

Ingenuity Pathway Analysis Plugin

USER MANUAL

User manual for Ingenuity Pathway Analysis 24.0.2

Windows, macOS and Linux

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This software is for research purposes only.

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Contents

1	Introduction	4
2	Uploading data to IPA using the Pathway Analysis tool	6
	2.1 Error handling	11
3	Import Expression Data	12
	3.1 Metadata and expression data matrix	14
4	Ingenuity Pathway Analysis workflows	16
	4.1 Analyze Count Matrix and Upload Comparisons to IPA	16
	4.1.1 Running the Analyze Count Matrix and Upload Comparisons to IPA Workflow	17
	4.2 Analyze Expression Data and Upload Comparisons to IPA	20
	4.2.1 Running the Analyze Expression Data and Upload Comparisons to IPA Workflow	21
5	Analyzing Data in IPA	24
6	Install and uninstall plugins	26
	6.1 Installation of plugins	26
	6.2 Uninstalling plugins	27

Introduction

The Ingenuity Pathway Analysis plugin provides the ability to upload Statistical comparison data generated using the RNA-Seq tools from CLC Genomics Workbench to Ingenuity Pathway Analysis (IPA). In addition it provides support for import of expression data provided as a count matrix.

IPA provides valuable biological insight into the results of gene expression experiments by uncovering enriched signaling and metabolic pathways, activated and inhibited upstream regulators and effects on downstream diseases, functions, and phenotypes. IPA can visualize at the isoform level for human genes.

The plugin comes with two tools and two template workflows (figure 1.1):





- The Pathway Analysis tool uploads statistical comparison data (generated by the tool Differential Expression for RNA-Seq) to IPA. The Pathway Analysis tool has been implemented to succeed on many aspects such as usage of the new IPA API, stability, error handling, and user feedback during the upload process. The tool will output one or more Statistical Comparison tracks.
- The **Import Expression Data** tool which handles import of expression count data provided as a data matrix and produces an expression track per entry in the table. The tool is

workflow enabled and requires metadata. It handles import of raw counts, TPM and RPKM.

- The template workflow **Analyze Expression Data and Upload Comparisons to IPA**, which takes expression data as input. The workflow analyzes them using the RNA-Seq Analysis tools, and submits the comparisons to IPA using the Pathway Analysis tool.
- The template workflow **Analyze Count Matrix and Upload Comparisons to IPA**, which takes an expression matrix as input. The workflow imports the counts and analyzes them using the RNA-Seq Analysis tools, and submits the comparisons to IPA using the Pathway Analysis tool.

It is possible to use gene and transcript based RNA-Seq experiments as basis for the analysis, but also microarrays from Illumina and Affymetrix are supported. You can also upload small RNA based experiments (Statistical Comparison Table format) where the seeds are most appropriate to upload.

Once the experiment data are ready, it is possible to annotate with any of the supported statistics:

- Transformed and normalized foldchange
- Baggerley's test
- Kal's Z test
- ANOVA
- edgeR

Uploading data to IPA using the Pathway Analysis tool

Launch the Pathway Analysis tool from the toolbox:

Toolbox | Ingenuity Pathway Analysis | Pathway Analysis

Use one or several statistical comparison(s) as input (A,) (figure 2.1), and click Next.

Gx	Ingenuity Pathway Anal	rsis X Select statistical analysis tracks
1.	Choose where to run	Navigation Area Selected elements (1)
2.	Select statistical	Q ▼ <enter search="" term=""> = Δ, Tumor vs. Normal</enter>
	analysis tracks	CLC_Data
з.	Set configuration	Example Data
4.	Set upload parameters	Cloning
1919	O The ansate une	Primers V
5.	Set analysis parameters	< >>
	Help Reset	Previous Next Finish Cancel



Under **Set configuration** (figure 2.2), you get the following options:

- **IPA server location** Select the IPA server relevant for your account.
- **IPA user login** Click the **Log in** button to open a new browser (or new tab) where you can log in. This gives the workbench permission to upload data to IPA on your behalf.
- **Project Name** This will be the name of the project in IPA once created. {1} will be substituted with a date stamp. It is also possible to create a custom project name by typing in the desired name in this field.
- **Upload only / Upload and analyse** Select "Upload only", if you only wish to create a dataset in IPA. Select "Upload and analyse", if you want to create an analysis from the dataset as well.

🐼 Ingenuity Pathway Analysis				
1. Choose where to run Set configuration 2. Select statistical analysis tracks IPA US server O IPA China server IPA China server				
 Set configuration Set upload parameters 	IPA user login Log in Not logged in			
5. Set analysis parameters	Project name Project {1} O Upload only Image: Configuration of the second s			
Help Reset	Previous Next Finish Cancel			

Figure 2.2: Configure the tool to upload and potentially analyze the statistical comparison data in IPA.

Click **Next** to go to the next wizard step (figure 2.3).

In this wizard step, the cutoff values for what should be uploaded to IPA can be specified. Only features that pass the cutoffs that have been specified at this step will be sent to IPA and be part of the dataset that can be seen in IPA.

