analytikjena
An Endress+Hauser Company

Operating Manual

qPCRsoft

Software for REAL-TIME PCR Thermal Cycler

Manufacturer

Analytik Jena AG
Konrad-Zuse-Str.1
07745 Jena · Germany
Phone + 49 3641 77 70
Fax + 49 3641 77 9279
E-mail info@analytik-jena.de

Service

Analytik Jena AG
Konrad-Zuse-Str. 1
07745 Jena · Germany
Phone + 49 3641 77 7407
Fax + 49 3641 77 7449
E-mail service@analytik-jena.com

General information

http://www.analytik-jena.com

Documentation number 10-3107-012-23

Edition A (06/2020)

Implementation of the Technical Documentation Analytik Jena AG

Contents

1 About qPCRsoft Software ... 7
1.1 How to use this manual ... 7
1.2 Installation of qPCRsoft ... 7
1.3 Starting and exiting qPCRsoft ... 8
1.4 The main window in qPCRsoft ... 9
1.4.1 Menu commands overview ... 10
1.4.2 Overview of the tools in the toolbar ... 13
1.4.3 Project explorer components ... 15
1.4.4 Project interface with project window ... 17
1.4.5 Help function ... 17
1.4.6 Version number of the software ... 18

2 Managing projects and templates ... 19
2.1 Creating a new project or opening a project ... 19
2.2 Saving a template ... 21
2.3 Saving a project ... 21
2.4 Importing/exporting analyses ... 21
2.5 Creating a project template from a transfer file ... 22
2.6 Performing a Multigene-/Multiplate-Analysis ... 22
2.7 Closing project windows ... 22
2.8 Printing ... 22

3 Settings for a Real-Time PCR experiment ... 24
3.1 Entering general information on the project ... 24
3.2 Creating a PCR protocol ... 25
3.2.1 Entering information in the program header ... 26
3.2.2 Overview about the PCR protocol ... 27
3.2.3 Inserting a new temperature step / deleting a temperature step ... 28
3.2.4 Entering the target temperature, hold time and heating/cooling rates ... 28
3.2.5 Defining loops ... 29
3.2.6 Entering increments/decrements for temperature and hold time ... 29
3.2.7 Arranging a fluorescence measurement ... 30
3.2.8 Adding a melting curve ... 31
3.2.9 Programming the temperature gradient (for devices with gradient function only) ... 32
3.2.10 Graphical display and programming the PCR protocols ... 33
3.3 Defining the parameters for the fluorescence measurement ... 34
3.3.1 Manually defining the scan region ... 36
3.3.2 Color compensation ... 37
3.4 Editing the sample table ... 39
3.4.1 Entering sample properties in the layout ... 41
3.4.2 Entering sample properties into the sample table ... 43
3.4.3 Entering a sample layout for a multiplex assay ... 44
3.4.4 Entering a sample layout for a singleplex assay ... 46
3.4.5 Automatic generation of dilution series/replicates ... 48
3.4.6 Defining groups ... 50
3.4.7 Layout preview ... 51
3.4.8 Copying the layout ... 52
3.4.9 Exporting or importing the layout in Excel ... 53
3.4.10 Functions for creating and editing a plate layout ... 53

4 Monitoring ... 56
4.1 Starting the PCR protocol ... 56
4.2 Display options for monitoring ... 57
4.2.1 Default settings for the Monitoring view ... 58

4.2.2 Adjusting the view in the Monitoring project window ... 59
4.2.3 Displaying and hiding measurement results for individual wells ... 59
4.2.4 Exporting fluorescence data ... 61
4.3 Monitoring the PCR run ... 61
4.4 Displaying product accumulation curves and calculating Ct values ... 63
4.5 Displaying melting curves and calculating the melting temperatures T_m ... 63

5 Evaluation ... 65
5.1 General functions in the evaluation project window ... 66
5.1.1 Making basic settings ... 66
5.1.2 Activating/deactivating samples for evaluation ... 67
5.1.3 Exporting fluorescence data ... 69
5.1.4 Configure results table ... 69
5.1.5 Export results ... 70
5.2 Absolute quantification ... 71
5.2.1 Creating an evaluation for an absolute quantification ... 71
5.2.2 Setting parameter for absolute quantification ... 72
5.2.3 Displaying the fluorescence curves for absolute quantification ... 73
5.2.4 Display of the mean Ct values or mean concentrations ... 74
5.2.5 Displaying the standard curve and results of an absolute quantification ... 75
5.2.6 Exporting results of calculations ... 77
5.2.7 Importing the standard curve ... 78
5.2.8 Deleting the evaluation of an absolute quantification ... 78
5.3 Relative quantification ... 79
5.3.1 Creating a new evaluation for a relative quantification ... 79
5.3.2 Parameter settings for relative quantification ... 80
5.3.3 Displaying the fluorescence curves in the relative quantification ... 82
5.3.4 Display of the normalised relative concentrations ... 83
5.3.5 Displaying the standard curves and the results of a relative quantification ... 83
5.3.6 Importing the standard curve for relative quantification ... 85
5.3.7 Deleting the evaluation of a relative quantification ... 86
5.4 ΔΔCt method ... 86
5.4.1 Creating a new evaluation for a ΔΔCt method ... 86
5.4.2 Parameter settings for the ΔΔCt method ... 87
5.4.3 Displaying the fluorescence curves for the ΔΔCt method ... 88
5.4.4 Display of the normalized relative expression or relative quantities ... 89
5.4.5 Setting the calculation mode for the standardized expression ... 90
5.4.6 Displaying the validation curves and values ... 92
5.4.7 Deleting a ΔΔCt method evaluation ... 94
5.5 Melting curve analysis ... 95
5.5.1 Creating a new melting curve analysis ... 95
5.5.2 Parameter settings for melting curve analysis ... 96
5.5.3 Displaying fluorescence curves/melting curves ... 97
5.5.4 Display of melting temperatures ... 99
5.5.5 Displaying the result table for the melting curves ... 99
5.5.6 Deleting a melting curve analysis ... 100
5.6 Genotyping ... 101
5.6.1 Creating a new evaluation for genotyping ... 101
5.6.2 Parameter settings for genotyping ... 102
5.6.3 Specifying genotyping options ... 104
5.6.4 Displaying the fluorescence curves, scatter plot and bar graph ... 104
5.6.5 Display of the values for the genotyping evaluation ... 107
5.6.6 Deleting a genotyping ... 109
5.7 POS/NEG analysis at the end point ... 109
5.7.1 Starting end point analysis ... 109
5.7.2 Parameters settings for endpoint analysis ... 110
5.7.3 Displaying results in end point analysis ... 112

6 Multigene-/Multiplate-Analysis ... 116
6.1 Start Multigene-/Multiplate-Analysis ... 116
6.2 The window for Multigene-/Multiplate-Analysis ... 116
6.3 Overview of menu commands ... 117
6.4 Overview of the tools in the toolbar ... 118
6.5 Help function ... 118
6.6 Managing Multigene-/Multiplate-Analysis files ... 118
6.6.1 Start new Multigene-/Multiplate-Analysis ... 118
6.6.2 Open saved Multigene-/Multiplate-Analysis ... 118
6.6.3 Save Multigene-/Multiplate-Analysis ... 119
6.6.4 Close Multigene-/Multiplate-Analysis ... 119
6.6.5 Print Multigene-/Multiplate-Analysis ... 119
6.7 Load Project Files ... 119
6.8 Activate/Deactivate project files for Multigene-/Multiplate-Analysis ... 120
6.9 Activate/Deactivate samples for Multigene-/Multiplate-Analysis ... 120
6.10 Define Interplate-Standards for Multigene-/Multiplate-Analysis ... 121
6.11 Set threshold and PCR-efficiencies for Multigene-/Multiplate-Analysis ... 122
6.12 Evaluation of the Multigene-/Multiplate-Analysis ... 124

7 MIQE documentation ... 131

8 Functions in Extras menu ... 133
8.1 Device initialization ... 133
8.2 Editing color modules ... 133
8.3 Connecting the device to the PC ... 134
8.4 General settings in the qPCRsoft software ... 134

9 User management ... 136
9.1 Basic settings for password, login and logout ... 136
9.2 Managing user profiles / user groups ... 137
9.3 Edit password ... 141

10 Optional 21 CFR Part 11 module ... 142
10.1 Activation ... 142
10.2 Audit trail ... 144
10.3 Login monitoring ... 145
10.4 Digital signatures ... 146
10.4.1 Signing a document ... 146
10.4.2 Displaying signatures ... 147

11 Appendix A – Short instruction ... 148

12 Appendix B - How to save communication data ... 154

13 Appendix C – Creating a project template from the transfer file (LIMS) ... 155

14 Appendix D – Entries in the Audit Trail ... 156

15 Appendix E – Available Color Modules and Dyes to be detected ... 168

1 About qPCRsoft Software

The qPCRsoft software can be used to create and perform PCR and Real-Time PCR experiments. This section describes the basic setup and the layout of the operating elements of the software.

The short instructions also offer a quick entrance on the operation of qPCRsoft in the section "Appendix A – Short instruction" p. 148.

Described software version

This description is based on the version qPCRsoft V. 4.1.

Supported devices

The program supports the device control and data analysis of the following devices:

- TOptical / TOptical G
- qTOWER 2.0 / qTOWER 2.2
- qTOWER^3 / qTOWER^3 G

Functions that are only available for certain devices or that are not available are marked in the text.

New file formats

Starting with qPCRsoft 4.0, new file formats for projects, templates and multi-gene analyses with the extensions "*.rtpx", "*.rtsx" or "*.mgax" are used. The software is backwards compatible, i. e. the files of type "*.rtp", "*.rts" and "*.mga" can be opened and edited. However, the new data formats cannot read by older versions of qPCRsoft. When saving, you can select in which format the files are tob e saved. To ensure the further use of the data, we recommend that you also save files from previous software versions in the new format.

1.1 How to use this manual

The following symbols and conventions are used to facilitate orientation in the manual:

→	Cross reference to other sections and/or figures.
Formatting	In the description of the operating procedures, menu commands, dialog boxes, buttons, options, etc. are highlighted in small capitals. Menu commands of a command sequence are separated by "▸", e.g. **File ▸ Open project**. Buttons are additionally written in square brackets, e.g. **[Save]**.

1.2 Installation of qPCRsoft

Administrator rights on the operating system are required for installing the program.

System requirements for installing qPCRsoft

For using qPCRsoft to control the Real-Time PCR device, your PC must fulfill the following minimum requirements:

Operating system	Windows 10

Processor	Min. Intel Core i3
RAM	2 GB
Available hard disk space	Min. 300 MB
Interfaces	Min. USB 2.0

Installation procedure

qPCRsoft is delivered on CD-Rom.

1. Insert the CD in the CD-Rom drive. Normally, the installation's start window opens automatically.
   If this is not the case, start the "setup.exe" file on the CD.
   A selection dialog window for installing the device driver or the user management or for viewing the PDF files of the manuals appears.
2. Activate your language version.
3. Select the device type.
4. Click on **[Install]**.
   The installation routine begins.
5. Follow the further instructions of the installation program.
6. Switch on the device at the power switch. Start qPCRsoft.

Note:
The software will only be installed correctly if it has been started once with administrator rights. A password for the program administrator must be entered during this first start.

Set up administrator

After the software installation, an administrator password must be assigned and an administrator must be specified.

1. Start qPCRsoft.
2. Enter and confirm the password for the adminstrator in the window **Login.**
3. Set up the user profiles (→ section "User management" p. 136).
   If the user mangement is not necessary, disable the user management via **Extras ▸ Options / Usermanagement**.

1.3 Starting and exiting qPCRsoft

Starting qPCRsoft

1. To start qPCRsoft, click on the **[Start]** button on the Windows desktop. Then go to the **Programs** folder and search for qPCRsoft. Click on qPCRsoft there.
2. Alternatively, you may click on the qPCRsoft icon on the Windows desktop.
   qPCRsoft is started.

If the user management has been installed, you will be prompted to enter username and password. The qPCRsoft workspace will only become accessible if the entry of this data was successful.

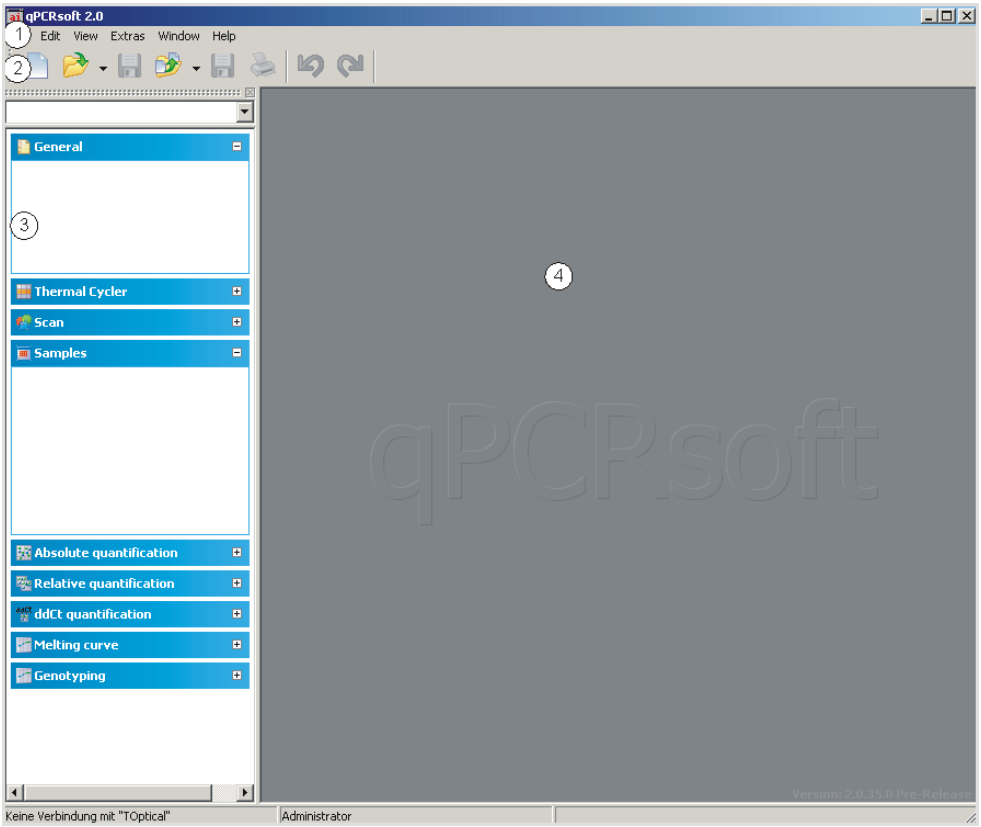
Note:
An administrator and password must be defined during the first program start. Only the administrator can set up further user accounts or disable the user management.

Note:
At the first start of the software the color modules installed in the device must be defined in the software (→ section "Editing color modules" p. 133). Instructions can be found in chapter 8.4. of this manual. A table of all cur-rently available color modules can be found on the website of Analytik Jena AG https://www.analytik-jena.com and this manual (→ section "Appendix E – Available Color Modules and Dyes to be detected" p. 168).

Exiting qPCRsoft

1. To exit the qPCRsoft application, activate the **File ▸ Exit** menu command.
2. The program will display a message if any projects that have not yet been saved are still open at this point.
3. If you want to save these projects, click on **[Yes]**. Save the projects in the **Save as** standard window
4. Then call up the **File ▸ Exit** menu command again to exit qPCRsoft.

1.4 The main window in qPCRsoft



Main window of the qPCRsoft software

After starting the qPCRsoft software the main window opens. It has the following sections:

Menu bar (1)	The menu bar contains the menu commands for, e.g., opening, editing and saving projects, managing user profiles, setting basic software options and a help function.

Toolbar (2)	Commands for editing projects are arranged in the toolbar. The commands offered in the toolbar may change according to context.
Project explorer (3)	In the project explorer a drop-down menu provides a quick overview of the most important information on the current open project.
Project interface (4)	The project interface is used to process projects. As soon as a new project is created or an existing project is loaded, a window opens in which all relevant settings for the respective project can be made.

1.4.1 Menu commands overview

The menu bar is context-sensitive and is automatically adapted to the program tasks. Menu items that are not necessary for the current work interface are automatically hidden. The following menu commands are available in the qPCRsoft software:

Menu	Function	Description
File	**New**	Opens a new project.
	Open template	Opens a template.
	Open project	Opens a project.
	Open autom. saved project	Opens an automatically saved project.
	Save template	Saves a template file in the qPCRsoft standard folder.
	Save template as	Saves a template file in any user-selected folder.
	Save project	Saves a project file in the qPCRsoft standard folder.
	Save project as	Saves a project file in any user-selected folder.
	Import analyses	Opens an analysis file.
	Export analyses	Saves an analysis file.
	Import LIMS	Imports a transfer file, with which the software of another program can be configured, e. g. LIMS.
	MultiGene	Starts the **Multi Gene** analysis for analyzing experiments that comprise multiple PCR plates and multiple genes.
	Close	Closes a template or a project.
	Close all	Closes all open projects or templates.
	Print	Prints a project.
	Exit	Closes the software.
Edit	**Undo**	Reverses the last text modification (up to 10 steps).
	Redo	Restores the last deleted text item (up to 10 steps).
	Cut	Cuts a marked text area.
	Copy	Copies an active and/or marked text area.
	Paste	Pastes atext area copied to the clipboard.
	Delete	Deletes an active and/or marked text area.
	Mark all	Marks a complete text area.

	User management	Opens the window for creating user profiles and changing the password. (Only available if the user management function is activated)
View	**Project explorer**	Switches the project explorer view in the main window on or off.
	Toolbar	Switches the toolbar view in the main window on or off.
Scan	**Set color compensation**	Opens the window for creating files for spectral color compensation.
Extras	**Device initialization**	Resets the connected device to the initial state.
	Device identifcation	Activates the connected device
	Edit color modules	Opens the window for configuring the system with color filter modules.
	Transport lock	Prepares the device for transport (not for qTOWER).
	Options	Opens the window for general basic software settings.
Window	**Tile horz**	Arranges project windows horizontally.
	Tile vert	Arranges project windows vertically.
	Cascade	Arranges project windows in a cascaded fashion.
Help	**Content**	Opens the table of contents of the help function.
	Info	Displays software information.
Cycler	**Add empty step**	Adds a new step.
	Delete step	Deletes a step.
	Cut step	Cuts a step and copies it to the clipboard.
	Copy step	Copies the parameters in one step into the clipboard.
	Paste step	Inserts a copied step.
Scan	**Edit color compensation**	Opens the window for creating files for spectral color compensation.
Samples	**Edit layout**	Edit sample table.
	Copy layout	Copy the area of the sample table.
	Paste layout	Insert the copied area of the sample table.
	Preview layout	Detailed view of the plate assignment
Monitoring	**Start qPCR run**	Start the PCR run.
	Stop qPCR run	Stop the PCR run.
	Pause qPCR run	Pause the PCR run.
	Display options	Display options for the product accumulation curves
	QBase Export	Exports measured values as a CSV or an XLS file in qBase format (available after Ct calculations)

AbsQuant	**Add abs. quantification**	Create new evaluation.
	Delte abs. quantification	Delete evaluation.
	Optionsabs. quantificationg	Opens a window for basic evaluation settings.
	Automa. Threshold	Automatic determination of the fluorescence threshold for detecting Ct values.
	Import Standard curve	Import a saved standard curve.
	Qbase export	Exports measured values as a CSV or an XLS file in qBase format.
RelQuant	**Add rel. quantification**	Create new evaluation.
	Delte rel. quantification	Delete evaluation.
	Optionsrel. quantificationg	Opens a window for basic evaluation settings.
	Automa. Threshold/Cut off	Automatic determination of the fluorescence threshold for detecting Ct values.
	Import Standard curve	Import a saved standard curve.
DeltaDeltaCt	**Add ΔΔCt quantification**	Create new evaluation.
	Delte ΔΔCt. quantification	Delete evaluation.
	OptionsΔΔCt. quantificationg	Opens a window for basic evaluation settings.
	Automa. Threshold	Automatic determination of the fluorescence threshold for detecting Ct values.
Melting Curve	**Add melting curve**	Create new evaluation.
	Delte melting curve	Delete evaluation.
	Optionsmelting curve	Opens a window for basic evaluation settings.
	Automa. Threshold	Determine threshold automatically.
Genotyping	**Add Genotyping**	Create new evaluation.
	Delete Genotyping	Delete evaluation.
	Options Genotyping	Opens a window for basic evaluation settings.
	Autom. Threshold\Cut off	Automatic determination of the fluorescence threshold for detecting Ct values.
Endpoint	**Add Endpoint**	Create new evaluation.
	Delete Endpoint	Delete evaluation.

	Options Endpoint	Opens a window for basic evaluation settings.
	Auto Threshold\Cut off	Automatic determination of the cut-off fluorescence value for decision POS/NEG
MIQE	**Import MIQE Documentation**	Imports MIQE information from other project.

1.4.2 Overview of the tools in the toolbar

The buttons in the toolbar are mostly context-sensitive. The program automatically adjusts the toolbar to the window content and adds buttons provided that this is required and useful for the current project window view. Buttons not accessible for the current contents of the workspace are hidden.

You can display or hide the toolbar via the **View ▸ Toolbar** menu command.

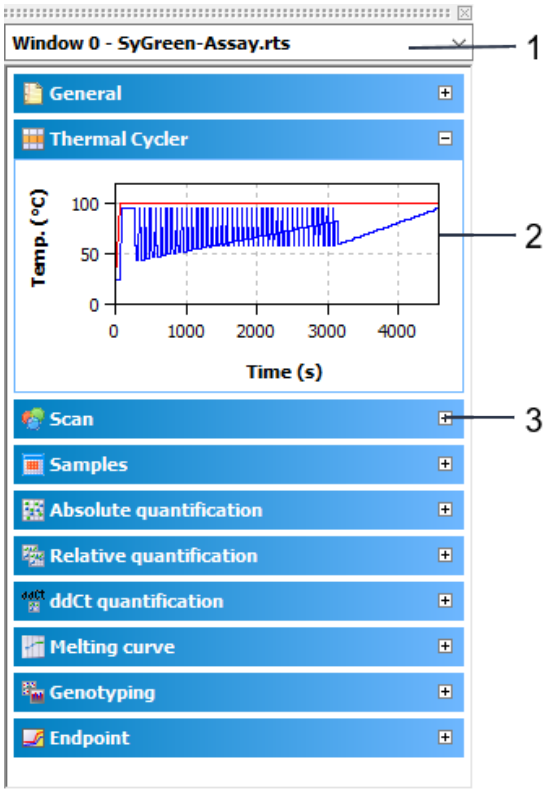
Button	Command	Function
General		
	New	Opens a new project.
	Open template	Opens a template.
	Save template	Saves a template.
	Open project	Opens a project.
	Save project	Saves a project.
	Print project	Prints a project.
	Undo	Reverses the last modification.
	Redo	Restores the last deleted modification.
	Cut	Cuts a marked area.
	Copy	Copies an active and/or marked area.
	Paste	Pastes an area copied into the clipboard.
	Delete	Deletes an active and/or marked area.
	MultiGene	Startet die MultiGene- und Multi-Platten-Analyse.
	Englisch	Schaltet zur englischen Sprachversion der Software um.
PCR protocol		
	Add empty step	Adds a new step.
	Delete step	Deletes a step.
	Activate melting curve	Adds a step for melting curve determination.

	Cut step	Cuts a step and copies it to the clipboard.
	Copy step	Copies the parameters in one step into the clipboard.
	Paste step	Pastes a copied step.
Color		
	Edit color compensation	Opens the window for creating files for spectral color compensation.
Samples		
	Edit layout	Assigns changes that were made to the sample table.
	Copy layout	Copies an area in the sample table.
	Paste layout	Pastes a copied area from the sample table.
	Preview layout	Displays a complete view of the plate assignment.
MIQE		
	Import MIQE Documentation	Imports MIQE information from other project.
Monitoring		
	Start PCR protocol	**Starts** the PCR run.
	Stop PCR protocol	Ends the PCR run.
	Pause PCR protocol	Breaks the PCR run.
	Options	Display options of the product accumulation curves.
	qBase Export	Exports measured values as a CSV or an XLS file in qBase format.
Evaluation/absolute quantification		
	New	Creates a new evaluation
	Delete	Deletes an evaluation
	Options	Opens a window for basic evaluation settings.
	Automatic threshold	Automatic determination of the fluorescence threshold value for Ct value determination.
	Import standard curve	Imports a saved standard curve.
	qBase Export	Exports measured values as a CSV or an XLS file in qBase format.
Evaluation/relative quantification		
	New	Creates a new evaluation.
	Delete	Deletes an evaluation.

	Options	Opens a window for basic evaluation settings.
	Automatic threshold	Automatic determination of the fluorescence threshold value for Ct value determination.
	Import standard curve	Imports a saved standard curve.
Evaluation/ΔΔCt analysis		
	New	Creates a new evaluation.
	Delete	Deletes an evaluation.
	Options	Opens a window for basic evaluation settings.
	Automatic threshold	Automatic determination of the fluorescence threshold value for Ct value determination
Evaluation/melting curve		
	New	Creates a new evaluation.
	Delete	Deletes the current evaluation.
	Options	Opens a window for basic settings for the evaluation.
	Automatic threshold	Automatically determines the threshold.
Evaluation/Genotyping		
	New	Creates a new evaluation.
	Delete	Deletes the current evaluation.
	Options	Opens a window for basic settings for the evaluation.
	Auto threshold/cut off	Automatically determines the threshold.
Evaluation/Endpoint		
	New	Creates a new evaluation.
	Delete	Deletes the current evaluation.
	Options	Opens a window for basic settings for the evaluation.
	Auto threshold/Cut off	Automatic determination of the cut-off fluorescence value for decision POS/NEG

1.4.3 Project explorer components

Different menus (2) in the project explorer offer a quick overview of the currently processed project. Individual projects can be selected via a selection list (1). The information on the individual menus can be displayed or hidden via the **[+]** and **[-]** (3) buttons.



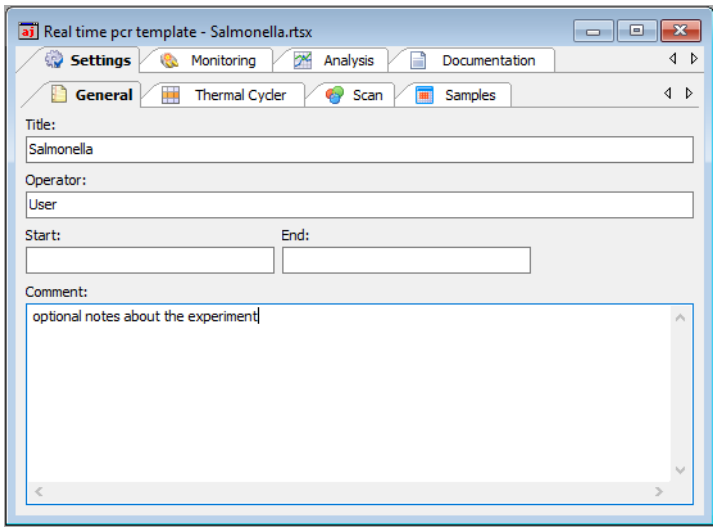
Organization of the project explorer

Menu	Information
General	Project title, user, date, timeand device
Thermocycler	Graphic display of the history of the PCR program in the active project
Scan	Overview of the colors and areas of the PCR plate that are being scanned
Samples	Displays a short info text on the plate layout. Used for activation or deactivation of samples during measurement and evaluation (→ sections "Display options for monitoring" p. 57 and "Activating/deactivating samples for evaluation" p. 67). The edit mode for the plate layout displays detailed information on the selected well.
Absolute quantification	Graphic display Ct against log concentration
Relative quantification	Graphic display Ct against log concentration
ΔΔCt	Graphical display dCt(V) compared to log concentration
Melting Curve	Graphical display of melting curve compared to temperature
GenoTyping	Graphical display of dRn Genotype 1 compared to dRn Genotype 2 as a scatter plot or bar graph
Endpoint	Graphical display of endpoint fluorescences as a bar chart for the GOI and the IPC

You can display or hide the project explorer via the **View ▸ Project explorer** menu command.

1.4.4 Project interface with project window

The project interface is initially empty when starting the program. Only when a new project is created or a saved project and/or a template is loaded the project window opens.



View of a project window

In the project window all parameters, measuring data and evaluations for one PCR plate are combined. The basic functions are arranged on the four main tabs on the top:

Tab	Function
Settings	Contains all functions required for defining Real-Time PCR runs.
Monitoring	Contains different tools for monitoring Real-Time PCR runs.
Evaluation	Includes the evaluation algorithms implemented in the software for analyzing acquired data.
Documentation	Opens the input mask for MIQE compliant documentation of Real-Time PCR experiments.

These four tabs are always visible. The view of the project window changes depending on the selection of a function tab. To indicate the tab the descriptions refer to, the tabs and list sheets are listed in the order in which they were activated and divided by a slash, e.g. **Settings / Thermocycler / Table**.

1.4.5 Help function

You can get help on the operation of qPCRsoft via the menu command **Help ▸ Content**.

While working with project windows, you can activate context-sensitive help by pressing function key **[F1]**.

The program pops up brief information (tool tips) on buttons of toolbar while you move the mouse pointer across the button.

1.4.6 Version number of the software

By means of the **Help ▸ Info** menu command, you open the window with information on the version number of the software program.

2 Managing projects and templates

The qPCRsoft software saves all experiments in the **project** files. A project contains different information required to perform a Real-Time PCR experiment:

- Description of the experiment
- PCR protocol
- Scan settings of the optical system
- Plate assignment with detailed information on each sample
- Measuring results and the corresponding evaluation after the experiment has been performed

All basic information required for performing an experiment that is stored in the project window on the **Settings** tab, such as the description of the experiment, the PCR protocol, scan settings of the optical system and the plate assignment, can be saved as a **template**.

Following file extensions are used in the software:

Extension	File type	Description
*.rtp	real time project file	Project file with settings of the PCR run, analysis and measurement results
*.rtpx	real time project file	File format for projects from qPCRsoft V 4.0
*.rts	real time settings file	Templates with settings of the PCR run and the analysis
*.rtsx	real time settings file	File format for templates from qPCRsoft V 4.0
*.mga	real time multigen analysis file	Multigene/Multiplate analysis
*.mgax	real time multigen analysis file	File format for Multigene/Multiplate analysis from qPCRsoft V 4.0
*.rta	real time analysis file	Export/import file format for analysis parameters of a project

The new data formats cannot read by older versions of the qPCRsoft. When saving, you can select in which format the files are tob e saved. To ensure the further use of the data, we recommend that you also save files from previous software versions in the new format.

2.1 Creating a new project or opening a project

A project is always indicated in a project window in a section of the project interface of the main window.

Creating a new project

To create a new project, click on or call up the **File ▸ New** menu command.

A new project with standard presets is created in the project window.

Creating a new project based on a template

A new project can be opened with saved presets (template):

1. Click on or call up the **File ▸ Open template** menu command.
2. In the standard window, select the desired template to open files and confirm the selection with **[OK]**.

A new project with the parameter settings of the template is created in the project window.

Opening a saved project

1. Click on or call up the **File ▸ Open project** menu command.
2. In the standard window, select the desired project to open files and confirm the selection with **[OK]**.

The project with the parameter settings, measurement results and evaluations is created in the project window.

Opening an automatically saved project

The qPCRsoft programs enables you to automatically save the last completed Real-Time PCR run to a folder of your choice and thus prevents data loss due to unexpected terminations of a PCR run.

1. Recovere the terminated measurement with the **File ▸ Open autom. saved project** menu command.
2. Save the file under a different name as a project file.

To change the storage location of the file, proceed as follows:

1. Select **Extras ▸ Options** to open the window of the same name.
2. Open the **General** tab.
3. Click **[...]** and select a storage location.

The **autom. save** option enables you to switch the automatic save function on and off.

Viewing the projects

You can open several projects at a time. Each project is displayed in its own project window. With the commands from the **Window** menu, the project windows can be arranged:

Command	Description
Tile horz	In the horizontal layout the project windows are shown below each other. Dependent on their number they are additionally arranged in two columns next to each other for more than 4 project windows.
Tile vert	In the vertical layout the project windows are shown next to each other. For more than 4 project windows they are additionally divided into two columns.
Cascade	In the cascading layout the project windows are placed on top of each other with an offset.

Changes can only be made in the respective active window.

2.2 Saving a template

All basic information required for performing an experiment that is stored in the project window on the **Settings** tab, such as the description of the experiment, the PCR protocol, scan settings of the optical system and the plate assignment, can be saved as a template.

1. Call up the **File ▸ Save template as** command.
2. To save files enter the name of the template in the standard window and save the template with **[OK]**.

The changes in a template can be saved with the **File ▸ Save template** menu command.

Optionally, you can click on the symbol in the toolbar.

2.3 Saving a project

You can save the project with all parameters of the PCR run, the fluorescence curves and evaluations.

1. Call up the **File ▸ Save project as** command.
2. To save files enter the name of the template in the standard window and save the template with **[OK]**.

The changes in a project can be saved with the **File ▸ Save project** menu command.

Optionally, you can click on the symbol in the toolbar.

In the window **Options / general** (menu command **Extras ▸ Options**) you can define how the file will be saved and how the file name is to be assigned:

- Automatically by date or name and an index number:
  The results of the PCR run are saved without user interaction. The file name consists of the date or the selected name and a three-digit index.
- Manually when stating the PCR run:
  The user is promted to enter the file name of the project when starting the project.
- Manually at the end of the PCR run (default):
  After the PCR run, the user is prompted to enter a file name of the project.

2.4 Importing/exporting analyses

Settings for the data evaluations of a project can be saved (exported) and later transferred (imported) to an open project. The evaluations are applied to the open project when they are imported.

1. Select the **File ▸ Export analyses** menu command.
2. To save files enter the name of the analysis and save the files with **[OK]**.

Analyses are saved with the extension "*.rta".

1. Select the **File ▸ Import analyses** menu command.

2. Select the name of the analysis in the default window for opening files and import the analysis into the current project by clicking **[OK]**.

2.5 Creating a project template from a transfer file

With the **File ▸ Import LIMS** menu command, a project template is created from a transfer file and a PCR run can be started (→ section "Appendix C – Creating a project template from the transfer file (LIMS)" S. 155).

2.6 Performing a Multigene-/Multiplate-Analysis

The **File ▸ MultiGene** menu command allows the analysis of experiments that required multiple PCR runs and that comprise multiple genes (→ section "Multigene-/Multiplate-Analysis" p. 116).

2.7 Closing project windows

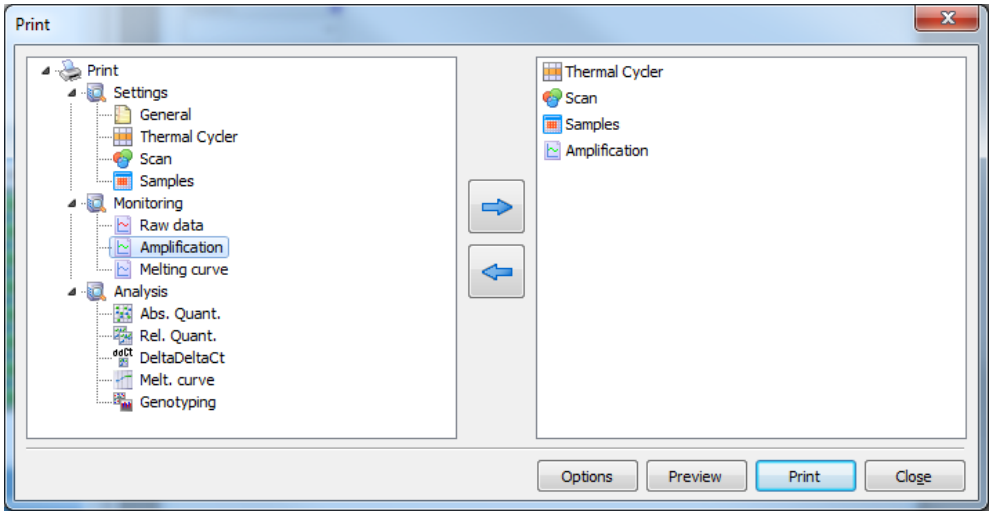
The **File ▸ close** menu command closes the active project window. To close all project windows, select the **File ▸ close all** menu command. If any unsaved changes have been made in project windows, a confirmation prompt appears.

2.8 Printing

You can specify the desired contents for printing a project in a selection list:

1. Select the **File ▸ Print** menu command.
2. Manage the print output using the displayed lists. Select the desired information in the list on the left and click to transfer it to the print list on the right. To remove undesired information from the print list, click .
3. Click **[Print]** to start the printout.

Select **[Options]** to configure the printout, or **[Preview]** to display a page view of the print image.



Print window

The individual print modules are sorted into the **Settings, Monitoring** and **Analysis** subgroups in the **Print** window.

3 Settings for a Real-Time PCR experiment

If you want to start a new project, create a new project or open a template:

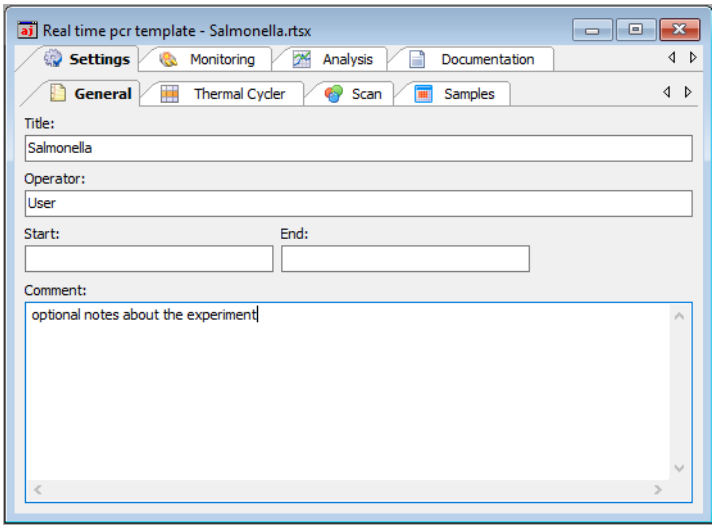
- Create an empty project with a click on in the toolbar.
- Optionally, open a template with to use the previously stored parameters for the new project or to modify them.

All functions necessary to create a new project are combined under the **Settings** tab. Additional tabs on the second level are assigned to the **Settings** tab:

Tab page	Function
General	Allows you to enter general information and remarks
Thermocycler	Used for programming PCR protocols
Scan	Definition of the colors to be measured and settings for the measuring parameters
Samples	Opens the sample table in which detailed information on each sample can be saved and groups of experiments defined

3.1 Entering general information on the project

You can save general information on each project. The entries can be made on the **General** tab:



Real-time PCR run – General information

Command	Description
Title	Analysis title
Operator	User If you are using a user management option, the user name you signed in with will be entered automatically.

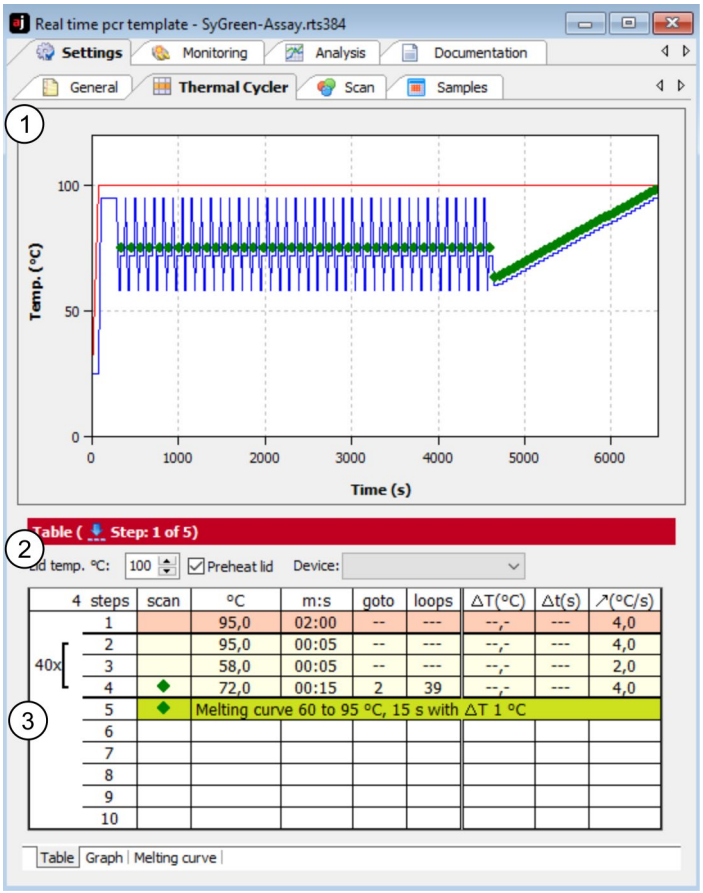
Start / End	Start and end of the qPCR run. Remains empty in a template.
Comment	Comment input

Note:

For text inputs on the tab **General** being able to use the usual commands for copying, cut out and insert of texts. These commands are arranged in the menu **Edit**.

3.2 Creating a PCR protocol

A PCR protocol must be programmed for each Real-Time PCR experiment. All necessary functions are combined in the **Settings / Thermocycler** project window. The screen is divided in three sections: graphical program preview (1), program header (2) and program table (3).



Program table for creating PCR protocols

The program preview illustrates the history of the PCR protocol. The program header defines the general conditions for the PCR protocol, such as the programmed lid temperature, the lid-heating mode and the thermocycler's block type. The programming table offers a clear representation of the individual steps of the program.

The **Thermocycler** tab contains three list sheets. The tabs for switching between the list sheets are located at the bottom of the window.

List sheet	Function
Table	Contains a table for programming PCR programs.
Graph	Offers the option for graphical programming of PCR programs.
Melting curve	Is used to enter parameters for measuring a melting curve.

Editing a PCR protocol

Programs can be edited in the tabular or graphical representation.

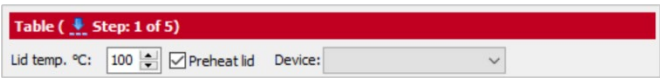
You can switch between the two alternative representations via the **Table** and **Graph** screens. To edit one step (the current active step is highlighted light red) you can use the corresponding function from the **Cycler** menu in the menu bar or the corresponding symbol from the toolbar:

Symbol	(Cycler/...) menu command	Description
	Add empty step	Inserts a new step behind the active step
	Delete step	Deletes the active step
	Cut step	Cuts the active step
	Copy step	Copies the active step
	Paste step	Inserts a copied step behind the active step

3.2.1 Entering information in the program header

The program header contains several general, device specific program settings:

- Options for lid heating
- Temperature control method
- Stand-by mode activation after the PCR is complete



Program header of the PCR program (example for qTower settings)

Option/List	Description
Lid temp.	Sets the lid temperature. The temperature of the heating lid should generally be slightly above the maximum block temperature to prevent liquids from evaporating from the reaction mixture and condensing at the walls or lid of the reaction cups.

Preheat lid	If activated, this option preheats the lid to the set **Lid temperature** before the actual PCR program starts. This is the recommended standard setting to ensure the formation of a homogeneously tempered air cushion between the sample containers. This leads to an improved temperature uniformity between the samples. While the lid is being heated, the block is kept at a constant 25°C. If the option is deactivated, the PCR program starts while the lid is still being heated. Adjustable lid temperature: 30 – 110 °C **Note:** After the heating lid has reached the target temperature, this is followed by a 40-second equilibration before the program is started.
Select block type (for TOptical)	The installed block module is automatically detected by the TOptical thermocycler during device initialization (with/without gradient function). When creating a new program this module is accepted as default. However, it is possible to create a program for a different block format than that currently installed (for example, a program with a temperature gradient although no block capable of a gradient has currently been inserted). The desired block type can be selected from the list.
Device selection	The installed qTOWER (with /without gradient function) is automatically detected during initialization. When creating a new program this type is accepted as default. However, it is possible to create a program for a different device type than that currently installed (for example, a program with a temperature gradient although no block capable of a gradient has currently been inserted). To do so, select the type from the devices list.

3.2.2 Overview about the PCR protocol

The PCR program is entered into the program table of the **Table** list sheet. One row of the table contains the parameters of a temperature step.

You can navigate in the program table by using the mouse or the four arrow keys **([←] [→] [↑] [↓])** on the keyboard. Each entry is confirmed with the **[Enter]** key or the **[→]** arrow key. The cursor jumps into the corresponding field in the adjoining column. If the cursor is in the last row, an additional temperature step is inserted with **[↓]**. Click in the next empty row for inserting an additional temperature step.

	4 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
	1		95,0	02:00	--	---	--,-	---	3,0
40x	2		95,0	00:05	--	---	--,-	---	3,0
	3		58,0	00:05	--	---	--,-	---	2,0
	4	◆	72,0	00:15	2	39	--,-	---	3,0
	5	◆	Melting curve 60 to 95 °C, 15 s with ΔT 1 °C						
	6								
	7								
	8								
	9								
	10								

Program table for a PCR run

The following values are entered into the table or calculated from the default values:

Value	Description

Scan	Measures the sample fluorescence during this step, if marked
Steps	Number of the step in the temperature program Is automatically numbered consecutively
°C	Enter the target temperature of the step in °C
m:s	Enter the hold time of the target temperature
goto / loop	Define the loop with the number of repetitions for a cycle
ΔT(°C)	Enter the increment or decrement of the target temperature within the PCR run
Δt(s)	Enter the increment or decrement of the hold time within the PCR run
↗(°C/s)	Enter the heating or cooling rate to reach the target temperature in the temperature step

3.2.3 Inserting a new temperature step / deleting a temperature step

- You can insert an additional temperature step to the program table with one of following functions:
  - Set the cursor in the last row of the temperature program and press the **[↓]** arrow key.
  - Select the menu command **Cycler ▸ Add empty step.**
  - Click on .
  - Click with the mous on the next row under the program.

