Data Analysis Guide

For the miRCURY LNA™ Universal RT microRNA Ready-to-Use PCR panels using Exiqon GenEx software

Version 2.0 (June 2011)

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The data analysis guide version 2

This data analysis guide will provide an instruction on how to export data from the most common real-time PCR cyclers, import and annotation of data in GenEx using the Exiqon data import wizard, and how to perform the data pre-processing. The data pre-processing described here involves steps such as interplate calibration, setting cut-off, handling missing data points, removing outliers, checking for sample and assay quality, averaging technical replica, normalizing to global mean or stable reference genes (including selection), scaling and log transforming the data. The guide will also provide a brief introduction to basic statistical analysis using either T-test or ANOVA to show whether any of the samples are significantly different from one another, including a list of the most regulated microRNAs across the samples. Finally, it will be mentioned how to easily create advanced publication-ready figures such as heatmap and principal component analysis, and retrieve further information on analyzed microRNAs from online databases.

The Exiqon data import wizard applies to data generated on miRCURY LNA™ Universal RT microRNA Ready-to-Use PCR panels whereas GenEx data analysis module can be used for data generated on both Ready-to-Use PCR panels as well as miRCURY LNA™ Universal RT microRNA individual assays.

Considerations on Normalization

Why normalize?

The purpose of a qPCR experiment is usually the detection or verification of differential, biologically relevant expression levels in a set of samples.

In setting up an experiment, small differences in replicate performance cannot be avoided, even when protocols are standardized: sampling may not be identical, storage may have different effects on each sample, RNA extraction may not be 100% reproducible, PCR inhibitors may be present in some but not all samples, pipetting may not be accurate, and real-time cyclers may show run-to-run variation. In order to get biologically relevant data, it is important to filter out technical variation. For this purpose, different approaches to normalization may be employed.

Controls and normalization assays

In the design of the plate-layout for the miRCURY LNA™ Universal RT microRNA PCR, Ready-to-Use PCR panels, we have incorporated several options for data normalization.

Inter-plate calibrator

Since each assay is present only once on each plate, replicates must be performed using separate plates. This raises the issue of run-to-run differences. To allow for simple inter-plate calibration, we have designed a calibration assay with a companion template (annotated as UniSp3 IPC in the plate layout files).

Three wells have been assigned for inter-plate calibration to provide triplicate values with the possibility for outlier removal. In each of these wells, both the primers and the DNA template are present, giving high reproducibility. The inter-plate calibrator is independent of cDNA quality in order to give a signal (but may be affected by PCR inhibitors in the sample) and can therefore be used to quality control each plate run.

Sample spike-in

Some sample types may contain PCR inhibitors, which sometimes survive RNA purification. This may result in different reverse transcription or PCR efficiencies between compared samples. One way to control for differences in efficiencies is by adding a known RNA spike-in to the sample during cDNA synthesis. We have designed an RNA spike-in, UniSp6, for this purpose. The UniSp6 RNA template is provided with the cDNA synthesis kit. One well in the Ready-to-Use PCR plates contains the matching primer set. A UniSp6 PCR primer set is also provided with the SYBR® green master mix kit, which is to be used with our non-plate based PCR primer set products. We do not recommend normalizing to the synthetic spike-in – it should be used for sample quality control.

Global Mean Normalization

Variation due to sample differences and handling prior to cDNA synthesis can be normalized using endogenously expressed miRs. In large screening studies, it is often recommendable to normalize data against the global mean; i.e., the average of all microRNAs expressed in all samples [1]. This can be the best option when screening samples with a high call-rate (number of expressed microRNAs) and a high proportion of essentially unregulated microRNAs, but should be used with caution in validation studies where most of the genes are chosen precisely for being differentially expressed. Global normalization is also not a good option between samples in which the overall microRNA expression level is changed.

Reference gene Normalization

The use of endogenous reference genes can be another approach to normalize against variation due to sample differences and handling prior to cDNA synthesis. Though this is a good and recommended approach, great caution should be taken in the selection of reference genes. The danger of using endogenous reference genes lies in the assumption that a specific gene is expressed at the exact same level in all sample types. This is rarely true. The selection of reference genes should therefore be made with care, and should be specific to the sample set you are working with. This selection can be performed using NormFinder and/or geNorm, both incorporated into GenEx. For validation studies based on panel screening, the reference genes may be selected during the screening process, based on expression behavior most resembling the global mean.

In our panels, we have pre-assigned 6 wells for 6 different genes which have stable expression levels over a wide range of sample types. Three of these are microRNAs which are often stably expressed, and the other three are small RNA reference genes. Once qPCR data has been obtained, the appropriateness of these references can be analyzed for your specific samples and the optimal number of references can be selected. When applicable, we recommend choosing stably expressed microRNAs over other small RNA reference genes, since microRNA best resemble the behavior of microRNA both biologically and during experimental handling (extraction, RT etc.)

How to get started with GenEx

Introduction to GenEx

Exiqon has partnered with MultiD to provide a software for qPCR data analysis specifically adapted to our miRCURY LNA™ Universal RT microRNA PCR products. GenEx offers advanced methods to analyze real-time qPCR data with simple clicks of the mouse. The methods are suitable to select and validate reference genes, classify samples, group genes, monitor time dependent processes and much more.

Possibly the most important part of qPCR experiments is the pre-processing of raw data for subsequent statistical analysis. Pre-processing steps need to be performed with consistence, in the correct order and with confidence. GenEx has a streamlined and user-friendly interface which aids data handling. Powerful presentation tools present professional illustrations of even the most complex experimental designs.

GenEx is intuitive and easy to use, and with the Exiqon qPCR plate import wizard, data import and merge becomes simple. Furthermore, GenEx has the advantage of incorporating both NormFinder and geNorm in the software. Thus, you get both of these algorithms on which to base your choice of reference genes in one software installation. GenEx also offers advanced statistical solutions for post-normalization data analysis. Current features include parametric and non-parametric statistical tests, clustering methods, principal component analysis, artificial neural networks, and much more.

Download and install GenEx

To install the Exiqon version of GenEx, go to http://www.exiqon.com/qpcr-software and the “software download” tab.

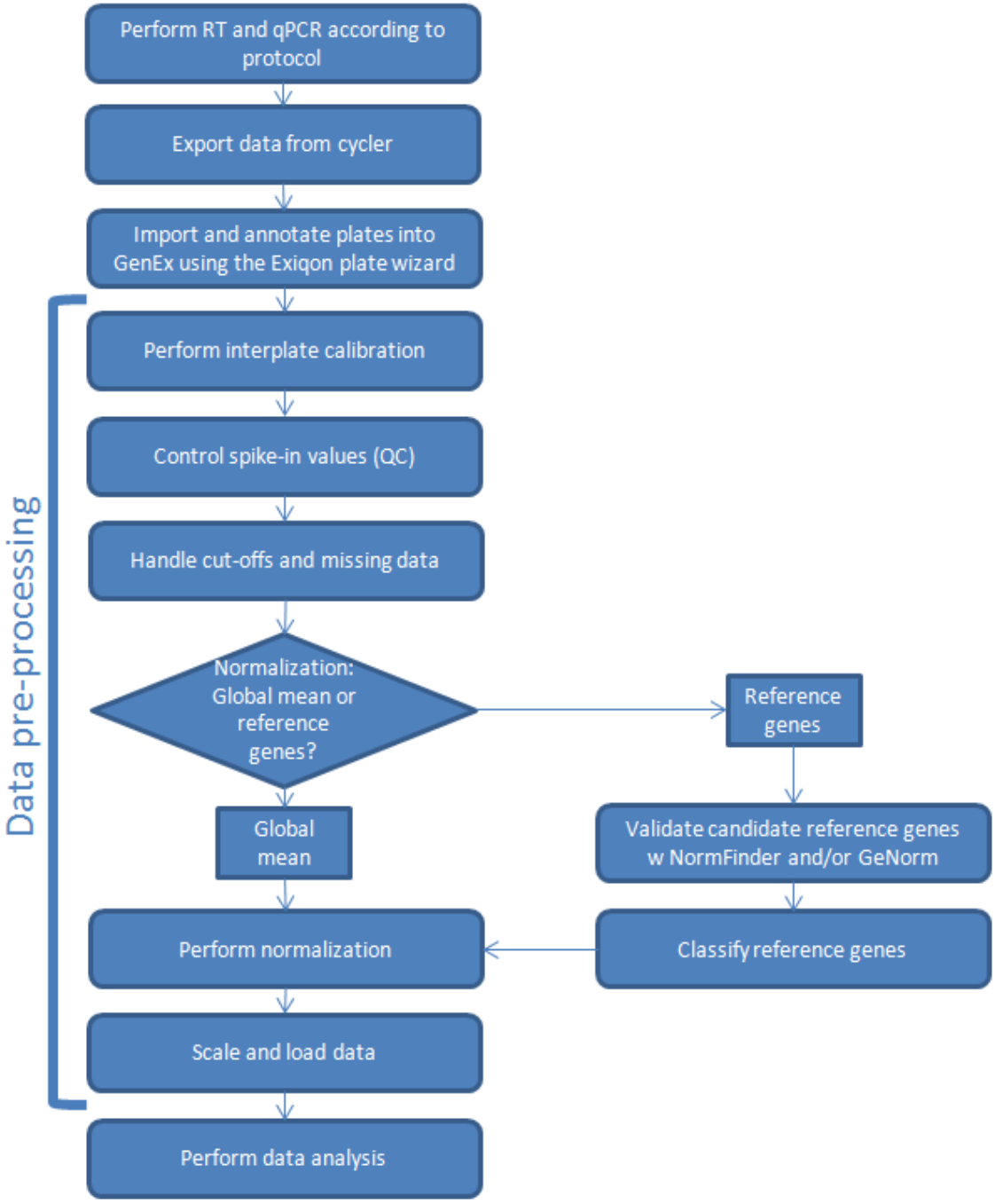
In the dialogue box choose run, and follow the installation guide. If you have purchased a GenEx license, use the license key provided by email. Otherwise you may use a free demo license for 30 days.

Once installed, remember to check for updates. MultiD continuously works to improve the software, and updates are periodically released, so check for updates routinely.

The GenEx manual is available within the software under the Help menu.

Flow of data analysis

Before you get started with setting up your experiments, it may be useful to consider the shown data analysis flow:



How to get the raw data from your cycler into GenEx

Export the run data from your cycler

Run your experiment according to the protocol.

Important: For ABI 7900 users. Prior to performing the experiment, it is possible to download run template files (.sdt files) from the Exiqon website (http://www.exiqon.com/sds). These files specify proper cycling and critical analysis settings, and can be used when setting up the experiment. In the SDS software, simply open the appropriate template file and start the run.

For your own convenience, it may be useful to annotate the run with assay and sample names – but this is not necessary for data analysis when using Exiqon Ready-to-use plates. Annotation will take place during the data import using the wizard.

Below, we have given examples of how to export your data from some of the major cycler types.

