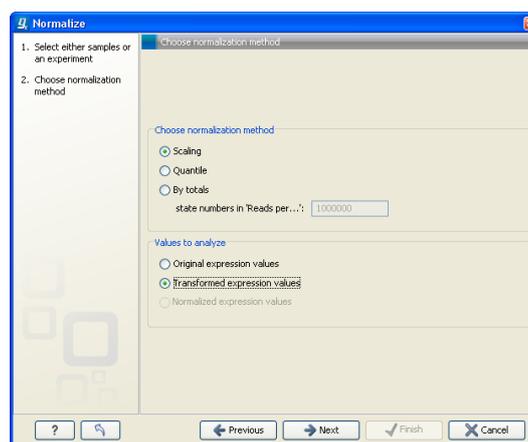


Figure 25.28: Choosing normalization method.

At the top, you can choose three kinds of normalization (for mathematical descriptions see [Bolstad et al., 2003]):

- **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 RNA-seq, 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 25.29 and 25.30 show the effect on the distribution of expression values when using scaling or quantile normalization, respectively.

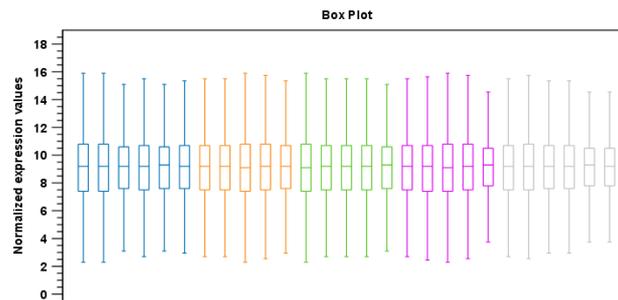


Figure 25.29: Box plot after scaling normalization.

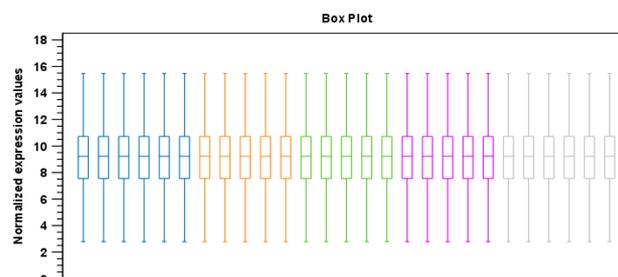


Figure 25.30: Box plot after quantile normalization.

At the bottom of the dialog in figure 25.28, you can select which values to normalize (see section 25.3.1).

Clicking **Next** will display a dialog as shown in figure 25.31.

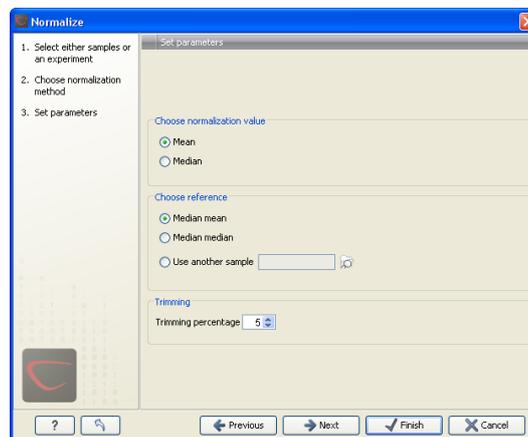


Figure 25.31: Normalization settings.

The following parameters can be set:

- **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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

25.4 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.

25.4.1 Creating box plots - analyzing distributions

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 its 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:

Toolbox | Transcriptomics 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 25.32.

Here you select which values to use in the box plot (see section 25.3.1).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Viewing box plots

An example of a box plot of a two-group experiment with 12 samples is shown in figure 25.33.

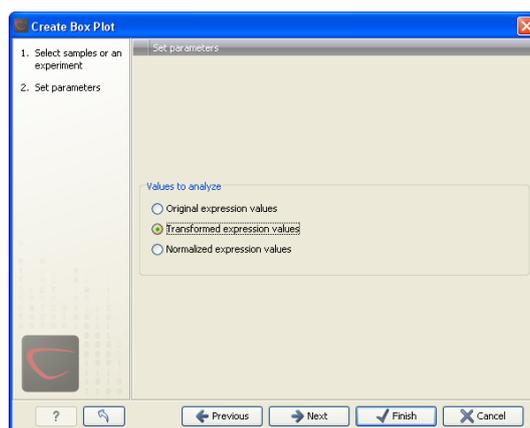


Figure 25.32: Choosing values to analyze for the box plot.

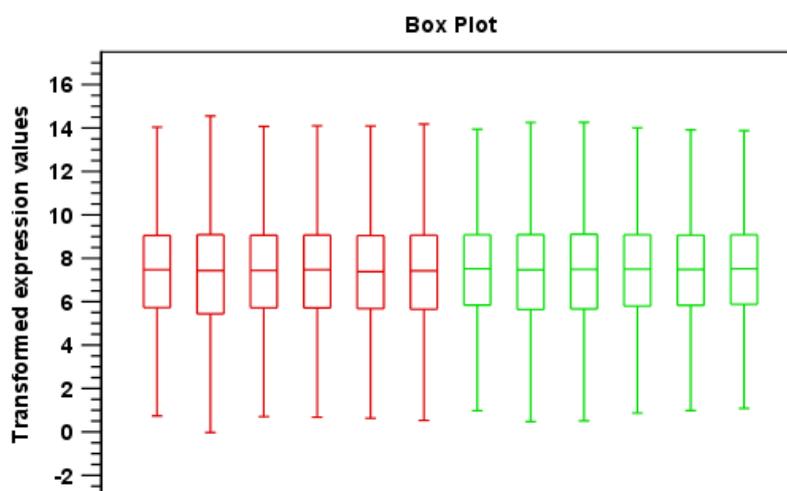


Figure 25.33: A box plot of 12 samples in a two-group experiment, colored by group.

Note that the boxes per default 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 (note that sample names are not shown in figure 25.33).

Per default the box includes the IQR values (from the lower to the upper quartile), the median is displayed as a line in the box, and the whiskers extend 1.5 times the height of the box.

In the **Side Panel** to the left, there is a number of options to adjust this view. Under **Graph preferences**, you can adjust the general properties of the box plot (see figure 25.34).

- **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.
 - Outside

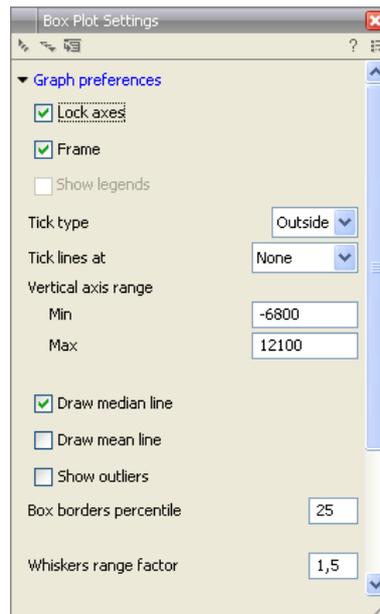


Figure 25.34: Graph preferences for a box plot.

- Inside
- **Tick lines at.** Choosing Major ticks will show a grid behind the graph.
 - None
 - Major ticks
- **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 25.35).

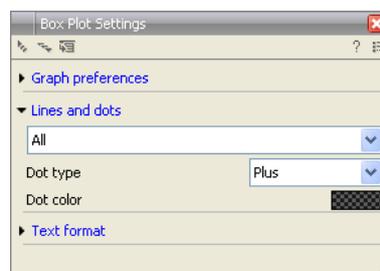


Figure 25.35: Lines and dot preferences for a box plot.

- **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**
 - None
 - Cross
 - Plus
 - Square
 - Diamond
 - Circle
 - Triangle
 - Reverse triangle
 - Dot
- **Dot 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 box plot, you need to save the settings of the **Side Panel** (see section 5.6).

Interpreting the box plot

This section will show how to interpret a box plot through a few examples.

First, if you look at figure 25.36, you can see a box plot for an experiment with 5 groups and 27 samples.



Figure 25.36: 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 25.37 shows a box plot for the same experiment after quantile normalization: the distributions have been brought into par.

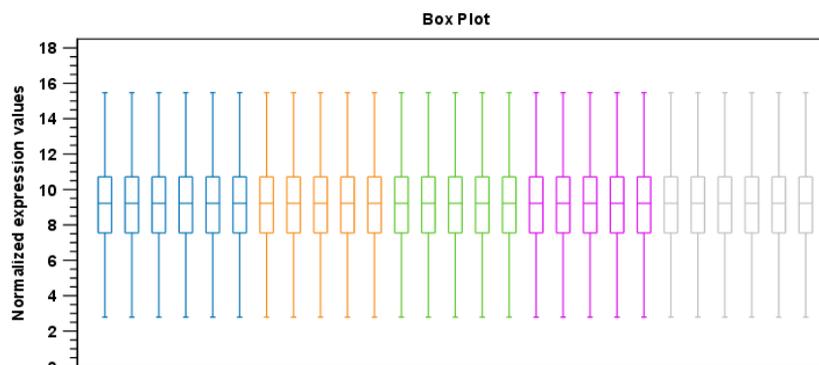


Figure 25.37: Box plot after quantile normalization.

In figure 25.38 a box plot for a two group experiment with 5 samples in each group is shown.

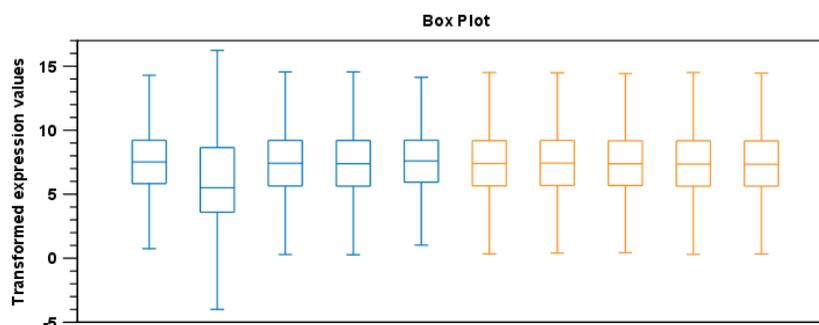


Figure 25.38: Box plot for a two-group experiment with 5 samples.

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.

25.4.2 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:

Toolbox | Transcriptomics 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 25.39. 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.

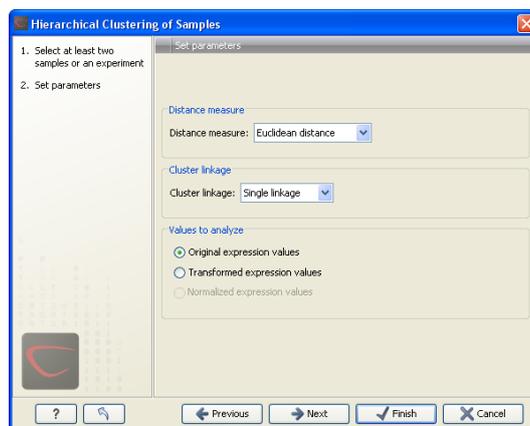


Figure 25.39: Parameters for hierarchical clustering of samples.

At the top, you can choose three kinds of **Distance measures**:

- **Euclidean distance.** The ordinary distance between two points - the length of the segment connecting them. If $u = (u_1, u_2, \dots, u_n)$ and $v = (v_1, v_2, \dots, v_n)$, then the Euclidean distance between u and v is

$$|u - v| = \sqrt{\sum_{i=1}^n (u_i - v_i)^2}.$$

- **1 - Pearson correlation.** The Pearson correlation coefficient between two elements $x = (x_1, x_2, \dots, x_n)$ and $y = (y_1, y_2, \dots, y_n)$ is defined as

$$r = \frac{1}{n-1} \sum_{i=1}^n \left(\frac{x_i - \bar{x}}{s_x} \right) * \left(\frac{y_i - \bar{y}}{s_y} \right)$$

where \bar{x}/\bar{y} is the average of values in x/y and s_x/s_y is the sample standard deviation of these values. It takes a value $\in [-1, 1]$. Highly correlated elements have a high absolute value of the Pearson correlation, and elements whose values are un-informative about each other have Pearson correlation 0. Using $1 - |\text{Pearson correlation}|$ as distance measure means that elements that are highly correlated will have a short distance between them, and elements that have low correlation will be more distant from each other.

- **Manhattan distance.** The Manhattan distance between two points is the distance measured along axes at right angles. If $u = (u_1, u_2, \dots, u_n)$ and $v = (v_1, v_2, \dots, v_n)$, then the Manhattan distance between u and v is

$$|u - v| = \sum_{i=1}^n |u_i - v_i|.$$

Next, you can select the cluster linkage to be used:

- **Single linkage.** The distance between two clusters is computed as the distance between the two closest elements in the two clusters.
- **Average linkage.** The distance between two clusters is computed as the average distance between objects from the first cluster and objects from the second cluster. The averaging is performed over all pairs (x, y) , where x is an object from the first cluster and y is an object from the second cluster.
- **Complete linkage.** The distance between two clusters is computed as the maximal object-to-object distance $d(x_i, y_j)$, where x_i comes from the first cluster, and y_j comes from the second cluster. In other words, the distance between two clusters is computed as the distance between the two farthest objects in the two clusters.

At the bottom, you can select which values to cluster (see section 25.3.1).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Note: the workflow execution of this tool does not end up modifying the input experiment. Instead, a standalone heat map is created.

Result of hierarchical clustering of samples

The result of a sample clustering is shown in figure 25.40.

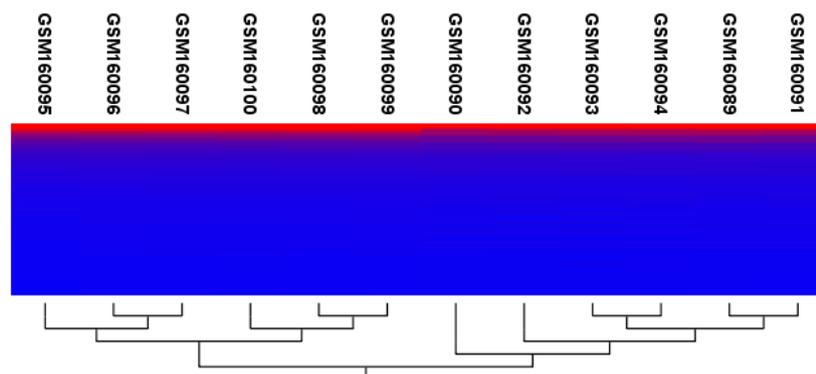


Figure 25.40: Sample clustering.

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 25.41).



Figure 25.41: 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 25.40, 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 25.6.1).

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, researchers 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 unclear 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 25.42).

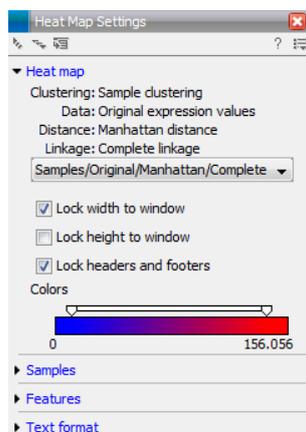


Figure 25.42: Side Panel of heat map.

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 25.56).

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

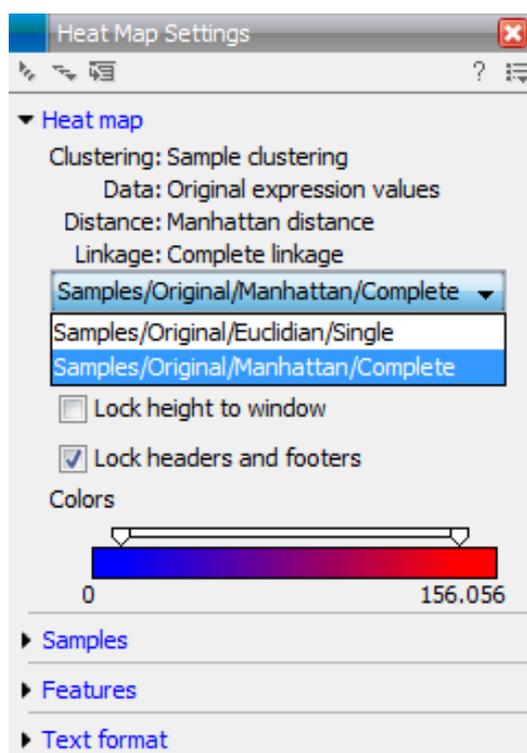


Figure 25.43: When more than one clustering has been performed, there will be a list of heat maps to choose from.

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 heatmap. 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.

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 5.6).

25.4.3 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:

Toolbox | Transcriptomics 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 25.44.

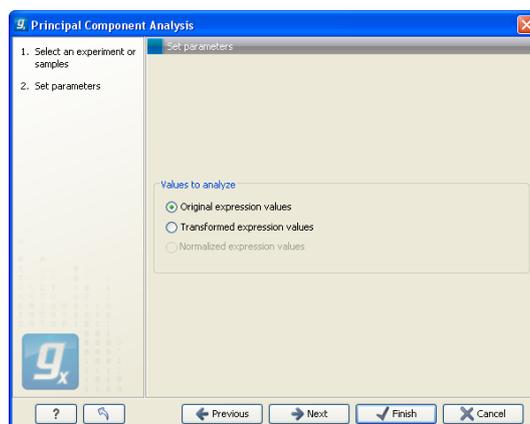


Figure 25.44: Selecting which values the principal component analysis should be based on.

In this dialog, you select the values to be used for the principal component analysis (see section 25.3.1).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Principal component analysis plot

This will create a principal component plot as shown in figure 25.45.