  The total number of steps and the current processed step are displayed in the protocol header.

- To delete a program step move the cursor to the program row and click on .

  Optionally, select the **Cycler ▸ Delete step** menu command.

3.2.4 Entering the target temperature, hold time and heating/cooling rates

- Enter the target temperature of the temperature step into the **°C** column.
- Into the **m:s** column, enter the hold time in the format "minutes:seconds" (e.g. hold time of a duration of 1 min 20 s: 1:20).
- For special applications or the transfer of protocols from other devices it may be required to adjust the heating and cooling rates.
  Enter the average heating and cooling rate for each individual step in the **↗(°C/s)** column.

  Note:
  The value in the **↗(°C/s)** column defines the speed at which the target temperature is reached. If the temperature is be heated (or cooled) at a speed of 3°C per second between step 2 and step 3 the value 3.0 must be entered for step 3.

  Note:
  If the speed is to be reduced for the whole program the heating or cooling rates of all steps must be adjusted. The settings are only valid for that one program.

4 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
1		95,0	02:00	--	---	--,-	---	4,0
2		95,0	00:05	--	---	--,-	---	4,0
3		58,0	00:05	--	---	--,-	---	2,0
4	◆	72,0	00:15	2	39	--,-	---	4,0
5	◆	Melting curve 60 to 95 °C, 15 s with ΔT 1 °C						
6								
7								
8								
9								
10								

40x

Entering the target temperature, hold time and heating/cooling rates in the PCR table

3.2.5 Defining loops

Program sequences that are repeated regularly can be summarized in **loops**. Generally, a loop is then defined by a target step for the return ("**goto**" and the number or repetitions ("**loops**").

4 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
1		95,0	02:00	--	---	--,-	---	4,0
2		95,0	00:05	--	---	--,-	---	4,0
3		58,0	00:05	--	---	--,-	---	2,0
4	◆	72,0	00:15	2	39	--,-	---	4,0
5	◆	Melting curve 60 to 95 °C, 15 s with ΔT 1 °C						
6								
7								
8								
9								
10								

40x

Programming a loop in the PCR table

1. Place the cursor on the last step of the future loop (step 4 in the example above).
2. Enter the number of the target step into the **goto** column ("2" in the example above).
3. In the **loops** column enter the number of repetitions ("39" in the example above).

After entering the target step and the repetitions the programmed loop is displayed as a bracket on the left side of the table.

Note:
The total number of loops displayed in the bracket is determined from the number of programmed repetitions plus 1, as the corresponding sequence of steps prior to reaching the loop has already cycled once.

3.2.6 Entering increments/decrements for temperature and hold time

By programming **increments/decrements** the temperature or hold time can be modified by a specific amount from one cycle to the other within a loop.This technology is for instance used for the "Touch Down" PCR.

3 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
40x[1		95,0	02:00	--	---	--,-	---	3,0
2		95,0	00:05	--	---	--,-	---	3,0
3		68,0	00:15	2	39	-1,0	5	3,0
4	◆	Melting curve 60 to 95 °C, 15 s with ΔT 1 °C						
5								
6								

Entering increments for temperature and hold time

- Enter the desired changes for the temperature step the values of which you wish to modify within the loop. Use the (-) sign to specify a decrement, i.e. temperature or hold time are reduced from cycle to cycle by the specified amount. No sign or (+) mark a decrement with the result that the parameter increases by the specified amount from cycle to cycle.
- To modify the target temperature in steps, enter the changes in the **ΔT(°C)** column.
- To modify the hold temperature in steps, enter the changes in the **Δt(s)** column.

Note:
The modified step must be within a loop. Otherwise, the entries in the columns **ΔT(°C)** and **Δt(s)** have no effect.

Note:
The extension of the hold time of one step affects the total run time of a protocol. A program with many cycles and significant hold time increases takes substantially longer than a comparable program without a programmed extension.

3.2.7 Arranging a fluorescence measurement

- To define the measurement of the sample fluorescence in a temperature step of the PCR protocol, click on the **Scan** column of the temperature step. A green diamond (◆) indicates that the measurements is active.

4 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
40x[1		95,0	02:00	--	---	--,-	---	4,0
2		95,0	00:05	--	---	--,-	---	4,0
3		58,0	00:05	--	---	--,-	---	2,0
4	◆	72,0	00:15	2	39	--,-	---	4,0
5	◆	Melting curve 60 to 95 °C, 15 s with ΔT 1 °C						
6								
7								
8								
9								
10								

Defining a fluorescence measurement in the PCR run

Define the parameters for the fluorescence measurement on the **Scan** tab (→ section "Defining the parameters for the fluorescence measurement"p. 34).

Note:
If a step for melting curve determination is added, the scanning process is automatically activated for this step. For all other steps of the PCR protocol the allocation must be made manually.

3.2.8 Adding a melting curve

For experiments with intercalating dyes we recommend to check the specificity of the products by measuring a melting curve. The device can be programmed to add the corresponding step in the PCR protocol. Activate the option **active** on the **Melting curve** tab.

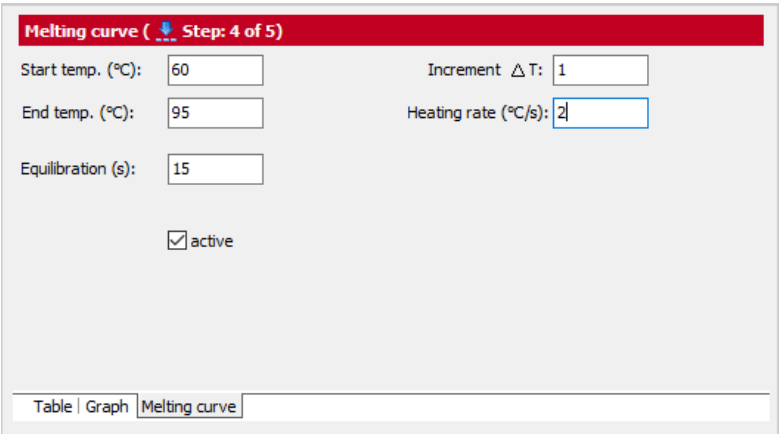
4 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
1		95,0	02:00	--	---	--,-	---	4,0
2		95,0	00:05	--	---	--,-	---	4,0
3		58,0	00:05	--	---	--,-	---	2,0
4	◆	72,0	00:15	2	39	--,-	---	4,0
5	◆	Melting curve 60 to 95 °C, 15 s with ΔT 1 °C						
6								
7								
8								
9								
10								

40x

PCR protocol with a step for determining the melting curve

- A melting curve can be added to the program table by activating the option **active** on **Melting curve** tab.

  The melting curve is added to the last temperature step in the table.
- To remove a melting curve from the program table deactivate the option **active** on melting curve tab.
- Set the individual parameters of the melting curve step on the **Meltingcurve** tab:



DNA melting curve screen

The following parameters can be modified:

Parameter	Description
Start temp. (°C)	Start temperature of the melting curve
End temp. (°C)	End temperature of the melting curve
Equilibration (s)	Time for equilibration of the sample in a temperature before a measurement is performed
Increment ΔT	Difference between two adjoining temperature steps in °C
Heating rate (°C/s)	Heating rate of the block
active	Add a melting curve on the end of the PCR protocol.

The fluorescence measurement is automatically defined when the melting curve is recorded.

Note:
If you want to record a melting curve without previous amplification you must add a separate step prior the melting step in the cycler program. This step must bring the block temperature to the start temperature of the melting curve and a fluorescence measurement must be performed. Recording a melting curve without at least one previous temperature step is not possible.

3.2.9 Programming the temperature gradient (for devices with gradient function only)

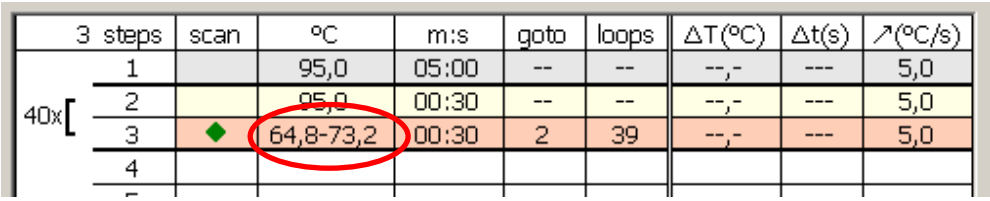
The gradient function is for following devices available:

- TOptical 96 Gradient
- qTOWER 2.2
- qTOWER³ G

Gradients can be programmed over the whole temperature range of the thermoblock between 3.0 °C and 99 °C. The gradient range can be max. 40 °C.

Temperature gradient with margin rows

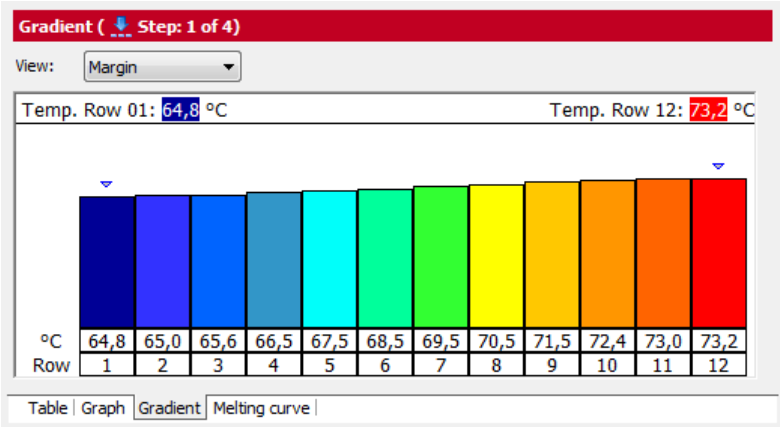
- Gradients are defined in the program table by entering two temperature values separated by a dash. The first value entered corresponds to the temperature in column 01 (left block side), the second value to the temperature in column 12 (right block side).



	3 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
40x[	1		95,0	05:00	--	--	--,-	---	5,0
	2		95,0	00:30	--	--	--,-	---	5,0
	3	◆	64,8-73,2	00:30	2	39	--,-	---	5,0
	4								

Entering the temperature gradient

The progression of the temperature gradient can be reviewed on the **Gradient** tab. The temperatures in the individual columns of the block are summarized in a table below the bar graph.



Gradient tab with Margin gradient function

- Adjust the gradient progression by entering temperature values for the first (**Temp. Row 01**) and last (**Temp. Row 12**) column of the block.

  The values displayed in the table are then updated.

- Alternatively you can click on the top end of column 1 or column 12 and change the height of the column with the left mouse button depressed. Moving the columns changes the respective temperature value accordingly.

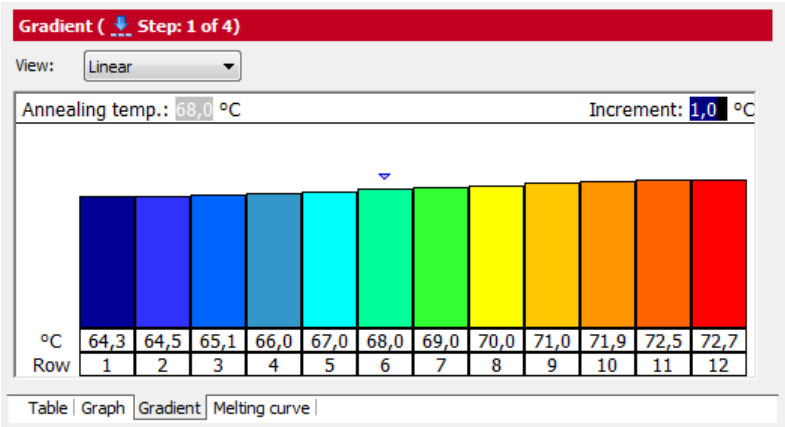
Programming a linear gradient

In addition to the option of defining a gradient by entering temperature values for columns 1 and 12, the gradient can also be programmed starting with an annealing temperature in the center of the block using fixed temperature increments.

- On the **Gradient** tab, select the **Linear** option from the **View** list.
- Enter the temperature for column 6 in the **Annealing temp.**field and the desired temperature change from column to column in the **Increment** field.

  The values displayed in the table are updated after the values have been entered.

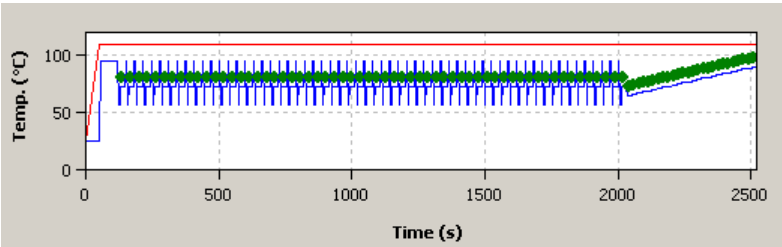
- Another option for adjusting the gradient is to click on the top end of column 6 and change the height of the column with the left mouse button depressed. Moving the column the changes the annealing temperature accordingly.



Programming a linear gradient

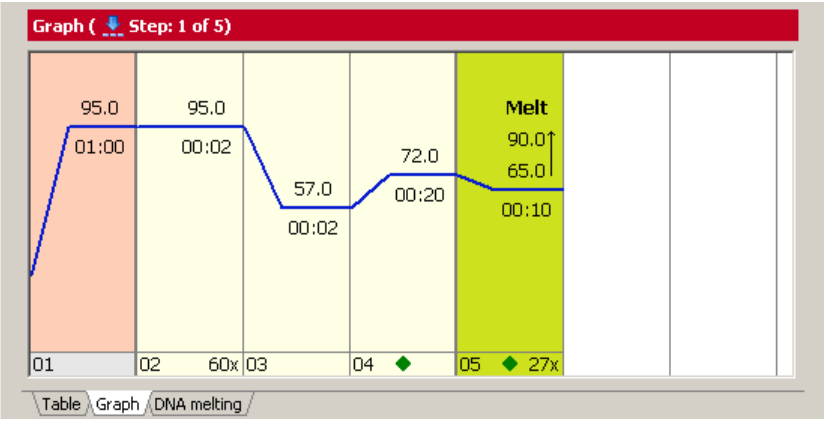
3.2.10 Graphical display and programming the PCR protocols

In the **Settings / Thermocycler** project window, the history of a programmed PCR protocol is displayed graphically in the **Table** and **Graph** screens. It represents the temperature curve of the block (blue line) and the heating lid (red line) over time. The green diamond symbolizes steps during which the fluorescence is measured.



Graphical representation of a PCR protocol

Programs are generally created in the table view, which allows new steps to be programmed quickly and provides a summarized overall survey of the protocol structure. Some programming options are only available in the table view. The graphical programming mode additionally offers a schematic representation of the temperature profile and the option to adjust protocols quickly. In the **Settings / Thermocycler** project window you can switch between the table view and the graphical mode via the **Table** or **Graph** sheets.



Graphical programming mode

The graphical programming is generally performed in the same way as in the programming table.

- By selecting a step (clicking on it) it becomes active and is highlighted light red.
- The bottom part of the display shows the number of the corresponding protocol step. Next to it, the number of repetitions in loops (right) and scanning processes (middle) can be programmed in this field.
  The number of repetitions is indicated as a figure (e.g. 40x) and can be edited by clicking on it with the left mouse button.
  Planned measurements are displayed by means of a green diamond in the middle of the field and can also be selected or deselected with the left mouse button.
- Temperatures and hold times are indicated as numerical values above or below the blue line that displays the corresponding temperature level at the individual steps. The values can be modified by clicking with the left mouse button. Melting curve steps are marked with the addition **Melt.** Additionally, a symbol of an arrow pointing upwards is displayed with the melting curve steps (↑).

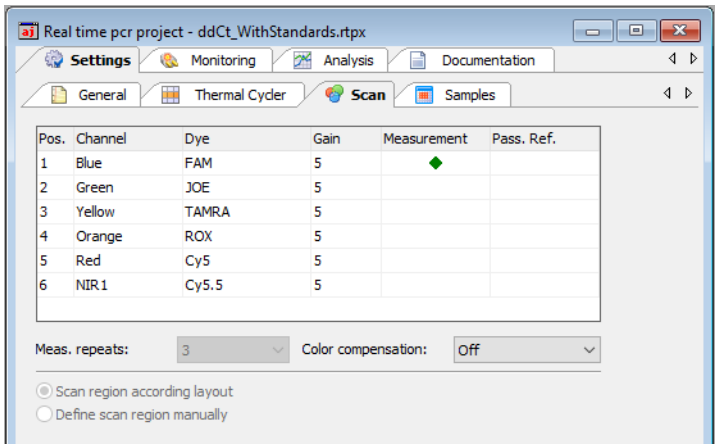
  Note:
  The number of repetitions in melting curve steps cannot be modified.
- The temperature value at each step can be modified using the mouse. For this purpose, press and hold the left mouse button to move the blue line of the temperature curve up or down.

3.3 Defining the parameters for the fluorescence measurement

The product accumulation is measured by means of the increase in fluorescence in the Real-Time PCR. The following measurement parameters must be defined for that purpose:

- Dyes to be measured
- Temperature step of the PCR protocol during which a measurement is to take place
- The area on the PCR plate that is to be scanned

The colors to be measured are defined in the **Settings / Scan** project window:



Project window with settings for the fluorescence measurement

Up to six color channels with different excitation and detection wavelengths can be used for the fluorescence measurement. The parameters of the fluorescence measurement apply for all layout samples on which a measurement is to be performed.

The **Scan** tab contains a table with different parameters for defining the scan properties:

Table parameters	Description
Pos.	Color module position in the device
Channel	Color channel description
Dye	Define the dye to be measured for the corresponding channel in the table by means of a selection list
Gain	Regulation of signal intensity The signal intensity can be adjusted in steps between 0 and 10. The higher the value, the higher the fluorescence signal in the corresponding channel. Standard value: 5
Measurement	Activates dye measurement An active measurement is indicated with a green diamond (◆)
Pass. Ref.	The LED technology of the device does not require a passive reference. If you wish to measure a reference dye anyway, place a checkmark in this column.

The information on position, channel and the selection list of the available dyes cannot be modified in this table. Modifications of these general settings can only be made in the **Edit color modules** window (→ section "Editing color modules" p.133).

Following lists and options are available on **Scan** tab:

Option	Descritption
Meas. repeats	Enter the number of repetitions of the fluorescence measurement Possible values: 1 to 16
Color compensation	Activate spectral compensation (→ section "Color compensation" p. 37).
Scan region according layout	Sample measurement according to the layout of the samples on the **Samples**tab (→ section "Editing the sample table" p. 39).
Define scan region manually	Sample measurement according to manual settings (→ section "Manually defining the scan region" p. 36).

Set the following parameters for each channel you wish to make a measurement for:

1. Select the dye to be measured in the **Dye** column. Click in the cell and mark the dye in the list that opens up.

   Note:
   The number of measured dyes does not have an influence on the scan time.

2. Set the signal quality in the **Gain** column.
   The standard setting is "3".

3. Activate the fluorescence measurement in the channel in the "Measurement" column by placing a green diamond (◆).
   Channels that are not marked with a diamond will not be measured.

4. If necessary, activate the reference dye measurement by placing a (✓) in the **Pass. Ref.** column.

5. Enter the number of repetitions for the fluorescence measurements in the **Meas. repeats** field.

   Note:
   An increased number of repeat measurements reduces the measurement value distribution but also creates longer scanning times and thus longer protocolling times.

6. Select one of the options for the **scan region** (**manuallyor according to layout**).

3.3.1 Manually defining the scan region

The scan region can be defined according to the plate layout in the sample table (→ section "Editing the sample table" p. 39) or manually. The scan region for the thermocycler is always defined per column. It must always consist of connected columns.

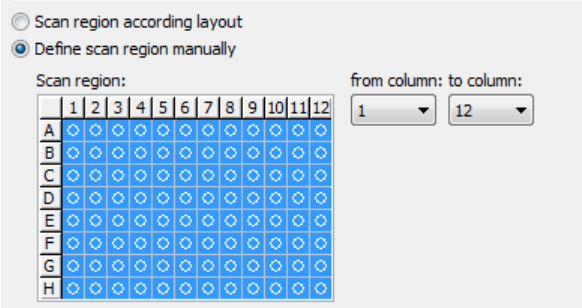
1. For a manual sample selection, select the **Define scan region manually** option on the **Settings / Scan**tab.

2. A graphical representation of the sample block is opened.

3. Enter the first and last column of the region to be scanned into the **From column** and**To column** fields.

   Optionally, you can use the mouse to select the columns. To select an individual column click directly into that column. If you wish to select several columns, press and hold the left mouse button and move the cursor over the corresponding area.

   Active columns are highlighted blue in the diagram.



Manual adjustment of the scan region

3.3.2 Color compensation

If you are using several dyes per reaction mixture the result may be a fluorescence crosstalk. This means that a second dye is excited and measured next to the desired dye at the same time. To subtract the fluorescence quotient of the second dye, you can use the color compensation function on **the Settings / Scan** project tab.

qPCRsoft offers two different variants for color compensation on measurement data:

- Using the standard color compensation **Standard1** or **Standard 2**
  Test which of the two compensations gives the best result. If the standard compensations do not give a satisfactory result, you will need to record a custom color compensation.
- Recording und selection of a customer-specific color compensation **Selection**

Color compensation: Off

The standard setting for the color compensation is **Off**. This is due to the fact that color compensation is not required for the most common applications (only one active measuring channel or dyes that are spectrally widely spaced, such as FAM and ROX).

Color compensation: Standard

Bei der Standard-Farbkompensation wird eine Kompensationsmatrix auf die Messdaten angewendet, die bei der Gain-Einstellung "5" in allen Farben eine ausreichende Kompensation des Übersprechens ermöglicht.

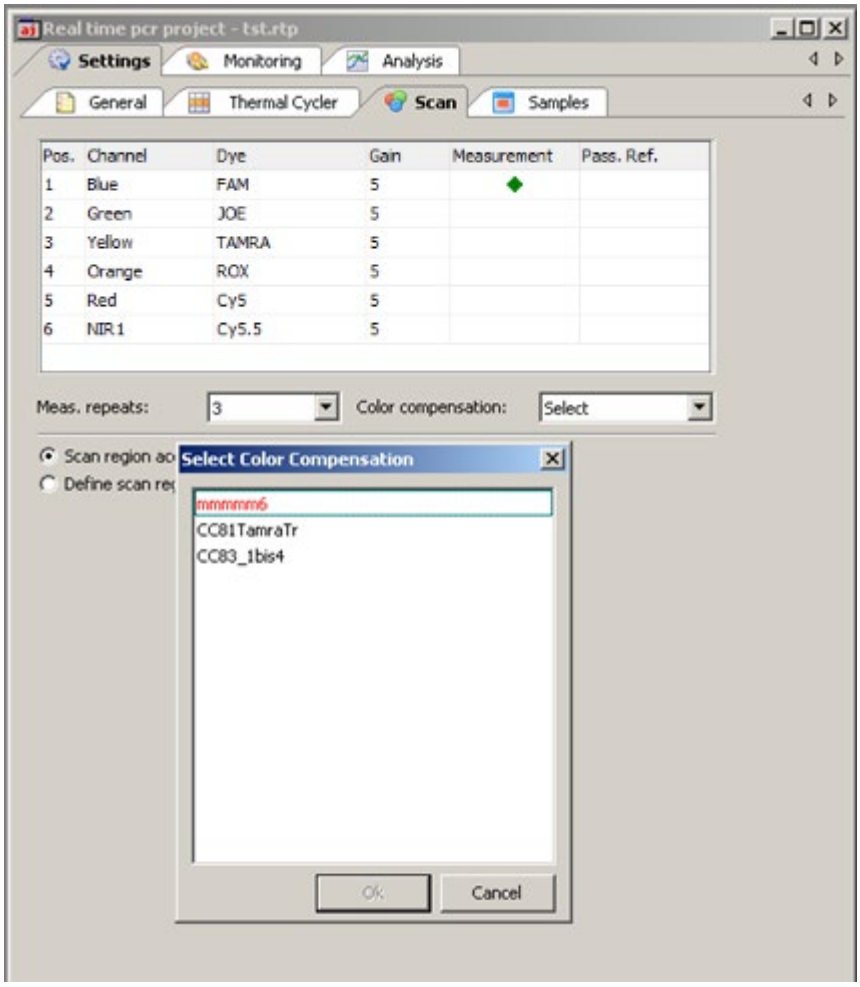
Using the standard color compensation, a compensation matrix is applied to the measurement data whichpermits a sufficient compensation of the crosstalk in all colors in the case of the gain setting "5".

Select S**tandard** from the **Color compensation** list for using the standard color compensation.

Color compensation: Selection

A better compensation is attained when a compensating matrix is recorded with the used dyes (→ "Spectral calibration" below).

Select **Select** from the **Color compensation** list for using this color compensation. A window appears, in which color compensations that have already been recorded can be opened and used again. In the window only those compensation data are printed in black color which meet the settings on the card **Scan**. Invalid compensation data appear in red color and cannot be selected.

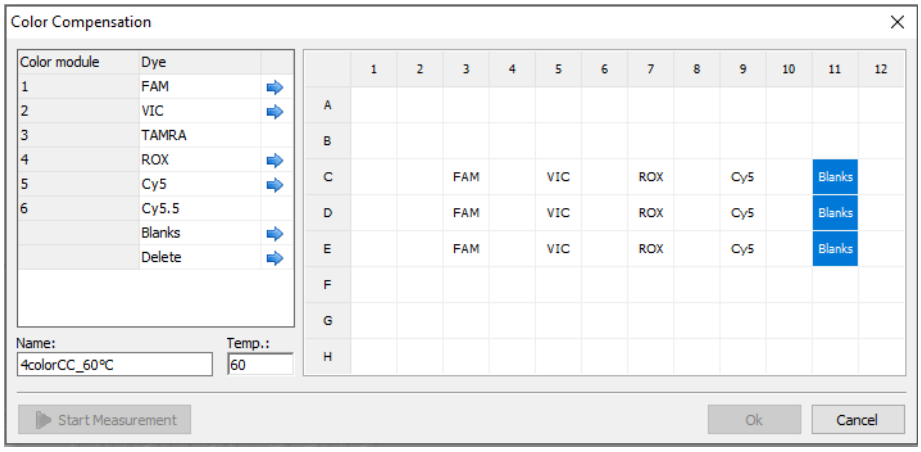


Window for selection of color compensation data

Select a color compensation for the current project with the left mouse button and press **[OK]** to activate that compensation.

Spectral calibration

With the symbol or the menu command **Scan ▸ Edit color compensation**, you can create a new color compensation by measurement. This process is called spectral calibration. A new window opens, in which all the required settings can be made. The window is divided in a selection list for dyesand a plate diagram.



Color compensation for spectral calibration window

To record the calibration data the dyes required for a color compensation must be available individually in solution. You could for example use the sensors for the calibration measurement that are to be used in the later PCR experiment. The dye concentration for the calibration measurement should be approx. 0.1 µmol/l.

In the displayed plate diagram the wells that contain the calibration samples are now marked individually for each dye and the dye in each of the samples is assigned to the marked well by clicking on the blue arrow. The dyes offered for selection are those that were selected in the **Settings / Scan** project tab.

For an exact calibration measurement we recommend that each dye be created at least as a triple replicate. It is also recommended to use blanks, i.e. samples which do not contain dyes. Blanks can be either your NTC samples or wells filled with buffer solution. In addition, the temperature at which the calibration measurement should be performed can be set. This should be the same temperature at which the fluorescence measurements are performed during the PCR run.

The calibration measurement is started by clicking on **[Start measurement]**.

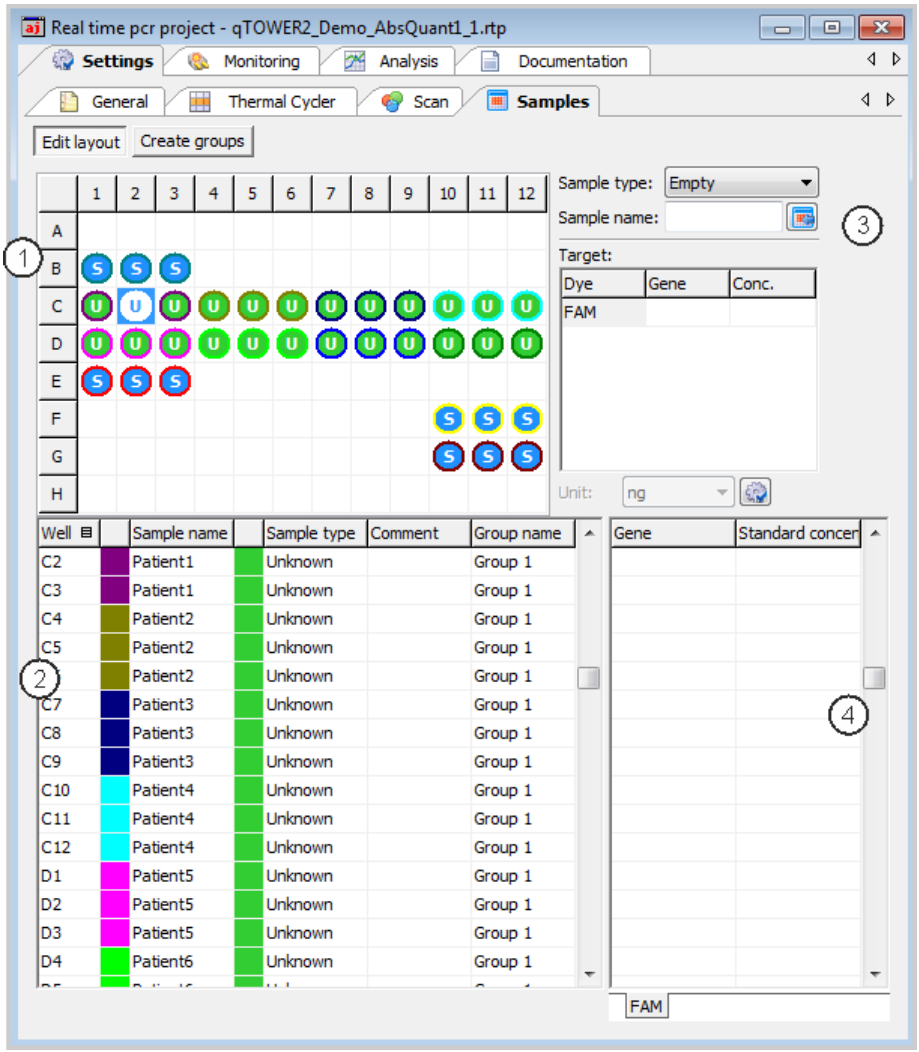
Note:
The selection of available dyes cannot be modified in this table. Modifications can only be made on **Scan** tab.

The new color compensation needs to be assigned a description in the name field. It is included in the selection list after pressing **[OK]** and will be displayed in the corresponding window. Templates that are no longer in use can be deleted with **[Delete]**.

3.4 Editing the sample table

The sample table defines which sample is in which position of the block. These details are required for using the evaluation functions of qPCRsoft. Here, a sample can be described by means of its properties, such as name, gene, type, concentration and dye. Furthermore, samples from different experimental approaches can be combined in groups.

The necessary entries can be made on the **Samples** tab after pressing the **[Edit layout]** button. The corresponding window is divided into different sections:



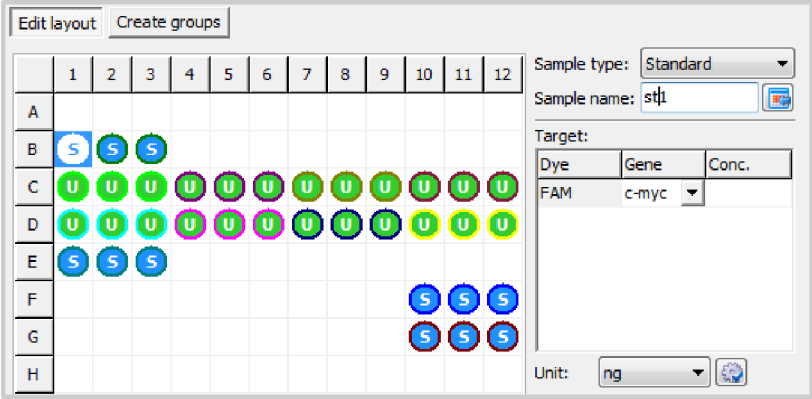
Window for editing the sample table

Range	Function
Layout view (1)	Graphical display of the well assignment on the microplate
Edit area (3)	Edit area for the sample properties: ▪ Sample name ▪ Sample type ▪ Concentration of standard samples ▪ Allocation of the dye and the analyzed gene
Sample table (2)	Summary of the information on each sample
Dyes (4)	Dyes and assigned genes for each sample

Note:
The sample table can also be edited after the Real-Time PCR run has been completed.

3.4.1 Entering sample properties in the layout

You can define the properties for the samples in the wells on the **Settings / Samples** project tab in the layout view and the edit area next to it.



Layout view for the sample type

Note

The color code of the inner circle that defines the different sample types and the color code of the outer ring that defines replicates can be modified in menu **Extras ▸ Options ▸ colors**.

The following sample types can be defined:

Sample type	Symbol	Definition
Empty		Describes an empty position on the PCR plate
Unknown	U	Sample of unknown concentration or dilution (measuring sample)
Standard	S	Sample of known concentration or dilution
Calibrator	K	Sample the target gene expression level of which is set to 1
No template control (NTC)	N	Complete reaction mixture but without template strand
Positive control	+	Positive control assay for which a reaction product can be expected
Negative control	-	Negative control assay for which no reaction product is to be expected

Samples with identical sample properties (sample name, sample type, same gene-dye-assignments) are viewed as replicates. For a better overview replicates are indicated by the same color code of the outer ring.The individual values of these samples are averaged and their mean value is used for the remaining calculations.

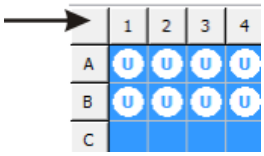
With a singleplex assay, samples can have the same sample name and sample type but differ as far as the gene-dye-assignment is concerned. These samples are identified as associated samples due to the same name. The evaluation, however, is performed individually.

- Mark the sample position to be edited by clicking into the layout. To select adjacent positions press the left mouse button and define an area. To select non-adjacent

positions press the Ctrl key on the keyboard plus the left mouse button at each position.

To mark rows or columns, click on the corresponding row or column name [A-H] or [1-12].

You can mark all sample positions in the layout by clicking on the gray button in the top left of the layout (between 1 and A1).



Button for marking the complete layout

- Enter the following sample parameters in the adjacent edit area:

Parameter	Description
Sample name	Sample description
Sample type	Selection of the sample type (see top of table)
Target table	
Gene column	In the dye row, enter the gene to be analyzed
Conc. column	For standards Enter the concentration of the gene to be analyzed.

- You can assign sample properties to the marked positions by clicking on or by pressing ENTER.
  For emptying on ore more wells, press the DEL key.

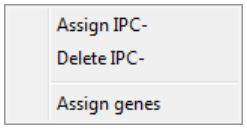
  Note:
  The entries for the selected area will only be applied by the program after they have been assigned. Entries or modifications that are not assigned will be lost.

- To display defined sample properties in the edit area, double-click on a well.

  You can edit the information and assign them to the well again by clicking or by pressing ENTER.

  To assign these sample properties to other wells, mark the desired wells and then click or press ENTER.

- By using the context menu it is possible to assign gene names to the selected wells afterwards without changing the other properties which had already been assigned. The context menu can also be used to assign and to remove sample wells which do not contain an internal positive control (IPC-). The context menu is displayed by right clicking the PCR plate scheme.



Context menu for assigning gennames and ICP- samples

The entry of a sample layout is described by means of an example for a singleplex assay and a multiplex assay (→ sections "Entering a sample layout for a singleplex assay" p. 46 and "Entering a sample layout for a multiplex assay" p. 44).

3.4.2 Entering sample properties into the sample table

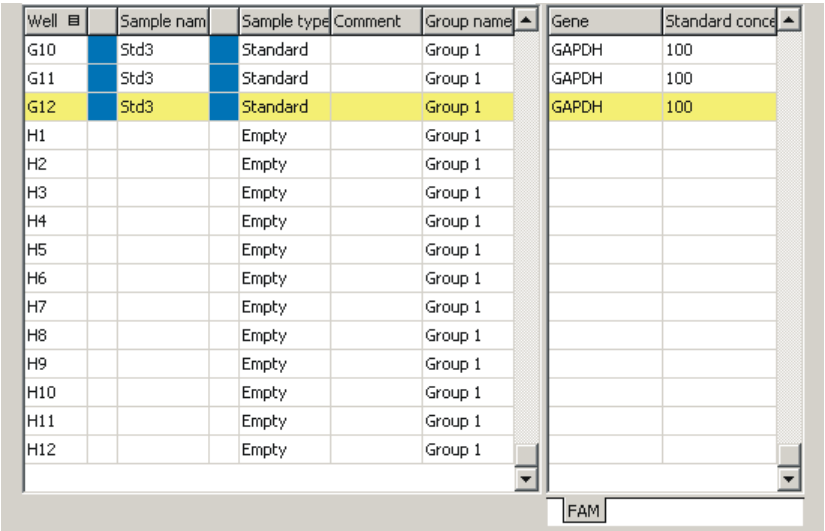
You can also make entries in the sample table itself.

- Select the desired position in the layout view or a field directly in the sample table.

  The corresponding row is then highlighted yellow.

- Enter descriptions and/or values directly in the designated cells.

  The sample table is edited cell by cell. Multiple selections and the associated assignment of parameters to several cells or rows at a time are not possible.



Well		Sample nam		Sample type	Comment	Group name	Gene	Standard conce
G10		Std3		Standard		Group 1	GAPDH	100
G11		Std3		Standard		Group 1	GAPDH	100
G12		Std3		Standard		Group 1	GAPDH	100
H1				Empty		Group 1		
H2				Empty		Group 1		
H3				Empty		Group 1		
H4				Empty		Group 1		
H5				Empty		Group 1		
H6				Empty		Group 1		
H7				Empty		Group 1		
H8				Empty		Group 1		
H9				Empty		Group 1		
H10				Empty		Group 1		
H11				Empty		Group 1		
H12				Empty		Group 1		

FAM

Sample table for entering sample properties

The genes and, in the case of standard samples, their concentration are summarized separately by dye in the second part of the sample table. A list sheet is assigned to each dye. It is thus possible to use different standard concentrations for each gene. The number of displayed sheets depends on which dyes have been activated on the **Settings / Scan** project tab for this measurement.

- Enter for each gene the desired standard concentration.

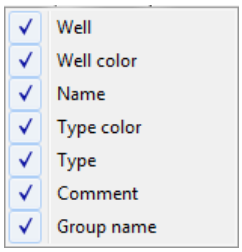
Selecting the table view

Depending on the selected window size and the number of dyes to be measured, two buttons with left and right arrows appear. They can be used to move between the different tabs.

The number of columns that are displayed in the sample table can be user-defined:

- Right-click on a column header to display and mark the desired columns in the context menu.

- The order of the columns can be changed by clicking on the table header and dragging with the mouse.



Selection field for defining the columns displayed in the sample table

Selecting curve colors

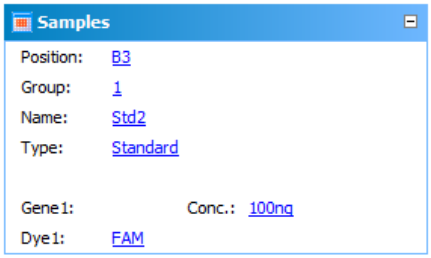
Note: The general color settings can be made in the **Options / Color** window (**Extras ▸ Options** menu command). This is where the colors for wells, replicates and sample types are determined.

In the graphic representation, the amplification curve is highlighted in the color shown in the 2nd column of the sample table. You can change the color individually:

- Double-click on the color cell to be changed in the sample table and select the desired color in the **Color** window.
- In order to reset the color of a well, press the Shift key (⇧) and double-click on the color cell at the same time. The color is reset to the default setting in the **Options / Color** window.
- To allocate the same color to multiple wells, press the Ctrl key and double-click on the color cell of a well.
  Mark the applicable wells in the plate layout in the **Edit colors** window and select the shared color. Click **[Accept]** to assign the color to the wells. Click **[Reset]** to reset the color changes in the marked wells to the default settings in the **Options / Color** window.

Displaying sample properties in the project explorer

Moving the mouse pointer over a well on the **Sample** project tab in the layout view will display the properties of the well in the **Samples** menu item in the project explorer.



Project explorer Samples window showing the well properties

3.4.3 Entering a sample layout for a multiplex assay

The following example shows the definition of four samples and standards with three repeat measurements each in the layout. The GAPDH gene is analyzed with the FAM dye and the c-myc gene with the VIC dye. The two dyes are selected on the **Settings / Scan** project tab and activated for measurement. The indicated sample names and standard concentrations serve as examples only.

1. Activate the **[Edit layout]** button.

Emptying sample layout

2. Empty the plate layout to make sure that no unintentional entries remain:
   - Mark the complete plate by clicking on the gray button in the top left of the layout.
   - In the **Sample type** list select the option **Empty**.
   - Click on or press ENTER.

Defining samples

3. Mark the three wells A1 - A3.
4. Make the following settings:

Parameter	Entered value
Sample name	Sample 1
Sample type	Unknown
FAM	GAPDH
VIC	c-myc

Note:
The genes are allocated to the corresponding dye by entering the name of the gene or by selecting it from the displayed list in the **Target** table.

5. Click on or press ENTER, to assign the sample properties to the three wells.
6. Repeat steps (3) - (5) for the other samples.
   Use the following parameters:

Wells	Sample name	Sample type	FAM	VIC
A4 – A6	Sample 2	Unknown	GAPDH	c-myc
B1-B3	Sample 3	Unknown	GAPDH	c-myc
B4-B6	Sample 4	Unknown	GAPDH	c-myc

Defining the standard samples

7. Mark the three wells C1 - C3.
8. Make the following settings:

Parameter	Entered value
Sample name	Std 1
Sample type	Standard

9. Make the following entries in the **Target** table:

Dye	Gene	Conc.
FAM	GAPDH	100
VIC	c-myc	50

Note:
The **Conc.** column in the **Target** table is only available for the **Standard** sample type.

10. From the **Unit**list, choose a concentration or mass unit. You can select from the following units:ng, ng/µl, ng/ml, pg/µl, copies, copies/µl, copies/ml, mg/ml, IU/µl, IU/ml or %.

11. Click on or press ENTER, to assign the sample properties to the three wells.

12. Repeat steps (7) - (10) for the other three standards.
    Use the following parameters:

Wells	Sample name	Conc.	
		FAM	VIC
C4 – C6	Std 2	50	5
D1 - D3	Std 3	10	1
D4 - D6	Std 4	0.1	0.05

- As sample type, select the **Standard** option.
- Assign the genes to the dyes as described in step (6).

✓ The plate layout for a multiplex assay is complete.

3.4.4 Entering a sample layout for a singleplex assay

The following example shows the definition of four samples and four standards with three repeat measurements each in the layout. The GAPDH and c-myc genes are analyzed with the FAM dye with the help of two sensors. The FAM dye was selected on the **Settings / Scan** project tab and activated for the measurement. The indicated sample names and standard concentrations serve as examples only.

1. Activate the **[Edit layout]** button.

Emptying sample layout

2. Empty the plate layout to make sure that no unintentional entries remain:
   - Mark the complete plate by clicking on the gray button in the top left of the layout.
   - In the **Sample type** list select the option **Empty**.
   - Click on or press ENTER.

Defining samples

3. Mark the three wells A1 - A3.
4. Make the following settings:

Parameter	Entered value
Sample name	Sample 1
Sample type	Unknown
FAM	GAPDH

5. Click on or press ENTER, to assign the sample properties to the three wells.
6. Mark the three wells A4 - A6.
7. Make the following settings:

Parameter	Entered value
Sample name	Sample 1
Sample type	Unknown
FAM	c-myc

8. Click on or press ENTER., to assign the sample properties to the three wells.
9. Repeat steps (3) - (8) for the other three samples.
   Use the following parameters:

Wells	Sample name	Sample type	Gene /FAM
B1-B3	Sample 2	Unknown	GAPDH
B4-B6	Sample 2	Unknown	c-myc
C1-C3	Sample 3	Unknown	GAPDH
C4-C6	Sample 3	Unknown	c-myc
D1-D3	Sample 4	Unknown	GAPDH
D4-D6	Sample 4	Unknown	c-myc

Defining the standard samples

10. Mark the three wells E1 - E3.
11. Make the following settings:

Parameter	Entered value
Sample name	Std1
Sample type	Standard

12. Make the following entries in the **Target** table:

Dye	Gene	Conc.
FAM	GAPDH	100

Note:
The **Conc.** column in the **Target** table is only available for the **Standard** sample type.