ABI 7900

If you have used an ABI 7900 cycler for you experiment, please note that it is necessary to verify that the data analysis settings have been correct.

First, it is important that the experiment has been run as an AQ experiment, not RQ (the SDS 2.4 version of the software allows you to convert between the two formats post-run).

Second, it is important that you have indicated whether or not you have used ROX passive reference dye in the experiment, and that baseline and threshold settings are set manually and correctly. For directions on how to analyze the data, please refer to our miRCURY LNA™ Universal RT microRNA PCR Instruction manual, tip 10. If you have used one of our run templates, the settings should be correct – but it is always a good idea to verify that the threshold is adequately set.

Once all settings are correct, you are ready to export the data. In the File menu, choose Export... . In the dialog box, choose Export: Results Table, All Wells and SDS 2.3 And Above. Then Export to the folder of your choice.

Tip: with SDS 2.4 it is possible to perform batch export without opening the files to export. In the File menu, choose Batch Export... . In the dialog box, choose Assay Type : Standard Curve (AQ) and Add the files you wish to export. Select the destination folder with Export To :, then Export. Please note that this method is only valid if you are sure that all the files have been analyzed correctly first.

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ABI 7500 and StepOne

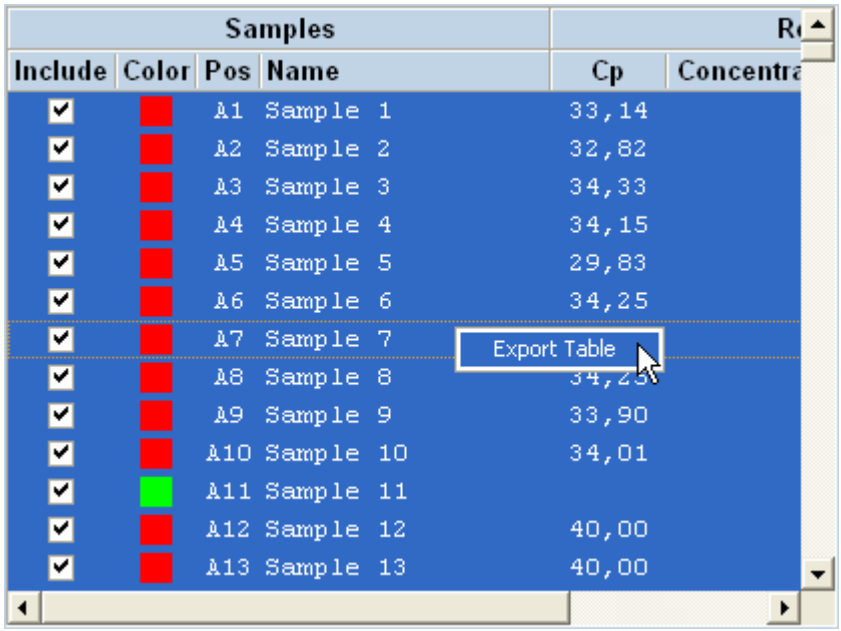
If you have used an ABI 7500, 7500 FAST or StepOne cycler for you experiment, please note that it is necessary to verify that the data analysis settings have been correct.

It is important that you have indicated whether or not you have used ROX passive reference dye in the experiment, and that baseline and threshold settings are set manually and correctly. For directions on how to analyze the data, please refer to the miRCURY LNA™ Universal RT microRNA PCR Instruction manual, tip 10.

Once all settings are correct, you are ready to export the data. In the File menu, choose Export... . In the dialog box, tick only Results , and select 2. Select one file or separate files: One File . Assign file name and location, and select File Type: (*.txt) . Then Start Export .

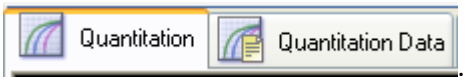
Roche LC480

If you have used a Roche LC480 for your experiment, go to Analysis , choose the 2nd derivative analysis method, and make sure that the Cq values have been calculated (if this has not been done yet, press Calculate). To export the data, right-click on the data table and choose Export table, browse to the location where you wish the file, and save.

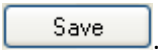


Tip: For high throughput, it may be worthwhile to create a macro for running the experiment and automatically export the data at the end of the run. How to program such a macro goes beyond the scope of this guide, and should be learned from the LC480 manual.

BioRad CFX

If you have a BioRad CFX cycler, you can choose to analyse either as regression or single threshold. Once the Cq calculations have been performed, data can be exported either from the Quantitation or Quantitation Data window 

. Simply place the cursor over the data table in the window, right-click and in the menu popping up choose 

. Browse to the folder of choice, name the file and 

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Stratagene Mx3000P/3005P

If you have used a Stratagene cycler for your experiment, we recommend verifying that the data analysis settings (baseline and threshold) have been correct. For directions on how to analyze the data, please refer to our miRCURY LNA™ Universal RT microRNA PCR Instruction manual, tip 10.

Once all settings are correct, you are ready to export the data. In the 

menu choose 

. Browse to your folder of choice, name the file and 

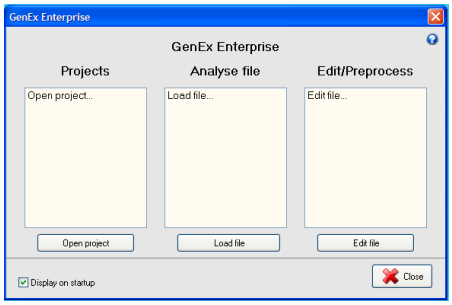
.

Importing and merging your instrument export files using the Exiqon import wizard

Starting the wizard:

Open GenEx.

If you have the start-up window active, close this.



Click the Exiqon qPCR plate import wizard button 

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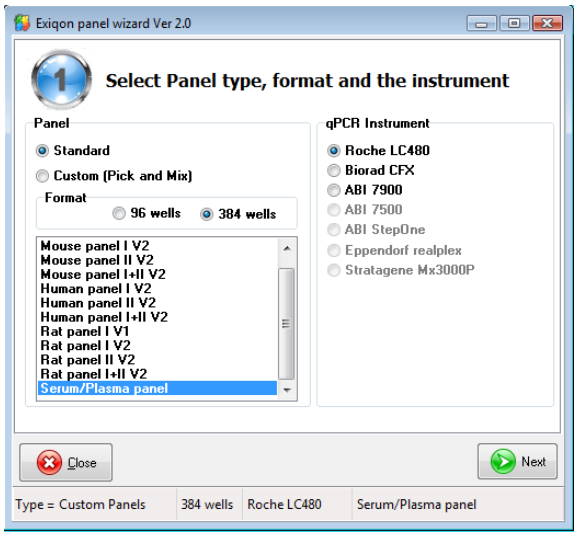
In the pop-up window, click start.

Step 1: Select panel type, format and instrument

The first choice to make is whether you have been running standard or custom panels, and whether the format is 96-well or 384-well.

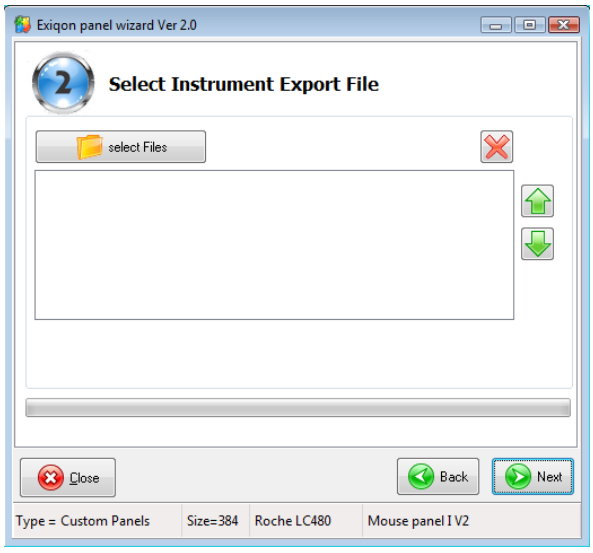


If you choose Standard, then a list of the available Exiqon standard panels (including focus panels) in the chosen format will appear. If you have run a set of two panels, please select the set from the list rather than just one of the plates as this will open the function for merging panel I and II of the same sample in the next window.
If you choose Custom, then a list of the different possible layouts in the chosen format will appear. Choose the layout that you have used.

Next, choose the cycler type used for performing the experiment. Click 

Step 2: Select instrument export files

You now need to select your instrument export files (.txt or .xml, depending on cycler type), and in the case of custom Pick&Mix panels you will in addition also have to select the plate layout file (.xls) that was provided when you configured your custom panel.
If you have run only one plate type (e.g.only Human Panel I V2), then a single pane will appear for file selection.



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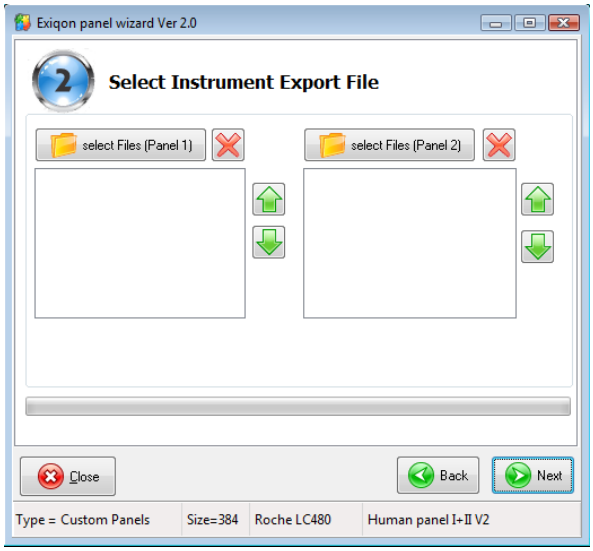
Click 

and browse to the location where you have your files. While using shift or ctrl for multiple file selection, choose and open your files. If the file naming has not automatically resulted in the desired sample order, you can move a file up or down in the list using the arrow buttons  .

If you have run a set of plates (e.g. Human Panel I and II V2) two panes will open.

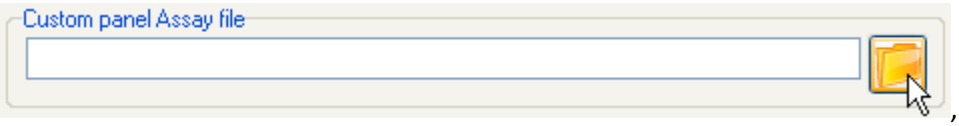


Each pane has a 

button. In the left panel open the files for panel I and in the right pane open the files of panel II.
Important: The files for panel I and II must have the same sample order, so that the two files for the same sample are aligned. If the file naming has not automatically resulted in the correct order, you can move a file up or down in the list using the arrow buttons  .

If the experiment was performed using custom Pick and Mix panels, you must also select the Excel layout file you received from Exiqon when placing the order, as this file will tell GenEx which assays are located in which wells.
If you left some wells empty when designing the plate, you may use these wells to add any assay of your choice. If you have done so, please make sure that the assay names have been entered into the appropriate cells in the Excel layout files thereby ensuring that GenEx gets the information.