Under Set upload parameters you get the following options:

- **Ignore features with mean expression values below** This value is used to filter genes/transcripts before uploading them to IPA. Features with 'Max group mean' values below this limit will not be uploaded.
- **Upload rows with value <=** Maximum p-value for feature (gene or transcript) to be uploaded. Features with a p-value above this number will not be uploaded. It is possible to choose between different types of p-Values: Standard, Bonferroni, and FDR. Note that when a feature has a standard p-Value but a missing Bonferroni or FDR p-Value, then these missing p-Values will be set to 1.0.
- **Upload rows with absolute value >=** Minimum absolute fold change for feature to be uploaded. Features with a fold change/log2 ratio below this number will not be uploaded. It is possible to choose between different types of fold changes: Fold change, and log₂-ratio.
- **Upload Summary** This summary shows how many features the tool will upload to IPA for each statistical comparison. The values are updated, when the user changes any of the upload parameters. In this way, the user can easily check the effect of the filtering (for instance to avoid setting the filters such that no features will be uploaded)

If you had selected "Upload only" in the first step, click **Finish** to start the tool. But if you had selected "Upload and analyze", click Next to see the dialog shown in figure 2.4.

Under Set analysis parameters, you get the following options:

Gx Ingenuity Pathway Analysis	×			
 Choose where to run Select statistical analysis tracks 	Set upload parameters Maximum of group mean expression upload filter Ignore features with mean expression values below 10.0			
 3. Set configuration 4. Set upload parameters 5. Set analysis parameters P-Value Standard Bonferroni FDR Upload rows with value <= 0.05 Fold change Fold change 				
	Upload rows with absolute value >= 1.0 Upload summary Element Features uploaded			
Help Reset	Tumor vs. Normal 68 Previous Next Finish Cancel			

Figure 2.3: Configure the parameters for uploading the data to IPA.

- Maximum of group mean expression analysis filter | Analysis cutoff Minimum group mean expression value for feature (gene or transcript) to be used in analysis. Features with a group mean expression value below this number will be uploaded, but will be ignored in the analysis.
- **p-Value | Analysis cutoff** Maximum p-value for feature (gene or transcript) to be used in analysis. Features with a p-value above this number will be uploaded, but will be ignored in the analysis. It is possible to choose between different types of p-values: Standard, Bonferroni, and FDR. Note that when a feature has a standard p-Value but a missing Bonferroni or FDR p-Value, then these missing p-Values will be set to 1.0.
- **Fold change | Analysis cutoff** Minimum absolute fold change for feature to be used in analysis. Features with a fold change/log₂ ratio below this number will be uploaded, but will be ignored in the analysis. It is possible to choose between different types of fold changes: Fold change, and log₂-ratio.
- **Fold change | Automatically calculate fold change cutoff** Automatically calculate fold change when uploading observation. The fold change cutoff will be set so that the number of features to include in the analysis gets as close to the targeted number as possible (see below). When this option is used, it is not necessary to set the "Fold change | Analysis cutoff", since it is automatically calculated by the tool for each statistical comparison. When using this option, the fold change analysis cutoff can be different for each statistical comparison.

Gx Ingenuity Pathway Analysis	×			
 Choose where to run Select statistical analysis tracks 	Set analysis parameters Maximum of group mean expression analysis filter Analysis cutoff 0.0			
 Set configuration Set upload parameters Set analysis parameters 	P-Value Standard Donferroni FDR			
6. Set analysis reference data	Analysis cutoff 0.05			
Fold change Automatically calculate fold change cutoff Target number of analysis features 3,000 Fold change Log2 ratio Analysis cutoff 1.5				
Upload and analysis summary				
Element Features uploaded Features analyz Tumor vs. Normal 68 61				
Help Reset	Previous Next Finish Cancel			

Figure 2.4: Set the parameters for the analysis of the data in IPA.

- **Fold change | Target number of analysis features** Enabled only when using automatically calculated fold change. The fold change cutoff will be set so that the number of features to include in the analysis gets as close to the targeted number as possible
- **Upload and analysis summary** This summary shows how many features the tool will upload to IPA for each statistical comparison, and how many features that will be included in each analysis. The values are updated when the user changes any of the analysis parameters. In this way, the user can easily check the effect of the filtering (e.g. avoid setting the filters such that no features will be analyzed).

The **Upload and analysis summary** table at the bottom of the dialog warns the user when too restrictive filters have been set (figure 2.5).

Click Next to choose the reference as seen in figure 2.6.

The reference can be:

- Ingenuity Knowledge Base (Genes only) IPA recognizes gene ids for several species, see https://qiagen.my.salesforce-sites.com/KnowledgeBase/KnowledgeNavigatorPage?id=kA41i000000L6BTCA0&catego
 IPA for a full list. Successive uploads to IPA are attempted, until the upload is successful:
 - If the statistical comparison contains gene ids from a recognized database (Ensembl, Entrez, Hugo, or RefSeq), the ids are uploaded to IPA using the corresponding IPA

🐼 Ingenuity Pathway Analysis 🛛 🕹				
1. Choose where to run	Set analysis parameters Maximum of group mean expression analysis filter			
 Select statistical analysis tracks 	Analysis cutoff 0.0			
3. Set configuration	P-Value			
4. Set upload parameters) Bonferroni			
5. Set analysis parameters	⊖ FDR			
6. Set analysis reference data	Analysis cutoff 0.05			
	Fold change			
	Automatically calculate fold change cutoff			
	Fold change			
	⊖ Log ₂ ratio			
	Analysis cutoff 800000			
	Upload and analysis summary			
	Element Features uploaded Features analyzed			
	Tumor vs. Normal 68 U			
Help Reset	Previous Next Finish Cancel			

Figure 2.5: A warning highlight in red analyses for which the cutoff is too restrictive.