13. From the **Unit** list, choose a concentration or mass unit. You can select from the following units:ng, ng/µl, ng/ml, pg/µl, copies, copies/µl, copies/ml, mg/ml, IU/µl, IU/ml or %.
14. Click on or press ENTER, to assign the sample properties to the three wells.
15. Repeat steps (10) - (13) for the other standards.
    Use the following parameters:

Wells	Sample name	Gene	Conc.
E4 – E6	Std. 1	c-myc	100
F1 – F3	Std. 2	GAPDH	75

F4 – F6	Std. 2	c-myc	75
G1 – G3	Std. 3	GAPDH	50
G4 – G6	Std. 3	c-myc	50
H1 – H3	Std. 4	GAPDH	10
H4 – H6	Std. 4	c-myc	10

16. Click on or press ENTER, to assign the sample properties to the three values.

Note:
Connected samples must have the same sample name.

✓ The plate layout for a singleplex assay is complete.

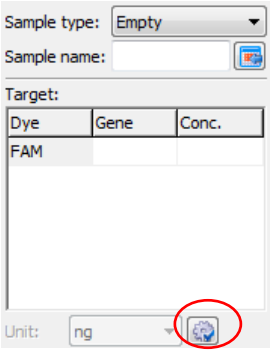
3.4.5 Automatic generation of dilution series/replicates

If in an experiment dilution series or replicates are measured, the layout creation can be automated.

1. Mark the well at which the dilution series or the sample replicate sequence should start (start at well) or mark an area on the PCR plate for the dilution series or replicates.

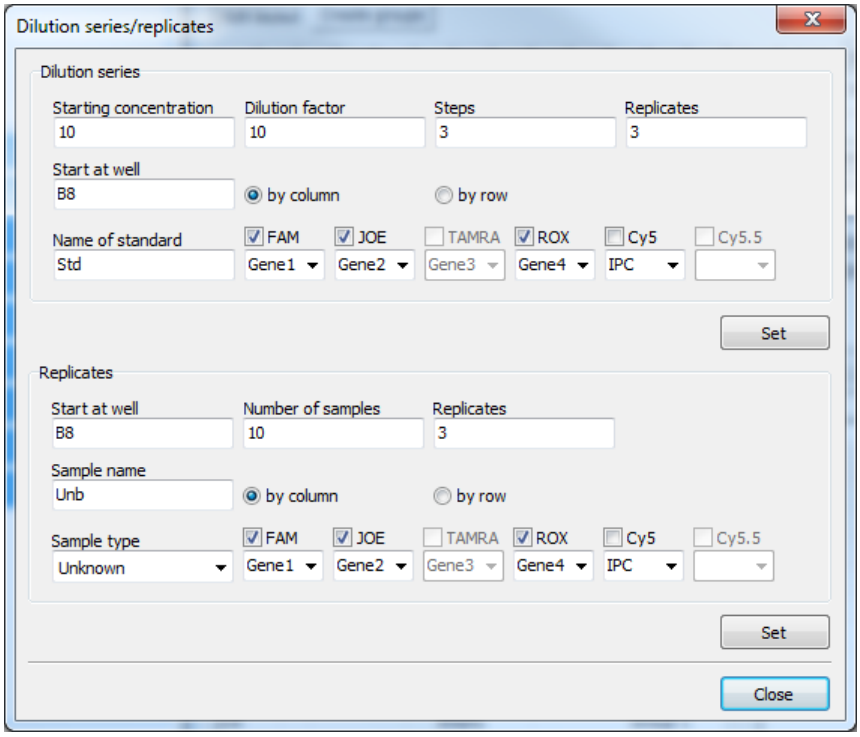
   If no area is preselected, the plate is always automatically filled to the rims.

2. Click on the button on the **Settings / Samples** project tab in the layout view.



Button for automatic creation of dilution series or replicates

In the window **Dilution series/replicates** parameters for automatic dilution series and replicates can be defined.



Window for creation of automatic dilution series/replicates

Creating a dilution series

1. The starting concentration, the dilution factor and the number of dilution steps and replicates.
2. Specify the start point (**Start at** well) and select whether the entry in the layout table should be done line by line or column by column.

   For determination of the start point, the software automatically takes over the currently active position in the layout or the first position at the upper left from a group of selected wells. Alternatively, the start point can also be defined by manual entry in the corresponding field.
3. Enter a name for the standard sample (**Name of standard**) and assign dyes to be measured (multiple selections are allowed). The standard name for each replicate is added by one digit (GAPDH1, GAPDH2, etc.).
4. Activate the dyes for which dilution series should be created. Select the names genes for the standards. A separate treatment of dyes (targets) is possible in such a way.
5. Click on **[Set]**.

   ✓ The dilution series is created automatically by the software and the corresponding data is displayed in the layout and the sample table.

Creating replicates

1. Enter the start point (**Start at well**) and the **Number of samples** and the **Number of replicates**.

   For determination of the start point, the software automatically takes over the currently active position in the layout or the first position at the upper left from a group of selected wells. Alternatively, the start point can also be defined by manual entry in the corresponding field.

2. Enter a **Sample name** and select whether the entry in the layout table should be done line by line or column by column. The sample name for each replicate is added by one digit (Test1, Test2, etc.)
3. Specify the **Sample type**.
4. Activate the dyes for which replicates should be created. Select the names genes for the samples. A separate treatment of dyes (targests) is possible in such a way.
5. Click on **[Set]**.
   ✓ Replicates are created automatically by the software and the corresponding data is displayed in the layout and the sample table.

3.4.6 Defining groups

Several experiments can be performed on one microplate at the same time. The samples that are part of one experiment are combined in a group. A group contains a number of reaction mixtures that will be evaluated together later on. You can define a maximum of 12 such groups.

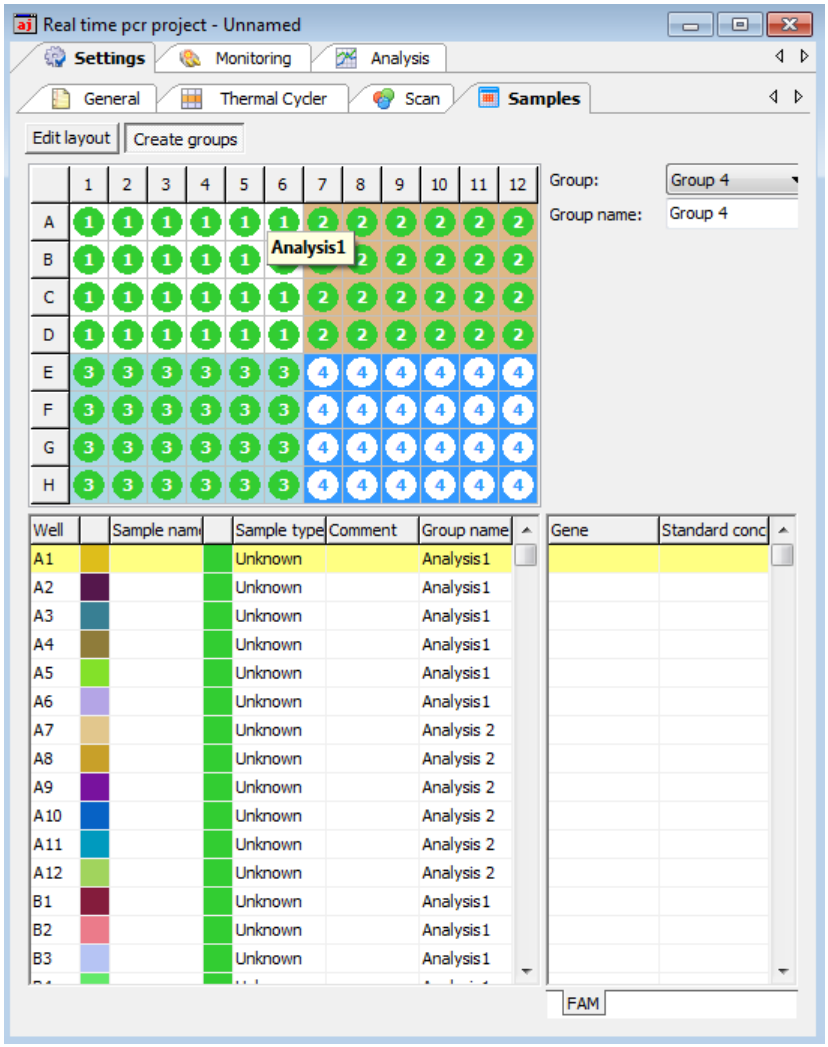
The groups are defined in the **Settings / Samples** project window.

1. In the **Settings / Samples** project window click on the **[Create groups]** button.

   The **Group** list and the **Group name** field are activated.
   In the layout, all samples are marked with the number 1. This means they have been assigned to group 1.
2. In the layout, select the samples that are part of one experiment. To select adjacent positions press the left mouse button and define an area. To select non-adjacent positions press the Ctrl key on the keyboard plus the left mouse button at each position
3. Select the next group from the **Group** list.
4. Enter the description for the experiment in the **Group name** field. You may select any group name.
5. Click on or press ENTER, to assign the group properties to the samples.

   The samples that belong together are marked with the group number in the layout.

   The descriptions are displayed in the sample table in the **Group name** column.



Sample layout with four groups

3.4.7 Layout preview

The layout preview provides a complete overview of the layout of the PCR plate with samples and the corresponding information that has been saved for the samples.

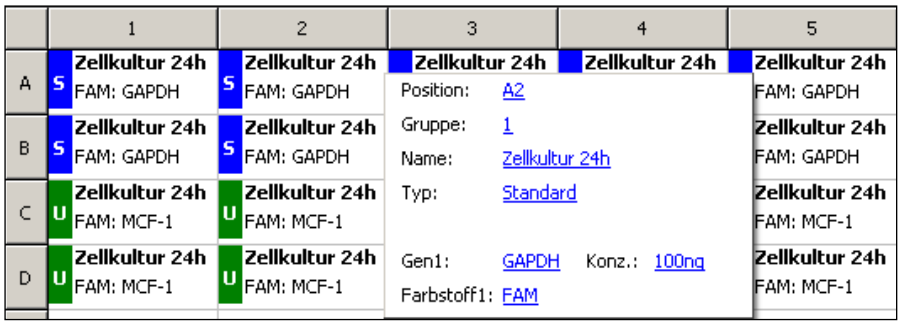
- Open the layout preview by clicking on the symbol in the toolbar.
  Optionally, call up the **Samples ▸ Preview layout** menu command.

  The layout preview is displayed in the **View sample plate** window.

The layout preview provides an overview of the following properties:

- Position on the PCR plate
- Genes to be determined
- Sample type by means of color marking at the edge
- Underlined in color to indicate group affiliation

If you move the cursor to a specific position, all known settings for this position, such as sample names, sample type and group and all genes and dyes to be measured for the sample as well as the concentration in the case of standards are displayed in detail.



Section from the layout preview table

The table can be printed and used, for example, as a template for pipetting the samples or for documenting the experiment.

- Press the **[Print]** button in the **View sample plate** window to print the table.

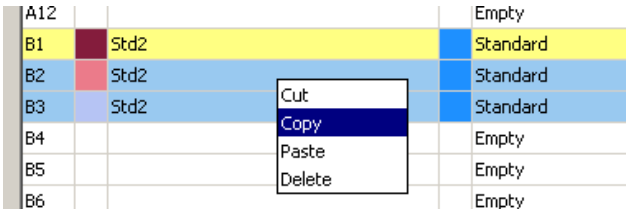
3.4.8 Copying the layout

The layout view or parts of the layout can be copied and inserted into another project.

1. Use your mouse to mark the area in the layout view to be copied.
2. Transfer the information to the clipboard by clicking on the symbol in the toolbar.
   Optionally, call up the **Samples ▸ Copy layout** menu command.
3. Select the target project.
4. Paste the information using the symbol.
   Optionally, you can use the **Samples / Paste layout** menu command.
   ✓ The copied areas become inserted at the same position in the target project like their position in the source file.

The way described to edit the layout is related to the graphical presentation of the PCR plate in the upper part of the project window. If the layout table shall be edited by copy, cut and paste this can be done by using the right hand mouse button and keeping the Ctr-button pressed. I this way, also areas within the same project can be copied.

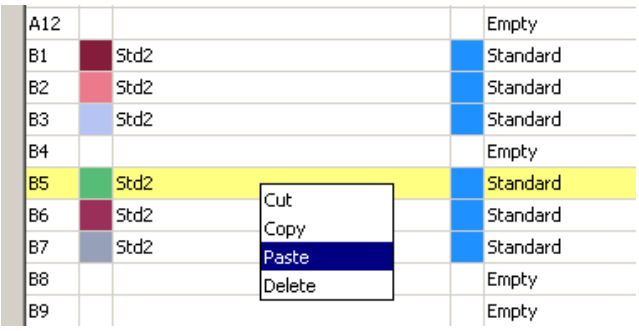
1. Press the Ctr-button and hold it pressed during the whole operation.
2. Mark the lines to be copied or cut by left-klicking the mouse and drawing it up or down.
3. Press the right mouse button and select the desired function



The standard samples in the wells B1, B2 and B3 are copied to the clipboard

4. Select the line by left clicking the mouse from which the copied samples shall be inserted. By right clicking the mouse the paste menu appears.

✓ In the example the standard Std2 is now a six fold replicate by their identical sample name.



The selected standard samples including their properties are now copied to the wells B5, B6 and B7

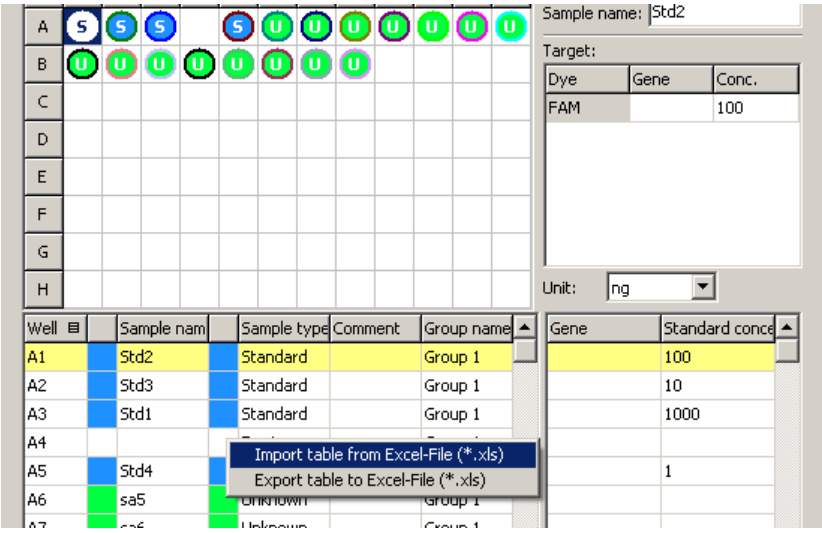
3.4.9 Exporting or importing the layout in Excel

The layout can be exported or imported as an Excel file (*.xls). The exported data can be edited in Excel and then reimported.

- Right-click on the sample table.

  A context menu with the **Import table from Excel file (*.xls)** and **Export table to Excel file (*.xls)** menu commands opens.

  Select the desired menu command.



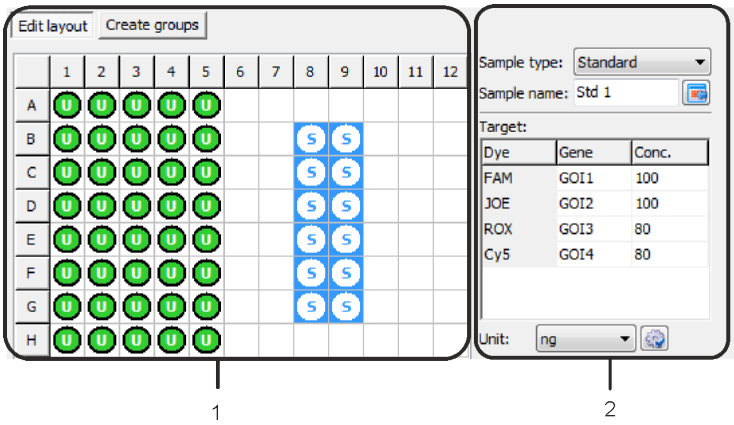
Excel export and import function for the sample layout

3.4.10 Functions for creating and editing a plate layout

Action	Where	Function
Left click a well	PCR plate scheme	Selects this well
Double left click a well	PCR plate scheme	Shows the information assigned to this well in the edit fields

Left click and drag	PCR plate scheme	Selects consecutive wells
Ctrl + left click	PCR plate scheme	Selects this well additionally
Ctrl + left click and drag	PCR plate scheme	Selects consecutive wells additionally
Right click selected wells	PCR plate scheme	Opens context menu for: ▪ Assigning and deleting wells which do not contain an internal positive control (IPC-, only relevant to end point analysis) ▪ Assigning genes afterwards (The gene names shown in the edit field will be assigned to the selected wells.)
ENTER	Keyboard	Complies with function **assig** (symbol placed near the edit field **Sample name** or in the toolbar)
DEL	Keyboard	Deletes the information assigned to the well and changes sample type to "Empty"
Function button F5	Keyboard / edit field "Gene"	Removes the selected gene from the displayed list of genes
Left click on the table header, column **Well**	Table	Changes sort sequence from "by line" to "by column" or vice versa
Right click on the table header	Table	Opens context menu for selection of the colums to be displayed in the table
Right click and drag on the table header	Table	Changes the sequence of the colums
Left click on a line	Table	Highlights this line an allows for data input or selection of sample types, directly in the table
Right click on a line	Table	Opens context menu for: exporting the layout table to Excel importing the layout table from Excel
Ctrl + right click (+ drag) on a line of the table (holding Ctrl pressed)	Ttable	Opens context menu to copy, cut, paste or delete the contents of the selected table lines
Symbol below the target table	Edit field	Opens dialog for automatically creating dilution series and replicates
Double-click on the color cell in the table row	Table	Opens the **Color** window for selecting the color of the amplification curve.

Shift key and double-click on color cell	Table	Resets the color setting of the amplification curve to the default setting in the **Options / Color** window.
Ctrl + double-click on color cell	Table	Opens the **Edit color** window. A selected color can be allocated to the amplification curves of multiple wells at the same time.



1 PCR plate scheme
2 Edit fields

4 Monitoring

All functions required for starting and monitoring a Real-Time PCR run are combined on the **Monitoring** project tab.

Note

After the PCR run is finished you have the choice to save the project or to continue without saving. If you save the project you cannot change the settings anymore and, for data integrity, it is not possible to start a new PCR run from that project. If you want to start a new PCR run with the same settings you must first generate a template from the project and then open it.

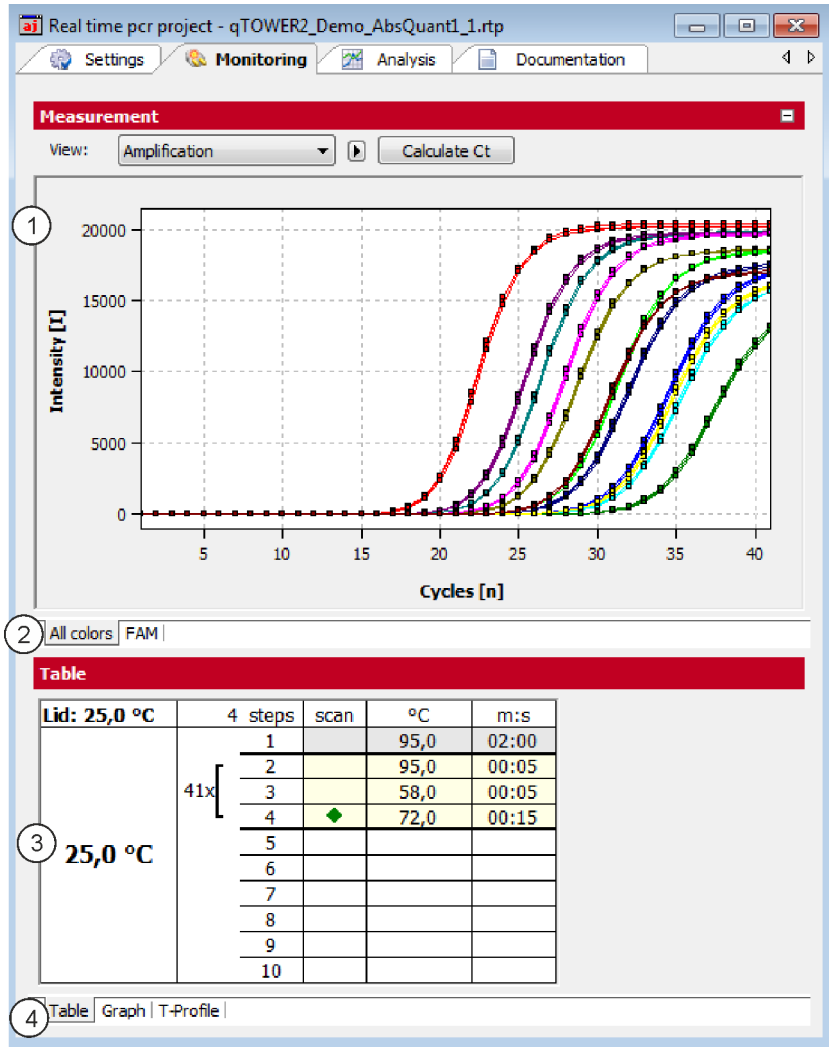
4.1 Starting the PCR protocol

The toolbar displays the icons for starting the PCR protocol defined in the **Settings** project window.

Symbol	Monitoring / ... menu command	Description
	Start qPCR run	Start the PCR run
	Stop qPCR run	The PCR run is interrupted and will not be continued. The data recorded up to this point is saved and can be evaluated.
	Pause qPCR run	The PCR run is interrupted. The symbol flashes during the break. The PCR run can be continued by clicking again.
	View options	Defines default settings for the Monitoring view.

4.2 Display options for monitoring

The **Monitoring** project window is divided into the following areas:



Monitoring project window for monitoring PCR runs

Area	Function
Measuring results (1)	Displays the measured fluorescence data. The fluorescence intensity is plotted against the cycle.
Colors tabs (2)	Switches between the fluorescence accumulation curves that were measured for the individual dyes.
PCR protocol (3)	Displays the PCR protocol. The active step is indicated by a green arrow.
Protocol view tabs (4)	Switches between different views of the PCR protocol (tabular, graphic, temperature profile).

The fluorescence measurements are displayed in the top area. On the different list sheets you can choose between the overlapping display of the measurement results of all dyes or the display of the individual dyes.

In the **View** list you can switch between the **Amplification, Melting curve** and **Raw data** views. The Ct value can be calculated for the amplification and the melting temperature T**m** for the melting curve.

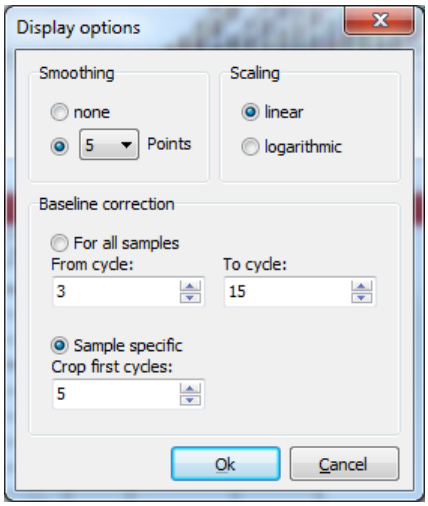
The current PCR protocol is displayed at the bottom of the table in the project window. The active step is marked by means of a green arrow during the PCR run. On the different sheets you can choose between a tabular or graphical view of the PCR protocol or the temperature profile. The view of the PCR protocol is described in the section "Monitoring the PCR run" p. 61.

Note: For each amplification curve a short information is displayed when the mouse pointer is placed it (mouse-over function).

4.2.1 Default settings for the Monitoring view

For all views in the **Monitoring** project window it is generally possible to choose between linear and logarithmic scaling for the graphical display of the data. The setting for the baseline correction can also be changed.

- Select the **Monitoring ▸ Display options** menu command or click in the toolbar.



Display options window for changing the default settings of the Monitoring view

Parameter / Option	Description
Smoothing	Setting the smoothing conditions for the measured data
Scaling	Scaling options for the data (**linear** or **logarithmic**)

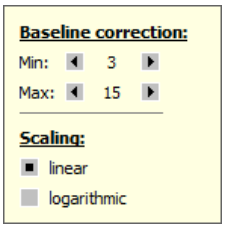
Baseline correction	There are two options for the baseline correction: ▪ **For all samples** If this option is selected, the baseline for every sample in the same range is determined. The upper and lower range limit must be set in the **From cycle** and **To cycle** fields. ▪ **Sample specific** Select this option if the curves have significantly different Ct values. The lower range limit for determining the baseline is set in the **Crop first cycles** field for all samples**.** The upper range limit is determined separately for each sample by an algorithm. **Note:** The **type** of baseline correction can only be set in this dialog. The range limits for the correction can also be adjusted in the project window.

4.2.2 Adjusting the view in the Monitoring project window

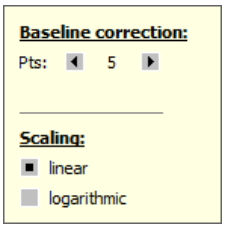
In the Monitoring project window, you can adjust the preset parameters (**Monitoring ▸ Display options** menu command) for the display of the scaling as well as the range limits for the baseline correction.

1. Click on the button above the chart.

   A selection field for setting the display options and entering the baseline parameters opens.



For all samples baseline correction parameter



Sample specific baseline correction parameter

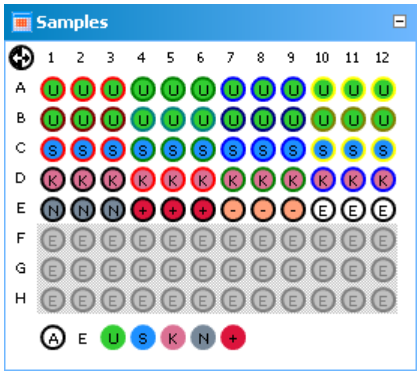
Parameter settings in the Monitoring project window

2. Change the baseline correction limits and activate the **linear** or **logarithmic** option for the desired view of the fluorescence curves.

4.2.3 Displaying and hiding measurement results for individual wells

The sample view in the **Monitoring** project window is controlled via the **Samples** menu item in the project explorer. Measurement results in the individual wells can be hidden or shown.

Note:
The selection in the project explorer only influences the display of the fluorescence data but not the measurement.



Project explorer Samples window

The color code for each sample type can be modified in menu **Extras / Options ▸ colors**.

The marking of the sample assignment corresponds to the marking on the **Samples** project tab:

Sample type	Symbol	Definition
Empty	E	Describes an empty position on the PCR plate
Unknown	U	Sample of unknown concentration or dilution (measuring sample)
Standard	S	Sample of known concentration or dilution
Calibrator	K	Sample whose gene of interest expression level is set as 1
No template control (NTC)	N	Complete reaction preparation but without matrix strand
Positive control	+	Positive control preparation for which a reaction product is expected
Negative control	-	Negative control preparation for which no reaction product is expected

Active wells (i.e. displayed wells) are marked with their sample type symbol. For deactivated wells the position is grey and the fluorescence data is hidden. Empty wells are marked "E". By default measurement data for empty wells is not shown. For control by activating empty wells the measurement data can be displayed.

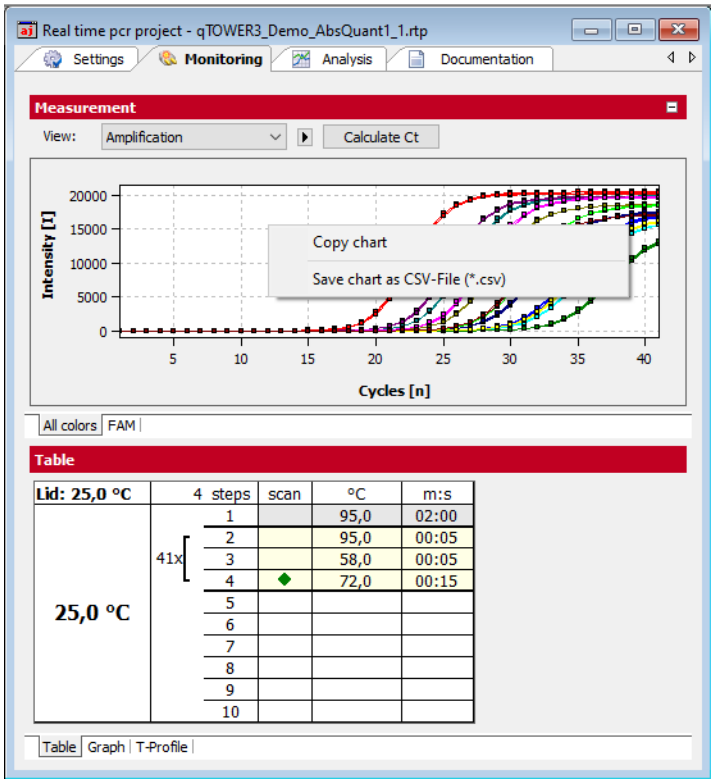
- Click with the mouse to switch. The activation changes with each click on a well.
- You can change adjacent wells by holding the mouse button and moving the cursor over the wells. To select non-adjacent positions press the Ctrl key on the keyboard plus the left mouse button at each position.
- Complete rows and columns can be inverted by clicking on the letter or number of the row [A-H] or column [1-12].
- The activation status of the complete plate can be inverted by clicking on on the top left between A and 1.
- To activate all wells click on the symbol below the chart.

- To activate only samples of a specific type click on the corresponding symbol below the chart. To activate multiple sample types at the same time keep the Ctrl key pressed when clicking on the sample types.

4.2.4 Exporting fluorescence data

The data from the fluorescence measurement can be exported as ".csv" files. In addition, the graphical display of the measurement results can be copied to the clipboard as a hardcopy and is hereby made available for other programs.

- Right-click on the graph.
- A selection window for export and hardcopy appears.
- Click on **Copy chart** to copy the chart to the clipboard.
- Select the **Save chart** option to export the fluorescence data.
  The **Save as** standard window opens.
  Enter a file name and confirm with **[OK]**.



Context menu for exporting the fluorescence data in the Monitoring project window

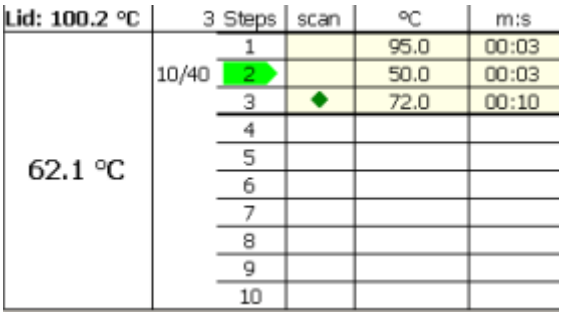
4.3 Monitoring the PCR run

The running PCR protocol is shown in the bottom part of the **Monitoring** project tab. It is generally possible to to choose between the three different views (list sheets) **Graph**, **Table** and **T-Profile** via tabs.

In addition to the display on the list sheets a status bar shows further information on the protocol, such as the plateau time, the calculated remaining time and, in programmed loops, the step number and the number of loops.

Status bar of the Monitoring project tab

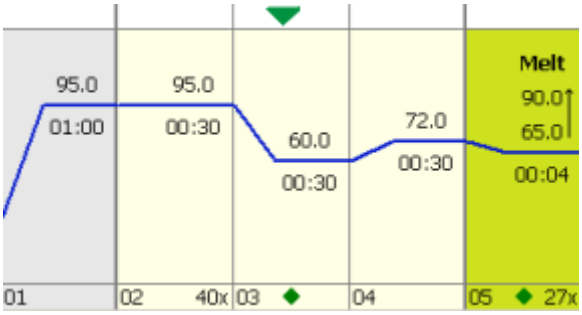
Table list sheet



Monitoring project window with PCR protocol table

Element	Description
Lid	Current lid temperature
Temperature display	Current block temperature
Steps	Temperature steps in the PCR protocol The active step is marked by a green arrow.
°C	Target temperature of the step
m:s	Hold time, format: min:s

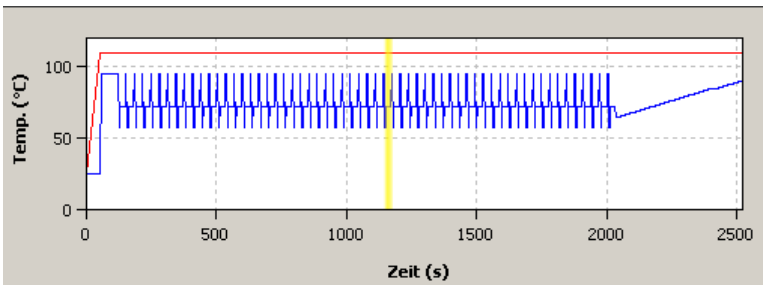
Graph list sheet



Monitoring project window with PCR protocol table

The **Graph** list sheet contains the same elements of the graphical representation of the PCR protocol as the **Settings / Thermocycler / Graph** project window. Once again, the active step is marked by a green arrow.

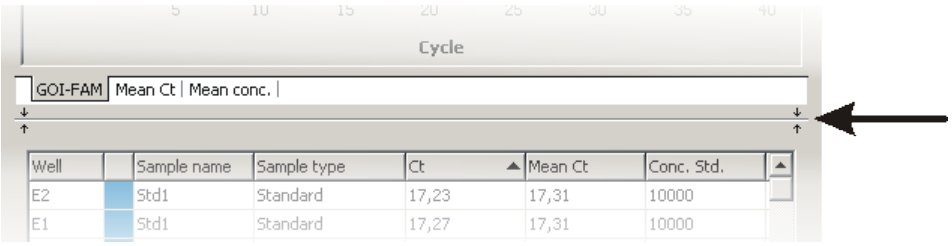
T profile list sheet



Monitoring project window with T profile

In the representation of the temperature profile a yellow progress bar indicates the step that is currently being performed.

The size of the area displayed for fluorescent curves and results table or standard curves can be adjusted by the slider control that is located between the two areas.



Slide control to adjust the size of the displayed areas

4.4 Displaying product accumulation curves and calculating Ct values

The product accumulation is documented by means of fluorescence measurements during the PCR run in the project window **Monitoring**.

Displaying the product accumulation curve

- Select the **Amplification** or **Raw data** option from the **View** list to display measurement curves for the product accumulation.

  In the chart the fluorescence intensity [I] is plotted against the number of cycles in relative units. The color of the curve that is being displayed corresponds to the color assigned to each well in the sample table (**Settings / Sample** project window).

- Selected the measurement results for the individual dyes via the corresponding list sheets.

  You have the option to display the measurement results of all dyes together (**all colors** list sheet) or only the results of an individual dye.

The display options for the product accumulation curves are described in the section "Display options for monitoring" p. 57.

Calculating the Ct values

After the PCR run, the Ct values can be calculated directly from the amplification curves, without having to create an analysis such as **Absolute quantification**.

- Select the **Amplification** or **Raw data** option from the **View** list and click **[Calculate Ct]**.

  The amplification curves are normalized and displayed individually or together on the list sheets for the dyes. The table below shows the Ct values of the individual samples and the mean values of the replicates.

  The threshold value for the individual dyes can be set on the applicable list sheet. The parameters set in the **Options / Analysis** window are factored in.

- After clicking on **[Data]**, you return to the display of the fluorescence intensities.

4.5 Displaying melting curves and calculating the melting temperatures T_m

Displaying melting curves

The course of the melting curve after the PCR can be monitored in the **Monitoring** project window.

- To display the melting curves, select the **Melting curve** option from the **View** list.

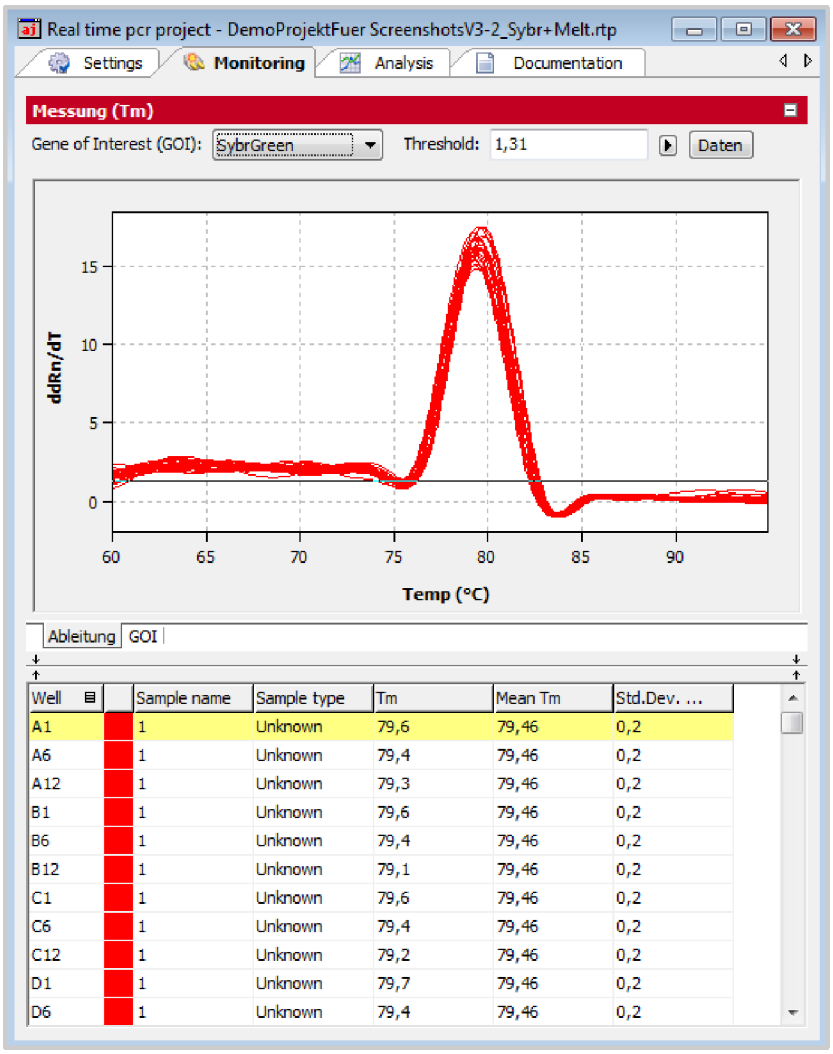
The display options for the product accumulation curves are described in the "Display options for monitoring" p. 57.

Calculating the melting temperature T_m

For a PCR run with included melting curve, the melting temperatures can be calculated in the Monitoring window without having to create a **Melting curve** analysis.

- In the **Monitoring** project window, select the **Melting curve** option in the **View** list.
- Click on **[Calculate Tm]**.
- Select the gene to be examined in the **Gene of Interest (GOI)** list.

  The melting temperature is calculated taking the parameters set in the **Options / Analysis** window into account, and the diagram and the results table are displayed.
- As an option, a threshold value can be set on the **Derivative** tab, with which significant peaks can be distinguished from insignificant ones.
- After clicking on **[Data]**, you return to the display of the fluorescence intensities.

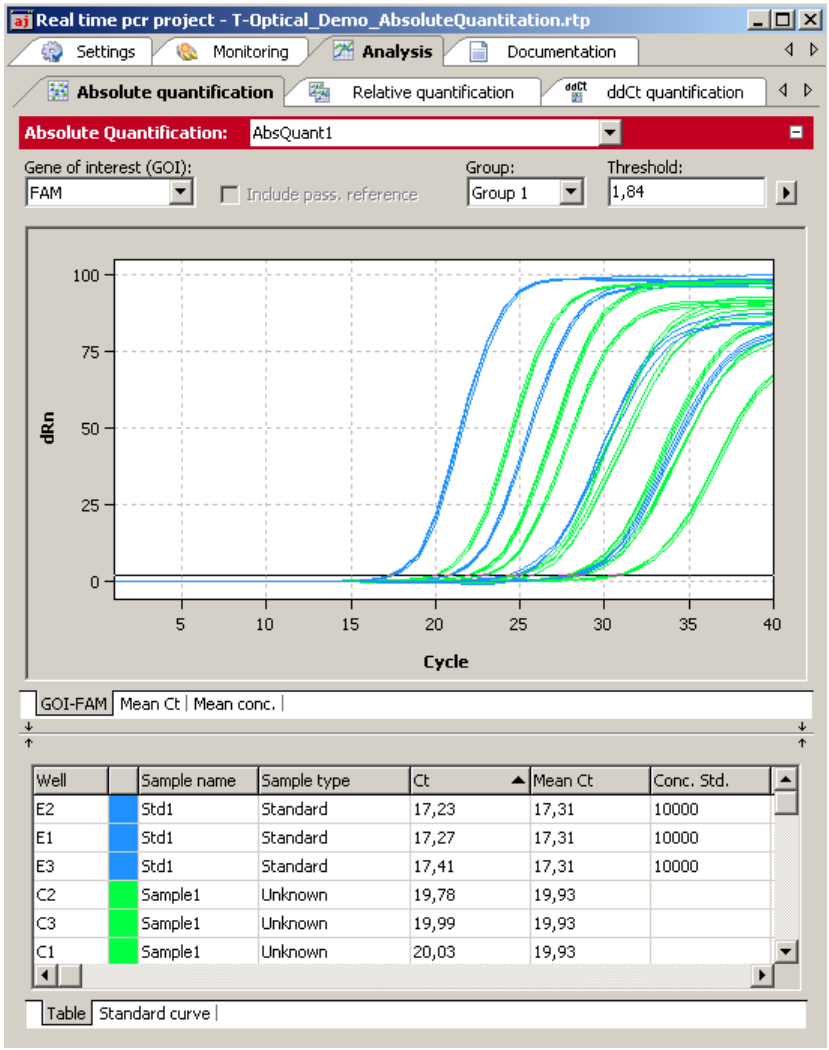


Monitoring project window with calculation of the melting temperature

5 Evaluation

On the **Analysis** tab of the project window the following methods are available for evaluating Real-Time PCR experiments on four cards:

- Absolute quantification
- Relative quantification
- ΔΔCt method
- Melting curve determination
- Genotyping
- POS/NEG analysis at the end point



Analysis project window

The individual evaluation methods can be accessed via the subordinated tabs. For each selection method different evaluations can be created.

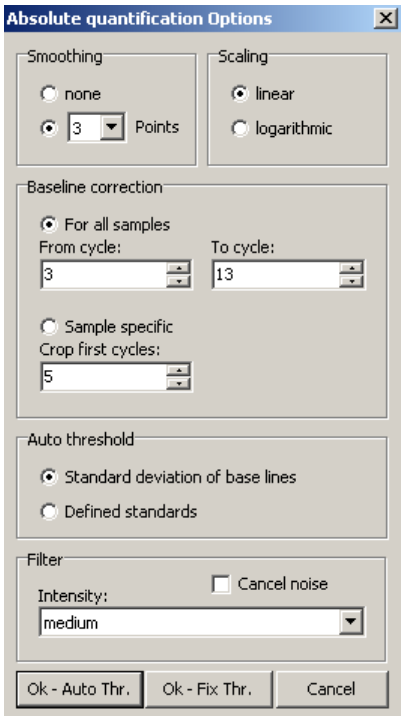
Toolbar and menu commands are adjusted to the requirements of the selected method tab.

5.1 General functions in the evaluation project window

5.1.1 Making basic settings

Presets can be made for some evaluation parameters.

1. Click on in the toolbar.
2. Set up the following parameters.

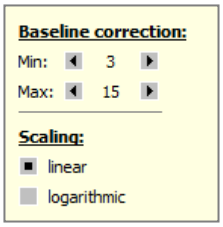


Window for basic evaluation settings

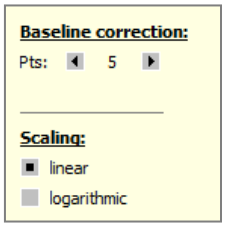
Option	Function
Smoothing	Smoothing the fluorescence curves on the basis of the calculated moving average over a range of 2 to 12 measuring points or representation without smoothing
Scaling	Linear or logarithmic representation of fluorescence curves
Baseline correction	At the correction of the base line you can choose between two options: ▪ **For all samples:** At this correction the base line is determined for every sample in the same area. The lower and upper area limit has to be edited in the fields **From cyncle** and **To cycle**. ▪ **Sample specific:** This correction should be chosen if the curves have very different Ct values. The lower area limit for the determination of the base line will adjusted in the field **Crop first cycles** for all samples. The upper area limit is separately found out by an algorithm for each sample. **Note:** The manner of the base line correction can be selected only in this dialog. The area limits can, however, be adapted for the correction in the project window.

Autom. threshold	Calculation of the threshold as a deviation of x times of the standard deviation of the baselines (factor can be adjusted under **Extras / Options / Analysis** in the main menu) or based on defined standards with the goal to get the maximum value for the coefficient of determination R^2
Filter	Digital filter for smoothing the fluorescence curves. Adjustable in steps: **slight, medium** and **strong**
Cancel noise	Curves with high background noise that are not interpreted as amplification curves by the software are set to 0 and no Ct values are calculated.
Auto Threshold	The threshold line is calculated new when changes to the basic settings are made.
Fix Threshold	The set threshold line is maintained when changes to the basic settings are made.

