

# **Ingenuity Pathway Analysis** Plugin

USER MANUAL

# User manual for Ingenuity Pathway Analysis 24.0.2

Windows, macOS and Linux

February 10, 2025

This software is for research purposes only.

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



# **Contents**

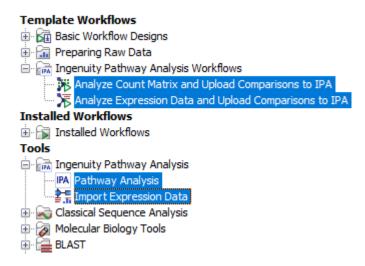
1	Introduction	4
2	Uploading data to IPA using the Pathway Analysis tool	6
	2.1 Error handling	11
3	Import Expression Data	12
	3.1 Metadata and expression data matrix	14
4	Ingenuity Pathway Analysis workflows	16
	4.1 Analyze Count Matrix and Upload Comparisons to IPA	16
	4.1.1 Running the Analyze Count 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

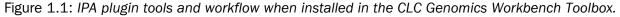
### Introduction

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

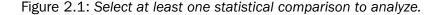
# Uploading data to IPA using the Pathway Analysis tool

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.

	Ingenuity Pathway Anal	vsis X Select statistical analysis tracks
1.	Choose where to run	Navigation Area Selected elements (1)
2.	Select statistical	Q <sup>▼</sup> <enter search="" term=""> = Δ<sub>1</sub><sup>™</sup>, Tumor vs. Normal</enter>
	analysis tracks	CLC_Data
з.	Set configuration	Example Data
4.	Set upload parameters	
A.M.	O Maranta una	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.

Gx Ingenuity Pathway Analy	/sis X
<ol> <li>Choose where to run</li> <li>Select statistical analysis tracks</li> <li>Set configuration</li> </ol>	Set configuration Server and User Account information IPA US server IPA china server IPA user login Log in Not logged in
<ol> <li>Set upload parameters</li> <li>Set analysis parameters</li> </ol>	Configuration Project name Project {1} Upload only  Upload and analyze
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 log<sub>2</sub>-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:

1. Choose where to run       Set upload parameters         2. Select statistical analysis tracks       Maximum of group mean expression upload filter         3. Set configuration       Ignore features with mean expression values below 10.0         4. Set upload parameters	Gx Ingenuity Pathway Analysis X					
4. Set upload parameters <ul> <li>Standard</li> <li>Bonferroni</li> <li>FDR</li> </ul>	Choose where to run     Maximum of group mean expression upload filter     Maximum of group mean expression values below     10.0					
Fold change Fold change C Log <sub>2</sub> ratio	4. Set upload parameters	<ul> <li>Standard</li> <li>Bonferroni</li> <li>FDR</li> <li>Upload rows with value &lt;= 0.05</li> </ul> Fold change Image: Im				
Upload rows with absolute value >= 1.0         Upload summary         Element       Features uploaded         Tumor vs. Normal       68         Help       Reset         Previous       Next		Upload summary Element Features uploaded Tumor vs. Normal 68				

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/log<sub>2</sub> 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 log<sub>2</sub>-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 X					
<ol> <li>Choose where to run</li> <li>Select statistical analysis tracks</li> </ol>	Set analysis parameters Maximum of group mean expression analysis filter Analysis cutoff 0.0				
<ol> <li>Set configuration</li> <li>Set upload parameters</li> <li>Set analysis parameters</li> </ol>	P-Value  Standard  Donferroni  FDR				
6. Set analysis reference data					
a a a	Fold change         Automatically calculate fold change cutoff         Target number of analysis features         3,000         Fold change         Log <sub>2</sub> ratio         Analysis cutoff         1.5				
	Upload and analysis summary				
A State of the second s	Element     Features uploaded     Features analyzed       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

