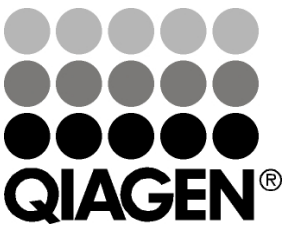
September 2019

RT² Profiler PCR Arrays & Assays Data Analysis Handbook

For analyzing gene expression data from Cataloged and Custom RT² Profiler PCR Arrays and RT² qPCR Assays



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Intended Use

The RT^2 Profiler PCR Arrays & Assays Data Analysis software is for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at https://www.qiagen.com/us/service-and-support/qa-qc-safety-data/safety-data-sheets-new/ where you can find, view, and print the SDS for each QIAGEN kit and kit component.

24-hour emergency information

Chemical emergency or accident assistance is available 24 hours a day from:

CHEMTREC

USA & Canada ▪ Tel: 1-800-424-9300

Outside USA & Canada ▪ Tel: +1-703-527-3887 (collect calls accepted)

Introduction

This document contains a Quick Reference Guide for experienced users and a Detailed Protocol for new users of the RT^2 Profiler PCR Arrays & Assays Data Analysis software. This document does not provide bench protocols. To obtain bench protocols, please refer to the appropriate handbook. If additional assistance is required, please contact a local QIAGEN technical support representative.

Important Notes

- Please set the screen resolution to 1024×768 or greater, if possible.
- Turn off any window pop-up blockers. Some features of the software will launch separate windows.
- Data to be uploaded must be saved as an .XLSX formatted Excel file. Files in the .XLS format are no longer compatible with the software.

Quick Reference Guide

This guide is intended for experienced users of the RT^2 Profiler PCR Arrays & Assays Data Analysis software only. New users should reference the Detailed Reference Guide.

Formatting data for the RT^2 Profiler PCR Arrays & Assays Data Analysis software

As guides, use the "Sample Upload Data Excel Files" available for download from, and image on, the "Upload/Select Dataset" page of the software after making the required selections there.

Upload/Select Project: Upload/Select Dataset

1. Select the "Product Type" used: "Cataloged Array", "Custom Array", or "Assay".
2. Select the "Species" specificity of the "Product Type" and Samples used.
3. Either select the "Catalog Panel" "Catalog" number used or enter the "Custom Panel" "Catalog" number used.
4. Select the "Plate Format" used: "96-Well", "384-Well", "Rotor-Gene Q 100-Well Ring", or "Fluidigm Biomark System".
5. Click the "Browse" button to select the Excel file prepared in the above section, and then click the "Submit" button.

Upload/Select Project: Select Job ID

Click the "Job ID" of the desired previously started and/or completed Job.

Setup

1. Job Details: Review the parameters associated with the analysis Job.
2. Uploaded Data: Confirm that the displayed "Gene Symbol", raw C_T values, Sample names, and Group definitions were uploaded correctly.
3. Sample Manager
   a. Use the "Group Definition" section to assign each Sample to their different Groups ensuring that Samples are placed into the "Control Group" and at least one test Group. Or, choose to "Exclude Sample".
   b. Click the checkbox if a PreAMP Primer Mix Kit was used.
   c. "Set C_T Cut-off": Use the default unless directed by Technical Support.
   d. Click the "Submit" button.
4. Data QC: Review the results of the Positive PCR Controls (PPC), Reverse Transcription Controls (RTC), and Genomic DNA Contamination (GDC) controls from all Samples.
5. Normalization: Provide the analysis Job with an "Analysis Name". Select the desired "Normalization Method" and reference genes using the appropriate dropdown menu and checkbox selections. Click the "Submit" button to proceed to "Analysis".

Analysis: Results

Review the "Fold Regulation", "Fold Change", "Average ΔC_T", and "2 ^ – ΔC_T" pages for the results and intermediate calculations generated by the software from the Upload Data Excel File based on the chosen "Setup" parameters.

Analysis: Plots & Charts

1. Scatter Plot & Volcano Plot
   Define the "Test Group" and "Control Group" to compare, "Fold Regulation" threshold, "p-Value" threshold (Volcano Plot only) and "Color Scheme" using the appropriate dropdown menus and dialog box(es).
   Deselect or reselect the right-hand table checkboxes to remove or add back genes to the plot.
   Click the "Update Chart" button to refresh the plot based on the new selections.
   Click the "Export Data" button to download the results as an Excel file.
   Click on the top right camera icon to save a PNG image of the plot.

2. Clustergram
   The default settings are the recommended settings. However, you may also:
   Choose to cluster "Sample" by "Array" or "Group".
   Choose to cluster by genes only or by genes and samples by selecting the "1-D" or "2-D" "Dimension".
   Choose a different linkage method by selecting a different "Join Type" option.
   Select how the plot should be "Color Coded" by selecting to use the average data across "Genes", "Samples", or the "Entire Dataset".
   Choose to make the "Item names visible" (Gene Symbols) or not.
   Deselect or reselect the right-hand table checkboxes to remove or add back genes to the plot.
   Click the "Update Chart" button to refresh the plot based on the selected parameters.
   To save an image of the plot, use your internet browser's functionality.

3. Heat Map
   Define the "Test Group" and "Control Group" to compare.
   Choose to display untransformed data by deselecting the "Log2 transform fold change" checkbox; however, the default selection is recommended.
   Click the "Update Chart" button to refresh the plot based on the new selections.
   Click the "Export Data" button to download the results as an Excel file.
   Click on the top right camera icon to save a PNG image of the plot.

4. Multigroup Plot
   Choose to plot "Average delta(C_T)", "2 ^ -delta(C_T)", or "Fold Change" on the "Y-Axis" by clicking the appropriate radial button.
   Choose to plot a "Line Chart" or "Column Chart" using the options in the "Chart Type" dropdown menu.
   Choose to display error bars by clicking the "Show Uncertainty" checkbox.
   Deselect or reselect the right-hand table checkboxes to remove or add back genes to the plot.
   Click the "Update Chart" button to refresh the plot based on the new selections.
   Click on the top right camera icon to save a PNG image of the plot.

What's next

Each module starts with the same first four steps:

Step 1: Define the "Experimental Group" (Test Group) and "Control Group" to compare using the appropriate dropdown menus.
Step 2: Use the appropriate radial button to analyze genes with "Decreased" or "Increased" expression or "Both".
Step 3: Define the desired fold regulation and p-value thresholds using the appropriate dialog boxes.
Step 4: Click the "Show Selected Genes" button.

Continue to follow the instructions for each module to identify the reagents needed for the next best experiment to follow up on the differentially expression genes with the help of the latest bioinformatics tools. The following modules are available: Gene Expression, miRNA Regulation, DNA Methylation, Transcription Factor / Histone, Protein Detection, and Somatic Mutation.

Export

1. Export Excel Report
   Select the desired data tables using the checkboxes and click the "Submit" button to download an Excel file with each data table in a different worksheet.

2. Export PDF Report
   Select the desired data tables to include in the PDF Report as well as the desired "Color Coding Scheme" using the checkboxes in the left-hand table. "Remove" or "Add More" pairwise Group comparisons to include in the PDF Report using the appropriate buttons on the right-hand side. Define the "Test Group", "Control Group", "Fold Change" threshold, and "p-Value" threshold for each pairwise Group comparison.
   Click the "Submit" button to generate and download the PDF Report.

Detailed Protocol

Formatting data for the RT^2 Profiler PCR Arrays & Assays Data Analysis software

The RT^2 Profiler PCR Arrays & Assays Data Analysis software analyzes C_T values to calculate changes in gene expression. For the software to correctly import and read data, the C_T values must be exported from the qPCR instrument and then organized in a specific fashion into a new .XLSX formatted Excel spreadsheet. The software only accepts Excel spreadsheets for analysis. The data from all plates and wells to be analyzed at the same time must be copied into a single worksheet. As guides, please use the downloadable "Sample Upload Data Excel Files" and displayed images on the "Upload/Select Dataset" page of the software and read their descriptions below.

For all plate formats, start by exporting the data from the real-time instrument into an Excel-compatible file. After exporting, check the exported file to ensure that the C_T values are arranged in the correct order, as shown in Figure 1 below.

Correct			Incorrect	
Well	**C_T**		**Well**	**C_T**
A01	33.4		A1	33.4
A02	21.2		A10	27.2
A03	26.7		A11	23.5
…			…	
H12	22.0		H9	34.4
…			…	
P24	19.2		P9	37.2

Figure 1. Correctly versus incorrectly ordered C_T values in the data file exported from the real-time PCR instrument.

1. Cataloged RT^2 Profiler PCR Arrays: 96 RT^2 qPCR Assays in a 96-Well or Rotor-Gene Q 100-Well Ring Plate Format or a Cataloged 384HT RT^2 Profiler PCR Array

These instructions apply to Cataloged RT^2 Profiler PCR Arrays including 96 RT^2 qPCR Assays either in the 96-Well Plate Format (A, C, D, F) or the Rotor-Gene Q 100-Well Ring Plate Format (R) or including 384 RT^2 qPCR Assays in the 384-Well Plate Format (E, G), the 384HT RT^2 Profiler PCR Arrays with 3000 series catalog numbers.