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*

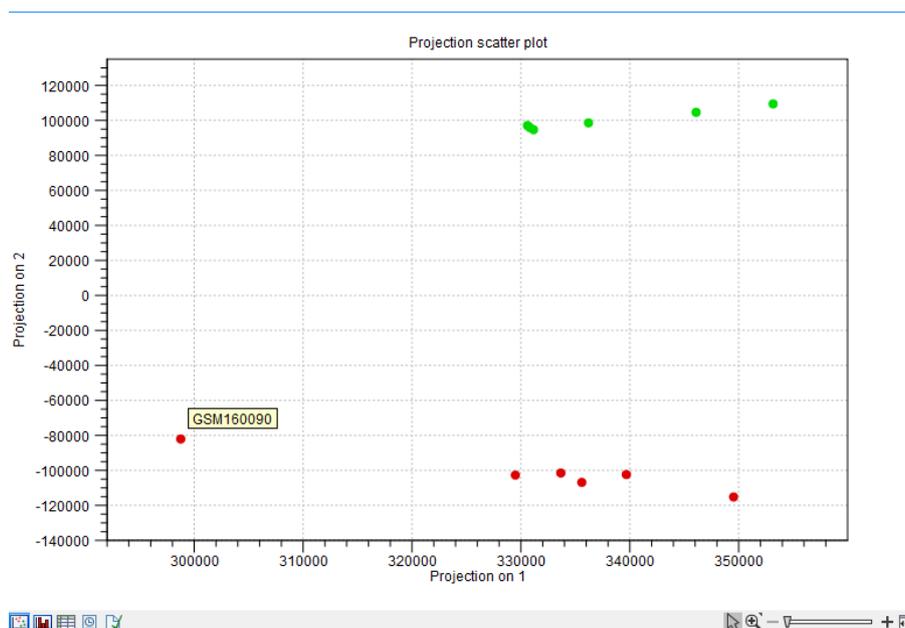


Figure 25.45: A principal component analysis colored by group.

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 25.45 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.
 - Outside
 - Inside
- **Tick lines at.** Choosing Major ticks will show a grid behind the graph.

- None
- Major ticks
- **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
 - * Wide
 - **Line type**
 - * None
 - * Line
 - * Long dash
 - * Short dash
 - **Line color.** Allows you to choose between many different colors. Click the color box to select a color.

Below the general preferences, you find the **Dot properties**:

- **Drop down menu** In this you choose which of the samples (that is, which 'dots') the choices you make below should apply to. You can choose between 'All', a particular group in your experiment, or a particular samples in your experiment.
- **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**
 - None
 - Cross
 - Plus
 - Square
 - Diamond

- Circle
 - Triangle
 - Reverse triangle
 - Dot
- **Dot color.** Allows you to choose between many different colors. 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 5.6).

Scree plot

Besides the view shown in figure 25.45, 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.
 - Outside
 - Inside
- **Tick lines at.** Choosing Major ticks will show a grid behind the graph.
 - None
 - Major ticks
- **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**

- None
- Cross
- Plus
- Square
- Diamond
- Circle
- Triangle
- Reverse triangle
- Dot

- **Dot color.** Allows you to choose between many different colors. Click the color box to select a color.

- **Line width**

- Thin
- Medium
- Wide

- **Line type**

- None
- Line
- Long dash
- Short dash

- **Line color.** Allows you to choose between many different colors. 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 5.6).

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 section 25.5.2) and tests that assume that the data is real-valued, has Gaussian distributions and compare means (described in section 25.5.3).

To run the statistical analysis:

Toolbox | Transcriptomics Analysis () | **Statistical Analysis** | **Empirical Analysis of DGE** ()

Toolbox | Transcriptomics Analysis (📊) | Statistical Analysis | Proportion-based Statistical Analysis (📊)

or **Toolbox | Transcriptomics 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 Empirical analysis of DGE, 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 Empirical analysis of DGE

The Empirical analysis of DGE tool implements the 'Exact Test' for two-group comparisons developed by Robinson and Smyth [Robinson and Smyth, 2008] and incorporated in the EdgeR Bioconductor package [Robinson et al., 2010]. The test is applicable to count data only, and is designed specifically to deal with situations in which *many* features are studied simultaneously (e.g. genes in a genome) but where only a *few* biological replicates are available for each of the experimental groups studied. This is typically the case for RNA-seq expression analysis. The test is based on the assumption that the count data follows a Negative Binomial distribution, which in contrast to the Poisson distribution has the characteristic that it allows for a non-constant mean-variance relationship. The test is also appropriate for larger numbers of samples.

The 'Exact Test' of Robinson and Smyth is similar to Fisher's Exact Test, but also accounts for overdispersion caused by biological variability. Whereas Fisher's Exact Test compares the counts in one sample against those of another, the 'Exact Test' compares the counts in one set of count samples against those in another set of count samples. This is achieved by replacing the Hypergeometric distributions of Fisher's Exact Test by Negative binomial distributions, whereby the variability within each of the two groups of samples compared is taken into account. This only works if the dispersions in the two groups compared are identical. As this cannot generally be assumed to be the case for the *original* (nor for the normalized) data, pseudodata for which the dispersion *is* identical is generated from the original data, and the test is carried out on this pseudodata. The generation of the pseudodata is performed simultaneously with the estimation of the dispersion, in an iterative procedure called quantile-adjusted conditional maximum likelihood. Either a single common dispersion for all features may be assumed (as in [Robinson and Smyth, 2008]), or it may be assumed that the dispersion for each feature (e.g. gene) is a 'weighted average' of the common dispersion and feature (e.g. gene) specific dispersions (as suggested in [Robinson and Smyth, 2007]). The weight given to each of the components depends on the number of samples in the groups: the more samples there are in the groups, the higher the weight will be given to the gene-specific component.

The Exact Test in the EdgeR Bioconductor package provides the user with the option to set a large number of parameters. The implementation of the 'Empirical analysis of DGE' algorithm in the Genomics Workbench uses for the most parts the default settings in the edgeR package, version 3.4.0. A detailed outline of the parameter settings is given in section 25.5.1).

Empirical analysis of DGE - implementation parameters

The 'Empirical analysis of DGE' algorithm in the *CLC Main Workbench* is a re-implementation of the "Exact Test", available as part of the EdgeR Bioconductor package.

The parameter values used in the *CLC Main Workbench* implementation are the default values for the equivalent parameters in the EdgeR Bioconductor implementation in all but one case. The exception is the `estimateCommonDisp` parameter, where the default is more stringent than that of EdgeR. The advantage of using a more stringent value for this parameter is that the results will be more accurate. The disadvantage is that the algorithm will be slightly slower, however according to our performance tests, this change has only a marginal impact on the run time of the tool. Overall, the user has a somewhat compromised run time but gains greater confidence in the results at the end.

The parameter values used in the *CLC Main Workbench* implementation, with reference to the EdgeR function names for clarity, are provided in the table below.

| Function in BioC package | Parameter name | Value used and comments |
|--------------------------|------------------|--|
| calcNormFactors | method | "TMM" |
| | refColumn | NULL (automatically selected) |
| | logratioTrim | 0.3 |
| | sumTrim | 0.05 |
| | doWeighting | TRUE |
| | Acutoff | -1e10 |
| estimateCommonDisp | tol | 1e-14 (default in edgeR: 1e-6) |
| | rowsum.filter | Set by user in wizard ("Total count filter cutoff", default 5) |
| estimateTagewiseDisp | prior.df | 10 |
| | trend | "movingave" |
| | span | NULL |
| | method | "grid" |
| | grid.length | 11 |
| | grid.range | c(-6, 6) |
| mglmOneGroup | maxit | 50 |
| | tol | 1e-10 |
| aveLogCPM | prior.count | 2 |
| | dispersion | 0.05 |
| exactTest | pair | Set by user in wizard ("Exact test comparisons") |
| | dispersion | "auto" (tagwise if available, otherwise common) |
| | rejection.region | "doubletail" |
| | big.count | 900 |
| | prior.count | 0.125 |

Running the Empirical analysis of DGE

First, find the **Empirical analysis of DGE** tool:

Toolbox | Transcriptomics Analysis (📁) | Statistical Analysis | Empirical Analysis of DGE (🔍)

The original count data for a full expression experiment are the expected input to the Empirical

Analysis of DGE tool.

When Experiments created within the Workbench are used as input, the original count values are always used. Columns of such Experiments that contain transformed or normalized values are ignored.

If expression values are being imported from outside the Workbench for use with this test, the data should be original (non-transformed, non-normalized) counts.

Whether the data has been generated in the Workbench or outside the Workbench and imported, the full set of expression results should be used. Please do not run this test on a subset of values from the original sample data.

The reason that the complete set of original count data for samples should be used as input to this test is that the algorithm assumes that the counts on which it operates are Negative Binomially distributed. It implicitly normalizes and transforms these counts, so if the counts have been altered prior to submitting them to the Empirical Analysis of DGE tool, this assumption is likely to be compromised.

When running the Empirical analysis of DGE tool in the Genomics workbench, the user is asked to specify two parameters related to the estimation of the dispersion (figure 25.46). Of these, the 'Total count filter cut-off' specifies which features should be considered when estimating the common dispersion component. Features for which the counts across all samples are low are likely to contribute mostly with noise to the estimation, and features with a lower cumulative count across samples than the value specified will be ignored. When the check-box 'Estimate tag-wise dispersions' is checked, the dispersion estimate for each gene will be a weighted combination of the tag-wise and common dispersion, if the check-box is un-ticked the common dispersion will be used for all genes.

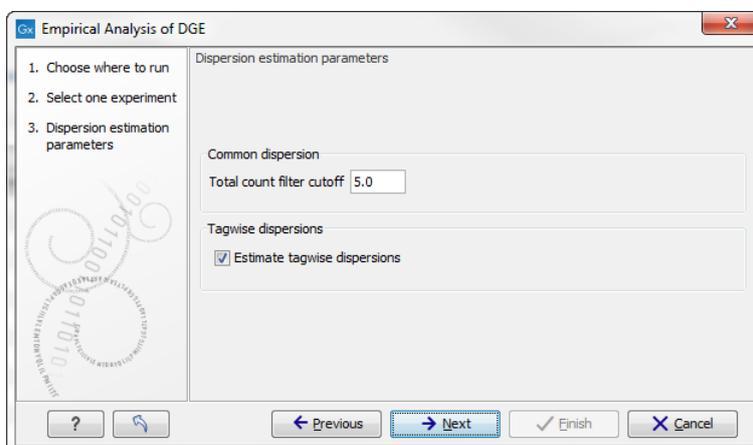


Figure 25.46: Empirical analysis of DGE: setting the parameters related to dispersion.

The Empirical analysis of DGE may be carried out between all pairs of groups (by clicking the 'All pairs' button) or for each group against a specified reference group (by clicking the 'Against reference' button) (figure 25.47). 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). For example, the All pairs option should be selected when you wish to perform the test of equality for group means for all of the pairs, e.g. if you would like to compare different tissues where each tissue is represented in a group. In this case there is no reference group, so the following comparisons will be performed:

- liver vs heart
- liver vs lung
- heart vs lung

The Against reference option should be selected when you wish to perform the test of equality for group means against one group, the reference, rather than all groups as above. Against reference could be used if you have a wild type and some mutant groups, e.g. Wild type, Mutant 1 and Mutant 2. In this case you might be interested in comparing the mutants to the wild type, but comparing the mutants to each other is not of interest. In this case the Wild Type group is considered the reference and the comparisons will be performed:

- Wild type vs Mutant 1
- Wild type vs Mutant 2

Note that with the Against reference option fewer comparisons are made, as in the above example where Mutant 1 vs. Mutant 2 is not considered.

The user can specify if Bonferroni and FDR corrected p-values should be calculated (see Section 25.5.4). 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].

When the Empirical analysis of DGE is run three columns will be added to the experiment table for each pair of groups that are analyzed: the 'P-value', 'Fold change' and 'Weighted

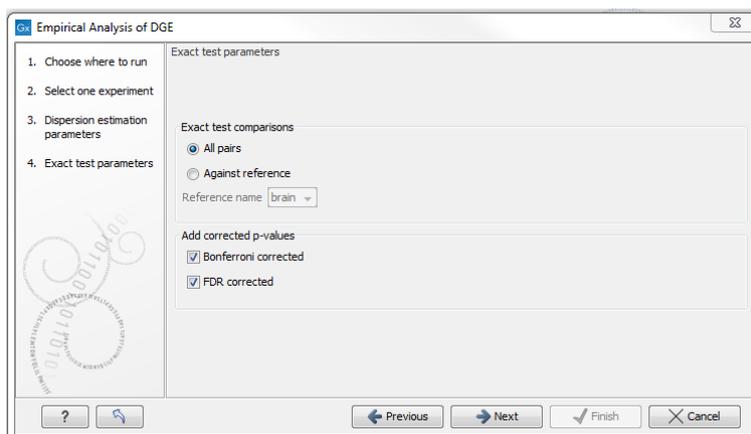


Figure 25.47: Empirical analysis of DGE: setting comparisons and corrected p-value options.

difference' columns. The 'P-value' holds the p-value for the Exact test. The 'Fold Change' and 'Weighted difference' columns are both calculated from the estimated relative abundances, which are derived internally in the Exact Test algorithm. They depend on both the sizes (depth of coverage/library size) of the samples, the magnitude of the counts and on the estimated negative binomial dispersion, so they cannot be obtained from the original counts by simple algebraic calculations.

The 'Fold Change' will tell you how many times bigger the relative abundance of group 2 is relative to that of group 1. If the relative abundance of group 2 is bigger than that of group 1 the fold change is the relative abundance of group 2 divided by that of group 1. If the relative abundance of group 2 is smaller than that of group 1 the fold change is the relative abundance of group 1 divided by that of group 2 with a negative sign. The 'weighted difference' column contains the difference between the relative abundance of group 2 and the relative abundance of group 1. In addition to the three automatically added columns, columns containing the Bonferroni and FDR corrected p-values will be added if that was specified by the user.

25.5.2 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 RNA-Seq or tag profiling. 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 your 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 (see 25.5.4).

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 (see 25.5.4).

25.5.3 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 25.48.

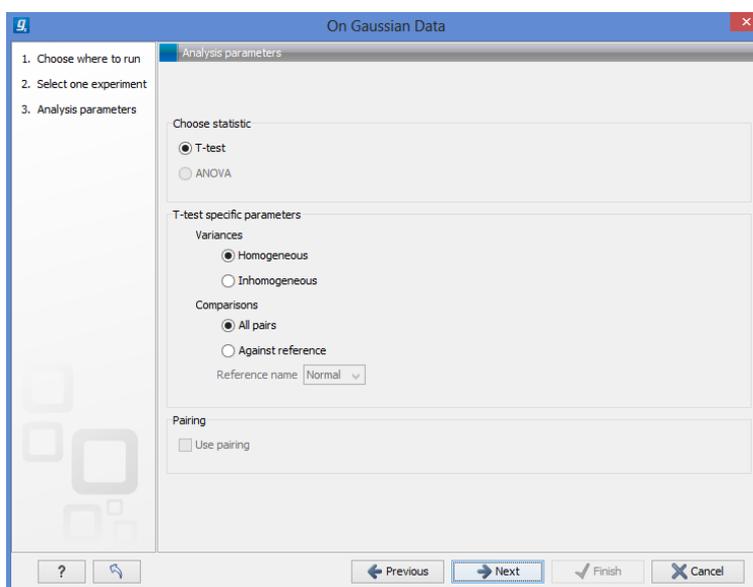


Figure 25.48: Selecting a t-test.

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 (see 25.5.4).

ANOVA

For experiments with more than two groups you can choose **T-test** as described above, or **ANOVA** as shown in figure 25.49.

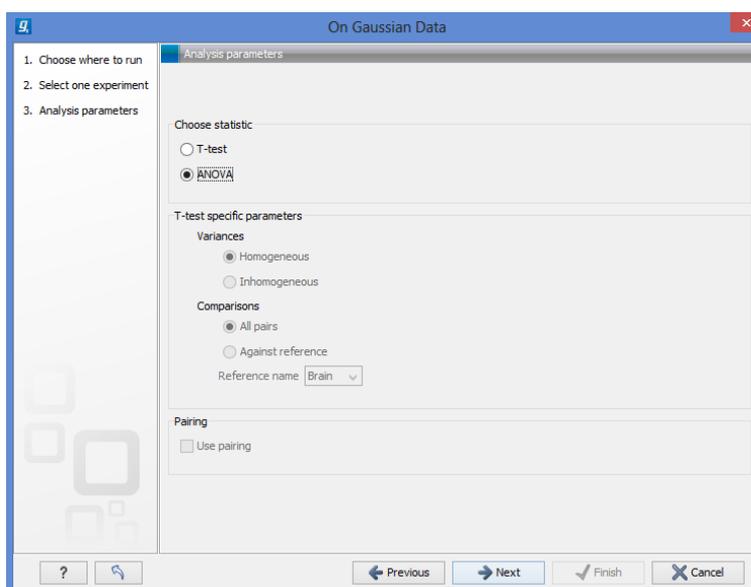


Figure 25.49: Selecting ANOVA.

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 (see 25.5.4).

25.5.4 Corrected p-values

Clicking **Next** will display a dialog as shown in figure 25.50.

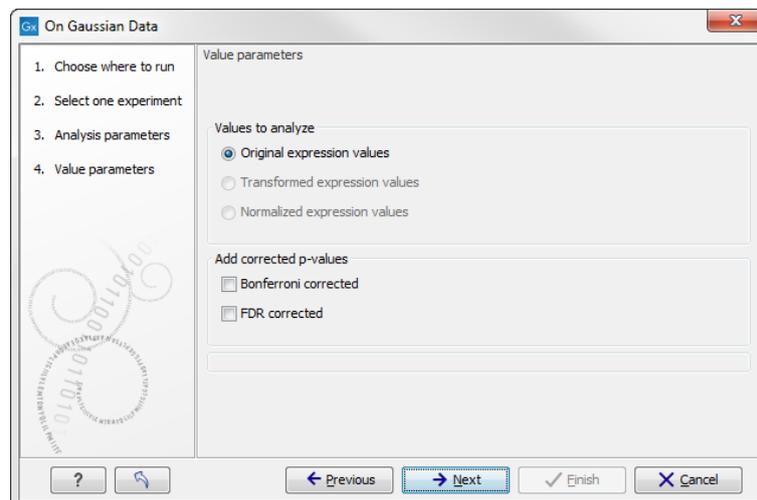


Figure 25.50: Additional settings for the statistical analysis.

At the top, you can select which values to analyze (see section 25.3.1).

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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Note that if you have already performed statistical analysis on the same values, the existing one will be overwritten.

25.5.5 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 9.3).

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 presented in section 25.1.4, 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 25.51.

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 your data (Read the note on fold change in section 25.1.2).