Click the folder button



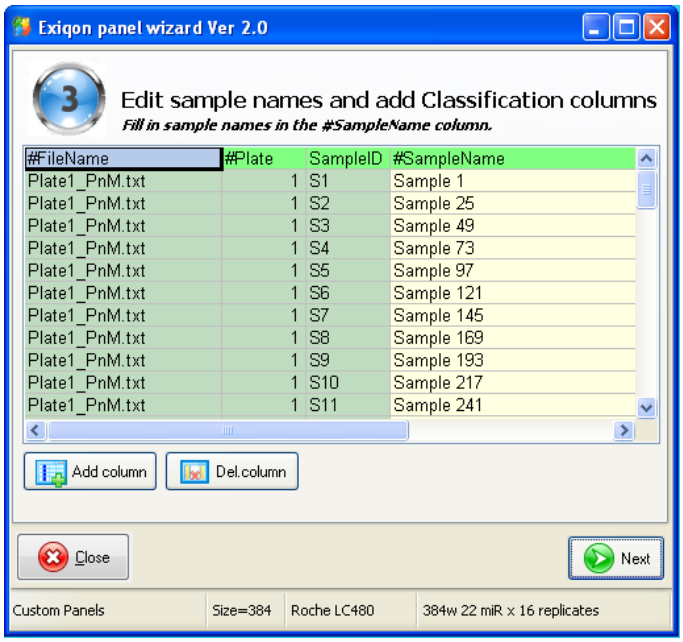
, browse to the location of your layout file, and open the file.

Click 

Please note that once you click next at this step a table is created based on the imported files. It is not possible to go back without re-starting the wizard.

Step 3: Edit sample names and add classification columns

The table generated after file import contains 4 predefined, automatically generated columns called classification columns.



Classifier is a term used in GenEx to group samples that belong to the same category (i.e. on same PCR plate, technical replicates, biological groups or negative controls). Sample classifiers are found in classification columns. Classifiers are also used to identify assays used for specific purposes (i.e reference genes and spike-in controls). In that case, the classifiers are found in classification rows, but that function is not relevant until step 4 – step 3 deals only with sample annotation which is done in columns.

The way a sample classifier works, is that samples belonging to the same category or group get the same number in the classification column. For example, negative control samples should be assigned 1 in a negative control classifier, treated samples could be assigned 1 and non-treated 2 in a treatment classifier, and RT replicates of the same RNA sample should be assigned the same number

in an RT classifier. A classification column is recognized as having a # as the first symbol in the header name. If you want GenEx to consider groups in the further data analysis you need to let GenEx know which ones these are by adding a column for each classifier.

As mentioned above, Step 3 automatically adds 4 pre-defined classification columns. The first three automatically assigned columns should not be edited:

#FileName serves to help identify which plates/samples came from which instrument export file, making it easier to assign correct sample names.

#Plate is a classifier identifying which samples were run on the same plate.

SampleID identifies a sample according to its position in the Exiqon plate layout file. Again, this should make it easier to assign proper sample names.

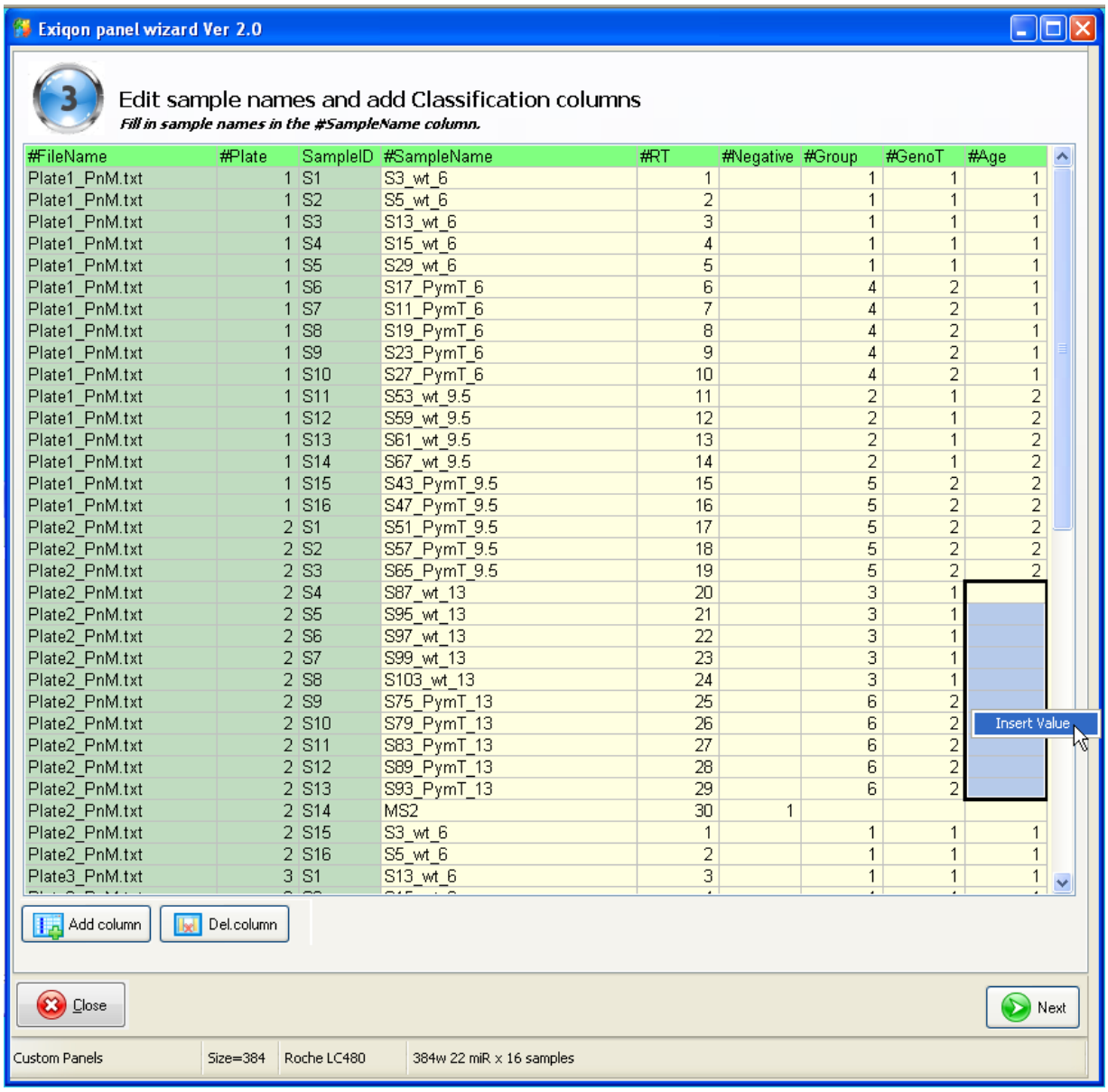
The only pre-defined column to edit is the #SampleName. If correct sample names have not been assigned already in the cycler, the sample name assigned by your cycler will appear (this may vary between cycler types). Using the file name and sample ID to identify each sample, assign each sample name to be used during further downstream analysis. This can be done by selecting and filling each cell individually or by copy-paste from an Excel spread-sheet containing a sample overview. The latter method requires that the plates/samples appear in the same order in the spread-sheet as in the wizard.

It is now possible to add additional classification columns. To the extent it is relevant in your experiment, we recommend adding classifiers to identify at least technical replicates, negative controls and biological groups. Additional classifications may in some cases be relevant, i.e. to identify sub-groups.

For each classifier you wish to add, just click 

, name the new column (starting with #), and assign the classifications starting from 1 and onwards for each group or category. Multiple cells can be selected using shift or ctrl, and assigned the same classifier by right-clicking and select insert value (this function does not work for #SampleName). Negative control samples should be given the classification value 1, while unknowns are left empty or assigned 0 in the negative control classifier. Again, copy-paste can also be used if a spread-sheet has been created with sample overview.

For the sake of keeping the overview, it is at this point possible to re-size the wizard window.



#FileName	#Plate	SampleID	#SampleName	#RT	#Negative	#Group	#GenoT	#Age
Plate1_PnM.txt	1	S1	S3_wt_6	1		1	1	1
Plate1_PnM.txt	1	S2	S5_wt_6	2		1	1	1
Plate1_PnM.txt	1	S3	S13_wt_6	3		1	1	1
Plate1_PnM.txt	1	S4	S15_wt_6	4		1	1	1
Plate1_PnM.txt	1	S5	S29_wt_6	5		1	1	1
Plate1_PnM.txt	1	S6	S17_PymT_6	6		4	2	1
Plate1_PnM.txt	1	S7	S11_PymT_6	7		4	2	1
Plate1_PnM.txt	1	S8	S19_PymT_6	8		4	2	1
Plate1_PnM.txt	1	S9	S23_PymT_6	9		4	2	1
Plate1_PnM.txt	1	S10	S27_PymT_6	10		4	2	1
Plate1_PnM.txt	1	S11	S53_wt_9.5	11		2	1	2
Plate1_PnM.txt	1	S12	S59_wt_9.5	12		2	1	2
Plate1_PnM.txt	1	S13	S61_wt_9.5	13		2	1	2
Plate1_PnM.txt	1	S14	S67_wt_9.5	14		2	1	2
Plate1_PnM.txt	1	S15	S43_PymT_9.5	15		5	2	2
Plate1_PnM.txt	1	S16	S47_PymT_9.5	16		5	2	2
Plate2_PnM.txt	2	S1	S51_PymT_9.5	17		5	2	2
Plate2_PnM.txt	2	S2	S57_PymT_9.5	18		5	2	2
Plate2_PnM.txt	2	S3	S65_PymT_9.5	19		5	2	2
Plate2_PnM.txt	2	S4	S87_wt_13	20		3	1	
Plate2_PnM.txt	2	S5	S95_wt_13	21		3	1	
Plate2_PnM.txt	2	S6	S97_wt_13	22		3	1	
Plate2_PnM.txt	2	S7	S99_wt_13	23		3	1	
Plate2_PnM.txt	2	S8	S103_wt_13	24		3	1	
Plate2_PnM.txt	2	S9	S75_PymT_13	25		6	2	
Plate2_PnM.txt	2	S10	S79_PymT_13	26		6	2	
Plate2_PnM.txt	2	S11	S83_PymT_13	27		6	2	
Plate2_PnM.txt	2	S12	S89_PymT_13	28		6	2	
Plate2_PnM.txt	2	S13	S93_PymT_13	29		6	2	
Plate2_PnM.txt	2	S14	MS2	30	1			
Plate2_PnM.txt	2	S15	S3_wt_6	1		1	1	1
Plate2_PnM.txt	2	S16	S5_wt_6	2		1	1	1
Plate3_PnM.txt	3	S1	S13_wt_6	3		1	1	1

If you accidentally added too many classification columns, they can easily be removed again. Simply select the column you wish to delete, and click 