More setting options may be available depending on the analysis method used. They are explained separately in the respective sections. All items displayed on the **Analysis** project tab can also be accessed quickly via the settings area of the baseline and displayed as linear or logarithmic representations. For this purpose, a selection for display options can be opened in the corresponding window via the right-arrow button ▶:



For all samples baseline correction parameter



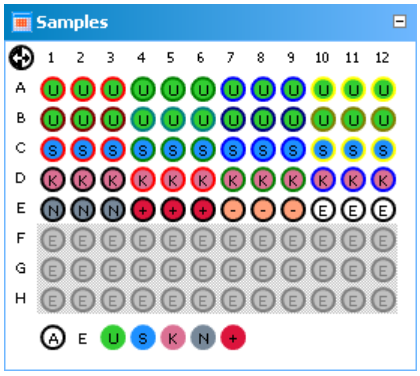
Sample specific baseline correction parameter

Individual setting of the baseline correction and fluorescence curve scaling during the evaluation

5.1.2 Activating/deactivating samples for evaluation

Samples from individual wells can be activated or deactivated for evaluation in the project explorer **Samples** menu item. This enables you, for example, not to include excessive values when calculating mean values.

Note:
The selection in the project explorer only influences the analysis of the fluorescence data. Measured data will not be deleted.



Project explorer Samples window for (de-)activating the samples in the evaluation

The marking of the sample assignment corresponds to the marking on the **Samples** project tab. The color code for each sample type can be modified in menu **Extras / Options ▸ colors**.

Sample type	Symbol	Definition
Empty	E	Describes an empty position on the PCR plate
Unknown	U	Sample of unknown concentration or dilution (measuring sample)
Standard	S	Sample of known concentration or dilution
Calibrator	K	Sample whose gene of interest expression level is set as 1
No template control (NTC)	N	Complete reaction preparation but without matrix strand
Positive control	+	Positive control preparation for which a reaction product is expected
Negative control	-	Negative control preparation for which no reaction product is expected

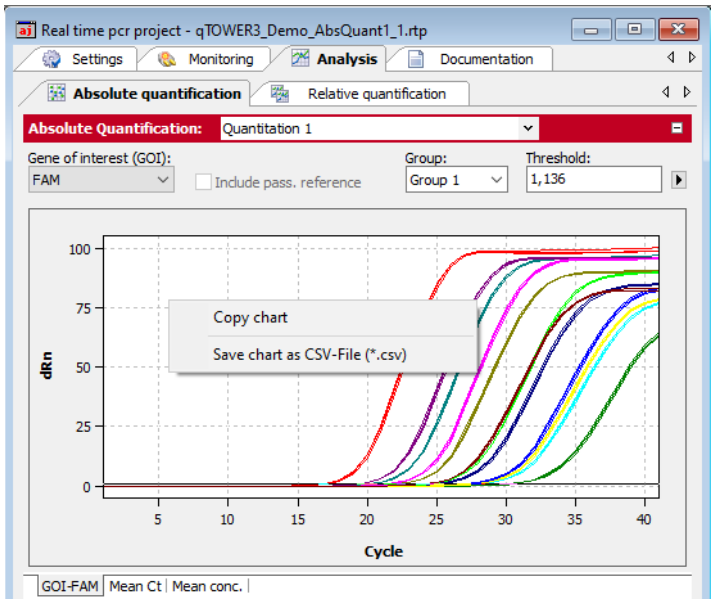
Active wells (i.e. wells included in the analysis) are marked with their sample type symbol. For deactivated wells the position is grey and the fluorescence data is hidden. Empty wells are marked "E".

- Click with the mouse to switch. The activation changes with each click on a well.
- You can change adjacent wells by holding the mouse button and moving the cursor over the wells.
- Complete rows and columns can be inverted by clicking on the letter or number of the row [A-H] or column [1-12].
- The complete plate can be inverted by clicking on on the top left between A and 1.
- To activate all wells click on the symbol below the chart.
- To activate only samples of a specific type click on the corresponding symbol below the chart. To activate multiple sample types at the same time keep the Ctrl key pressed when clicking on the sample types.

5.1.3 Exporting fluorescence data

The data from the fluorescence measurement can be exported as ".csv" files. In addition, the graphical display of the measurement results can be copied to the clipboard as a hardcopy and is hereby made available for other programs.

- Right-click on the graph.
- A selection window for export and hardcopy appears.
- Click on **Copy chart** to copy the chart to the clipboard.
- Select the **Save chart** option to export the fluorescence data.
  The **Save as** standard window opens.
  Enter a file name and confirm with **[OK]**.



Exporting data from the evaluation

5.1.4 Configure results table

For each analysis the results are summarized in a table that is accessible by the tab TABLE.

Well		Sample name	Sample type	Ct	Mean Ct	Conc. Std.
B1		Std2	Standard	20,58	20,7	1000
B2		Std2	Standard	20,78	20,7	1000
B3		Std2	Standard	20,76	20,7	1000
C1		Sample1	Unknown	20,03	19,93	
C2		Sample1	Unknown	19,78	19,93	
C3		Sample1	Unknown	19,99	19,93	

Table | Standard curve

Results table

Depending on the analysis method the results table contains different data sets but for each table the view and selection of columns to be displayed can be configured user-defined:

- By click with the right mouse button on a column header a box is displayed in which single columns can be selected or deselected to be shown in the table.

- By dragging a columns header with pressed left mouse button columns can be exchanged against each other and the arrangement modified.
- By click with the left mouse button on the left or right border of a columns header the width can be modified.
- By click with the left mouse button on a columns header the data becomes arranged ascending or descending (by number or alphabetically).
- The colors of the amplification curves can be changed after double-clicking on the color cell in the table row.
  Press the Ctrl key and double-click the **Edit color** window to set the color for multiple wells (→ section "Entering sample properties into the sample table" p. 43).
- By click with the left mouse button on the column "Well" the display of data switches between column- or row-wise representation of the results. The column- or row-wise representation is based on the arrangement of samples in the layout.

Symbol	Meaning
Well	Display of data by columns
Well	Display of data by rows

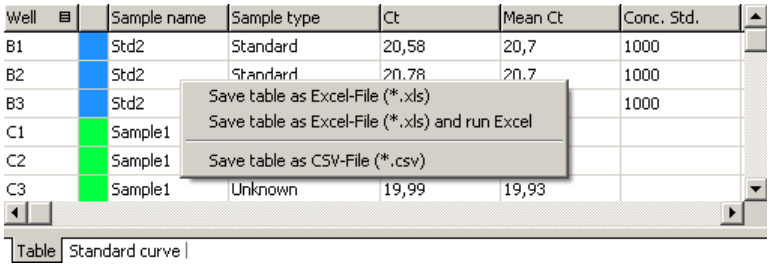
5.1.5 Export results

The results table can be exported as Excel-file (*.xls) or "*.csv"-file.

- Click with the right mouse button on the results table.

  A context menu opens with the commands SAVE TABLE AS EXCEL-FILE (*.XLS), SAVE TABLE AS EXCEL-FILE (*.XLS) AND RUN EXCEL and SAVE TABLE AS CSV-FILE (*.CSV).
- Select the corresponding command.
- The standard Windows dialog SAVE AS opens. Enter a name for the file and confirm the settings with [OK].

Note:
User defined configurations of the results table are included in the exported data set (→ section "Configure results table" p. 69).



Export results

5.2 Absolute quantification

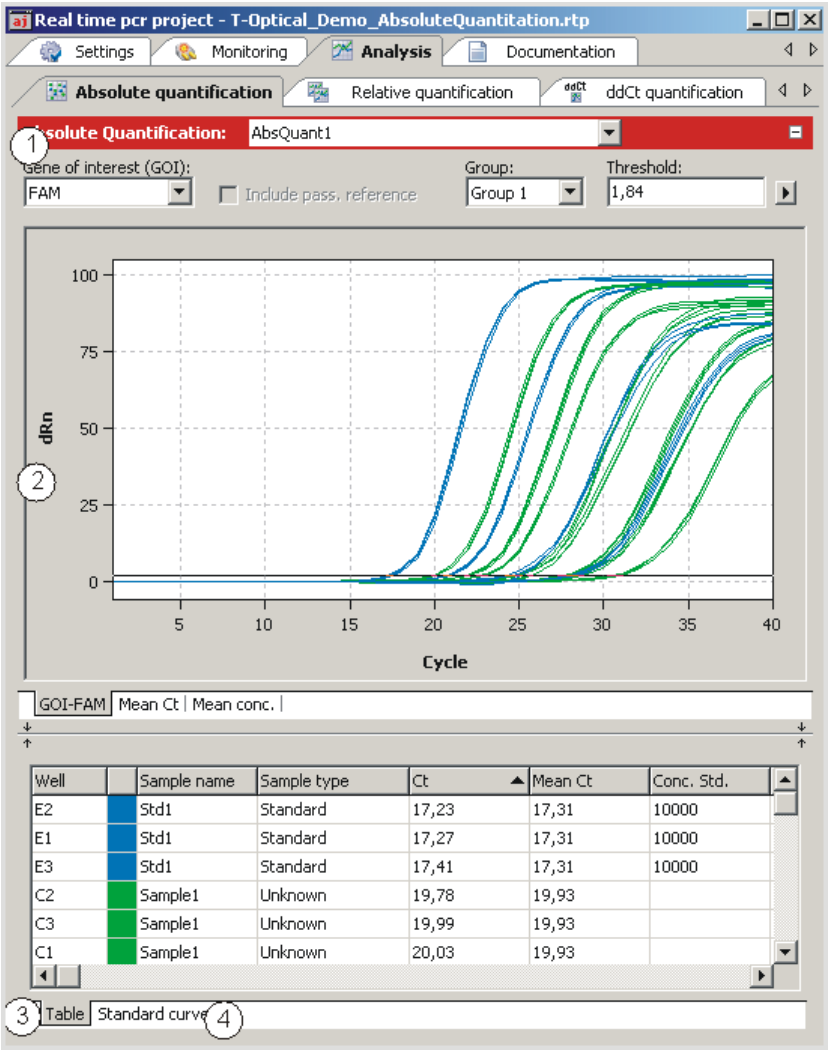
The absolute quantification is used to determine absolute copy numbers in samples with the help of the comparison with standards with known concentrations.

5.2.1 Creating an evaluation for an absolute quantification

1. Go to the **Analysis / Abs quant** project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the symbol in the toolbar.
   Optionally, call up the **AbsQuant ▸ Add abs. quantification** menu command.
3. An input window appears. Enter the description for the current evaluation.

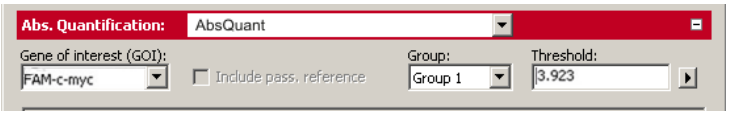
On the **AbsQuant** tab the following information is activated:

- Parameter settings (1)
- Display of fluorescence spectra (2)
- Display of result table with measurement results (3)
- Display of the standard curves and the calculated coefficients (4)



Window for absolute quantification

5.2.2 Setting parameter for absolute quantification



Parameter settings for absolute quantification

Set the following parameters for the absolute quantification:

Option	Description
Selection list	Selection of an evaluation created for the experiment
Gene of interest (GOI)	Selection list of target gene/dye combinations According to the selection, the fluorescence and regression curves for the concentration are displayed.
Incloud pass. reference	Only active if a dye has been defined as a passive reference on the **Settings /Scan** project tab If this option is activated, the fluorescence of the dye that has been set as a passive reference is used for standardization.

Group	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed. (→ section "Defining groups" p. 50).
Threshold	Manually adjust threshold value The threshold value must be between 1 and 100, depending on the standardized representation of the fluorescence curves (dRn). **Note:** The threshold value can be calculated automatically or set manually in the chart (see also "Setting the threshold value" below).
▸	Opens the selection window with display options (→ section "Displaying the fluorescence curves for absolute quantification" p. 73).

Setting the threshold value

To determine Ct values for the evaluation, a threshold value needs to be determined for each experiment first.

You have several options for setting the threshold value:

- In the general options (→ section "Making basic settings" p. 66).
- Manually in the parameters for the respective evaluation (see table above)
- Graphically in the fluorescence curves representation:
  In the chart, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Ct values in the result table are updated.

  Note:
  Due to the further spread of the early exponential area of the product accumulation curves, a logarithmic representation is better suited for setting the threshold manually in the display range than a linear representation.
- By automatic calculation:

  The automatic calculation of the threshold value is activated by clicking on the symbol.
  Alternatively, you can call up the **AbsQuant ▸ Autom. Threshold** menu command.

Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the corresponding **Threshold** input field.

Fix Threshold

The threshold value becomes recalculated by the software each time basic settings for the analysis are changed. For the analysis the option **Fix Threshold** can be used, so that the threshold value is retained if the basic settings for the analysis are changed (→ section "Making basic settings" p. 66).

5.2.3 Displaying the fluorescence curves for absolute quantification

In the display range, the measured data, standardized to the value 100 for highest fluorescence intensity, is plotted against the cycle for the selected target gene.

The respective fluorescence curves are displayed by switching to another target gene/dye combination.

The fluorescence data is displayed as a linear or logarithmic representation depending on the selected display option. For both display options the program shows brief information on the sample if the cursor is placed on one of the curves.

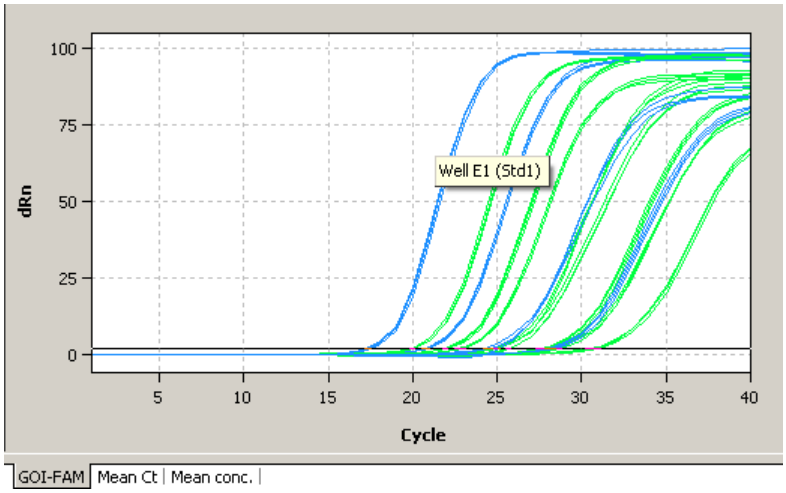
Switching the display options for the chart

1. Click on the button in the parameter bar.

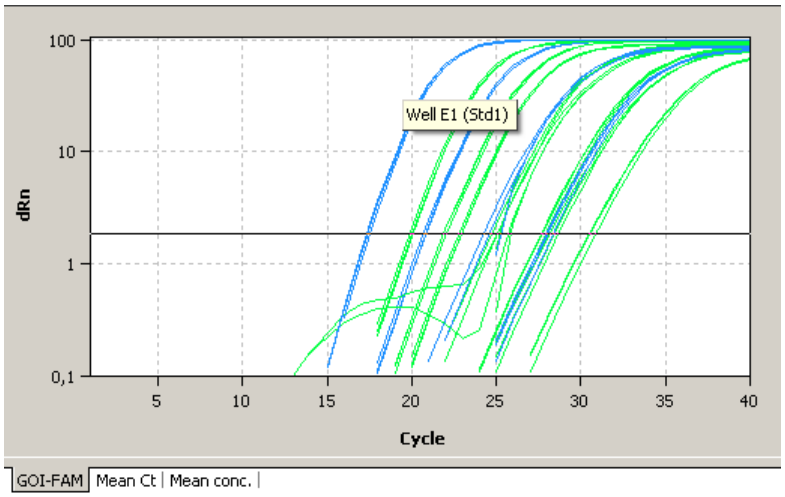
   A selection window for the display options opens.

2. Select the **Scaling logarithmic** or **linear** option.

   Click next to the selection window. The changes are applied.



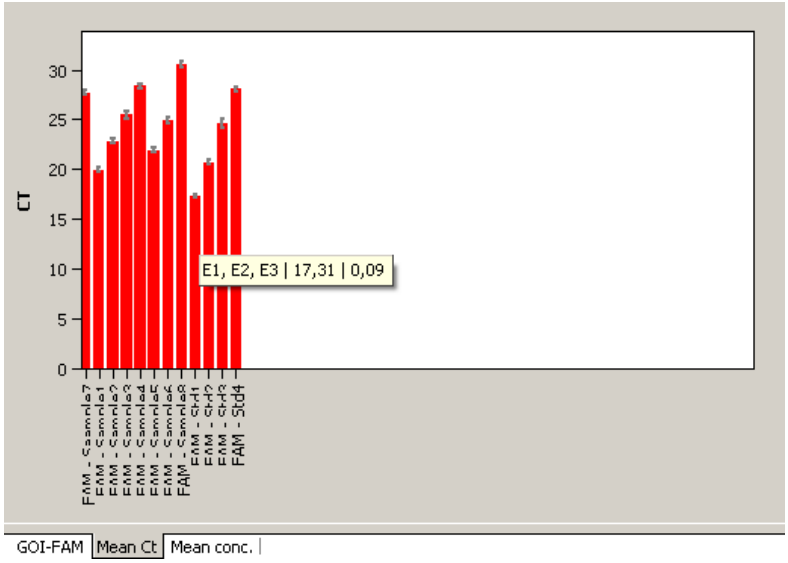
Linear representation of the fluorescence curve for the absolute quantification



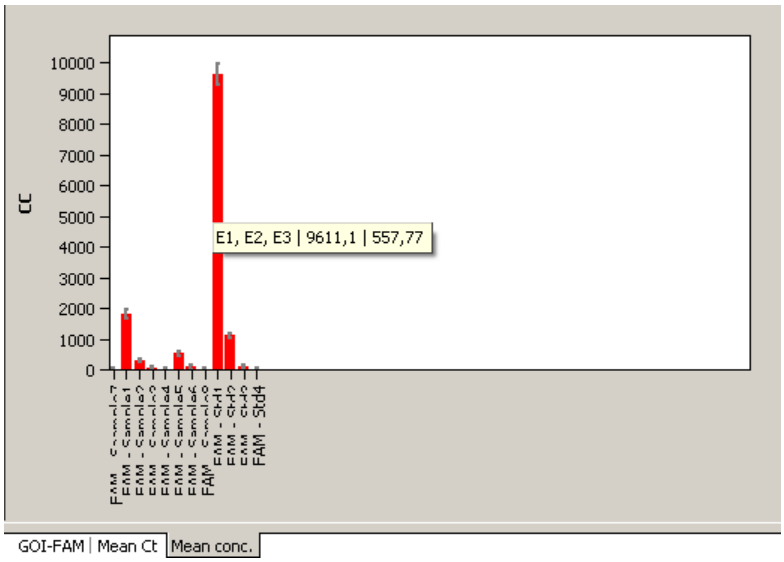
Logarithmic representation of the fluorescence curve with horizontal threshold line

5.2.4 Display of the mean Ct values or mean concentrations

After selecting the tab MEAN. CT or MEAN. CONC. the display changes to a presentation of the results as bar chart. The respective sample name is given below each bar. The height of the bar is determined by the mean Ct-value or the calculated mean concentration of replicates. For each bar some short information about the position of the samples, the mean value and the calculated standard deviation is shown if the mouse pointer is placed on it. The size of the standard deviation is shown as error bar on top of each bar. Since for large numbers of samples not all bars can be displayed in the screen the diagram can be moved horizontally with pressed left mouse button and dragged to the desired position.



Bar chart for the display of mean Ct values of replicates



Bar chart for the display of mean concentrations of replicates

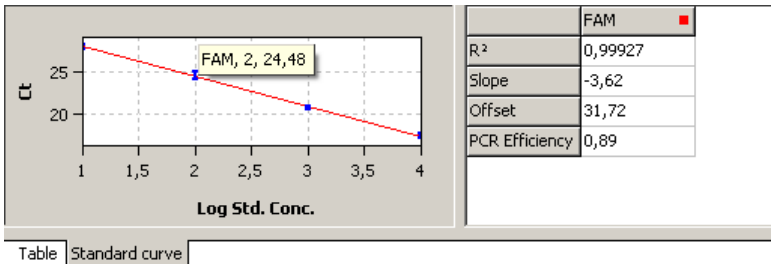
5.2.5 Displaying the standard curve and results of an absolute quantification

In the bottom **Standard curve**section of the **Analysis** project window, you can switch between the calculated standard curve and the result table via the **Curve** and **Table** list sheets.

For the display of the standard curve the Ct values of the standard samples are plotted graphically against the logarithm of their concentration. In the value range on the right the following calculated data is displayed:

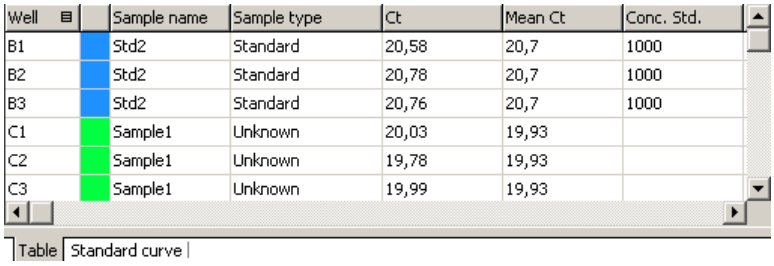
- the coefficient of determination R^2 of the linear equation
- the standard curve gradient
- the intersection of the curve with the y-axis at x=0 (offset)
- The PCR efficiency

The standard curve and the values are automatically calculated by the qPCRsoft software and updated in case of settings modifications.



Standard curve for absolute quantification

The result table for the absolute quantification contains all data and the associated measurement values for the samples.



Well		Sample name	Sample type	Ct	Mean Ct	Conc. Std.
B1		Std2	Standard	20,58	20,7	1000
B2		Std2	Standard	20,78	20,7	1000
B3		Std2	Standard	20,76	20,7	1000
C1		Sample1	Unknown	20,03	19,93	
C2		Sample1	Unknown	19,78	19,93	
C3		Sample1	Unknown	19,99	19,93	

Table | Standard curve

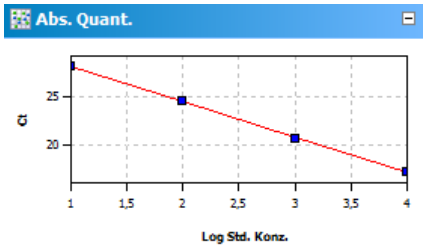
Result table for absolute quantification

For the absolute quantification the results table contains the following information:

Column	Description
Well	Position of sample
color of curve	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
Sample name	Name of sample
Sample type	Type of sample
Group	Assignment of the sample to an experimental group
Gene	Name of gene measured in the sample
Ct	Ct value of sample
Mean. Ct	Mean Ct value of replicates
Conc. Std	Concentration of the standard sample
Mean conc.	Concentration determined from the standard curve on the basis of the mean Ct value
StdDev. Ct	Standard deviation of the Ct values between replicates
%CV Ct	Variation coefficient of the Ct values between replicates
StdDev. Mean conc.	Standard deviation of the mean concentration

Display in the project explorer

A shortened representation of the standard curve calculated by the software is displayed in the project explorer under **Abs. quant.** The image displays the graphical plot of the Ct values of the standard samples against the logarithm of their concentration:



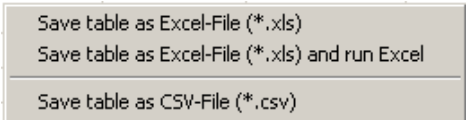
Representation of the standard curve in the project explorer

5.2.6 Exporting results of calculations

Exporting measurement data and results of calculations enables you to perform more extended data analysis by using dedicated software.

Excel and CSV

You can design the results table according to your requirements by setting type, sequence and width of the columns to be displayed. You can also adjust the sort sequence of data in the column (alphabetical, numerical, by column, by row). The table configured this way can be exported as Excel or CSV file by right clicking the table.



Context menu for data export

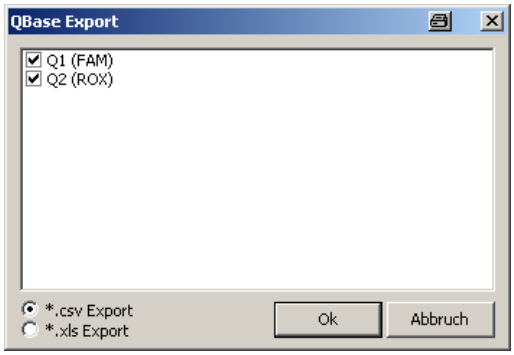
Export to qBASE+

qBASE+ is a software which allows for a more detailed statistical analysis of your qPCR data. For information see www.biogazelle.com. Using the analysis module 'Absolute Quantification' of qPCRsoft you can export qPCR data to a file which can be read and analyzed by qBASE+.

1. In order to export qPCR data for use in qBASE+ click the symbol in the toolbar. Alternatively you can select the menu ABSOLUTE QUANTIFICATION / QBASE EXPORT.
2. Choose the targets shall be exported and the data format shall be used (qBASE+ is able to read both formats) in next dialogue.

The following information is sent to qBASE+:

- Well
- sample type
- sample name
- gene name
- Ct value
- concentrations of standards
- sample active/inactive



Window QBase Export fort he data export to qBase+

Note:
Only those genes are displayed in the export dialogue which were set as GOI before. Therefore in multiplex assays for each GOI, i.e. each dye, an absolute quantification must be defined. To do so repeat the steps explained in section "Creating an evaluation for an absolute quantification" p. 71, topic 2 and 3, for each GOI.

5.2.7 Importing the standard curve

Next to the option to measure a standard curve in the experiment, the qPCRsoft software can also be used to determine the concentration of the samples based on the saved standard curve. You can use the import function for this purpose.

1. Use the symbol in the toolbar to open the **Import standard curve** window.

   Optionally, you can call up the **AbsQuant / Import standard curve** menu command.

   The mathematical equation that the standard curve is based on and the associated dye are each displayed in the list fields of the window.

2. Select one of the import options from the **Import standard curve** window and make the corresponding entries:

Option	Meaning
Import from this run	Imports a standard curve from the current open project. If several standard curves are saved in one project, all curves are displayed and you can make a selection.
Import from saved run	Imports a standard curve from a saved project. If several standard curves have been saved, select the corresponding curve from the list.
Manual input	Standard curve coefficients are entered manually. Enter the gradient and the intercept for the equation Ct = gradient * log(conc) + intercept.
Delete External Standards	Deletes imported or entered standard curves so that they are no longer used for analysis

5.2.8 Deleting the evaluation of an absolute quantification

An evaluation that is no longer required can be removed.

1. Activate the evaluation by selecting its description in the evaluation list of the method tab.
2. Click on in the toolbar.Optionally, you can call up the **Absolute quantification ▸ Delete evaluation** menu command.

   The evaluation is removed.

5.3 Relative quantification

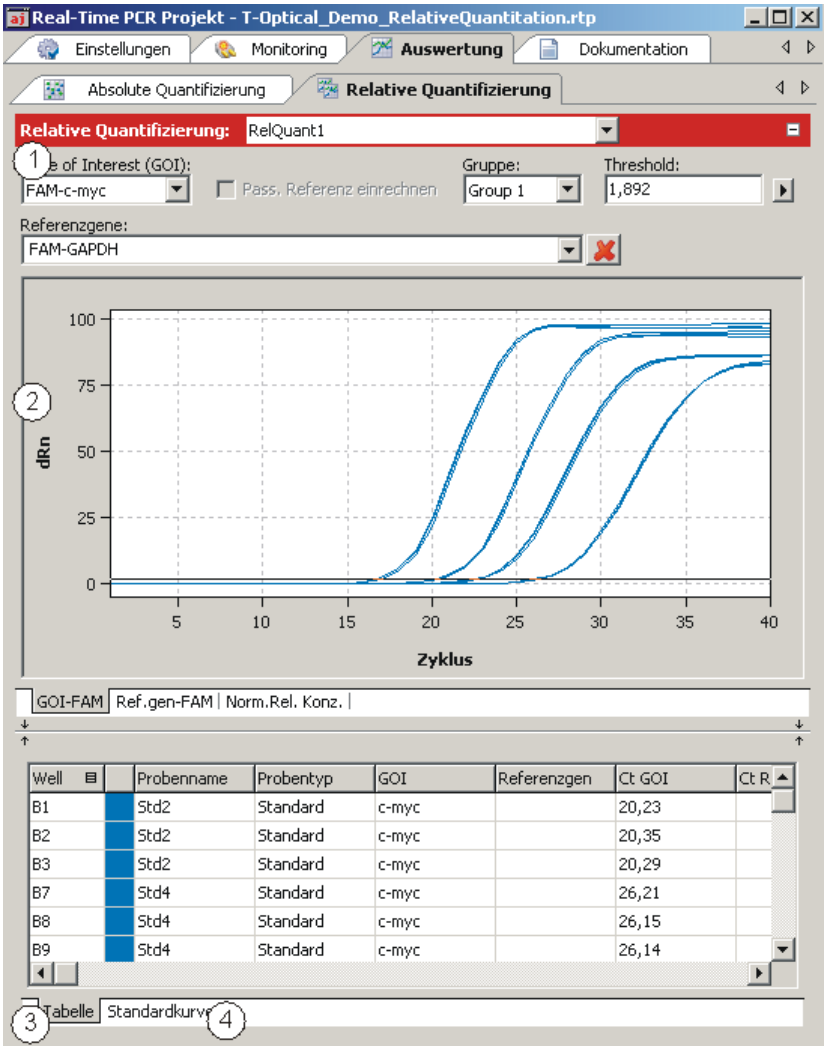
Relative quantitation allows for determination of the relative expression level of a gene of interest in relation to one or more reference genes (often housekeeping genes). If one of the samples is denoted as the calibrator , the expression level of that sample is set to unity and the relative expression levels of all the other samples are given in relation to the calibrator sample. For relative quantification standard dilution series are required for the gene of interest as well as for the reference genes.

5.3.1 Creating a new evaluation for a relative quantification

1. Go to the **Analysis / RelQuant** project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the symbol in the toolbar.
   Alternatively, call up the **RelQuant ▸ Add rel. quantification** menu command.
3. An input window appears. Enter the description for the current evaluation.

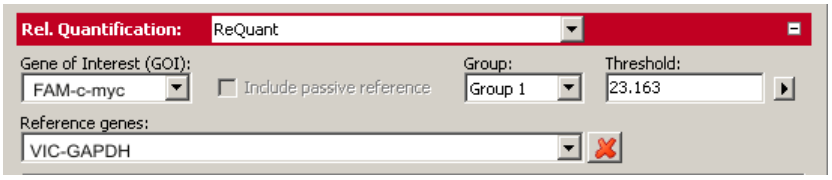
On the **Rel. Quant.** tab the following information is activated:

- Parameter settings (1)
- Display of the fluorescence curves for the target gene and the reference gene (2)
- Display of the result table with the added values (3)
- Display of the standard curves for the target gene and the reference gene and the calculated coefficients (4)



Window for relative quantification

5.3.2 Parameter settings for relative quantification



Parameter settings for relative quantification

The following parameters must be set for the relative quantification:

Option	Description
Selection list	Selection of an evaluation created for the experiment
Gene of interest (GOI)	Selection list of target gene/dyecombinations According to the selection, the fluorescence and regression curves for the concentration are displayed. Only one target gene at a time can be selected.

Reference genes	Reference gene selection list In contrast to the target gene, several reference genes can be selected at the same time. The number of list sheets in the display range therefore grows with each reference gene. The symbol is used to remove all reference gene settings from the evaluation.
Include pass. reference	Only active if a dye has been defined as a passive reference on the **Settings / Scan** project tab. If this option is activated, the fluorescence of the dye that has been set as a passive reference is used for standardization.
Group	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed. (→ section "Defining groups" p. 50).
Threshold	Manually adjust threshold values The threshold value must be between 1 and 100, depending on the standardized representation of the fluorescence curves (dRn). Note: The threshold value can be calculated automatically or set in the graph.
	Opens the selection window with display options

Setting the threshold value

To determine Ct values for the evaluation, a threshold value needs to be determined for each experiment first.

You have several options for setting the threshold value:

- In the general options (→ section "Making basic settings" p. 66)
- Manually in the parameters for the respective evaluation (see table above)
- Graphically in the fluorescence curves representation:
  In the chart, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Ct values in the result table are updated.

  Note:
  Due to the further spread of the early exponential area of the product accumulation curves, a logarithmic representation is better suited for setting the threshold manually in the display range than a linear representation.
- By having it calculated automatically:

  The automatic calculation of the threshold value is activated by clicking on the symbol.
  Alternatively, you can call up **the RelQuant ▸ Autom. Threshold** menu command.

Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the corresponding **Threshold iput** field.

Fix Threshold

The threshold value becomes recalculated by the software each time basic settings for the analysis are changed. For the analysis the option **Fix Threshold** can be used, so that the threshold value is retained if the basic settings for the analysis are changed (→ section "Making basic settings" p. 66).

5.3.3 Displaying the fluorescence curves in the relative quantification

In the display range, the measured data, standardized to the value 100 for highest fluorescence intensity, is plotted against the cycle for the selected target gene. The target gene/dye and the reference gene/dye combinations are each assigned a list sheet that can be activated by clicking on the gene/dye tab on the bottom.

Since only one target gene/dye combination is permitted at a time, the fluorescence curves of the selected combination are displayed whenever a new combination is selected. The number of the available list sheets depends on the number of the selected reference genes.

The fluorescence data is displayed as a linear or logarithmic representation depending on the selected display option. For both display options the program shows brief information on the sample if the cursor is placed on one of the curves.

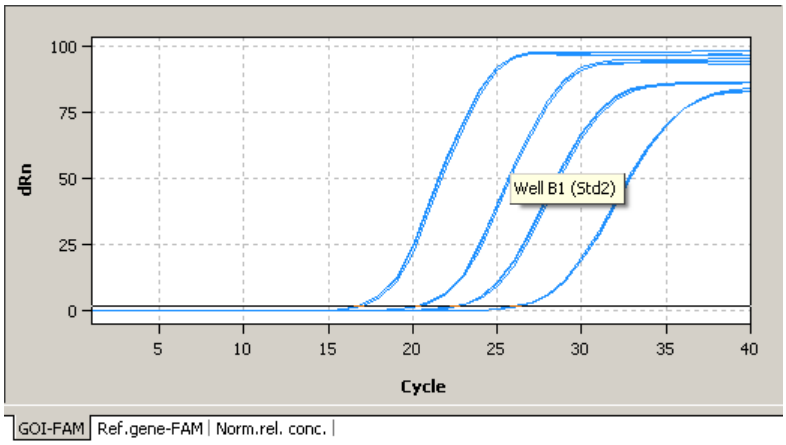
Switching the display options for the chart

1. Click on the button in the parameter bar.

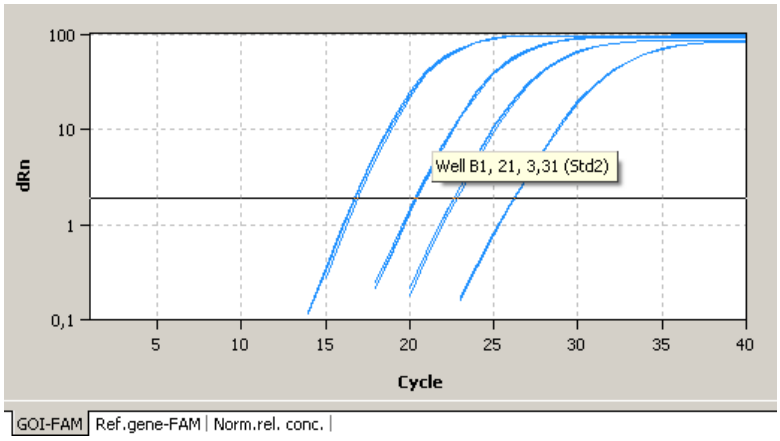
   A selection window for the display options opens.

2. Select the **Scaling logarithmic** or **linear** option.

   Click next to the selection window. The changes are applied.



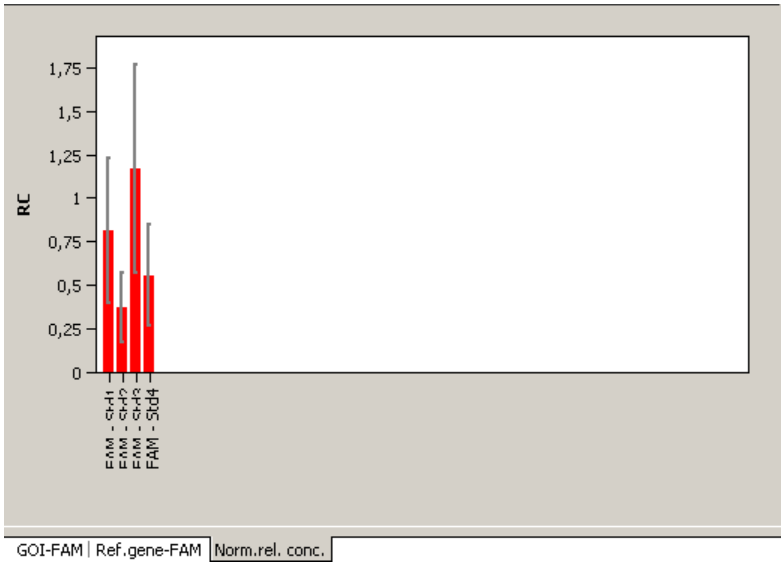
Linear representation of the fluorescence curve for the relative quantification



Logarithmic representation of the fluorescence curve for the relative quantification with horizontal threshold line

5.3.4 Display of the normalised relative concentrations

After selecting the tab **Norm. rel. conc.** the display changes to a presentation of the results as bar chart. The respective sample name is given below each bar. The height of the bar is determined by the mean Ct-value or the calculated mean concentration of replicates. For each bar some short information about the position of the samples, the mean value and the calculated standard deviation is shown if the mouse pointer is placed on it. The size of the standard deviation is shown as error bar on top of each bar. Since for large numbers of samples not all bars can be displayed in the screen the diagram can be moved horizontally with pressed left mouse button and dragged to the desired position.



Bar chart for display of normalised relative concentrations

5.3.5 Displaying the standard curves and the results of a relative quantification

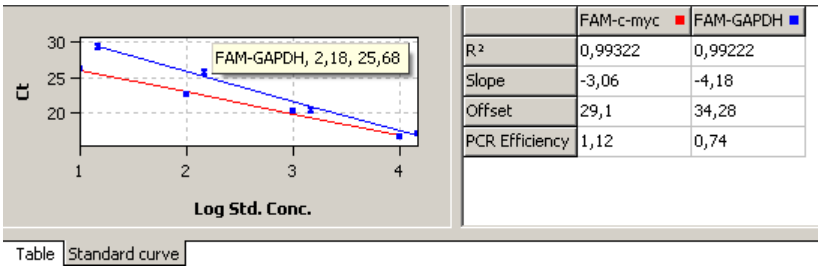
In the upper area of the project window you can switch between the display of the calculated standard curves for the target gene and all selected reference genes and the result table for the relative quantification using the **Standard curve** and **Table** list sheets.

For the display of the standard curve the Ct values of the standard samples are plotted graphically against the logarithm of their concentration. For the respective data points errors bar are shown that indicate the standard deviation between replicates. For each data point some short information about the sample name and the size of the standard deviation of replicates is shown if the mouse pointer is placed on it. In the value range on the right the following calculated data is displayed:

- the coefficients of determination R^2 of the linear equation
- the standard curve gradients
- the intersections of the curves with the y-axis at x=0 (offset)
- the PCR efficiency

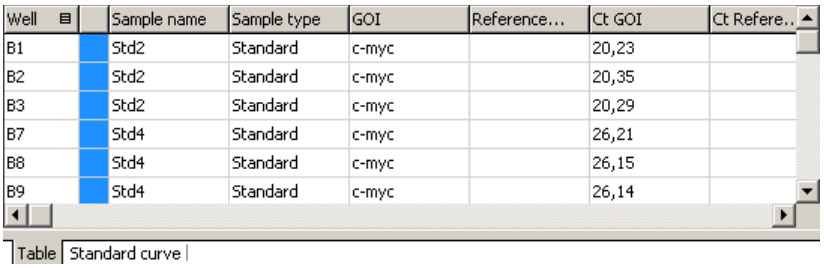
When more than one standard curve is displayed each curve has its individual color. Accordingly each table has a color code in the header that reflects the assignment to the respective standard curve. The standard curve and the values are automatically

calculated by the qPCRsoft software and updated in case of settings modifications. According to the number of genes used a scroll bar appears under the table. It can be used to navigate through the table's columns.



Standard curves for relative quantification

The result table for the relative quantification contains all data and the associated measurement values for the samples.



Well		Sample name	Sample type	GOI	Reference...	Ct GOI	Ct Refere...
B1		Std2	Standard	c-myc		20,23	
B2		Std2	Standard	c-myc		20,35	
B3		Std2	Standard	c-myc		20,29	
B7		Std4	Standard	c-myc		26,21	
B8		Std4	Standard	c-myc		26,15	
B9		Std4	Standard	c-myc		26,14	

Result table for relative quantification

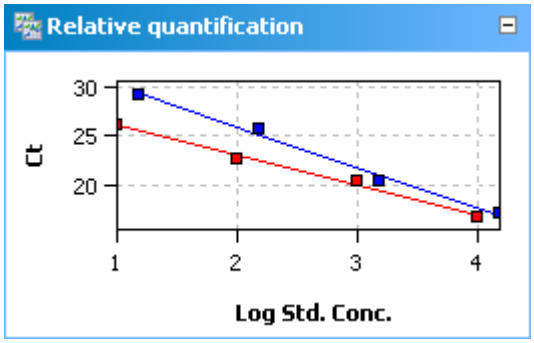
For the relative quantification the results table contains the following information:

Column	Meaning
Well	Position of sample
Color of curve	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
Sample name	Name of sample
Sample type	Type of sample
Group	Assignment of the sample to an experimental group
GOI	Gene of interest
Reference gene	Reference gene
Ct GOI	Ct value of gene of interest
Ct Reference gene	Ct value of reference gene
Mean. Ct GOI	Mean Ct value of replicates of the gene of interest
Mean. Ct Ref.gen	Mean Ct value of replicates of the reference gene
Conc. Std. GOI	Concentration of the standard for the gene of interest
Conc. Std Ref.gen	Concentration of the standard for the reference gene
Mean Conc. GOI	Concentration for the gene of interest determined from the standard curve on the basis of the mean Ct value

Mean Conc.. Ref. gen	Concentration for the reference gene determined from the standard curve on the basis of the mean Ct value
Std.Dev. Ct GOI	Standard deviation of the Ct values between replicates of the gene of interest
Std.Dev. RefGen	Standard deviation of the Ct values between replicates of the reference gene
%CV Ct GOI	Variation coefficient of the Ct values between replicates of the gene of interest
%CV Ct RefGen	Variation coefficient of the Ct values between replicates of the reference gene
Relative Conc.	Relative (x-fold) expression level of the gene of interest in relation to the reference gene
Norm. Rel. Conc.	Relative (x-fold) expression level of the gene of interest in relation to the reference gene, standardized to the expression of the calibrator (if defined)
Std.Dev. Relative Conc.	Standard deviation of the relative concentrations
Std.Dev. Norm. Rel. Conc.	Standard deviation of the normalised relative concentrations

Display in the project explorer

A shortened representation of the standard curves calculated by the software is displayed in the project explorer under **Relative quantification**. The image displays the graphical plot of the Ct values of the standard samples against the logarithm of their concentration.



Representation of the standard curves in the project explorer

5.3.6 Importing the standard curve for relative quantification

Next to the option to measure a standard curve in the experiment, the qPCRsoft software can also be used to determine the concentration of the samples based on the saved standard curve. You can use the import function for this purpose.

1. Use the symbol in the toolbar to open the **Import standard curve** window.

   Optionally, you can call up the **RelQuant ▸ Import standard curve** menu command.

2. All additional setting are analogous to the settings for the absolute quantification (→ section "Importing the standard curve" p. 78).

5.3.7 Deleting the evaluation of a relative quantification

An evaluation that is no longer required can be removed.

1. Activate the evaluation by selecting its description in the evaluation list of the method tab.
2. Click on the symbol in the toolbar.
   Alternatively, call up the **RelQuant ▸ Delete rel. quantification** menu command.

   The evaluation is removed.