The larger the difference in expression of a feature, the more extreme its 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

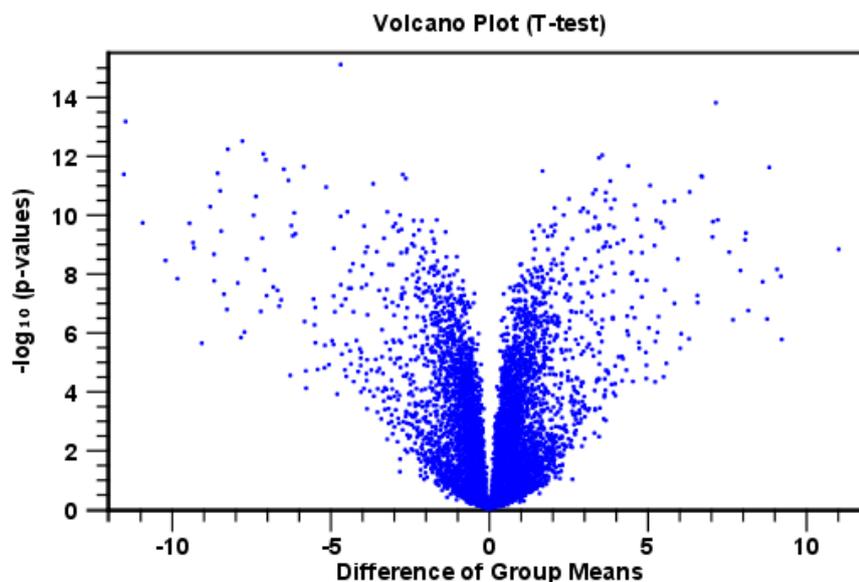


Figure 25.51: *Volcano plot.*

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 3.2).

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.
 - Outside
 - Inside
- **Tick lines at.** Choosing Major ticks will show a grid behind the graph.
 - None
 - Major ticks

- **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**
 - None
 - Cross
 - Plus
 - Square
 - Diamond
 - Circle
 - Triangle
 - Reverse triangle
 - Dot
- **Dot color.** Allows you to choose between many different colors. 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 [5.6](#)).

25.6 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.

25.6.1 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:

Toolbox | Transcriptomics 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 25.52. 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.

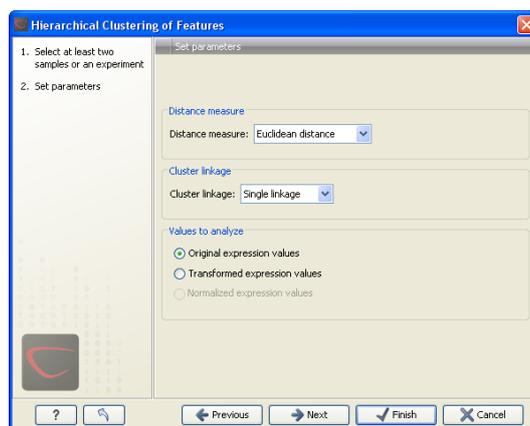


Figure 25.52: Parameters for hierarchical clustering of features.

At the top, you can choose three kinds of **Distance measures**:

- **Euclidean distance.** The ordinary distance between two points - the length of the segment connecting them. If $u = (u_1, u_2, \dots, u_n)$ and $v = (v_1, v_2, \dots, v_n)$, then the Euclidean distance between u and v is

$$|u - v| = \sqrt{\sum_{i=1}^n (u_i - v_i)^2}.$$

- **1 - Pearson correlation.** The Pearson correlation coefficient between two elements $x = (x_1, x_2, \dots, x_n)$ and $y = (y_1, y_2, \dots, y_n)$ is defined as

$$r = \frac{1}{n-1} \sum_{i=1}^n \left(\frac{x_i - \bar{x}}{s_x} \right) * \left(\frac{y_i - \bar{y}}{s_y} \right)$$

where \bar{x}/\bar{y} is the average of values in x/y and s_x/s_y is the sample standard deviation of these values. It takes a value $\in [-1, 1]$. Highly correlated elements have a high absolute value of the Pearson correlation, and elements whose values are un-informative about each other have Pearson correlation 0. Using $1 - |Pearsoncorrelation|$ as distance measure means that elements that are highly correlated will have a short distance between them, and elements that have low correlation will be more distant from each other.

- **Manhattan distance.** The Manhattan distance between two points is the distance measured along axes at right angles. If $u = (u_1, u_2, \dots, u_n)$ and $v = (v_1, v_2, \dots, v_n)$, then the Manhattan distance between u and v is

$$|u - v| = \sum_{i=1}^n |u_i - v_i|.$$

Next, you can select different ways to calculate distances between clusters. The possible cluster linkage to use are:

- **Single linkage.** The distance between two clusters is computed as the distance between the two closest elements in the two clusters.
- **Average linkage.** The distance between two clusters is computed as the average distance between objects from the first cluster and objects from the second cluster. The averaging is performed over all pairs (x, y) , where x is an object from the first cluster and y is an object from the second cluster.
- **Complete linkage.** The distance between two clusters is computed as the maximal object-to-object distance $d(x_i, y_j)$, where x_i comes from the first cluster, and y_j comes from the second cluster. In other words, the distance between two clusters is computed as the distance between the two farthest objects in the two clusters.

At the bottom, you can select which values to cluster (see section 25.3.1). Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

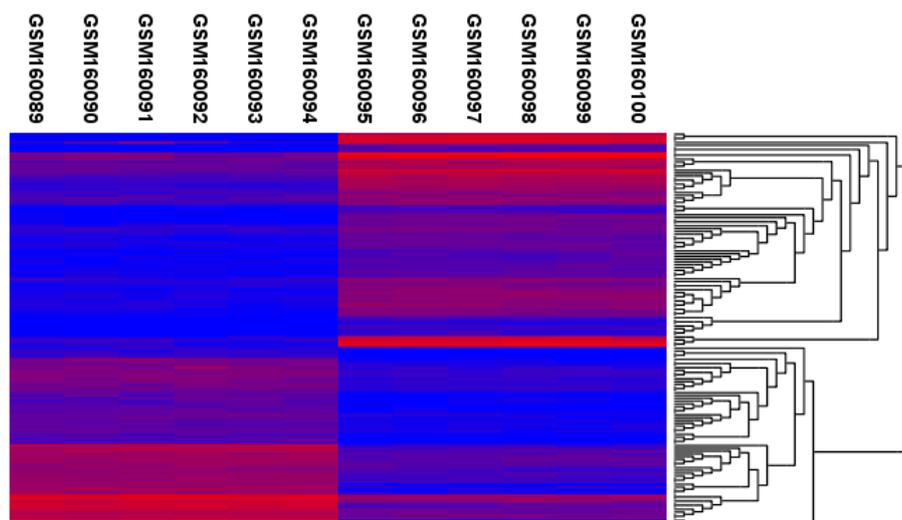


Figure 25.53: Hierarchical clustering of features.



Figure 25.54: Showing the hierarchical clustering of an experiment.

Result of hierarchical clustering of features

The result of a feature clustering is shown in figure 25.53.

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 25.54).

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 25.53). In the heatmap each row corresponds to a feature and each column to a sample. The 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 25.55).

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 25.56).

Note that if you perform an identical clustering, the existing heat map will simply be replaced.

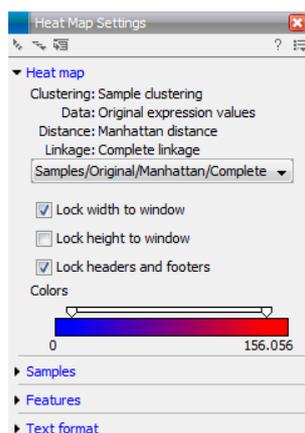


Figure 25.55: Side Panel of heat map.

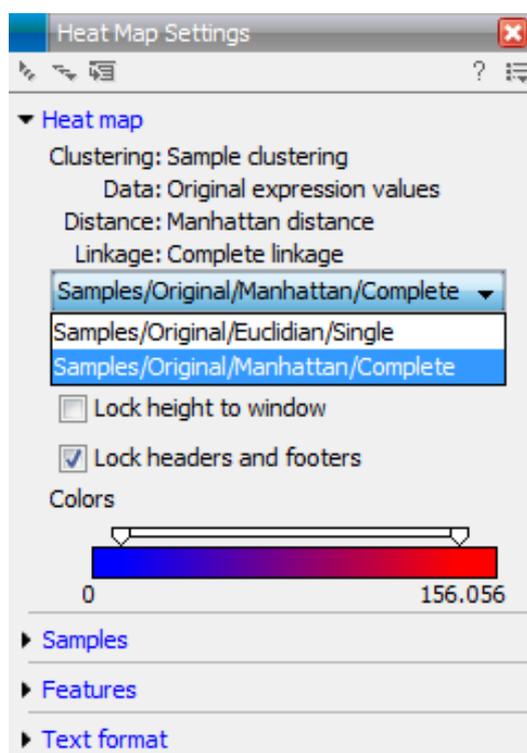


Figure 25.56: When more than one clustering has been performed, there will be a list of heat maps to choose from.

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 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 heatmap. 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.

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 5.6).

25.6.2 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.

Toolbox | Transcriptomics 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 25.57.

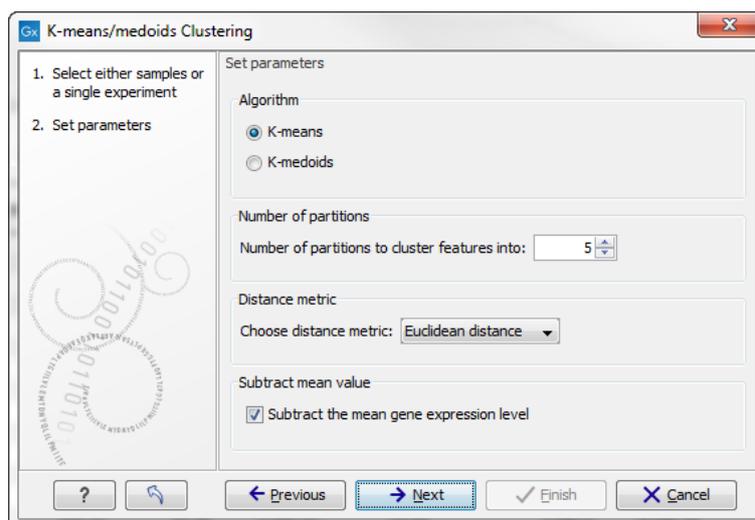


Figure 25.57: Parameters for k-means/medoids clustering.

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 $S_i, i = 1, 2, \dots, k$ 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 $S_i, i = 1, 2, \dots, k$ 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-algorithm is described in [Kaufman and Rousseeuw, 1990].

- **Number of partitions.** The number of partitions to cluster features into.
- **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 $u = (u_1, u_2, \dots, u_n)$ and $v = (v_1, v_2, \dots, v_n)$, 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 $u = (u_1, u_2, \dots, u_n)$ and $v = (v_1, v_2, \dots, v_n)$, then the Manhattan distance between u and v is

$$|u - v| = \sum_{i=1}^n |u_i - v_i|.$$

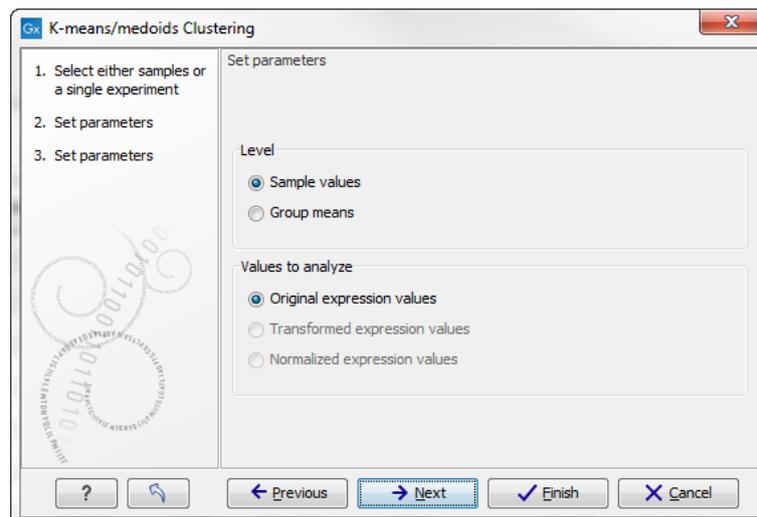


Figure 25.58: Parameters for *k*-means/medoids clustering.

- **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 25.58.

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 25.3.1).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

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 25.57) - there is one graph per cluster. Using drag and drop as explained in section 3.1.6, you can arrange the views to see more than one graph at the time.

Figure 25.59 shows an example where four clusters have been arranged side-by-side.

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 than 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.

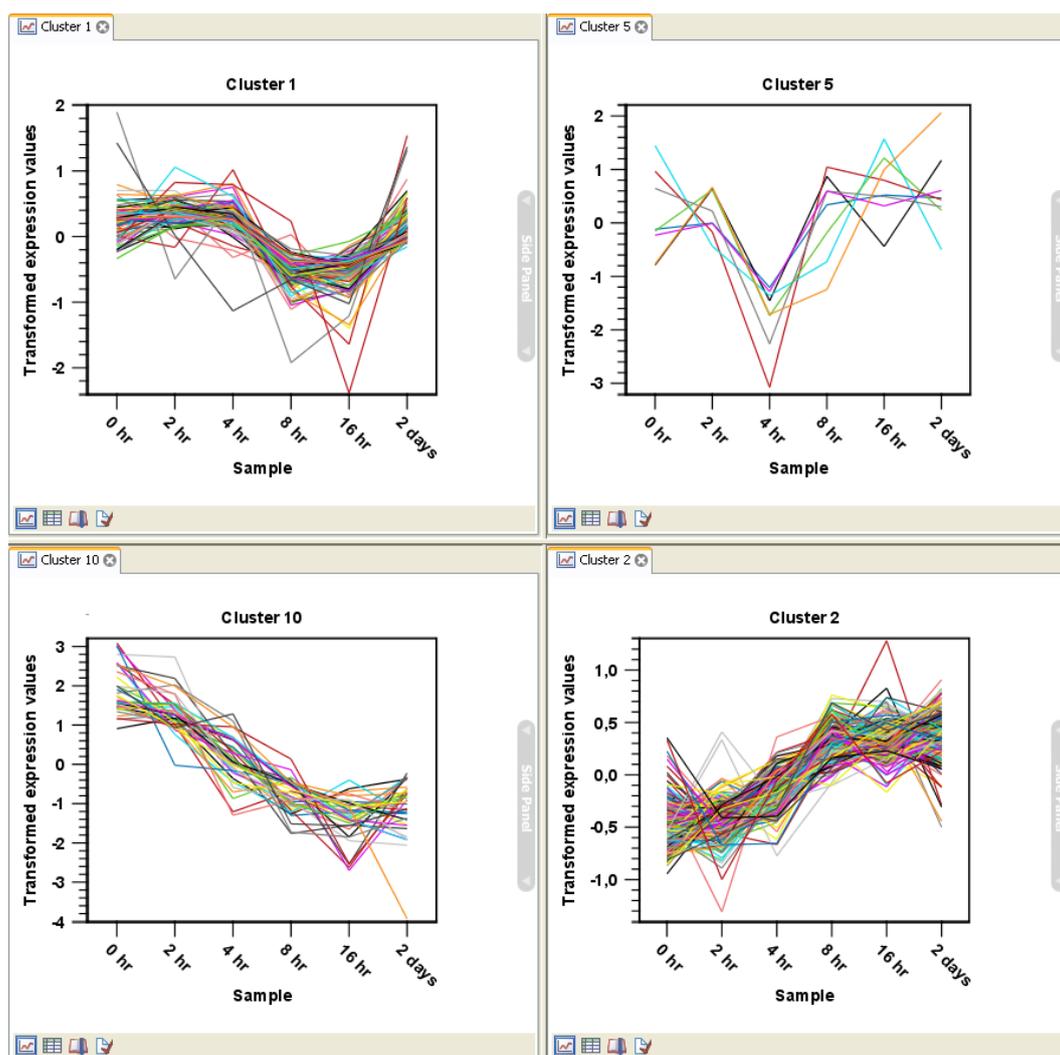


Figure 25.59: Four clusters created by *k*-means/medoids clustering.

25.7 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](#).

25.7.1 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].

Toolbox | Transcriptomics 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 25.60.

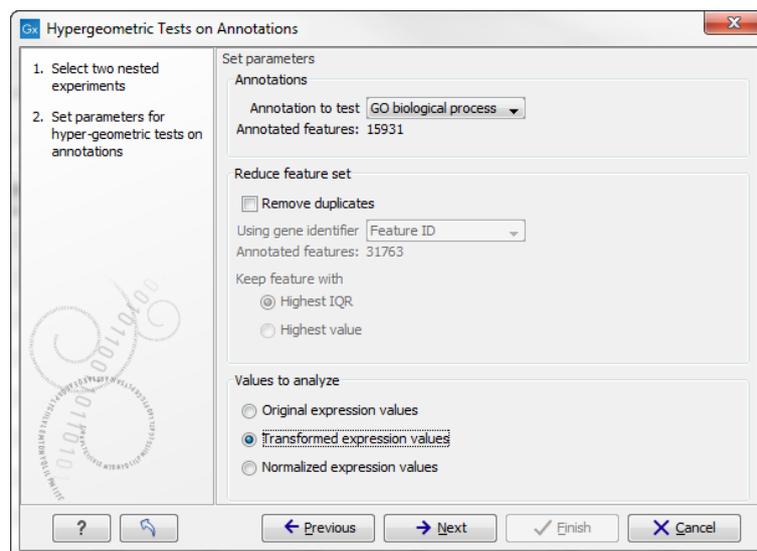


Figure 25.60: Parameters for performing a hypergeometric test on annotations.

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 25.3.1). 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.

The final number of tests ... features used for the test is reported in this history view of the test results.

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Result of hypergeometric tests on annotations

The result of performing hypergeometric tests on annotations using GO biological process is shown in figure 25.61.

| 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 25.61: The result of testing 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.
- **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.

25.7.2 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 that 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 of differential expression, prior to running GSEA on it.

Toolbox | Transcriptomics Analysis (📁) | Annotation Test | Gene Set Enrichment Analysis (GSEA) (🖼️)

Select an experiment and click **Next**.

Click **Next**. This will display the dialog shown in figure 25.62.