Start with the "96-Well Cataloged PCR Array" or "384-Well Cataloged PCR Array" "Sample Upload Data Excel Files" available on the "Upload/Select Dataset" page of the software and follow the instructions below. See Figure 2 for an example of a correctly formatted Upload Data Excel File for a Cataloged RT^2 Profiler PCR Array in the 96-well Plate Format.

a. Delete all Sample and Group names and data from the Sample Upload Data Excel File. Do not delete well numbers in Column A (POS).
b. Copy and paste the C_T values exported from the instrument into the template starting from first Cell B3 then C3 then D3, and so on. Each column of data will contain data from one Sample lined up with the correct well location (POS) on the PCR plate.
c. Above the raw data, add the Samples' names to Row 1. Optionally, add Group designations to Row 2. The Group names must precisely read as "Control Group", "Test Group 1", "Test Group 2", etc. If defining Groups in the software after uploading the data is preferred, simply leave Row 2 blank, and use the "Sample Manager" described below.
d. Save the Upload Data Excel File in the.XLSX format and use it to upload the data into the software.

	A	B	C	D	E
1		Resting 6 h	6 h Stimulation	Resting 24 h	24 h Stimulation
2	POS	Control Group	Test Group 1	Test Group 2	Test Group 3
3	A01	29.08	29.89	28.86	27.39
4	A02	32.02	31.15	33.33	31.89
5	A03	33.83	31.57	35.7	37.93
6	A04	33.95	31.3	33.05	29.21
7	A05	Undetermined	Undetermined	Undetermined	Undetermined
8	…	…	…	…	…
9	H12	19.9	20.65	20.09	19.85

Figure 2: A correctly formatted Upload Data Excel File for a Cataloged RT^2 Profiler Array in the 96-well Plate Format is displayed.

2. Cataloged RT² Profiler PCR Arrays: 96 RT² qPCR Assays and 4 samples in the 384-Well Plate Format

These instructions apply to RT² Profiler PCR Arrays with 96 RT² qPCR Assays replicated 4 times in the 384-Well Plate Format (E, G). These instructions do not apply to the 384HT RT² Profiler PCR Arrays with 3000 series catalog numbers.

Each Sample's data must first be reorganized into 4 individual columns before pasted into the correct format as indicated by the "96-Well Cataloged PCR Array" Sample Upload Data Excel File. Use the "384-Well (4x96) Cataloged Array Patch" available for download from the "Upload/Select Dataset" page of the software and displayed in Figure 3.

384-Well Format		96-Well Format Equivalent				
Well #	C_T	Position	Sample 1	Sample 2	Sample 3	Sample 4
1	A01	A01	A01	A02	B01	B02
2	A02	A02	A03	A04	B03	B04
3	A03	A03	A05	A06	B05	B06
4	A04	A04	A07	A08	B07	B08
5	A05	A05	A09	A10	B09	B10
6	A06	A06	A11	A12	B11	B12
...	...					
384	P24					

Figure 3. The "384-Well (4x96) Cataloged Array Patch" is displayed.

a. Open the "384-Well (4x96) Cataloged Array Patch" Excel file.
b. Copy the C_T values exported from the real-time PCR instrument and Paste Special Values into the yellow cells starting at cell A10 of the Excel file. The 384-well data will automatically be reorganized into 4 separate columns one for each sample in the white cells to the right.
c. Copy the content of the white cells into an Upload Data Excel File as described above for Cataloged RT² Profiler PCR Arrays with 96 RT² qPCR Assays in the 96-Well Plate Format

See Figure 2 for an example of a correctly formatted Upload Data Excel File for a Cataloged RT² Profiler PCR Array in the 96-well Plate Format.

3. Custom RT^2 Profiler PCR Arrays

For Custom RT^2 Profiler PCR Arrays in the 96-Well or 384-Well Plate Formats that include replicates of less than 96 or 384 RT^2 qPCR Assays, respectively, to characterize multiple Samples on the same plate, each Sample's data must first be reorganized into individual columns before pasting the data into an Upload Data Excel File. Use the "Universal Custom PCR Array Patch" available on the "Upload/Select Dataset" page of the software as described below.

a. Open the "Universal Custom PCR Array Patch" Excel file. In the default "Data_Entry" worksheet, select "RT^2 Profiler PCR Arrays", select the Plate Format used (either "96-Well Plate" or "384-Well Plate" or "Rotor-Disc 100"), and the plate layout based on its combination of "genes" and "samples", each from the respective dropdown menus. Finally, click hyperlink that appears.
b. Into the new worksheet, copy the C_T values exported from the real-time PCR instrument and Paste Special Values into the yellow cells starting at cell A05. The data will be automatically reorganized into a separate column for each sample in the white cells to the right.
c. Copy the content of the white cells and paste it into the correct cells of an Upload Data Excel File as described below.

For Custom RT^2 Profiler PCR Arrays where only one sample is analyzed on only one plate in either the 96-Well or 384-Well Plate Format, the C_T values exported from the real-time PCR instrument may be pasted directly into either the "96-Well Custom PCR Array" or "384-Well Custom PCR Array", respectively, Upload Data Excel File as described below.

See Figure 4 for an example of a correctly formatted Upload Data Excel File for a 96-assay Custom RT^2 Profiler PCR Array in the 96-Well Plate Format.

To complete the Upload Data Excel File for a Custom RT^2 Profiler PCR Array:

a. Open either the "96-Well Custom PCR Array" or "384-Well Custom PCR Array" Upload Data Excel File based on the Plate Format.
b. Delete all Sample and Group names and data from the Sample Upload Data Excel File. Do not delete well numbers in Column A (POS).
c. Paste the data from each Sample into the Upload Data Excel File starting from first Cell D3 then E3 then F3, and so on. The data in each column should line up with the correct well location (POS) on the PCR array.
d. Copy the gene content of the custom PCR array into column B starting at Cell B3, and copy the RT^2 qPCR Assay catalog numbers of the custom array into column C starting at Cell C3. This information may be obtained from the Custom RT^2 Profiler PCR Array sign-off form used for ordering.
e. Above the raw data, add the Samples' names to Row 1. Optionally, add Group designations to Row 2. The Group names must precisely read "Control Group", "Test Group 1", "Test Group 2", etc. If defining Groups in the software after uploading the data is preferred, simply leave Row 2 blank, and use the Sample Manager as described below.
f. Save the Upload Data Excel File in the .XLSX format and use it to upload the data into the software.

	A	B	C	D	E	F	G
1				Resting 6 h	6-h Stimulation	Resting 24 h	24-h Stimulation
2	POS	Gene Symbol	Assay Catalog #	Control Group	Test Group 1	Test Group 2	Test Group 3
3	A01	BMP1	PPH00515B	29.08	29.89	28.86	27.39
4	A02	BMP2	PPH00549C	32.02	31.15	33.33	31.89
5	A03	BMP3	PPH00513F	33.83	31.57	35.7	37.93
6	A04	BMP4	PPH00546A	33.95	31.3	33.05	29.21
7	A05	BMP5	PPH00509A	29.00	26.67	29.38	26.32
…	…			…	…	…	…
98	H12	PPC	PPX63339A	19.9	20.65	20.09	19.85

Figure 4. A correctly formatted Upload Data Excel File for a 96-well Custom RT^2 Profiler PCR Array is displayed.

4. Individual RT^2 qPCR Assays

For experiments using a small number of individual RT^2 qPCR Assays, start with the "Individual Assays" Sample Upload Data Excel File downloadable from the "Upload/Select Dataset" page of the software, and follow the instructions below. See Figure 5 for an example of a correctly formatted Upload Data Excel File for analyzing data from individual RT^2 qPCR Assays.

a. Delete all Sample and Group names and data from the Sample Upload Data Excel File. Do not delete the "Gene #" data in Column A.
b. Copy and paste the C_T values exported from the real-time PCR instrument into the Upload Data Excel File starting from first Cell D3 then E3 then F3, and so on. Each column of data should contain the data from one sample lined up with the correct "Gene #" in Column A.
c. Copy the gene list into column B starting at Cell B3, and copy the RT^2 qPCR Assay catalog number list into column C starting at Cell C3.
d. Above the raw data, add the Samples' names to Row 1. Optionally, add Group designations to Row 2. The Group names must precisely read "Control Group", "Test Group 1", "Test Group 2", etc. If defining Groups in the software after uploading the data is preferred, simply leave Row 2 blank, and use the Sample Manager as described below.
e. Save the Upload Data Excel File in the .XLSX format and use it to upload the data to the software.