Gx	Ingenuity Pathway Analys	is	×
1. 2. 3. 4. 5.	Choose where to run Select statistical analysis tracks Set configuration Set upload parameters Set analysis parameters Set analysis reference data	Set analysis reference data Select reference data for analysis Reference set Ingenuity Knowledge Base (Genes Only) Ingenuity Knowledge Base (Genes Only) Uploaded dataset	
	Help Reset	Previous Next Finish Cancel	

Figure 2.6: Choose the reference to be used for the analysis of the data in IPA.

identifier type. Otherwise, the 'Name' column is uploaded with IPA identifier types for Ensembl, Entrez, GenBank, miRBase (mature) and RefSeq.

 If the previous upload fails, it could be because the gene identifiers of the uploaded species are not supported by IPA. Gene names are often conserved across species, so uploads are attempted with gene names formatted according to the IPA human (upper case e.g. BRCA1) or mouse/rat (capitalized e.g. Brca1) gene names formats, using the corresponding IPA gene symbol identifier types. Uploads are performed in decreasing order of the number of unformatted gene names matching the the human and mouse/rat formats.

Note that upload is successful even if just one gene has been successfully identified by IPA. All performed uploads and their error messages from IPA for failed uploads are written to the log.

If all upload attempts fail, the upload errors from IPA will be displayed. The error "The identifier type that you selected may be incorrect. [...]" indicates that the species is not supported by IPA and the gene names did not match any of the human, mouse and rat genes.

• Uploaded dataset The data uploaded will be analyzed using itself as a reference.

Click **Finish** to start the tool.

2.1 Error handling

Concurrent IPA sessions

When submitting data to IPA, the upload may fail, if several sessions are established concurrently using the same username. This can happen when the IPA application is running while data is being uploaded using the IPA tool, or if the IPA tool is part of a workflow with several IPA uploads running at the same time.

To deal with this issue, the IPA tool has a retry functionality, such that the upload is attempted again, if it failed due to a problem with concurrent sessions. The waiting time between retry attempts is increased with a random factor for each attempt, to avoid that two processes continue to block each other.

Upload multiple statistical comparisons

If the IPA tool encounters an error when uploading multiple statistical comparisons, it will in most case continue uploading the remaining statistical comparisons. However, if the tool gets one of the errors below, it stops uploading immediately, because it cannot expect that they will be resolved before the next upload:

- Login error (invalid or expired login secret)
- User agreement not accepted
- License expired
- Upload limit exceeded
- Analysis limit exceeded

Import Expression Data

Import Expression Data enables import of individual expression tracks from an expression data matrix. The data matrix needs to conform to the following formatting:

- The matrix should be constructed in Excel or csv format.
- Columns represent samples and rows represent genes. See figure 3.1 for an example of correct formatting.
- Feature ID (gene ID or transcript ID) should be in the first column and samples in the following.
- Only one feature ID is supported. It should be unique, i.e. Ensembl or geneID, not a mixture.
- Three types of expression values are supported: Raw counts, TPM, an RPKM. Only one of these values should be supplied. We recommend to use raw counts when available.
- If the matrix has been filtered for low count entries before upload, the provided calculation
 of TPM or RPKM needs to be on the filtered matrix as well, otherwise the counts will not be
 properly translated.
- Import of other normalization types are not supported.

To launch the Import Expression Data tool, go to:

Toolbox | Ingenuity Pathway Analysis | Import Expression Data

Figure 3.2 shows the Import Expression Data dialog.

In the Expression Data section of the dialog that opens, first select the data matrix by using the **Browse** button.

Select the expression values that matches the expression data type. All value types must be non-negative values:

- Counts
- TPM

	А	В	С	D	E
1	Name	23T	23N	26T	26N
2	DDX11L1	0	0.09587955	0	0
3	WASH7P	0.53207257	0.73096019	0.67368009	0.21937677
4	MIR1302-10	0	0	0	0
5	FAM138A	0.12064385	0.06905845	0.904455	0
6	OR4G4P	0	0	0	0
7	OR4G11P	0	0	0	0
8	OR4F5	0	0	1.12094619	0
9	RP11-34P13.7	0.14422163	0.02358707	1.66816069	0.02548434
10	RP11-34P13.8	0.61323481	0	0.50153024	0
11	CICP27	0	0	0	0
12	AL627309.1	1.18823541	1.5185073	0.80103692	3.2129422
13	RP11-34P13.15	0	0	0	0
14	RP11-34P13.16	0	0	0	0

Figure 3.1: RPKM count matrix using Ensembl gene names and representing 4 samples in a Tumor Normal design.

• RPKM

When selecting TPM or RPKM, the expected minimum count must be specified. The value must be the smallest count value that was present in the expression matrix when calculating the TPMs or RPKMs values. In unfiltered data this value will typically be 1 (default).

Under References, specify how expression values were generated. This is for defining whether it was generated as a gene or transcript matrix as well as to specify how the TPM/RPKM were calculated.

- **Genes with accompanying transcripts** Matches imported values against genes. Transcripts are used for identifying exon length when translating between counts and TPM/RPKM.
- **Genes** Matches imported values against genes. Gene length are used when translating between counts and TPM/RPKM.
- **Transcripts** Matches imported values against transcripts and uses exon length when translating between counts and TPM/RPKM.