5.4 ΔΔCt method

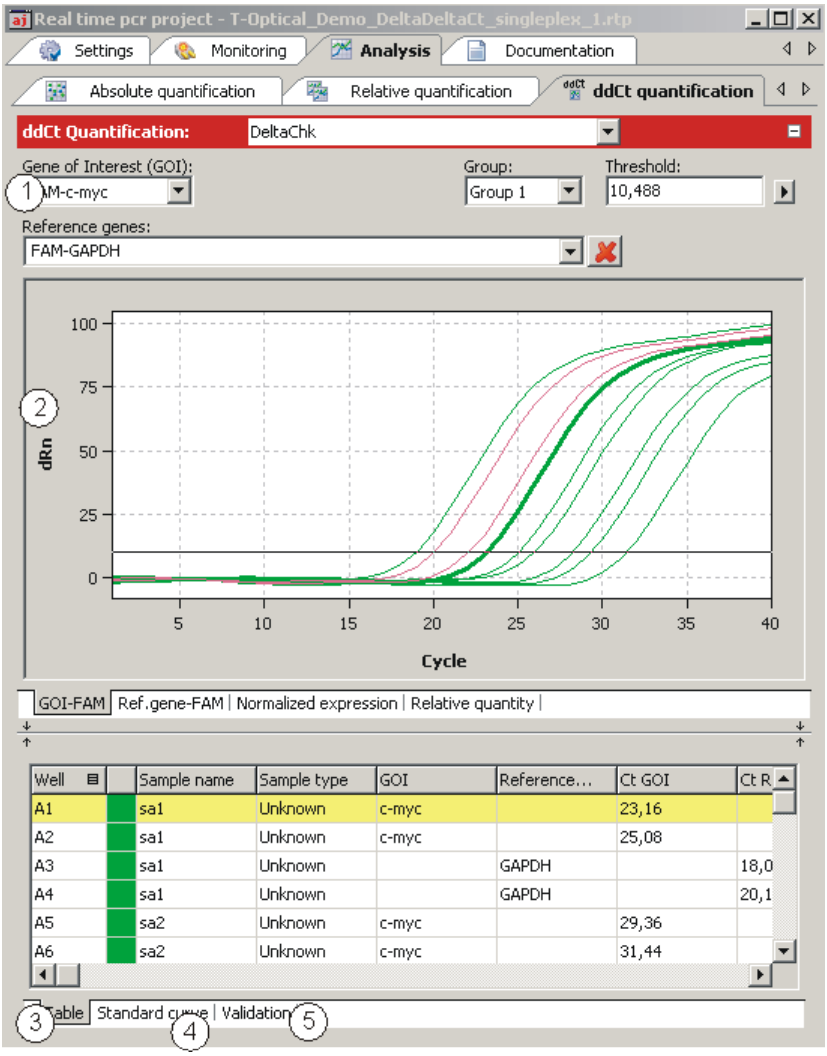
The ΔΔCt-method allows for determination of the relative expression level of a gene of interest in relation to one or more reference genes (often housekeeping genes). One of the samples has to be denoted as the calibrator. The expression level ofthe calibrator is set to unity and the relative expression levels of all the other samples are given in relation to the calibrator sample. In ΔΔCt-method there is no need to measure standard dilution series. However, if the ΔΔCt-method shall be validated within the same PCR run standard dilution series must be defined.

5.4.1 Creating a new evaluation for a ΔΔCt method

1. Go to the **Analysis / ddCt quantification** project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the symbol in the toolbar.
   Alternatively, call up the **DeltaDeltaCt / Add ddCt quantification** menu command.
3. An input window appears. Enter the description for the current evaluation.

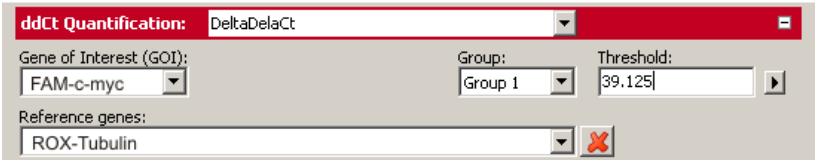
On the **DeltaDeltaCt** tab the following information is activated:

- Parameter settings (1)
- Display of the fluorescence curves for the target gene and the reference gene (2)
- Display of the result table with the added values (3)
- If standards have been defined, display of the standard curve (4) and the validation curve (5) for the expression level of the gene of interest in relation to the reference gene and the calculated factors



Project window for the ΔΔCt method

5.4.2 Parameter settings for the ΔΔCt method



Parameter settings for the ΔΔCt method

The following parameters must be set for the ΔΔCt method:

Option	Description
Selection list	Selection of an evaluation created for the experiment
Gene of interest (GOI)	Selection list of combinations of measured dyes and target genes to be quantified Only one target gene at a time can be selected.

Reference genes	Selection list for the reference genes In contrast to the target gene, several reference genes can be selected at the same time. The number of list sheets in the display range therefore grows with each reference gene. With the symbol you can remove all selected reference genes from the selection.
Group	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed. (→ section "Defining groups" p. 50).
Threshold	Manually adjust threshold values The threshold value must be between 1 and 100, depending on the standardized representation of the fluorescence curves (dRn). Note: The threshold value can be calculated automatically or set in the graph.
	Opens the selection window with display options

Setting the threshold value

To determine Ct values for the evaluation, a threshold value needs to be determined for each experiment first.

You have several options for setting the threshold value:

- In the general options (→ section "Making basic settings" p. 66).
- Manually in the parameters for the respective evaluation (see table above)
- Graphically in the fluorescence curves representation:
  In the chart, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Ct values in the result table are updated.

  Note:
  Due to the further spread of the early exponential area of the product accumulation curves, a logarithmic representation is better suited for setting the threshold manually in the display range than a linear representation.
- By having it calculated automatically:

  The automatic calculation of the threshold value is activated by clicking on the symbol.
  Optionally, you can call up the **DeltaDeltaCt ▸ Autom. Threshold** menu command.

Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the corresponding **Threshold iput** field.

Fix Threshold

The threshold value becomes recalculated by the software each time basic settings for the analysis are changed. For the analysis the option **Fix Threshold** can be used, so that the threshold value is retained if the basic settings for the analysis are changed (→ section "Making basic settings" p. 66).

5.4.3 Displaying the fluorescence curves for the ΔΔCt method

In the display range, the measured data, standardized to the value 100 for highest fluorescence intensity, is plotted against the cycle for the selected target gene. The

gene/dye and the reference gene/dye combinations are each assigned a list sheet that can be activated by clicking on the gene/dye tab on the bottom.

In the evaluation you can always use only one target gene but several reference genes. The number of the available list sheets depends on the number of the selected genes.

The fluorescence data is displayed as a linear or logarithmic representation depending on the selected display option. For both display options the program shows brief information on the sample if the cursor is placed on one of the curves.

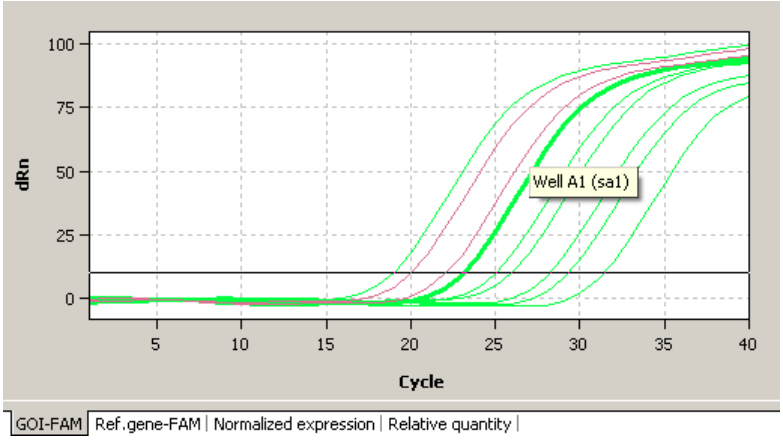
Switching the display options for the chart

1. Click on the button in the parameter bar.

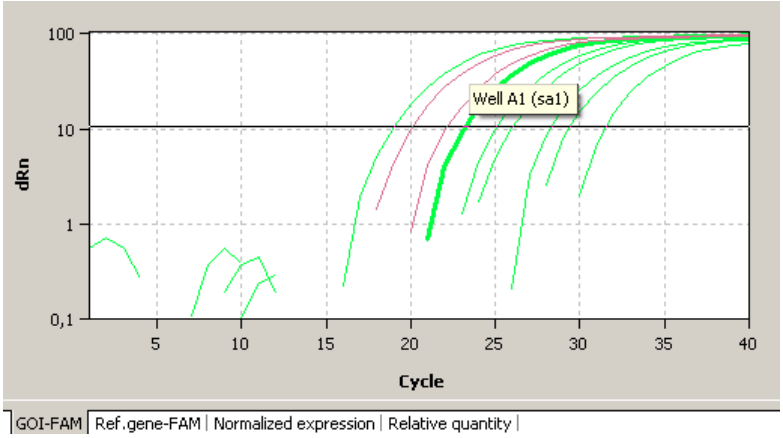
   A selection window for the display options opens.

2. Select the **Scaling logarithmic** or **linear** option.

   Click next to the selection window. The changes are applied.



Linear representation of the fluorescence curve for the ΔΔCt method

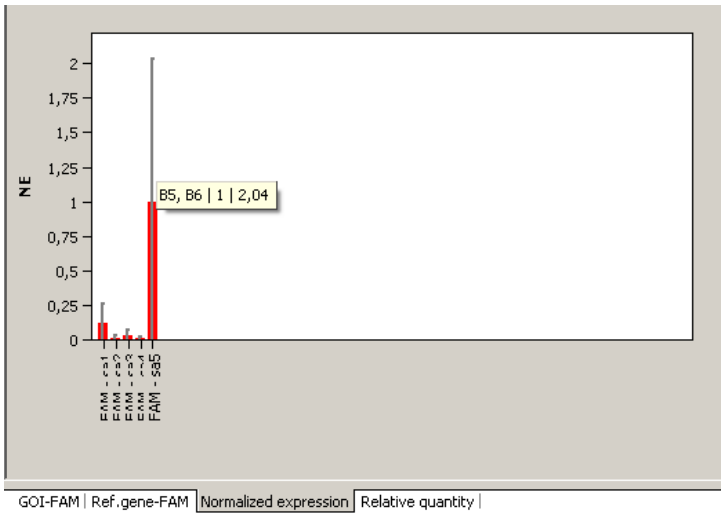


Logarithmic representation of the fluorescence curve for the ΔΔCt method

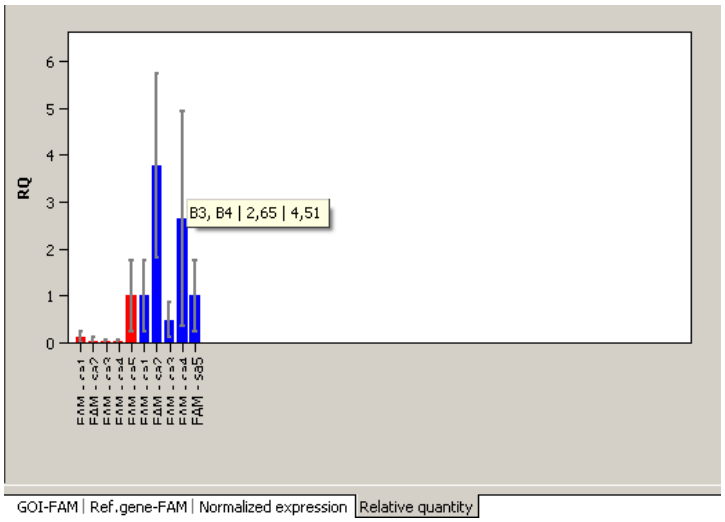
5.4.4 Display of the normalized relative expression or relative quantities

After selecting the tab **Normalized Expression** or **Relative Quantity** the display changes to a presentation of the results as bar chart. The respective sample name is given below each bar. The height of the bar is determined by the calculated normalized expression or the calculated relative quantity of replicates. For each bar some short information about the position of the samples, the mean value and the calculated standard deviation is

shown if the mouse pointer is placed on it. The size of the standard deviation is shown as error bar on top of each bar. For the representation of the relative quantities the results for the target gen and reference gene the bars are colored differently. Since for large numbers of samples not all bars can be displayed in the screen the diagram can be moved horizontally with pressed left mouse button and dragged to the desired position.



Bar chart for display of normalized expression of replicates



Bar chart for display of relative quantities of replicates

5.4.5 Setting the calculation mode for the standardized expression

The qPCRsoft software enables you to calculate the standardized expression SE with two different methods:

- Without PCR efficiency calculation (Livak method)
- With PCR efficiency calculation for GOI and reference genes (Pfaffl method)

To calculate the standardized expression SE, one sample must be defined as the calibrator.

Calculating the standardized expression SE without efficiency calculation (Livak method)

The Livak method assumes that the PCR efficiencies of the gene of interest (GOI) and the reference gene (RefG) are equal. The following applies:

$$NE = 2^{-\Delta\Delta Ct}$$

where $\Delta\Delta Ct = \Delta Ct(Calibrator) - \Delta Ct(Sample)$

and $\Delta Ct(Sample) = Ct(GOI, Sample) - Ct(RefGene, Sample)$

$\Delta Ct(Calibrator) = Ct(GOI, Calibrator) - Ct(RefGene, Calibrator)$

Calculating the standardized expression SE with efficiency calculation (Pfaffl method)

The Pfaffl method considers the efficiencies determined for the gene of interest (GOI) and the reference gene (RefGene). The efficiencies (E(GOI) and E(RefGene)) can be calculated from dilution series or specified in the software. The following applies:

$$NE = \frac{[1 + E(GOI)]^{\Delta Ct(GOI)}}{[1 + E(RefGene)]^{\Delta Ct(RefGene)}}$$

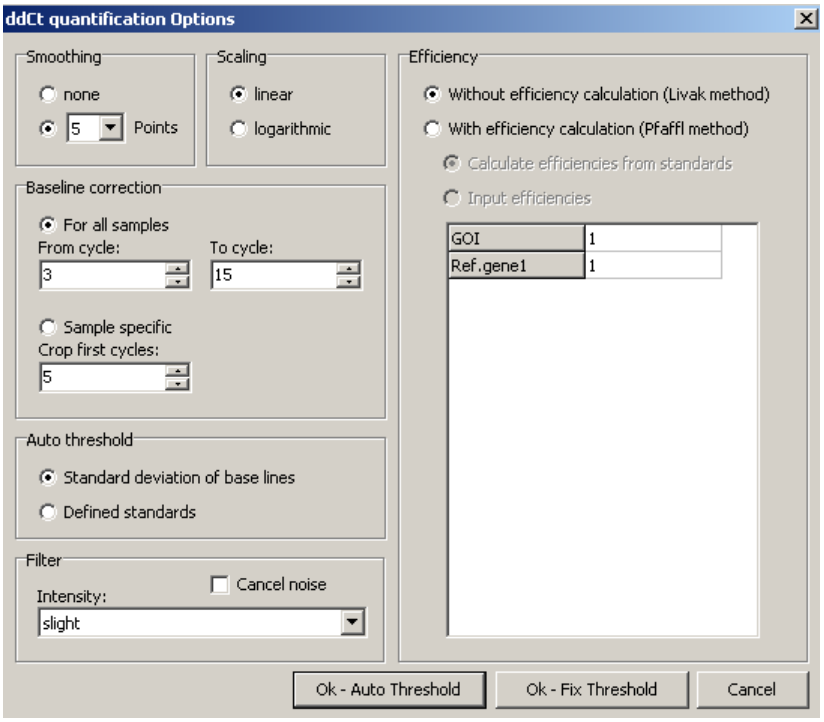
where $\Delta Ct(GOI) = Ct(GOI, Calibrator) - Ct(GOI, Sample)$

and $\Delta Ct(RefGene) = Ct(RefGene, Calibrator) - Ct(RefGene, Sample)$

The Pfaffl method is generally preferred as the basic assumption of the Livak method (equal efficiency when amplifying the gene of interest and the reference gene) is rarely the case in practice and the calculation can therefore lead to incorrect values.

The calculation method is selected in the **ddCt Options** window.

- Click on the symbol in the toolbar.
  Alternatively, call up the **DeltaDeltaCt** **ddCt Quant. options** menu command.
- Select the desired calculation method.
  For the Pfaffl method, the efficiency values can be determined automatically from the standard curves (dilution series) for the gene of interest and the reference gene (if standards have been defined) or the values can be entered manually in the respective fields.



ddCt Options window for presetting the analysis of the ΔΔCt method

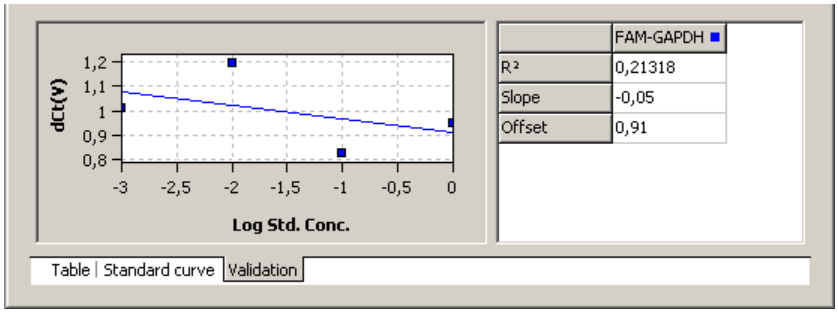
5.4.6 Displaying the validation curves and values

To calculate the ΔΔCt values there is no need to determine a validation curve. However, it might be useful to consult it when checking the data quality.Prerequisite for creating a validation curve is measuring a standard series with different dilution levels of target gene and reference gene. If standard series have been measured for the target and reference gene, the expression ratio between target and reference gene is represented graphically in the **Validation curve** display. For this purpose, the mean Ct value of the target gene is subtracted from the mean Ct value of the reference gene for the corresponding dilution level and the resulting dCt(V) value is plotted against the logarithm of the concentration.

In the value range on the right the following calculated data is displayed:

- The coefficient of determination R^2
- The gradient of the validation straight line
- the intersection of the curve with the y-axis at x=0 (offset)

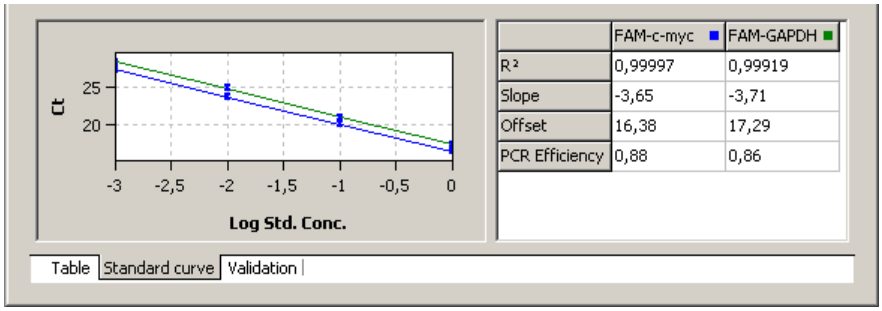
The gradient of the curve should not exceed a value of ± 0.1. The assumption then applies that the efficiencies of the amplification of the gene of interest and the reference gene are almost identical and the calculation of the ΔΔCt values produces valid data.



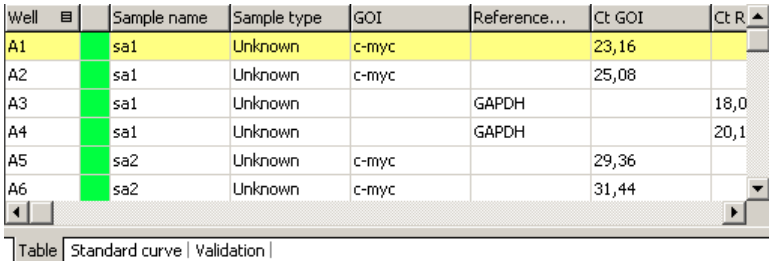
Validation curves view for the ΔΔCt method

You can switch between the ΔΔCt calculation views using the Table, Standard curve and Validation tabs. The validation curves and the values are automatically calculated by the qPCRsoft software and updated if the settings change. For both, validation curves as well as standard curves the respective data points errors bar are shown that indicate the standard deviation between replicates. For each data point some short information about the sample name and the mean Ct value of replicates is shown if the mouse pointer is placed on it.

When more than one standard curve is displayed each curve has its individual color. Accordingly each table has a color code in the header that reflects the assignment to the respective standard curve.



Validation curves view for the ΔΔCt method



Well		Sample name	Sample type	GOI	Reference...	Ct GOI	Ct R
A1		sa1	Unknown	c-myc		23,16	
A2		sa1	Unknown	c-myc		25,08	
A3		sa1	Unknown		GAPDH		18,0
A4		sa1	Unknown		GAPDH		20,1
A5		sa2	Unknown	c-myc		29,36	
A6		sa2	Unknown	c-myc		31,44	

Result table view for the ΔΔCt method

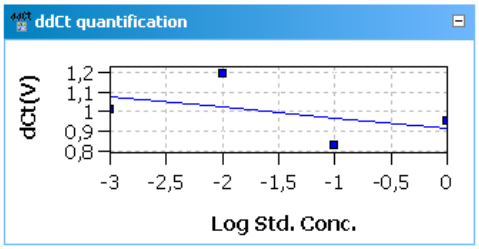
For the ΔΔCt-quantification the results table contains the following information:

Column	Meaning
Well	Position of sample*
Color of curve	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
Sample name	Name of sample*
Sample type	Type of sample*
Group	Assignment of the sample to an experimental group*

GOI	Gene of interest
Reference gene	Reference gene
Ct GOI	Ct value of gene of interest
Ct Ref.gene	Ct value of reference gene
Mean Ct GOI	Mean Ct value of replicates of the gene of interest
Mean Ct Ref.gene	Mean Ct value of replicates of the reference gene
Std.Dev. Ct GOI	Standard deviation of the Ct values between replicates of the gene of interest
Std.Dev. Ct Ref.gene	Standard deviation of the Ct values between replicates of the reference gene
%CV Ct GOI	Variation coefficient of the Ct values between replicates of the gene of interest
%CV Ct Ref.gene	Variation coefficient of the Ct values between replicates of the reference gene
dCt GOI	Delta Ct value for replicates of the gene of interest
dCt Ref.gene	Delta Ct value for replicates of the reference gene
RQ GOI	Calculated relative amount for replicates of the gene of interest in the original sample
RQ Ref.gene	Calculated relative amount for replicates of the reference gene in the original sample
Mean RQ Ref.gene	Average calculated relative amount for replicates of the reference gene in the original sample
Norm. Expression	Standardized relative (x-fold) expression level of the gene of interest in the sample in relation to the calibrator

Display in the project explorer

A shortened representation of the validation curves calculated by the software is displayed in the project explorer under **DeltaDeltaCt**. The image displays the graphical plot of the dCt(V) values against the logarithm of the sample concentration:



Representation of the validation curves in the project explorer

5.4.7 Deleting a ΔΔCt method evaluation

An evaluation that is no longer required can be removed.

1. Activate the evaluation by selecting its description in the evaluation list of the method tab.

2. Click on the symbol in the toolbar.
   Optionally, call up the **DeltaDeltaCt ▸ Delete ddCt quantification**
3. menu command.

   The evaluation is removed.

5.5 Melting curve analysis

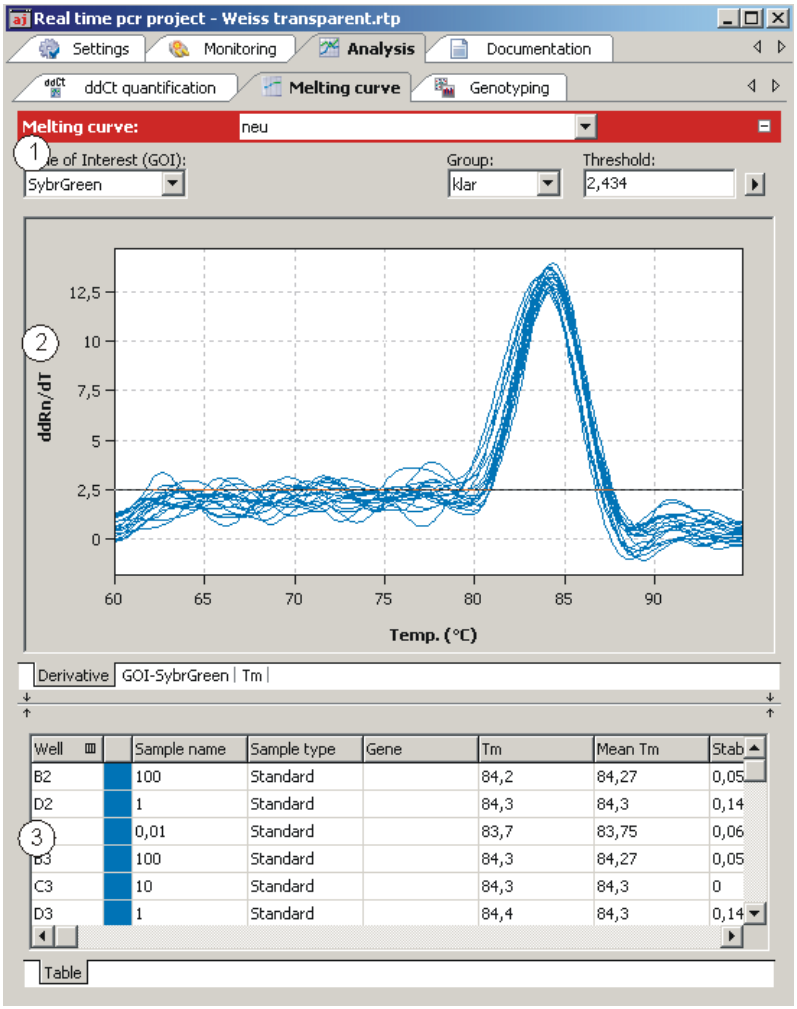
When performing a melting curve analysis the temperature in the reaction mixture is increased successively until the PCR product is denatured. The dissociation of the fragment in single strands will result in the release of a intercalating dye. The associated reduction of the fluorescence intensity is measured and recorded by the device. By forming the first derivative of the fluorescence curve you will get a peak that describes the melting point and the approximate concentration of the PCR fragment. By means of melting curve analysis you can differentiate whether the reaction has caused the formation of a specific PCR product or whether unspecific by-products, such as primerdimers, were produced.

5.5.1 Creating a new melting curve analysis

1. Go to the **Analysis ▸ Melt. curve** project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the symbol in the toolbar.
   Optionally, call up the **Melting curve ▸ Add melting curve**menu command.
3. An input window appears. Enter the description for the current evaluation.

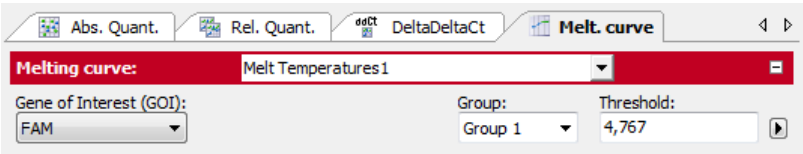
On the **MeltCurve** tab the following information is activated:

- Parameter settings (1)
- Display of the fluorescence values as a function of the temperature and/or its first derivative (2)
- Display of the result table (3)



Window for melting curve analysis

5.5.2 Parameter settings for melting curve analysis



Parameter settings for melting curve quantification

Set the following parameters for the meltings curve quantification:

Option	Description
Selection list	Selection of a selection created for the experiment
Gene of interest (GOI)	Selection list of target gene/ dye combinations Generally, an intercalating dye must be selected for the target gene for the melting curve analysis. According to the selection, the fluorescence and regression curves for the concentration are displayed.
Group	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed. (→ section"Defining groups" p. 50).

Threshold	Manually adjust threshold values The threshold is now effective on the **Derivative** tab. Only curves whose maximum dRn/dT is greater than the threshold are analyzed. **Note:** The threshold value can be calculated automatically or set manually in the chart (see also "Setting the threshold value" below).
	Opens the selection window with display options

Setting the threshold value

For the correct evaluation a threshold value must be determined for the melting curves.

You have several options for setting the threshold value:

- In the general options (→ section "Making basic settings" p. 66)
- Manually in the parameters for the respective evaluation (see table above)
- Graphically in the representation of the fluorescence curves derivative:
  In the chart**Derivative**, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Tm values in the result table are updated.
- Automatic calculation:

  The automatic calculation of the threshold value is activated by clicking on the symbol.
  Optionally, you can call up the **Melting curve / Autom. Threshold** menu command.

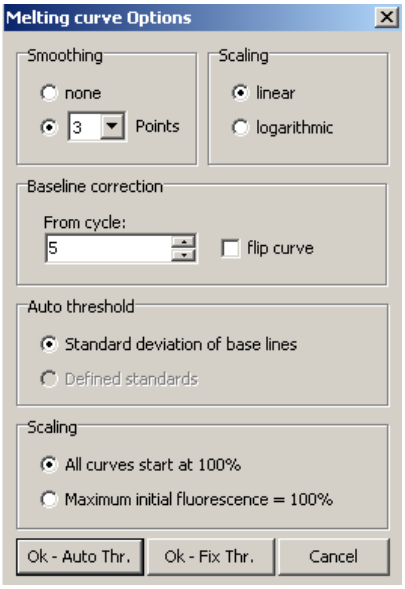
Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the corresponding **Threshold iput** field.

Fix Threshold

The threshold value becomes recalculated by the software each time basic settings for the analysis are changed. For the analysis the option **Fix Threshold** can be used, so that the threshold value is retained if the basic settings for the analysis are changed (→ section "Making basic settings" p. 66).

5.5.3 Displaying fluorescence curves/melting curves

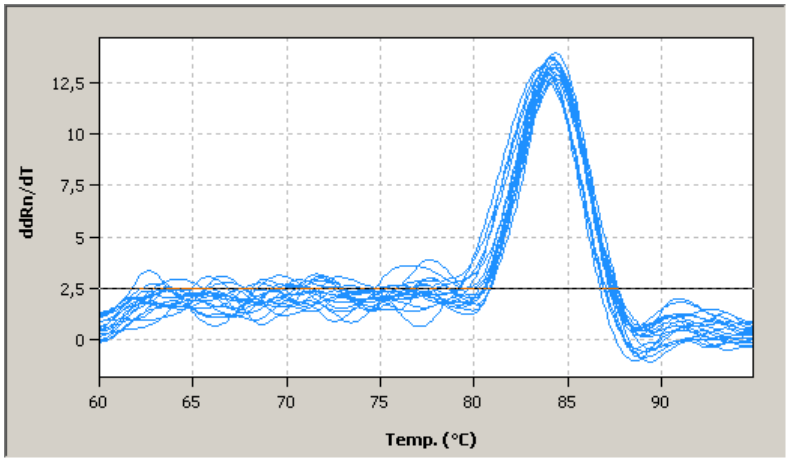
In the display area, the measured fluorescence curves are shown in relation to the temperature and are either standardized to the highest fluorescence value or both standardized to a target value of 100, depending on the settings made in the **Melt. Curve Options** window. The data is displayed as a linear or logarithmic representation depending on the selected display option.



Display options for melting curve analysis

The program shows brief information on the sample if the cursor is placed on one of the curves.

The melting temperature Tm is determined by forming the first derivative of the melting curves from the maxima of the forming peaks. To evaluate fluorescence data from protein stability measurements, the melting curves can be reversed. The reversal of the melting curves can be activated via the **flip curve** option.



View of the melting curves derivative with threshold line

You can switch between the melting curves display and the derivatives via the tab in the bottom left corner of the display range.

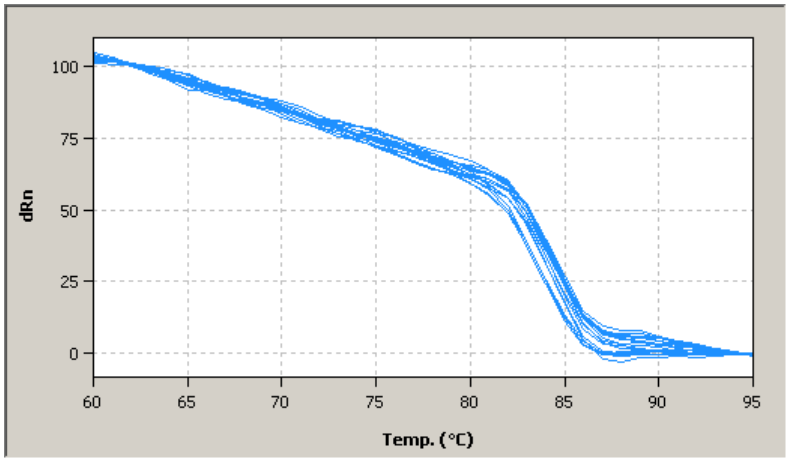
Switching the display options for the chart of fluorescence curves

1. Click on the button in the parameter bar.

   A selection window for the display options opens.

2. Select the **Scaling logarithmic** or **linear** option.

   Click next to the selection window. The changes are applied.
   The **Derivative** tab cannot be displayed logarithmically.

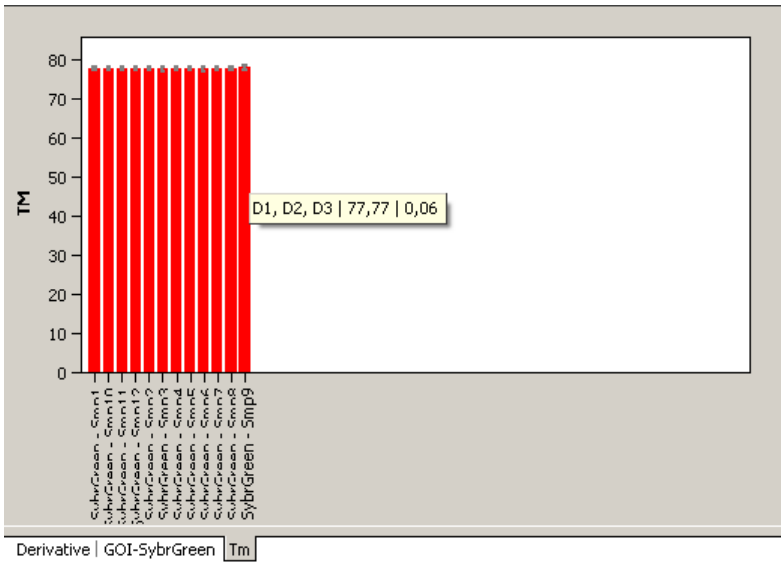


Fluorescence curves display (linear view)

The linear display does not show a threshold line.

5.5.4 Display of melting temperatures

After selecting the tab TM the display changes to a presentation of the results as bar chart. The respective sample name is given below each bar. The height of the bar is determined by the measured melting temperature of replicates. For each bar some short information about the position of the samples, the mean value and the calculated standard deviation is shown if the mouse pointer is placed on it. The size of the standard deviation is shown as error bar on top of each bar. Since for large numbers of samples not all bars can be displayed in the screen the diagram can be moved horizontally with pressed left mouse button and dragged to the desired position.



Bar chart for display of melting curves

5.5.5 Displaying the result table for the melting curves

The result table for melting curves contains all data and the associated measurement values for the samples.

Well		Sample name	Sample type	Gene	Tm	Mean Tm	Stab
B2		100	Standard		84,2	84,27	0,05
D2		1	Standard		84,3	84,3	0,14
F2		0,01	Standard		83,7	83,75	0,06
B3		100	Standard		84,3	84,27	0,05
C3		10	Standard		84,3	84,3	0
D3		1	Standard		84,4	84,3	0,14

Table

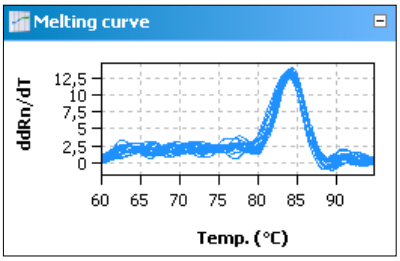
Result table for melting curve analysis

For melting curves the results table contains the following information:

Column	Meaning
Well	Position of sample
Color of curve	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
Sample name	Name of sample
Sample type	Type of sample
Group	Assignment of the sample to an experimental group
Gen e	Name of gene measured in the sample
Tm	Melting temperature of the sample
Mean Tm	Mean melting temperature of replicates
Stabw. Mittl. Tm	Standardabweichung der durchschnittlichen Schmelztemperatur von Replikaten

Display in the project explorer

A shortened representation of the melting curves calculated by the software is displayed in the project explorer under "Melt Curve". This representation shows the graphical plot of the first derivative of the fluorescence values against the temperature.



Representation of the melting curve derivatives in the project explorer

5.5.6 Deleting a melting curve analysis

A melting curve analysis that is no longer required can be removed.

1. Activate the evaluation by selecting its description in the evaluation list of the method tab.
2. Click on the symbol in the toolbar.
   Alternatively, call up the **Melting curve** ▸ **Delete melting curve** menu command.

   The evaluation is removed.

5.6 Genotyping

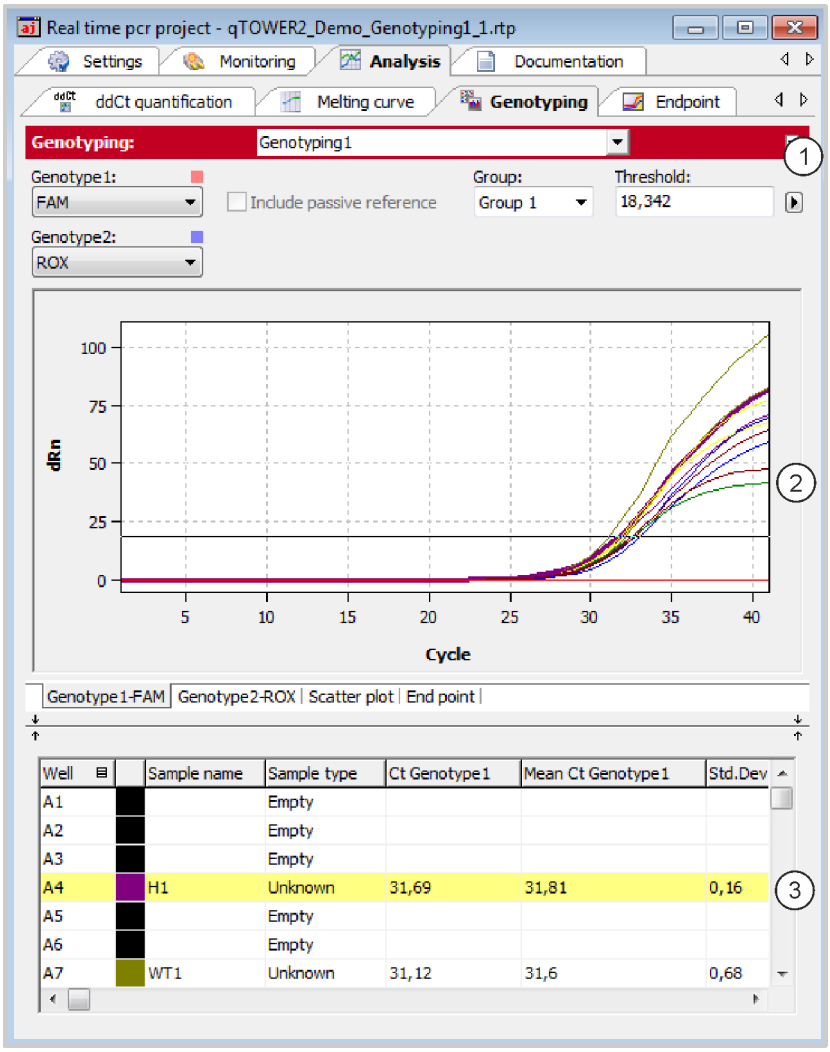
Genotyping serves to determine sequence differences between a sample and a standard. The standard is defined as the reference sequence (genotype 1); the genetic condition of the sample is to be determined in the experiment. Genotyping shows which alleles an individual has inherited from its parents.

5.6.1 Creating a new evaluation for genotyping

1. Go to the **Analysis / Genotyping** project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the symbol in the toolbar.
   Alternatively, call up the **Genotyping / Add genotyping** menu command.
3. Enter the description for the current evaluation in the input window.

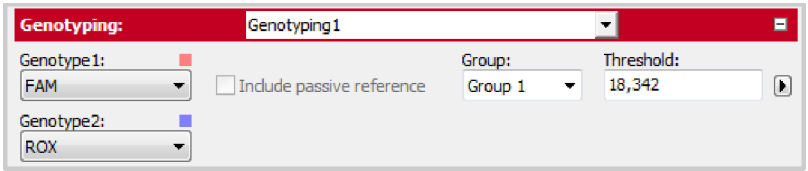
The following information is activated on the **Genotyping** tab:

- Parameter setting for defining the threshold and selecting the dye with which the wild type and the mutant were measured (1)
- Display of the fluorescence curves of wild type and mutantand/or display of the results as a scatter plot or bar graph
- Display of the result table with the results (3)



Genotyping window

5.6.2 Parameter settings for genotyping



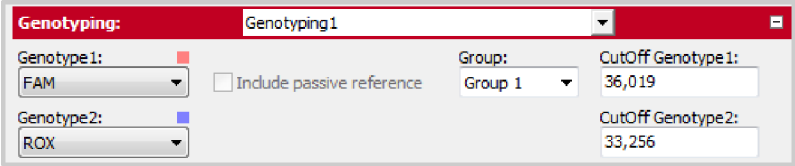
Parameter settings for genotyping for the fluorescence curve display

Set the following parameters for the absolute quantification:

Option	Description
Selection list	Selection of an evaluation created for the experiment
Genotype 1	Selection list for the dye with which the genotype 1 was measured.
Genotype 2	Selection list for the dye with which the genotype 2 was measured.

Include passive reference	Only active if a dye has been defined as a passive reference on the **Settings / Scan** project tab. If this option is activated, the fluorescence of the dye that has been set as a passive reference is used for standardization.
Group	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed (→ section "Defining groups" p. 50).
Threshold	Manually adjust threshold value. The threshold value must be between 1 and 100, depending on the standardized representation of the fluorescence curves (dRn). **Note** The threshold value can be calculated automatically or set manually in the chart (→ see also "Setting the threshold value" below).
▶	Opens the selection window with display options (→ section "Displaying the fluorescence curves for absolute quantification" S. 73).

When changing to the scatter plot or end point bar graph, two fields are displayed for the **CutOff** value for the **Genotype 1** and the **Genotype 2** instead of the Threshold field:



Parameter settings for the scatter plot and the bar graph of a genotyping

Setting the threshold value

To determine Ct values for the evaluation a threshold value must be determined for each experiment.

You have several options for setting the threshold value:

- In the general options (→ section "Making basic settings" p. 66)
- Manually in the parameters for the respective evaluation (see table above)
- Graphically in the fluorescence curves representation:
  In the chart, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Ct values in the result table are updated.

  Note:
  The logarithmic view is more suitable for placing the threshold manually in the display area than the linear view because of the wider spread of the early exponential area of the product accumulation curves.
- By having it calculated automatically:

  To activate the automatic calculation of the threshold value, click on the symbol. Alternatively, you can call up the **Genotyping / Autom. threshold** menu command.

Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the **Threshold** input field.

Fix Threshold

The threshold value becomes recalculated by the software each time basic settings for the analysis are changed. For the analysis the option **Fix Threshold** can be used, so that

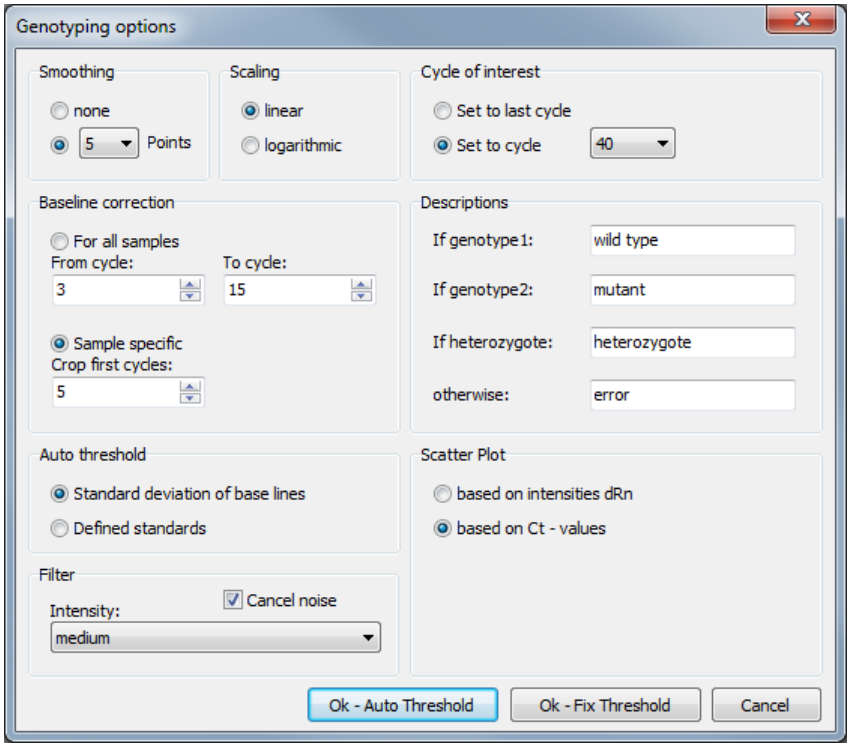
the threshold value is retained if the basic settings for the analysis are changed (→ section "Making basic settings" p. 66).

5.6.3 Specifying genotyping options

Special evaluation options are available for genotyping.

- Click on in the toolbar to open the **Genotyping Options** window.

  Alternatively, you can call up the **Genotyping / Genotyping Options** menu command.



GenoTyping Options window for genotyping settings

Option	Description
Cycle of interest	Select the cycle for evaluation. This can preferably be the last cycle (end point) but also any other cycle of the PCR run. The respective cycle can be selected from a list.
Name	Input fields for own names for the genotype1, genotype 2, heterozygote categories or otherwise
Scatter Plot	Generation of the scatter plot based on the fluorescence intensities of the analyzed cycle and/or the Ct values

5.6.4 Displaying the fluorescence curves, scatter plot and bar graph

The respective combination of genotype 1 dye or genotype 2 dye displayed is shown on the tabs in the bottom left corner of the area. The entry for the respective active combination on the tab is highlighted in white. The fluorescence data is displayed as a linear or logarithmic representation depending on the selected display option. For both

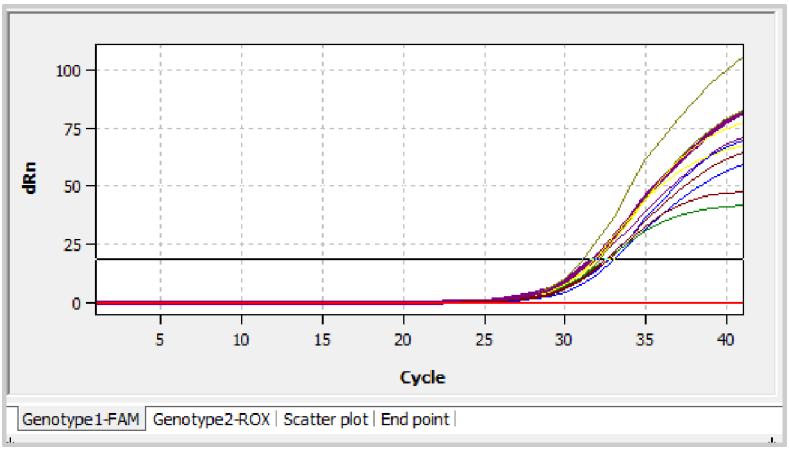
view types a brief information is shown as soon as the mouse pointer is positioned on one of the curves.