	A	B	C	D	E	F	G
				Resting 6 h	6-h Stimulation	Resting 24 h	24-h Stimulation
1	Gene #	Gene Symbol	Assay Catalog #	Control Group	Test Group 1	Test Group 2	Test Group 3
2	1	BMP1	PPH00515B	29.08	29.89	28.86	27.39
3	2	BMP2	PPH00549C	32.02	31.15	33.33	31.89
4	3	BMP3	PPH00513F	33.83	31.57	35.7	37.93
5	4	BMP4	PPH00546A	33.95	31.3	33.05	29.21

Figure 5. A correctly formatted Upload Data Excel File for individual RT^2 qPCR Assays is displayed.

Upload/Select Project

1. Upload/Select Dataset

The "Upload/Select Dataset" page contains links to download this Handbook and the "Sample Upload Data Excel Files" and patches described above needed based on the selections made as described below. This page also provides an image of a properly formatted Upload Data Excel File again based on the selections made as described below.

To start the analysis on this page, follow these instructions:

a. Product Type

Select whether analyzing data from a "Cataloged Array", a "Custom Array", or individual "Assays" from this dropdown menu.

b. Species

Select from this dropdown menu the model system used and the specificity of the arrays or assays to be analyzed.

c. Catalog

If the "Cataloged Array" "Product Type" was selected, select the species-specific product used based on its catalog number and description from this dropdown menu.
If the "Custom Array" "Product Type" was selected, enter the provided catalog number for the custom array into the dialog box. In this case, the use of this feature is optional.
If the "Assays" "Product Type" was selected, this option is unnecessary and will not be available.

d. Plate Format

Select whether the array or assays were used on a "96-Well" plate, a "384-Well" plate, a "Rotor-Gene Q 100-Well Ring", or the "Fluidigm BioMark System" from this dropdown menu.

e. Upload File

Click the "Browse" button to navigate your computer to find the Upload Data Excel File prepared in the above section. Select the file and click "Open".

f. Submit

Finally, click the "Submit" button to upload the Upload Data Excel File and automatically navigate to the "Setup" page.

2. Select Job ID

The "Select Job ID" page lists all previously initiated analysis Jobs. The table includes columns for the "Job ID", "Product Type", "Analysis Name", "Plate Format", "Catalog #", "Start Time", and "Status". Some of these terms are defined above, while the other terms are defined below.

Click on the "Job ID" number link to finish or review that Job's analysis starting on the "Setup" page.

Setup

After submitting the Upload Data Excel File or selecting a previous "Job ID", the software will navigate to the "Setup" page that includes the following pages listed, and accessible from the links, in the left-hand navigation panel: "Job Details", "Uploaded Data", "Sample Manager", "Data QC", and "Normalization".

1. Job Details

Job ID: The software automatically assigns this number specifically to each analysis Job.

Analysis Name: This name is manually defined on the "Normalization" page described below. Otherwise, this field will read "not provided".

Product Type, Catalog Number, Plate Format: The selections made on the "Upload/Select Dataset" page for this analysis Job are relisted here.

Start Time: The date and time at which the Upload Data Excel File was submitted is displayed.

Status: This field describes the analysis Job's state of progress, specifically:

File is uploaded: The Upload Data Excel File has been submitted, but nothing else has been done.
Sample manager information set up: The Upload Data Excel File and the "Sample Manager" information have been submitted, but nothing else has been done.
Job is running: The software is processing the raw data in the Upload Data Excel File based on the submitted "Sample Manager" and "Normalization" information. A Job should never have this status for an extended period. If a Job is stuck at this point, contact a QIAGEN Technical Support representative.
Job is done: The analysis Job's results and the other software functionality may be reviewed.

2. Uploaded Data

This page displays the raw data as provided in the Upload Data Excel File. Ensure that the displayed "Gene Symbol", raw C_T values, Sample names, and Group definitions were uploaded as intended.

3. Sample Manager

a. Group Definition

If Samples were already assigned to Groups in the submitted Upload Data Excel File, then those assignments will already be defined in the software and displayed here.

If not or if changes need to be made, then assign which Samples belong to which Group by choosing the correct Group from the dropdown menu next to each Sample name. Biological and/or technical replicate Samples obtained under the same control or various test experimental conditions should be placed in the same Group.

The software supports the analysis of up to as many Groups as there are Samples; however, the Analysis and Export Excel Report and Clustergram pages will use the same "Control Group" to compare its data with the data from each numbered test Group when calculating the Fold Change, Fold Regulation, and p-value results. If more than one "Control Group" is to be used, separate analyses must be performed for each different "Control Group" in separate Jobs. Other pages (all other Plots & Charts and the Export PDF Report) allow any Group to be compared to any other Group.

To exclude a Sample from the data analysis, select the "Exclude Sample" option from the Samples' dropdown menu. Such an assignment is useful for outlier Samples or Samples with failed "Data QC" described below.

b. Select Reference Genes and Selected Reference Genes

This feature is only available for the "Custom Array" and "Assays" "Product Type" and not for the "Cataloged Array" "Product Type".

Define the genes to be used for manual or automatic reference gene selection on the "Normalization" page described below. Scroll down the genes in the "Select Reference Genes" list to find the desired reference genes. Select a gene by clicking it and then click the "Add" button to include it in the "Selected Reference Genes" list that appears when the first gene or genes are added. Hold down the CTRL key and click on multiple genes to select and add them all at the same time. To remove a gene from the "Selected Reference Genes" list, click on it and then the "Remove" button.

If present, the standard set of species-specific reference genes included on cataloged arrays will be automatically added to the "Selected Reference Genes" list.

c. Click if a PreAMP Primer Mix Kit was used

Select this checkbox if a pre-amplification of the cDNA was performed before using cataloged or Custom RT^2 Profiler PCR Arrays. This option only affects the interpretation of the QC control assays on the "Data QC "page described below and does not affect the rest of the analysis.

d. Set C_T Cut-Off

The "Set C_T Cut-Off" function defines the lower limit of detection by defining the upper limit of useful C_T values in calculating Fold Change results. All raw undetermined C_T values (e.g., Undetermined, ND, N/A, 0, etc.) or C_T values greater than the "Set C_T Cut-Off" value are changed to that value before any of other calculations described below are performed.

For most applications, the default "Set C_T Cut-Off" value will not need to be changed. Only under special circumstances specifically recommended by QIAGEN Technical Support should the "Set C_T Cut-Off" value be adjusted to C_T values ranging from 30 to 40.

4. Data QC

A key benefit of the RT^2 Profiler PCR Arrays is the proprietary quality control assays included on all cataloged arrays (and highly recommended for custom arrays) to provide information on the technical quality of the results. These controls are the Genomic DNA Control (GDC) control, which measures the potential contribution of genomic DNA to the raw C_T values; the Reverse Transcription Control (RTC), which measures the relative efficiency of the reverse transcription reaction; and the Positive PCR Control (PPC), which measures how well the overall PCR performed. The "Data QC" page compares the specific results of the control assays to established pass/fail criteria and provides information on whether the controls passed QC or not.

If all Samples passed all three quality controls, then only a table reporting this fact will be displayed. If any Sample did not pass any of the three quality controls, a table will display which Sample(s) did not pass which control(s). Another table will then display the raw C_T values of all controls from all Samples that did not pass at least one control. This second table's legend describes the pass/fail criteria for each control. Consult the appropriate RT^2 Profiler PCR Array Handbook or contact a QIAGEN Technical Support representative for further troubleshooting.

a. Genomic DNA Contamination

Each species-specific GDC control assay very sensitively detects a unique non-coding region within the species' genome far removed from any known transcription start sites. The GDC control assay effectively replaces the need for a separate control reverse transcription reaction lacking the reverse transcriptase enzyme. If the GDC control assay's C_T value is lower than the limit of detection defined with the "Set C_T Cut-Off" feature on the "Sample Manager" page, then the "Data QC" page reports "Pass" for this control. Otherwise, it reports "Inquiry", and re-purification of the Samples' RNA with genomic DNA removal followed by a re-analysis of the Samples will be required.

b. Reverse Transcription Efficiency

The RT^2 First Strand cDNA synthesis kit reaction includes an artificial RNA molecule with a poly-A tail that is not homologous to any mammalian or bacterial sequence. This transcript is then reverse transcribed along with the RNA Samples. The RTC assay detects the cDNA synthesized from the spiked-in RNA molecule as a measure of overall reverse transcription efficiency of the RNA Samples. RNA quality influences reverse transcription efficiency the most. If the difference between the average RTC assays' C_T values and of the average PPC assays' C_T values (described below) is less than or equal to 5, then the "Data QC" page reports "Pass" for this control. Otherwise, it reports "Inquiry", and re-purification of the Samples' RNA followed by a re-analysis of the Samples will be required.

c. PCR Array Reproducibility

The PPC assay is designed to assess the reliability of the PCR itself. It contains a small amount of template DNA with an artificial sequence and primers designed to amplify this sequence. The PPC assay's template DNA is not homologous to any mammalian or bacterial sequence or to the RNA molecule spiked into the RT^2 First Strand cDNA synthesis kit assay described above. Extensive testing and validation of the PPC assay has determined that its C_T values should always lie with a specific range and be consistent across Samples. If the average of replicate PPC assays' C_T values for a Sample is 20 ± 2 and if the difference between the average PPC assays' C_T values from any two Samples is not greater than 2, then the Data QC page reports "Pass" for this control; otherwise, it reports "Inquiry", meaning that the PCR itself was likely negatively impacted.