The key is that you specify the Gene and mRNA tracks that were used to generate the expression values. When selecting **Genes with accompanying transcripts** as parameter you can choose to calculate expression for genes without transcript. This will result in the generation of a transcript that is expected to have the length of the full gene. Enabling this option allows calculation of TPM and RPKM when counts have been supplied.

At the bottom of the dialog, specify how unmatched genes or transcripts should be handled. An unmatched gene/transcript is either not found or ambiguous in the provided track. Unmatched gene/transcripts can be ignored or cause the import to fail. When importing raw counts, they can also be included. However, when importing TPM or RPKM, a match in the track is needed for translating the expression to counts.

The Import Expression Data tool outputs one expression track per samples.

Gx Import Expression Da	ta			
1. Choose where to run	Parameters Expression Data			
2. Parameters	Table file Count_Tumor_Normal.xlsx Browse			
3. Result handling	Table has headers			
	Counts			
	ОТРМ			
	Minimum count 1			
	References			
 Genes with accompanying transcripts 				
◯ Genes				
	◯ Transcripts			
	Gene track 🚓 Homo_sapiens_ensembl_v99_hg38_no_alt_analysis_set_Genes 😡			
mRNA track 🔆 Homo_sapiens_ensembl_v99_hg38_no_alt_analysis_set				
Ce.	Calculate expression for genes without transcripts			
(US)	Unmatched genes/transcripts			
Martin Contractor				
11	Ignore			
10 1	◯ Fail			
P O MANANAUM				
Help Res	set Previous Next Finish Cancel			

Figure 3.2: Parameters available in the Import Expression Data tool. Select the Table file containing the expression matrix and select the type of data matching the values in the file (in this case it contains count data). Add references to import against appropriate gene or transcript annotations. Select how to handle unmatched genes or transcripts.

3.1 Metadata and expression data matrix

The expression data matrix can be accompanied with metadata in the form of another Excel or CSV file. A header row must be present in the expression matrix to link the sample to the identifier in the metadata. The link needs to be an exact match.

Importing metadata is however optional.

- When running in a workflow It is possible to skip metadata import and still iterate over the samples, in which case the iteration will be for each imported expression track. If the import connects to tools that requires metadata, e.g., Differential Expression for RNA-Seq, then metadata is required. Note that importing metadata directly from the Import Expression Data tool is only available when running in a workflow.
- When running tool first import the expression tracks and then create and associate metadata using the Import Metadata tool.

For an example of a metadata table that matches the expression matrix described above, see figure 3.3.

	А	В	С
1	Sample	group	Treatment
2	26T	Tumor	0
3	23T	Tumor	1
4	26N	Normal	0
5	23N	Normal	0

Figure 3.3: Metadata describing the samples from figure 3.1.

An example of a workflow using the Import Expression Data tool is described in the next section.

Ingenuity Pathway Analysis workflows

Ingenuity Pathway Analysis Plugin includes two workflow, Analyze Count Matrix and Upload Comparisons to IPA and Analyze Expression Data and Upload Comparisons to IPA. The workflow takes expression data provided either as single samples or as a expression matrix, and analyzes them using the tools in the RNA-Seq Analysis folder. It then submits the comparison to IPA using the Pathway Analysis tool.

You find the workflows among the Template Workflows in the toolbox, lower right corner of the CLC Genomics Workbench. The workflow is located in the Ingenuity Pathway Analysis Workflow folder as shown in figure 4.1 below:



Figure 4.1: Location of the template workflows in the Toolbox.

4.1 Analyze Count Matrix and Upload Comparisons to IPA

The workflow Analyze Count Matrix and Upload Comparisons to IPA imports expression data from an Expression count matrix, and analyzes them using the tools in the RNA-Seq Analysis folder. It then submits the comparison to IPA using the Pathway Analysis tool.

The purpose of the workflow is to make it as easy as possible for the user to get from Sample to Insight. The user only has to provide the expression count matrix, sample metadata as well as mRNA and Genes as input, and the workflow performs import, statistical analyses and data interpretation using capabilities available via CLC Genomics Workbench and IPA.

Opened in the workflow editor, the workflows looks like this (see figure 4.2 below):

The expression data from the count matrix data are imported by the Import Expression Data tool that splits each sample into a track. The expression tracks are then sent to three tools:

• Create Heat Map for RNA-Seq The tool creates a two dimensional heat map of expression



Figure 4.2: Layout of the Analyze Count Matrix and Upload Comparisons to IPA workflow.

values. Each column corresponds to one sample, and each row to a feature (a gene or a transcript). The samples and features are both hierarchically clustered.

- **Differential Expression for RNA-Seq** The tool performs a statistical differential expression test for a set of Expression Tracks. It's outputs are used as inputs for the IPA tool and for Create Venn Diagram for RNA-Seq (see below).
- **PCA for RNA-Seq** The tool creates a PCA plot, which is a projection of a high-dimensional dataset (where the number of dimensions equals the number of genes or transcripts) onto two of three dimensions. This helps in identifying outlying samples for quality control, and gives a feeling for the principal causes of variation in a dataset.

The outputs from the tools are saved in the chosen output folder for the workflow. The outputs from the Differential Expression for RNA-Seq tool are furthermore used for processing by these two tools:

- Pathway Analysis The tool uploads the comparisons to IPA. See chapter 2 for details.
- **Create Venn Diagram for RNA-Seq** The tool makes it possible to compare two or more statistical comparison tracks. The Venn diagram comparison visualizes the overlap between the differentially expressed genes or transcripts in the selected statistical comparison tracks. The genes considered to be differentially expressed can be controlled by setting appropriate p-value and fold change thresholds.