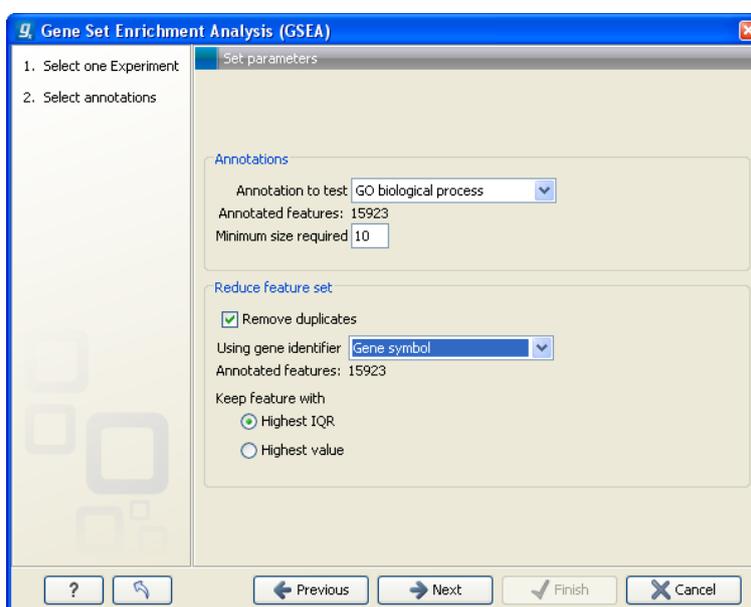


Figure 25.62: Gene set enrichment analysis on GO biological process

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.

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 25.63.

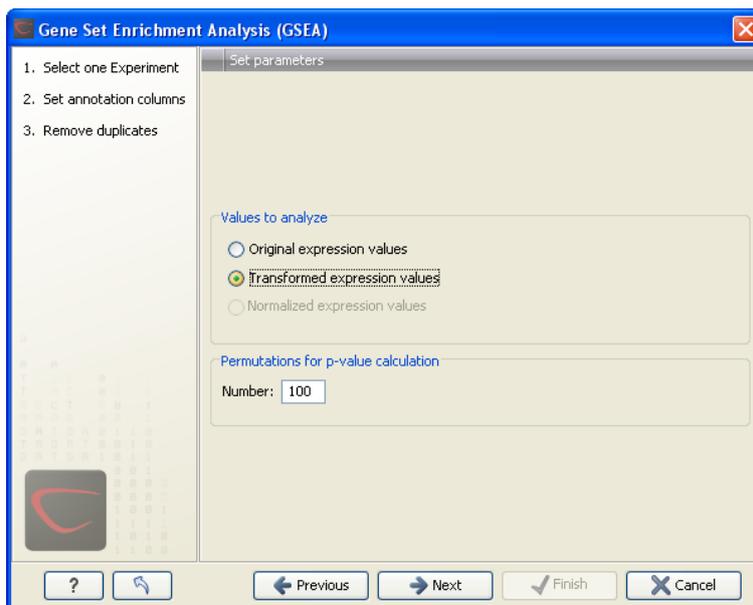


Figure 25.63: Gene set enrichment analysis parameters.

At the top, you can select which values to analyze (see section 25.3.1).

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).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Result of gene set enrichment analysis

The result of performing gene set enrichment analysis using GO biological process is shown in figure 25.64.

The table shows the following information:

| Category | Description | Size | Test statistic | Lower tail | Upper tail |
|----------|---------------------|------|----------------|------------|------------|
| 0006412 | translation | 204 | -13,92 | 0,00 | 1,00 |
| 0006941 | striated muscle ... | 15 | -28,52 | 0,00 | 1,00 |
| 0006936 | muscle contract... | 26 | -37,56 | 0,00 | 1,00 |
| 0006937 | regulation of m... | 17 | -30,63 | 0,00 | 1,00 |
| 0007519 | skeletal muscle ... | 30 | -20,45 | 1E-4 | 1,00 |
| 0005977 | glycogen meta... | 19 | -19,62 | 2E-4 | 1,00 |
| 0007517 | muscle develop... | 21 | -15,18 | 1,7E-3 | 1,00 |
| 0001501 | skeletal develo... | 42 | -14,10 | 2,1E-3 | 1,00 |
| 0006094 | gluconeogenesis | 16 | -14,19 | 3,5E-3 | 1,00 |
| 0009749 | response to glu... | 37 | -12,23 | 5,4E-3 | 0,99 |
| 0006414 | translational el... | 12 | -13,48 | 6E-3 | 0,99 |
| 0001756 | somitogenesis | 13 | -12,34 | 6,8E-3 | 0,99 |
| 0007528 | neuromuscular ... | 13 | -12,81 | 7,8E-3 | 0,99 |
| 0005978 | glycogen biosy... | 11 | -12,03 | 8,8E-3 | 0,99 |

Figure 25.64: The result of gene set enrichment analysis on GO biological process.

- **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.

25.8 General plots

The last folder in the **Expression Analysis** (📁) folder in the **Toolbox** is **General Plots**. Here you find three general plots that may be useful at various point of your analysis work flow. The plots are explained in detail below.

25.8.1 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:

Toolbox | Transcriptomics 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 25.65.

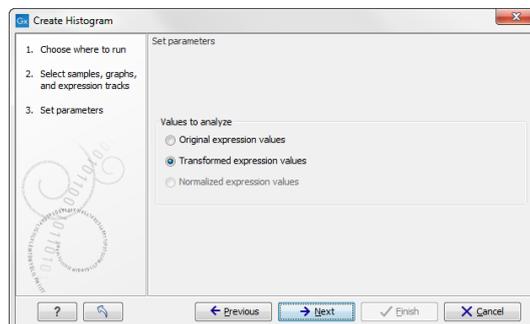


Figure 25.65: Selecting which values the histogram should be based on.

In this dialog, you select the values to be used for creating the histogram (see section 25.3.1). Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Viewing histograms

The resulting histogram is shown in a figure 25.66

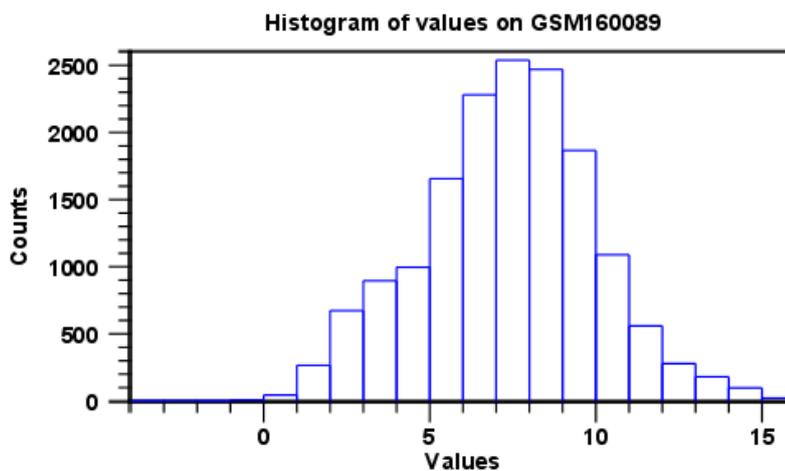


Figure 25.66: Histogram showing the distribution of transformed expression values.

The histogram shows the expression value on the x axis (in the case of figure 25.66 the transformed expression values) and the counts of these values on the y axis.

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.

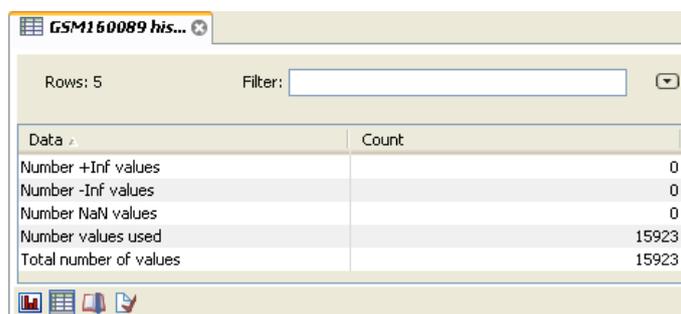
- **Lock axes.** This will always show the axes even though the plot is zoomed to a detailed level.
- **Frame.** Shows a frame around the graph.

- **Show legends.** Shows the data legends.
- **Tick type.** Determine whether tick lines should be shown outside or inside the frame.
 - Outside
 - Inside
- **Tick lines at.** Choosing Major ticks will show a grid behind the graph.
 - None
 - Major ticks
- **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 5.6).

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 25.67.



| Data | Count |
|------------------------|-------|
| Number +Inf values | 0 |
| Number -Inf values | 0 |
| Number NaN values | 0 |
| Number values used | 15923 |
| Total number of values | 15923 |

Figure 25.67: 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**

25.8.2 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:

Toolbox | Transcriptomics Analysis (📄) | General Plots | Create MA Plot (📊)

Select two samples ((📄), (📄) or (📄)). Clicking **Next** will display a dialog as shown in figure 25.68.

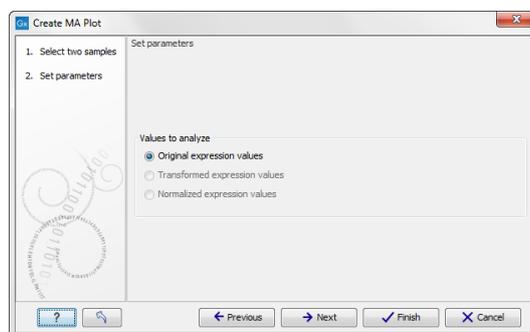


Figure 25.68: Selecting which values the MA plot should be based on.

In this dialog, you select the values to be used for creating the MA plot (see section 25.3.1).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

Viewing MA plots

The resulting plot is shown in a figure 25.69.

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 25.69 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 25.3.2).

Figure 25.70 shows the same two samples where the MA plot has been created using log₂ transformed values.

The much more symmetric and even spread indicates that the dependence 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.

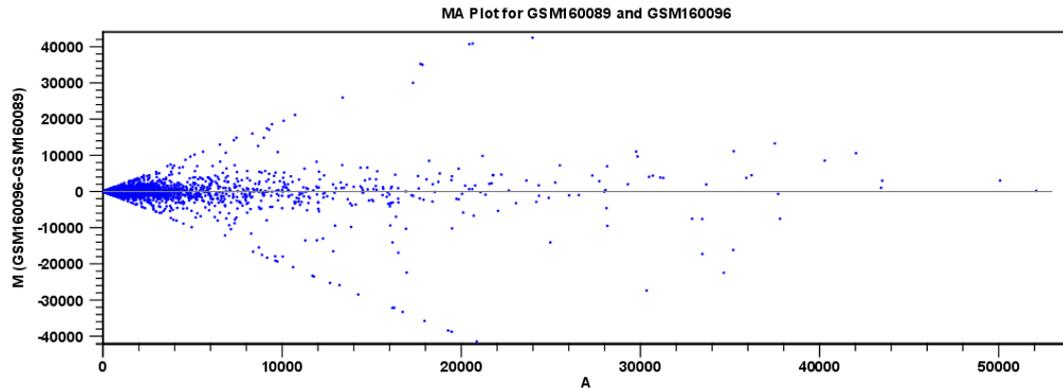


Figure 25.69: MA plot based on original expression values.

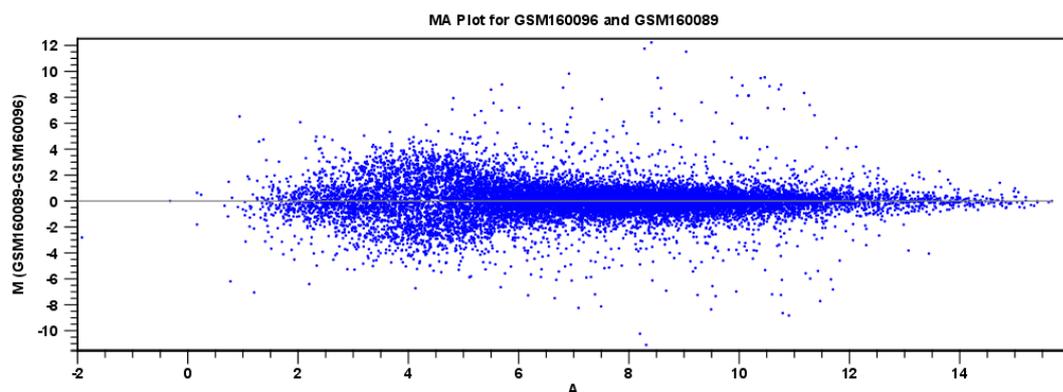


Figure 25.70: MA plot based on transformed expression values.

- **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.
 - Outside
 - Inside
- **Tick lines at.** Choosing Major ticks will show a grid behind the graph.
 - None
 - Major ticks
- **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
- * Wide

- **Line type**

- * None
- * Line
- * Long dash
- * Short dash

- **Line color.** Allows you to choose between many different colors. Click the color box to select a color.

- **Line width**

- Thin
- Medium
- Wide

- **Line type**

- None
- Line
- Long dash
- Short dash

- **Line color.** Allows you to choose between many different colors. 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**

- None
- Cross
- Plus
- Square
- Diamond
- Circle
- Triangle
- Reverse triangle
- Dot

- **Dot 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 scatter plot, you need to save the settings of the **Side Panel** (see section 5.6).

25.8.3 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:

Toolbox | Transcriptomics Analysis (📁) | General Plots | Create Scatter Plot (📊)

Select two samples (🇩🇪), (🇺🇸) or (🇺🇸). Clicking **Next** will display a dialog as shown in figure 25.71.

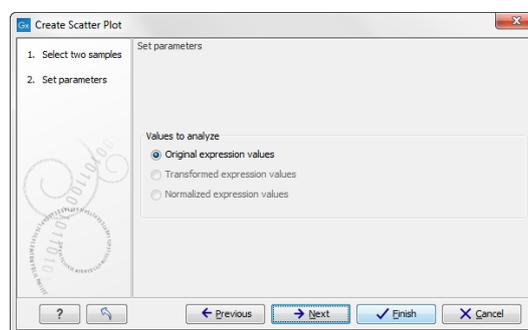


Figure 25.71: *Selecting which values the scatter plot should be based on.*

In this dialog, you select the values to be used for creating the scatter plot (see section 25.3.1).

Click **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

For more information about the scatter plot view and how to interpret it, please see section 25.1.4.

Chapter 26

BLAST search

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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 26.6.

Figure 26.9 shows an example of a BLAST result in the *CLC Main Workbench*.

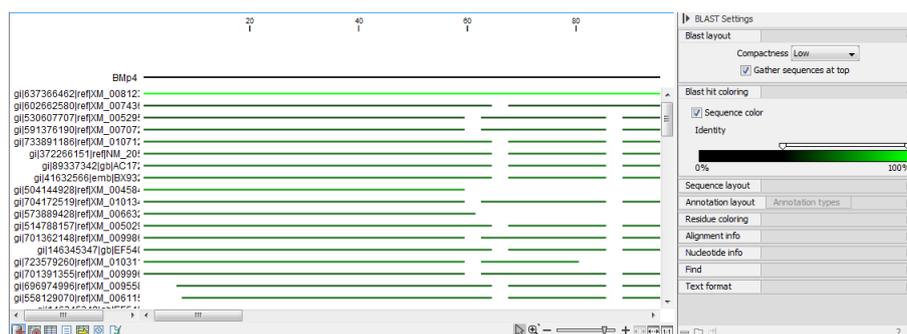


Figure 26.1: Display of the output of a BLAST search. At the top is there a graphical representation of BLAST hits with tool-tips showing additional information on individual hits. Below is a tabular form of the BLAST results.

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 (<http://www.ncbi.nlm.nih.gov/>) 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:

Toolbox | 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

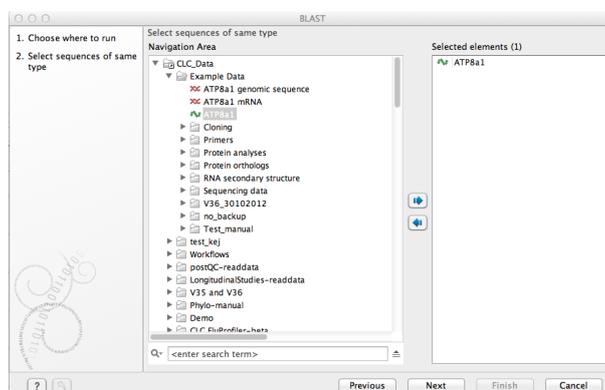


Figure 26.2: Choose one or more sequences to conduct a BLAST search with.

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. See 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 choose among to run. A complete list of these databases can be found in Appendix C. Here you can also read how to add additional databases available the NCBI to the list provided in the dropdown menu.

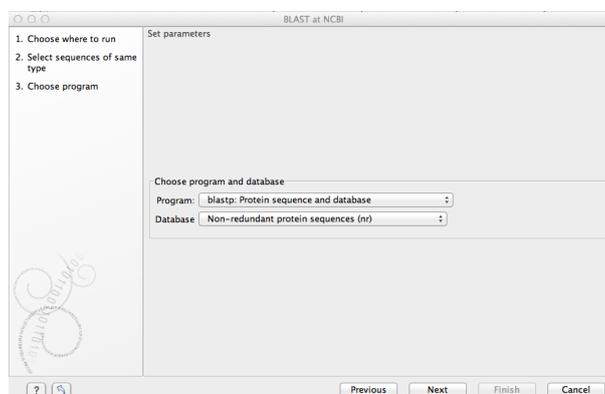


Figure 26.3: Choose a BLAST Program and a database for the search.

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.

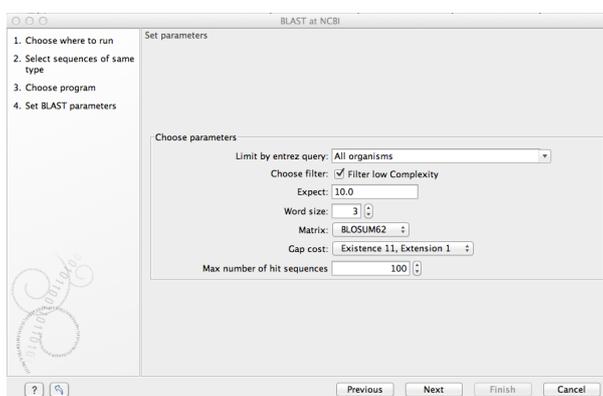


Figure 26.4: Parameters that can be set before submitting a BLAST search.

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.

The following description of BLAST search parameters is based on information from <http://www.ncbi.nlm.nih.gov/BLAST/blastcgihelp.shtml>.

- **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 http://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.
- **Choose filter.** You can choose to apply **Low-complexity**. 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 default value is 10, meaning that under the circumstances of this search, 10 matches are expected to be found merely by chance according to the stochastic model of Karlin and Altschul (1990). Details of how E-values are calculated can be found at the NCBI: <http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html> If the E-value ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT 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 of E less than one can be entered as decimals, or in scientific notation. 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 **Next** if you wish to adjust how to handle the results (see section 9.2). If not, click **Finish**.

26.1.2 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.