Again, if any samples return "Inquiry" for any of these three controls, consult the appropriate RT^2 Profiler PCR Array Handbook or contact a QIAGEN Technical Support representative for further troubleshooting assistance.

Please note that if a pre-amplification of the cDNA was performed, the QC assays' criteria for passing or failing will be different than un-amplified cDNA Samples. If any of the controls fails in any Sample, those altered criteria will be provided in the legend of the second table described above.

5. Normalization

Reference genes are defined as those expressed at relatively constant levels under most experimental conditions. This constant expression level proves to be ideal for normalizing raw RT-PCR C_T values to control for small differences in Sample amount used. All cataloged RT^2 Profiler PCR Arrays include a standard set of five species-specific reference genes. The reference genes chosen to be included in Custom RT^2 Profiler PCR Arrays or in an analysis using individual RT^2 qPCR Assays should have been defined on the "Sample Manager" page described above before arriving at the "Normalization" page. If specific reference genes to be used for normalization were not previously included in the panel or defined on the "Sample Manager" page, then only the "Automatic Selection from Full Panel" "Normalization Method" described below may be chosen and used.

Three methods for data normalization are available: "Manual Selection", "Automatic Selection from HKG Panel", and "Automatic Selection from Full Panel". For all normalization methods, be sure to click the "Submit" button before proceeding. Otherwise, the software will not allow navigation to any other page. Once the calculations are complete, the software will automatically navigate to the "Analysis" page.

a. Manual Selection

To normalize the uploaded raw data for analysis, this method allows for the selection of all or a subset of either the built-in reference genes on cataloged arrays or the reference genes from custom arrays or sets of assays as defined in the "Sample Manager".

De-select or re-select the "Control Gene" checkboxes next to each "Gene Symbol" to visualize the effects on the "Arithmetic Mean" and "Geometric Mean" to be used as normalization factors. Continue evaluating the possible normalization factors until those values differ as little as possible across the samples, ideally by less than one.

Using the "Normalized By" dropdown menu, select whether to use either the "Arithmetic Mean" or "Geometric Mean" as the normalization factor. Finally, click the "Submit" button below the table to initiate the Job's calculations, or select a different "Normalization Method" from the above dropdown menu.

b. Automatic Selection from HKG Panel

To normalize the uploaded raw data for analysis, this method automatically selects an optimal set of reference genes from either the built-in reference genes on cataloged arrays or the reference genes from custom arrays or sets of assays as defined in the "Sample Manager".

The software identifies the reference gene set yielding "Geometric Means" that differ as little as possible across the Samples, ideally by less than one.

If satisfied with the result, click the "Submit" button below the table to initiate the Job's calculations, or select a different "Normalization Method" from the above dropdown menu.

c. Automatic Selection from Full Panel

To normalize the uploaded raw data for analysis, this method automatically selects an optimal set of reference genes from the entire cataloged array, custom array, or set of assays.

The software identifies the reference gene set yielding "Geometric Means" that differ as little as possible across the samples, ideally by less than one.

If satisfied with the result, click the "Submit" button below the table to initiate the Job's calculations, or select a different "Normalization Method" from the above dropdown menu.

Analysis

1. Results

Fold change calculations (gene expression ratios) are calculated using the classic, well-established, and widely adopted $\Delta\Delta C_T$ method originally published in Livak K.J. and Schmittgen T.D. (2001) Methods **25**, 402. The "Analysis" page includes several "Results" pages listed, and accessible from the links, in the left-hand navigation panel: "Fold Regulation" (default view), "Fold Change", "Average ΔC_T". and "2 ^ $-\Delta C_T$".

a. Fold Regulation and Fold Change

"Fold Change" is calculated as the ratio of the relative gene expression between the "Control Group" and each test Group. Numbers greater than 1 indicate increased gene expression, numbers between 0 and 1 indicate decreased gene expression, and a Fold Change value of 1 indicates no change. For example, a Fold Change value of 0.25 occurs when the normalized expression of a gene in a test Group is four times lower than the normalized gene expression in the "Control Group".

First, ΔC_T values, also called the normalized data, for each gene are calculated by subtracting the normalization factor (as selected on the "Normalization" page) from the C_T value of each gene of interest (GOI).

$$\Delta C_T = C_{T\,(GOI)} - C_{T\,(Reference\ Gene)} \text{ or } \Delta C_T = C_{T\,(GOI)} - \text{Average } (C_{T\,(Reference\ Genes)})$$

Next, the gene-specific ΔC_T values for all Samples in each Group are averaged. If there is only 1 sample per group, this step is skipped.

$$\text{Average } \Delta C_T = (\Delta C_{T\,(Sample1)} + \Delta C_{T\,(Sample2)} + \ldots \Delta C_{T\,(Sample\ n)}) \,/\, n \text{ Samples}$$

The $\Delta\Delta C_T$ value is calculated by subtracting the gene-specific average ΔC_T value of each test Group from the corresponding value of the Control Group.

$$\Delta\Delta C_T = \text{Average } \Delta C_{T\,(Group\ n)} - \text{Average } \Delta C_{T\,(Control\ Group)}$$

The Fold Change value is then calculated by converting the $\Delta\Delta C_T$ from a $\log_2$ scale to a linear scale using the following equation:

$$\text{Fold Change} = 2^{(-\Delta\Delta C_T)}$$

"Fold Regulation" conveys the same information as "Fold Change", but "Fold Regulation" results are easier to read and interpret. If a Fold Change value greater than 1, the Fold Regulation and Fold Change values are the same. For Fold Change values (X) less than 1, the Fold Regulation is the negative inverse of the Fold Change value ($-\frac{1}{X}$). For example, a Fold Change value of 0.25 corresponds with a Fold Regulation value of –4.0. Fold Regulation values greater than 1 indicate increased gene expression, Fold Regulation values less than zero indicate decreased gene expression, and a Fold Regulation value of 1 indicates no change. For example, a Fold Regulation value of –4.0 occurs when the normalized expression of a gene in the test Group is four times lower than the normalized gene expression in the Control Group.

p-Values

The "p-Value" is calculated based on a Student's t-test of the replicate $2^{-\Delta C_T}$ values (or linearized normalized gene expression levels) for each gene in each "Control Group" and test Group comparison. The p-value calculation used is based on parametric, unpaired, two-sample equal variance, two-tailed distribution – a method widely accepted in scientific literature. Both Groups in each pairwise comparison must contain at least 3 samples for the software to calculate p-values for that comparison. Otherwise, "nan" is reported.

On all software pages and in all exported files, all "Fold Change" and "Fold Regulation" results are listed with 2 significant digits, while "p-Value" results are listed with 6 significant digits. On the "Results" pages, "Fold Change" results greater than 2 or less than 0.5 and "Fold Regulation" results greater than 2 or less than -2 are highlighted in blue, while "p-Value" results less than 0.05 are highlighted in red.

Comments

The "Comments" provide disclaimers on "Fold Change" and "Fold Regulation" results based on the original average C_T values in the Groups compared. Given the higher degree of variability that may be observed with lower copy number templates (e.g. C_T>30), care needs to be taken when interpreting such results and deciding on next steps such as validation and functional studies, etc. The "Comments" provide insight into the relative abundance of the transcripts based on their C_T values used in the Fold Change calculations. Table 1 describes the "Comments".

Table 1. Definition of Comments

Comment	Explanation
[No Comment displayed]	The gene's expression is relatively high in both the test and control group. The threshold cycle (C_T) is <30.
A	The gene's expression is relatively low or not detected in either the test or control group. Low expression means that the average threshold cycle value is high (C_T>30) for that group. The expression in the other group is then relatively high (C_T<30). Because the detection of gene expression can be limited by the original amount of mRNA, the real Fold Change value is at least as large as reported. This result may also have variations in p-value (>0.05). Therefore, it is important to have enough biological replicates to verify the results for this gene.
B	The gene's relative expression level is low in both samples. Low expression means having a high average threshold cycle (C_T>30). In addition, the p-value is either not available or relatively high (>0.05). The Fold Change or Fold Regulation result may be more variable due to the low levels of expression. Therefore, it is important to have enough biological replicates to verify the results for this gene.
C	This gene is not detected in either sample. The gene's average threshold cycle (C_T) is either not determined or greater than the C_T Cut-Off defined by the Set C_T Cut-Off feature on the Sample Manger page. While the software tries to calculate a Fold Change or Fold Regulation value after normalization, the Fold Change may be erroneous.

b. Average ΔC_T

This page tabulates the average ΔC_T (normalized) values for each gene within each Group using 2 significant digits.

c. 2^-ΔC_T

This page provides tabulates the average $2^{-\Delta C_T}$ value, or linearized normalized expression level, for each gene within each Group using 6 significant digits.