4.1.1 Running the Analyze Count Matrix and Upload Comparisons to IPA Workflow

The workflow can be started from the toolbox, or by using the Launch button (Q).

In the first step choose the reference data, if default is selected no elements are configured and you will have to fill in the desired reference elements in the next two steps. Note, that you should be very specific with the chosen reference data that needs to match the genes in the Expression matrix that is imported, otherwise you might lose genes that cannot be matched on import.

Next select the tables file with the expression data and specify what type of count data you provide (raw count, TPM or RPKM are allowed) as well as how they were processed. Finally, select the metadata table. See figure 4.3.

Gx Analyze Count Matrix and Upload Comparisons to IPA					
1. Choose where to run	Import Expression Data Configurable Parameters				
2. Select reference data set	Table file Count_Tumor_Normal.xlsx Browse				
3. Genes	Type of values Counts ~				
4. mRNA	Type of values Genes with accompanying transcripts 🗸				
5. Import Expression Data	Calculate expression for genes without transcripts Unmatched genes/transcripts Ignore V				
6. Differential Expression for RNA-Seg	Metadata file Count_Tumor_Normal.xlsx Browse				
7. Pathway Analysis	Locked Settings				
8. Result handling					
9. Save location for new elements					
Help Reset	Previous Next Finish Cancel				

Figure 4.3: Selecting table file to import including specifying type of value (Gene/TPM/RPKM) and Gene/Transcript, as well as choosing the location of the sample metadata file.

Metadata is required for organization of the imported counts and essential to the experimental design in the Differential Expression for RNA-Seq tool. In addition it and can be used to add extra layers of insight in the Create Heat Map and PCA for RNA-Seq tools. To learn more about how to create a metadata table, how to import a metadata table, or how to associate data elements with metadata, see http://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Metadata.html.

Following this, the parameters for the Differential Expression for RNA-Seq need to be specified, see figure 4.4.

Specify the following

Test differential expression due to Select the factor to be tested for differential expression.

while controlling for Select the factor to be controlled for.

- **Comparisons** Select groups to be compared. It is possible to choose between "Across groups", "All group pairs", and "Against control group".
- **Control group** If "Against control group" was selected in "Comparisons", a control group must be selected.

In the next step, the parameters for the Pathway Analysis tool need to be set. Setting the parameters in a workflow context is not as user-friendly as when run stand-alone, because the parameters from the different pages are grouped on the same page in workflow execution. To help clarify this, it is indicated in figure 4.5 which parameters pertain to the IPA upload, and which pertain to the IPA analysis.

Gx	🐼 Analyze Count Matrix and Upload Comparisons to IPA 🛛 🗙 🗙						
	Differential Expression for RNA-Seq						
1.	Choose where to run	Configurable Parameters					
2.	Select reference data set	Test differential expression due to group	•				
3.	Genes	While controlling for Treatment	•				
4.	mRNA	Comparisons Against control group	\sim				
5.	Import Expression Data	Control group Normal	•				
6.	Differential Expression for RNA-Seq	Locked Settings					
7.	Pathway Analysis						
8.	Result handling						
9.	Save location for new elements						
	Help Reset	Previous Next Finish Canc	el				

Figure 4.4: Set up the Differential Expression analysis by selecting metadata and choosing parameters.

In the final step, standard result handling is performed: The selected parameters can be previewed, and an output location must be chosen.

Gx Analyze Count Matrix and Upload Comparisons to IPA X							
1 Choose where to run							
1. Choose where to run	Configurable Parameters						
2. Select reference data set	IPA Server IPA US server	~					
3. Genes	IPA user login Log in Not logged in	l i i i i i i i i i i i i i i i i i i i					
4. mRNA	Project name Project {1}						
5 Import Expression Data	Analysis type Upload and analyze	\sim					
	Ignore features with mean expression values below 10.0						
 Differential Expression for RNA-Seq 	P-value type Upload Standard	~					
	Upload rows with value <= parameters 0.05						
7. Pathway Analysis	Upload rows with absolute value >= 1.0						
8. Result handling	Analysis cutoff 0.0						
9. Save location for new	P-value type Analysis Standard	~					
elements	Analysis cutoff 0.05						
() e	Automatically calculate fold change cutoff						
(CS)	Target number of analysis features 3,000						
The second second	Fold change type Fold change	~					
01	Analysis cutoff 1.5						
10	Reference set Ingenuity Knowledge Base	(Genes Only) ${\scriptstyle\bigvee}$					
ALL MARKAGENER CONTRACTOR	Locked Settings						
Help Reset	Previous Next Finish	Cancel					

Figure 4.5: Selecting the Pathway Analysis tool parameters for upload to IPA and analysis in IPA.

4.2 Analyze Expression Data and Upload Comparisons to IPA

The workflow Analyze Expression Data and Upload Comparisons to IPA takes expression data as input, and analyzes them using the tools in the RNA-Seq Analysis folder. It then submits the comparison to IPA using the Pathway Analysis tool.

The purpose of the workflow is to make it as easy as possible for the user to get from Sample to Insight. The user only has to provide expression data as input, and the workflow generates all available statistical analyses and data interpretation capabilities available via CLC Genomics Workbench and IPA.

