QPCR Tutorial

Version 3.0

Create Date Sep 10, 2008

Last modified May 25, 2009 by Stephan Pabinger

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1 Introduction

QPCR is a web application designed for storing, parsing, managing, and analyzing qPCR data. Including several different algorithms it can facilitate the analysis of qPCR results.

1.1 Purpose

This tutorial is written to give you an example of how to use the presented application. It shows the typical analysis path which starts with the export of files and ends with print ready charts. For this tutorial files of two different vendors are used:

- Applied Biosystems – Abi Prism SDS 7000 (abbreviated as SDS)
- Roche Lightcycler – LC 4.05 (abbreviated as LC)

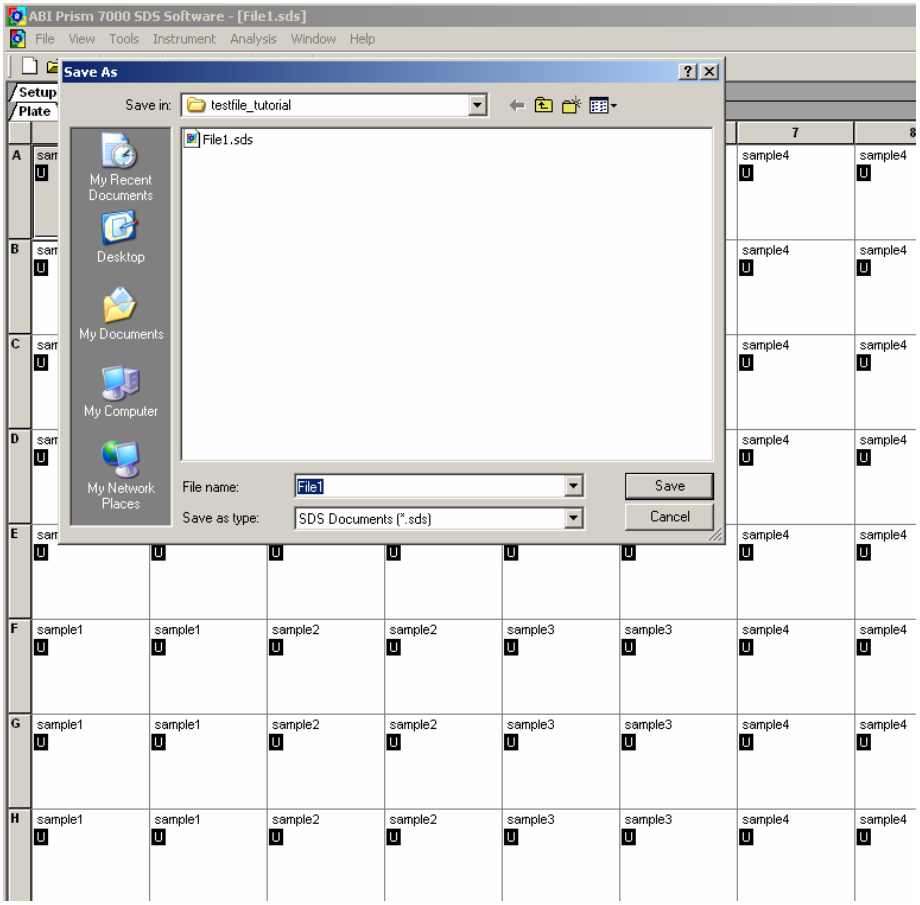
Both files and the generated runs and experiments are available in the QPCR application if you log in as *user*: guest; *password*: guest. Moreover you can download them from https://rtpcr.genome.tugraz.at/rtpcr/info/infoindex.html

2 Save and Export Files

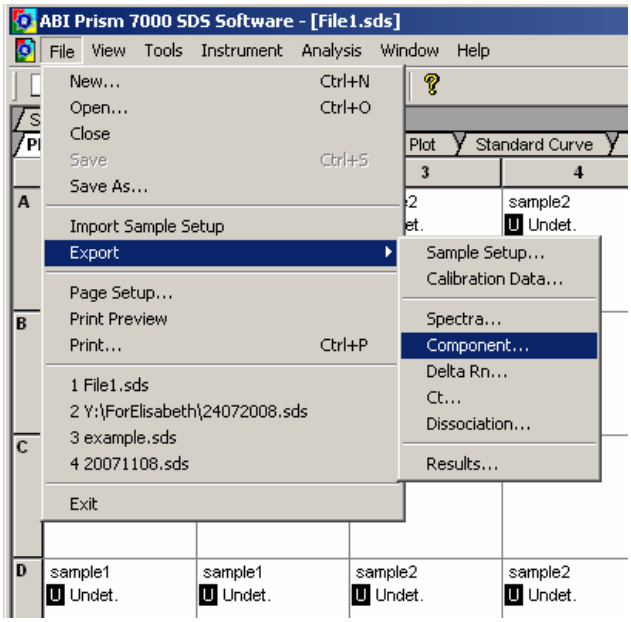
The first step in the analysis pipeline is to create the files that should be analyzed later on.

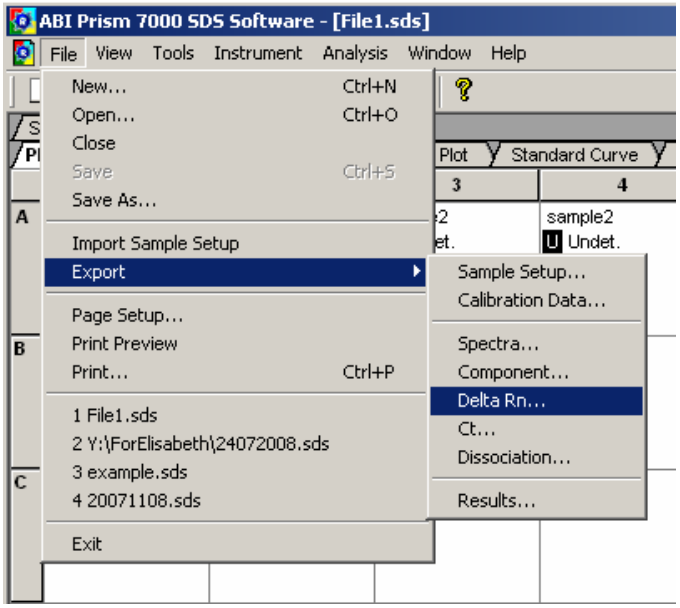
2.1 SDS

After performing the qPCR experiment the file needs to be saved. Go to `file` -> `save` and save the `.sds` file.



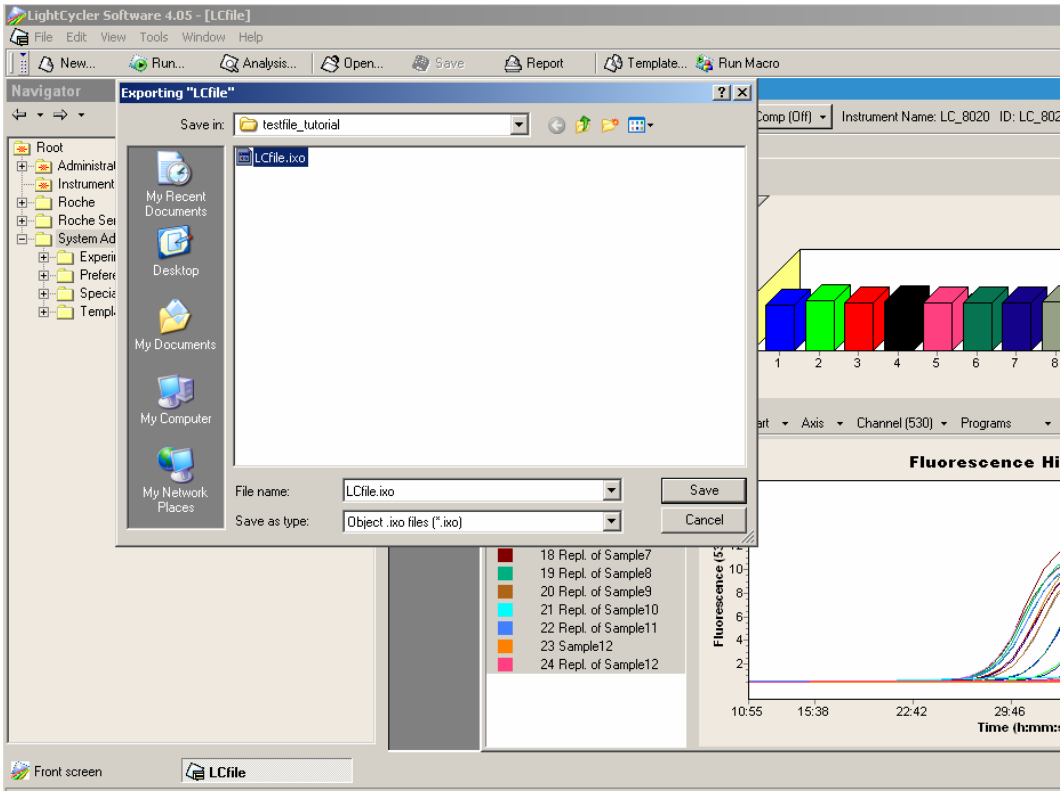
Next you need to export the component and deltaRn values which hold the values that are needed to analyze the experiment. These files will be later uploaded to the QPCR application.