After reviewing the "Results", continue to generate "Plots & Charts" by clicking on the option in the left-hand navigation pane.

2. Plots & Charts

a. Scatter Plot

The "Scatter Plot" compares the normalized expression level of each gene between 2 Groups. Every the $\log_{10}$ of each gene-specific $2^{-\Delta C_T}$ value in one Group is plotted on one axis against the corresponding value in another Group on the other axis to neatly visualize gene expression differences. Diagonal lines are used to clearly delineate gene expression differences of interest for upregulated and downregulated genes above and below a manually selected "Fold Regulation" threshold. The central diagonal line indicates unchanged gene expression. The upper left section of the "Scatter Plot" (above the upper diagonal line) contains genes upregulated in the y-axis Group relative to the x-axis Group. The lower right section of the "Scatter Plot" (below the lower diagonal line) contains genes downregulated in the y-axis Group relative to the x-axis Group.

The default "Scatter Plot" compares the "Control Group" to "Group 1" with a "Fold Regulation" threshold of 2 and a "Blue-Yellow" "Color Scheme" (for downregulated and upregulated genes, respectively).

i. Modify the Scatter Plot

Using the dropdown menus at the top of the page, select the "Test Group" and "Control Group" to be plotted on the y-axis and x-axis, respectively.
Enter the desired "Fold Regulation" threshold to move the diagonal lines by entering a new value in the dialog box or using its arrows to change the value by 0.1 increments.
Select the desired "Color Scheme" from the dropdown menu: "Blue-Yellow", "Blue-Red", or "Green-Red" for downregulated and upregulated genes, respectively.
After modifying these parameters as desired, click the "Update Chart" button to view the new "Scatter Plot".

ii. Identify genes on the Scatter Plot

The right-hand table lists the plate position ("POS"), "Gene" Symbol, and $\log_{10}$ normalized gene expression levels in the two Groups used to generate the Scatter Plot. Mouse over the data points in the "Scatter Plot" to visualize their array positions and Gene Symbols.

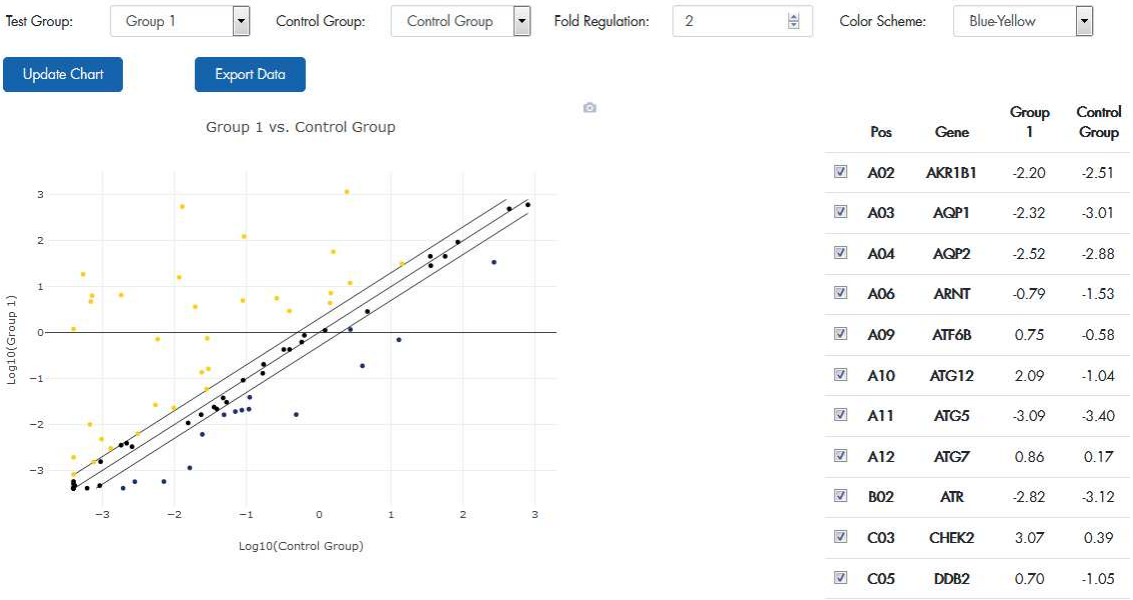


Figure 6. A Scatter Plot from the RT^2 Profiler PCR Arrays & Assays Data Analysis software is displayed.

iii. Exclude genes from the Scatter Plot

In the right-hand table, deselect the checkbox of any gene to be removed from the "Scatter Plot". Conversely, reselect any gene-specific checkbox to add it back to the "Scatter Plot". Click the "Update Chart" button to view the new "Scatter Plot". By default, the GDC control, RTC, and PPC assays are not displayed in the "Scatter Plot".

iv. Export Scatter Plot results and image

Click the "Export Data" button to generate, download, and save to a local computer an Excel file containing the list of upregulated and downregulated genes and their associated results for the displayed pairwise Group comparison based on selected "Fold Regulation" threshold.

Mouse over and click on the camera icon in the upper right hand corner of the "Scatter Plot" to download and save a PNG file of the "Scatter Plot" image to a local computer.

b. Volcano Plot

The "Volcano Plot" helps evaluate gene expression changes in the context of their statistical significance. Based on chosen pairwise Group comparison, the graph plots the $\log_2$ of each gene's "Fold Change" value on the x-axis and the negative $\log_{10}$ of each gene's "p-Value" on the y-axis. The default plot compares the "Control Group" with "Group 1", or the first Group with at least three Samples. The center vertical represents no change in gene expression ($\log_2(1) = 0$). The data points to the right of the center vertical line indicate upregulated genes, while the data points left of the solid vertical line indicate downregulated genes. The outer vertical lines represent a manually selected "Fold Regulation" absolute value threshold. The default setting is 2. The horizontal line represents a selected "p-Value" threshold. The default setting is 0.05. Data points above the horizontal line are statistically significant, while data points below the horizontal line are not.

NOTE: Because the Volcano plot utilizes p-values, it can only use Groups with 3 or more replicate Samples. A popup alert is displayed if trying to use a Group with less than 3 replicate Samples for the plot. If no Group includes 3 or more replicate Samples, then the Volcano Plot link is disabled.

i. Modify the Volcano plot

Select the "Test Group" and "Control Group" to be compared in the "Volcano Plot" using the respective dropdown menus.
Enter the desired "Fold Regulation" and "p-Value" thresholds into the respective dialog boxes or use their arrows to change their values by 0.1 and 0.01 increments, respectively.
Select the desired "Color Scheme" from the dropdown menu: "Blue-Yellow", "Blue-Red", or "Green-Red" for downregulated and upregulated genes, respectively.
After modifying these parameters as desired, click the "Update Chart" button to view the new "Volcano Plot".

ii. Identify genes on the Volcano Plot

The right-hand table lists the plate position ("POS"), "Gene" Symbol, and their "p-Value" and "Fold Change" results used to generate the "Volcano Plot". Mouse over the data points in the "Volcano Plot" to visualize their array positions and Gene Symbols.

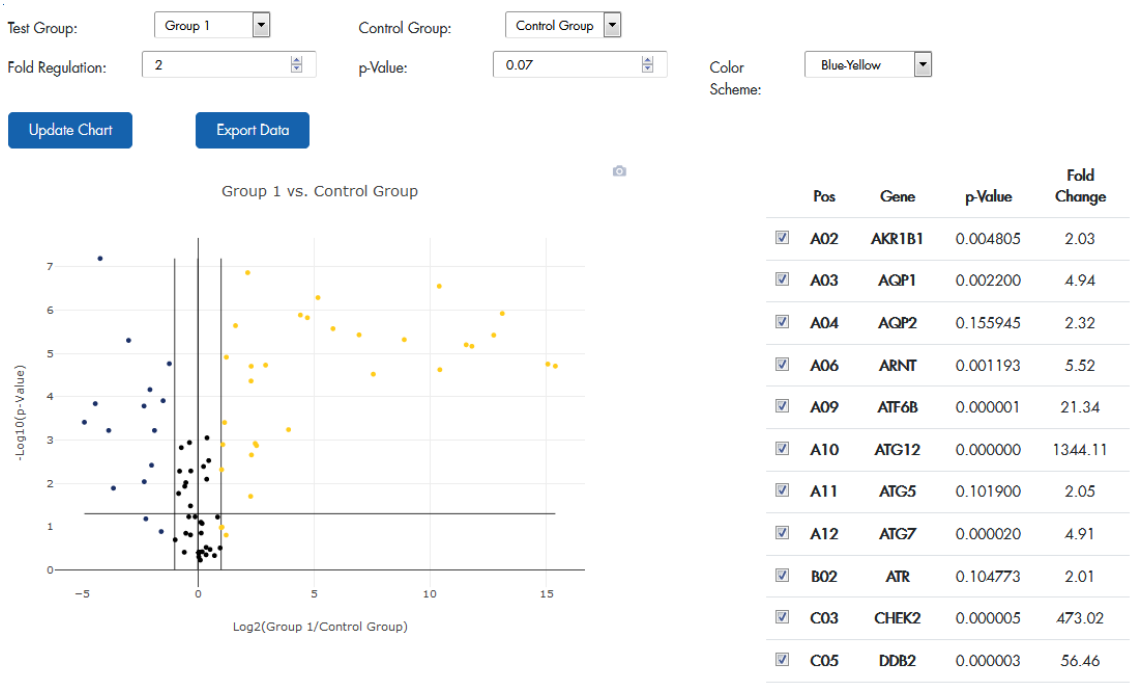


Figure 7. A Volcano Plot from the RT² Profiler PCR Arrays & Assays Data Analysis software is displayed.