Opened in the workflow editor, the workflows looks like this (see figure 4.6 below):

The expression tracks are sent to three tools:

- **Create Heat Map for RNA-Seq** The tool creates a two dimensional heat map of expression values. Each column corresponds to one sample, and each row corresponds to a feature (a gene or a transcript). The samples and features are both hierarchically clustered.
- **Differential Expression for RNA-Seq** The tool performs a statistical differential expression test for a set of Expression Tracks. Its outputs are used as inputs for the IPA tool and for Create Venn Diagram for RNA-Seq (see below).
- **PCA for RNA-Seq** The tool creates a PCA plot, which is a projection of a high-dimensional dataset (where the number of dimensions equals the number of genes or transcripts) onto



Figure 4.6: Layout of the Analyze Expression Data and Upload Comparisons to IPA workflow

two of three dimensions. This helps in identifying outlying samples for quality control, and gives a feeling for the principal causes of variation in a dataset.

The outputs from the tools are saved in the chosen output folder for the workflow. The outputs from the Differential Expression for RNA-Seq tool are furthermore used for processing by these two tools:

- Pathway Analysis The tool uploads the comparisons to IPA. See chapter 2 for details.
- Create Venn Diagram for RNA-Seq The tool makes it possible to compare two or more statistical comparison tracks. The Venn diagram comparison visualizes the overlap between the differentially expressed genes or transcripts in the selected statistical comparison tracks. The genes considered to be differentially expressed can be controlled by setting appropriate p-value and fold change thresholds.

4.2.1 Running the Analyze Expression Data and Upload Comparisons to IPA Workflow

The workflow can be started from the toolbox, or by using the Launch button (Q).

Choose the expression data to be analyzed and uploaded (see figure 4.7).

Gx Analyze Expression Data and Upload Comparisons to IPA X						
1. Choose where to run	Select input for Expression Tracks					
2. Select Expression Tracks	Select files for import: CLC Format	\sim				
 Differential Expression for RNA-Seq 	Navigation Area Selected elements (5)					
4. Pathway Analysis	Q ▼ <enter search="" term=""> The search term> The searc</enter>					
5. Result handling	2: SRR 1543488 (GE) 2: SRR 1543532 (GE) 2: SRR 154368 (TE)					
 Save location for new elements 	SRR 1543627 (GE)					
	Batch					
Help Reset	Previous Next Finish Cancel					

Figure 4.7: Selecting input parameters in the IPA plugin workflow.

Following this, the parameters for the Differential Expression for RNA-Seq need to be specified (see figure 4.8):

Gx	Gx Analyze Expression Data and Upload Comparisons to IPA X							
1.	Choose where to run	Differential Expression for RNA-Seq						
2.	Select Expression Tracks	Metadata table	😥 Samples	à				
3.	Differential Expression for RNA-Seq	Test differential expression due to	Sample type	•				
4.	Pathway Analysis	While controlling for	Patient_ID	*				
5.	Result handling	Control group	N	•				
6.	Save location for new elements	 Locked Settings 						
	Help Reset Previous Next Finish Cancel							

Figure 4.8: Selecting parameters for Differential Expression for RNA-Seq.

- **Metadata table** Select a metadata object that associates the selected input objects to metadata used by the RNA-Seq analysis.
- Test differential expression due to Select the factor to be tested for differential expression.
- **Comparisons** Select groups to be compared. It is possible to choose between "Across groups", "All group pairs", and "Against control group".
- **Control group** If "Against control group" was selected in "Comparisons", a control group must be selected.

An example of a metadata table is shown in figure 4.9.

I Samples ×				
Rows: 6				Filter Ţ
Read_ID	Patient_ID	Sample_ID	Sample type	Cancer
23N_R1	23	23N	N	Normal
23T_R1	23	23T	т	Esophagus
26N_R1	26	26N	N	Normal
26T_R1	26	26T	т	Esophagus
27N_R1	27	27N	N	Normal
27T_R1	27	27T	т	Esophagus

Figure 4.9: An example of a metadata table.

Metadata is required when defining the experimental design in the Differential Expression for RNA-Seq tool, and can be used to add extra layers of insight in the Create Heat Map and PCA for RNA-Seq tools. To learn more about how to create a metadata table, how to import a metadata table, or how to associate data elements with metadata, see http://resources.giagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Metadata.html.

In the next step, the parameters for the Pathway Analysis tool need to be set. Setting the parameters in a workflow context is not as user-friendly as when run stand-alone, because the

parameters from the different pages are grouped on the same page in workflow execution. To help clarify this, it is indicated in figure 4.10 which parameters pertain to the IPA upload, and which pertain to the IPA analysis.

Gx Analyze Count Matrix and	I Upload Comparisons to IPA	×				
Pathway Analysis						
1. Choose where to run	Configurable Parameters					
2. Select Expression Tracks	IPA Server	IPA US server \sim				
3. Differential Expression	IPA user login	Log in Not logged in				
for RNA-Seq	Project name	Project {1}				
4. Pathway Analysis	Analysis type	Upload and analyze \sim				
5. Result handling	Ignore features with mean expression values below	10.0				
6. Save location for new	P-value type Upload	Standard 🗸				
elements	Upload rows with value <= parameters	0.05				
	Upload rows with absolute value >=	1.0				
	Analysis cutoff	0.0				
	P-value type Analysis	Standard 🗸				
0	Analysis cutoff parameters	0.05				
() ()	Automatically calculate fold change cutoff					
(US)	Target number of analysis features	3,000				
Margal and	Fold change type	Fold change \checkmark				
01	Analysis cutoff	1.5				
0	Reference set	Ingenuity Knowledge Base (Genes Only) $$				
The second	 Locked Settings 					
Help Reset	Previous	Next Finish Cancel				

Figure 4.10: Selecting the Pathway Analysis tool parameters for upload to IPA and analysis in IPA.