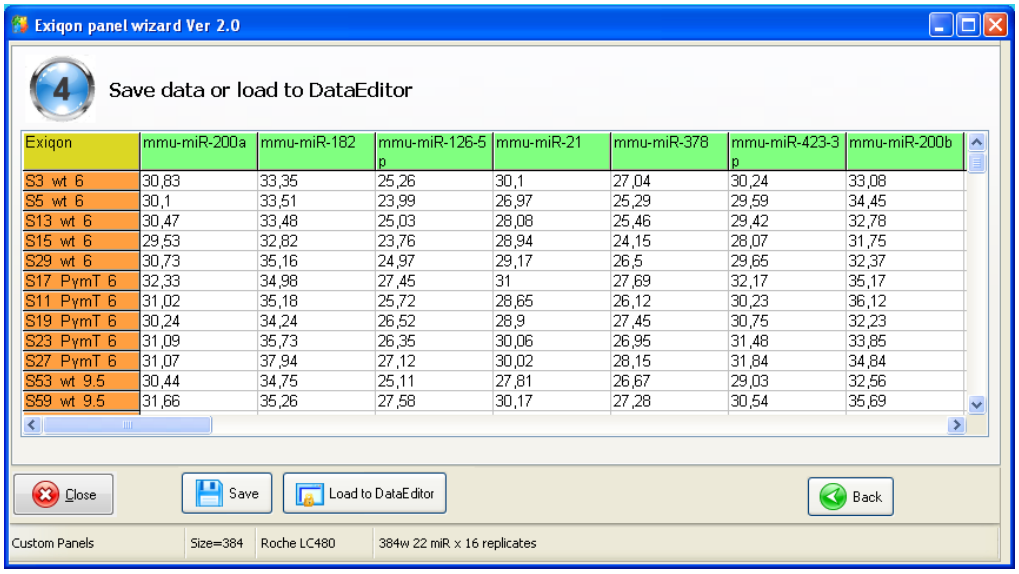
. Note that the original 4 columns are necessary, and cannot be deleted.

Once all sample names and classifiers have been assigned, click 

.

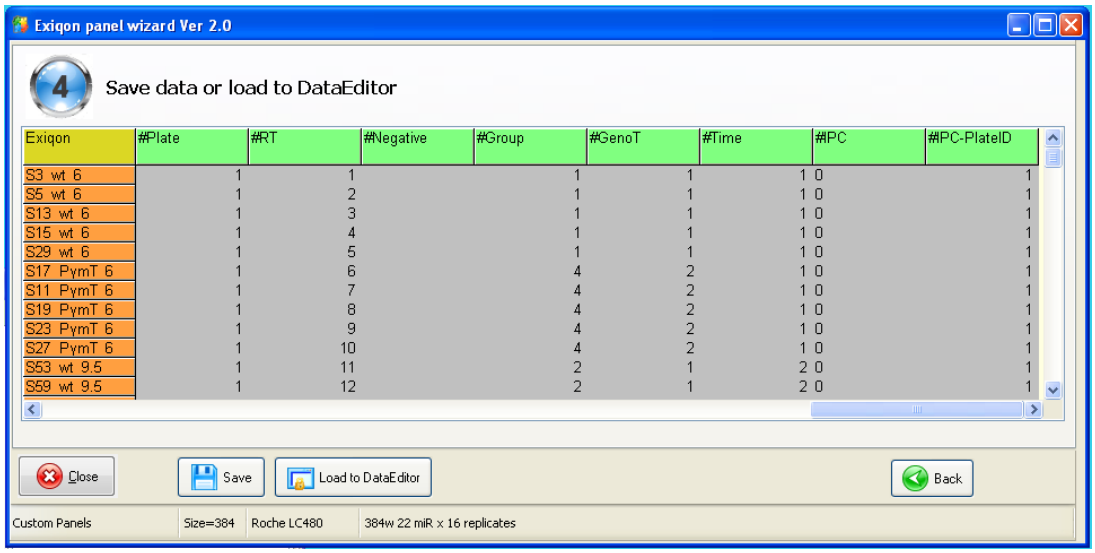
Step 4: Save data or Load to data editor

You are now ready to load your data into GenEx and iinitiate pre-processing or alternatively save the data for later loading. During pre-processing, GenEx will need classification columns for some of the steps. In step 4, you get a chance to verify that all sample names and necessary classification columns have been assigned correctly before loading to GenEx and commencing the pre-processing.



Exiqon	mmu-miR-200a	mmu-miR-182	mmu-miR-126-5p	mmu-miR-21	mmu-miR-378	mmu-miR-423-3p	mmu-miR-200b
S3 wt 6	30,83	33,35	25,26	30,1	27,04	30,24	33,08
S5 wt 6	30,1	33,51	23,99	26,97	25,29	29,59	34,45
S13 wt 6	30,47	33,48	25,03	28,08	25,46	29,42	32,78
S15 wt 6	29,53	32,82	23,76	28,94	24,15	28,07	31,75
S29 wt 6	30,73	35,16	24,97	29,17	26,5	29,65	32,37
S17 PymT 6	32,33	34,98	27,45	31	27,69	32,17	35,17
S11 PymT 6	31,02	35,18	25,72	28,65	26,12	30,23	36,12
S19 PymT 6	30,24	34,24	26,52	28,9	27,45	30,75	32,23
S23 PymT 6	31,09	35,73	26,35	30,06	26,95	31,48	33,85
S27 PymT 6	31,07	37,94	27,12	30,02	28,15	31,84	34,84
S53 wt 9.5	30,44	34,75	25,11	27,81	26,67	29,03	32,56
S59 wt 9.5	31,66	35,26	27,58	30,17	27,28	30,54	35,69

If you scroll all the way to the right, you will see the classification columns.



Exiqon	#Plate	#RT	#Negative	#Group	#GenoT	#Time	#PC	#PC-PlateID
S3 wt 6	1	1		1	1	1	0	1
S5 wt 6	1	2		1	1	1	0	1
S13 wt 6	1	3		1	1	1	0	1
S15 wt 6	1	4		1	1	1	0	1
S29 wt 6	1	5		1	1	1	0	1
S17 PymT 6	1	6		4	2	1	0	1
S11 PymT 6	1	7		4	2	1	0	1
S19 PymT 6	1	8		4	2	1	0	1
S23 PymT 6	1	9		4	2	1	0	1
S27 PymT 6	1	10		4	2	1	0	1
S53 wt 9.5	1	11		2	1	2	0	1
S59 wt 9.5	1	12		2	1	2	0	1

Note that the #FileName and SampleID columns have disappeared since they have served their purpose. Instead, two new columns have appeared automatically: #IPC and #IPC-PlateID .These are needed for later use in interplate calibration, and since the wizard knows the position of the interplate calibrators from the plate layout they were automatically assigned. The difference between #Plate and #IPC-PlateID is mainly found in experiments where two plates make a pair (as in

human panel I+II). In this case #Plate identifies the plate pair after merge. #IPC-PlateID instead identifies the individual plates, to allow for correct interplate calibration of each individual plate.

If you scroll down, you will notice a number of almost empty rows named IPC. These are needed for later interplate calibration, and will disappear once this task has been performed. For some plate types, you may also notice that a #RefGene and/or #Spike row has been added.

#RefGene	0	0
#Spike	0	0

The #RefGene row is automatically assigned only for standard plate layouts, and identifies Exiqon's suggestion for candidate reference genes. For custom panels, reference genes must be manually identified. This, however, is not done in the wizard but during the subsequent pre-processing (explained in a later section).

The #Spike will automatically be assigned in all plate layouts containing the UniSP6 CP primers for detection of the RNA spike-in supplied with the cDNA synthesis kit, and can be used for sample QC (explained in a later section).

The order of assays in the table depends on the instrument export file from your cycler. Many cycler types (i.e. Roche LC480) export the assays in the order of the plate, sorted by row. However, some cyclers (i.e. ABI 7900) re-arrange the assay order alphabetically. The order of assays has no effect on down-stream processing, and can be changed once the data has been loaded to the Data Editor.

If all looks well, 

and/or 

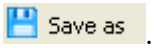
.

Your data is now ready for pre-processing in the Data editor.

How to pre-process and normalize your data in GenEx

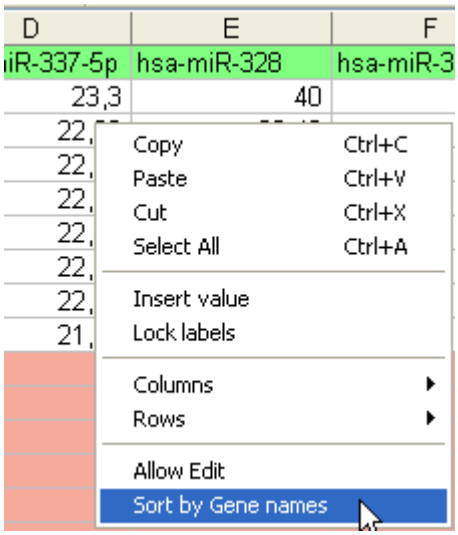
In this section we go through all the steps necessary to get your data ready, including normalization and generation of relative values (e.g. “delta delta Cq” calculation), for subsequent statistical analysis.

If at some point during the pre-processing you wish to break from the work and continue later, save the file using the data editor 

menu and 

. When you wish to contiue the work later, be sure to import the file using edit file  - otherwise data will loaded to the control panel rather than a data editor, and pre-processing will not be an option.

The order of the assays will at this point appear in the order specified by the cycler. If you wish to sort the assays alphabetically, you can do so at any time during the pre-processing. Simply right-click anywhere in the Data Editor table, and in the menu choose Sort by Gene names.

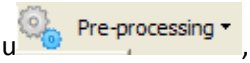


Futhermore, it may be easier to read the table if you fix the labels. Click  in the Data editor tool bar.

Inter-plate calibration

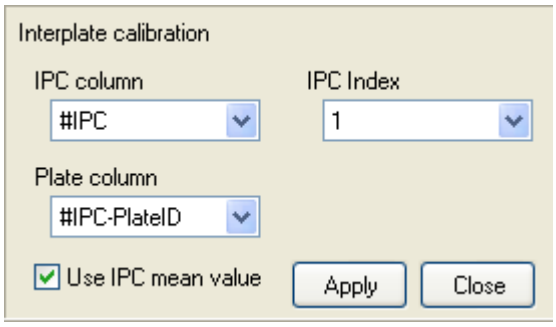
When comparing multiple plates, as for full panels, the first thing you want to do is calibrate your data between the plates. Keep in mind that some cyclers are so robust that run-to-run variation is negligible – in this case inter-plate calibration may not be necessary. However, skipping inter-plate calibration requires knowledge of negligible run-to-run variation.

During the plate import using the Exiqon plate wizard, interplate calibrators have automatically been identified.

In the pre-processing menu 

, choose 

.

Fill in the dialog box as shown here: 

Then 

.

You should notice that the rows and columns containing IPC and Plate classifiers disappear, since they have now fulfilled their purpose. This is a general theme: once you no longer need a row or column, it will disappear from the table.