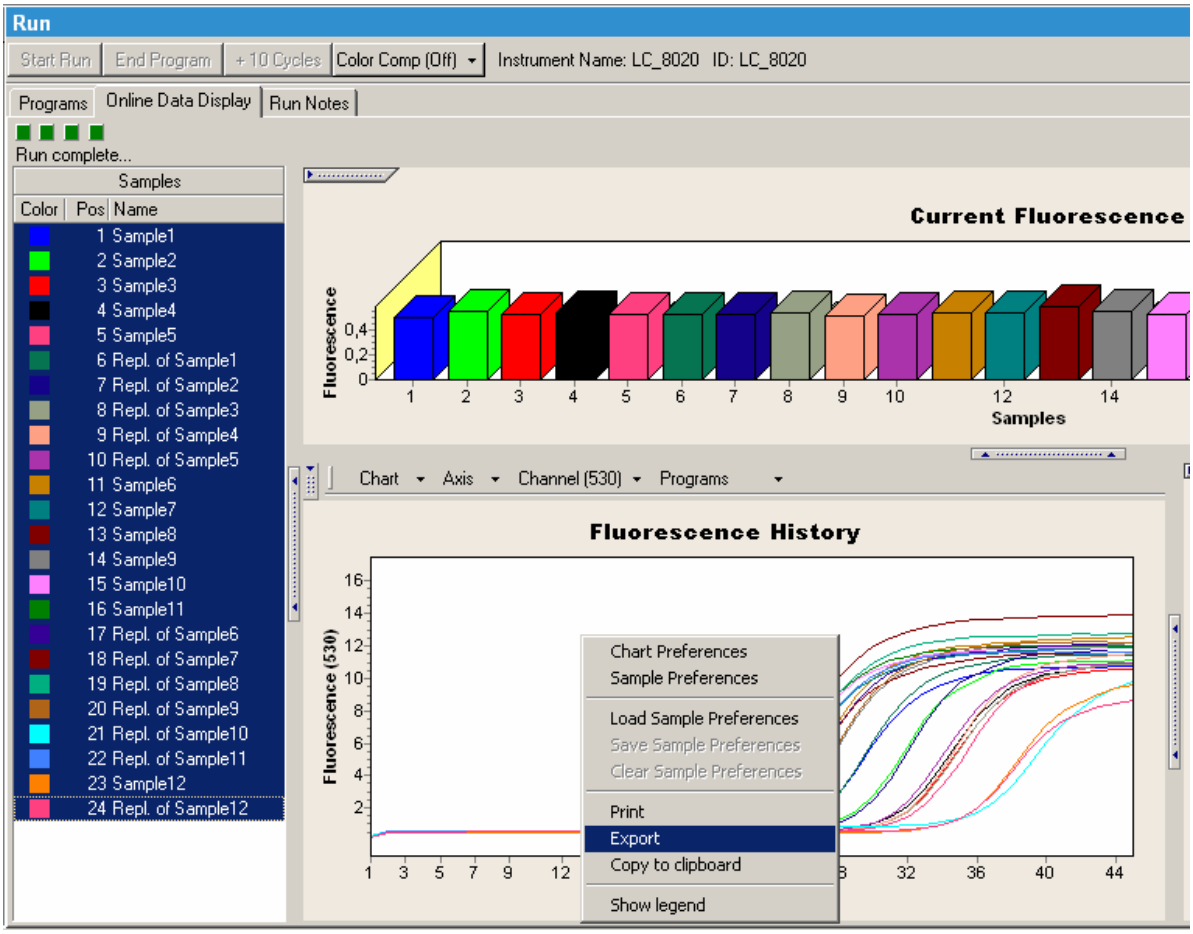


2.2 LC

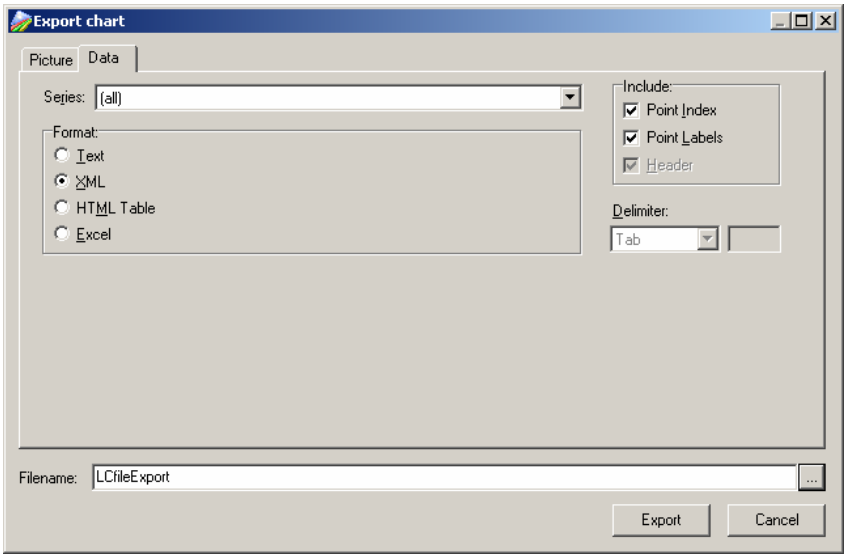
After performing the qPCR experiment the file is exported in the .IXO format. Select `file -> export` and save the experiment.



In addition the fluorescence values need to be exported. Therefore go to `Run` -> `Online Data Display`. Select **all** samples, choose `fluorescence history`, and select `Fluorescence over Cycles`. Right click on the chart and select `Export`.



Select the `Data` tab and tick `XML` as the file format. Then save the fluorescence values.



2.3 CSV

If the QPCR does not support the files produced by your thermocycler you can upload your results using the generic CSV file format.

An example file and the corresponding description can be found at

Information about the CSV file format

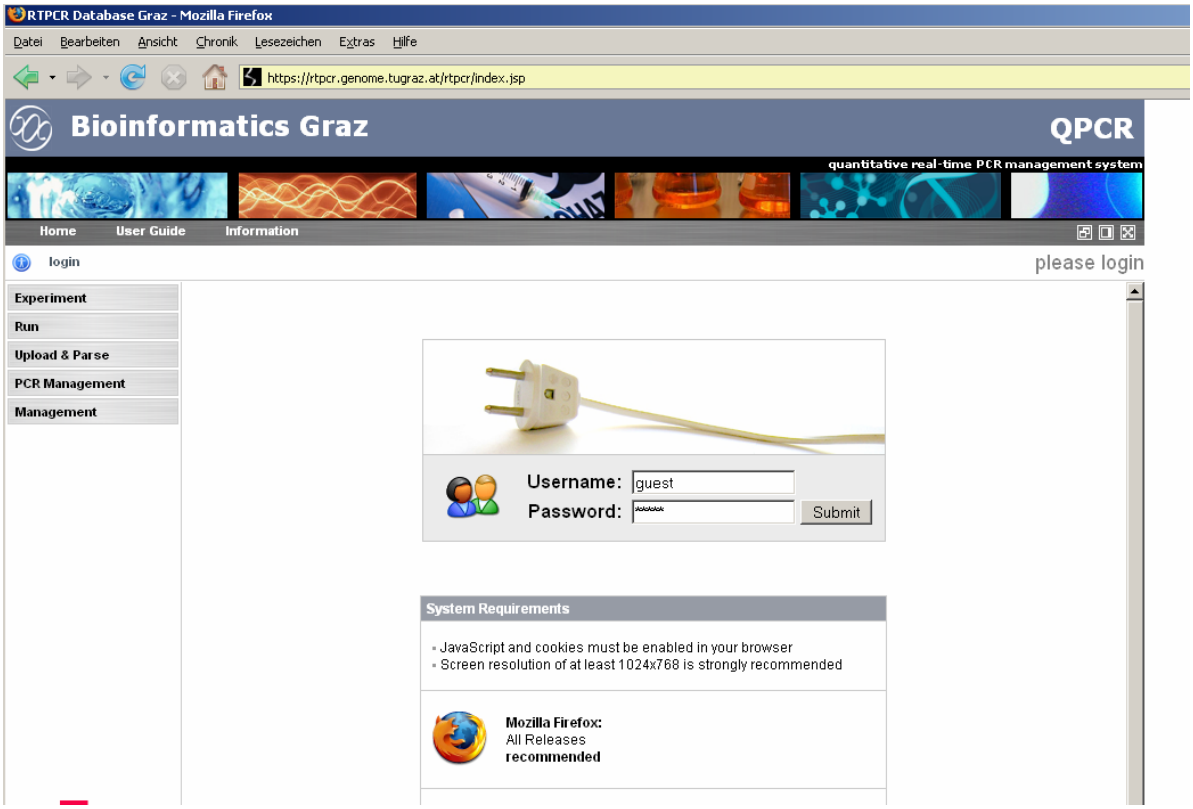
For further information consult the user guide.

3 Start QPCR and log in

After performing the qPCR experiments and exporting the results you are ready to use the QPCR application. Therefore start your browser (e.g.: firefox) and go to

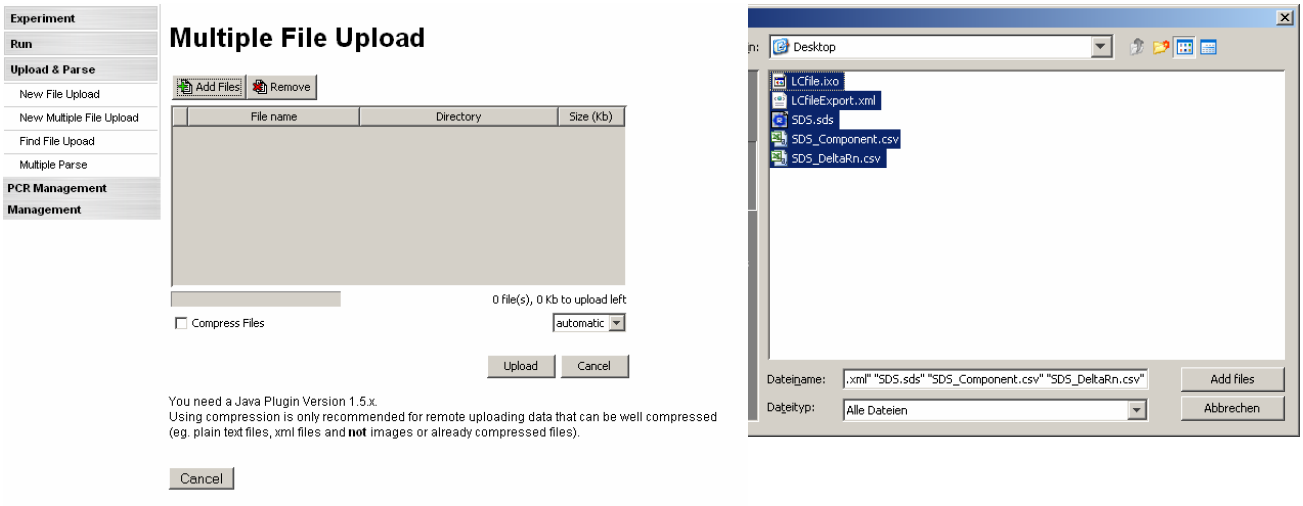
http://rtpcr.genome.tugraz.at/ .

Next you have to log in with your provided username and password (guest, *guest*).

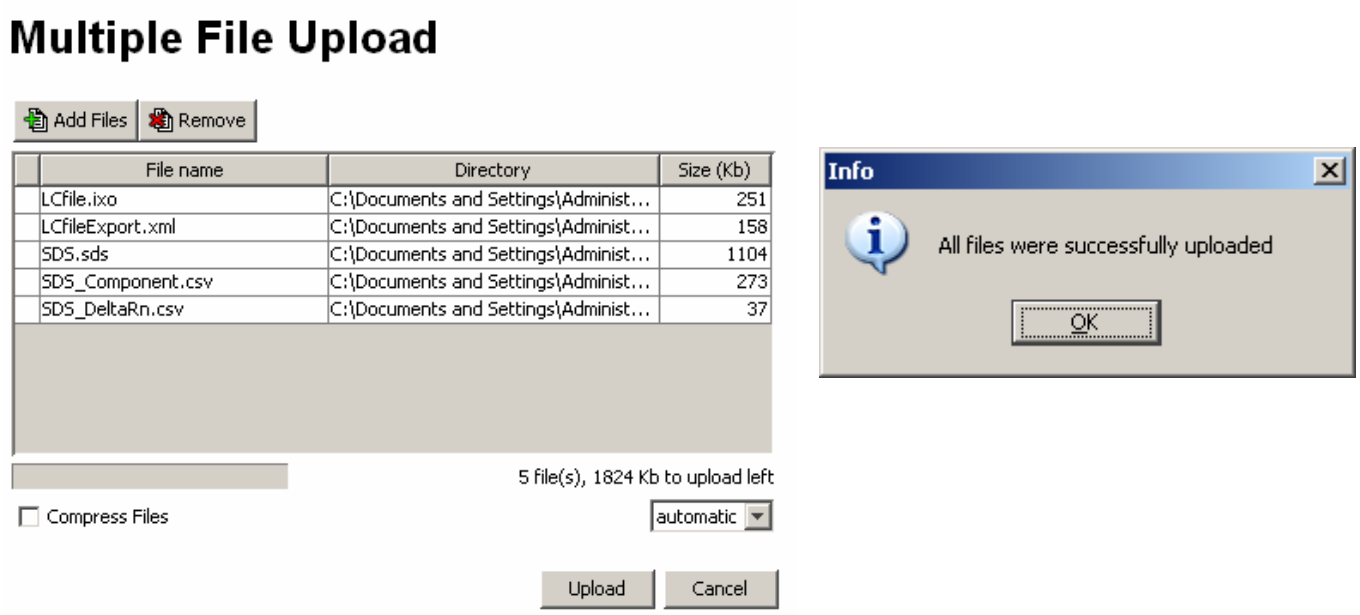


4 Upload files

To upload the created files into the QPCR application, go to Upload & Parse and select New Multiple File Upload. Press Add Files and select the newly created files.



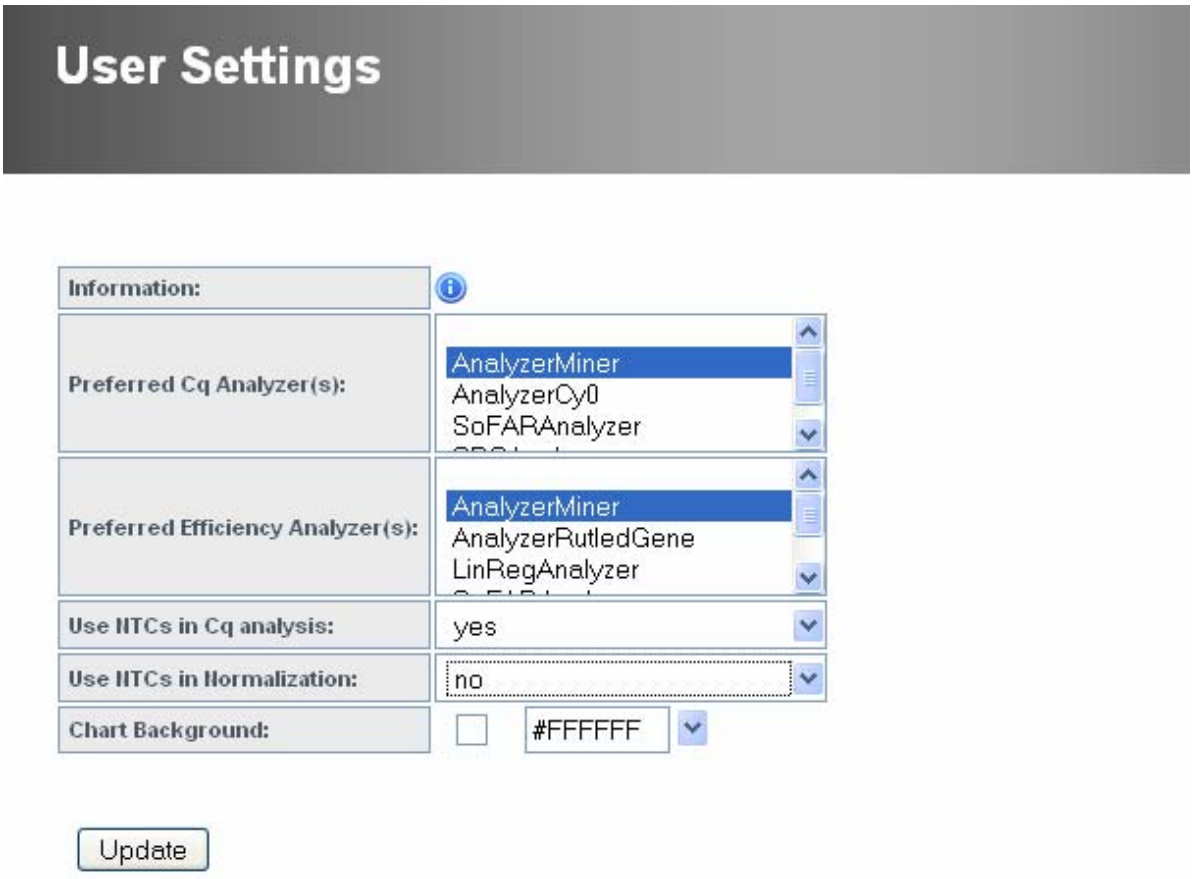
After pressing `Upload` the files are transmitted to the QPCR application and now available for further analysis steps.