In the final step, standard result handling is performed: The selected parameters can be previewed, and an output location must be chosen.

Analyzing Data in IPA

Ingenuity Pathway Analysis enables you to analyze and visualize RNA-Seq datasets, eliminating the obstacles between data and biological insight. For example, IPA can predict upstream regulation and downstream outcomes from your expression data, and identify relevant signaling and metabolic pathways. Figure 5.1 shows a newly opened analysis in IPA.

§ IPA	A ready to the field of the second second					
<u>File E</u> dit <u>V</u> iew <u>W</u> indow <u>H</u> elp						
NEW 8		Genes and Chemicals	Diseases and Functions	Pathways and Tox Lists	SEAR	H Advanced Search
Project Manager 🛛 🛛 🕅	Normal vs. Tumor (TE) 2016-03-03 02:41:06					් ව
A-Z SORT SEARCH REFRESH	Summary Canonical Pathways Upstream Ana	ysis 🛛 Diseases & Function	s \ Regulator Effects \ Netw	orks \Lists \My Pathways \N	Molecules \	
Project 2016-03-03 test						Export: 🔯 🔯 💼
Analyses	> Analysis Settings					
Normal vs. Tumor (TE) 2016-03-03 02:41:06 Definition Analyses	V Top Canonical Pathways					
Biomarker Filter Results	Name				n-value	Overlap
Biomarker Comparison Analyses MicroRNA Target Filter Results	FXR/RXR Activation				• 1.96E-15	30.2 % 38/126
BioProfiler Results	Atherosclerosis Signaling				3 96F-14	29.0 % 36/124
- 🛅 IsoProfiler Results	I PS/II -1 Mediated Inhibition of RXR Fund	tion			• 1.01E-13	22 3 % 49/220
	LXB/RXR Activation				. 811E-11	25.6 % 31/121
My Lists	Acute Phase Perpage Signaling				2,625.00	20,7 % 25/160
				1 2 3 4 5 6 7	8.9 >	20,0 70 33,203
	✓ Top Upstream Regulators					
	Upstream Regulator				p-value of overlap	Predicted Activation
	TGFB1				 1,42E-25 	Inhibited
	IL1B				 5,09E-24 	Inhibited
	TNF				 1,00E-23 	Inhibited
	lipopolysaccharide				 2,34E-23 	Inhibited
	HNF1A				 6,59E-23 	Inhibited
				1 2 3 4 5 6 7	89 >	
	\sim Top Diseases and Bio Functions					
	$\scriptstyle{lash}$ Diseases and Disorders					
	Name				p-value range	# Molecules
	Cancer				9,56E-05 - 3,67E-39	1316
	Organismal Injury and Abnormalities				9,56E-05 - 3,67E-39	1331
	Dermatological Diseases and Conditions			100.00	+ 7,10E-05 - 2,70E-35	789
	Gastrointestinal Disease				9,56E-05 - 2,37E-23	1128
	Immunological Disease			- C.A.A.	8,16E-05 - 9,69E-19	232
				1 2 3 4 5 6 7	80 >	
	Molecular and Cellular Functions					
	Name				p-value range	# Molecules
	Vame Cellular Movement				p-value range 6,91E-05 - 2,41E-25	# Molecules 370

Figure 5.1: The first window when opening an analysis in IPA

Figure 5.2 shows some of the visual capabilities of IPA.

For more information about IPA, please visit https://digitalinsights.qiagen.com/ products-overview/discovery-insights-portfolio/analysis-and-visualization/



Figure 5.2: Another IPA application screenshot

qiagen-ipa/.

Install and uninstall plugins

Ingenuity Pathway Analysis is installed as a plugin.

6.1 Installation of plugins

Note: In order to install plugins and modules, the *CLC Workbench* must be run in administrator mode. On Windows, you can do this by right-clicking the program shortcut and choosing "Run as Administrator". On Linux and Mac, it means you must launch the program such that it is run by an administrative user.

Plugins and modules are installed and uninstalled using the Workbench Plugin Manager. To open the Plugin Manager, click on the **Plugins (button** in the top Toolbar, or go to the menu option:

Utilities | Manage Plugins... (💕)

The Plugin Manager has two tabs at the top:

- **Manage Plugins** An overview of your installed plugins and modules is provided under this tab.
- **Download Plugins** Plugins and modules available to download and install are listed in this tab.

To install a plugin, click on the **Download Plugins** tab (figure 6.1). Select a plugin. Information about it will be shown in the right hand panel. Click on the **Download and Install** button to install the plugin.