Gx Ingenuity Pathway Analysis X					
1. Choose where to run	Set analysis parameters Maximum of group mean expression analysis filter				
<ol> <li>Select statistical analysis tracks</li> </ol>	Analysis cutoff 0.0				
3. Set configuration	P-Value				
4. Set upload parameters					
5. Set analysis parameters	⊖ 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					
⊖ Log <sub>2</sub> ratio					
	Analysis cutoff 800000				
	Upload and analysis summary				
	Element Features uploaded Features analyzed				
	Tumor vs. Normal 68 0				
The second					
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	×
2. 3. 4. 5.	Set upload parameters	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 Data X				
1. Choose where to run	Parameters Expression Data	_		
2. Parameters	Table file Count_Tumor_Normal.xlsx Browse			
3. Result handling	Table has headers			
	Ounts			
	ОТРМ			
	○ RPKM			
	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				
Cre.	Calculate expression for genes without transcripts			
(US)	Unmatched genes/transcripts	_		
Martin Contractor	○ Indude			
011	● Ignore			
1	⊖ Fail			
ATTEND TO				
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:

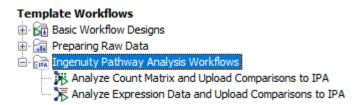


Figure 4.1: Location of the template workflows in the Toolbox.

#### 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

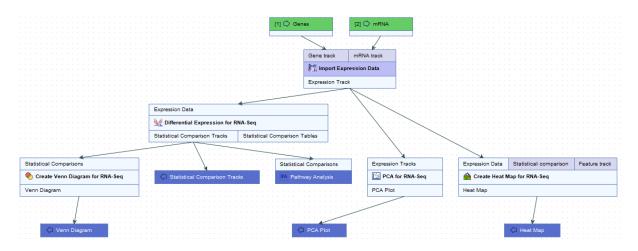


Figure 4.2: Layout of the Analyze Count Matrix and Upload Comparisons to IPA workflow.

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	Jpload Comparisons to IPA X
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 <a href="http://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Metadata.html">http://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Metadata.html</a>.

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		
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 Cance	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	Pathway Analysis					
1. Choose where to run	Configurable Parameters					
2. Select reference data set	IPA Server	IPA US server $\checkmark$				
3. Genes	IPA user login	Log in Not logged in				
4. mRNA	Project name	Project {1}				
5. Import Expression Data	Analysis type	Upload and analyze $\sim$				
5. Import Expression Data	Ignore features with mean expression values below	10.0				
<ol> <li>Differential Expression for RNA-Seq</li> </ol>	P-value type Upload	Standard 🗸				
lorran beq	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 parameters	Standard 🗸				
elements	Analysis cutoff	0.05				
Oe	Automatically calculate fold change cutoff					
( ST	Target number of analysis features	3,000				
Start Maria	Fold change type	Fold change $\checkmark$				
07	Analysis cutoff	1.5				
6	Reference set	Ingenuity Knowledge Base (Genes Only) $$				
ALL AND	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

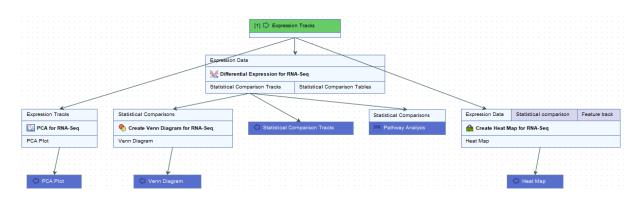


Figure 4.6: Layout of the Analyze Expression Data and Upload Comparisons to IPA workflow

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						
1. Choose where to run	Select input for Expression Tracks					
2. Select Expression Tracks	Select files for import: CLC Format					
<ol> <li>Differential Expression for RNA-Seq</li> </ol>	Navigation Area Selected elements (5)					
4. Pathway Analysis	Q▼ <enter search="" term="">           □         □           □         □           □         □           □         □           □         □           □         □           □         □           □         □           □         □           □         □           □         □           □         □</enter>					
5. Result handling						
<ol> <li>Save location for new elements</li> </ol>	SRR 1543519 (GE)     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	6			
3.	Differential Expression for RNA-Seq	Test differential expression due to	Sample type	*			
4.	Pathway Analysis	While controlling for Comparisons	Patient_ID Against control group	+			
5.	Result handling	Control group	N	4			
6.	Save location for new elements	<ul> <li>Locked Settings</li> </ul>					
	Help Reset	Previous	Next Finish Cance	1			

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.