5 User Settings

Before the uploaded files are parsed and analyzed it is necessary to take a look at the user settings, which can be found by pressing `User Settings` in the top bar.

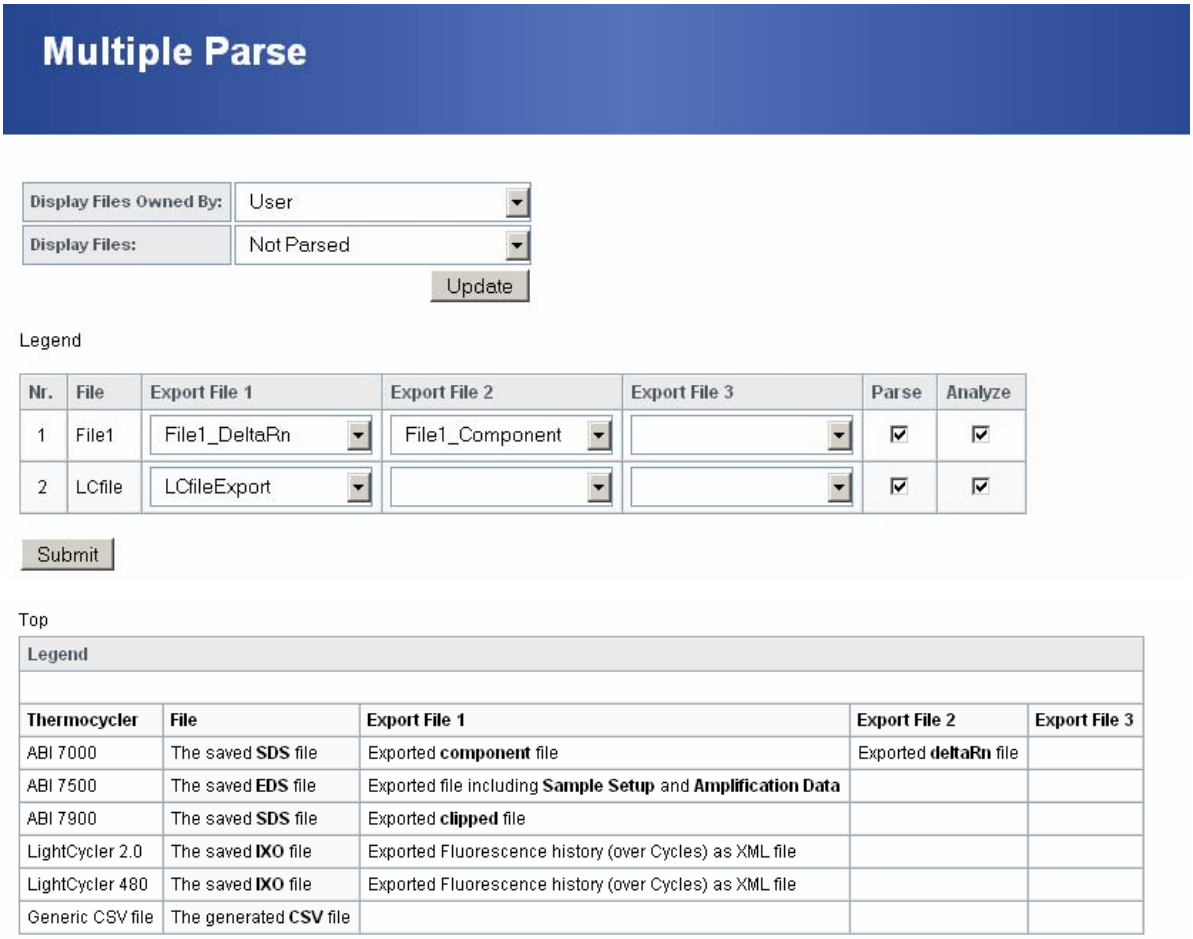
Here you can select your preferred Cq and Efficiency Analyzers, the NTC settings, and the chart background color. For more information click on the information icon.



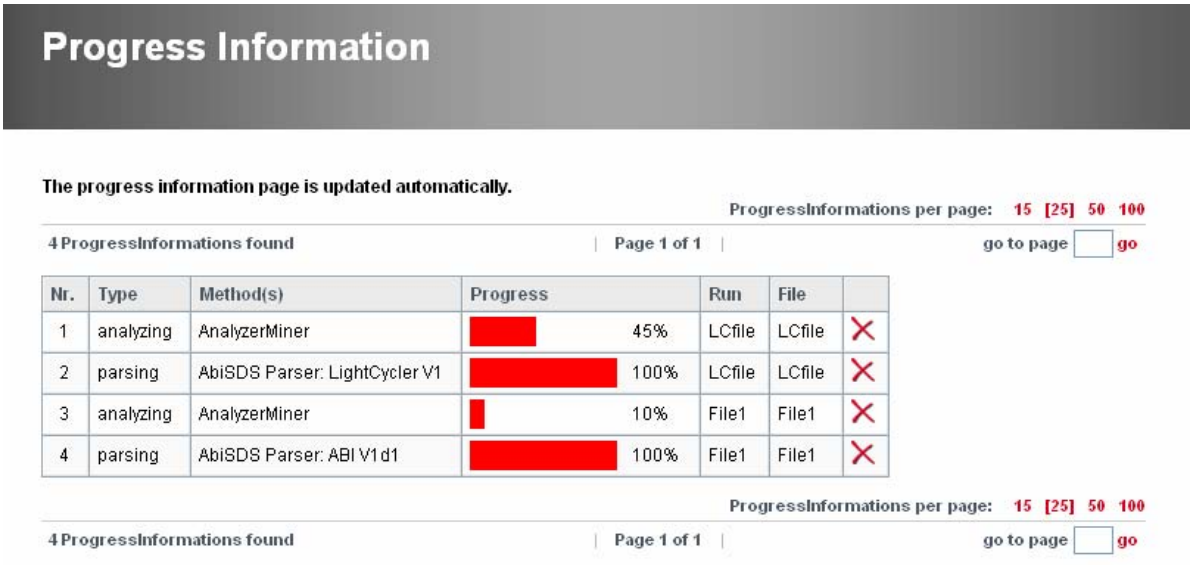
6 Multiple Parse

The (`Multiple) Parse` window helps you to parse and analyze your uploaded files. It automatically detects all files that have not been parsed and displays them in a list. If the export file contains the name of the main file (e.g.: SDS -> SDS.sds, Export -> SDS_component.txt), it is automatically assigned to the corresponding main file. For each file/export file combination you can choose whether you want to parse or parse and analyze it.

By clicking on the submit button the files are sent to the parse and analyze services. Using the `Progress Information` page (accessible through a link in the top menu) you can keep track of the ongoing processes.



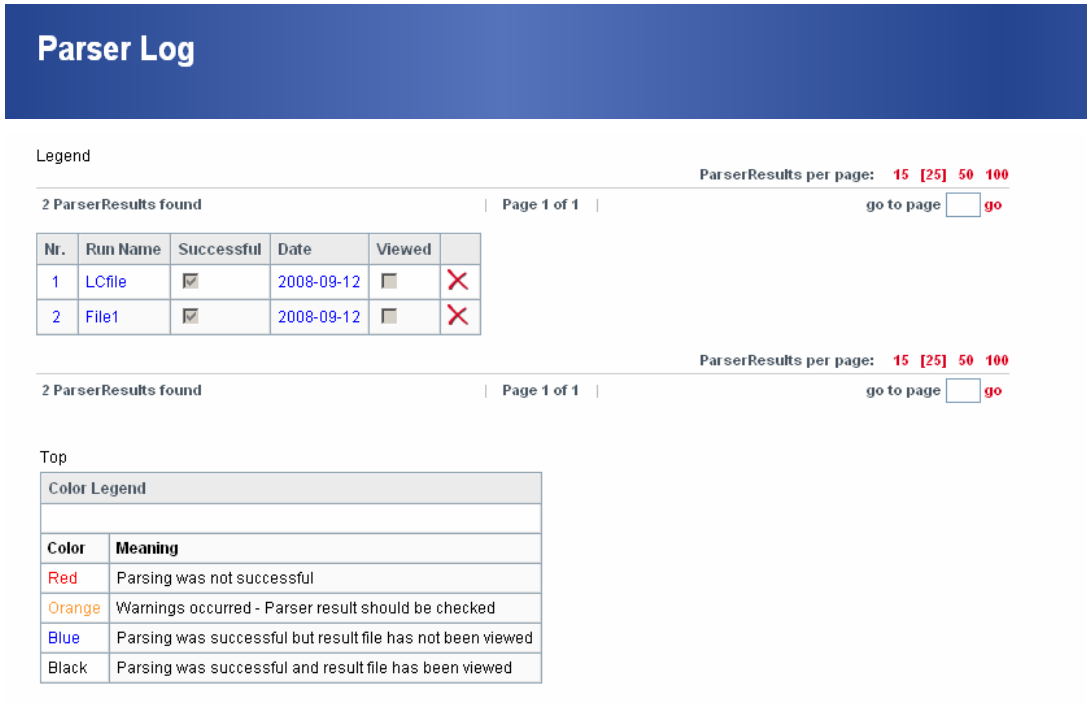
To view the progress information click on `Progress Information` in the top menu bar.



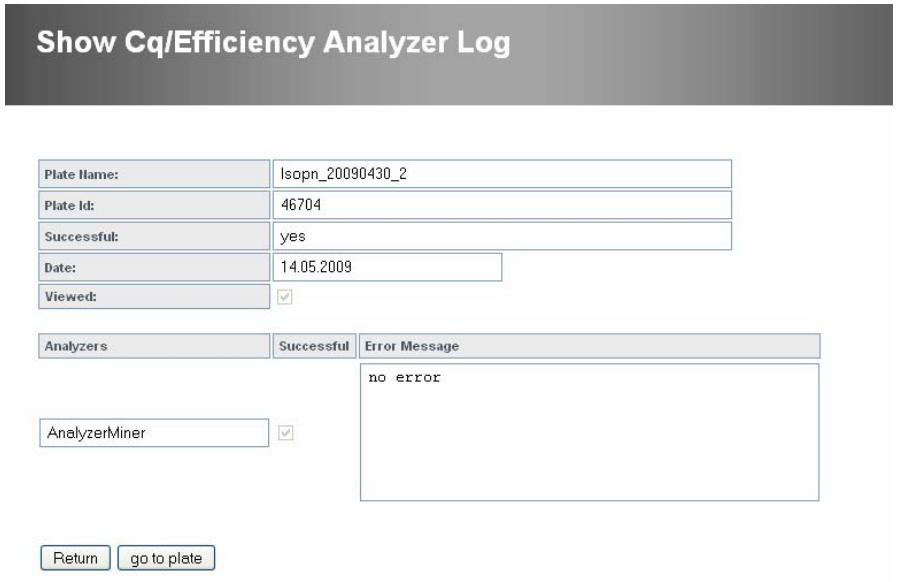
7 Parser and analyzer logs

After completing the parsing and analyzing processes the results can be view by clicking on `New Parser Log` and `New Analyzer Log` in the top menu bar.