Accepting the license agreement

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

Installing a cpa file

 Manage Plugins 		
PM		
Manage Plugins		
(P) Provider: OIAGEN Aarh	us h	
Support contact: ts-bioir	nformatics@qiagen.com	
Version: 21.0 (Build: 201. Perform alignments with ClustalO	ClutalW and MUSCLE	
Size: 8.5 MB	Developing and Testall	
	Download and Install	
P Provider: QIAGEN Aarh	nile us	
Annotate with GFF if Provider: QIAGEN Aarh Support contact: ts-bioir Version: 21.0 (Build: 201) Using this plug-in it is possible to a annotations forourd in a GFF file Located in the Toolbox.	file us formatics@qiagen.com 17-0903-212955) annotate a sequence from lat of	
Annotate with GFF f Provider: QIAGEN Aarh Support contact: tribior Version: 21.0 (Build: 201 Using this plug-in it is possible to annotations found in a GFF file Located in the Toolbox. Size: 320.9 kB	file us formatics@qiagen.com 172903-212953) annotate a sequence from lat of Download and Install	
Annotate with GFF Provder: QLAGEN Aarh Support contact: the bid Version: 21.0 (Build: 201: Using this pulcy: a is possible to annotation found in a GFF file Located in the Toolbox. Size: 320.9 kB MC ChLST Module Provider: QLAGEN Aarh Support: contact: the bid Version: 21.0 (Build: 201: The CLC MLST Module makes at e	hie us 17-0903-221953) annotate a sequence from lat of Download and Install formatics@quiagen.com 124-1953-221959) 124-1953-221959	
Annotate with GFF [Provider: QLAGEN Arth Support contact: the biol Arsign of the specific contact: the biol Located in the Toolback Size: 32:0.9 kB CLNLST Module Provider: QLAGEN Arth Support contact: the biol Provider: QLAGEN Arth Support contact: the biol Provider: QLAGEN Arth Support contact: the biol Support contact: the biol	hie us formatica@qlagen.com 127-0903-221953) annotate a sequence from list of Download and Install us formatica@qlagen.com 124-1053-221959) aay and fast to type bacterial species	
Annotate with GFF (Provider: QIAGEN Asin's Support contact: the bio's Version: 21.0 (Buid? 202). Using this plug-in: it is possible to i annotations found in a GFF file Located in the Toolbox. Siter: 220.9 kB CLC MLST Module Provider: QIAGEN Asin's Support contact: the bior Version: 21.0 (Buid?: 201). The CLC MLST Module makes it e from Sanger sequencing data. Nugin requires registration.	Ne us formatice@piagen.com 127-9903-221953) annotate a sequence from list of Download and Install Us formatice@piagen.com 121-1053-221959 asy and fast to type bacterial species	
Annotate with GFF (Provider: QIAGEN Aarh Support contact the bioling Version: 21.0 (Build': 2010) Using this plug-in it is possible to a annotations found in a GFF file Located in the Toolbox. Size: 320.9 kB CL MLST Module Movider: QIAGEN Aarh Support contact: the bioling Version: 21.0 (Build: 2010) The CLC MLST Module makes it efform Sanger sequencing data. Plugin requires registration. Commercial plugin - 14 day evalue: The 32.04 ML	hie us formatice@plagen.com 127-9903-221953) annotate a sequence from list of Download and Install us formatice@plagen.com 141-053-221959) asy and fast to type bacterial species atom license available.	

Figure 6.1: Plugins and modules available for installation are listed in the Plugin Manager under the Download Plugins tab.

If you have a .cpa installer file for Ingenuity Pathway Analysis, you can install it by clicking on the **Install from File** button at the bottom of the Plugin Manager.

If you are working on a system not connected to the internet, plugin and module .cpa files can be downloaded from https://digitalinsights.qiagen.com/products-overview/plugins/using a networked machine, and then transferred to the non-networked machine for installation.

Restart to complete the installation

Newly installed plugins and modules will be available for use after restarting the software. When you close the Plugin Manager, a dialog appears offering the opportunity to restart the *CLC Workbench*.

6.2 Uninstalling plugins

Plugins and modules are uninstalled using the Workbench Plugin Manager. To open the Plugin Manager, click on the **Plugins (button** in the top Toolbar, or go to the menu option:

Utilities | Manage Plugins... (💱)

This will open the Plugin Manager (figure 6.2). Installed plugins and modules are shown under the Manage Plugins tab of the Plugins Manager.

To uninstall a plugin or module, click on its entry in the list, and click on the **Uninstall** button.

Plugins and modules are not uninstalled until the Workbench is restarted. When you close the Plugin Manager, a dialog appears offering the opportunity to restart the *CLC Workbench*.

Disabling a plugin without uninstalling it

If you do not want a plugin to be loaded the next time you start the Workbench, select it in the

Gx Manage Plugins				X
P M Manage Plugins				
Provider: QIAGEN Aa Support contact: ts-b Version: 1.1 (Build: 1)	nics Analysis rhus ioinformatics@qiagen.com 90328-1503-191404)	<u>.</u>		
Biomedical Genomics Analysis				Uninstall Disable
CLC MLST Module Provider: QIAGEN Aa Support contact: ts-b Version: 1.9 (Build: 12	rhus ioinformatics@qiagen.com 81115-1337-185442)			Update available
MLST Module makes it easy a	nd fast to do MultiLocus Sequence	e Typing.		\smile
			Update Import License	Uninstall Disable
CLC Microbial Gene Provider: QIAGEN Aa Support contact: ts-b Version: 4.1 (Build: 1	omics Module rhus ioinformatics@qiagen.com 90129-1433-1883333)			
CLC Microbial Genomics Modu	le			
			Import License	Uninstall Disable
Help Proxy Settings	Check for Updates	install from File		Close

Figure 6.2: Installed plugins and modules are listed in the Plugins Manager under the Manage Plugins tab.

list under the Manage Plugins tab and click on the **Disable** button.