1. Click on the button in the parameter bar.

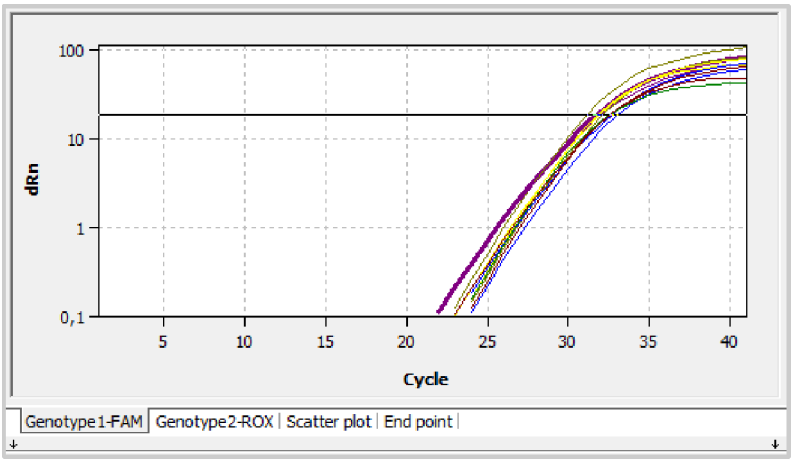
   A selection window for the display options opens.

2. Select the **Scaling logarithmic** or **linear** option.

   Click next to the selection window. The changes are applied.



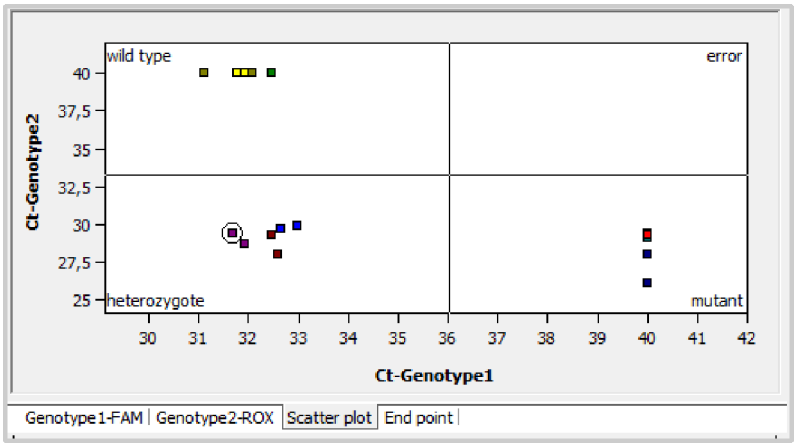
Linear representation of the fluorescence curve for genotyping



Logarithmic representation of the fluorescence curve with horizontal threshold line

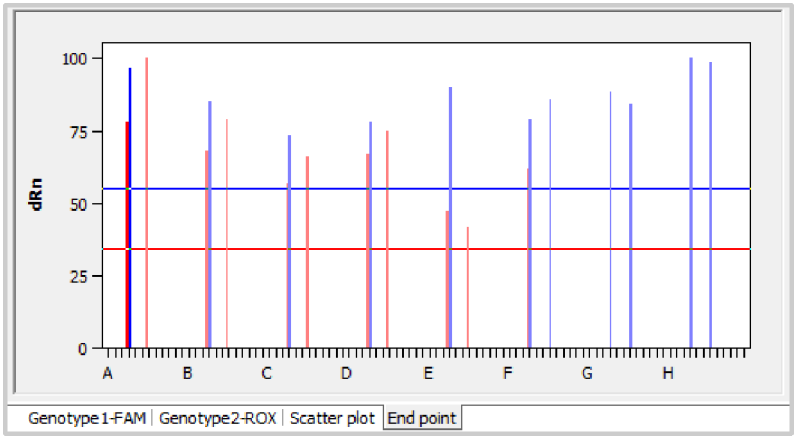
To determine Ct values for the analysis a threshold value must be determined for the fluorescence curves. This can be automated but is also possible manually in the display area (→ section "Parameter settings for genotyping" p. 102).

In addition to the fluorescence curves representation, the results can also be displayed as a scatter plot or bar graph. This can be selected via the respective tabs below the display area.



Scatter plot for genotyping

The scatter plot is divided into four quadrants for genotype1, genotype 2, heterozygote and error. The samples are assigned to one of the quadrants based on the measured relative fluorescence or the Ct values of the two dyes. The respective cutoff value for the sample assignment is represented by two black lines in the scatter plot view. To change the position of the lines and thus change the cutoff value, select the lines and keep the left mouse button pressed. Alternatively, the respective cutoff value for genotype 1 and genotype 2 can also be entered in the respective fields in the selection area (→ "Parameter settings for genotyping" p. 102).



Bar chart for genotyping

The bar graph shows the measured relative fluorescences as bars. The x-axis is scaled from A to H based on the rows of the block, i.e. the first twelve samples correspond to positions A1 to A12 in the block, the next twelve samples correspond to positions B1 to B12 etc. The cutoff value is set by moving the red or blue horizontal line up or down with the left mouse button pressed. Alternatively, the respective cutoff value for mutant and wild type can also be entered in the respective fields in the selection area of this view (→ "Parameter settings for genotyping" p. 102).

The cutoff values are thresholds after which the question whether a sample shows a reaction is answered with "Yes" (see **Reaction genotype 1** and **Reaction genotype 2** table columns).

5.6.5 Display of the values for the genotyping evaluation

The result table for the genotyping combines all data and corresponding measurements for the samples. The columns shown in the result table differ depending on the tab selected in the display area. The table for the fluorescence curves provides a summary which includes the measuring data of both dyes. If the fluorescence intensity at the end point is evaluated, the result table for the scatter plot is the same as the one for the bar graph. The data summarized in the result table for the fluorescence curves, however, partly differ from the data in the scatter plot or bar graph evaluation.

Well		Sample name	Sample type	Genotyp	Reaction ...	Reaction ...	Genc
A4		H1	Unknown	heterozygote	yes	yes	hete
B4		H1	Unknown	heterozygote	yes	yes	hete
C4		H2	Unknown	heterozygote	yes	yes	hete
D4		H2	Unknown	heterozygote	yes	yes	hete
E4		H3	Unknown	heterozygote	yes	yes	hete
F4		H3	Unknown	heterozygote	yes	yes	hete
G4		M1	Unknown	mutant	no	yes	muta

Results table for genotyping

For the genotyping the results table contains the following information:

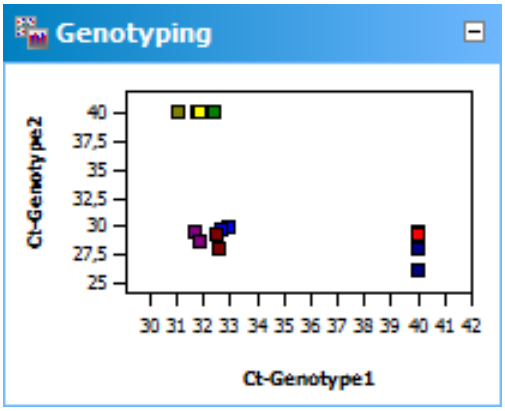
Column	Meaning
Well	Position of sample*
Color of curve	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
Sample name	Name of sample*
Sample type	Type of sample*
Group	Assignment of the sample to an experimental group*
Ct Genotype 1	Ct value of genotype 1
Mean Ct Genotype 1	Mean Ct value of replicates of the genotype 1
Std.Dev. Ct Genotype 1	Standard deviation of the Ct values between replicates of the genotype 1
Ct Genotype 2	Ct value of genotype 2
Mean Ct Genotype 2	Mean Ct value of replicates of the genotype 2
Std.Dev. Ct Genotype 2	Standard deviation of the Ct values between replicates of the genotype 2
Genotype	Assign the sample according to genotype 1, genotype 2, heterozygote or error
Reaction Genotype 1	Yes or no, depending on the end point fluorescence or Ct value
Reaction Genotype 2	Yes or no, depending on the end point fluorescence or Ct value

Genotype Replicates	Assign replicates according to genotype 1, genotype 2, heterozygote or error ("?" symbol)
dRn Genotype 1	Standardized fluorescence intensity of the genotype 1reaction
Mean dRn Genotype 1	Standardized fluorescence intensity between replicates of the genotype 1 reaction
Std.Dev. dRn Genotype 1	Standard deviation of the standardized fluorescence intensity between replicates of the genotype 1reaction
dRn Genotype 2	Standardized fluorescence intensity of the genotype 2reaction
Mean dRn Genotype 2	Standardized fluorescence intensity between replicates of the genotype 2reaction
Std.Dev. dRn Genotype 2	Standard deviation of the standardized fluorescence intensity between replicates of the genotype 2reaction

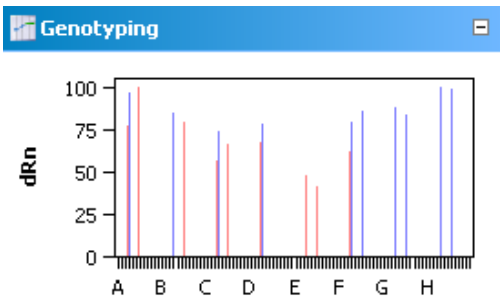
Individual columns can be shown or hidden by selection or deselection. The layout of the columns can also be freely modified. By dragging a column heading with the left mouse button depressed columns can be swapped. The display of the results in the table can thus be adjusted as desired.

Display in the project explorer

Optional the scatter plot or the end-point analysis calculated by the software can be displayed in short form in the project explorer under the point Genotyping. The scatter plot is divided into for quadrants for mutant, heterozygous, wild-type and error. The samples are assigned to a quadrant by the relative fluorescence or the Ct values determined for both fluorescent dyes. For the end-point analysis the measured relative fluorescence is plotted as bar chart. Die height of the bars defines the samples as genotype 1, genotype 2, heterozygous or error.



Representation of results for genotyping as scatter plot.



Representation of results for genotyping as bar chart.

5.6.6 Deleting a genotyping

An evaluation that is no longer required can be removed.

1. Activate the evaluation by selecting its name in the evaluation list of the method tab.
2. Click on in the toolbar.
   Alternatively, call up the **Genotyping ▸ Delete Genotyping** menu command.

   The evaluation is removed.

5.7 POS/NEG analysis at the end point

Positive / negative analysis at the end point of a qPCR is used to decide, whether or not a target gene is present in the reaction mix. Such an analysis can be configured as singleplex or multiplex experiment using fluorescence data at the end point of a PCR run, i.e. after amplification is finished. The position of the end point – with respect to the cycle number – as well as the cycles to be included can be set by you. Using the NTC samples a cutoff value is calculated which discerns positive and negative for each individual sample. The software also accounts for internal positive controls (IPC) which can be added to each sample in order to avoid false negative results thus increasing the confidence level of the experiment.

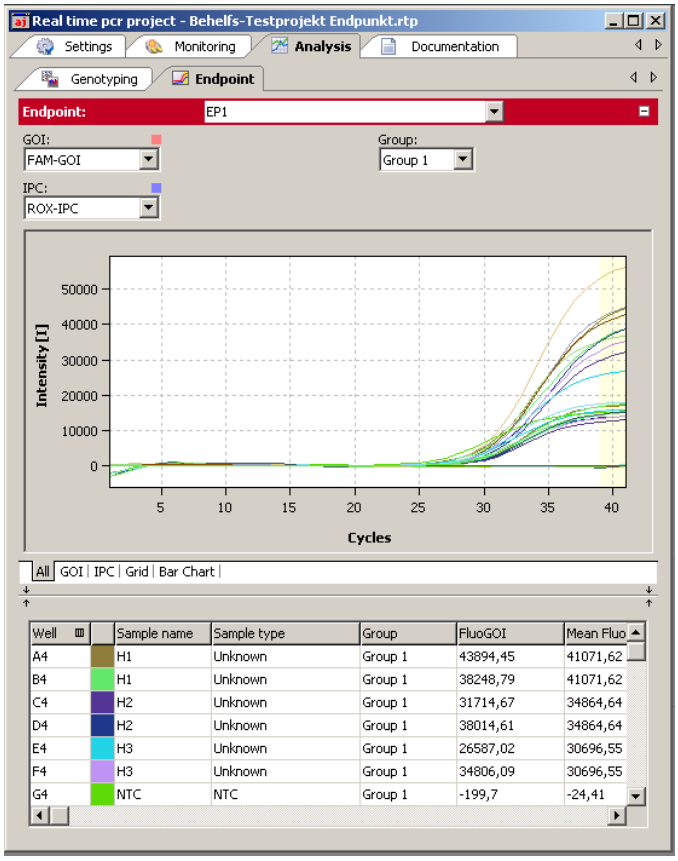
5.7.1 Starting end point analysis

1. Go to the **Analysis / Endpoint** project tab. If it is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click the symbol in the toolbar. Alternatively you can select the menu ENDPOINT / ADD ENDPOINT.
3. An input window appears. Enter the description of the current evaluation.

Note:
An end point analysis can only be opened if the plate layout contains at least one NTC-sample (→ section "Editing the sample table" p. 39).

On the Endpoint tab the following information is activated:

- Edit fields for parameter settings
- Amplification curves of the selected GOI and the internal positive control IPC in a combined diagram.
- Amplification curves of the selected GOI and the internal positive control IPC in separate diagrams which allow for setting the cutoff.
- Display of results in a PCR plate scheme
- Display of results in a bar chart
- Results table with measurement data and classification of the unknown samples as POS, NEG, ??? , CHECK



Window for end point anaylis

5.7.2 Parameters settings for endpoint analysis

Parametersettings for endpoint analysis

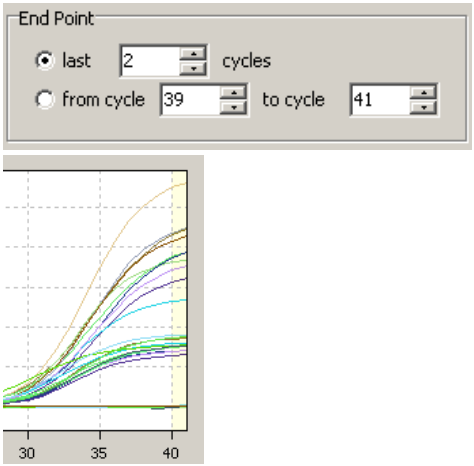
1. Set the following parameters for endpoint analysis:

Option	Meaning
Shortlist	Selection of an evaluation created for this experiment
Gene of Interest (GOI)	Selection list of the gene/dye combination of interest
Internal Positive Control (IPC)	Selection of the gene/dye combination which is used for IPC
Group	If several experiments were carried out on the plate, select of experiments to be analyzed.
Cutoff	Cutoff defines the fluorescence value above which the sample is considered to be positive

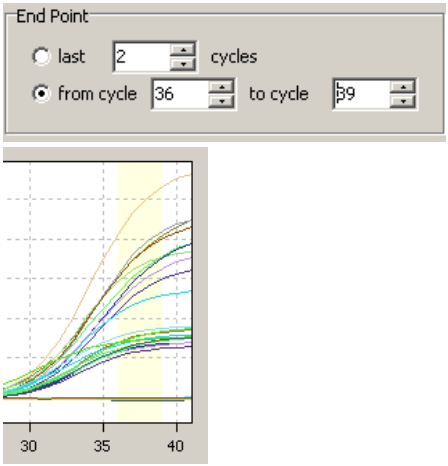
2. Define the cycle numbers which shall be considered as endpoint.

By default the mean fluorescence of the last two cycles is taken for calculations. You can define more than 2 cycles at the end or an interval of cycles of the qPCR run as source of endpoint fluorescence data.

The selected cycles are highlighted in the chart by a yellowish bar.



Last 2 cycles are marked



Cycle 36 to 39 are marced

Area selection for endpoint analysis

Setting Cutoff values

Cutoff-values for GOI and IPC can be set manually or calculated automatically. You can select different methods for automatic calculation in the options dialogue for endpoint analysis.

Manual methods

- Enter a number for Cutoff directly in the edit field **Cutoff** or in the options dialogue
- Shift the Cutoff line using the mouse in the tabs GOI and IPC

Automated methods

- With negative control or NTC:
  The Cutoff-value is calculated from the mean fluorescence of the NTC samples plus the fraction (given in percent) from the difference between maximal sample fluorescence and mean fluorescence of the NTC samples. Fluorescence data is taken from the specified end point cycles.
- With internal positive control IPC and NTC:
  Cutoff values for NTC and IPC are calculated separately. The standard deviation of the fluorescence of all NTC samples (or IPC- samples, i.e. samples which do not contain an internal positive control) is multiplied with a factor T which is read from a table and is dependent from the desired confidence interval and the number of samples.
- You can start the automatic calculation of Cutoffs according to the settings in the options by clicking . Alternatively you can choose the command ENDPOINT / AUTO THRESHOLD CUTOFF from the menu.

Note:
If the plate layout does not contain samples which are labeled as IPC- , the option "with internal positive control and NTC" will not be available. On the tab **Settings / Samples**

selected wells can be labeled as IPC- by right clicking the highlighted wells and select **Assign IPC-** from the context menu (→ section "Editing the sample table" p. 39).

5.7.3 Displaying results in end point analysis

The results of the analysis (POS, NEG, ???, CHECK) are evaluated according to the following rules:

Without IPC

Fluoescence of a single GOI sample	Result
> Cutoff (GOI)	POS (positive)
≤ Cutoff (GOI)	NEG (negative)

With IPC

Fluoescence of a single GOI sample	Fluoescence of a single IPC sample	Result
> Cutoff (GOI)	> Cutoff (IPC)	POS (positive)
≤ Cutoff (GOI)	> Cutoff (IPC)	NEG (negative)
> Cutoff (GOI)	≤ Cutoff (IPC)	??? (problematic)
≤ Cutoff (GOI)	≤ Cutoff (IPC)	??? (problematic)

Bewertung bei Replikaten

Samples which are present in replicates (having the same sample name) are considered to be POS or NEG if all replicates of that sample are POS or NEG. If this is not the case CHECK will be displayed. It is possible to manually deselect samples in the project explorer which are recognized as outliers.

Fluoescence of replicates	Result of sample
all POS	POS (positive)
all NEG	NEG (negative)
otherwise	CHECK (eliminate outliers or repeat sample)

After creation of a new analysis or after having changed options or cutoff values the results are re-calculated and charts and table will be refreshed.

Results table

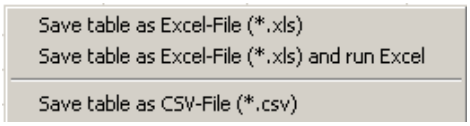
Well		Sample name	Sample type	Group	FluoGOI	Mean FluoGOI	Std.Dev. FluoGOI	FluoIPC	Mean FluoIPC	Std.Dev. FluoIPC	Status GOI	Status IPC	Result sa...	Result replicates
A4		H1	Unknown	Group 1	43894,45	41071,62	3992,08	17019,34	15944,93	1519,44	POS	POS	POS	POS
B4		H1	Unknown	Group 1	38248,79	41071,62	3992,08	14870,52	15944,93	1519,44	POS	POS	POS	POS
C4		H2	Unknown	Group 1	31714,67	34864,64	4454,74	12812,05	13328,08	729,78	POS	POS	POS	POS
D4		H2	Unknown	Group 1	38014,61	34864,64	4454,74	13844,11	13328,08	729,78	POS	POS	POS	POS
E4		H3	Unknown	Group 1	26587,02	30696,55	5811,76	15718,75	14758,92	1357,41	POS	POS	POS	POS
F4		H3	Unknown	Group 1	34806,09	30696,55	5811,76	13799,08	14758,92	1357,41	POS	POS	POS	POS
G4		NTC	NTC	Group 1	-199,7	-24,41	247,89	15239,33	16507,95	1794,1	NEG	POS	NEG	NEG
H4		NTC	NTC	Group 1	150,88	-24,41	247,89	17776,58	16507,95	1794,1	NEG	POS	NEG	NEG
A7		WT1	Unknown; IPC-	Group 1	55748,83	50011,56	8113,73	-238,58	-213,27	35,8	POS	NEG		
B7		WT1	Unknown; IPC-	Group 1	44274,29	50011,56	8113,73	-187,95	-213,27	35,8	POS	NEG		
C7		WT2	Unknown; IPC-	Group 1	36536,85	39388,36	4032,65	-246,75	-149,28	137,84	POS	NEG		
D7		WT2	Unknown; IPC-	Group 1	42239,87	39388,36	4032,65	-51,81	-149,28	137,84	POS	NEG		
F7		M2	Unknown	Group 1	-346,38	-411,01	91,4	15201,38	15105,65	135,39	NEG	POS	NEG	NEG
G7		M2	Unknown	Group 1	-475,65	-411,01	91,4	15009,91	15105,65	135,39	NEG	POS	NEG	NEG
H7		M3	Unknown	Group 1	-85,52	-85,52	0	17320,61	17320,61	0	NEG	POS	NEG	NEG

Result table of endpoint analysis

For the end point the results table contains the following information:

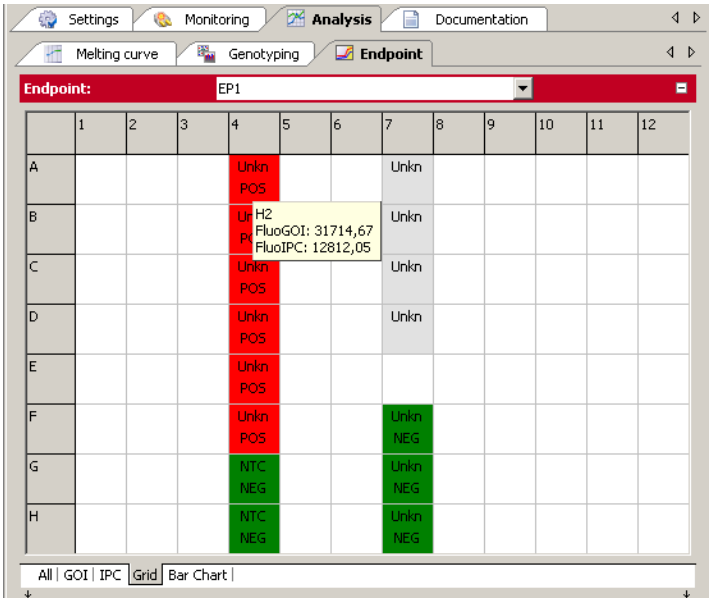
Column	Meaning
Well	Position of sample
Color of curve	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
Sample name	Name of sample
Sample type	Type of sample*
FluoGOI	Fluorescence of the GOI samples at the endpoint
Mean FluoGOI	Mean fluorescence of the GOI replicates at the endpoint
Std.Dev. FluoGOI	Standard deviation of the GOI replicates at the endpoint
FluoIPC	Fluorescence of the IPC samples at the endpoint
Mean FluoIPC	Mean fluorescence of the IPC replicates at the endpoint
Std.Dev.FluoIPC	Standard deviation of the IPC replicates at the endpoint
Status GOI	**POS** if FLUOGOI > CUTOFF, else **NEG** (for each sample well)
Status IPC	**POS** if FLUOIPC > CUTOFF, else **NEG** (for each sample well)
Result sample	Rating **POS/NEG/???** for each well
Result replicates	Rating **POS/NEG/CHECK** of the replicates

The results table displays all calculated numerical data and the evaluation of single samples and replicates. You can design the results table according to your requirements by setting type, sequence and width of the columns to be displayed. You can also adjust the sort sequence of data in the column (alphabetical, numerical, by column, by row). The table configured this way can be exported as Excel or CSV file by right clicking the table.



Context menu for data export

PCR plate scheme (Grid)

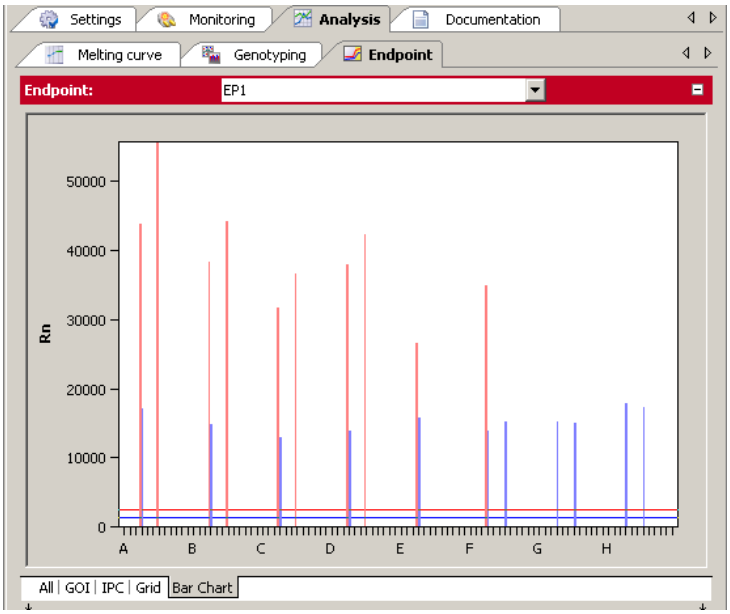


Results of endpoint analysis on the plate scheme

For qTOWER³ 84, the columns from 1 to 24 and rows from A to P are available using the scroll bars.

The PCR plate scheme offers a quick overview of the results obtained in each single well. If the mouse pointer is moved across a well a hint box is displayed showing the sample name and the end point fluorescence of GOI and IPC for that well. You can change the colors which code for POS, NEG and ??? in EXTRAS / OPTIONS / COLORS.

Bar Chart



Bar graph of endpoint analysis results

The bar chart displays the end point fluorescence of the GOI together with the IPC as well as the corresponding cutoff values as horizontal lines. Red lines denote the GOI and blue ones the IPC. If the mouse pointer is moved across a bar a hint box is displayed showing the sample name and the end point fluorescence of GOI and IPC for the corresponding well.

Note:
Cutoff lines can not be changed in the bar chart. To do so, please switch to the tabs GOI or IPC.

6 Multigene-/Multiplate-Analysis

The Multigene-/Multiplate-Analysis is used to analyze real-time PCR data of multiple target genes in parallel or to analyze data from multiple project files if, for example, several PCR plates have been used for an experiment. The Multigene-/Multiplate-Analysis is executed as dialog in a separate window independend of the surface of the qPCRsoft software. For the Multigene-/Multiplate-Analysis project files generated by the qPCRsoft software are used. The project files must include a ΔΔCt-analysis for Multigene-/Multiplate-Analysis.

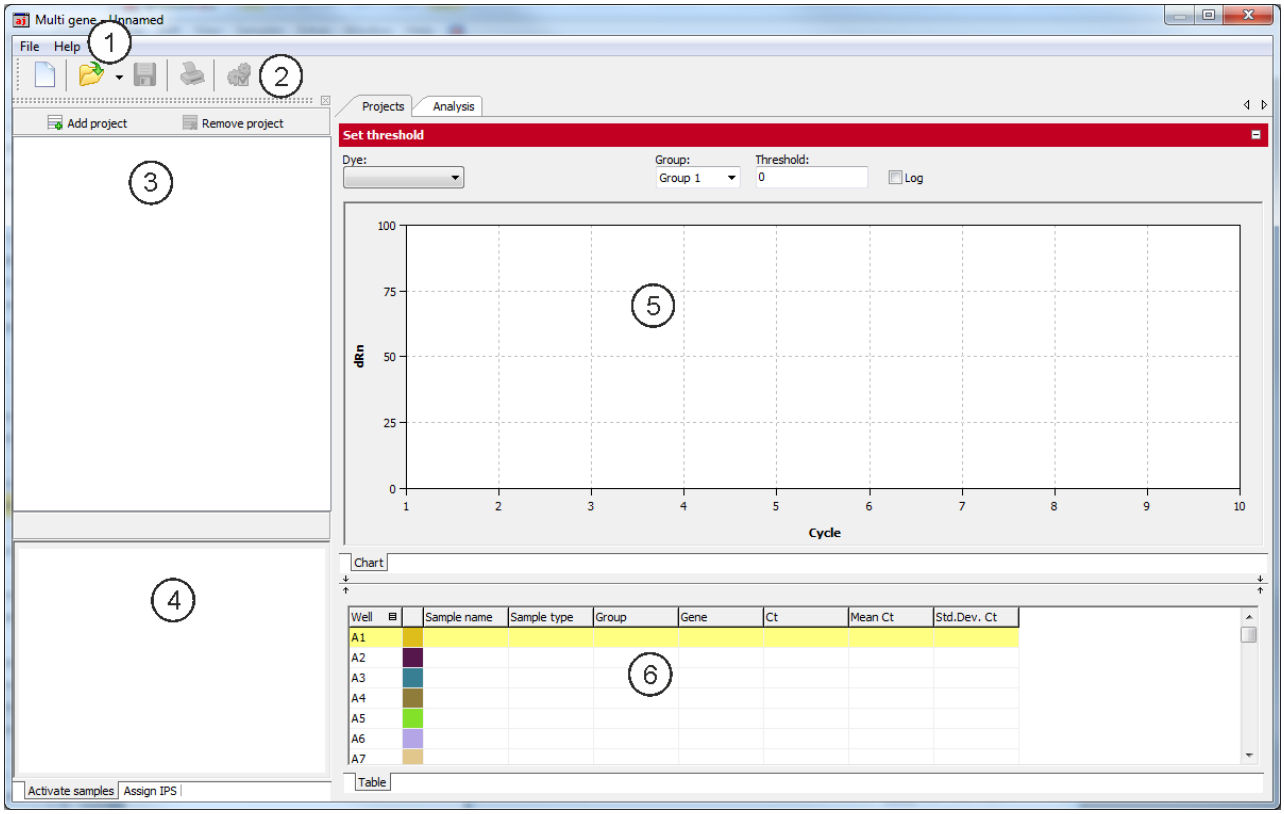
6.1 Start Multigene-/Multiplate-Analysis

To start the Multigene-/Multiplate-Analysis click on the button in the toolbar. A new window for Multigene-/Multiplate-Analysis appears.

6.2 The window for Multigene-/Multiplate-Analysis

After starting the dialog the window for Multigene-/Multiplate-Analysis opens. It is devided into the following sections:

Menu bar (1)	The menu bar contains the menu commands for, e.g., opening, editing, saving and printing of Multigene-/Multiplate-Analysis files and a help function.
Toolbar (2)	In the toolbar commands for editing Multigene-/Multiplate-Analysis files are arranged.
Project list (3)	In the project list projects that are used for Multigene-/Multiplate-Analysis are listed and administrated.
Samples (4)	In the layout the measurement data of samples can be selected or unselected. Only data of selected samples becomes included to the analysis and is used for the results calculation. Furthermore in the layout the position of interplate standards (IPS) can be defined.
Data display (5)	In the data display amplification curves of imported project files are shown or the results of the Multigene-/Multiplate-Analysis are plotted as bar chart.
Results table (6)	In the results table the imported measurement data of the activated project file or the results of the Multigene-/Multiplate-Analysis are shown.



Windows Multi gene for Multigene-/Multiplate analysis

6.3 Overview of menu commands

In the Multigene-/Multiplate-Analysis window the following menu commands are available:

Menu	Function	Description
File	**New Multigene-/Multiplate-Analysis**	Opens a new Multigene-/Multiplate-Analysis
	Open Multigene-/Multiplate-Analysis	Opens a Multigene-/Multiplate-Analysis.
	Save Multigene-/Multiplate-Analysis	Saves a Multigene-/Multiplate-Analysis in the qPCRsoft standard folder
	Save Multigene-/Multiplate-Analysis as	Saves a Multigene-/Multiplate-Analysis in any user-selected folder
	Print Multigene-/Multiplate-Analysis	Prints the results of a Multigene-/Multiplate-Analysis
	Close	Closes the window for Multigene-/Multiplate-Analysis

6.4 Overview of the tools in the toolbar

Button	Command	Function
	NEw	Opens a new Multigene-/Multiplate-Analysis
	Open	Opens a Multigene-/Multiplate-Analysis
	Save	Saves a Multigene-/Multiplate-Analysis
	Print	Prints a Multigene-/Multiplate-Analysis
	Options	Allows the input of efficiencies of PCR reactions for single genes of interest

6.5 Help function

You can get help on the operation of the Multigene-/Multiplate-Analysis via the menu command **Help ▸ Content**.

While working with the Multigene-/Multiplate-Analysis, you can activate the help function by pressing function key [F1].

The program pops up brief information (tool tips) on buttons of toolbar while you move the mouse pointer across the button.

6.6 Managing Multigene-/Multiplate-Analysis files

After clicking on the Multigene-/Multiplate-Analysis dialog starts in a separate window and contains no data.

6.6.1 Start new Multigene-/Multiplate-Analysis

To start a new Multigene-/Multiplate-Analysis press on or call up the **File ▸ New** menu command. An already loaded Multigene-/Multiplate-Analysis will be closed. If the analysis has been modified and the changes are not yet saved, a prompt opens.

Note:
Only one Multigene-/Multiplate-Analysis can be opened.

6.6.2 Open saved Multigene-/Multiplate-Analysis

1. Click on or call up the **File ▸ Open Multigene-/Multiplate-Analysis** menu command.
2. In the standard window, select the desired file to open and confirm the selection with [OK].

Note:
If the file type is linked to the qPCRsoft application in **Extras ▸ Options** the dialog for Multigene-/Multiplate-Analysis starts automatically by a double click on the selected file.

The Multigene-/Multiplate-Analysis including the project list, sample layout, measurement data and analysis opens.

6.6.3 Save Multigene-/Multiplate-Analysis

The Multigene-/Multiplate-Analysis including all loaded project files and analysis is stored.

1. Select the menu command **File ▸ Save Multigene-/Multiplate-Analysis**.
2. To save files enter the name of the template in the standard window and save the template with **[OK]**.

Changes in a Multigene-/Multiplate-Analysis can be saved with the **File ▸ Save Multigene-/Multiplate-Analysis** menu command. Optionally, you can click on the symbol in the toolbar.

6.6.4 Close Multigene-/Multiplate-Analysis

The **File ▸ Close Multigene-/Multiplate-Analysis** menu command closes the Multigene-/Multiplate-Analysis window. If any unsaved changes have been made in analysis, a confirmation prompt appears.

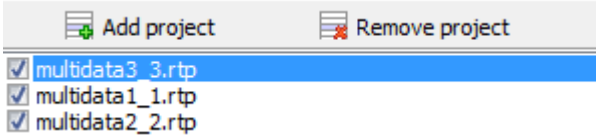
6.6.5 Print Multigene-/Multiplate-Analysis

The data and results of a Multigene-/Multiplate-Analysis can be printed using the **Print** function. For this purpose, an analysis must be applied and in the data display the tab **Analysis** must be selected (→ section "The window for Multigene-/Multiplate-Analysis" p. 116). By using the command **File ▸ Print Multigene-/Multiplate-Analysis** or the icon in the toolbar the print function is started.

6.7 Load Project Files

If a new Multigene-/Multiplate-Analysis is started, at first a blank window is displayed. To perform an analysis project files have to be loaded. In the project files a ΔΔCt-analysis must be applied to evaluate them by Multigene-/Multiplate-Analysis.

1. To load a project file click on the button **[Add project]**.
2. In the standard window select one or more stored project files and confirm your selection with [OK]. Loaded projects are listed in the project list.

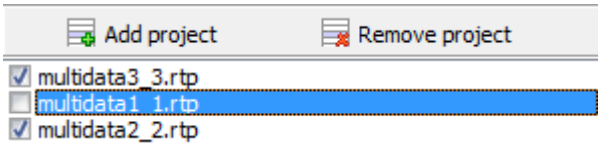


Projekt list for Multigene-/Multiplate-Analysis

1. To remove a project file mark the File in the project list.
2. Click on button **[Remove project]**.

6.8 Activate/Deactivate project files for Multigene-/Multiplate-Analysis

In the project list, a check box is show next to each loaded project. By the check box projects can be activated or deactivated. Only data from activated projects is used for analysis.



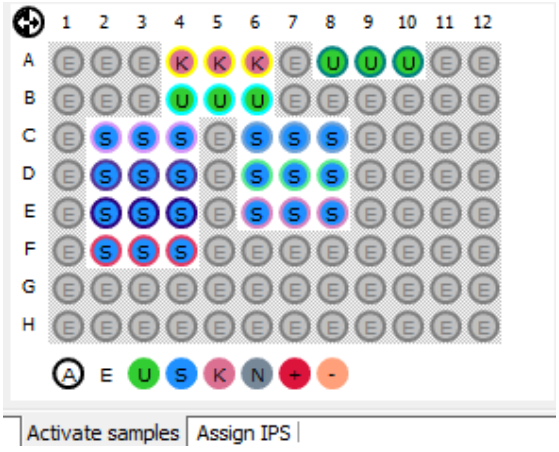
Projekt list for Multigene-/Multiplate-Analysis with activated/deactivated samples

The data of deactivated project files remain loaded in the background and after re-activation are used for analysis again.

6.9 Activate/Deactivate samples for Multigene-/Multiplate-Analysis

In the data display for Multigene-/Multiplate-Analysis activate the tab **Projects** (→ section "The window for Multigene-/Multiplate-Analysis" p. 116). On the tab **Activate samples** of the data display samples of single wells can be activated or deactivated for analysis. By deactivation of samples outliers are eliminated and not included in the calculation of mean values.

Note:
The sample selection has only influence on the analysis of data, no measurement data is deleted.



Sample layout for Multigene-/Maultiplate-Analysis

The sample layout is taken from the loaded project files. The color code for each sample type can be modified in menu EXTRAS / OPTIONS ▸ COLORS.

Sample type	Symbol	Definition
Empty	E	Describes an empty position on the PCR plate
Unknown	U	Sample of unknown concentration or dilution (measuring sample)
Stnadard	S	Sample of known concentration or dilution
Calibrator	K	Sample whose gene of interest expression level is set as 1
No Template Control (NTC)	N	Complete reaction preparation but without matrix strand
Positive Control	+	Positive control preparation for which a reaction product is expected
Negative control	-	Negative control preparation for which no reaction product is expected

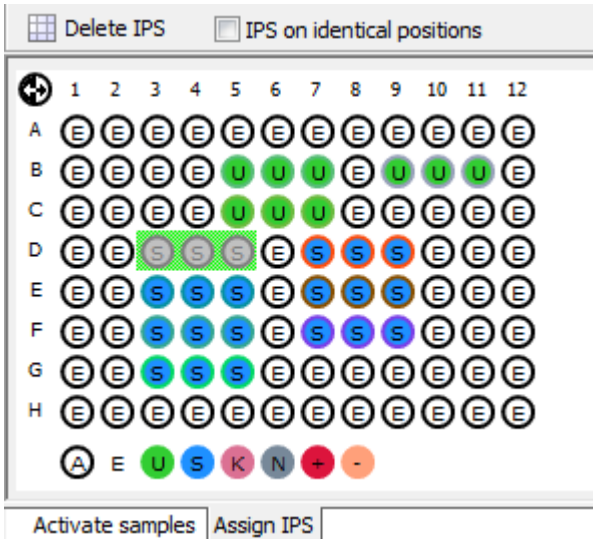
Active wells (i.e. displayed wells) are marked with their sample type symbol. For deactivated wells the position is grey and the fluorescence data is hidden. Empty wells are marked "E".

- Click with the mouse to switch. The activation changes with each click on a well.
- You can change adjacent wells by holding the mouse button and moving the cursor over the wells. To select non-adjacent positions press the Ctrl key on the keyboard plus the left mouse button at each position.
- Complete rows and columns can be inverted by clicking on the letter or number of the row [A-H] or column [1-12].
- The activation status of the complete plate can be inverted by clicking on on the top left between A and 1.
- To activate all wells click on the symbol below the chart.
- To activate only samples of a specific type click on the corresponding symbol below the chart. To activate multiple sample types at the same time keep the Ctrl key pressed when clicking on the sample types.

6.10 Define Interplate-Standards for Multigene-/Multiplate-Analysis

Activate the tab **Projects** in the data display for Multigene-/Multiplate-Analysis (→ section "The window for Multigene-/Multiplate-Analysis" p. 116). For the Multigene-/Multiplate-Analysis Interplate-Standards (IPS) are used on each PCR plate and the deviations among themselves are determined and calculated.

1. To define Interplate-Standards activate the tab **Assign IPS** in the sample display.

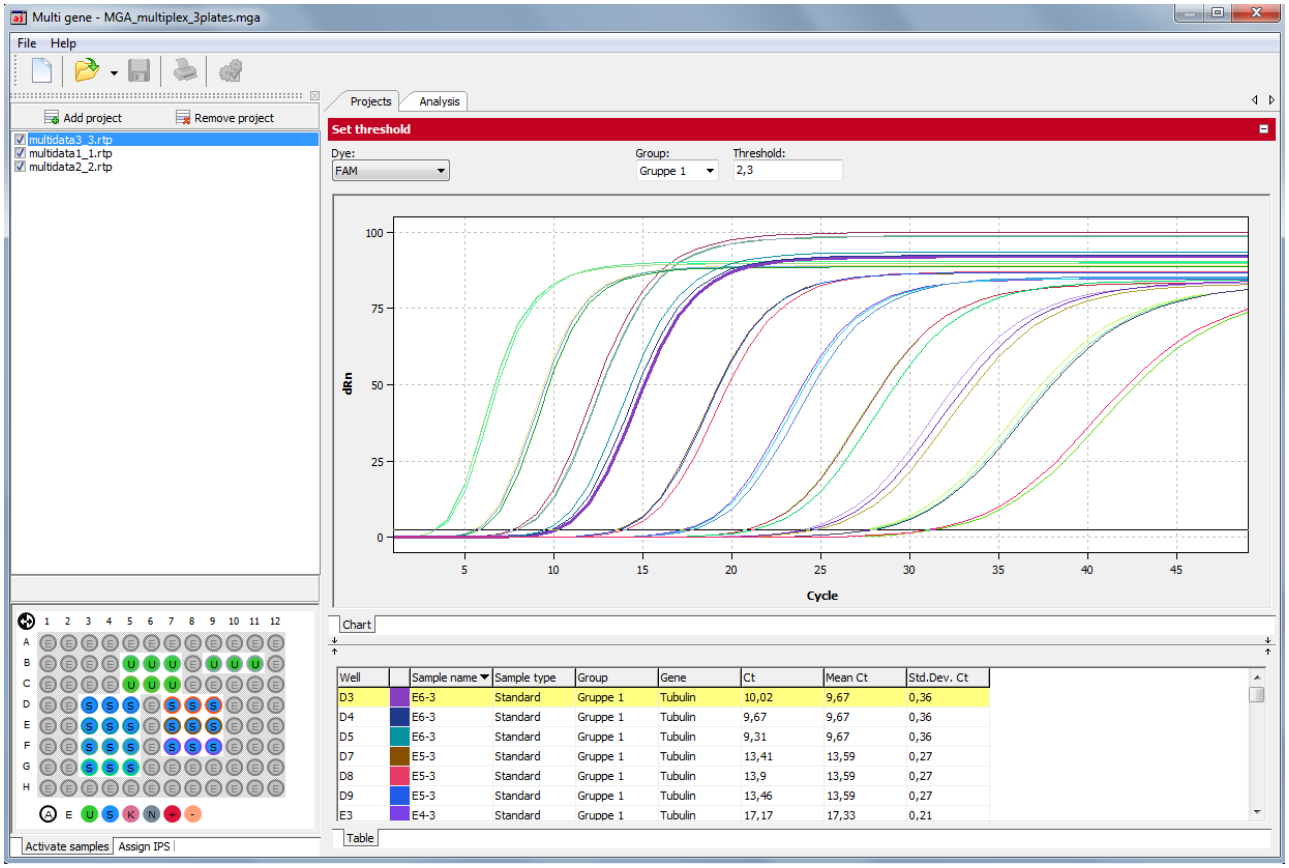


Defining IPS

2. Select the position of Interplate-Standards:
   - Press the left mouse button and drag the area that contains the IPS samples: Selected samples are displayed in grey against a green background. For not selected samples only the sample type icon is displayed. Empty wells are marked with "E". To select samples see also section "Activate/Deactivate samples for Multigene-/Multiplate-Analysis" p. 120.
   - If the IPS position is the same on all plates the current selection is transferred to all loaded projects by activating the checkbox [IPS on identical positions].
   - Click on the button **[Delete IPS]** to delete the IPS in all loaded projects.

6.11 Set threshold and PCR-efficiencies for Multigene-/Multiplate-Analysis

After loading project files the measurement data for the active project is shown in the display area. The active project is marked blue in the project list on the left. By clicking on the name in the project list another project can be activated and thus switched between different projects.