The parser logs are shown in a list and are colored according to their result. The legend provides an explanation for each used color.



For each analyzed file an Analyzer Log is provided which displays information about the performed analysis.



8 Run

8.1 List

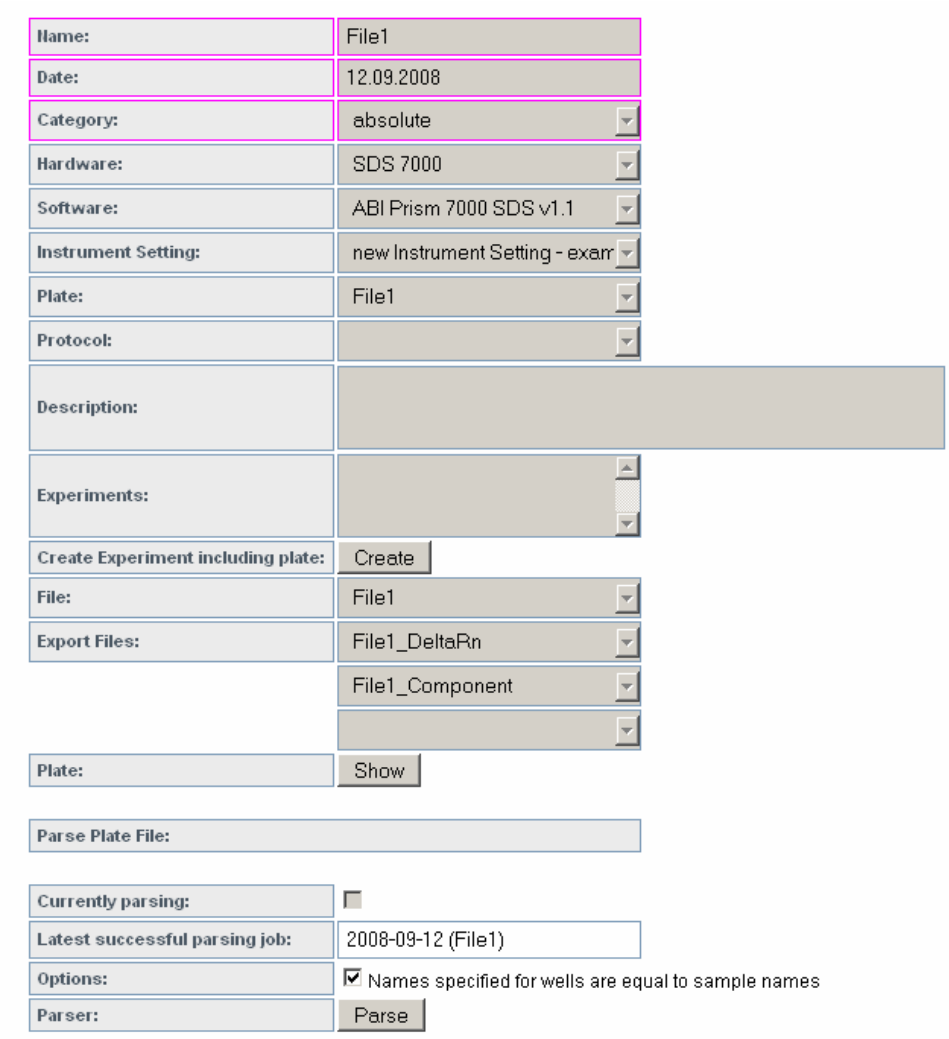
Now you can have a look at the created runs; go to `Run -> Find Runs`. Each parsed file is associated with a run which represents a performed qPCR run (qPCR experiment). The first three symbols are used to inform the user that the run is currently `analyzed`, `parsed`, or `deleted`. By clicking on the gird icon you are linked to the plate layout. The chart symbol is a direct link to the charts of the run.

8.2 Information

By clicking on the name of a run information about the used software, hardware, and instrument setting is displayed. Moreover this page provides information about the category, the used files, and the latest successful parsing job.

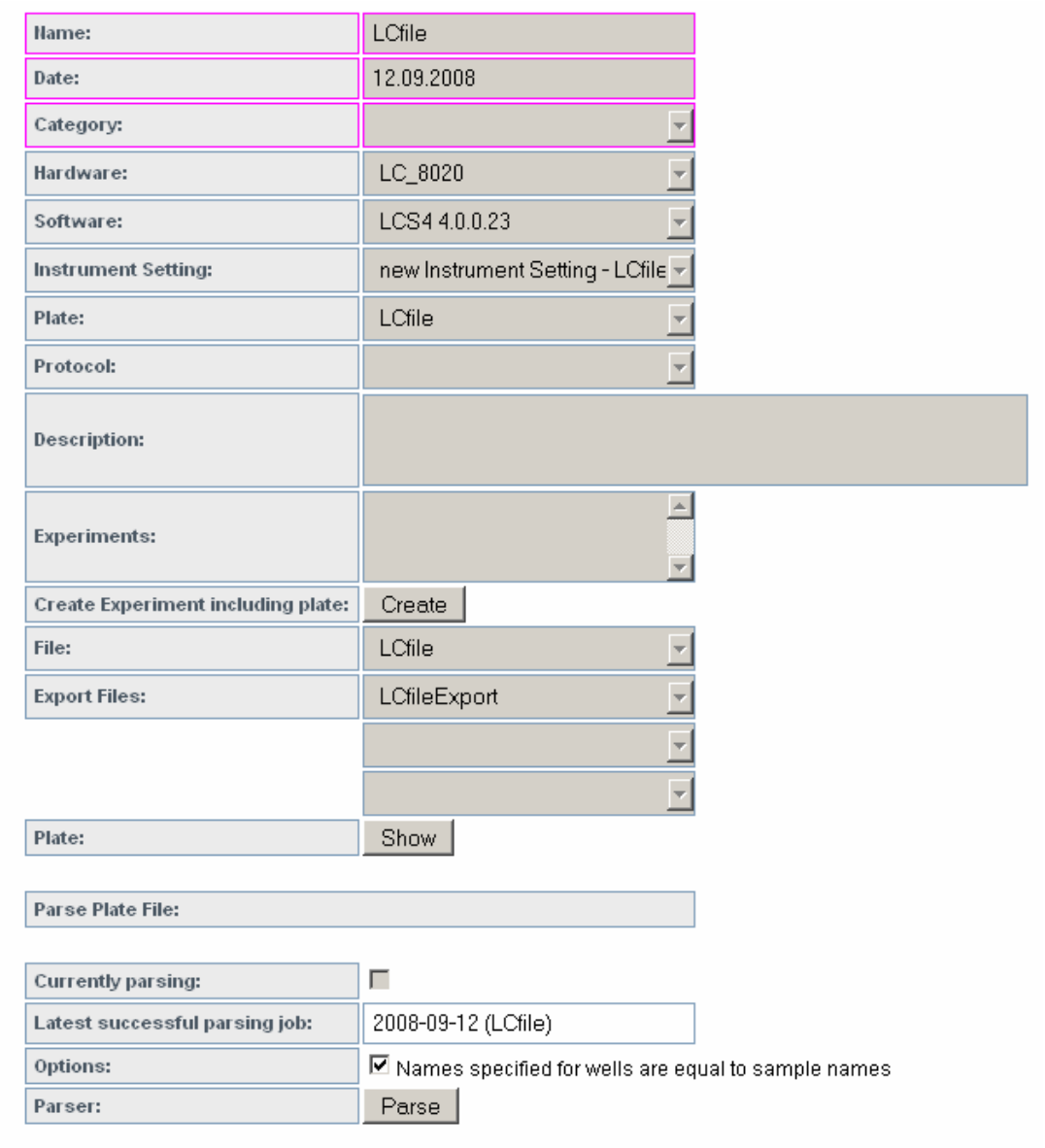
8.3 SDS

Displayed is the information page of the SDS run.



8.4 LC

Displayed is the information page of the LC run.

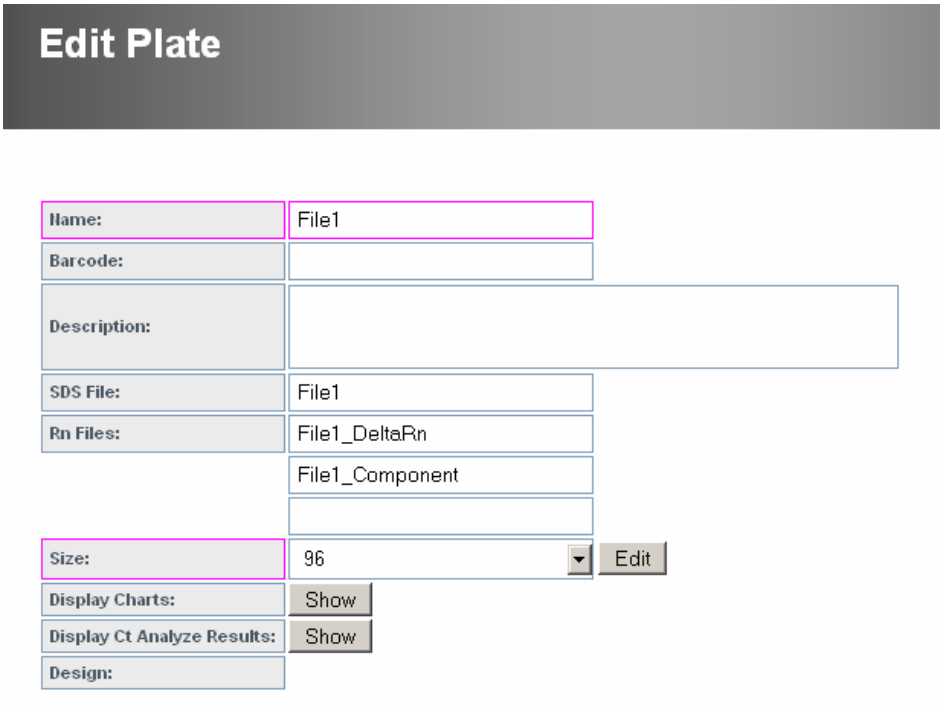


9 Plate

9.1 Information

The plate view (accessed by clicking on `Show` next to `Plate` in the run view) is used to display general information about the plate (barcode, description, used files) and to provide a list showing all wells or capillaries. By clicking on a well detailed information about this well is displayed.

9.2 SDS



Nr.	Well Number	Omitted	Passive Reference	Target(s)	cDNA	Task	
1	A1		ROX	detector1	sample1	Sample	
2	A2		ROX	detector1	sample1	Sample	
3	A3		ROX	detector1	sample2	Sample	
4	A4		ROX	detector1	sample2	Sample	
5	A5		ROX	detector1	sample3	Sample	
6	A6		ROX	detector1	sample3	Sample	
7	A7		ROX	detector1	sample4	Sample	
8	A8		ROX	detector1	sample4	Sample	
9	A9		ROX	detector1	sample5	Sample	
10	A10		ROX	detector1	sample5	Sample	
11	A11		ROX	detector1	sample6	Sample	
12	A12		ROX	detector1	sample6	Sample	
13	B1		ROX	detector2	sample1	Sample	
14	B2		ROX	detector2	sample1	Sample	
15	B3		ROX	detector2	sample2	Sample	
16	B4		ROX	detector2	sample2	Sample	
17	B5		ROX	detector2	sample3	Sample	
18	B6		ROX	detector2	sample3	Sample	
19	B7		ROX	detector2	sample4	Sample	
20	B8		ROX	detector2	sample4	Sample	
21	B9		ROX	detector2	sample5	Sample	
22	B10		ROX	detector2	sample5	Sample	
23	B11		ROX	detector2	sample6	Sample	

9.3 LC

Name:	LCfile
Barcode:	
Description:	
SDS File:	LCfile
Rn Files:	LCfileExport
Size:	24 Edit
Display Charts:	Show
Display Ct Analyze Results:	Show
Design:	