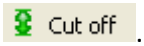
Interplate calibration, as well as several subsequent pre-processing steps, involves averaging. This creates additional decimals, which are difficult to look at and have no real meaning (the precision of measure is certainly not to the third or fourth decimal). At any point during pre-processing, it is possible to specify the number of decimals displayed. In the Data editor 

menu, choose 

and then the number of decimals you wish.

Cut off

You may wish to define a cut off value indicating that data with a Cq higher than this value should be considered background.

In the pre-processing menu, choose 

. In the dialog box, indicate your chosen cut-off (e.g. 37), and for now replace with a blank (just leave that cell empty).

Tick 

to remove all empty rows and undetected miRs will be automatically removed. Partially empty rows and columns can be removed at a later step, using the validate sheet option. Click 

.

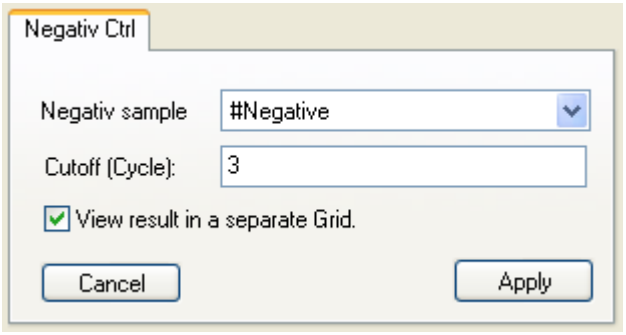
Quality control

Negative control

A few assays may have a tendency to form primer-dimers at a lower Cq than the chosen overall cut-off. For these assays it will be useful to set an individual cut-off based on the negative control Cq values. GenEx is able to set such an individual cut-off in a simple operation if you have performed one or more negative control(s), and uploaded and annotated these together with the rest of the data.

The negative controls used for this purpose may be either no template control (no RNA template added), noRT control (no RT enzyme added in the RT reaction), or mock RT (RT reaction on carrier RNA not containing microRNA). If your study consist of ready-to-use panels, you should run at least one entire panel with the negative control.

To set the cut-off for affected assays, from the pre-processing menu choose Quality control ▸, then Negativ Control. In the dialog box, select the classifier desribing your negative control samples in the pull-down menu, and the desired delta-Cq for cut-off. In the example below, the individual cut-off will be set as the average of the negative controls for the affected assay minus 3 Cq. Values too close to the individual cut-off will be deleted.



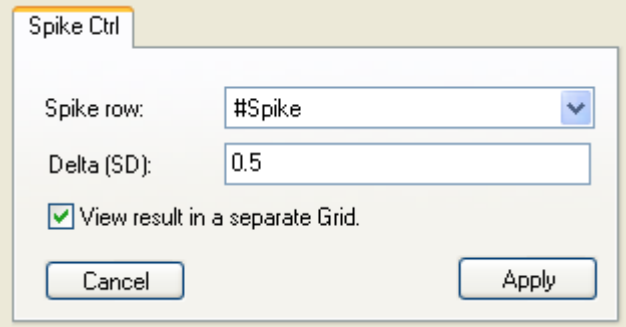
If you tick ☑ View result in a separate Grid., a new table will give you an overview of affected assays. Click Apply.

RNA spike-in control

With the cDNA synthesis kit, an RNA spike-in control is included. This spike-in can be added either in the RNA extraction or in the cDNA synthesis. It is our recommendation to apply the spike-in as a quality check on the cDNA synthesis and PCR by spiking it into the cDNA synthesis reaction. If there is great divergence across the different samples, it may be worthwhile to inspect the dataset for the outlier samples to estimate whether there might be inhibitors in the sample. We do not recommend normalizing to the spike-in control.

During plate import using the Exiqon qPCR plate import wizard, a spike-in classification row was automatically added if the UniSP6 CP assay was present in the plate-layout (always the case for standard panels, optional for custom panels).

In the pre-processing menu, choose Quality control, then Spike Control. Set the accepted standard deviation for delta Cq from each sample spike to the average of spike values in all samples.



Click Apply.

If the spike-in value of any sample(s) deviate beyound the assigned standard deviation, the affected samples will now appear in a new grid. These samples should be manually inspected for signs of inhibition.

Inhibition will be seen as several assays amplifying with later Cq's than in un-inhibited samples, and with lower amplification efficiencies. Note that not all assays will be equally affected, and thus inhibited samples cannot be rescued by normalization. Inhibited samples should be disregarded.

Before discarding samples, please keep in mind that the spike-in is a small RNA, and thus susceptible to RNA degrading factors such as repeated freeze-thaw cycles or presence of RNases. It is therefore important to evaluate whether any discrepancies in Cq are due to spike-in degradation in which case only the spike-in will deviate from other samples, or sample inhibition resulting in several assays deviating. The outlier detection described below may assist in this task, since inhibition should result in an unusual large amount of outliers, all from the affected samples.

Outlier detection

Sometimes a single well has technical problems and produces an erroneous Cq value or even a missing value. This is the reason why we recommend running replicates. Replicates allow you to detect and deselect a value that is an outlier. GenEx uses the Grubb's test for this detection.

NOTE: The preferred experimental design is to include at least three biological replicates or, where this is not possible, three cDNA synthesis replicates followed by individual PCR. For more information please go to www.exiqon.com/mirna-pcr-analysis. You may also contact Exiqon Technical Support for help.

In the pre-processing menu, choose 

then 

. In the classification column pull-down, choose your replicate classifier (either technical or biological). Usually the default Confidence level (0.95) and cutoff SD (0.25) will be applicable, but you do have the freedom to change these if you so desire. You can now either choose the prudent way and have the outliers highlighted for manual inspection and deletion, or tick 

and have the software automatically delete all outliers. 

.

Inhibited samples should light up as having many assays with outlier values (in red) compared to the biological group. Such samples should now be removed from further analysis.

Outlier values should now be removed and not affect the averages, but should be filled in with reasonable values for later calculations. This should be done using the Missing data handling described below.

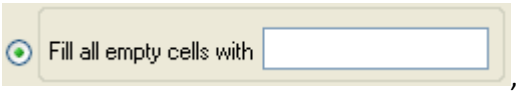
Missing data

Non-numerical values

Your real-time cycler software may have left cells empty or given values of 0 or a text (e.g. Undetermined) in wells with no signal. These usually appear as “NAN” in GenEx and will be color coded red. The subsequent pre-processing steps may not allow NAN values, so you need to replace these with empty cells. The missing signal can either be due to a technical “fall-out” (no detection in that replicate) or due to lack of expression. Fall-outs will be dealt with as outliers, so for now just treat all as follows

In the pre-processing menu choose 

.

Check 

, leave the cell empty, and 

.

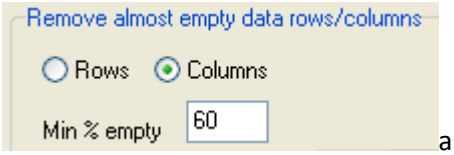
This will remove NaN values.

Assays with low call-rate

In order to make data analysis and interpretation easier, you can now remove all assays not detected in a certain proportion of samples.

In the pre-processing menu, choose 

. In the dialog box, specify the percentage of samples that should have missing values in order to render the assay undetected.



and 

. Be careful to set the % empty such that assays detected in one group but not another are not removed – such assays may indeed be important.

After the message specifying which assays were removed, 

the window.

Values missing due to technical issues

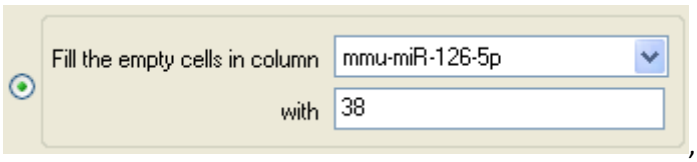
In the Missing Data dialog box, select 

and choose the classifier identifying the type of replicas you want to use for calculating the fill-in value. If RT replicates have been performed, this should be your first choice. If there are still missing values, the next choice should be the biological groups.

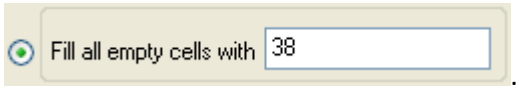
microRNAs detected only in a subset of samples

In case a given miR is detected in some groups but not in others, we recommend setting the non-detected groups to the value of the cut-off + 1. This can be done either by using the missing data function, or manually.

In the Missing Data dialog box,, either tick

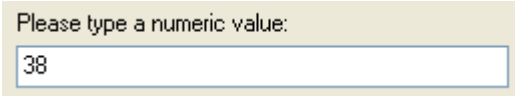


, choose the column with empty data, and the value to fill in

or tick 

.

For manual fill-in, select the cells to fill in (multiple cells can be selected by holding down shift or ctrl) and right-click. From the menu, select Insert value , fill in the dialog box



and click 

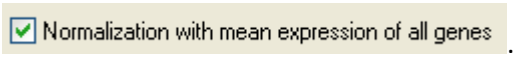
.

Normalization to global mean

As described in the introduction of this guide, variability arising from differences in RNA samples and handling of these samples prior to cDNA synthesis is minimized through normalization. The global mean of all expressed genes should be the preferred method of normalization in screening studies, where a large number of assays (>100) are analyzed without presumptions on which or how many are regulated. It requires that the majority of tested genes have little or no regulation.

From the pre-processing menu, choose 

.

If you wish to normalize to global mean, simply tick 

. Some genes may be expressed only in a subset of samples and filled in with above-cutoff values during the missing data handling, while genes expressed close to the limit of detection (i.e. cut-off) are known to have greater technical variation than genes expressed at lower Cq values. It is not desirable to include such values in a global mean normalization. In order to avoid using such genes for the global mean, tick 

, fill in the normalization cut-off of your choice, and 

.

Normalization to reference gene(s)

If the study is on a limited set of microRNAs chosen on the expectation that they should be regulated, then global mean normalization is not an option. In this case, normalization should be to a small set of stably expressed genes. In microRNA analysis, the preferred reference gene type would be stably expressed microRNA, but in many cases small non-coding RNA can also be used.

Step 1: Selecting candidate reference genes

From an Exiqon miRCURY LNA™ microRNA PCR panel screening study

If a screening study using the Exiqon miRCURY LNA™ microRNA PCR panels has been performed prior to a validation experiment, using the same sample types, then the choice of reference genes for use in the validation study may be based upon the screening results. In this case, from the screening study select the microRNAs most resembling the behavior of the global. These are recognized as the microRNAs having least variation after global mean normalization.

If this method of selection is used, then 2-3 reference genes can be chosen, and it is not necessary to further validate them since selection was based on global mean behavior during screening of the same sample type. In that case, **you can skip step 2, and continue with step 3: Normalizing to reference genes**.