26.1.3 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, the *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 26.4.3)
- You can add pre-formatted databases (see section 26.4.1)
- You can use sequence data from the **Navigation Area** directly, without creating a database first.

To conduct a local BLAST search, go to:

Toolbox | BLAST  | **BLAST** 

This opens the dialog seen in figure 26.5:

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.
- **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.

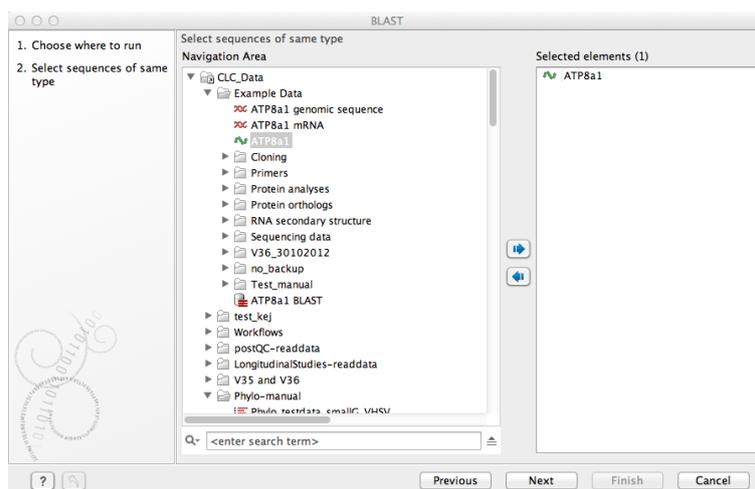


Figure 26.5: Choose one or more sequences to conduct a BLAST search.

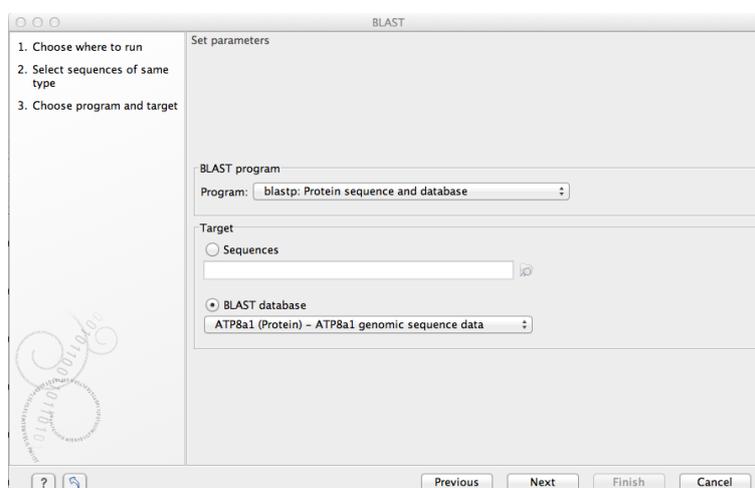


Figure 26.6: Choose a BLAST program and a target database.

- **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).

Click **Next**.

This dialog allows you to adjust the parameters to meet the requirements of your BLAST search (figure 26.7).

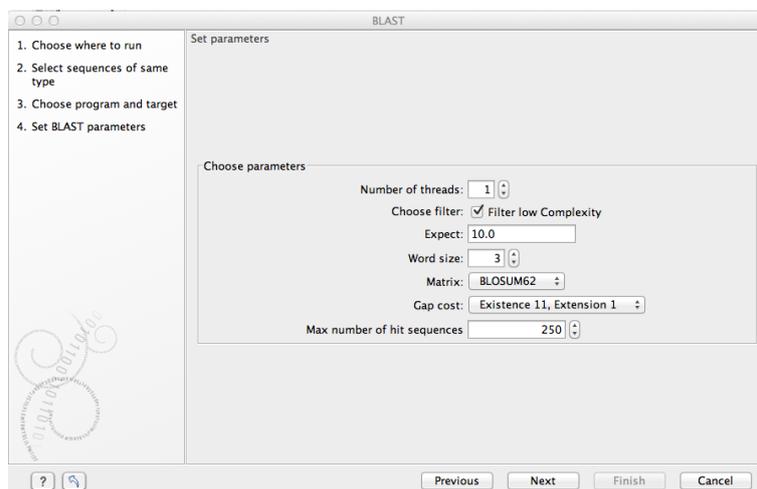


Figure 26.7: Parameters that can be set before submitting a local BLAST search.

- **Number of threads.** You can specify the number of threads, which should be used if your Workbench is installed on a multi-threaded system.
- **Choose filter.** You can choose to apply **Low-complexity**. 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 default value is 10, meaning that under the circumstances of this search, 10 matches are expected to be found merely by chance according to the stochastic model of Karlin and Altschul (1990). Details of how E-values are calculated can be found at the NCBI: <http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html> If the E-value ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT 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 of E less than one can be entered as decimals, or in scientific notation. 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.

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 BLAST database first, see section 26.4.3.
- **BLAST Database.** Select a database already available in one of your designated BLAST database folders. Read more in section 26.5.

When a database or a set of sequences has been selected, click **Next**.

This opens the dialog seen in figure 26.8:

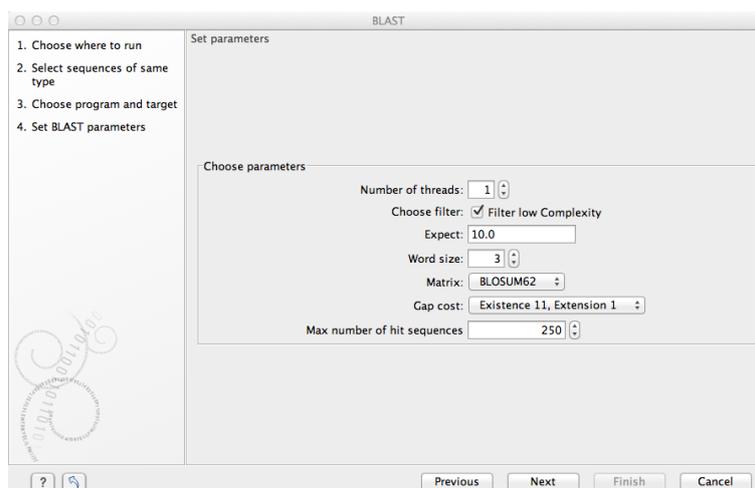


Figure 26.8: Examples of parameters that can be set before submitting a BLAST search.

See section 26.1.1 for information about these limitations.

26.1.4 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 26.9. In cases where only one sequence was entered into a BLAST search, such a graphical overview is the default output.

Figure 26.9 shows an example of a BLAST result for an individual query sequence in the *CLC Main Workbench*.

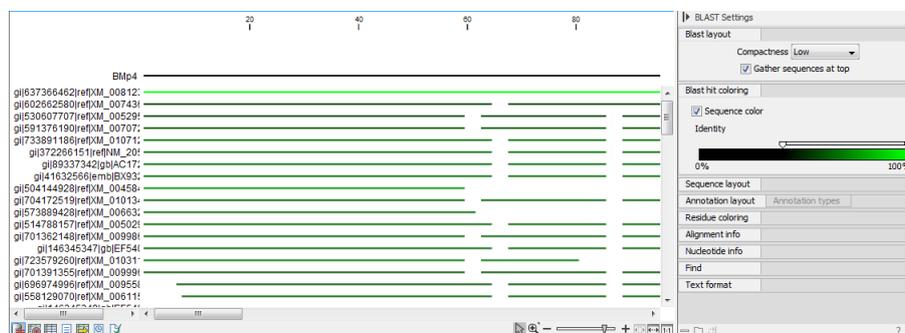


Figure 26.9: Default display of the output of a BLAST search for one query sequence. At the top is there a graphical representation of BLAST hits with tooltips showing additional information on individual hits.

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 26.10, there is one row for each query sequence. Each row represents the BLAST result for this query sequence.

The screenshot shows a web-based BLAST results interface. At the top, it says 'Rows: 2' and has a 'Filter' button. Below is a table with the following data:

| 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 |

At the bottom of the table, there are three buttons: 'Open BLAST Output', 'Extract Consensus', and 'Open Query Sequence'.

Figure 26.10: 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 [12.1](#).

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.

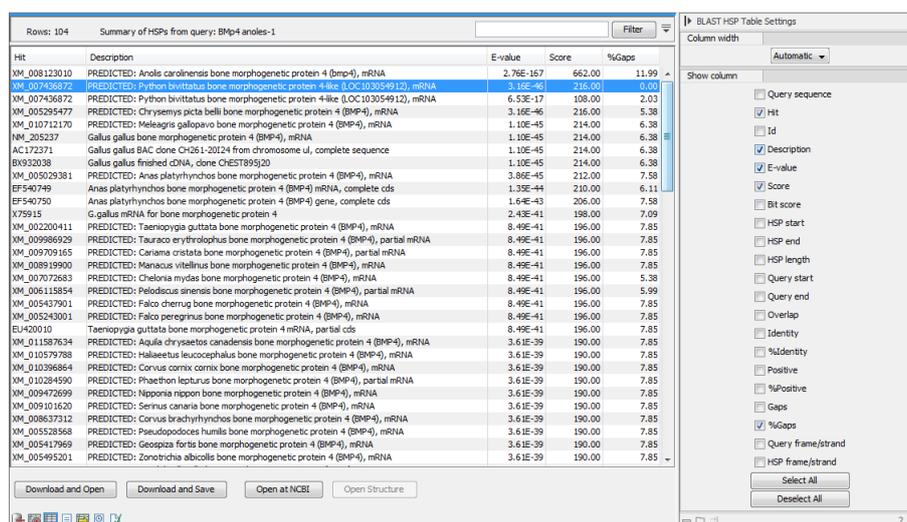
26.2.4 BLAST HSP table

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 26.11 is an example of a BLAST HSP Table.



| 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_005295777 | PREDICTED: Chrysemys picta bellii bone morphogenetic protein 4 (BMP4), mRNA | 3.10E-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 Ch1261-20124 from chromosome uJ, complete sequence | 1.10E-45 | 214.00 | 6.38 |
| BM932038 | Gallus gallus finished cDNA, clone CHEST89520 | 1.10E-45 | 214.00 | 6.38 |
| XM_005029381 | PREDICTED: Anas platyrhynchos bone morphogenetic protein 4 (BMP4), mRNA | 3.60E-45 | 212.00 | 7.58 |
| EF540789 | Anas platyrhynchos bone morphogenetic protein 4 (BMP4) mRNA, complete cds | 1.33E-44 | 210.00 | 6.11 |
| EF540790 | Anas platyrhynchos bone morphogenetic protein 4 (BMP4) gene, complete cds | 1.64E-43 | 206.00 | 7.58 |
| X75915 | G.gallus mRNA for bone morphogenetic protein 4 | 2.43E-41 | 198.00 | 7.09 |
| XM_002200411 | PREDICTED: Taeniopygia guttata bone morphogenetic protein 4 (BMP4), mRNA | 8.49E-41 | 196.00 | 7.85 |
| XM_00986929 | PREDICTED: Taurus erythraeops bone morphogenetic protein 4 (BMP4), partial mRNA | 8.49E-41 | 196.00 | 7.85 |
| XM_009709165 | PREDICTED: Cariana cristata bone morphogenetic protein 4 (BMP4), partial mRNA | 8.49E-41 | 196.00 | 7.85 |
| XM_008919900 | PREDICTED: Manacus vitellinus bone morphogenetic protein 4 (BMP4), mRNA | 8.49E-41 | 196.00 | 7.85 |
| XM_007072683 | PREDICTED: Chelonia mydas bone morphogenetic protein 4 (BMP4), mRNA | 8.49E-41 | 196.00 | 5.38 |
| XM_006115854 | PREDICTED: Pelodiscus amurens bone morphogenetic protein 4 (BMP4), partial mRNA | 8.49E-41 | 196.00 | 5.99 |
| XM_005437901 | PREDICTED: Falco cherrug bone morphogenetic protein 4 (BMP4), mRNA | 8.49E-41 | 196.00 | 7.85 |
| XM_005243001 | PREDICTED: Falco peregrinus bone morphogenetic protein 4 (BMP4), mRNA | 8.49E-41 | 196.00 | 7.85 |
| EU420010 | Taeniopygia guttata bone morphogenetic protein 4 mRNA, partial cds | 8.49E-41 | 196.00 | 7.85 |
| XM_011587634 | PREDICTED: Aquila chrysaetos canadensis bone morphogenetic protein 4 (BMP4), mRNA | 3.61E-39 | 190.00 | 7.85 |
| XM_010579788 | PREDICTED: Haliaeetus leucoccephalus bone morphogenetic protein 4 (BMP4), mRNA | 3.61E-39 | 190.00 | 7.85 |
| XM_010386864 | PREDICTED: Corvus corax cornix bone morphogenetic protein 4 (BMP4), mRNA | 3.61E-39 | 190.00 | 7.85 |
| XM_010284590 | PREDICTED: Phaeothorax lepturus bone morphogenetic protein 4 (BMP4), partial mRNA | 3.61E-39 | 190.00 | 7.85 |
| XM_009472699 | PREDICTED: Nipponia nippon bone morphogenetic protein 4 (BMP4), mRNA | 3.61E-39 | 190.00 | 7.85 |
| XM_009101620 | PREDICTED: Serinus canaria bone morphogenetic protein 4 (BMP4), mRNA | 3.61E-39 | 190.00 | 7.85 |
| XM_008537312 | PREDICTED: Corvus brachyrhynchos bone morphogenetic protein 4 (BMP4), mRNA | 3.61E-39 | 190.00 | 7.85 |
| XM_005328568 | PREDICTED: Pseudopodiceps humilis bone morphogenetic protein 4 (BMP4), mRNA | 3.61E-39 | 190.00 | 7.85 |
| XM_005417969 | PREDICTED: Geopelia fortis bone morphogenetic protein 4 (BMP4), mRNA | 3.61E-39 | 190.00 | 7.85 |
| XM_005495201 | PREDICTED: Zonotrichia albicollis bone morphogenetic protein 4 (BMP4), mRNA | 3.61E-39 | 190.00 | 7.85 |

Figure 26.11: *BLAST HSP Table*. The HSPs can be sorted by the different columns, simply by clicking the column heading.

The BLAST HSP Table includes the following information:

- **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 (3D view in *CLC Main Workbench* or *CLC Genomics Workbench*).

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.

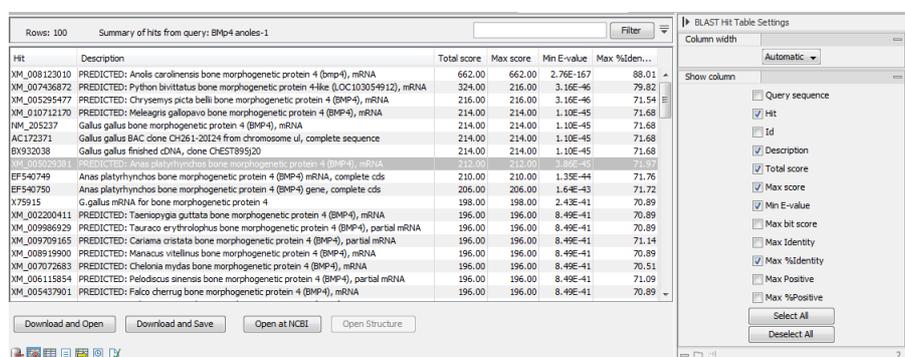
The table is integrated with the graphical view described in section 26.2.3 so that selecting a HSP in the table will make a selection on the corresponding sequence in the graphical view.

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 26.12 is an example of a BLAST Hit Table.



| Hit | Description | Total score | Max score | Min E-value | Max %Iden... |
|--------------|---|-------------|-----------|-------------|--------------|
| NM_008123010 | PREDICTED: Anolis carolinensis bone morphogenetic protein 4 (BMP4), mRNA | 662.00 | 662.00 | 2.76E-167 | 88.01 |
| NM_007936872 | PREDICTED: Python bivittatus bone morphogenetic protein 4-like (LOC103954912), mRNA | 324.00 | 216.00 | 3.16E-46 | 79.82 |
| NM_005295477 | PREDICTED: Chrysemys picta bellii bone morphogenetic protein 4 (BMP4), mRNA | 216.00 | 216.00 | 3.16E-46 | 71.54 |
| NM_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 |
| AC_123271 | Gallus gallus BAC clone CH261-20124 from chromosome u1, complete sequence | 214.00 | 214.00 | 1.10E-45 | 71.68 |
| EF932328 | Gallus gallus finished cDNA, clone CEST95920 | 214.00 | 214.00 | 1.10E-45 | 71.68 |
| NM_002929381 | PREDICTED: Scops polyborus bone morphogenetic protein 4 (BMP4), mRNA | 212.00 | 212.00 | 3.63E-45 | 71.62 |
| EF540749 | Anas platyrhynchos bone morphogenetic protein 4 (BMP4) mRNA, complete cds | 210.00 | 210.00 | 1.33E-44 | 71.76 |
| EF540750 | Anas platyrhynchos bone morphogenetic protein 4 (BMP4) gene, complete cds | 206.00 | 206.00 | 1.64E-43 | 71.72 |
| X79515 | G. gallus mRNA for bone morphogenetic protein 4 | 198.00 | 198.00 | 2.43E-41 | 70.89 |
| NM_002200411 | PREDICTED: Taeniopygia guttata bone morphogenetic protein 4 (BMP4), mRNA | 196.00 | 196.00 | 8.49E-41 | 70.89 |
| NM_00986929 | PREDICTED: Taurus erythrololus bone morphogenetic protein 4 (BMP4), partial mRNA | 196.00 | 196.00 | 8.49E-41 | 70.89 |
| NM_009709165 | PREDICTED: Cariacus cristata bone morphogenetic protein 4 (BMP4), partial mRNA | 196.00 | 196.00 | 8.49E-41 | 71.14 |
| NM_008919900 | PREDICTED: Manacus vitellinus bone morphogenetic protein 4 (BMP4), mRNA | 196.00 | 196.00 | 8.49E-41 | 70.89 |
| NM_00702863 | PREDICTED: Chelonia mydas bone morphogenetic protein 4 (BMP4), mRNA | 196.00 | 196.00 | 8.49E-41 | 70.51 |
| NM_006115854 | PREDICTED: Pelodiscus sinensis bone morphogenetic protein 4 (BMP4), partial mRNA | 196.00 | 196.00 | 8.49E-41 | 71.09 |
| NM_005437901 | PREDICTED: Falco cherrug bone morphogenetic protein 4 (BMP4), mRNA | 196.00 | 196.00 | 8.49E-41 | 70.89 |

Figure 26.12: BLAST Hit Table. The hits can be sorted by the different columns, simply by clicking the column heading.