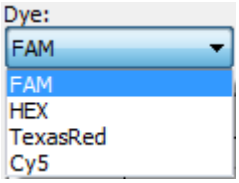
For the active project the fluorescence curves and results are shown under the tab "Projects"

From the loaded projects all measurement results and settings are taken. In the dialog for Multigene-/Multiplate-Analysis most settings cannot be modified, this is only possible in the individual projects in the qPCRsoft software.

Edit threshold

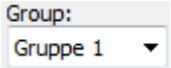
On the tab **Projects** the threshold for each dye can be adapted.

1. Activate the relevant project in the project list.
2. Select a dye from the list on the tab **Projects**.



Select list for dyes

3. If in the project file different groups are defined select the right group from the list.



Select list for groups

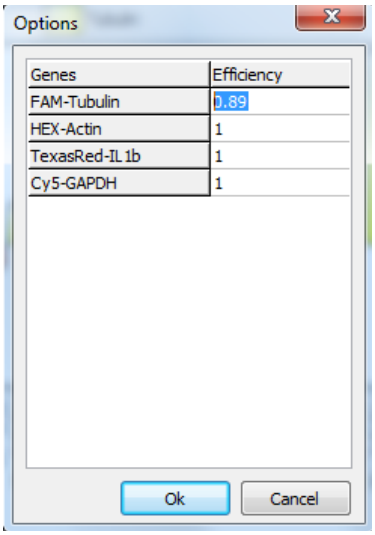
4. To modify the threshold value two different options are available:
   - In the chart, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so.
   - Enter a value in the field Threshold.

With the modification of the threshold the values in the result table displayed below are updated.

Edit PCR efficiency

Basically, PCR efficiencies are taken from the loaded project files but it is possible to set adjust the value for the considered genes.

1. Activate the tab **Analysis** in the data display and click the button in the toolbar.
2. Adjust for each gene the PCR efficiency in the window **Options**.

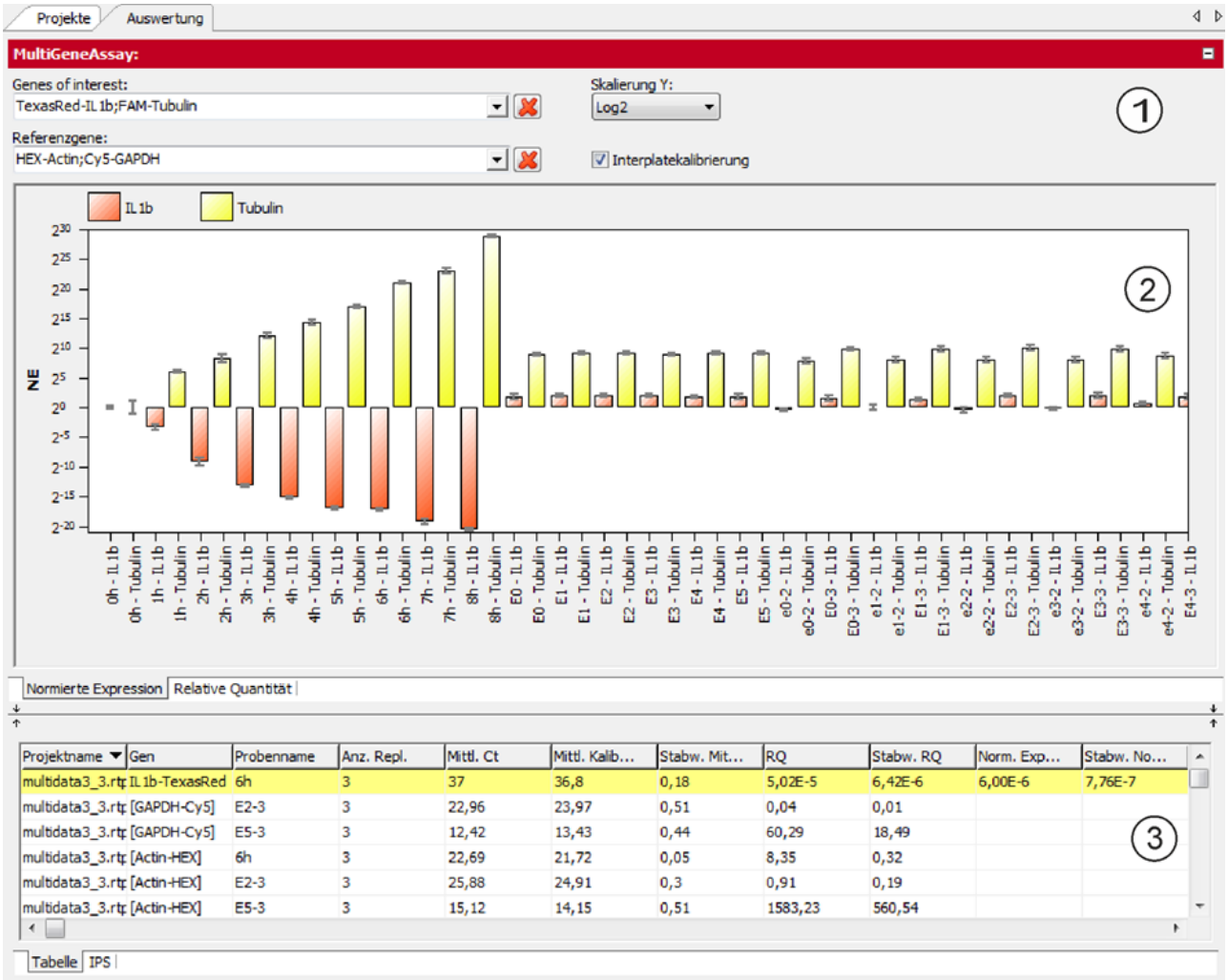


Window Options for editing of PCR efficiency

3. Enter a new value and confirm with [OK].

6.12 Evaluation of the Multigene-/Multiplate-Analysis

The results of the Multigene-/Multiplate-Analysis are summarized under the tab **Analysis** of the data display.

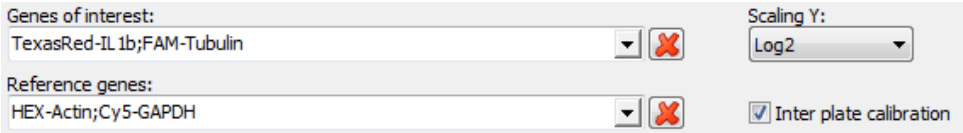


Results of the Multigene-/Multiplate-Analysis

The data display is divided into the following sections:

- Parameter settings (1)
- Graphical data display (2)
- Results table with measured values (3)

Parameter settings for Multigene-/Multiplate-Analysis



Parameter settings for Multigene-/Multiplate-Analysis

Option	Description
Genes of Interest (GOI)	Selection list of target gene/dye-combinations. Multiple target genes can be selected. The symbol is used to remove all target genes from the evaluation

Reference genes	Selection list of reference gene/dye-combinations. Multiple reference genes can be selected. The symbol is used to remove all reference genes from the evaluation.
Scaling Y	Selection of the scaling of the Y-axis.
Interplate calibration	With activated interplate calibration from the selected IPS samples of all plates a correction factor is determined* and from the **mean Ct values** of replicates the **corrected mean Ct values** are calculated (see results table). The **corrected mean Ct values** are then used to calculate the relative quantity and the normalized expression. Is the interplate calibration deactivated, the **corrected mean ct values** are equal to the **mean ct values**.

*Correction calculation

$$Ct_{i,p}^{corr} = Ct_{i,p}^{meas} - \overline{Ct}_p^{IPC} + \frac{1}{N}\sum_{p=1}^{N} Ct_p^{IPC}$$

with

$Ct_{i,p}^{corr} - \ corrected\ Ct - value\ for\ replicate\ i\ on\ plate\ p$

$Ct_{i,p}^{meas} -\ measured\ Ct - value\ for\ replicate\ i\ on\ plate\ p$

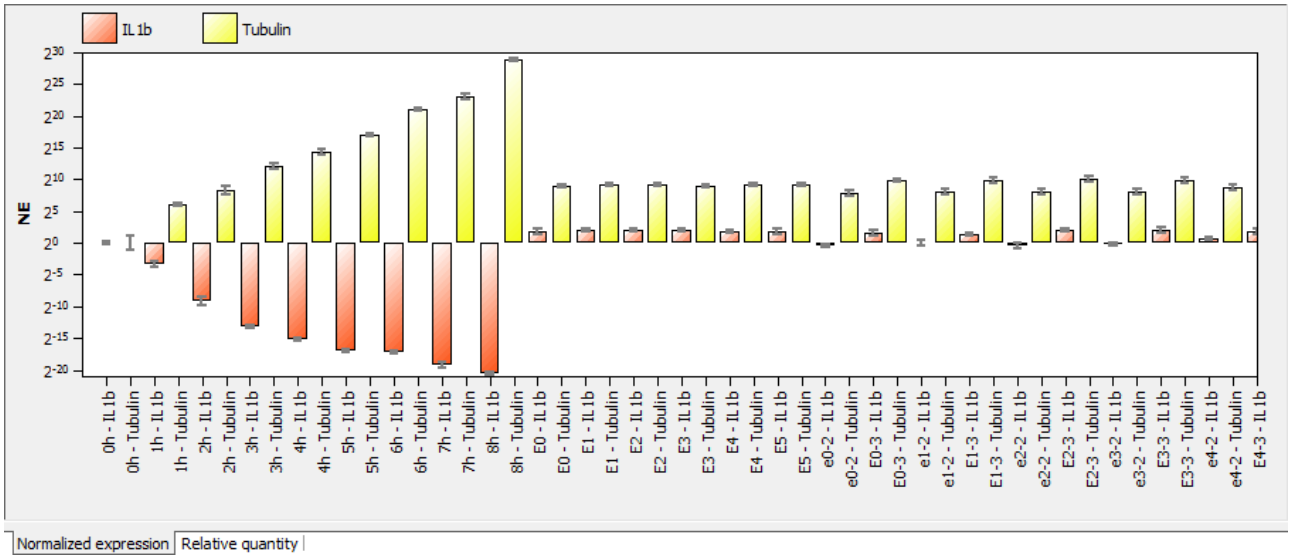
$\overline{Ct}_p^{IPC} \ -\ mean\ value\ of\ Ct - values\ of\ IPS - samples\ on\ plate\ p$

$\frac{1}{N}\sum_{p=1}^{N} Ct_p^{IPC} -\ mean\ value\ of\ Ct - values\ of\ all\ IPS - samples\ on\ all\ N\ plates$

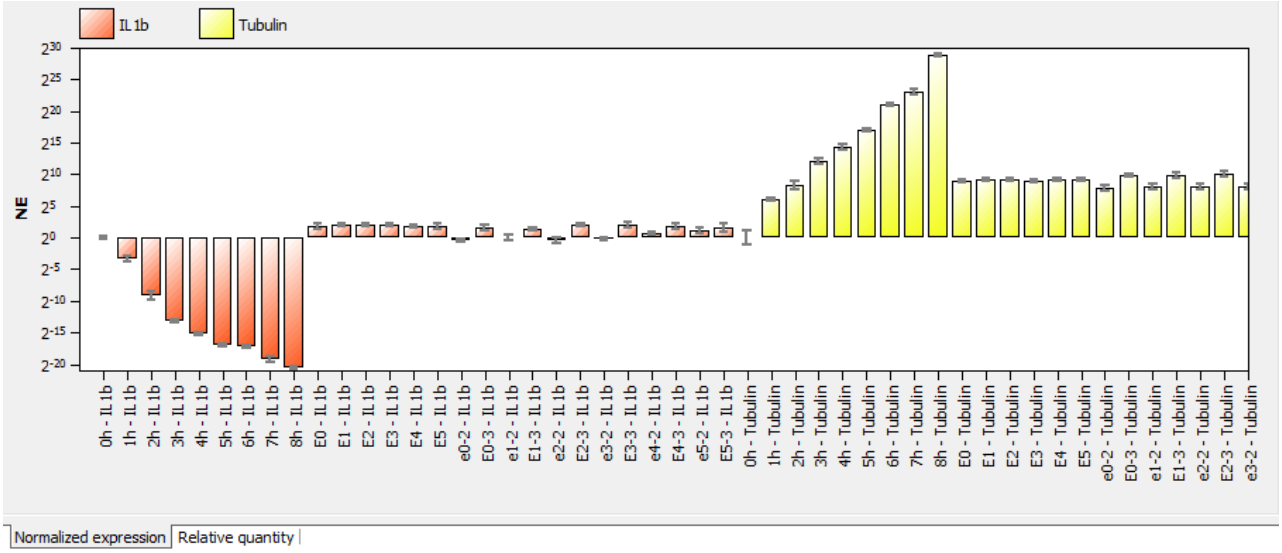
Results display for Multigene-/Multiplate-Analysis

The results of the Multigene-/Multiplate-Analysis are shown as bar charts. In the tab **Normalized Expression** the expression of selected genes of interest, normalized to the expression of the reference genes, is shown. In the second tab **Relative Quantity** the corresponding quantity for the genes of interest and reference genes is displayed. The respective sample name is given below each bar. The height of the bar is determined by the calculated normalized expression of replicates. For each bar some short information about the sample name, mean value and the calculated standard deviation is shown if the mouse pointer is placed on it. The standard deviation for the normalized expression is shown as error bar on top of each bar. Since for larger numbers of samples not all can be displayed in the screen the diagram can be moved horizontally with pressed left mouse button and dragged to the desired position. Using the mouse wheel allows to narrow or broaden the width of the representation. Alternatively the cursor keys ↑ and ↓ can be used.

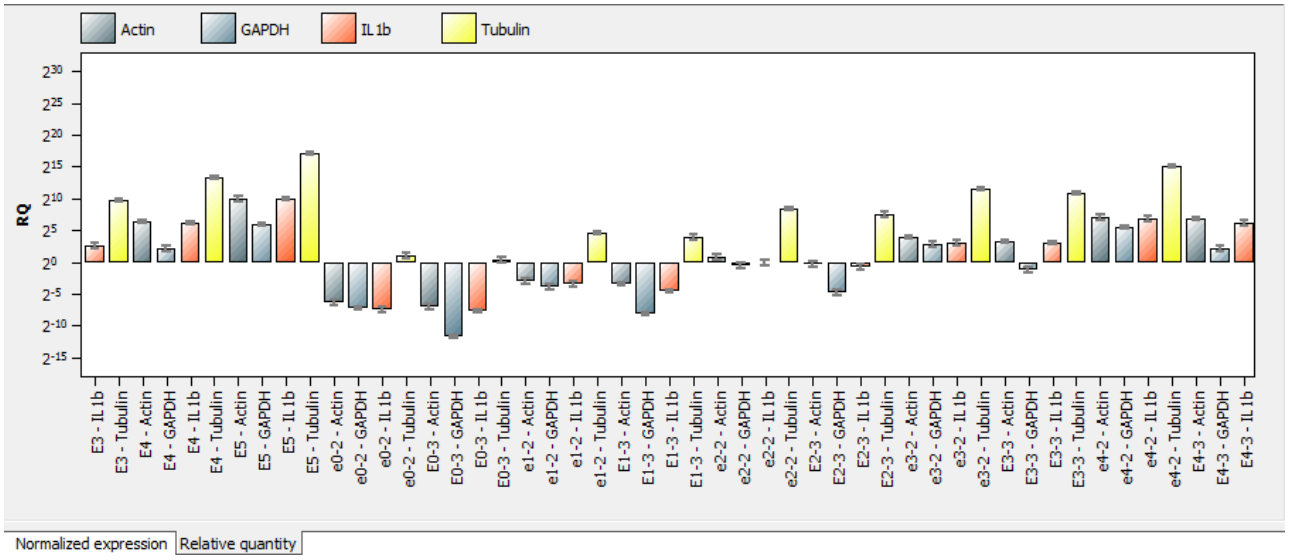
By a right-click on the chart the results are sorted by genes or samples names on the x-axis.



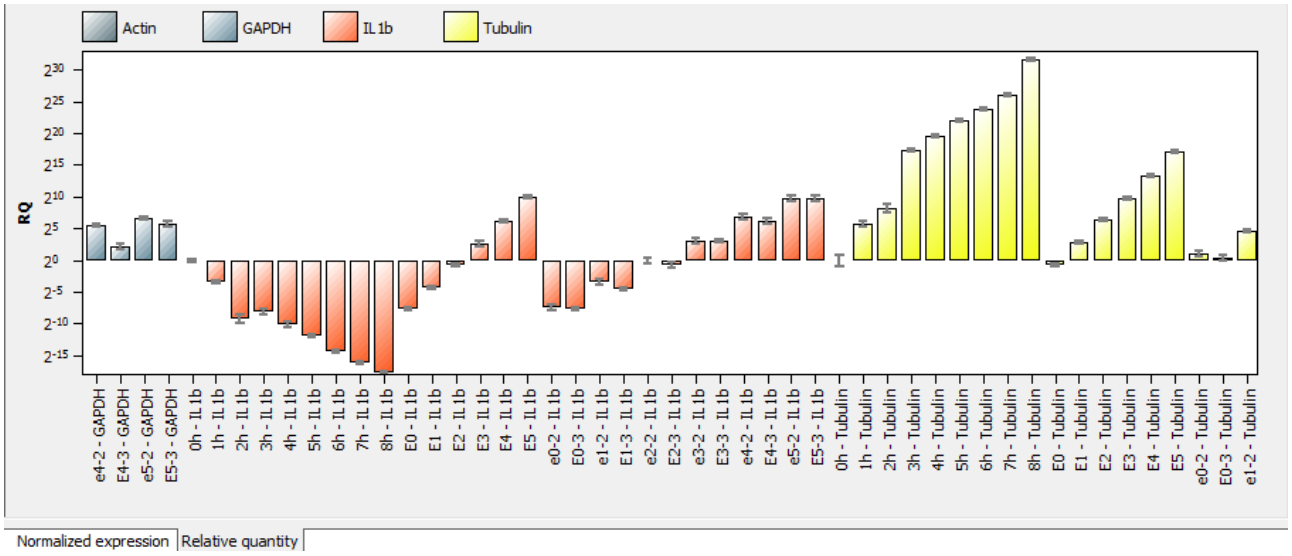
Representation of the normalized expression as bar chart sorted by sample name.



Representation of the normalized expression as bar chart sorted by gene name



Representation of the relative quantity as bar chart sorted by sample name.



Representation of the relative quantity as bar chart sorted by gene name

In the tab **Table** of the results table for Multigene-/Multiplate-Analysis all data and measurement values for the samples are summarized.

Project name	Gene	Sample name	No. of repl...	Mean Ct	Mean calib...	Std.Dev. ...	RQ	Std. Dev. ...	Norm. exp...	Std.Dev.n...
multidata1_1.rt	[GAPDH-Cy5]	0h	3	16,28	19,34	0,01	1	0,01		
multidata1_1.rt	IL 1b-TexasRed	0h	3	20,78	22,52	0,07	1	0,05	1	0,05
multidata1_1.rt	[Actin-HEX]	0h	3	22,13	24,78	0,03	1	0,02		
multidata1_1.rt	[GAPDH-Cy5]	0h	3	16,28	19,34	0,01	1	0,01		
multidata1_1.rt	[Actin-HEX]	0h	3	22,13	24,78	0,03	1	0,02		
multidata1_1.rt	Tubulin-FAM	0h	3	27,14	30,59	2,15	1	1,49	1	1,49

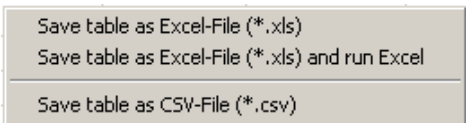
Table | IPS

Result table for Multigene-/Multiplate-Analysis

For the Multigene-/Multiplate-Analysis the results table contains the following information:

Column	Meaning
Project name	Name of the loaded project that contains the sample
Gene	Name of the investigated gene
Sample NAME	Name oft he sample
No of replicates	Number of replicates of the sample
Mean Ct	Mean Ct-value of the replicates of a sample
Mean Calib. Ct	Mean Ct-values of replicates of a sample calibrated by the IPS
Std. Dev. Mean Calib. Ct	Standard deviation of the mean calibrated Ct-values of replicates of a sample
RQ	Calculated relative quantity for replicates of the gen in the original sample
Std. Dev. RQ	Standard deviation of the calculated relative quantity for replicates of the gen in the original sample
Norm. Exp.	Normalized expression of the sample
Std. Dev. Norm. Exp.	Standard deviation of the normalized expression of the sample

You can design the results table according to your requirements by setting type, sequence and width of the columns to be displayed. You can also adjust the sort sequence of data in the column (alphabetical, numerical, by column, by row). The table configured this way can be exported as Excel or CSV file by right clicking the table.



Context menu for data export

In the tab IPS of the results table for Multigene-/Multiplate-Analysis all data for the Interplate Standards (IPS) is summarized.

Project name	Dye	Mean Ct (IPS, Project)	Mean Ct (IPS, all Projects)	Correction value
multidata3_3.rtp	FAM	9,67	8,48	-1,19
multidata3_3.rtp	HEX	11,35	10,38	-0,97
multidata3_3.rtp	TexasRed	8,95	8,75	-0,21
multidata3_3.rtp	Cy5	8,36	9,36	1
multidata1_1.rtp	FAM	5,03	8,48	3,45
multidata1_1.rtp	HEX	7,74	10,38	2,65

Table | IPS

Summarization of IPS data

Column	Meaning
Project name	Name of the loaded project that contains the IPS sample
Dye	Dye that has been used to determine the Ct value of the IPS sample
Mean Ct (IPS, Project)	Mean Ct value of IPS samples in the project (dependent upon the used dye)
Mean Ct (IPS, all Projects)	Mean Ct value of IPS samples in all projects (dependent upon the used dye)

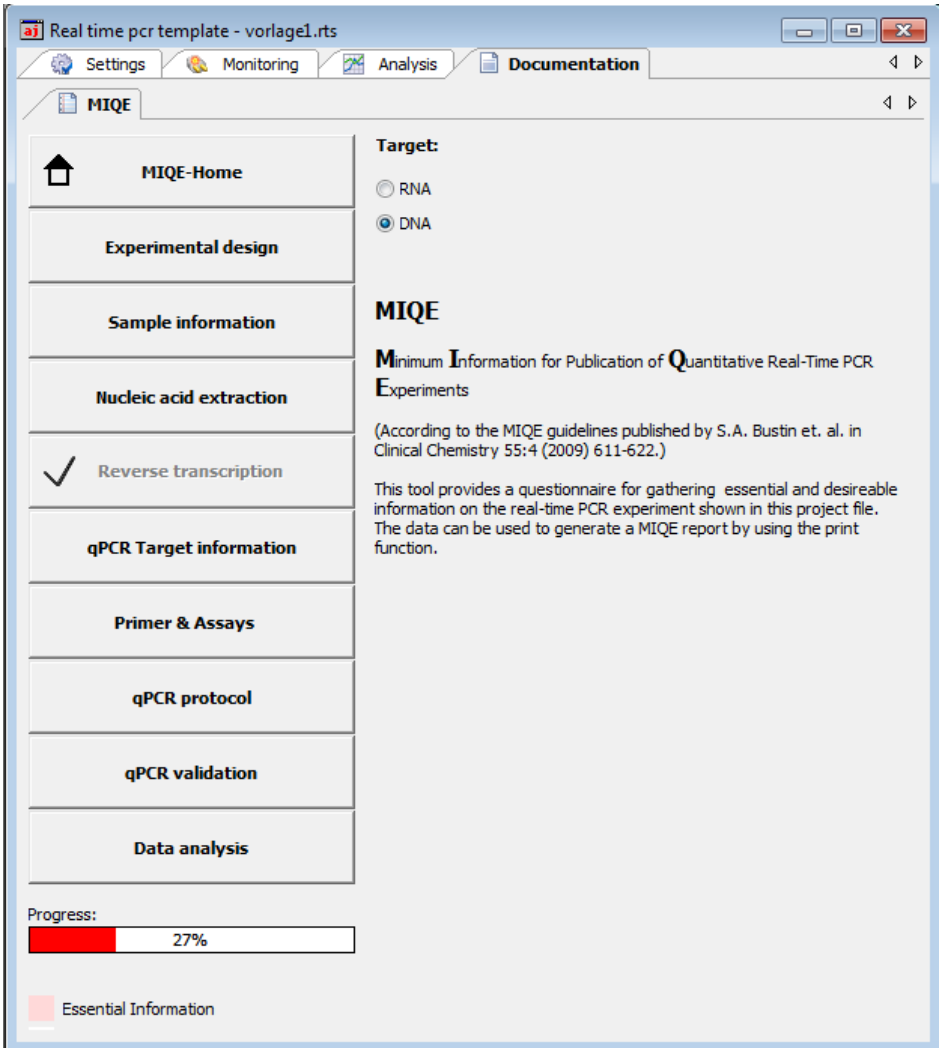
Correction value	Ct correction value that is applied for all samples of the project (column 1) and the dye (column 2)

7 MIQE documentation

In 2009 an international group of experts led by Prof. Steven Bustin developed guidelines for the publication of real-time PCR data (Bustin et al. 2009, Clinical Chemistry 55:4, 611-622). The aim was to prevent the publication of incomplete or incorrect real-time PCR data and to ensure comparability and reproducibility of experiments. The guidelines regulate the requirements regarding the minimum information necessary for the publication of data and have become known as MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments).

Instructions for completing the MIQE documentation

1. MIQE consists of a list of questions to 9 different topics about real-time PCR experiments. In the qPCRsoft software under the tab **MIQE** buttons for each topic buttons are present providing access to the corresponding list of questions. Additionally the button MIQE-HOME allows to jump back to the main menu from any sub menu.
2. In general at first by activating the corresponding radio button in the main screen it should be defined is DNA or RNA has been used as starting material. If the option DNA is activated the questions for topic REVERSE TRANSCRIPTION do not have to be answered and the corresponding button becomes inactive.
3. After clicking on a button the corresponding questionnaire opens. The number of questions differs between the topics. The user should answer as much questions as possible..
4. The answer to some questions is taken from the currently opened project and active project if the relevant information is available.
5. The completeness of the answers to the questions is presented by the software as progress bar and measured in %. The MIQE questionnaire distinguishes between essential questions that should be answered and additional optional questions that can be answered. The entry fields for essential questions are highlighted by a red colored background whereas additional questions have a white colored background. For the progress bar only the answered essential questions are ranked by the software. The total number of questions differs depending on whether DNA or RNA has been used as starting material. The software cannot evaluate the quality of the answers. It is up to user to fully answer the questions and with due diligence.
6. The qPCRsoft software offers to import MIQE data from other projects. Clicking on the button in the toolbar or by using the menu item **MIQE / Import MIQE Documentation** opens a dialog screen. After selecting the appropriate project the saved MIQE data is imported into the current project.
7. The questionnaire can be printed by the PRINT function of the qPCRsoft software. Select the point MIQE under DOCUMENTATION (→ section "Printing" pg. 22) and press PRINT. It is always the complete questionnaire printed.



Main menu of the MIQE input mask

8 Functions in Extras menu

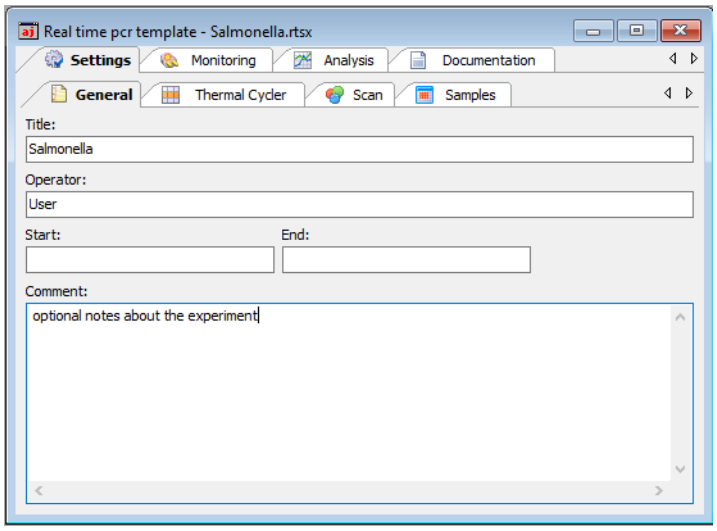
8.1 Device initialization

The device initialization sets the device to the original state. A device initialization is only required after an error has occurred.

- Call up the **Extras ▸ Device initialization** command.

8.2 Editing color modules

After inserting the color modules into the device's probe the color modules must be specified in the software.



Edit color modules window

1. Call up the **Extras ▸ Edit color modules** command.

   A window with the same name opens.

2. The available color modules for your device are displayed on the left.
3. From the list, select the module you have installed in the device, activate the **Properties** checkbox and select the position on which the module is mounted in the device. If necessary, you can also add dye names if these have not yet been added to the list. Click on **[Accept]**.
4. Repeat this for each color module you have installed.
5. If you wish to define a new color module that is not included in the list of available color modules, click **[Add]**. A new color module with the description COLOR.000.000.00.0 is created. You can define the properties on the right side of the dialog.
6. To delete a color module from the list, mark the module with the cursor and click on **[Delete]**.
7. To change the properties of a color module, proceed as follows:

- Activate the **Properties** checkbox.
- In the **Position** list select the position of the color module on the carrier in the fluorescence probe.
- Enter the dye that is detected with the color module.
  Click on **[+]**.
  The dye is added to the list below.
  You can remove a dye by marking it in the list and clicking on **[-]**.
- Click **[Accept]** to assign the properties to the marked color module.

Note
A dye can only be assigned to one module. If it is to be measured with a different module, it first has to be removed from the first module.

8.3 Connecting the device to the PC

The software qPCRsoft automatically recognizes which instrument is connected and whether it is switched on or not. The instrument may also be switched on and off during running qPCRsoft. Whether an active connection is established will be indicated in the lower left corner of the status line.

- If a connection cannot be obtained in between 30 seconds you can use the **Extras ▸ Device** identification in order to solve the problem

8.4 General settings in the qPCRsoft software

General settings for the qPCRsoft program can be made in the **Options** window.

Note
For most of the settings in the **Options** window you need to be logged into qPCRsoft as an administrator.

1. Close all projects.
2. Select the **Extras ▸ Options** menu command to open the window of the same name.
3. Make the following settings on the tabs:

Tab	Function
General	Define that the last measurement results are saved automatically (→ section "Opening an automatically saved project" p. 20). Define the saving mode of projects and their file names (→ section "Saving a project" S. 21).
Data format	Select decimal separator for the data export and number of decimal places to be displayed for different variables.
Language	Select language of the qPCRsoft program interface.

Measurement	**Sensitivity** Set the basic sensitivity for optical measurements. **Meas. rep. color comp.** Enter the measurement repetitions for recording the color compensation. **Show negative values resulting from color compensation** If activated, negative values are also displayed in the sequence of the color compensation, otherwise the output is "0". **STC (simulated tube control) active** If active, the temperature control of the sample block is performed in such a way to obtain the temperature profile desired immediately in the sample. This function is default when generating a new template.
Analysis	You can enter a factor for the quantitative evaluations (**Quant factor),** for the melting curve analysis (**Melting factor**) and for the genotyping (**Genotyping factor**) in the list fields. This factor will be used for the automatic calculation of the threshold. **Fix scaling to 100%** In all charts which display normalized fluorescence (fluorescence, dRn) the maximum value of the Y axis can be set to 100% permanently. There will be no auto scale of the Y axis. This option simplifies the evaluation of weak fluorescence data.
Device	Enables you to define a file name into which the device communication data will be written. The recorded data is used for error diagnosis (→ section "Appendix B - How to save communication data" p. 154). With the **Select device** function, the qPCRsoft program can be switched over to other real-time PCR devices offered by Analytik Jena AG, without the appropriate device being connected. Projects that were created with this device can thus be analyzed on a different PC than that used for measurement. If a device is connected via USB und turned on when starting the program, it is automatically selected.
File	If activated, the qPCRsoft program starts and the file opens if you select a file of this type in the file explorer of the operating system. See also information about file formats in section "Managing projects and templates" p. 19.
User management	Activate or deactivate the user management. If the user management is deactivated, no login prompt will appear at the program start. The functions for setting up the user management and for signing projects will not be available. If the module 21CFR Part11 is installed and active, the user management can't be de-activated.
Colors	Allows to select the color code of sample type and replicates and to let display the curves color according to sample type, well or replicates. The desired colors for wells, replicates, sample types and the marking of positive and negative analyses can also be set.

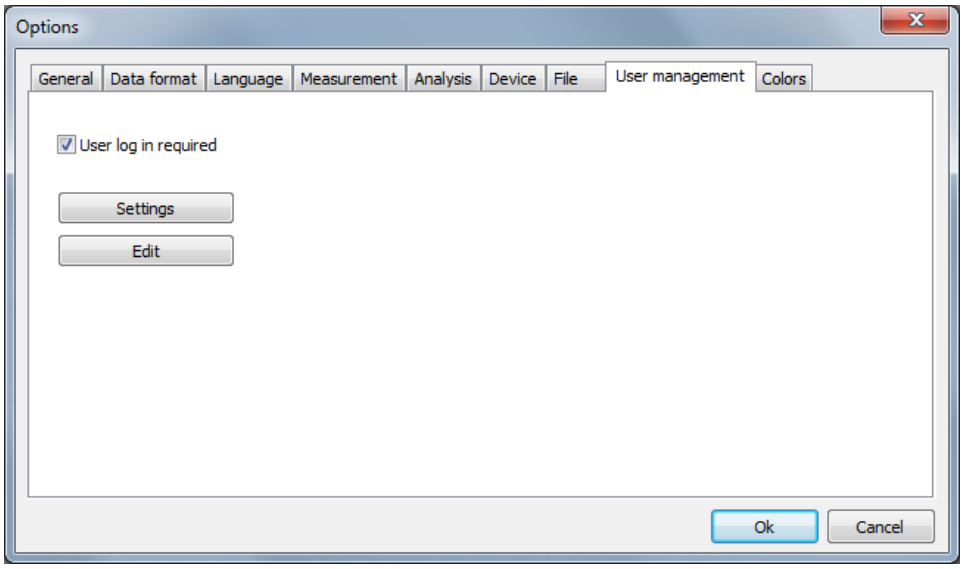
9 User management

Note on the general data security

Due to the encryption used, reading and changing project, template, evaluation and communication files generated by qPCRsoft is only possible with qPCRsoft.

The usermangement will be enabled in the window Options / User management.

Option	Description
User login is required	If enabled, the user management is effective at the next program start. Logging on to the program is then only possible with a valid user profile. Note: The first time the program is started after installation, an administrator with access to the user administration is created.
[Settings]	Settings for passwords, login and logout
[Edit]	Manage user profiles



Window Options / User management

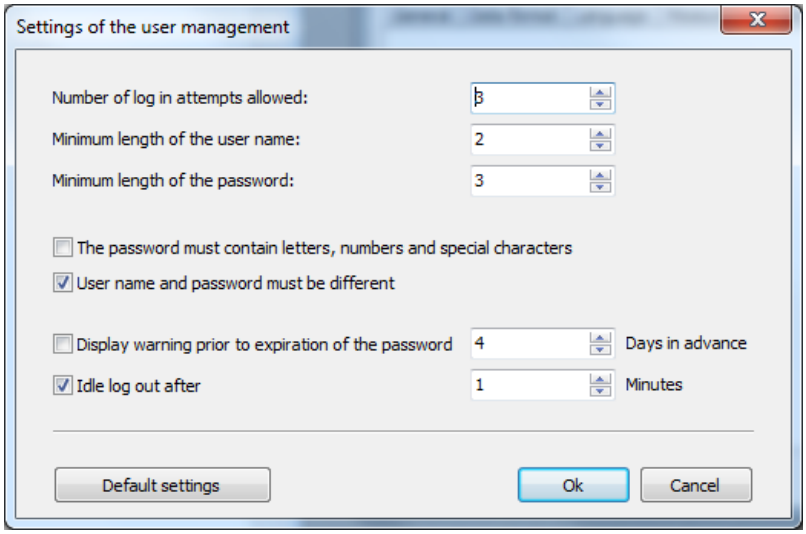
9.1 Basic settings for password, login and logout

To access the basic settings that apply to all users, select the **Extras ▸ Options** menu command. On the **User management** tab click on **[Settings]**.

You can make the following settings in user management:

- Number of login attempts: If the number of allowed login attempts to a user account is exceeded, i.e., if the attempts fail, the user account is deactivated. It can then only be reactivated by the administrator.
- Minimum length of the user name and the password
- Required characters in the password
- Warning prior to the password expiring
  The password expiring is set in the user profiles

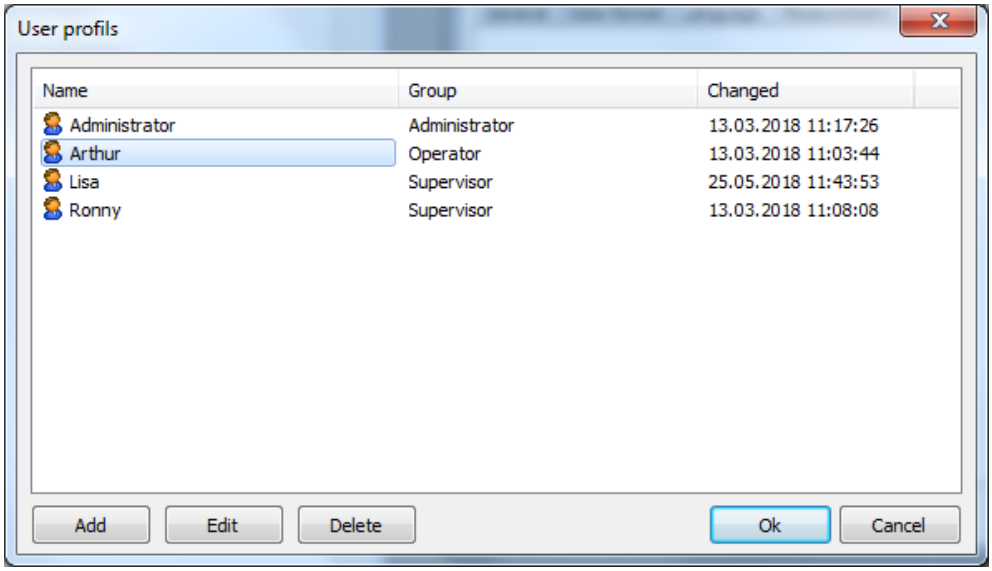
- Logout in case of inactivity: After the specified time has expired without mouse or keyboard activity, the program interface will be locked and the login window is displayed. The user first has to enter the password before the interface can be used again. If **[Cancel]** is selected in the login window, the program is closed. It is not possible to change the user at this point. Automatic logout does not occur if a qPCR run is active.



General settings in user management

9.2 Managing user profiles / user groups

To access user management, select the **Extras ▸ Options** menu command. On the **User management** tab click on **[Edit]**.



User management with the overview of the created user profiles

The following functions are available in the extended user management:

Function	Description
[Add]	Create a new user profile

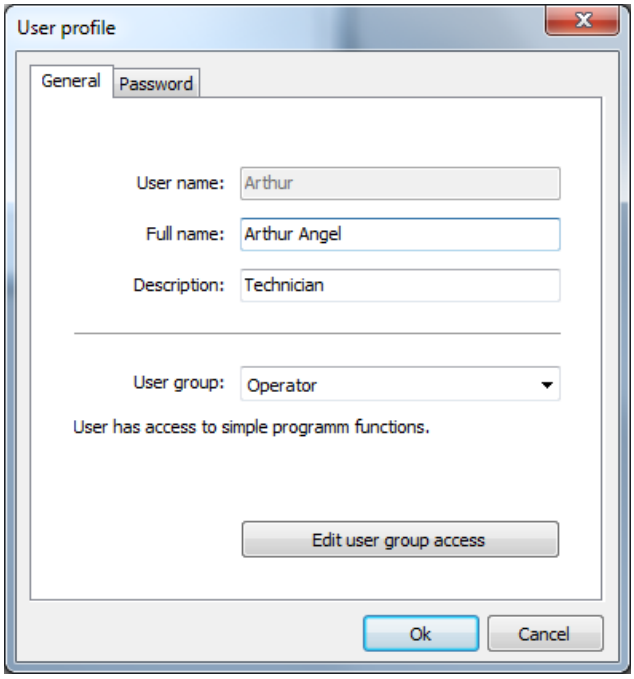
[Edit]	Edit a selected user profile
[Delete]	Delete a selected user profile

By default, these functions are only available to users belonging to the **Administrator** group. However, they can also be assigned to a **Supervisor** by editing the user rights.

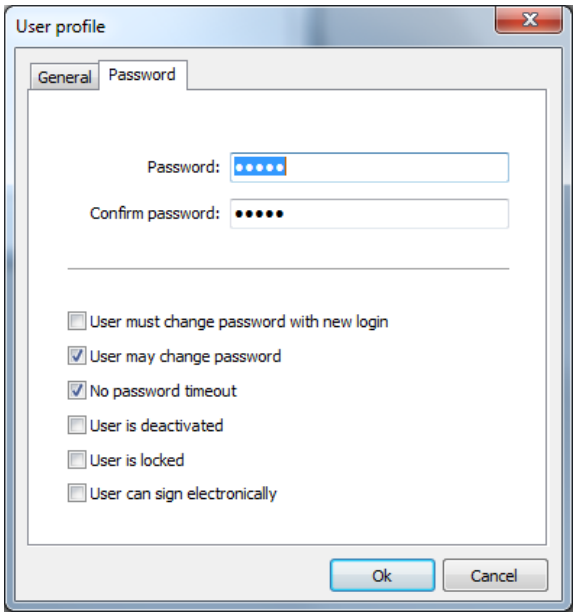
Add / edit user profile

- To create a new user profile, click on **[Add]**.
- To edit an existing user profile, mark the user profile in the list and click on **[Edit]**.
  - ✓ The window for editing the user profile appears.
- Enter the following data:

Option	Description
General tab	
User name	Login name at program start
Full name	Actual name (optional)
Description	Further description (optional)
User group	Assign a user group The rights of each individual user can be individually adjusted within the user group (see below).
Password tab	
Password	Enter the password
Confirm the password	Repeat the password
User must change password with new login	If activated, users must change their password at first login.
User may change password	Users are allowed to change their own password.
No password timeout	The password is valid without a time limit. If deactivated, the expiry date must be specified.
User is deactivated	The user profile was automatically locked after multiple failed login attempts or by an authorized user. Enter the number of possible login attempts in the settings (→ section "Basic settings for password, login and logout" p. 136). The time when the user was locked is displayed.
User is locked	The user profile has been locked by an authorized user. The user name no longer appears in the login dialog, however, the user remains created. The time when the user was deactivated is displayed.
User can sign electronically	The user is allowed to sign a project electronically (→ section "Digital signatures" p. 146). This optionis only available, if the optional module 21 CFR Part 11 is enabled.



Settings for the user name and the assigned user group



Settings for the password and the validity of the user profile

User groups

The following user groups are available:

Administrator:

- This user group has unlimited rights to all program functions
- Administrators can create, delete, lock and unlock users and assign rights to them
- They are allowed to change their own password and that of other users
- They can deactivate user management in **Extras ▸ Options ▸ User management**

Supervisor:

- This user group has the same rights as the administrator, but administrators can block certain rights for each user logged in as a supervisor

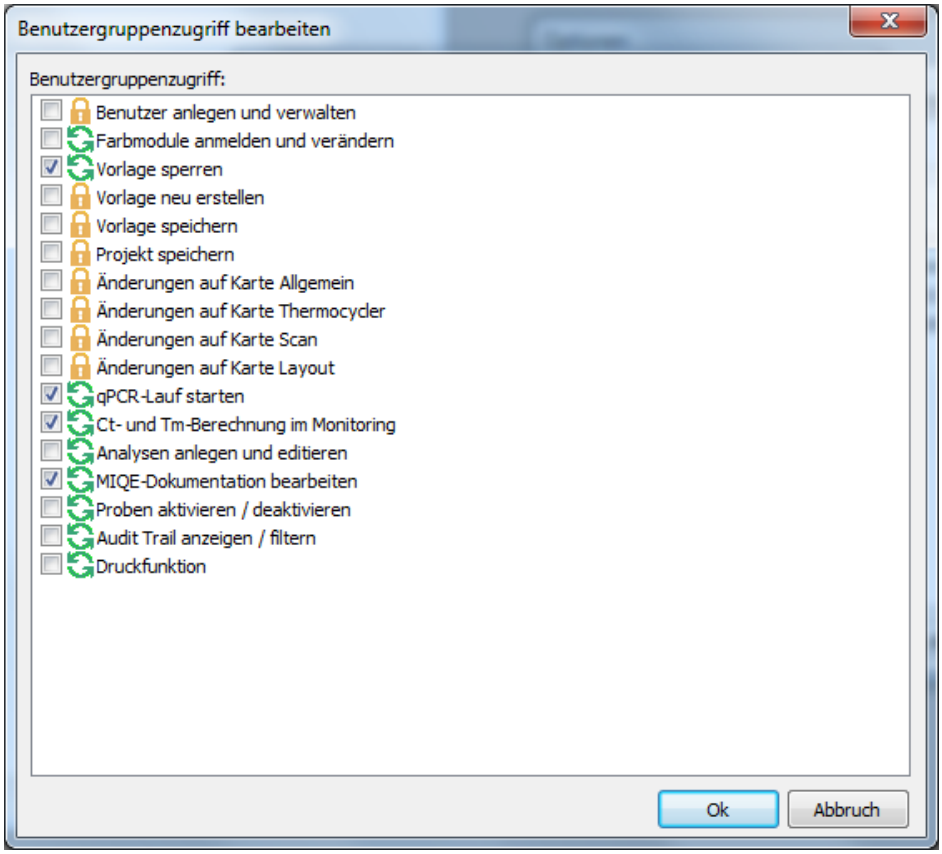
Operator:

The following rights cannot be assigned to an operator:

- Create and manage users
- Create and save templates
- Save projects
- Changes in the **Options** window on the **General**, **Thermal cycler**, **Scan**, and **Layout** tabs

With this selection you automatically assign a certain user role to the user and thus preset rights which you can additionally supplement or reduce using the **Edit user access** function. This allows individual rights to be defined for each user. It is also possible to set up several administrators with different rights.