iii. Exclude genes from the Volcano plot

In the right-hand table, deselect the checkbox of any gene to be removed from the "Volcano Plot". Conversely, reselect any gene-specific checkbox to add it back to the "Volcano Plot". Click the "Update Chart" button to view the new "Volcano Plot". By default, the GDC control, RTC, and PPC assays are not displayed in the "Volcano Plot".

iv. Export Volcano Plot results and image

Click the "Export Data" button to generate, download, and save to a local computer an Excel file containing the list of upregulated and downregulated genes and their associated results for the displayed pairwise Group comparison based on selected "Fold Regulation" and "p-Value" thresholds.

Mouse over and click on the camera icon in the upper right hand corner of the "Volcano Plot" to download and save a PNG file of the "Volcano Plot" image to a local computer.

c. Clustergram

The "Clustergram" performs non-supervised hierarchical clustering of the entire dataset to display a heat map with dendrograms indicating co-regulated genes across groups or individual samples. With PCR array data, the "Clustergram" provides an overview of what genes may be co-regulated by a common factor, e.g. a transcription factor, miRNA, methylation status, etc.

Clustering algorithms divide a set of objects (genes or Samples) into clusters so that gene expression patterns within a cluster are more similar than patterns across clusters. Cluster analysis includes hierarchical and non-hierarchical clustering methods. Hierarchical clustering is used to understand the relationship of each gene or Samples to every other gene or Samples, respectively. Hierarchical clustering is also used when little or no prior knowledge of how the data will be clustered is known, because hierarchical clustering does not set the number of clusters to form before the analysis begins. Hierarchical clustering analysis produces dendrograms, which are treelike figures used to graphically represent the hierarchy.

NOTE: The "Clustergram" does not cluster genes based on known functional or sequence homology.

The "Clustergram" is based on a hierarchical clustering method that:

- Assigns each gene and each array or group to its own cluster (agglomerative)
- Joins the nearest clusters
- Re-estimates the distance between clusters
- Repeats for each gene and each array or each group

Objects that are more similar are joined at lower heights in the dendrogram and those that are less similar are joined at higher heights. To create a hierarchical cluster, a distance/dissimilarity metric (here based on the magnitude of gene expression) and linkage method must be specified. The magnitude of gene expression is determined by calculating the $2^{-\Delta C_T}$ for each individual gene and normalizing to the average $2^{-\Delta C_T}$ of all genes across all Samples. The distance/dissimilarity metric is used to convert data points into clusters, while the linkage method is used to join the clusters to form a tree.

The software utilizes a correlation coefficient to express similarity of all genes across all Samples calculated based on the following equation for the correlation coefficient between two dimensional profiles:

$$\rho_{X,Y} = \frac{Cov(X,Y)}{\sigma_X \sigma_Y} = \frac{\sum_{i=1}^{n}(X_i - \mu_X)(Y_i - \mu_Y)}{\sqrt{\sum_{i=1}^{n}(X_i - \mu_X)^2 \cdot \sum_{i=1}^{n}(Y_i - \mu_Y)^2}}$$

The "Clustergram" page provides optional features to change the appearance of the display by selecting new options with the dropdown menus; however, the default settings provide the most easily interpreted representation.

i. Modify the default Clustergram

Select Array or Group for Sample

Selecting "Array" (the default setting) uses the gene-specific magnitude of gene expression from each Sample as the distance/dissimilarity metric in the analysis and displays the results from each Sample as a separate column of color-coded data. Selecting "Group" uses the average gene-specific magnitude of gene expression across Samples in the same Group ("Control Group", "Group 1", etc.) as the distance/dissimilarity metric in the analysis and displays the results from each "Group" as a separate column of color-coded data.

Select Dimension

Selecting "2-D" (the default setting) displays dendrograms on both the x-axis ("Array" or "Group") and y-axis (Genes). Selecting "1-D" only draws a dendrogram for the y-axis.

Select Join Type

See the Figure 8 for a visual demonstration of the difference between the following three "Join Type" methods.

Selecting "Maximum" or complete linkage defines the distance between clusters using the pair of genes with the greatest difference in their distance/dissimilarity metric. This method tends to produce dendrograms with shorter branches that clump together.

Selecting "Minimum" or single linkage defines the distance between clusters using the pair of genes with the smallest difference in their distance/dissimilarity metric. This method tends to produce dendrograms with longer branches.

Selecting "Average" linkage defines the distance between clusters using the average of the differences in the distance/dissimilarity metric between all pairs of genes in the two clusters.

NOTE: Only one individual gene from each cluster is considered when using "Maximum" or "Minimum", while using "Average" considers all genes in each cluster.

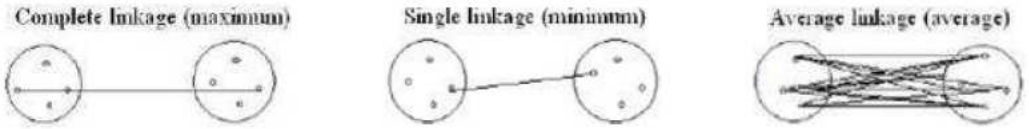


Figure 8. Differences between the three Join Type methods.

Select Color Coding

The colors in the cells of the "Clustergram" represent the relative magnitude of gene expression based on a selected method of calculating an average value. The black color represents the average magnitude of gene expression. The brightest green represents the smallest value, and the brightest red represents the highest value. See Figure 9.

Selecting "Genes" (the default setting) sets a different average value for each gene on the y-axis.

Selecting "Samples" sets a different average for each Sample on the x-axis, whether by "Array" or "Group".

Selecting "Entire Dataset" sets one average for all data used to generate the "Clustergram".

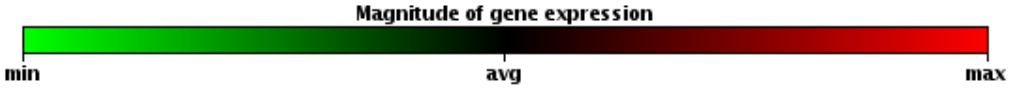


Figure 9. The Clustergram Color Coding legend is shown.

Select Item names visible

Selecting "Yes" (the default setting) labels the names of the "Arrays" or "Groups" and Genes on the "Clustergram". Selecting "No" hides the names of the "Arrays" or "Groups" and Genes on the "Clustergram".

Once all desired changes have been made to these settings, click the "Update Chart" button to visualize the new "Clustergram".

ii. Save the Clustergram image

Use your internet browser's functionality.

d. Heat Map

This feature is only available for the "Catalog Array" "Product Type".

The "Heat Map" provides a graphical and color-coded representation of Fold Regulation data between two Groups overlaid onto the PCR Array plate layout. The black color represents the average magnitude of gene expression. The brightest green represents the smallest value, and the brightest red represents the highest value.

The table below the "Heat Map" contains the Fold Regulation data used to generate the plot for each of the annotated Gene Symbols in its Well position. If any Fold Regulation results were annotated with "Comments" on the "Fold Regulation" page of the "Analysis" tab, then those "Comments" are also annotated in the "Heat Map" table. The definitions of the "Comments" are re-listed under the table. See Figure 10 for an example "Heat Map" image.

i. Modify the default Heat Map

Select the "Test Group" and the "Control Group" to compare in the "Heat Map".
Selecting "Log2 transform fold change" checkbox (the recommended default setting) uses the $\log_2$ of the Fold Regulation values to determine the color-coding scheme. Deselecting the checkbox uses the untransformed Fold Regulation values to determine the color-coding scheme.
Once all desired changes have been made to these settings, click the "Update Chart" button to display the new "Heat Map".

ii. Export the associated data table and save the Heat Map image

Click the "Export Data" button to generate and download an Excel file containing the data used to generate the "Heat Map". Save the file on a local computer.
Mouse over and click on the camera icon in the upper right hand corner of the "Heat Map" to download and save a PNG file of the "Heat Map" image to a local computer.



Figure 10. A Heat Map from the RT² Profiler PCR Arrays & Assays Data Analysis software is displayed.

e. Multigroup Plot

The "Multigroup Plot" displays either intermediate calculations across every Group or Fold Change results across every test Group compared to the Control Group. The plot can include results from up to 10 selected genes of interest either as dots connected by lines or as columns, each with or without error bars indicating the deviations in the y-axis values. This plot is best suited for following the trend of expression changes for a few genes across the various treatment conditions in the experiment, such as time-course or dose-response experiments to monitor changes over time or drug concentration, respectively.