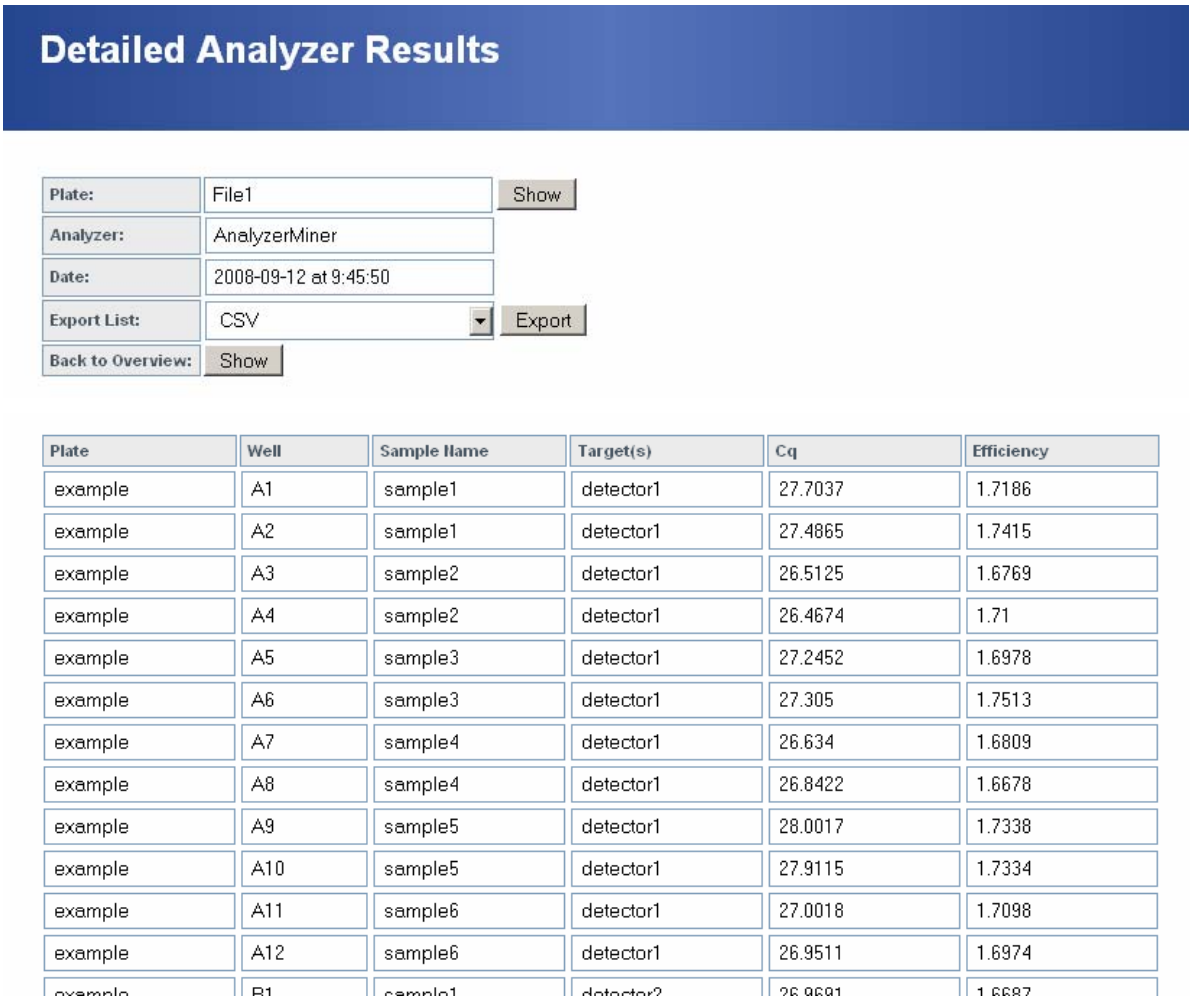
Page 1 of 1 Go to page 1 Go Items per page 96 Go

Nr.	Well Number	Omitted	Passive Reference	Detector(s)	cDNA	Task	
1	Pos 0	☐		Detector1	Sample1	Unknown	
2	Pos 1	☐		Detector1	Sample2	Unknown	
3	Pos 2	☐		Detector2	Sample3	Unknown	
4	Pos 3	☐		Detector2	Sample4	Unknown	
5	Pos 4	☐		Detector3	Sample5	Unknown	
6	Pos 5	☐		Detector1	Sample1	Unknown	
7	Pos 6	☐		Detector1	Sample2	Unknown	
8	Pos 7	☐		Detector2	Sample3	Unknown	
9	Pos 8	☐		Detector2	Sample4	Unknown	
10	Pos 9	☐		Detector3	Sample5	Unknown	

10 Cq Analyze Results

By clicking on the `Show` button next to `Display Cq Analyze Results` an overview page is shown, which lists the performed Cq analysis results. Each result can be exported or displayed in detail, shown in the next image.

Here you can check the calculated Cq and efficiency values.



Detailed Analyzer Results

Plate:	File1	Show
Analyzer:	AnalyzerMiner	
Date:	2008-09-12 at 9:45:50	
Export List:	CSV	Export
Back to Overview:	Show	

Plate	Well	Sample Name	Target(s)	Cq	Efficiency
example	A1	sample1	detector1	27.7037	1.7186
example	A2	sample1	detector1	27.4865	1.7415
example	A3	sample2	detector1	26.5125	1.6769
example	A4	sample2	detector1	26.4674	1.71
example	A5	sample3	detector1	27.2452	1.6978
example	A6	sample3	detector1	27.305	1.7513
example	A7	sample4	detector1	26.634	1.6809
example	A8	sample4	detector1	26.8422	1.6678
example	A9	sample5	detector1	28.0017	1.7338
example	A10	sample5	detector1	27.9115	1.7334
example	A11	sample6	detector1	27.0018	1.7098
example	A12	sample6	detector1	26.9511	1.6974
example	B1	sample1	detector2	26.9691	1.6687

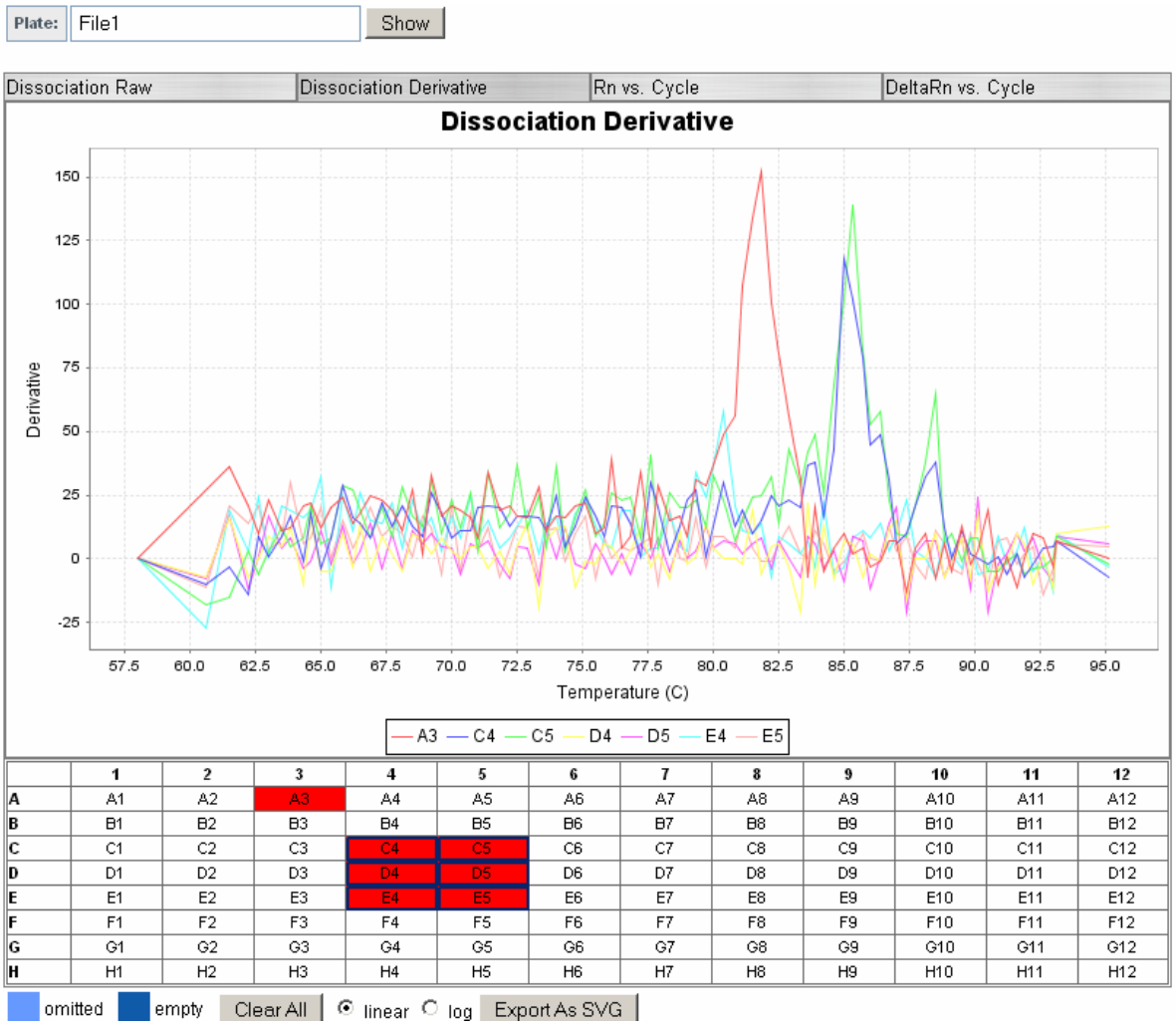
11 Charts

11.1 Information

The chart view (accessed by clicking on `Show` next to `Display Charts` in the plate view) is used to display graphs of dissociation and fluorescence data (if available). To switch between the different views click on the tabs in the top bar. Below the chart a grid/list is shown which represents the used plate layout. By clicking on a well, it is included/excluded from the chart which is then automatically updated. You can select multiple wells at once by holding the “ctrl” key while selecting them. Additionally you can click on every well individually. Here you can check whether the melting curve analysis was successful and you can get a rough overview of the shape of the fluorescence curves.

11.2 SDS

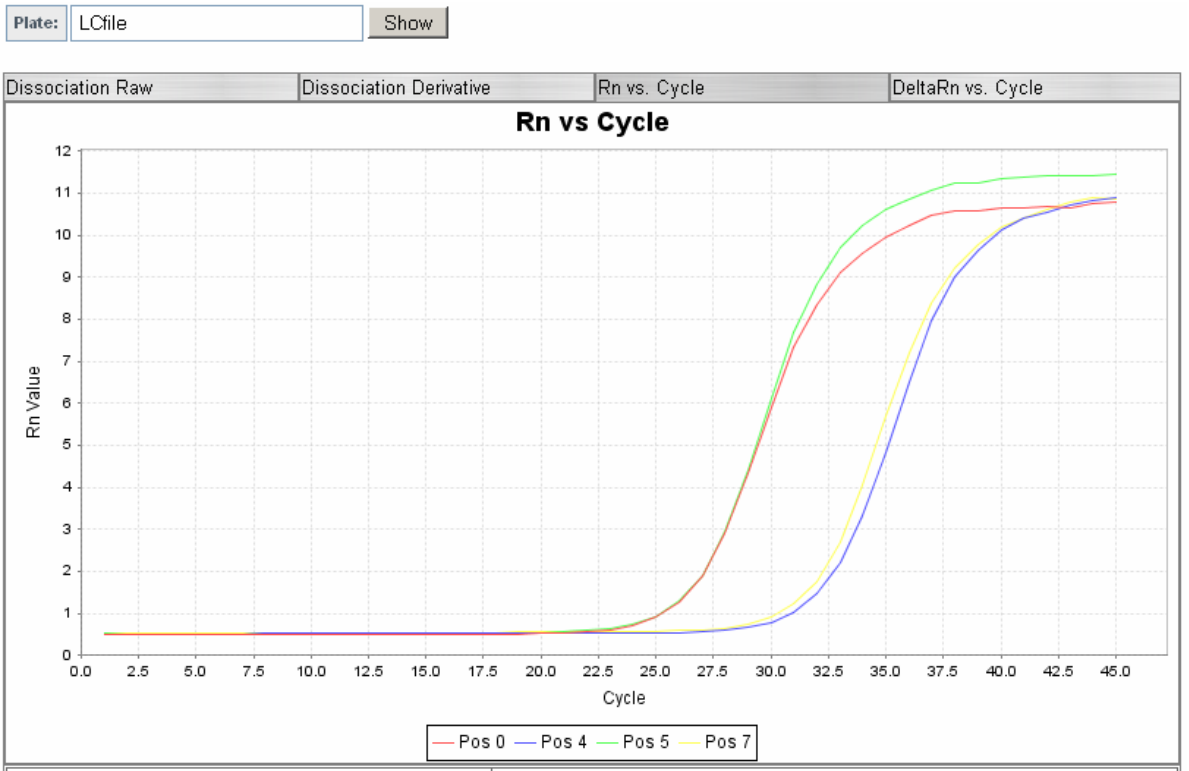
The grid beneath the chart displays the used plate layout. It colors empty and omitted wells and currently selected wells are colored in red.