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.
- **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.

26.3 Extract consensus sequence

You can extract a consensus sequence from a BLAST result. Clicking on the button Extract Consensus Sequence opens a dialog where you can decide how to handle regions with low coverage. The first step is to define a **threshold for when coverage is considered low**. The default value is 0, which means that low coverage is defined as no coverage (i.e. no reads align to the reference at this position). That means if you have one read covering a given position, it will only be that read that determines the consensus sequence. If you need more confidence that the consensus sequence is correct, we advise raising this value. Setting a higher low coverage threshold will require more mapped reads to construct the consensus sequence.

A consensus based on mapped reads cannot be generated in regions that meet or are below the value set for the low coverage threshold, there are several options for handling these low coverage regions:

- **Remove regions with low coverage.** When using this option, no consensus sequence is created for the low coverage regions. There are two ways of creating the consensus sequence from the remaining contiguous stretches of high coverage: either the consensus sequence is **split** into separate sequence when there is a low coverage region, or the low coverage region is simply ignored, and the high-coverage regions are directly **joined** (in this case, an annotation is added at the position where a low coverage region is removed in the consensus sequence produced, see below).
- **Insert 'N' ambiguity symbols.** This will simply add Ns for each base in the low coverage region. An annotation is added for the low coverage region in the consensus sequence produced (see below).
- **Fill from reference sequence.** This option will use the sequence from the reference to construct the consensus sequence for low coverage regions. An annotation is added for the low coverage region in the consensus sequence produced (see below).

In addition to deciding how to handle low coverage regions, you can also decide how to handle conflicts or disagreement between the reads when building a consensus sequence in regions above the low coverage threshold:

- **Vote.** Whenever the reads disagree on the base at a given position, the vote resolution will let the majority of the reads decide which base is correct. In addition, you can specify to let the voting use the base calling **quality scores** from the reads. This is done by simply adding all quality scores for each base and let the sum determine which one is correct. The base with the highest total quality scores will be chosen. If there are two bases that end up summing to the same total quality score for all reads at that location, A is preferred before

C, C before G, and G before T. An annotation with the complete information that was used to resolve the conflict will be added.

- **Insert ambiguity codes.** When this option is selected, read conflicts are addressed by using an ambiguity code representing all read bases represented at the reference location. The problem with the voting option is that it will not be able to represent true biological heterozygous variation in the data. For a diploid genome, if two different alleles are present in an almost even number of reads, only one will be represented in the consensus sequence. With the option to insert ambiguity codes, this can be solved. However, if an ambiguity code would always be inserted if just one read had a different base, there would be an ambiguity code whenever there was a sequencing error. In high-coverage NGS data that would be a big problem, because sequencing errors would be abundant. To solve this problem, you can specify a **Noise threshold**. The default value for this is 0.1 which means that for a base to contribute to the ambiguity code, it must be in at least 10 % of the reads at a given position. The **Minimum nucleotide count** specifies the minimum number of reads that are required before a nucleotide is included. Nucleotides below this limit are considered noise.
- **Use quality score.** The "Use quality score" checkbox option is available for conflicts regardless of whether "Vote" or "Insert ambiguity codes" has been selected. The "Use quality score" checkbox option allows you to use the base calling **quality scores** from the reads. This is done by simply adding all the quality scores for each base and let the sum determine which bases to consider. In other words, if quality scores are used, we will sum the quality score (instead of amount of reads) for each base on each position before applying the noise filters and finally call the consensus symbol.

Click **Next** to set the output option as shown in figure 26.13).

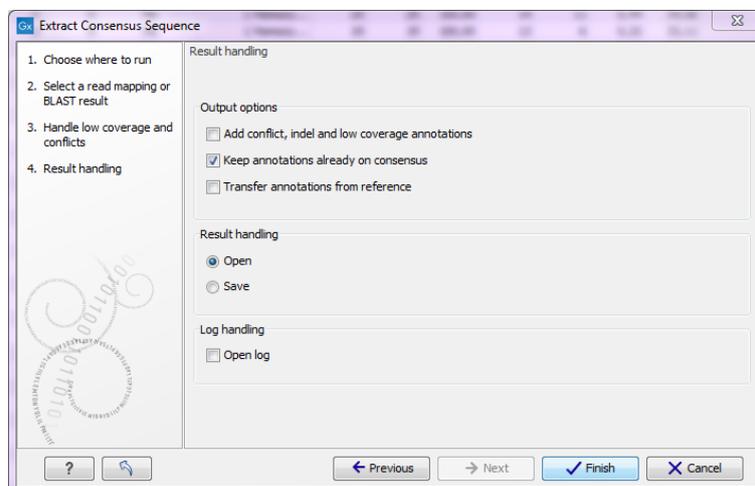


Figure 26.13: Choose to add annotations to the consensus sequence.

The annotations that can be added to the consensus sequence produced by this tool show both conflicts that have been resolved and low coverage regions (unless you have chosen to split the consensus sequence). Please note that for large data sets, this can amount to a very high number of annotations, which will cause the tool to take longer to complete, and the result will take up much more disk space.

It is also possible to transfer existing annotations to the consensus sequence produced. Please note that since the consensus sequence produced may be broken up, the annotations will also be broken up, and you cannot expect them to have the same length as before. In some cases, gaps and low-coverage regions will lead to differences in the sequence coordinates between the input data and the new consensus sequence. The annotations copied will be placed in the region on the consensus that corresponds to the region on the input data, but the actual coordinates might have changed.

Copied/transferred annotations will contain the same qualifier text as the original. That is, 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, not the new sequence, which may differ.

The resulting consensus sequence (or sequences) will have quality scores assigned if quality scores were found in the reads used to call the consensus. For a given consensus symbol X we compute its quality score from the "column" in the read mapping. Let Y be the sum of all quality scores corresponding to the "column" below X , and let Z be the sum of all quality scores from that column that supported X ¹. Let $Q = Z - (Y - Z)$, then we will assign X the quality score of q where

$$q = \begin{cases} 64 & \text{if } Q > 64 \\ 0 & \text{if } Q < 0 \\ Q & \text{otherwise} \end{cases}$$

26.4 Local BLAST databases

BLAST databases on your local system can be made available for searches via your *CLC Main Workbench*, (section 26.4.1). To make adding databases even easier, you can download pre-formatted BLAST databases from the NCBI from within your *CLC Main Workbench*, (section 26.4.2). You can also easily create your own local blast databases from sequences within your *CLC Main Workbench*, (section 26.4.3).

26.4.1 Make pre-formatted BLAST databases available

To use databases that have been downloaded or created outside the Workbench, you can either:

- Put the database files in one of the locations defined in the BLAST database manager (see section 26.5). All the files that comprise a given BLAST database must be included. This may be as few as three files, but can be more. See figure 26.14.
- Add the location where your BLAST databases are stored using the BLAST database manager (see section 26.5). See figure 26.18.

¹By supporting a consensus symbol, we understand the following: when conflicts are resolved using voting, then only the reads having the symbol that is eventually called are said to support the consensus. When ambiguity codes are used instead, all reads contribute to the called consensus and thus $Y = Z$.

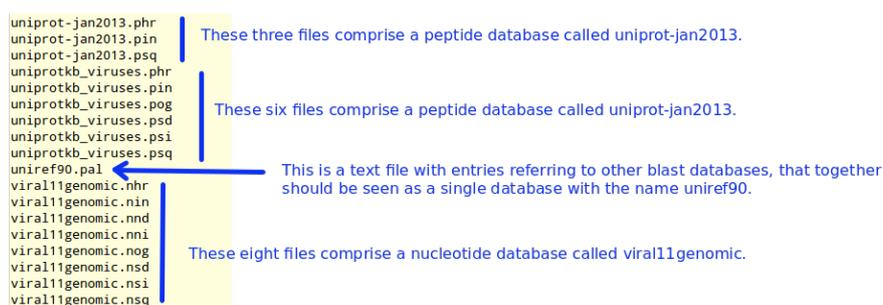


Figure 26.14: BLAST databases are made up of several files. The exact number varies and depends on the tool used to build the databases as well as how large the database is. Large databases will be split into the number of volumes and there will be several files per volume. If you have made your BLAST database, or downloaded BLAST database files, outside the Workbench, you will need to ensure that all the files associated with that BLAST database are available in a CLC Blast database location.

26.4.2 Download NCBI pre-formatted BLAST databases

Many popular pre-formatted databases are available for download from the NCBI. You can download any of the databases available from the list at <ftp://ftp.ncbi.nlm.nih.gov/blast/db/> from within your CLC Main Workbench.

You must be connected to the internet to use this tool.

To download a database, go to:

Toolbox | BLAST (📁) | Download BLAST Databases (🌐)

A window like the one in figure 26.15 pops up showing you the list of databases available for download.

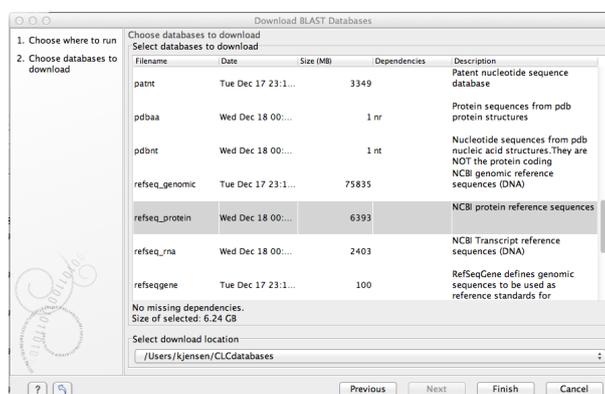


Figure 26.15: Choose from pre-formatted BLAST databases at the NCBI available for download.

In this window, you can see the names of the databases, the date they were made available for download on the NCBI site, the size of the files associated with that database, and a brief description of each database. You can also see whether the database has any dependencies. This aspect is described below.

You can also specify which of your database locations you would like to store the files in. Please see the **Manage BLAST Databases** section for more on this (section 26.5).

There are two very important things to note if you wish to take advantage of this tool.

- Many of the databases listed are very large. Please make sure you have space for them. If you are working on a shared system, we recommend you discuss your plans with your system administrator and fellow users.
- Some of the databases listed are dependent on others. This will be listed in the **Dependencies** column of the **Download BLAST Databases** window. This means that while the database you are interested in may seem very small, it may require that you also download a very big database on which it depends.

An example of the second item above is *Swissprot*. To download a database from the NCBI that would allow you to search just Swissprot entries, you need to download the whole *nr* database in addition to the entry for Swissprot.

26.4.3 Create local BLAST databases

In the *CLC Main Workbench* you can create a local database that you can use for local BLAST searches. You can specify a location on your computer to save the BLAST database files to. The Workbench will list the BLAST databases found in these locations when you set up a local BLAST search (see section 26.1.3).

DNA, RNA, and protein sequences located in the **Navigation Area** can be used to create BLAST databases from. Any given BLAST database can only include one molecule type. If you wish to use a pre-formatted BLAST database instead, see section 26.4.1.

To create a BLAST database, go to:

Toolbox | BLAST (📁) | Create BLAST Database (🛠️)

This opens the dialog seen in figure 26.16.

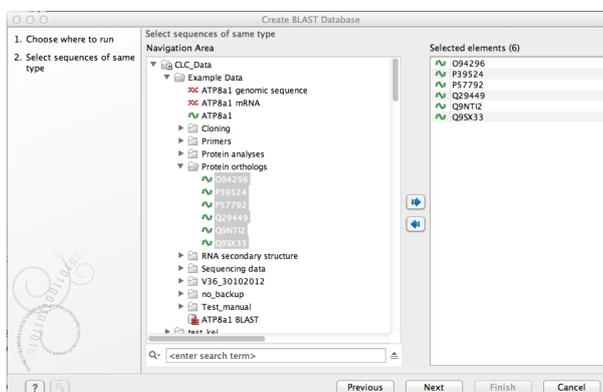


Figure 26.16: Add sequences for the BLAST database.

Select sequences or sequence lists you wish to include in your database and click **Next**.

In the next dialog, shown in figure 26.17, you provide the following information:

- **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.** You can add more details to describe the contents of the database.

- **Location.** You can select the location to save the BLAST database files to. You can add or change the locations in this list using the **Manage BLAST Databases** tool, see section 26.5.

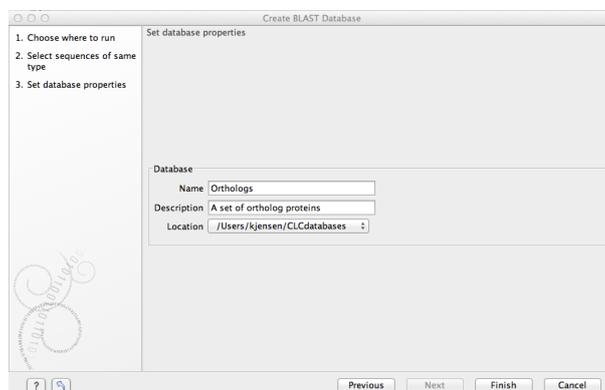


Figure 26.17: Providing a name and description for the database, and the location to save the files to.

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 26.5, and when running local BLAST (see section 26.1.3).

26.5 Manage BLAST databases

The BLAST databases available as targets for running local BLAST searches (see section 26.1.3) can be managed through the Manage BLAST Databases dialog (see figure 26.18):

Toolbox | BLAST (📁) | Manage BLAST Databases (🗑️)

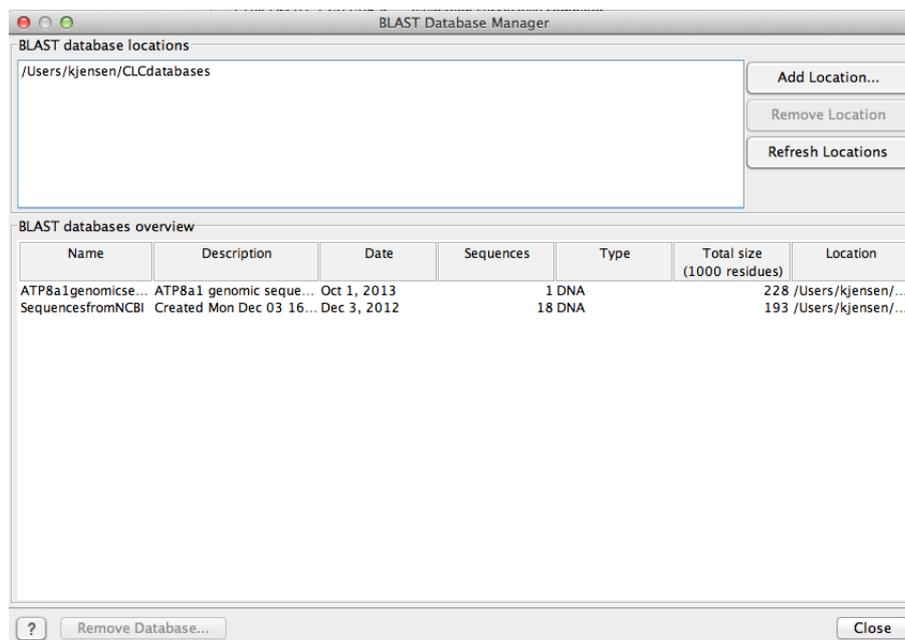


Figure 26.18: Overview of available BLAST databases.

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 from within the Workbench using the **Create BLAST Database tool**, see section 26.4.3, 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.

Note:The BLAST database location and all folders in its path should **not** have any spaces in their names on Linux or Mac systems.

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.

26.5.1 Migrating from a previous version of the Workbench

In versions released before 2011, the BLAST database management was very different from this. In order to migrate from the older versions, please add the folders of the old BLAST databases as locations in the BLAST database manager (see section 26.5). The old representations of the BLAST databases in the **Navigation Area** can be deleted.

If you have saved the BLAST databases in the default folder, they will automatically appear because the default database location used in *CLC Main Workbench 7.6.7* is the same as the default folder specified for saving BLAST databases in the old version.

26.6 Bioinformatics explained: BLAST

BLAST (Basic Local Alignment Search Tool) has become the *de facto* 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, a BLAST search identifies homologous sequences by searching one or more databases usually hosted by NCBI (<http://www.ncbi.nlm.nih.gov/>), on the query sequence of interest [McGinnis and Madden, 2004].

BLAST is an open source program and anyone can download and change the program code. This has also given rise to a number of BLAST derivatives; WU-BLAST is probably the most commonly used [Altschul and Gish, 1996].

BLAST is highly scalable and comes in a number of different computer platform configurations which makes usage on both small desktop computers and large computer clusters possible.

26.6.1 Examples of BLAST usage

BLAST can be used for a lot of different purposes. A few of them are mentioned below.

- **Looking for species.** If you are sequencing DNA from unknown species, BLAST may help identify the correct species or homologous species.
- **Looking for domains.** If you BLAST a protein sequence (or a translated nucleotide sequence) BLAST will look for known domains in the query sequence.
- **Looking at phylogeny.** You can use the BLAST web pages to generate a phylogenetic tree of the BLAST result.
- **Mapping DNA to a known chromosome.** If you are sequencing a gene from a known species but have no idea of the chromosome location, BLAST can help you. BLAST will show you the position of the query sequence in relation to the hit sequences.
- **Annotations.** BLAST can also be used to map annotations from one organism to another or look for common genes in two related species.

26.6.2 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.

After the BLAST search the user will receive a report specifying found homologous sequences and their local alignments to the query sequence.

26.6.3 How does BLAST work?

BLAST identifies homologous sequences using a heuristic method which initially finds short matches between two sequences; thus, the method does not take the entire sequence space

into account. After initial match, BLAST attempts to start local alignments from these initial matches. This also means that BLAST does not guarantee the optimal alignment, thus some sequence hits may be missed. In order to find optimal alignments, the Smith-Waterman algorithm should be used (see below). In the following, 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 26.19 for an illustration of words in a protein sequence.



Figure 26.19: 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 26.19). 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 26.20). The default scoring matrix for blastp is BLOSUM62 (for explanation of scoring matrices, see www.clcbio.com/be). The compilation of exact words and neighborhood words is then used to match against the database sequences.