From other sources

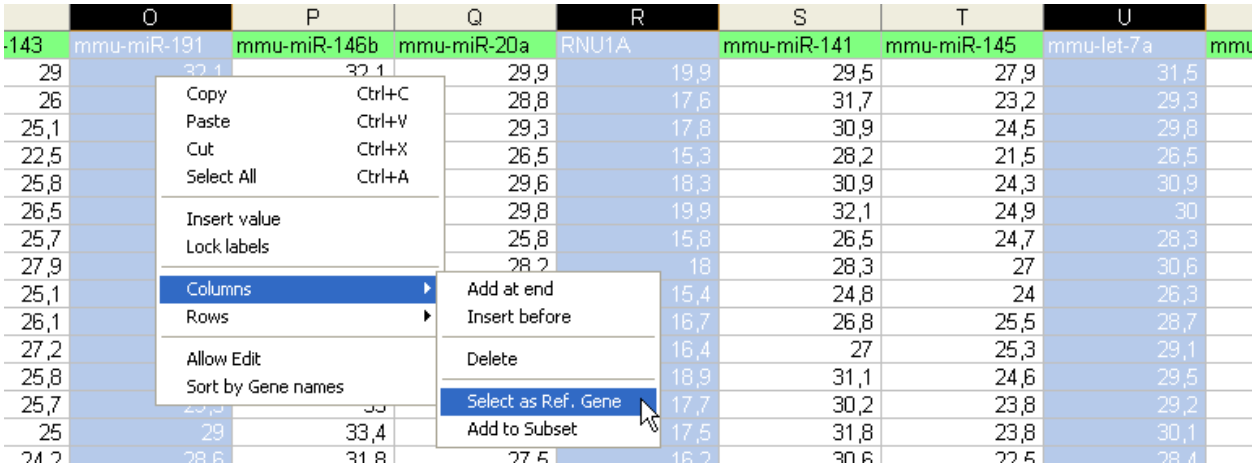
Candidate reference genes can also be chosen based on results from a different experimental platform (e.g. microarray), from previous studies which are similar but not identical, or from literature showing stable expression. In each of these cases, it is necessary to pick and test several candidate reference genes (typically 5-6) and use NormFinder and/or geNorm to pick the best reference gene(s) among the candidates.

NormFinder and geNorm both look at gene expression variance to choose the most stably expressed genes, but they use different algorithms to do so and may come up with slightly different results. It may be useful to perform both analyses, and then select reference genes they agree on.

Step 2: Choosing reference genes from candidates using NormFinder and/or geNorm

Before performing the NormFinder and/or geNorm analysis, you need to identify the candidate reference genes in your experiment in a manner that GenEx understands. For the full panels, Exiqon has already made a suggestion for candidate reference genes, and these were automatically classified as such. It may be desirable to add further candidates before proceeding with choosing the optimal genes. For Pick&Mix, no pre-decided information is available, which means that you need to specify the candidate reference genes to GenEx.

To specify candidate reference genes, simply select the columns containing candidate reference genes, (multi-gene selection is possible using shift or ctrl). Right-click and from the menu, choose Columns, then Select as Ref. Gene.

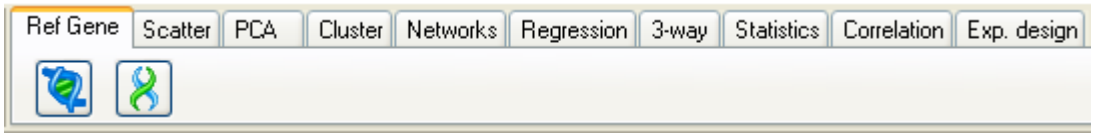


Once reference genes have been classified, a classification row will appear at the bottom of the Data editor table identifying candidate reference genes by a 1.

Now create a data subset containing only the candidate reference genes. In the Grid menu, choose Create Subset of then #RefGene row. The subset will open in a new data editor. Load the subset to the Control panel to perform NormFinder and/or geNorm analysis.

The following example illustrates how to select reference genes after creating a subset of the data from the panel.

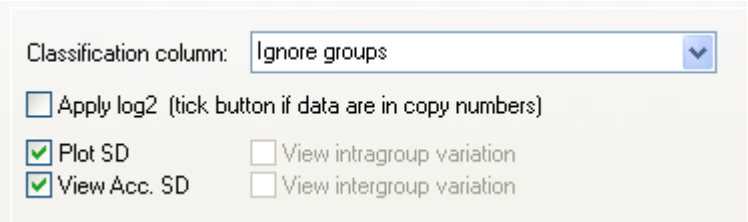
In the main window, there is a ref gene tab. Make sure that this is the active tab.



You can perform both NormFinder and geNorm analysis on the reference gene data. For NormFinder, you have the added option of looking at intergroup variation – i.e. whether assay variation is stable across groups and not just across samples.

NormFinder:

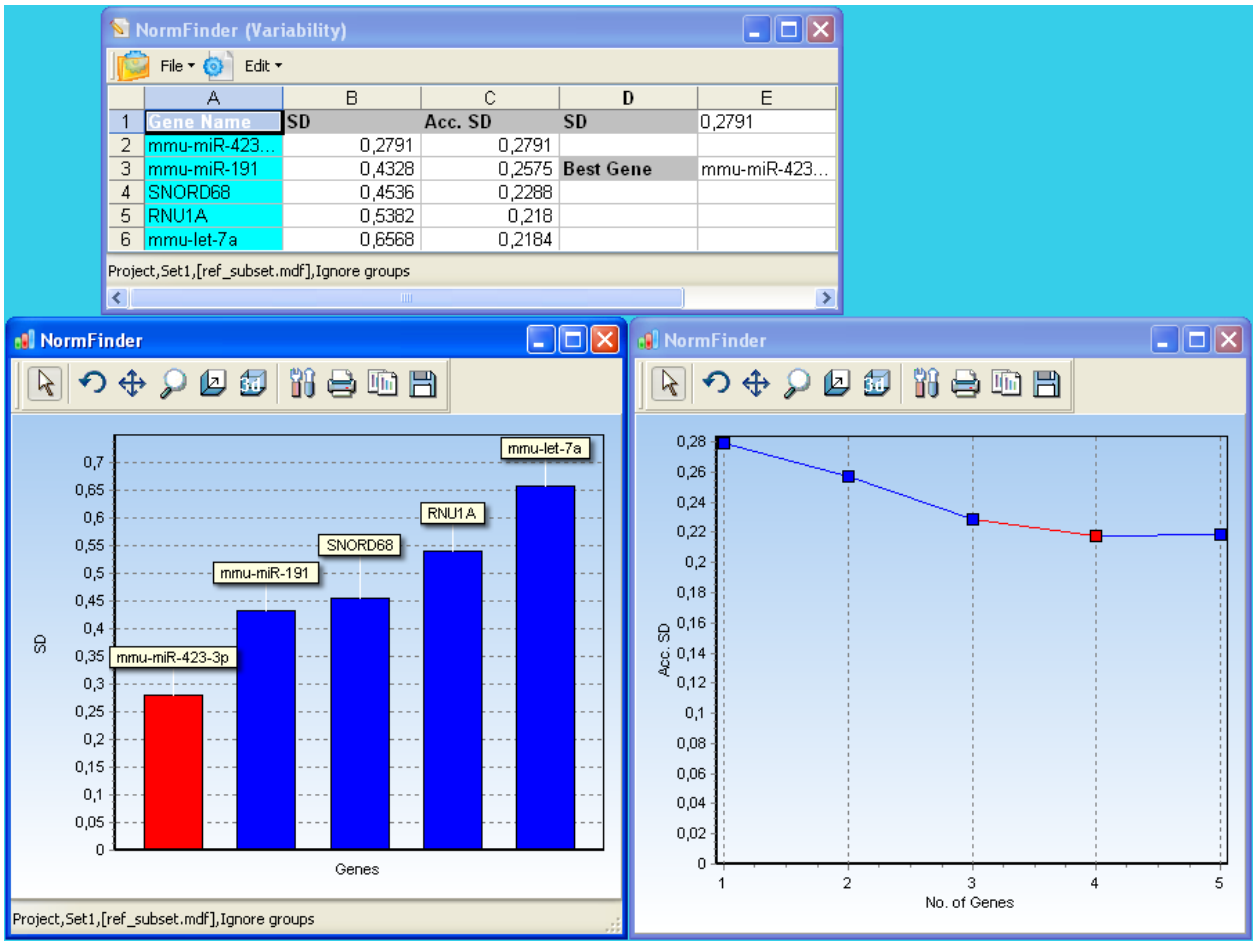
Click  , verify that Plot SD and View Acc. SD are ticked to get graphs of standard deviation and accumulated standard deviation, respectively.



then 

.

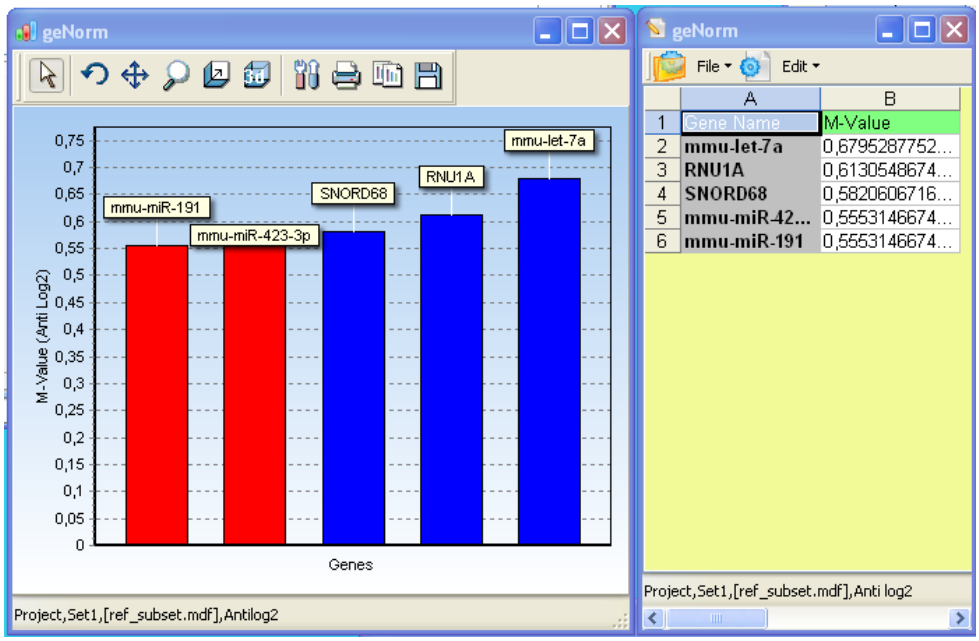
You should get an output consisting of 3 windows, similar to this:



geNorm:

Click  . is ticked by default and should not be changed. Click  .

You should get an output consisting of 2 windows, similar to this:



Selecting the reference genes

Both algorithms provide you with a table of values indicating the variability of each gene (M-value in geNorm, SD in NormFinder) and the best gene and/or best gene combination.

Both algorithms provide bar-charts showing which genes are most stably expressed (most stable gene to the left). Keep in mind that these are two different algorithms, so they may not always come up with the same answer. However, combined they should give a good indication of which genes are most stably expressed and which are less so. In this example, we would not choose let-7a as a reference gene.