	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

11.3 LC

Since the Lightcycler 2.0 uses a linear plate layout the grid beneath the chart changed to a list view.

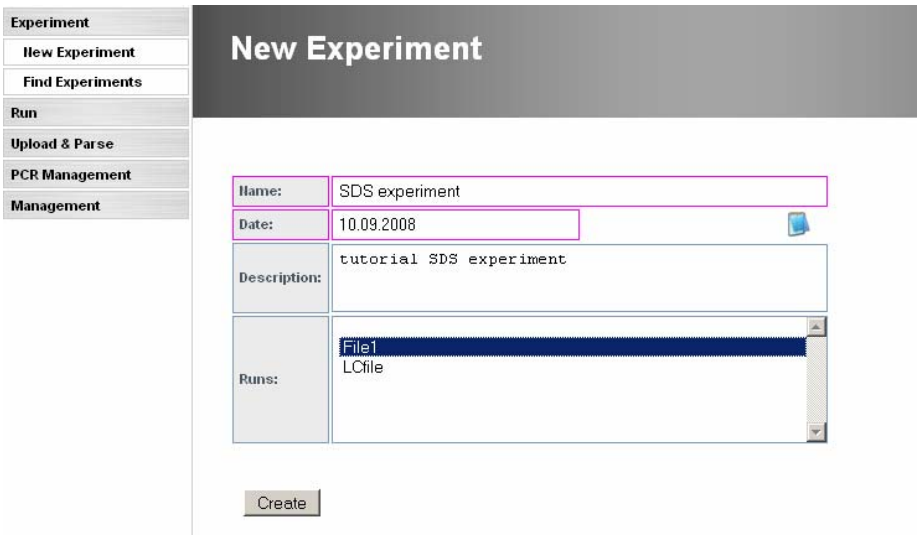


	1
1	Pos 0
2	Pos 1
3	Pos 2
4	Pos 3
5	Pos 4
6	Pos 5
7	Pos 6
8	Pos 7
9	Pos 8

12 Experiment

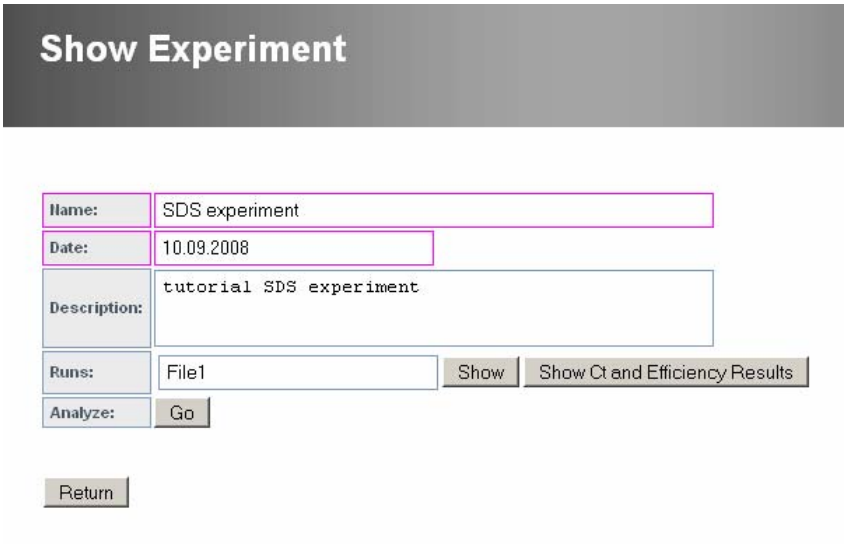
Since the upcoming steps are the same for the **SDS** and the **LC** files, only the **SDS** run is considered in the next steps. However you can view the results of the **LC** run by using the guest account of the QPCR application.

After you have taken a look at the generated runs and evaluated the parsed and analyzed results it is necessary to create a new experiment. Experiments consist of one or many runs (e.g.: experiment is spread over multiple plates because of the limited amount of wells on one plate) which are analyzed together. To create an experiment click on `Experiment` and select `New Experiment.` Define name, date, and description and choose the runs which should be in this experiment. Multiple runs are selected by holding the `ctrl` key.



Now you see the created experiment. The first icon links you directly to the analysis page and skips the experiment information page.

By clicking on the name of the experiment you are guided to the following screen which provides links to the runs in the experiment and the calculated Cq/efficiency results.



13 Analysis

To analyze an experiment you have to define several parameters. In this tutorial one way to analyze the experiment is shown. In order to get detailed information about the parameters please consult the user guide.

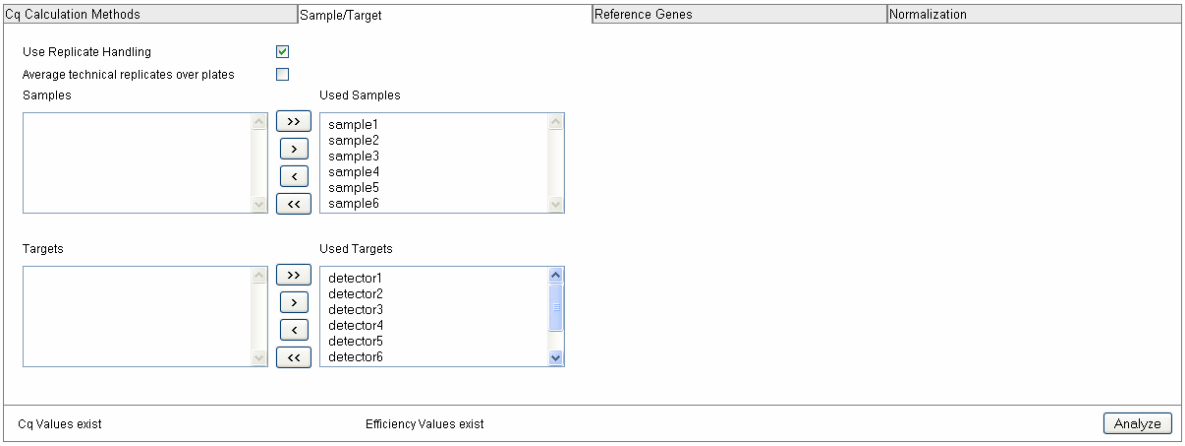
During the analysis relative quantities are calculated using averaging of technical replicates, normalization against reference genes, and inter-run calibration. For more details consult the paper “qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data” by Hellemans *et al.* (2007).

13.1 Cq Calculation Methods

Please pick the Cq calculation method you have selected in the user settings. Therefore the Cq values for the used runs exist and can be used in the upcoming analysis.

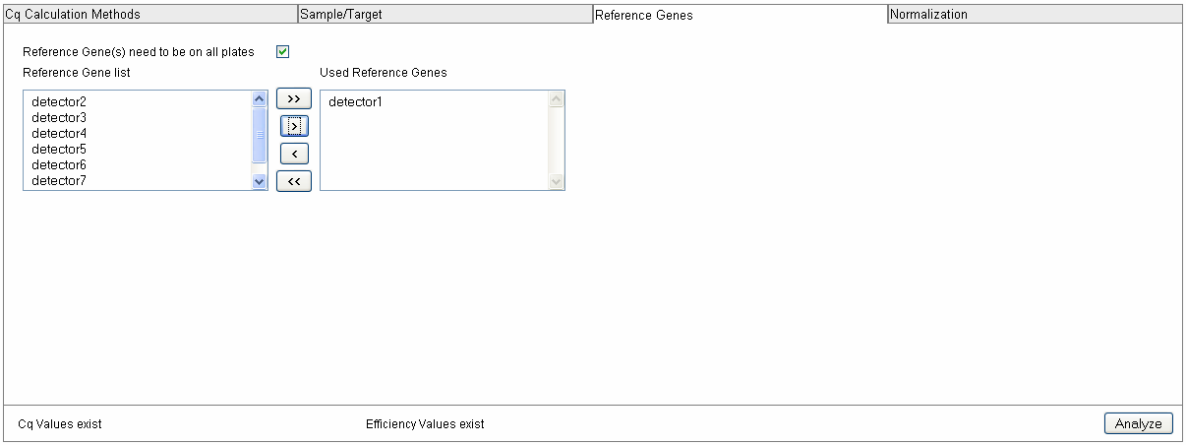
13.2 Sample/Detector

Here you can specify which samples and targets are used in the analysis. In this tutorial all samples and targets are used. Please tick “Use Replicate Handling” and leave “Average technical replicates over plate” unticked.



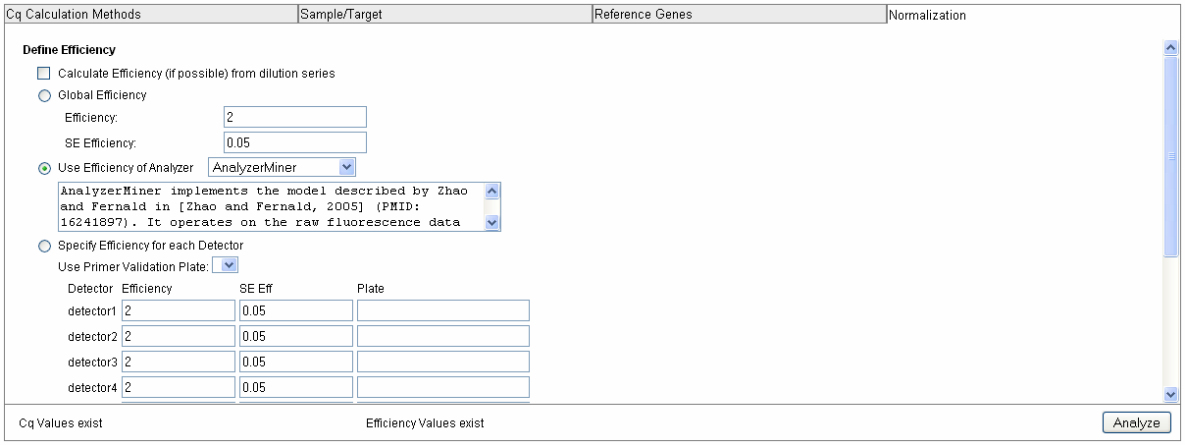
13.3 Reference Genes

This tab lets you choose which tragets should act as reference genes. It is possible to select multiple reference genes or analyze the experiment without a reference gene.



13.4 Normalization

In this view you can select which efficiency should be incorporated into the analysis. To follow the tutorial use `Use Efficiency of Analyzer` and select the analyzer you have picked in the user settings.

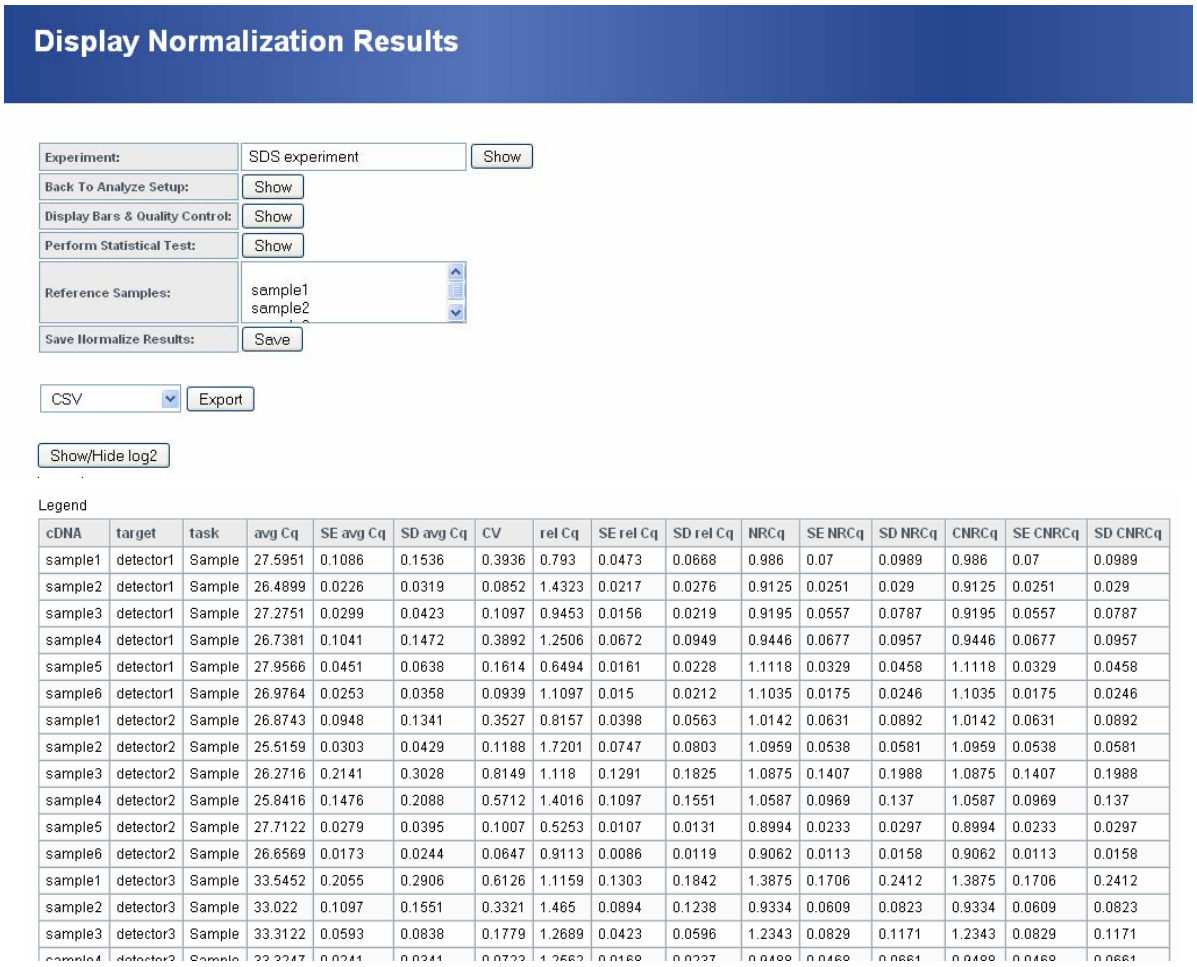


Now the setup is complete and you can press the `Analyze` button to start the analysis.