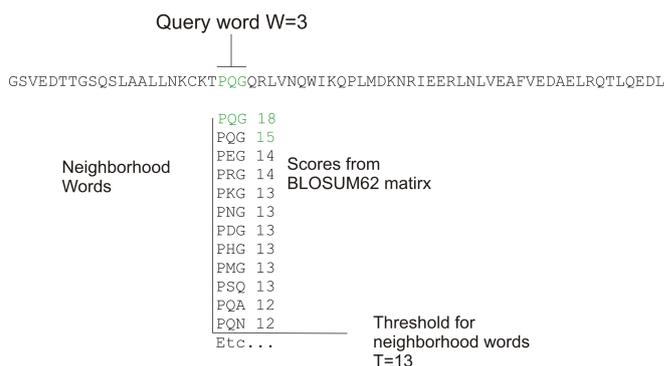


Figure 26.20: Neighborhood BLAST words based on the BLOSUM62 matrix. Only words where the threshold T exceeds 13 are included in the initial seeding.

After initial finding of words (seeding), the BLAST algorithm will extend the (only 3 residues long) alignment in both directions (see figure 26.21). 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 TLASVLDCTVTPMGSRLKRWLHMPVRDTRVLLERQQTIGA      330
  
```

Figure 26.21: 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 26.22.

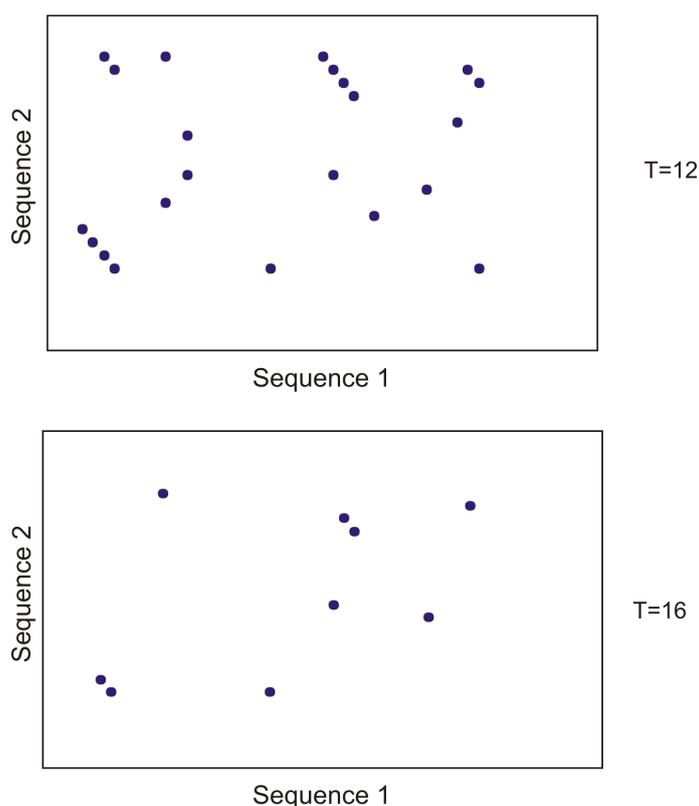


Figure 26.22: Each dot represents a word match. Increasing the threshold of T limits the search space significantly.

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.

26.6.4 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:

| 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.

26.6.5 Which BLAST options should I change?

The NCBI BLAST web pages and the BLAST command line tool offer a number of different options which can be changed in order to obtain the best possible result. Changing these parameters can have a great impact on the search result. It is not the scope of this document to comment on all of the options available but merely the options which can be changed with a direct impact on the search result.

The E-value

The *expect value* (E-value) can be changed in order to limit the number of hits to the most significant ones. The lower the E-value, the better the hit. The E-value is dependent on the length of the query sequence and the size of the database. For example, an alignment obtaining an E-value of 0.05 means that there is a 5 in 100 chance of occurring by chance alone.

E-values are very dependent 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. The default threshold for the E-value on the BLAST web page is 10. Increasing this value will most likely generate more hits. Below are some rules of thumb which can be used as a guide but should be considered with common sense.

- **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 protein is matched to the database.
- **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 homologue but it is a gray area.

- **E-value > 1** Proteins are most likely not related
- **E-value > 10** Hits are most likely junk 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 the 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 'fftflllss', 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

Change of 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.

Fortunately, the optimal search options for finding short, nearly exact matches can already be found on the BLAST web pages <http://www.ncbi.nlm.nih.gov/BLAST/>.

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. See *Bioinformatics Explained* on scoring matrices on <http://www.clcbio.com/be/>. The default scoring matrix for blastp is BLOSUM62.

26.6.6 Explanation of the BLAST output

The BLAST output comes in different flavors. On the NCBI web page the default output is html, and the following description will use the html output as example. Ordinary text and xml output for easy computational parsing is also available.

The default layout of the NCBI BLAST result is a graphical representation of the hits found, a table of sequence identifiers of the hits together with scoring information, and alignments of the query sequence and the hits.

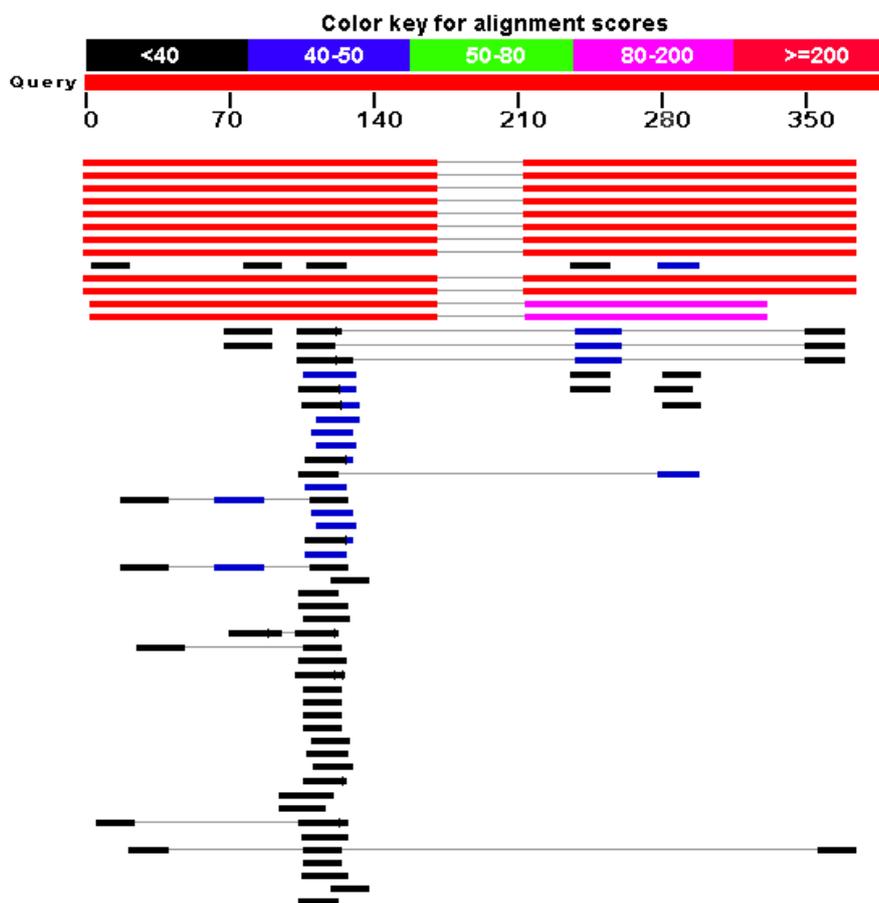


Figure 26.23: *BLAST* graphical view. A simple graphical overview of the hits found aligned to the query sequence. The alignments are color coded ranging from black to red as indicated in the color label at the top.

The graphical output (shown in figure 26.23) gives a quick overview of the query sequence and the resulting hit sequences. The hits are colored according to the obtained alignment scores.

Sequences producing significant alignments:
(Click headers to sort columns)

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident | Links |
|---------------------------------------|--|-----------|-------------|----------------|---------|-----------|---|
| Transcripts | | | | | | | |
| NM_174886.1 | Homo sapiens TGFβ-induced factor (TALE family homeobox) (TGIF) | 339 | 563 | 85% | 1e-90 | 100% | U E G M |
| NM_172210.1 | Homo sapiens TGFβ-induced factor (TALE family homeobox) (TGIF) | 339 | 563 | 85% | 1e-90 | 100% | U E G M |
| NM_172329.1 | Homo sapiens TGFβ-induced factor (TALE family homeobox) (TGIF) | 339 | 563 | 85% | 1e-90 | 100% | U E G M |
| NM_172311.1 | Homo sapiens TGFβ-induced factor (TALE family homeobox) (TGIF) | 339 | 563 | 85% | 1e-90 | 100% | U E G M |
| NM_172320.1 | Homo sapiens TGFβ-induced factor (TALE family homeobox) (TGIF) | 339 | 563 | 85% | 1e-90 | 100% | U E G M |
| NM_1723208.1 | Homo sapiens TGFβ-induced factor (TALE family homeobox) (TGIF) | 339 | 563 | 85% | 1e-90 | 100% | U E G M |
| NM_172695.2 | Homo sapiens TGFβ-induced factor (TALE family homeobox) (TGIF) | 339 | 563 | 85% | 1e-90 | 100% | U E G M |
| NM_003244.2 | Homo sapiens TGFβ-induced factor (TALE family homeobox) (TGIF) | 339 | 563 | 85% | 1e-90 | 100% | U E G M |
| NM_003246.2 | Homo sapiens thrombospondin 1 (THBS1), mRNA | 38.2 | 38.2 | 4% | 7.2 | 100% | U E G M |
| NM_172845.2 | Homo sapiens chromosome 8 open reading frame 37 (C8orf37) | 38.2 | 38.2 | 4% | 7.2 | 100% | U E G M |
| Genomic sequences [show first] | | | | | | | |
| NT_010859.14 | Homo sapiens chromosome 18 genomic contig, reference assembly | 339 | 602 | 85% | 1e-90 | 100% | |
| NW_926940.1 | Homo sapiens chromosome 18 genomic contig, alternate assembly | 339 | 602 | 85% | 1e-90 | 100% | |
| NT_011109.15 | Homo sapiens chromosome 19 genomic contig, reference assembly | 252 | 375 | 73% | 3e-67 | 94% | |
| NW_927217.1 | Homo sapiens chromosome 19 genomic contig, alternate assembly | 252 | 375 | 73% | 3e-67 | 94% | |

Figure 26.24: *BLAST* table view. A table view with one row per hit, showing the accession number and description field from the sequence file together with *BLAST* output scores.

The table view (shown in figure 26.24) provides more detailed information on each hit and furthermore acts as a hyperlink to the corresponding sequence in GenBank.

In the alignment view one can manually inspect the individual alignments generated by the *BLAST*

```

> ref|NM\_173209.1 UEGM Homo sapiens TGFB-induced factor (TALE family homeobox) (TGIF),
transcript variant 5, mRNA
Length=1382

Sort alignments for this subject sequence by:
E value Score Percent identity
Query start position Subject start position

Score = 339 bits (171), Expect = 1e-90
Identities = 171/171 (100%), Gaps = 0/171 (0%)
Strand=Plus/Plus

Query 1   ATTTGCACATGGGATTGCTAAAACAGCTTCCTGTTACTGAGATGCTTCAATGGAATACA 60
          |||
Sbjct 993  ATTTGCACATGGGATTGCTAAAACAGCTTCCTGTTACTGAGATGCTTCAATGGAATACA 1052

Query 61  GTCATTCCAAGAACTATAAACTTAAAGCTACTGTAGAAACAAGGGTTTTCTTTTTAAA 120
          |||
Sbjct 1053 GTCATTCCAAGAACTATAAACTTAAAGCTACTGTAGAAACAAGGGTTTTCTTTTTAAA 1112

Query 121 TGTTTCTGGTAGATTATTTCATAAATGTGAGATGGTCCCAATATCATGTGA 171
          |||
Sbjct 1113 TGTTTCTGGTAGATTATTTCATAAATGTGAGATGGTCCCAATATCATGTGA 1163

Score = 224 bits (113), Expect = 6e-56
Identities = 161/161 (100%), Gaps = 0/161 (0%)
Strand=Plus/Plus

Query 213 GACTGTGCAATACTTAGAGAACCCTATAGCATCTTCTCATTCCCATGTGGAACAGGATGCC 272
          |||
Sbjct 1205 GACTGTGCAATACTTAGAGAACCCTATAGCATCTTCTCATTCCCATGTGGAACAGGATGCC 1264

Query 273 CACATACTGTCTAATTAATAAAATTTCCATTTTTCAACAAAGTATGAATCTAGTTGG 332
          |||
Sbjct 1265 CACATACTGTCTAATTAATAAAATTTCCATTTTTCAACAAAGTATGAATCTAGTTGG 1324