Normfinder provides an additional graph of the accumulated SD, indicating in red the optimal number of reference genes, which may be from 1 to more than 5 (in this case 4, i.e. mmu-miR-423-3p, mmu-miR-191, SNORD68 and RNU1A).

Choosing the best reference genes now becomes an educated estimate – there is no final solution. geNorm selects the top pair of the most stably expressed reference genes, in this example mmu-miR-191 and mmu-miR-423-3p, and tells us that all candidates could be good choices. NormFinder indicates that 4 reference genes might be optimal, and in fact selects almost the same order of stability as geNorm. The option therefore would be to choose mmu-miR-423-3p, mmu-miR-191, SNORD68 and RNU1A as the best set of 4 reference genes. Both algorithms seem to be in agreement that these are not bad reference genes. But do keep in mind: more is not always better.

Step 3: Normalizing to reference genes

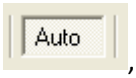
Once the final choice of reference genes has been made from the candidate reference gene subset, the candidate reference genes not used for normalization should be changed back to 0 in the

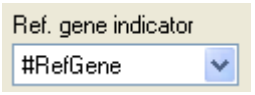
original data editor containing the full data set. To do so, activate the edit button  and simply edit the classifier value(s).

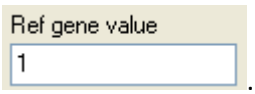
Now you are ready to perform the normalization.

In the 

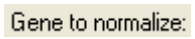
menu, choose 

. In the dialog box, activate 

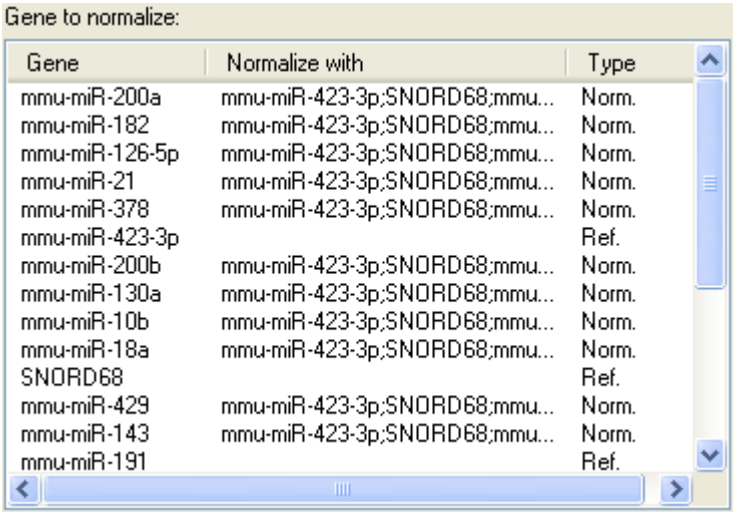
, select 

and set 

. Now click 

. The pane 

should now be filled in to indicate the chosen reference genes



Click 

NOTE: In the “delta delta Cq” calculation, this step is the first delta Cq

Average RT replicates

As recommended earlier, the reason for including replicate RT reactions is to get more robust data with the option of omitting potential outliers due to technical issues. But, in the end, we are interested in comparing just one value for each sample. This value is obtained by averaging the accepted technical replicates.

In the pre-processing menu, select 

.

In the Normalize by column pull-down select your replicate classifier and 

.

You will notice that the replicate rows have disappeared, leaving one row for each sample. These now contain the average of the replicates, after removal of the outliers.

Converting to relative quantities

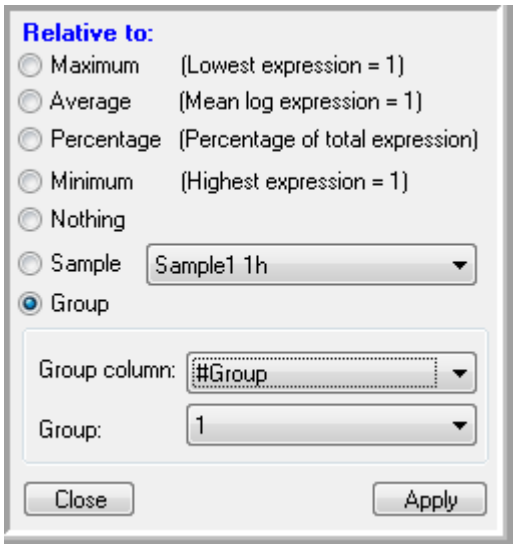
The numbers in the data table by now will not resemble the original Cq values. However, they will still be on the logarithmic scale of Cq values. It is therefore helpful to convert these values to relative quantities on the linear scale.

Step 1: Relative quantities

In the pre-processing menu select 

.

You now have several options for how you want values to be related:



In our example, we are comparing several groups with differential expression. This is most easily seen by calculating the relative quantity of each compared to a control group.

Tick 

, and in the expanded menu select the classifier specifying your control group

then 

.

NOTE: The data have now been converted to linear scale according to the following formula:

$N = 2^{(Cq-Cqrel)}$

where Cqrel is the Cq value you are relating to (i.e. normal, untreated, or average).

Step 2: Log conversion

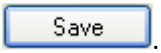
Many statistical tests, such as T-test and ANOVA, assume normal distribution. This is not always a true assumption when working with expression data at the linear scale, but by converting to log scale normal distribution is often obtained. Furthermore, some graphical representations are easier to view in the log scale.

In the 

menu you can choose between different log scales. Select 

.

Click 

, and in the dialog box click yes for saving your data. Choose a path and file-name, and 

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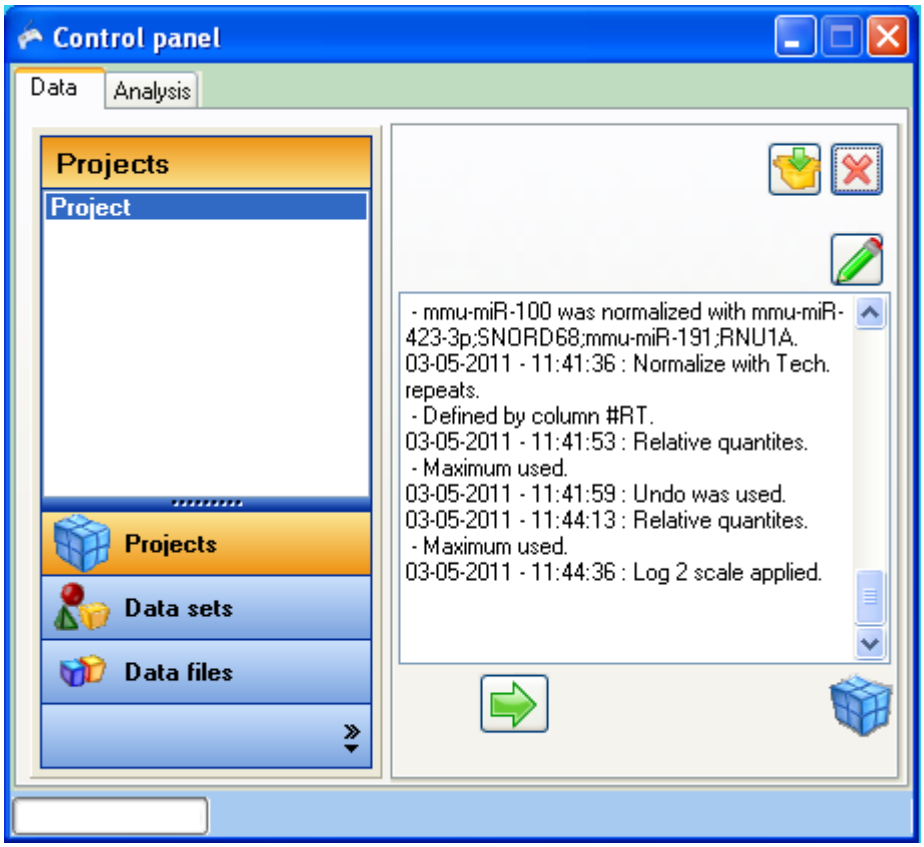
Your data has now been pre-processed and is ready for analysis by the multiple statistical features provided by GenEx.

Further data analysis using GenEx

After pre-processing is completed, you can either continue directly with analysis of the loaded data, or get back to this later. If you continue at a later time, you should import the pre-processed data set (saved at the end of pre-processing) using the load file function  . You should now have a project in the data manager, containing your pre-processed data set and log of pre-processing steps performed



At any time during data analysis, you can save your project with all analyses performed using the export project function  . You can then get back to the project at a later time by importing the projet to the control panels  .

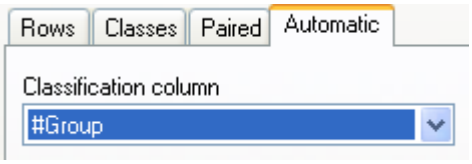
Using the Data manager

Before generating graphs or performing statistical analysis, you should open the “Data manager” for final data processing. If your data contains groups with biological replicates, you need to assign these groups now before you can perform any of the many statistical analyses possible in GenEx. Data manager also allows you to customize your graphs as you can assign different colors to samples, or groups of samples.

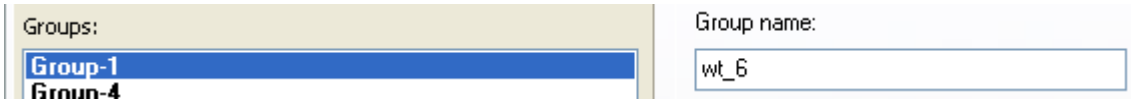
Once you have loaded your fully pre-processed data, click on  to open the Data manager. The Data manager has three tabs: Data selection, Colors and Symbols and Groups.

Under the Data selection tab you can select which data you want to analyze (typically all the data in the file, but if you want to perform analysis on a subset of your data this too is possible).

In the Groups tab, you define which samples belong to the same group (typically your biological replicates). If you have assigned group classifiers, you can use the automatic mode:
Select the tab Automatic and in the pull-down menu choose the classifier specifying your groups

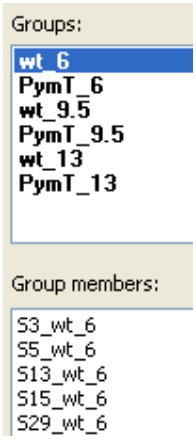


then click  . The groups have been given generic names (Group-1, -2 etc) but can be easily re-named to describe their content. Simply click the group name to edit, then write the new name in the Group name text box

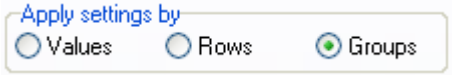


and click 

. Continue this procedure for each group.
When a group is selected, it is easy to inspect which samples have been assigned to the group; these appear in the Group members pane:



In the Colors and Symbols tab, you can choose the color of each sample or group for bar charts and the symbol for each sample or group for graphs such as PCA plots.

Tick 

, select each group in turn and assign colour and/or symol to the group.