14 Analysis results

14.1 Overview

The page displayed, after the analysis has been performed, lists the calculated results and the provided legend gives information about the meaning of each result. By clicking on one or more `Reference Samples` you can select the samples used as a reference. By clicking on the `Show/Hide log2` button you can display log2 values of the calculated results.



Legend

cDNA	target	task	avg Cq	SE avg Cq	SD avg Cq	CV	rel Cq	SE rel Cq	SD rel Cq	NRCq	SE NRCq	SD NRCq	CNRCq	SE CNRCq	SD CNRCq
sample1	detector1	Sample	27.5951	0.1086	0.1536	0.3936	0.793	0.0473	0.0668	0.986	0.07	0.0989	0.986	0.07	0.0989
sample2	detector1	Sample	26.4899	0.0226	0.0319	0.0852	1.4323	0.0217	0.0276	0.9125	0.0251	0.029	0.9125	0.0251	0.029
sample3	detector1	Sample	27.2751	0.0299	0.0423	0.1097	0.9453	0.0156	0.0219	0.9195	0.0557	0.0787	0.9195	0.0557	0.0787
sample4	detector1	Sample	26.7381	0.1041	0.1472	0.3892	1.2506	0.0672	0.0949	0.9446	0.0677	0.0957	0.9446	0.0677	0.0957
sample5	detector1	Sample	27.9566	0.0451	0.0638	0.1614	0.6494	0.0161	0.0228	1.1118	0.0329	0.0458	1.1118	0.0329	0.0458
sample6	detector1	Sample	26.9764	0.0253	0.0358	0.0939	1.1097	0.015	0.0212	1.1035	0.0175	0.0246	1.1035	0.0175	0.0246
sample1	detector2	Sample	26.8743	0.0948	0.1341	0.3527	0.8157	0.0398	0.0563	1.0142	0.0631	0.0892	1.0142	0.0631	0.0892
sample2	detector2	Sample	25.5159	0.0303	0.0429	0.1188	1.7201	0.0747	0.0803	1.0959	0.0538	0.0581	1.0959	0.0538	0.0581
sample3	detector2	Sample	26.2716	0.2141	0.3028	0.8149	1.118	0.1291	0.1825	1.0875	0.1407	0.1988	1.0875	0.1407	0.1988
sample4	detector2	Sample	25.8416	0.1476	0.2088	0.5712	1.4016	0.1097	0.1551	1.0587	0.0969	0.137	1.0587	0.0969	0.137
sample5	detector2	Sample	27.7122	0.0279	0.0395	0.1007	0.5253	0.0107	0.0131	0.8994	0.0233	0.0297	0.8994	0.0233	0.0297
sample6	detector2	Sample	26.6569	0.0173	0.0244	0.0647	0.9113	0.0086	0.0119	0.9062	0.0113	0.0158	0.9062	0.0113	0.0158
sample1	detector3	Sample	33.5452	0.2055	0.2906	0.6126	1.1159	0.1303	0.1842	1.3875	0.1706	0.2412	1.3875	0.1706	0.2412
sample2	detector3	Sample	33.022	0.1097	0.1551	0.3321	1.465	0.0894	0.1238	0.9334	0.0609	0.0823	0.9334	0.0609	0.0823
sample3	detector3	Sample	33.3122	0.0593	0.0838	0.1779	1.2689	0.0423	0.0596	1.2343	0.0829	0.1171	1.2343	0.0829	0.1171

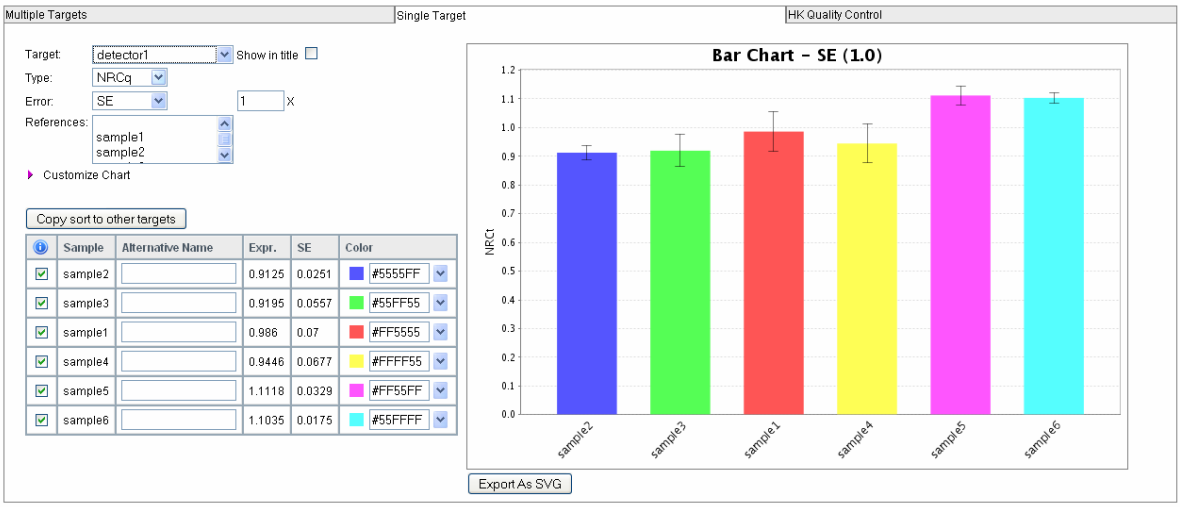
By clicking on `Display Bars & Quality Control` you are directed to the page which graphically displays the analysis results.

14.2 Multiple Targets

Here you can graphically view the calculated results and compare them for several targets. It allows you to customize it in many ways including error type, the used sample references, the grouping performed in the chart, title of the chart, and the samples displayed.

14.3 Single Target

The `Single Target` tab lets you view the results of a single selected traget. It provides the same customizability as the `Multiple Target` tab and additionally lets you choose the color of each sample and allows you to give each sample an alternative name. By using drag and drop you can rearrange the list of the displayed samples. In the customize Chart section you can edit the appearance of the chart to your needs.
The customize chart section lets you additionally adjust the chart.



14.4 Quality Control

Quality control for reference genes can only be performed by selecting multiple reference genes. An example is provided below. Please consult the user guide for more information.

Moreover quality controls are performed for NTCs and technical replicates.

	CV	M (geNorm)
detector1	9.04 %	0.2565
detector2	8.72 %	0.2565
Mean	8.88 %	0.2565

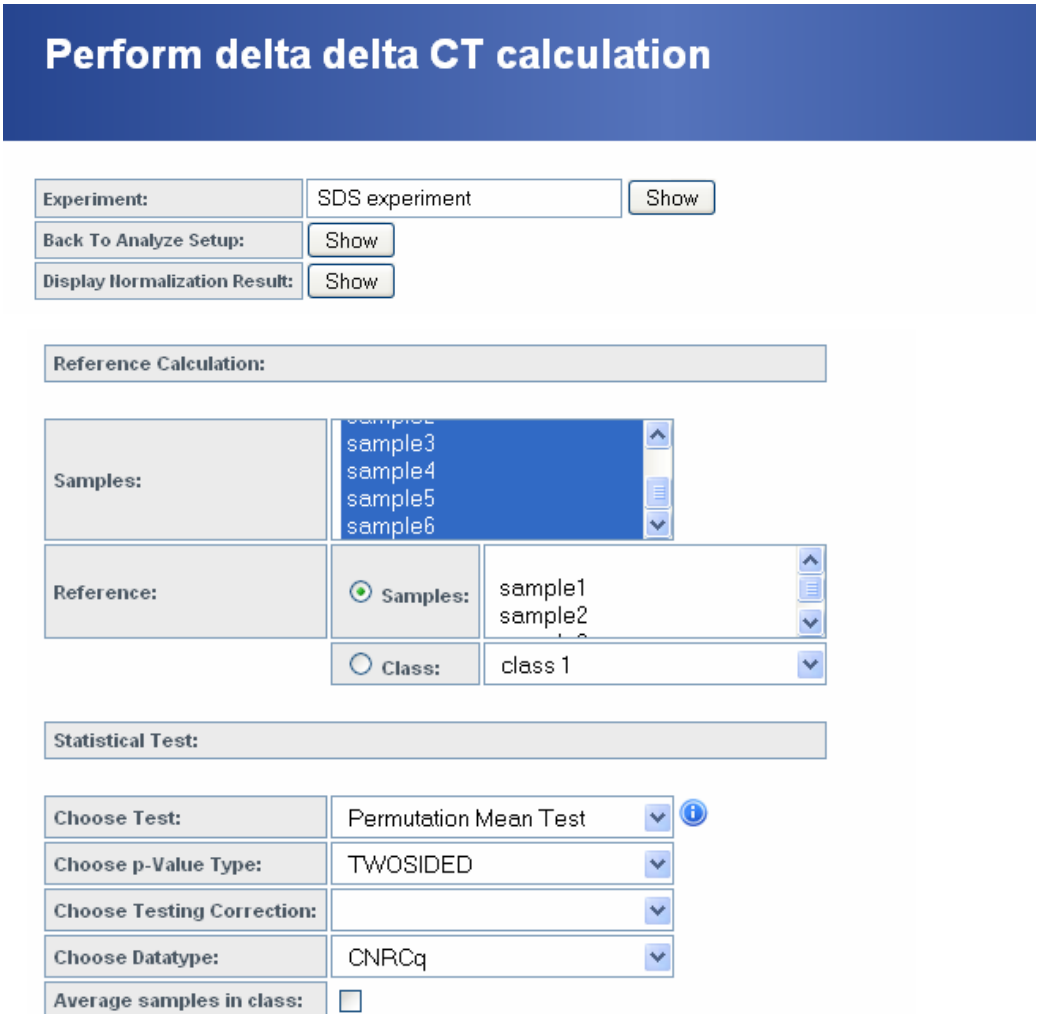
target	has NTC
detector1	false
detector2	false
detector3	false
detector4	false
detector5	false
detector6	false
detector7	false
detector8	false

target	cDNA	difference	#
detector8	sample6	0.1799	2
detector8	sample5	0.3121	2
detector8	sample4	0.1712	2
detector8	sample3	0.2569	2
detector8	sample2	0.0497	2
detector8	sample1	0.0477	2
detector7	sample6	0.2121	2
detector7	sample5	0.1909	2
detector7	sample4	0.2016	2
detector7	sample3	0.1536	2
detector7	sample2	0.2071	2
detector7	sample1	0.0564	2
detector6	sample6	0.0	1
detector6	sample5	0.3483	2
detector6	sample4	1.3124	2