Query 333 TTGATGCCTTTTTTTTCATGACATAATAAAGTATTTTCTTT 373
          |||
Sbjct 1325 TTGATGCCTTTTTTTTCATGACATAATAAAGTATTTTCTTT 1365

```

Figure 26.25: Alignment view of BLAST results. Individual alignments are represented together with BLAST scores and more.

algorithm. This is particularly useful for detailed inspection of the sequence hit found(sbjct) and the corresponding alignment. In the alignment view, all scores are described for each alignment, and the start and stop positions for the query and hit sequence are listed. The strand and orientation for query sequence and hits are also found here.

In most cases, the table view of the results will be easier to interpret than tens of sequence alignments.

26.6.7 I want to BLAST against my own sequence database, is this possible?

It is possible to download the entire BLAST program package and use it on your own computer, institution computer cluster or similar. This is preferred if you want to search in proprietary sequences or sequences unavailable in the public databases stored at NCBI. The downloadable BLAST package can either be installed as a web-based tool or as a command line tool. It is available for a wide range of different operating systems.

The BLAST package can be downloaded free of charge from the following location <http://www.ncbi.nlm.nih.gov/BLAST/download.shtml>

Pre-formatted databases are available from a dedicated BLAST ftp site <ftp://ftp.ncbi.nlm.nih.gov/blast/db/>. Moreover, it is possible to download programs/scripts from the same site enabling automatic download of changed BLAST databases. Thus it is possible to schedule a nightly update of changed databases and have the updated BLAST database stored locally or

on a shared network drive at all times. Most BLAST databases on the NCBI site are updated on a daily basis to include all recent sequence submissions to GenBank.

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 26.26). It is also much easier to batch download a selection of hit sequences for further inspection.

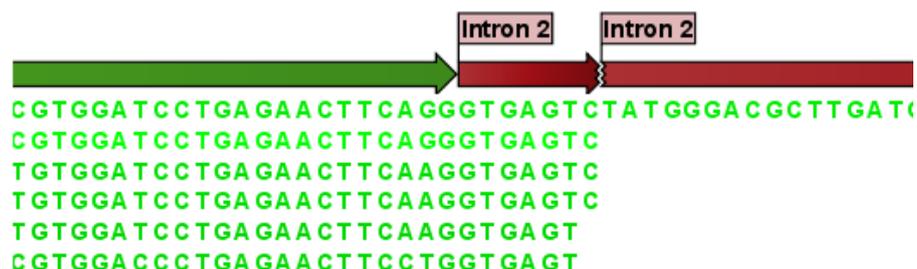


Figure 26.26: Snippet of alignment view of BLAST results from CLC Main Workbench. Individual alignments are represented directly in a graphical view. The top sequence is the query sequence and is shown with a selection of annotations.

26.6.8 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.

26.6.9 Other useful resources

The BLAST web page hosted at NCBI

<http://www.ncbi.nlm.nih.gov/BLAST>

Download pages for the BLAST programs

<http://www.ncbi.nlm.nih.gov/BLAST/download.shtml>

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/>

Explanation of scoring/substitution matrices and more

<http://www.clcbio.com/be/>

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Part IV

Appendix

Appendix A

Comparison of workbenches

Below we list a number of functionalities that differ between CLC Workbenches and the CLC Sequence Viewer:

- CLC Sequence Viewer (■)
- CLC Main Workbench (■)
- CLC Genomics Workbench (■)

| Data handling | Viewer | Main | Genomics |
|--|--------|------|----------|
| Add multiple locations to Navigation Area | | ■ | ■ |
| Share data on network drive | | ■ | ■ |
| Search all your data | | ■ | ■ |
| Assembly of sequencing data | Viewer | Main | Genomics |
| Advanced contig assembly | | ■ | ■ |
| Importing and viewing trace data | | ■ | ■ |
| Trim sequences | | ■ | ■ |
| Assemble without use of reference sequence | | ■ | ■ |
| Map to reference sequence | | ■ | ■ |
| Assemble to existing contig | | ■ | ■ |
| Viewing and edit contigs | | ■ | ■ |
| Tabular view of an assembled contig (easy data overview) | | ■ | ■ |
| Secondary peak calling | | ■ | ■ |
| Multiplexing based on barcode or name | | ■ | ■ |

| Next-generation Sequencing Data Analysis | Viewer | Main | Genomics |
|--|--------|------|----------|
| Import of 454, Illumina Genome Analyzer, SOLiD and Helicos data | | | ■ |
| Reference assembly of human-size genomes | | | ■ |
| De novo assembly | | | ■ |
| SNP/DIP detection | | | ■ |
| Graphical display of large contigs | | | ■ |
| Support for mixed-data assembly | | | ■ |
| Paired data support | | | ■ |
| RNA-Seq analysis | | | ■ |
| Expression profiling by tags | | | ■ |
| ChIP-Seq analysis | | | ■ |
| Expression Analysis | Viewer | Main | Genomics |
| Import of Illumina BeadChip, Affymetrix, GEO data | | ■ | ■ |
| Import of Gene Ontology annotation files | | ■ | ■ |
| Import of Custom expression data table and Custom annotation files | | ■ | ■ |
| Multigroup comparisons | | ■ | ■ |
| Advanced plots: scatter plot, volcano plot, box plot and MA plot | | ■ | ■ |
| Hierarchical clustering | | ■ | ■ |
| Statistical analysis on count-based and gaussian data | | ■ | ■ |
| Annotation tests | | ■ | ■ |
| Principal component analysis (PCA) | | ■ | ■ |
| Hierarchical clustering and heat maps | | ■ | ■ |
| Analysis of RNA-Seq/Tag profiling samples | | ■ | ■ |
| Molecular cloning | Viewer | Main | Genomics |
| Advanced molecular cloning | | ■ | ■ |
| Graphical display of in silico cloning | | ■ | ■ |
| Advanced sequence manipulation | | ■ | ■ |
| Database searches | Viewer | Main | Genomics |
| GenBank Entrez searches | ■ | ■ | ■ |
| UniProt searches (Swiss-Prot/TrEMBL) | | ■ | ■ |
| Web-based sequence search using BLAST | | ■ | ■ |
| BLAST on local database | | ■ | ■ |
| Creation of local BLAST database | | ■ | ■ |
| PubMed lookup | | ■ | ■ |
| Web-based lookup of sequence data | | ■ | ■ |
| Search for structures (at NCBI) | | ■ | ■ |

| General sequence analyses | Viewer | Main | Genomics |
|--|--------|------|----------|
| Linear sequence view | ■ | ■ | ■ |
| Circular sequence view | ■ | ■ | ■ |
| Text based sequence view | | ■ | ■ |
| Editing sequences | ■ | ■ | ■ |
| Adding and editing sequence annotations | | ■ | ■ |
| Advanced annotation table | | ■ | ■ |
| Join multiple sequences into one | ■ | ■ | ■ |
| Sequence statistics | ■ | ■ | ■ |
| Shuffle sequence | ■ | ■ | ■ |
| Local complexity region analyses | | ■ | ■ |
| Advanced protein statistics | | ■ | ■ |
| Comprehensive protein characteristics report | | ■ | ■ |
| Nucleotide analyses | Viewer | Main | Genomics |
| Basic gene finding | ■ | ■ | ■ |
| Reverse complement without loss of annotation | ■ | ■ | ■ |
| Restriction site analysis | ■ | ■ | ■ |
| Advanced interactive restriction site analysis | | ■ | ■ |
| Translation of sequences from DNA to proteins | ■ | ■ | ■ |
| Interactive translations of sequences and alignments | | ■ | ■ |
| G/C content analyses and graphs | | ■ | ■ |
| Protein analyses | Viewer | Main | Genomics |
| 3D molecule view | | ■ | ■ |
| Hydrophobicity analyses | | ■ | ■ |
| Antigenicity analysis | | ■ | ■ |
| Protein charge analysis | | ■ | ■ |
| Reverse translation from protein to DNA | | ■ | ■ |
| Proteolytic cleavage detection | | ■ | ■ |
| Prediction of signal peptides (SignalP) | | ■ | ■ |
| Transmembrane helix prediction (TMHMM) | | ■ | ■ |
| Secondary protein structure prediction | | ■ | ■ |
| PFAM domain search | | ■ | ■ |

| Sequence alignment | Viewer | Main | Genomics |
|---|--------|------|----------|
| Multiple sequence alignments (Two algorithms) | ■ | ■ | ■ |
| Advanced re-alignment and fix-point alignment options | | ■ | ■ |
| Advanced alignment editing options | ■ | ■ | ■ |
| Join multiple alignments into one | | ■ | ■ |
| Consensus sequence determination and management | ■ | ■ | ■ |
| Conservation score along sequences | ■ | ■ | ■ |
| Sequence logo graphs along alignments | | ■ | ■ |
| Gap fraction graphs | | ■ | ■ |
| Copy annotations between sequences in alignments | | ■ | ■ |
| Pairwise comparison | ■ | ■ | ■ |
| RNA secondary structure | Viewer | Main | Genomics |
| Advanced prediction of RNA secondary structure | | ■ | ■ |
| Integrated use of base pairing constraints | | ■ | ■ |
| Graphical view and editing of secondary structure | | ■ | ■ |
| Info about energy contributions of structure elements | | ■ | ■ |
| Prediction of multiple sub-optimal structures | | ■ | ■ |
| Evaluate structure hypothesis | | ■ | ■ |
| Structure scanning | | ■ | ■ |
| Partition function | | ■ | ■ |
| Dot plots | Viewer | Main | Genomics |
| Dot plot based analyses | | ■ | ■ |
| Phylogenetic trees | Viewer | Main | Genomics |
| Neighbor-joining and UPGMA phylogenies | ■ | ■ | ■ |
| Maximum likelihood phylogeny of nucleotides | | ■ | ■ |
| Pattern discovery | Viewer | Main | Genomics |
| Search for sequence match | ■ | ■ | ■ |
| Motif search for basic patterns | | ■ | ■ |
| Motif search with regular expressions | | ■ | ■ |
| Motif search with ProSite patterns | | ■ | ■ |
| Pattern discovery | | ■ | ■ |

| Primer design | Viewer | Main | Genomics |
|---|--------|------|----------|
| Advanced primer design tools | | ■ | ■ |
| Detailed primer and probe parameters | | ■ | ■ |
| Graphical display of primers | | ■ | ■ |
| Generation of primer design output | | ■ | ■ |
| Support for Standard PCR | | ■ | ■ |
| Support for Nested PCR | | ■ | ■ |
| Support for TaqMan PCR | | ■ | ■ |
| Support for Sequencing primers | | ■ | ■ |
| Alignment based primer design | | ■ | ■ |
| Alignment based TaqMan probe design | | ■ | ■ |
| Match primer with sequence | | ■ | ■ |
| Ordering of primers | | ■ | ■ |
| Advanced analysis of primer properties | | ■ | ■ |
| Molecular cloning | Viewer | Main | Genomics |
| Advanced molecular cloning | | ■ | ■ |
| Graphical display of in silico cloning | | ■ | ■ |
| Advanced sequence manipulation | | ■ | ■ |
| Virtual gel view | Viewer | Main | Genomics |
| Fully integrated virtual 1D DNA gel simulator | | ■ | ■ |

Appendix B

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.
 - Outside
 - Inside
- **Tick lines at.** Choosing Major ticks will show a grid behind the graph.
 - None
 - Major ticks
- **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.
- **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 **Lines and plots** below contains the following settings:

- **Dot type**

- None
- Cross
- Plus
- Square
- Diamond
- Circle
- Triangle
- Reverse triangle
- Dot

- **Dot color.** Allows you to choose between many different colors. Click the color box to select a color.

- **Line width**

- Thin
- Medium
- Wide

- **Line type**

- None
- Line
- Long dash
- Short dash

- **Line color.** Allows you to choose between many different colors. Click the color box to select a color.

For graphs with multiple data series, you can select which curve the dot and line preferences should apply to. This setting is at the top of the **Side Panel** group.

Note that the graph title and the 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 5.6).

Appendix C

BLAST databases

Several databases are available at NCBI, which can be selected to narrow down the possible BLAST hits.

C.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 <http://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 <http://www.rcsb.org/pdb/>.
- **env_nr.** Non-redundant CDS translations from env_nt entries.
- **month.** All new or revised GenBank CDS translations + PDB + SwissProt + PIR + PRF released in the last 30 days..

C.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.
- **refseq_rna.** mRNA sequences from NCBI Reference Sequence Project.
- **refseq_genomic.** Genomic 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.
- **month.** All new or revised GenBank+EMBL+DDBJ+PDB sequences released in the last 30 days.
- **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.
- **wgs.** Assemblies of Whole Genome Shotgun sequences.
- **env_nt.** Sequences from environmental samples, such as uncultured bacterial samples isolated from soil or marine samples. The largest single source is Sagarssso Sea project. This does overlap with nucleotide nr.

C.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: http://www.clcbio.com/wbsettings/NCBI_BlastNucleotideDatabases.zip
- Protein databases: http://www.clcbio.com/wbsettings/NCBI_BlastProteinDatabases.zip

Open the file you have downloaded into the `settings` folder, e.g. `NCBI_BlastProteinDatabases.properties` in a text editor and you will see the contents look like this:

```
nr[clcddefault] = 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 D

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 E

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: http://www.clcbio.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 MAC OS X, 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 F

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 <http://www.clcbio.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 [F.1](#)).

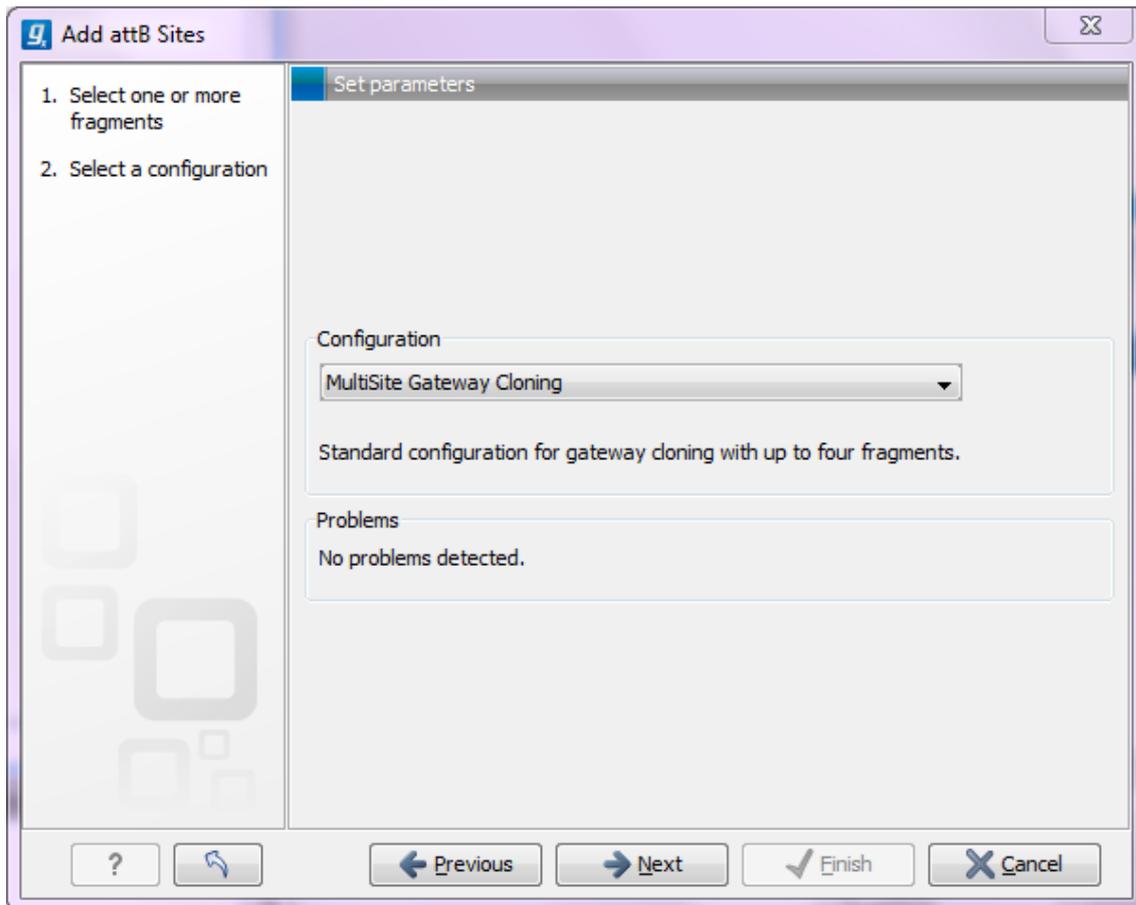


Figure F.1: Selecting between different gateway cloning configurations.

Appendix G

IUPAC codes for amino acids

(Single-letter codes based on International Union of Pure and Applied Chemistry)

The information is gathered from: http://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 Isoleucine |
| 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 |
| Z | Glx | Glutamic acid or Glutamine |
| X | Xaa | Any amino acid |

Appendix H

IUPAC codes for nucleotides

(Single-letter codes based on International Union of Pure and Applied Chemistry)

The information is gathered from: <http://www.iupac.org> and http://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 I

Formats for import and export

I.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 and trees.

I.1.1 Sequence data formats

| File type | Suffix | Import | Export | Description |
|---------------------------------|----------------|--------|--------|--|
| AB1 | .ab1 | X | | Including chromatograms |
| ABI | .abi | X | | Including chromatograms |
| 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 | .fsa/.fasta | X | X | Simple format, name & description |
| GCG sequence | .gcg | X | X | Rich information incl. annotations |
| GenBank | .gbk/.gb/.gp | X | X | Rich information incl. annotations |
| Gene Construction Kit | .gck | X | | |
| Lasergene | .pro/.seq | X | | |
| Nexus | .nxs/.nexus | X | X | |
| Phred | .phd | X | | Including chromatograms |
| PIR (NBRF) | .pir | X | X | Simple format, name & description |
| Raw sequence | any | X | | Only sequence (no name) |
| SCF2 | .scf | X | | Including chromatograms |
| SCF3 | .scf | X | X | Including chromatograms |
| 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 |
| Vector NTI archives* | .ma4/.pa4/.oa4 | X | | Archives in rich format |
| Vector NTI Database* | | X | | Special import full database |

*Vector NTI import functionality comes as standard within the CLC Main Workbench and can be installed as a plugin via the Plugins Manager of the CLC Genomics Workbench (read more in section [1.7.1](#)).

When exporting in fasta format, it is possible to remove sequence ends covered by annotations of type "Trim" (read more in section [21.2](#)).

I.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 |

I.1.3 Alignment formats

| File type | Suffix | Import | Export | Description |
|------------------|-------------|--------|--------|---|
| Aligned fasta | .fa | 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 | |

I.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 | |

I.1.5 Expression data formats

Read about technical details of these data formats in section [J](#).

| File type | Suffix | Import | Export | Description |
|-------------------------|------------|--------|--------|---------------------------------------|
| Affymetrix CHP | .chp/.psi | X | | Expression values and annotations |
| Affymetrix pivot/metric | .txt/.csv | X | | Gene-level expression values |
| Affymetrix NetAffx | .csv | X | | Annotations |
| 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 soft sample/series | .txt/.csv | X | | Expression values |
| Illumina | .txt | X | | Expression values and annotations |
| Table CSV | .csv | | X | Samples and experiments |
| Tab delimited | .txt | | X | Samples and experiments |

I.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 |

I.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 | .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 table [I.1.5 Expression data formats](#) for special cases of table imports.

I.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 Workbench and view these in the **Navigation Area**, but it is only the above mentioned formats whose *contents* can be shown in the Workbench.

I.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 [7.3](#) 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 J

Gene expression annotation files and microarray data formats

The 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 section J.5).

The workbench assumes that expression values are given at the gene level, thus probe-level analysis of e.g. Affymetrix GeneChips and import of Affymetrix CEL and CDF files is currently not supported. However, the workbench allows import of txt files exported from R containing processed Affymetrix CEL-file data (see section J.2).

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 section J.5).

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.

J.1 GEO (Gene Expression Omnibus)

The GEO (Gene Expression Omnibus) sample and series formats are supported. Figure J.1 shows how to download the data from GEO in the right format. GEO is located at <http://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
```

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,

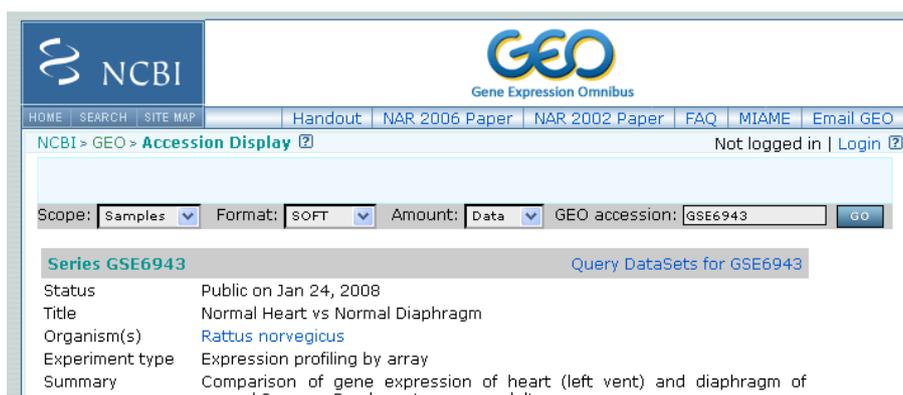


Figure J.1: Selecting Samples, SOFT and Data before clicking go will give you the format supported by the **CLC Main Workbench**.

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:

<http://www.clcbio.com/madata/GEOSampleFilesConcatenated.txt>

Below you can find examples of the formatting of the GEO formats.

J.1.1 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:

<http://www.clcbio.com/madata/GEOSampleFileSimple.txt>

J.1.2 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:

<http://www.clcbio.com/madata/GEOSampleFileAbsentPresent.txt>

J.1.3 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:

<http://www.clcbio.com/madata/GEOSampleFileAbsentPresentCallAndPValue.txt>

J.1.4 GEO sample file: using absent/present call and p-value columns for sequence information

The 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:

<http://www.clcbio.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:

<http://www.clcbio.com/madata/GEOSampleFileSimpleSequenceTagAndProbe.txt>

J.1.5 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:

<http://www.clcbio.com/madata/GEOSeriesFile.txt>

J.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 gene-level 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 gene expression levels to 'evals-txt'
```

The exported txt file (evals.txt) can be imported into the workbench using the Generic expression data table format importer (see section J.5; 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.

J.2.1 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):

<http://www.clcbio.com/madata/AffymetrixCHPandPSI.zip>

J.2.2 Affymetrix metrics 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 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 Workbench.

Download a small example sample file here:

<http://www.clcbio.com/madata/AffymetrixMetrics.txt>

J.2.3 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:

<http://www.clcbio.com/madata/AffymetrixNetAffxAnnotationFile.csv>

J.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.

J.3.1 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 Workbench. The AVG_Signal is used as the expression measure.

Download a small sample file here:

<http://www.clcbio.com/madata/IlluminaBeadChipCompact.txt>

J.3.2 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 Workbench. The AVG_Signal is used as the expression measure.

Download a small sample file here:

<http://www.clcbio.com/madata/IlluminaBeadChipExtended.txt>

J.3.3 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:

<http://www.clcbio.com/madata/IlluminaBeadStudioWithAnnotations.txt>

J.3.4 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:

<http://www.clcbio.com/madata/IlluminaBeadStudioMultipleSamples.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 workbench recognizes the 18 samples and their columns.

J.3.5 Illumina annotation files

The 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:

<http://www.clcbio.com/madata/IlluminaBeadChipAnnotation.txt>

J.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*. This can be used to annotate experiments as shown in section 25.1.3. See the complete list including download links at <http://www.geneontology.org/GO.current.annotations.shtml>.

This is an easy way to annotate your experiment with GO categories.

J.5 Generic expression and annotation data file formats

If you have your expression or annotation data in e.g. 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.

J.5.1 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 a this example as a file here:

<http://www.clcbio.com/madata/CustomExpressionData.txt>

J.5.2 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 identifiers 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:

<http://www.clcbio.com/madata/SimpleCustomAnnotation.csv>

<http://www.clcbio.com/madata/FullCustomAnnotation.csv>

To meet requirements imposed by special functionalities in the 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, ProbelD, 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 |
| Ensembl 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 | Transcript assignments |
| mma_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 K

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 <http://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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