Bamples >	<			
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 <a href="http://resources.giagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Metadata.html">http://resources.giagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Metadata.html</a>.

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	×			
1. Choose where to run	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 $\checkmark$			
elements	Upload rows with value <= parameters	0.05			
	Upload rows with absolute value >=	1.0			
	Analysis cutoff	0.0			
	P-value type Analysis Analysis cutoff parameters	Standard $\checkmark$			
0		0.05			
(De C)	Automatically calculate fold change cutoff				
(US)	Target number of analysis features	3,000			
Margalana .	Fold change type	Fold change $\sim$			
0	Analysis cutoff	1.5			
0	Reference set	Ingenuity Knowledge Base (Genes Only) $$			
and the second of the second s	<ul> <li>Locked Settings</li> </ul>				
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.

and the second second second	and the state had a little of the state of t		
<u>E</u> dit <u>V</u> iew <u>W</u> indow <u>H</u> elp			
	Genes and Chemicals Disease	es and Functions Pathways and Tox Lists	
v ×		· · · · · · · · · · · · · · · · · · ·	Advanced Search
· •		SEARCH	Advanced Search
ct Manager	Normal vs. Tumor (TE) 2016-03-03 02:41:06		- ď Ø
A-Z SORT SEARCH REFRESH		ator Effecte \ Natworke \ Litte \ My Dathwaye \ Moleculer \	
oject 2016-03-03 test	Summery Continues Factoriages (Opsiceant Analysis (Diseases & Factorias (Regul		
Dataset Files	<b></b>	E	ixport: 🔯 🔯 💷
Analyses	> Analysis Settings		
Normal vs. Tumor (TE) 2016-03-03 02:41:06	/ Analysis Setungs		
Comparison Analyses	✓ Top Canonical Pathways		
Biomarker Filter Results	Name	p-value	Overlap
Biomarker Comparison Analyses MicroRNA Target Filter Results	FXR/RXR Activation	• 1,96E-15	30,2 % 38/126
BioProfiler Results	Atherosclerosis Signaling	• 3,96E-14	29,0 % 36/124
IsoProfiler Results	LPS/IL-1 Mediated Inhibition of RXR Function	• 1,01E-13	22,3 % 49/220
My Pathways	LXR/RXR Activation	• 8,11E-11	25,6 % 31/121
My Lists			
	Acute Phase Response Signaling	2,63E-09	20,7 % 35/169
		123456789 >	
	✓ 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
	HNFIA	• 6,59E-23	Inhibited
		1 2 3 4 5 6 7 8 9 >	
	V Top Diseases and Bio Functions		
	✓ Diseases and Disorders		
	Name	p-value range	# Molecules
	Cancer		1316
	Organismal Injury and Abnormalities	9,56E-05 - 3,67E-39	1331
	Dermatological Diseases and Conditions	7,10E-05 - 2,70E-35	789
	Gastrointestinal Disease	9,56E-05 - 2,37E-23	1128
	Immunological Disease	8,16E-05 - 9,69E-19	232
	Immunological Disease	123458789 >	232
	V Molecular and Cellular Functions		
		n-value range	# Molecules
	V Molecular and Cellular Functions     Name     Cellular Movement	p-value range معادلة المعادية المعاد	# 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 <a href="https://digitalinsights.qiagen.com/">https://digitalinsights.qiagen.com/</a> products-overview/discovery-insights-portfolio/analysis-and-visualization/</a>