The x-axis plots all relevant Groups, and the y-axis plots either "Average delta (C_T)", "2 ^ -delta (C_T)", or "Fold Change", based on the selections made below. Each color for the points and lines or columns represents a different selected "Gene". The default "Multigroup Plot" displays the "Average delta (C_T)" of first two "Genes" in the list from all Groups using a "Line Chart" without error bars.

i. Modify the Multigroup Plot

Select the data to be plotted on the "Y-Axis" using the appropriate radial buttons. Selections include "Average delta (C_T)", "2 ^ -delta (C_T)", or "Fold Change".
Select the desired "Chart Type" from the dropdown menu to visualize the graphed data, either a "Line Chart" or a "Column Chart".
Click the "Show Uncertainty" checkbox to include error bars in the "Multigroup Plot". Table 2 lists how the uncertainty is calculated for each "Y-Axis" option chosen above.

Table 2. The calculations used to determine the error bars in the Multigroup Plot for each Y-Axis option is provided.

Y-Axis	Uncertainty
Average delta(C_T)	standard deviation of ΔC_T
2 ^ –delta(C_T)	standard deviation of $2^{-\Delta C_T}$
Fold Change	σFC = FC * ln2 * sqrt(σx2 / nx + σy2 / ny)

Define the "Genes" of interest to display by selecting or deselecting the checkboxes in the left-hand table. The maximum allowable number of selected "Genes" is 10.
Once all desired changes have been made to these settings, click the "Update Chart" button to visualize the new "Multigroup Plot".

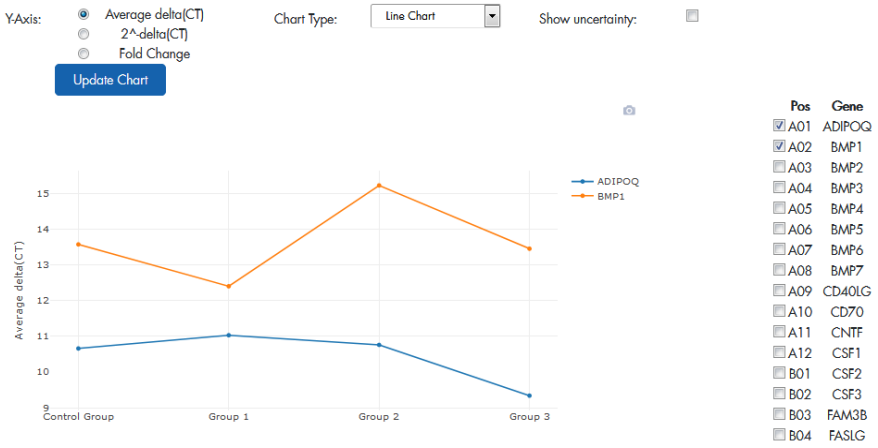


Figure 11. A Multigroup Plot generated by the RT² Profiler PCR Arrays & Assays Data Analysis software is displayed.

ii. Export the Multigroup Plot image

Mouse over and click on the camera icon in the upper right hand corner of the "Multigroup Plot" to download and save a PNG file of the "Multigroup Plot" image to a local computer.

What's Next

The RT^2 Profiler PCR Arrays & Assays Data Analysis software delivers a list of gene expression changes in the Samples from the supplied data. However, this result often only starts an investigation into the underlying biology. To assist in further analysis, the software utilizes the latest bioinformatics tools to analyze the results and suggest regulatory mechanisms and future experiments. Explore all the available tools using the links in the left-hand navigation panel.

Each "What's Next" tool first requires the desired search criteria to identify differentially expression genes be used in the analysis in the following manner:

Step 1: Which experimental groups from this study would you like to use?

Define the "Test Group" and "Control Group" to be compared from the respective dropdown menus.

Step 2: Would you like to investigate genes that are:

Specify whether "Decreased" or "Increased Expression in the Experimental Group versus Control" or "Both" are to be analyzed using the respective radial buttons.

Step 3: Set fold regulation and p-value thresholds for the selected genes:

Enter the desired "Fold Regulation" and "p-Value" thresholds into the respective dialog boxes or use their arrows to change their values by 0.1 and 0.01 increments, respectively. The "p-Value" entry is optional.

NOTE: If either of the two Groups in the comparison contain less than 3 Samples, a popup alert is displayed indicating that p-values could not be calculated and that the p-value threshold will be ignored.

1. Gene Expression

This tool will help define a panel of genes based of this experiment's results. This panel may represent a putative biomarker set, a target gene set or simply a collection of genes. The tool is designed to deliver a list of gene expression assays that would allow the user to follow-up the results of the analyzed experiment.

a. Step 4: Selected Genes

Once the parameters in "Step 1" through "Step 3" have been defined, click the "Show Selected Genes" button in "Step 4". A table will be displayed listing the "Position", "Gene Symbol", "Fold Regulation", "p value" (if applicable or nan if not), and the RT^2 qPCR "Assay Catalog" Number for each gene that meets the defined search criteria. The catalog number hyperlinks to the product's page on the QIAGEN website.

b. Step 5: Would you prefer to test these as individual assays or build a custom PCR Array?

Select either the "Individual Assays" or the "Custom PCR Array" radial button as desired.

c. Step 6: Export Data

Download all individual assays

For the "Individual Assays" option, click the "Export Data" button to generate, download, and save to a local computer an Excel file containing the table output from "Step 4".

For Custom RT^2 PCR Arrays, how many mRNAs would you like to analyze?

For the "Custom PCR Array" option, first select from the dropdown menu the number of gens from the "Step 4" table to include in the Custom RT^2 Profiler PCR Array. A limited number of options (8, 12, 16, 24, 32, 48, and 96) are available that match the offered the Custom RT^2 Profiler PCR Array layouts. The software automatically chooses the genes with the highest "Fold Regulation" absolute values. Click the "Export Data" button to generate and download an Excel file containing the recommended the Custom RT^2 Profiler PCR Array layout and the corresponding necessary RT^2 qPCR Assay catalog numbers. Save to a local computer.

2. miRNA Regulation

This tool will identify candidate miRNA regulators in your experimental system. The tool is designed to deliver a list of miRNAs that could be targeting the genes that had observed changes in expression in your selected Samples.

a. Step 4: Show Selected miRNAs

Once the parameters in "Step 1" through "Step 3" have been defined, click the "Show Selected Genes" button in "Step 4". A table will be displayed listing the "Position", "Gene Symbol", "Fold Regulation", and "p value" (if applicable or nan if not) for each gene that meets the defined search criteria.

b. Step 5: Search for miRNA Regulators

Click the "Search for miRNA Regulators" button to generate two tables, one for "miRNA Regulators for Genes with Decreased Expression" and one for "miRNA Regulators for Genes with Increased Expression". If genes only changing expression in one direction or the other are displayed in the "Step 4" table, then only the corresponding table will be displayed in "Step 5". The tables list the miRNAs predicted by the TargetScan algorithm to regulate the differentially expressed genes that met the search criteria.

For each miRNA predicted to regulate the differentially expressed genes, each table provides the following information in separate columns:

miRNA ID
Each row includes data from a single mature miRNA predicted to regulate one or more of the differentially miRNA.

of Target Genes This miRNA Targets
This data returns the number of genes with altered expression that are predicted to be regulated by this miRNA.

Total # of Target Sites Identified in Target Genes
This data returns the total number of target binding sites for this miRNA in all the targeted genes' mRNA.

Range of Strength Scores
This column lists the TargetScan derived strength score range (from minimum to maximum) used to help gauge the likelihood of the mRNA-miRNA interaction.

Is this miRNA expressed in your samples?

Order the miScript miRNA qPCR Assay using the provided catalog number to find out.

Enhance the miRNA Activity

Order the miScript miRNA Mimic using the provided catalog number.

Inhibit the miRNA Activity

Order the miScript miRNA Inhibitor using the provided catalog number.

Targeted Genes

This column lists the differentially regulated genes predicted to be regulated by the miRNA ID listed in the leftmost column.

Click "Export List of miRNAs" button on the top of either table to generate, download, and save to a local computer an Excel file containing all data from the "Step 4" and "Step 5" tables.

3. DNA Methylation

This tool will help define a panel of differentially expressed genes based on this experiment's results. Altered methylation patterns on the genes' promoters may be responsible for these gene expression changes. This tool is designed to deliver a list of available DNA methylation assays for those differentially expressed genes that would allow the user to follow-up their gene expression experiment with an epigenetic analysis.

a. Step 4: Show Selected Genes

Once the parameters in "Step 1" through "Step 3" have been defined, click the "Show Selected Genes" button in "Step 4". A table will be displayed listing the "Position", "Gene Symbol", "Fold Regulation", "p value" (if applicable or nan if not), and "Recommended EpiTect Methyl II qPCR Assay" (if available) for each gene that meets the defined search criteria. The assay catalog numbers hyperlink to the product's web page on the QIAGEN website.

b. Step 5: Would you prefer to test these as individual assays or build a custom PCR Array?