When you are finished remember to click 

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Statistical analysis in GenEx

GenEx offers a wide range of statistical analyses (some of which require specific experimental designs). In the Statistics tab you can choose from different analyses. We recommend as a minimum performing either a T-test (in case of only two groups) or an ANOVA (if multiple groups are tested):

 T-test compares two groups of data. This test will give a list of the tested assays, sorted by P value (statistical significance). Among other things, you will get the difference between the two groups and the statistical significance. If the P-value is significant even with Bonferroni correction, the P value is green. If significance is obtained by ignoring Bonferroni correction, the P value is yellow. I the difference is not statistically significant, the P value will be in red.

(wt_13) vs (P...	KS Group 1 (wt_13)	KS Group 2 (Py...	Difference bet...	P Value
mmu-miR-145	Not Determined	Not Determined	2,34825	1E-8
mmu-miR-31	Not Determined	Not Determined	-3,41075	3,1E-8
mmu-miR-429	Not Determined	Not Determined	-2,93575	3,73E-7
mmu-miR-200b	Not Determined	Not Determined	-2,98675	6,67E-7
mmu-miR-18a	Not Determined	Not Determined	-3,20875	7,74E-7
mmu-miR-378	Not Determined	Not Determined	3,05225	1,615E-6
mmu-miR-20a	Not Determined	Not Determined	-1,96075	1,89E-6
mmu-miR-146b	Not Determined	Not Determined	-3,79175	6,37E-6
mmu-miR-126...	Not Determined	Not Determined	1,57925	1,0334E-5
mmu-miR-200a	Not Determined	Not Determined	-2,61875	1,6481E-5
mmu-miR-141	Not Determined	Not Determined	-3,27375	3,1778E-5
mmu-miR-10b	Not Determined	Not Determined	2,71925	0,00024632
mmu-miR-182	Not Determined	Not Determined	-2,2109375	0,000346359
mmu-miR-143	Not Determined	Not Determined	1,94625	0,000382891
mmu-miR-130a	Not Determined	Not Determined	1,88225	0,001135011
mmu-miR-100	Not Determined	Not Determined	1,8290625	0,001651923
mmu-miR-21	Not Determined	Not Determined	-1,14075	0,005869913
mmu-let-7a	Not Determined	Not Determined	0,14925	0,695350322

ANOVA can be performed either as a  one-way ANOVA testing for just one parameter (e.g. different treatments), or as a  two-way ANOVA testing for two different parameters (e.g. genotype and time). A table showing the result for each tested microRNA will result.

In a one-way ANOVA a P-value will be given for significance of difference between groups.

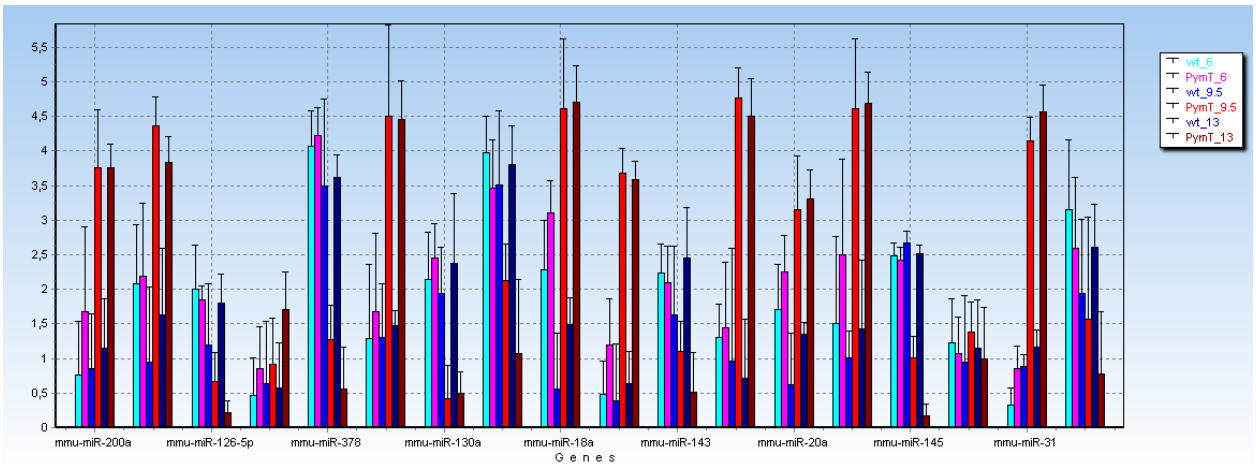
mmu-miR-200a					
Source	SS	df	MS	F	p-value
#Group	48,03322678	5	9,60664536	22,70092532	3E-8
Error	9,73320867	23	0,42318299		
Total	57,76643545	28			

The result of a two-way ANOVA indicates the significance of each factor, as well as the interaction of the two (i.e. whether the two factors influence each other).

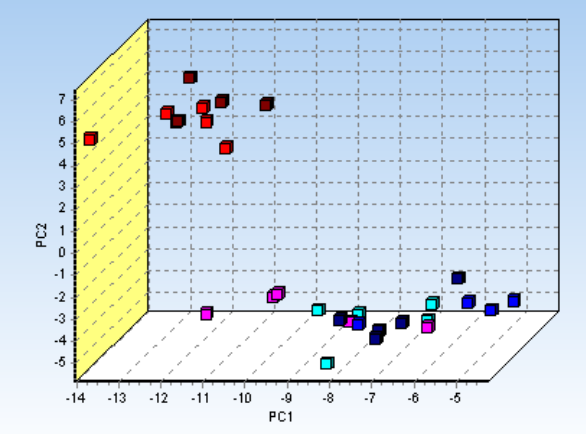
mmu-miR-200a					
Source	SS	df	MS	F	p-value
#GenoT	33,0547044	1	33,0547044	78,10971971	<1*10^-8
#Time	8,74788988	2	4,37394494	10,33582419	0,00062744
Interaction	5,59774808	2	2,79887404	6,61386241	0,00538143
Error	9,73320867	23	0,42318299		
Total	57,81117311	28			

Furthermore, several very nice graphical representations of the data set can be selected:

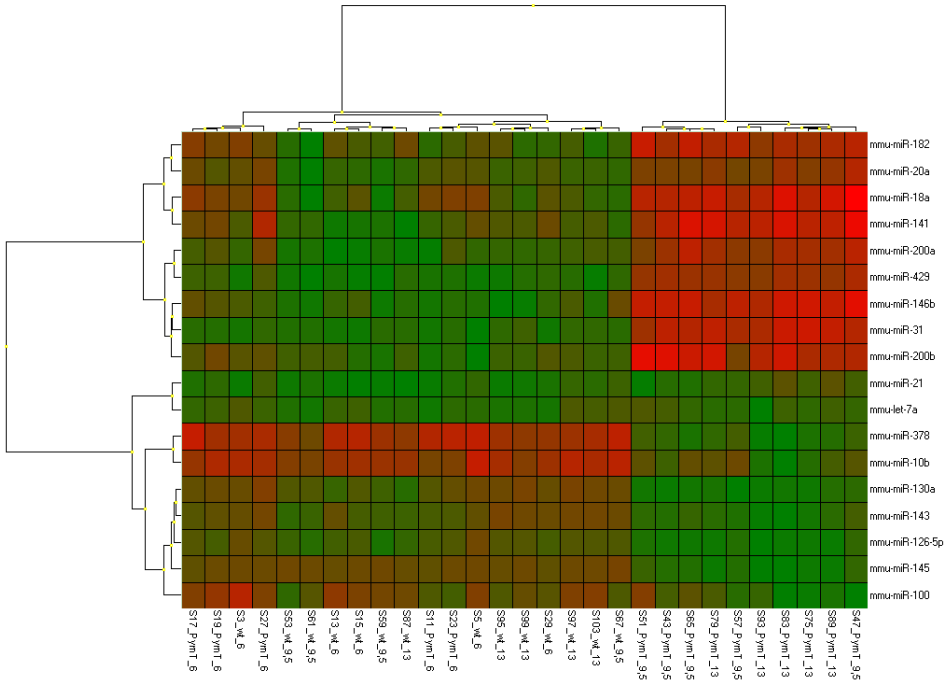
Descriptive Statistics gives a graphical representation of expression differences, with error bars as Standard deviation (STDEV), Standard error of the mean (SEM) or Confidence Intervals (CI) according to preference.



Principal component analysis shows how the samples group together. If colors have previously been assigned by grouping, it becomes easy to see whether the samples group as expected.



 Heat-map shows clustering, depicting grouping of microRNAs and samples



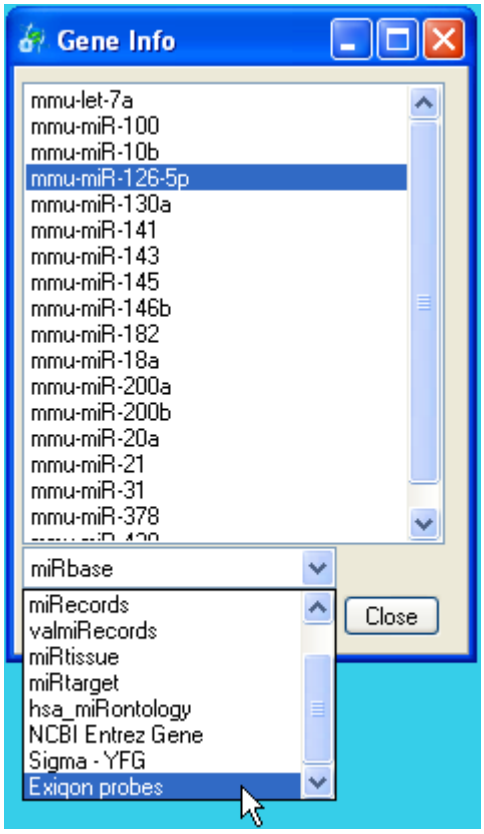
To find out more about these and other statistical analysis options, please see the GenEx Help function.

Many other types of plots and analysis possibilities are available, such as non-parametric statistical tests, Cohonen self-organizing maps, and nested ANOVA. The GenEx help function contains detailed explanations for these types of analyses

Gene info

GenEx can easily retrieve microRNA information on the assays used in and experiment from a number of relevant databases such as miRBase, miRecords and miRTarget (among others). You can also directly retrieve information on available probes from Exiqon's webshop, to ease the way into functional analysis.

Once the project has been loaded to the control panel, click  in the software toolbar menu. Then select your assay of interest in the dialog box appearing, choose the database of interest from the pull-down menu and click search.



GenEx will now start your internet browser, and find the selected microRNA in the chosen database.

Online help

For technical support please go to:

http://www.exiqon.com/contact

Join the GenEx online forum where you can ask other users and experts about qPCR data analysis and GenEx, please go to:

http://www.multid.se/forum.php

References

1. **A novel and universal method for microRNA RT-qPCR data normalization.**
Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J. **Genome Biol**. 2009;10(6): R64

2. **Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues.**
Peltier HJ, Latham GJ.**RNA**. 2008 14(5): 844-852

3. **Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer.**
Davoren PA, McNeill RE, Lowery AJ, Kerin MJ, Miller N. **BMC Mol Biol.** 2008 9: 76.