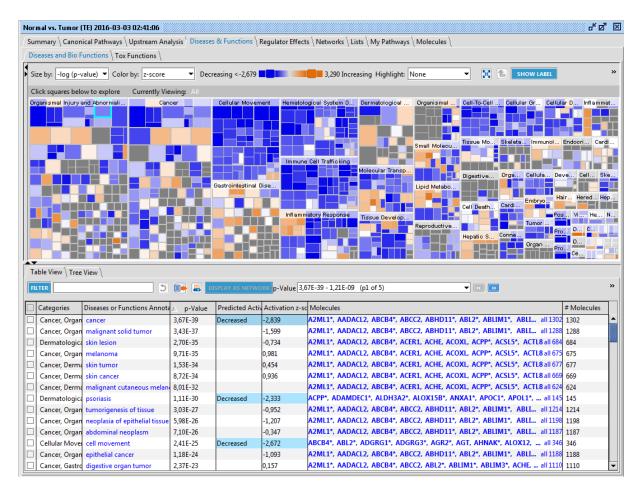


Figure 5.2: Another IPA application screenshot

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	Download Plugins	
<ul> <li>Additional Alignme</li> </ul>	nts A	
(P) Provider: QIAGEN Aarh	lus	
Support contact: ts-bioir Version: 21.0 (Build: 201		
Perform alignments with ClustalO		
Size: 8.5 MB	Download and Install	
Annotate with GFF		
Provider: QIAGEN Aarh Support contact: ts-bloir Version: 21.0 (Build: 201	nformatics@qiagen.com 217-0903-221953)	
Provider: QIAGEN Aarh Support contact: ts-bioir	nformatics@qiagen.com 217-0903-221953)	
Provider: QIAGEN Aarh Support contact: ts-bioir Version: 21.0 (Build: 201 Using this plug-in it is possible to annotations found in a GFF file	nformatics@qiagen.com 217-0903-221953)	
(P) Provider: QIAGEN Aarh Support contact: ts-biolic Version: 21.0 (Build: 201 Using this plug-in it is possible to: annotations found in a GFF file Located in the Toolbox.	Informatics@egagen.com 217:0903-21235 annotate a sequence from list of Download and Install	
Provider: QLAGEN Aarh Support contact: tre-biol Version: 21.0 (Build: 201 Uang this played: R is possible to annotations found in a GPF file Located in the Toolbox. Size: 32.0,8 kB CLC HLST Module Provider: QLAGEN Aarh Support contact: tre-biol Version: 21.0 (Build: 201 The CLC MLST Module makes te	Informatics@egagen.com 217:0903-21235 annotate a sequence from list of Download and Install	
Provider: QLAGEN Aeh Support contact: tro-biol version: 21.0 (Build: 201 Using this plug-in it is possible to amotations (source) as GFF file Located in the Toobox. Size: 32.0, kB Provider: QLAGEN Aah Support contact: tro-biol Version: 21.0 (Build: 201	Informaticu®quagen.com II:r093221253 annotate a sequence from list of Download and Install Nus nformaticu®quagen.com 214-1053-212595)	
Provider: QLAGEN Aarh Support contact: tre-biol Version: 21.0 (Build: 201 Uang this public and its possible to annotations found in a GFF file Located in the Toolbox. State: 32.0.9 kB CC MLST Module Provider: QLAGEN Aarh Support contact: tre-biol Version: 21.0 (Build: 201 The CLC MLST Module makes it e from Sanger sequencing data.	Informatic gelagen.com 12:0493-22:053 annotate a sequence from list of Download and Install Ius nformatics@elagen.com 21:41:053-22:595 Jaasy and fast to type bacterial species	
Provider: QLAGEN Aerh Support contact: trobio Version: 21.0 (Build: 201 Using this plug-in it is possible to annotations (sound in a GFF file Located in the Toobox. Size: 32.0 kB	Informatic gelagen.com 12:0493-22:053 annotate a sequence from list of Download and Install Ius nformatics@elagen.com 21:41:053-22:595 Jaasy and fast to type bacterial species	

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 <a href="https://digitalinsights.qiagen.com/products-overview/plugins/using">https://digitalinsights.qiagen.com/products-overview/plugins/using</a> 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				
Version: 1.1 (Build: 1	irhus pioinformatics@qiagen.com 90328-1503-191404)	1		<u>^</u>
Biomedical Genomics Analysis				Uninstall Disable
CLC MLST Module Provider: QIAGEN Aa Support contact: ts-t Version: 1.9 (Build: 1	pioinformatics@qiagen.com			Update available
MLST Module makes it easy a	nd fast to do MultiLocus Sequence	e Typing.		$\smile$
			Update Import License	Uninstall Disable
CLC Microbial Gen Provider: QIAGEN Aa Support contact: ts-t Version: 4.1 (Build: 1	irhus pioinformatics@qiagen.com			
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.