15 Statistical Test

15.1 Setup

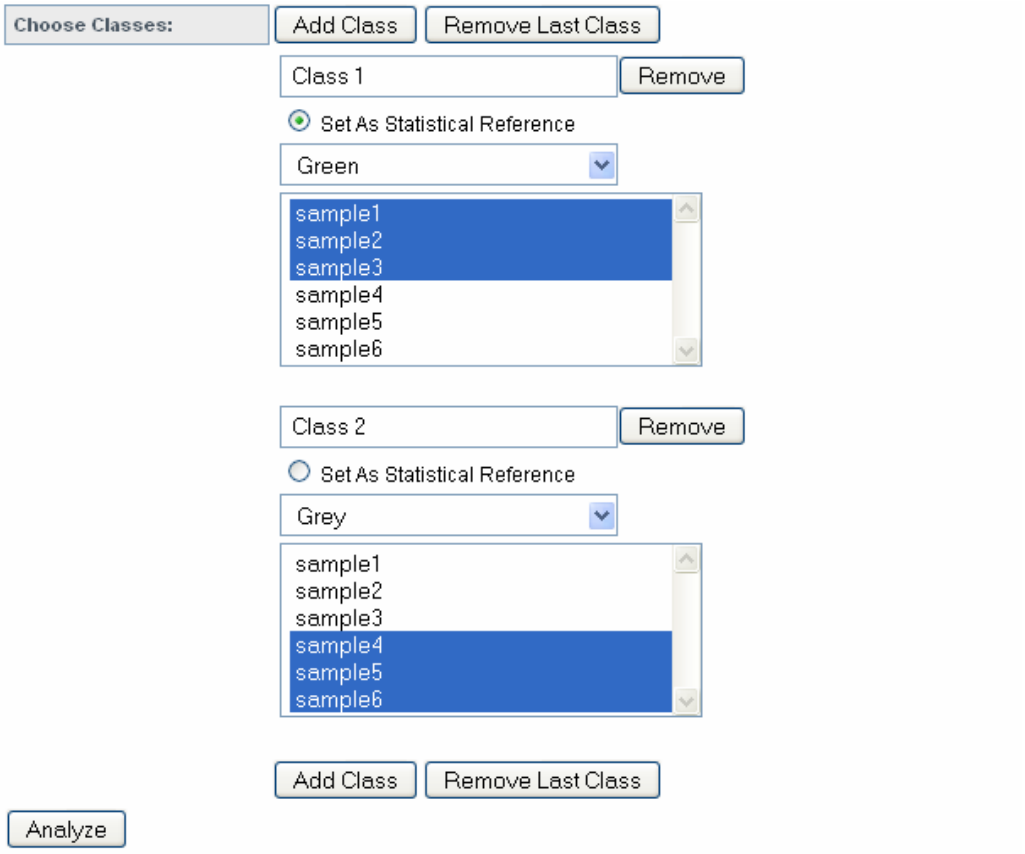
Statistical tests are used to test several groups (in the software named as class) of samples for significant difference between them. Here you can define which samples should be included in the test and which samples or which class should act as reference. Moreover you can choose the method and which p-Value type should be used. For more information click on the icon or consult the user guide. In this tutorial the standard settings are used.



You can add as many classes as you want to the statistical test. On class acts as the statistical reference (reference class) and all other classes are tested for their statistical significant difference to this reference class. Do not confuse this with the sample references which are used to reference the samples to a given set of samples (no statistical test).

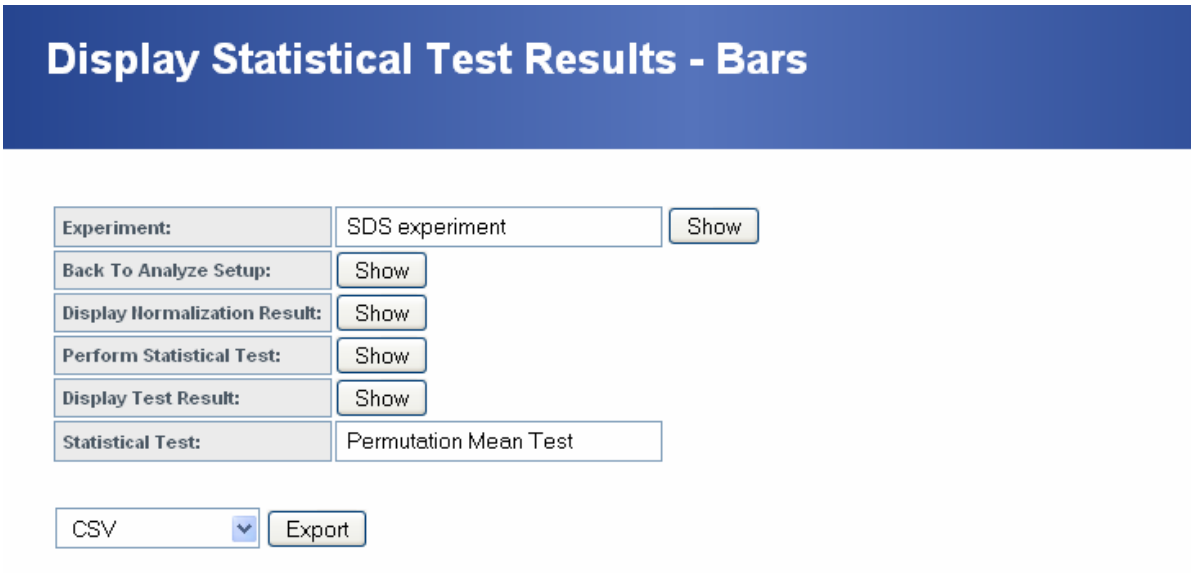
Each class has a color or pattern associated, is given a specific name, and needs to consist of at least one sample. In one class the property `Set As Statistical Reference` is set which specifies to which class all other classes are compared.

In this case the classes consist of 3 biological replicates and are therefore named “replicates 1” and “replicates 2”. Those replicates are then tested for their statistical significant difference.

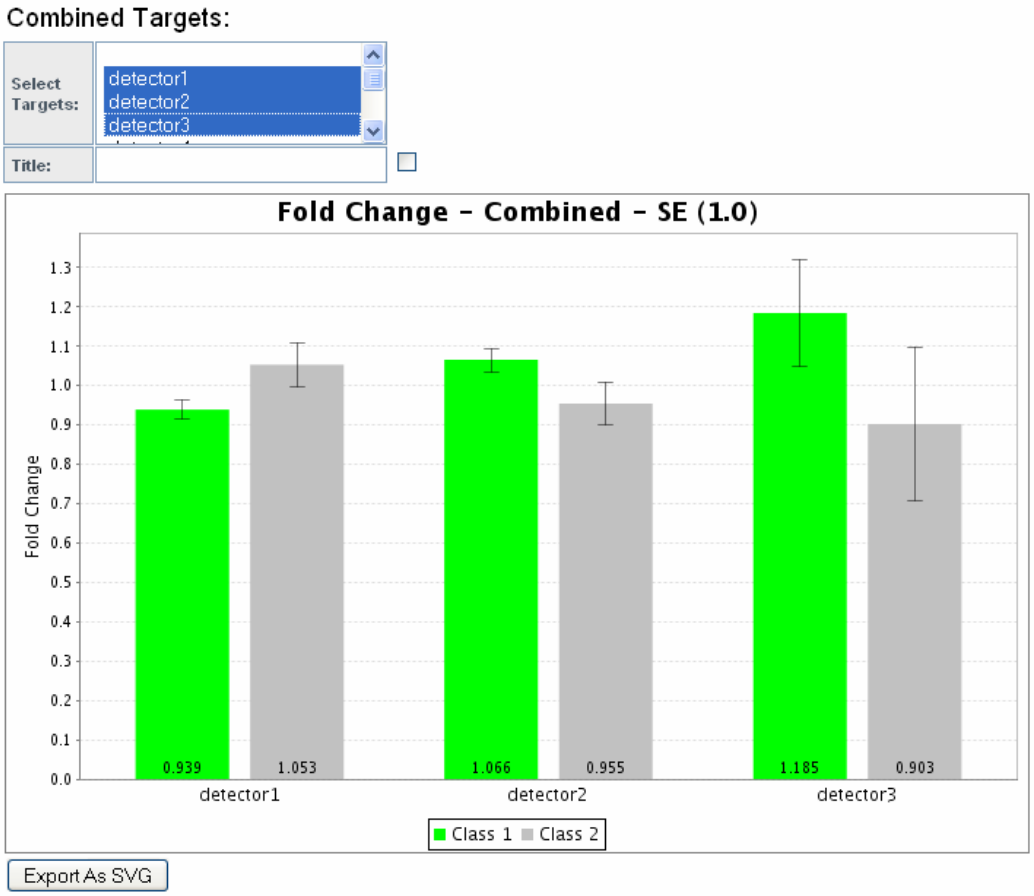


15.2 Result

The upper section of the statistical result page provides links back to the various analysis pages and gives you the opportunity to export the generated results.



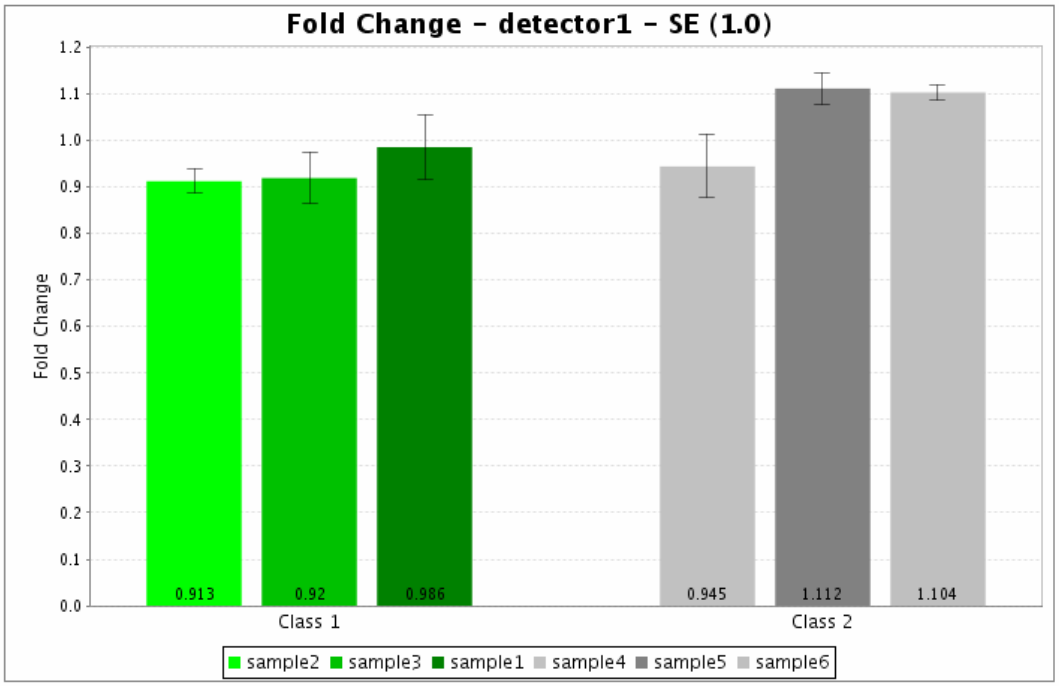
The combined targets view displays the averaged results of each class (in this case the classes replicates1 and replicates2) for the selected targets.



Next the results for each target are shown. As an example detector1 is presented in this document.

Target: detector1

Class	p-Value	Ø ddCq / reference	Ø SE ddCq / reference	Ø SD ddCq / reference	Is Statistical Reference
Class 1	-	-	-	-	true
Class 2	0.1997	1.1214	[+0.0655; -0.0655]	[+0.1134; -0.1134]	false



Export As SVG

In addition to the graphical view results of the statistical test can be displayed in text format. This view is accessed by clicking on `show` next to `Display Test Result`

detector1

Class	p-Value	Ø ddCt / reference	Ø SE ddCt / reference	Ø SD ddCt / reference	Is Statistical Reference
replicates 1	-	-	-	-	true

Sample	ddCt	SE ddCt	SD ddCt
sample1	1.0	0.023332	0.032996
sample2	1.0	0.028336	0.040074
sample3	1.0	0.05177	0.073213
Avg replicates 1	1.0	0.017272	0.029916

replicates 2	1.0	1.0	0.0306	0.053	false

Sample	ddCt	SE ddCt	SD ddCt
sample4	1.0	0.067074	0.094856
sample5	1.0	0.06139	0.086818
sample6	1.0	0.019126	0.027049
Avg replicates 2	1.0	0.025289	0.043801

16 Export

All relevant result can be exported using the provided mechanism.

In addition each graph can be saved by right clicking on it and selecting e.g.: “save image as”. If you want to save it as SVG file you can use the provided button right beneath the displayed image.

For more information about the QPCR application please consult the user guide.