Select either the "Individual Assays" or the "Custom PCR Array" radial button as desired.

c. Step 6: Export Data

Download all individual assays

For the "Individual Assays" option, click the "Export Data" button to generate, download, and save to a local computer an Excel file containing the table output from "Step 4".

For Custom EpiTect Methyl II PCR Arrays, how many genes would you like to analyze?

For the "Custom PCR Array" option, first select from the dropdown menu the number of genes from the "Step 4" table to include in the Custom EpiTect Methyl II PCR Array. A limited number of options (6, 8, 12, and 24) are available that match the offered the Custom EpiTect Methyl II PCR Array layouts. The software automatically chooses the genes with the highest Fold Regulation absolute values. Click the "Export Data" button to generate and download an Excel file containing the recommended the Custom EpiTect Methyl II PCR Array layout and the corresponding necessary EpiTect Methyl II qPCR Assay catalog numbers. Save to a local computer.

4. Transcription Factor / Histone

This tool will help define a panel of differentially expressed genes based on this experiment's results. Altered transcription factor binding activity on the genes' promoters may be responsible for these gene expression changes. Altered histone modification patterns on the genes' promoters may also be responsible for these gene expression changes. This tool is designed to deliver a list of the transcription factors that might regulate the selected differentially expressed genes as well as the available respective gene-specific real-time PCR assays for DNA from anti-transcription factor or anti-histone chromatin immunoprecipitations. These assays would then allow the user to follow-up their gene expression experiment with an epigenetic analysis.

a. Step 4: Show Selected Genes

Once the parameters in "Step 1" through "Step 3" have been defined, click the "Show Selected Genes" button in "Step 4". A table will be displayed listing the "Position", "Gene Symbol", "Fold Regulation", "p value" (if applicable or nan if not), "Transcription Factors" with predicted binding sites in the gene's promoter, and the "Recommended EpiTect ChIP qPCR Assay" for each gene that meets the defined search criteria. The assay catalog numbers hyperlink to the product's web page on the QIAGEN website.

b. Step 5: Would you prefer to test these as individual assays or build a custom PCR Array?

Select either the "Individual Assays" or the "Custom PCR Array" radial button as desired.

c. Step 6: Export Data

Download all individual assays

For the "Individual Assays" option, click the "Export Data" button to generate, download, and save to a local computer an Excel file containing the table output from "Step 4".

For Custom EpiTect ChIP PCR Arrays, how many mRNAs would you like to analyze?

For the "Custom PCR Array" option, first select from the dropdown menu the number of genes from the "Step 4" table to include in the Custom EpiTect ChIP PCR Arrays. A limited number of options (8, 12, 16, 24, 32, 48, and 96) are available that match the offered the Custom EpiTect ChIP PCR Arrays layouts. The software automatically chooses the genes with the highest Fold Regulation absolute values. Click the "Export Data" button to generate and download an Excel file containing the recommended the Custom EpiTect ChIP PCR Arrays layout and the corresponding necessary EpiTect ChIP PCR Assay catalog numbers. Save to a local computer.

5. Protein Detection

This tool will help define a panel of cytokines or chemokines based on this experiment's results. This panel may represent a putative biomarker set, a target gene set or simply a collection of genes. The tool is designed to deliver a list of ELISAs that would allow the user to follow-up the results of the analyzed experiment.

a. Step 4: Show Selected Genes

Once the parameters in "Step 1" through "Step 3" have been defined, click the "Show Selected Genes" button in "Step 4". A table will be displayed listing the "Position", "Gene Symbol", "Fold Regulation", "p value" (if applicable or nan if not), and the "Single Analyte ELISArray" catalog number (if available) for each gene that meets the defined search criteria. The catalog numbers hyperlink to the product's webpage on the QIAGEN website.

Click the "Export Data" button to generate, download, and save to a local computer an Excel file containing the table output from "Step 4".

6. Somatic Mutation

This tool will help define a panel of genes based on this experiment's results. Mutations in these genes may affect whether their expression changes have any effect or different effects on the experimental model system. The tool is designed to deliver a list of available somatic mutation assays for these differentially expressed genes that would allow the user to follow-up the results of the analyzed experiment.

a. Step 4: Show Selected Genes

Once the parameters in "Step 1" through "Step 3" have been defined, click the "Show Selected Genes" button in "Step 4". A table will be displayed listing the "Position", "Gene Symbol", "Fold Regulation", "p value" (if applicable or nan if not), and the "qBiomarker Somatic Mutation Assay" catalog number (if available) for each gene that meets the defined search criteria. The catalog numbers hyperlink to the product's webpage on the QIAGEN website.

Click the "Export Data" button to generate, download, and save to a local computer an Excel file containing the table output from "Step 4".

Export

1. Export Excel Report

Select the data tables to export using the corresponding checkboxes and click "Submit" button to download and save to a local computer an Excel file with each data table in a different worksheet.

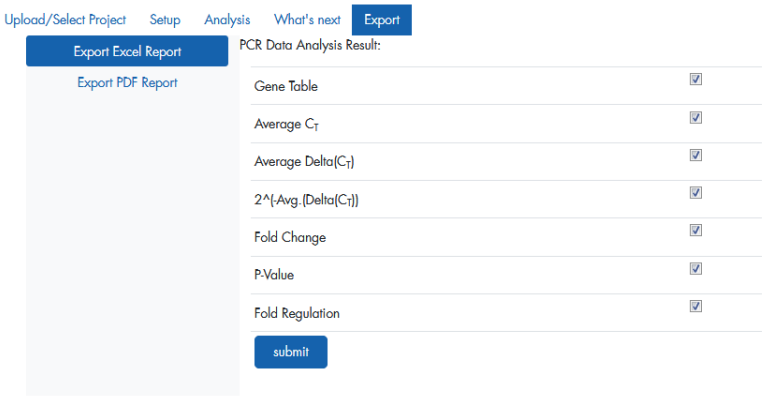


Figure 12. The Export Excel Report page is displayed.

2. Export PDF Report

Select the desired data tables to include in the PDF Report as well as the desired "Color Coding Scheme" using the checkboxes in the left-hand table. "Remove" or "Add more" pairwise Group comparisons to include in the PDF Report using the appropriate buttons on the right-hand side. Defined the "Test Group", "Control Group", "Fold Change" threshold, and "p-Value" threshold for each pairwise Group comparison. Click the "Submit" button to generate and download the PDF Report.

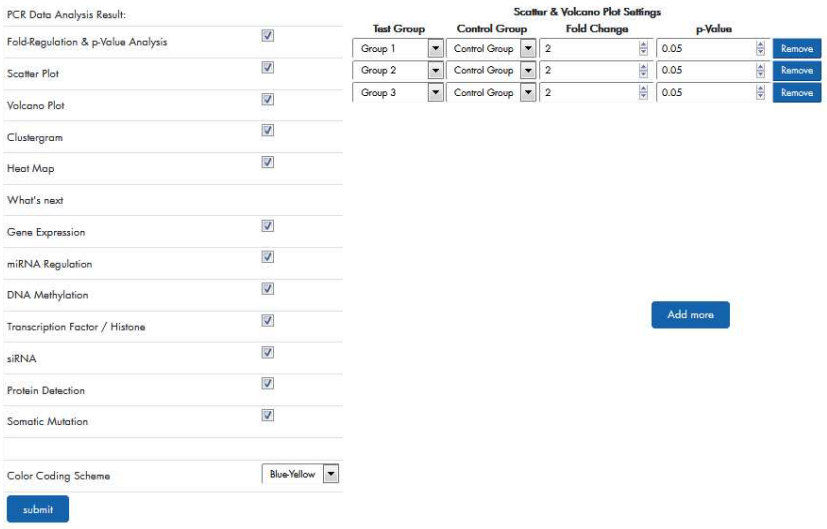


Figure 13. The Export PDF Report page is displayed.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

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Ordering Information

Product	Contents	Cat. no.
RT^2 Profiler PCR Arrays	For exceptionally sensitive and specific gene expression profiling using RT^2 qPCR Assays Ready-to-Use PCR Array of gene-specific assays in 96-well or 384-well format	330231
Custom RT^2 Profiler Custom Arrays	Design your own custom-formatted 96- or 384-well qPCR plates for the RT^2 Profiler PCR Array System Custom RT^2 Profiler PCR Arrays are provided in one ready-to-use 96- or 384-well plate	330171
Custom RT^2 Profiler Custom Arrays, Additional Plates	Design your own custom-formatted 96- or 384-well qPCR plates for the RT^2 Profiler PCR Array System Additional Custom RT^2 Profiler PCR Arrays; only available in addition to the base plates ordered through the core product	330181
RT^2 qPCR Assays	For extremely sensitive relative gene expression profiling Contains forward and reverse primers for 200 reactions	330001

Notes

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