- In the **User profile / General** window, click on **[Edit user group access]**.
  - ✓ The window of the same name containing the rights of the selected user appears.
- If a checkbox is activated by a checkmark, this right is granted to the user and the user can use the function.
- Checkboxes with a lock symbol cannot be changed.
- The number of these locked rights is determined by the selected **Administrator**, **Supervisor** or **Operator** user role and increases in this order. This means that an operator has fewer rights than a supervisor or administrator from the outset and can never be assigned all rights.
- An administrator has all rights in the program and these rights can only be restricted by removing the checkmarks. The right of an administrator to manage and create users cannot be locked, otherwise user management would no longer be possible.



Configuring an individual user profile within a user group

9.3 Edit password

If the user may change his password, he can open his user profile in the user management and change the password. Supervisors and operators can only do this in the usermanagement. They have not access to over functions in the usermangement.

1. Open with the menu point **Extras ▸ Options the window Options / User management.**
2. Open with **[Edit]** the window **User profiles**.
3. Select your user profile and click on **[Edit]**.
4. Change and confirm the password in the window **User profile / password.**
5. Confirm the settings and exit all windows with [OK].

10 Optional 21 CFR Part 11 module

Starting with version 4.1, the qPCRsoft software contains functions that make the program compatible with the requirements of 21 CFR Part 11. After fee-based activation, the following functions are available:

- **Audit Trail** in templates and projects, i.e., changes made to the project settings are continuously recorded.
- **Login Monitoring** during which attempts to log in to the program and also changes to the user settings are stored
- **Editors** for the evaluation of audit trails and log files using search functions, including a print function
- Creation and display of **Digital Signatures** in templates and projects with monitoring of their validity
- **Automatic Logout** in case of inactivity with the option of adjusting the time

To activate the functions, you need an activation code that can be ordered from your contact person or dealer. This fee-based activation code entitles you to use the software on a single computer (single-user license). If it is planned to use this optional module on several computers, an activation code is required for each computer.

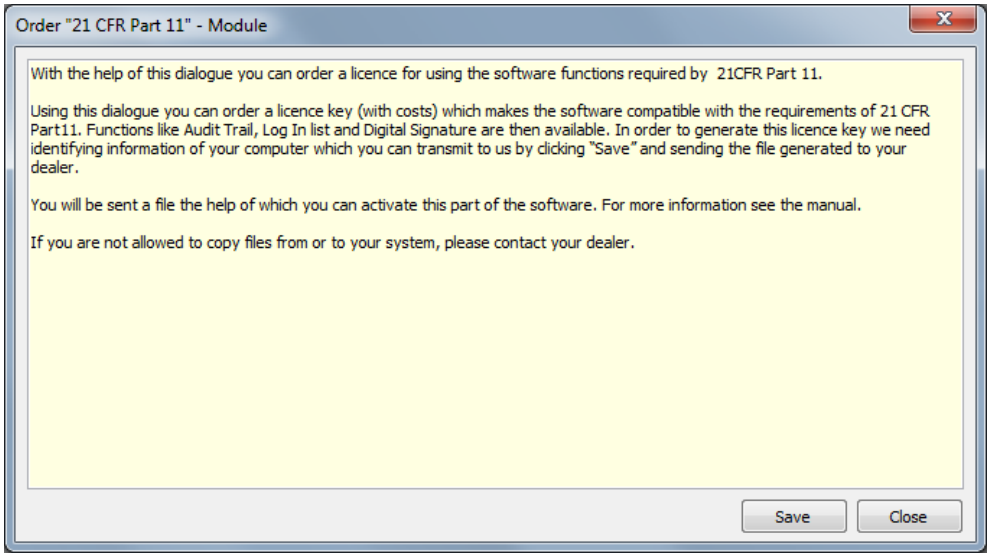
10.1 Activation

The module is activated in two steps:

- Send an order file (*.RTO) to Analytik Jena
- Import an activation file (*.RTL) sent by Analytik Jena

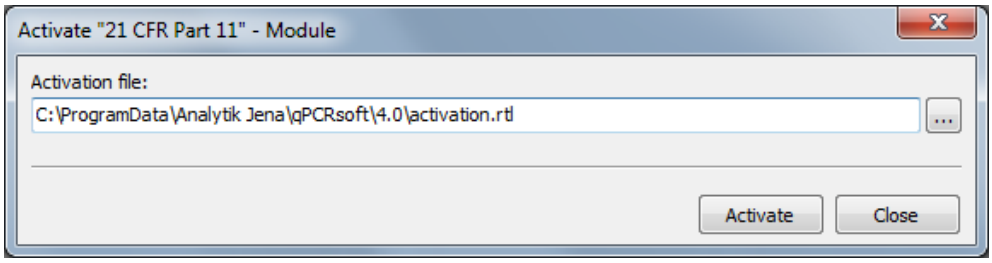
Automatic activation

- Select the **Help ▸ Additional modules ▸ Order "21 CFR Part 11" module** menu command.
  - ✓ A window containing information on how to continue appears.



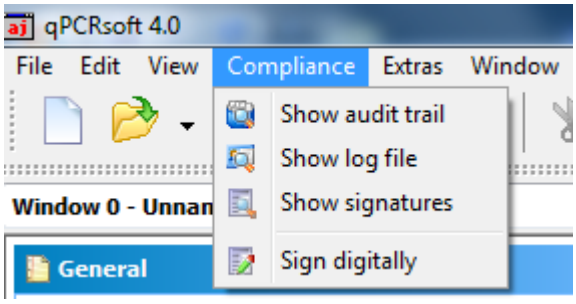
Creating the RTO file for ordering the activation code

- Click on **[Save]**.
  - ✓ This creates a file for generating the activation code.
- Save the file with the extension "*.rto" under any name on your computer and send this file by e-mail to your dealer or contact person.
- As soon as Analytik Jena has processed your order, you will receive a file for activating the 21 CFR Part 11 functions (license file). Copy the file to the hard drive on your computer.
- Start qPCRsoft and select the **Help ▸ Additional modules ▸ Activate "21CFR Part11" module** menu command.
- In the **Activate "21 CFR Part 11" module** window, enter the storage location for the license file and click on **[Activate]**.



Importing the RTL file for activation

  - ✓ A notice field indicates whether activation was successful.
- After completing activation, restart the software and check whether the new functions appear in the **Compliance** menu.
  Note: The functions are only active if user management is activated.
  - ✓ If the functions are displayed, activation has been successfully completed. If activation is not successful, contact your contact person or dealer.



Compliance menu for the "21 CFR Part 11" module

Note on data protection

The created RTO-file contains the name of the computer on which activation should take place and the MAC address of the network card in encrypted form. Analytik Jena uses this data in order to automatically generate the activation code for your computer. The data is processed in encrypted form. It is not used for other purposes or passed on to third parties.

Manual activation

If you are not allowed to copy or send files on your computer, a manual activation method is available. In this case, contact your contact person or dealer.

10.2 Audit trail

The audit trail records changes to metadata in templates and projects. Metadata determines how the results are calculated and displayed from the raw data of a qPCR run, and thus influences the result of the experiment. The raw data, on the other hand, always remains unchanged, meaning it is not part of the audit trail. If settings are changed in the project or the template, the new metadata is appended as a block to the original data when the file is saved. This process takes place automatically in the background. If a project is frequently opened and edited, the scope of the audit trail can increase considerably. It is not possible to delete contents in the audit trail.

Display and evaluate audit trail

Search functions are available for evaluating the (in some cases extensive) audit trail of a project or a template. These functions allow you to systematically search for and identify changes to metadata.

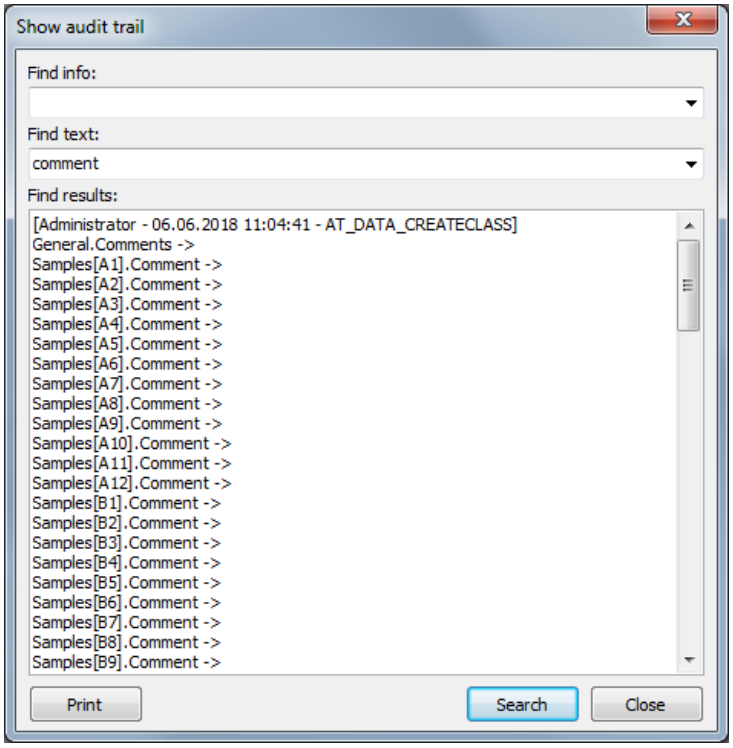
- Select the **Compliance ▸ Show audit trail** menu command.
- To start the search, click on **[Search]**.
- To print out the content that is displayed in the text field (the complete audit trail or reduced content as a result of a search), click on **[Print]**.

The following search options are available:

- The **Find info** and **Find text** input fields are empty: The entire audit trail is displayed.
- There is an entry in the **Find info** field: Search in the block headers, i.e., in the text in the square brackets. This makes it possible to track when the file was created, by whom, and how often it was edited.
- There is an entry in the **Find text** field: Search in the audit trail. A search can be carried out for terms that characterize certain settings for the qPCR run, the display

of results, the evaluations, the layout and general information. An overview of the terms and their meaning can be found in Annex D.

The input fields save search terms that have been previously used and make them available for reuse via the drop-down menu

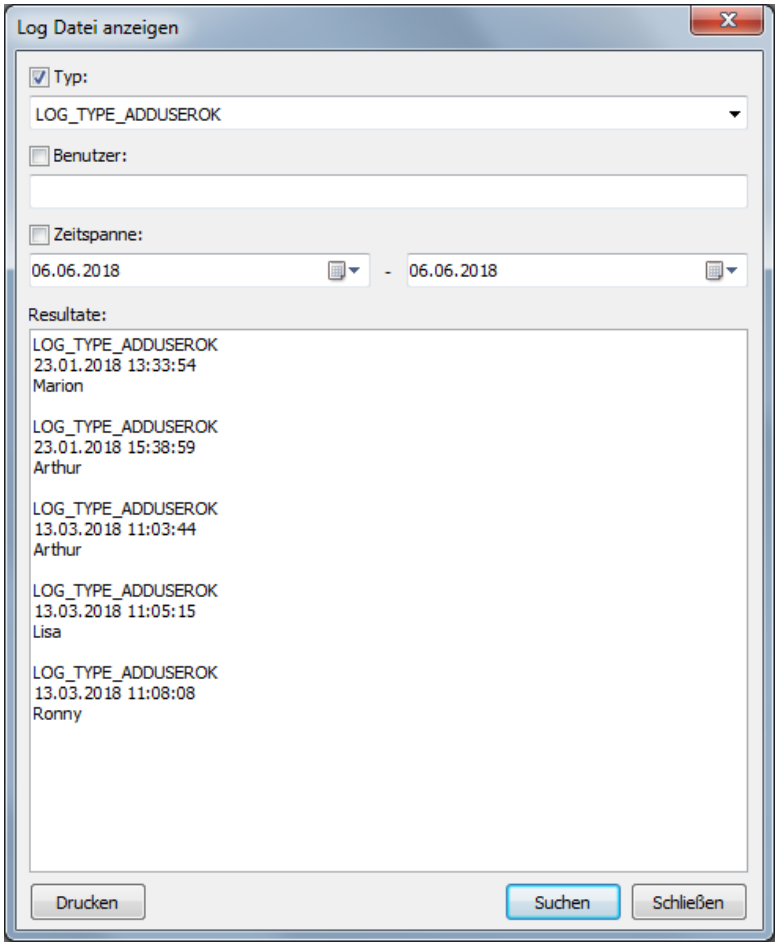


Audit trail for a project with a search

10.3 Login monitoring

All login processes in the qPCRsoft software are monitored and stored in encrypted form in a log file that is protected against manipulation by a checksum. This file also contains information about newly created users and digital signatures. The program records whether logins and signatures were successful or failed. In case of failed operations, the cause is also indicated (wrong password, wrong user name).

- Select the **Compliance ▸ show log file** menu command.
- Limit the display by activating the checkboxes and the selection in the corresponding lists.
- To print the displayed results, click on **[Print]**.



Log file with filter

10.4 Digital signatures

10.4.1 Signing a document

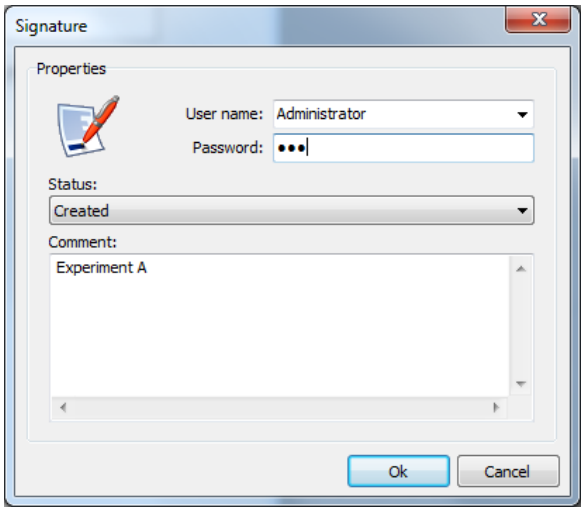
Registered users who also have the relevant authorization can digitally sign a template or a project (→ section "Managing user profiles / user groups" p. 137). The signature is equivalent to a handwritten signature because it can be clearly traced back to its creator.

Sign an active document

- Select the **Compliance ▸ Sign digitally** menu command.
  - ✓ The **Signature** window appears.
- Make the following settings:

Parameter	Description
User name	Select the registered users who are authorized to sign The user logged in to the program is preset in the selection
Password	Enter the user's password
Status	Select the signing status: **Created**, **Edited** and **Released**
Comment	Additional explanation of the signature (optional)

- Confirm your entries with **[OK]**.
  - ✓ The digital signature is saved in the project or the template. Digital signatures are valid as long as no changes are made to the project or the template.

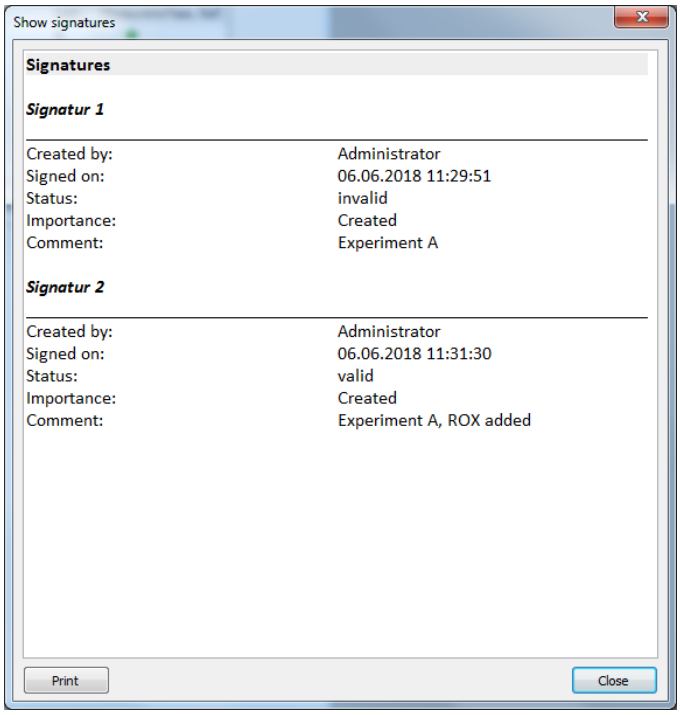


Window for signing a template or a project

10.4.2 Displaying signatures

You can view and print out the signatures assigned to a project or a template.

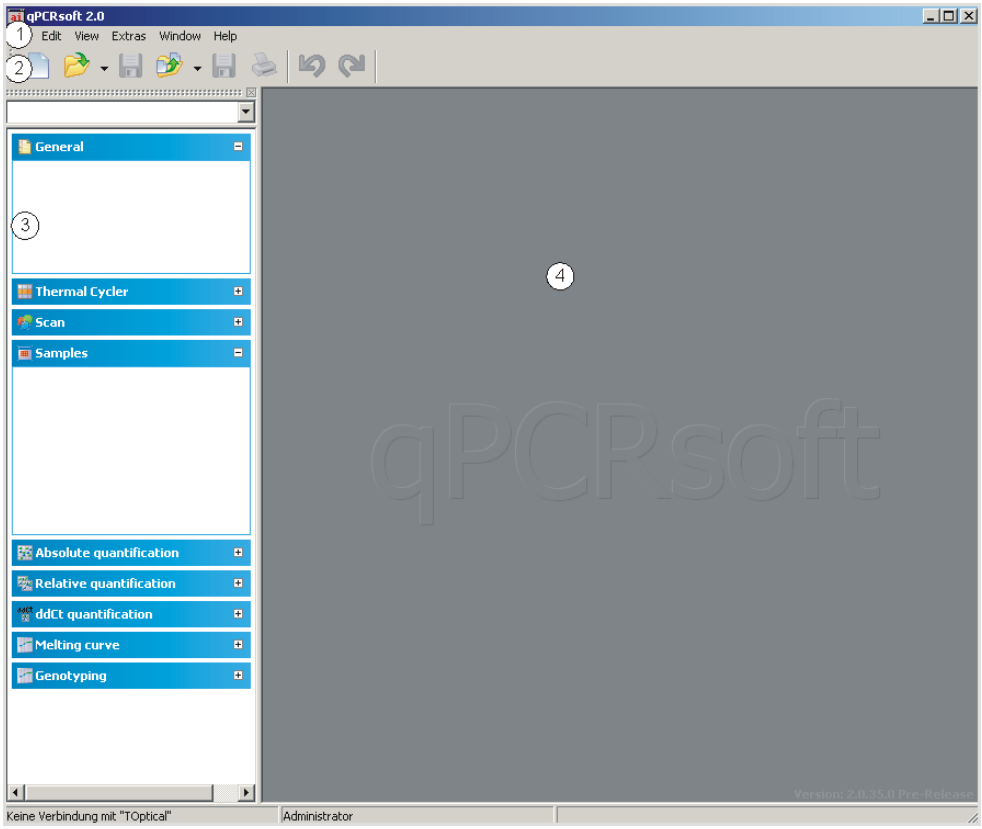
- Select the **Compliance ▸ Show signatures** menu command.
  - ✓ The **Show signatures** window appears. You can check the validity of the date and the creator of the signature.
- To print the displayed data, click on **[Print]**.



Overview of the signatures of a template or a project

11 Appendix A – Short instruction

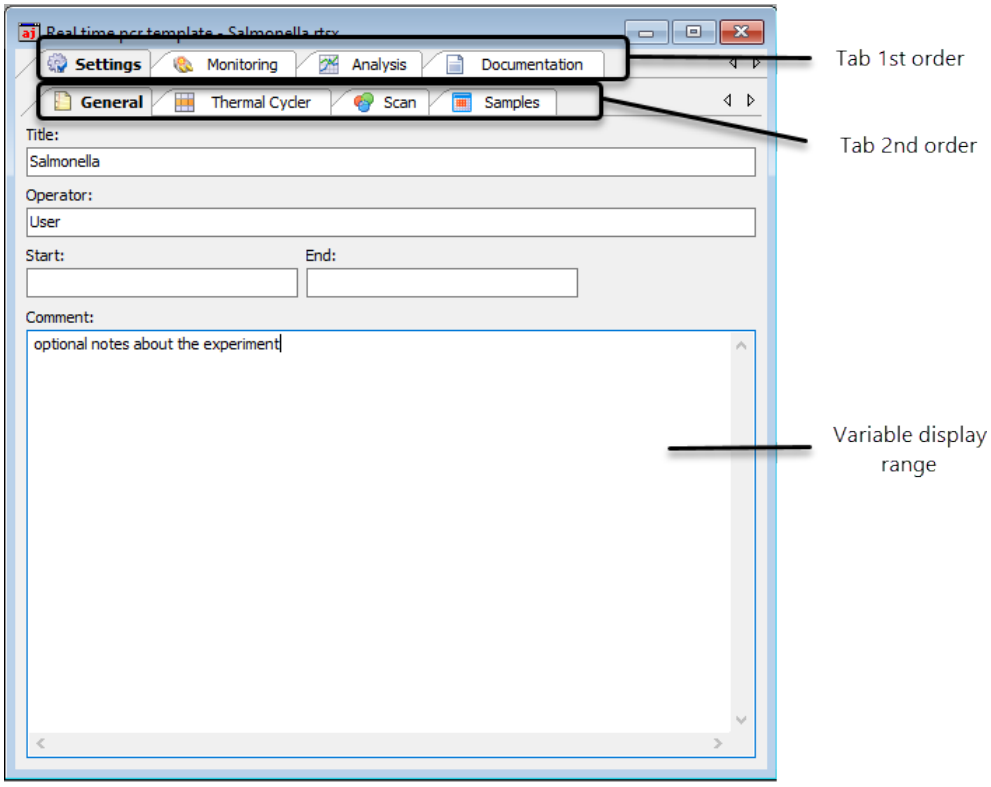
The qPCRsoft main window



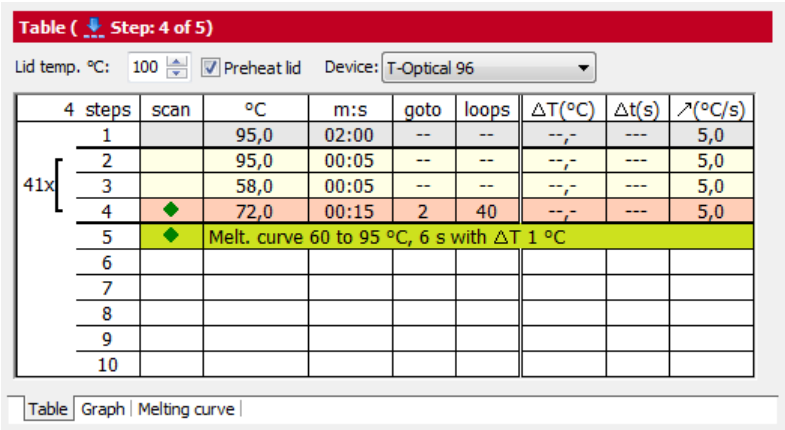
The main window is arranged in different areas: Menu bar (1), Toolbar (2), Project explorer (3), Project interface (4).

Project window - **Settings**

1. To create a new project select the menu command **File ▸ New** or press the button in the toolbar.
   Use the commands **File ▸Open project** or **File ▸Open template** to load saved files.
2. Open the **Settings** tab.

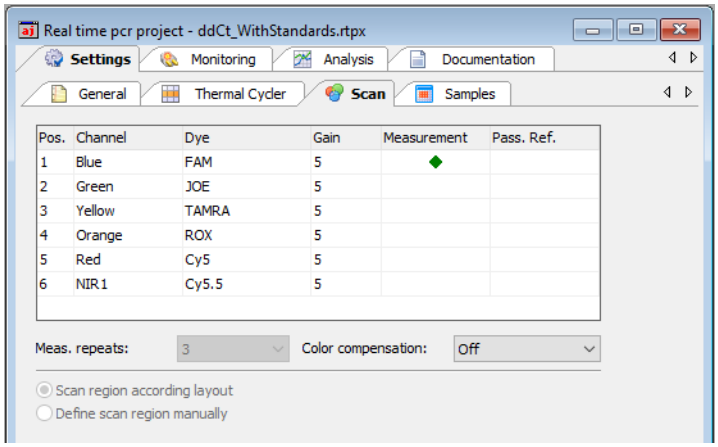


3. Enter general information for the project under tab **General**.
4. Enter PCR program under tab **Thermal Cycler**.



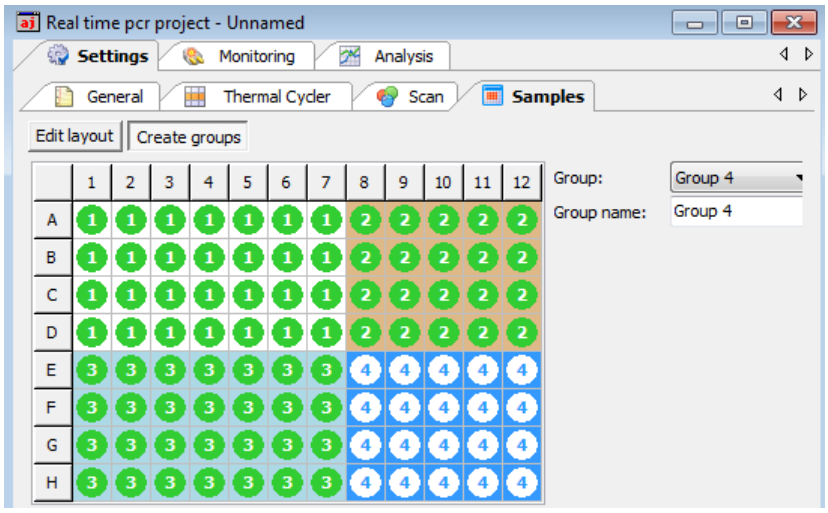
- Set lid temperature and activate or deactivate preheat mode.
  - Set temperature and time for each step. If desired define gradient (only for devices with gradient function).
  - For loops set the step number in column **goto** the program jumps back to and in the column **loops** define the number of measurement repeats.
  - If necessary define temperature or time increments or adjust the ramping rates.
  - If necessary activate the melting step and edit parameters.
  - In the column **scan** define at which step the fluorescence is measured.

5. Define the fluorescence measurement settings on the tab **Scan**:



- Set hash key in the column **Measurement** for each channel to measure
- Select dye to measure in column **Dye** and set LED intensity in column **Gain**.
- For passive reference check mark in column **Pass. Ref.**
- Set number of measurement repeats and define region to scan.
- If necessary activate color compensation.

6. Define plate layout under the tab **Samples** (can be done during the run):
   - Set sample name and sample type.
   - Enter name for the gene to be measured in column **Gene**.
   - For standards enter concentration in column **Conc.** and set unit
   - Select position or region in the plate layout.
   - Assign settings to the selected wells using Toolbar button .
7. If the plate contains samples from different experiments that have to be analyzed separately create groups:

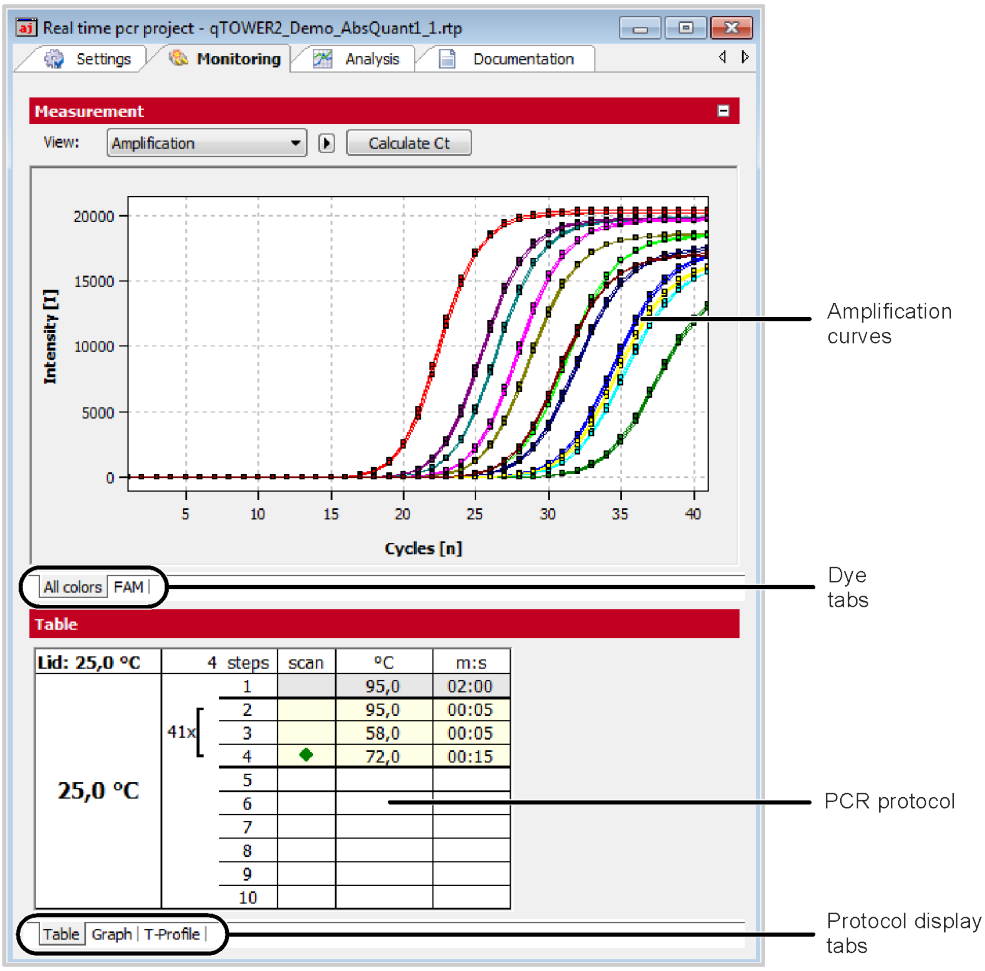


- Select group and set group name.
- Select position or region in the plate layout.
- Assign settings to the selected wells using Toolbar button .

8. The layout preview functions provide a comprehensive overview for the plate layout. To activate the layout preview use button .

Project window - **Monitoring**

- Press button to start the run
- In the **Monitoring** window the results of the run are displayed in real-time

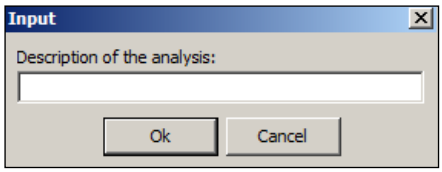


- For the results either the PCR accumulation curves or the melting curves can be displayed. Use selective list **View** to display the different results.
- Use dye tabs to select between the different dyes.
- The PCR protocol can be displayed tabular, graphical or as temperature profile.
- After the PCR run, Ct values and melting temperatures can be calculated for all samples without applying an evaluation. Click the **[Calculate Ct]** button in the amplification curve display or **[Calculate T_m]** in the melting curve display.

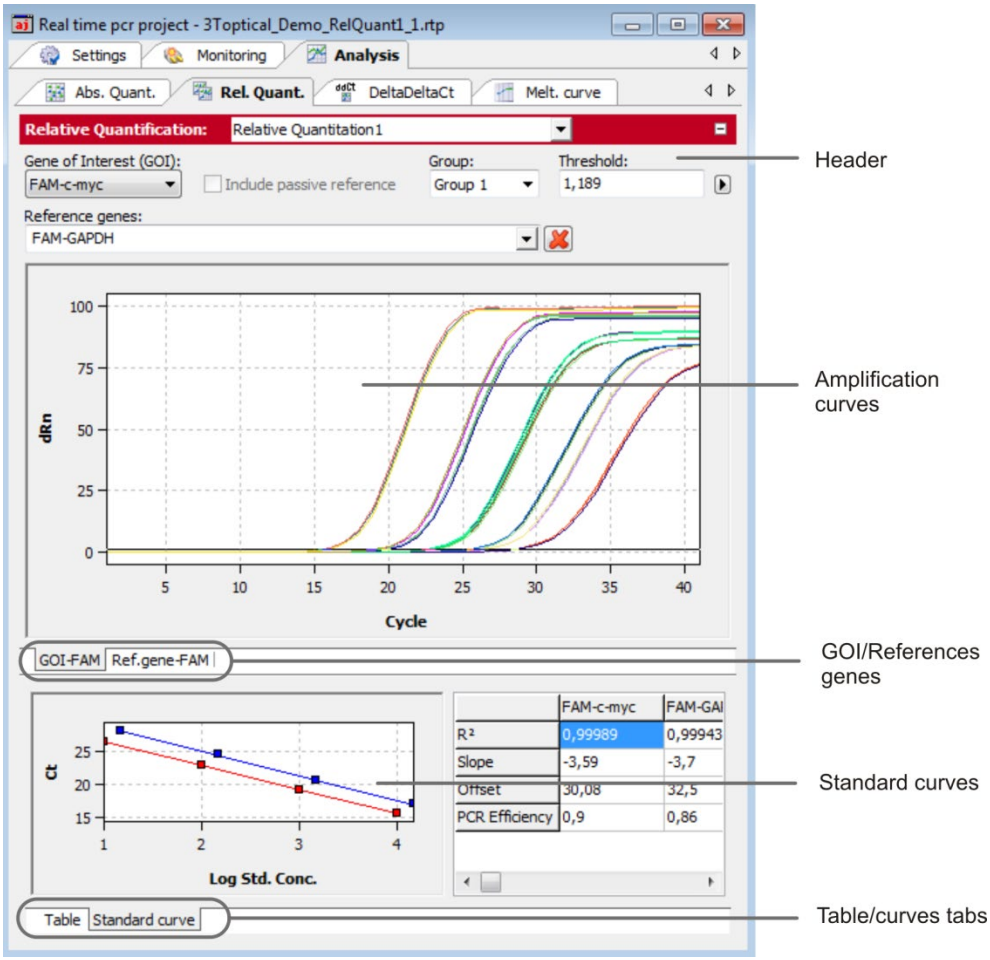
Project window - **Analysis**

For analysis different analysis methods for absolute quantification, relative quantification, ΔΔCt-method, genotyping and melting curve analysis are available.

- To start an analysis select corresponding tab and press button **[Add analysis]** in the toolbar. This button is labeled with a + -symbol (for example).
- In the window that opens enter a name for the analysis:

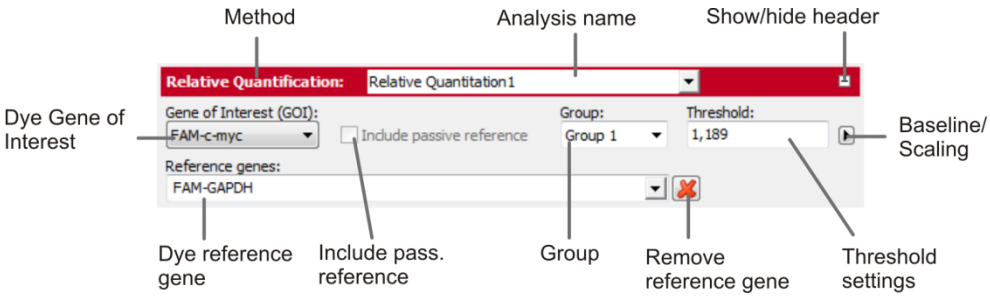


The analysis window is separated in a header for general settings, a graphical chart to display amplification curves and an area to display standard- or validation curves or the result table:



In the header various settings can be made that differ by the analysis methods:

- Select gene of interest (GOI).
- If applicable set passive reference (e.g. ROX) for normalization.
- Select between different experiments (groups).
- Select at least one reference gene to calculate a standard or validation curve.
- Set baseline correction.
- Set threshold (manually or automatically).
- Switch between linear and logarithmic display of the fluorescence intensities.

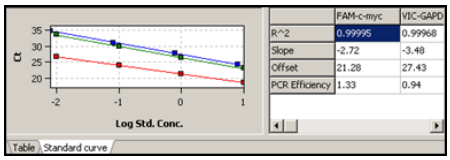


- In the amplification curves area the fluorescence intensity curves are shown:
- In the linear mode the base line correction can be checked.
- In the logarithmic mode the threshold line can be set manually. Move the threshold line up or down by using the mouse.
- Switch by the tabs between the different dyes.
- If the cursor is moved on a curve some short information for the sample is displayed.

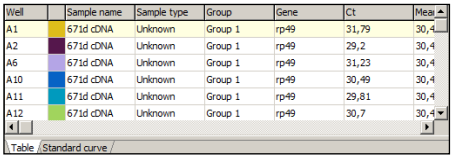
In the lower part of the analysis window the standard or validation curves are displayed:

- For the curves calculated values are displayed in a table.
- Use tabs to switch between the display of curves and of the result table.

Standard-/Validation curves



Results table



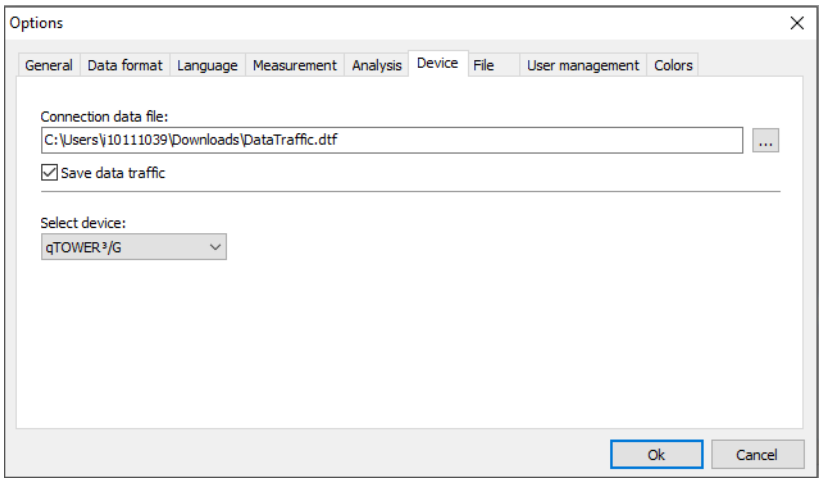
- Define columns to be displayed in the result table after right mouse click on a column header.
- Export data from the result table as *.csv files after right mouse click in the table.

12 Appendix B - How to save communication data

With problems with your device you can record the communication data of the device and send it to the service. This makes easier the distant diagnosis. For this purpose the recording must be activated and a file name to the memory of the data be agreed.

Note
To the activation of the recording of the data traffic you must be announced as an administrator.

1. Run qPCRsoft.
2. Choose the menu command **Extras ▸ Options** and open the **Device** tab.
3. Activate the checkbox **Save data traffic**.
4. Click the button [...], the **Open** dialog appears.
5. Type in a file name to which the communication data shall be sent. The extension of the traffic file is "*.dtf".
6. Confirm the dialog by clicking **[Open]**.



During the next PCR run the communication data between instrument and PC are recorded in the file denoted. Send this file to Analytik Jena upon request.

13 Appendix C – Creating a project template from the transfer file (LIMS)

The qPCRsoft software can be configured by another program (e.g. a Laboratory Information Management System" (LIMS)). In order to do this, the LIMS has to create a file that is imported by qPCRsoft via the **File ▸ Import LIMS** function. The so-called transfer file has a specific structure that can be provided by Analytik Jena if requir qPCRsoft soft creates a template with the transfer file, with which a PCR run can be started immediately.

The different export functions of qPCRsoft can be used to transfer the results of the PCR run to the LIMS, depending on which data the LIMS expects.

14 Appendix D – Entries in the Audit Trail

Audit-Trail print out	Description
[Administrator - 25.06.2018 08:47:00 - AT_DATA_CREATECLASS]	Who created this project and when? Default Audit Trail data set is following.
[Administrator - 04.07.2018 11:10:17 - AT_DATA_SAVEPROJECT]	Who saved this project and when? Altered Audit Trail data set is following.
General	
DEVICETYPE	device type: 3=qTOWER[3]
APPVERSION	version of qPCRsoft used
FIRMWAREVERSION	firmware version of the connected device
DataUser.Name -> Administrator	logged in user
DataUser.Level	general user role (Administrator, Supervisor, Operator)
DataUser.LevelBits	granted rights of this user, coded in bits
DataUser.Password	password of the logged in user (not displayed here)
DataUser.Userdependent	not used
General.Title	titel entered on the card General
General.Operator	operator
General.DateTime	date and time of project creation
General.Comments	comments entered on the card General
General.DeviceID	device identification data read from EEPROM
Thermal Cycler	

CyclerProgram -> ProgData.BlockType=14;ProgData.LidTemp=100;ProgData.HotStart=True; ProgData.Control=10;ProgData.Standby=False;ProgData.BlockTemp=12; ProgData.MeasTime=10;MeltData.StartTemp=60; MeltData.EndTemp=95;MeltData.Gradient=1;MeltData.Time=16.128;MeltData.Ramp=5; MeltData.Equilibration=6;MeltData.Active=False;StepCount=4;Head.ProgramNumber=1; Head.Gradient=False;Head.ProgramPath=TOP;Head.ProgramName=PCR; Head.ProgramDate.Day=11; Head.ProgramDate.Month=7;Head.ProgramDate.Year=12;Step1.ScanFalse;Step1.Temp=95; Step1.Time=02:00;Step1.Goto=-;Step1.Loops=-;Step1.TempInc=-;Step1.TimeInc=-;Step1.Ramp=5;Step2.ScanFalse; Step2.Temp=95;Step2.Time=00:05;Step2.Goto=-;Step2.Loops=-;Step2.TempInc=-;Step2.TimeInc=-; Step2.Ramp=5;Step3.ScanFalse;Step3.Temp=58;Step3.Time=00:05;Step3.Goto=-;Step3.Loops=-; Step3.TempInc=-;Step3.TimeInc=-;Step3.Ramp=5;Step4.ScanTrue;Step4.Temp=72;Step4.Time=00:15; Step4.Goto=2;Step4.Loops=40;Step4.TempInc=-;Step4.TimeInc=-;Step4.Ramp=5;	program of the thermal cycler used fo this experiment
Fluorescence Measurement	
ColorModule.Position -> 1	color module position 1
ColorModule.Code -> Blue.470.520.11.3	type of the color module used
ColorModule.Color -> FAM	dye selected
ColorModule.Gain -> 5	set gain
ColorModule.Meas -> -1	activated for measurement (-1) or not (0)
ColorModule.Refr -> 0	used as passive reference, Yes (-1), No (0)
ColorModule.Position -> 2	color module position 2
ColorModule.Code -> Green.515.545.11.2	type of the color module used
ColorModule.Color -> JOE	dye selected
ColorModule.Gain -> 5	set gain
ColorModule.Meas -> 0	activated for measurement (-1) or not (0)
ColorModule.Refr -> 0	used as passive reference, Yes (-1), No (0)
ColorModule.Position -> 3	color module position 3
ColorModule.Code -> Yellow.535.580.11.2	type of the color module used
ColorModule.Color -> TAMRA	dye selected
ColorModule.Gain -> 5	set gain
ColorModule.Meas -> 0	activated for measurement (-1) or not (0)
ColorModule.Refr -> 0	used as passive reference, Yes (-1), No (0)
ColorModule.Position -> 4	color module position 4

ColorModule.Code -> Orange.565.605.11.2	type of the color module used
ColorModule.Color -> ROX	dye selected
ColorModule.Gain -> 5	set gain
ColorModule.Meas -> 0	activated for measurement (-1) or not (0)
ColorModule.Refr -> 0	used as passive reference, Yes (-1), No (0)
ColorModule.Position -> 5	color module position 5
ColorModule.Code -> Red.630.670.11.1	type of the color module used
ColorModule.Color -> Cy5	dye selected
ColorModule.Gain -> 5	set gain
ColorModule.Meas -> 0	activated for measurement (-1) or not (0)
ColorModule.Refr -> 0	used as passive reference, Yes (-1), No (0)
ColorModule.Position -> 6	color module position 6
ColorModule.Code -> NIR1.660.705.11.1	type of the color module used
ColorModule.Color -> Cy5.5	dye selected
ColorModule.Gain -> 5	set gain
ColorModule.Meas -> 0	activated for measurement (-1) or not (0)
ColorModule.Refr -> 0	used as passive reference, Yes (-1), No (0)
Compensation.Name ->	name of the color compensation used
Compensation.Infos[0].Position through Compensation.Infos[5].Position	information about color modules, dyes and gains used for this color compensation, displayed for all 6 color module positions
Compensation.Infos[0].Color through Compensation.Infos[5].Color	
Compensation.Infos[0].Gain through Compensation.Infos[5].Gain	
Compensation.Matrix[0, 0] through Compensation.Matrix[5,5]	elements of the color compensation matrix
Scan.Repetitions -> 3	number of measurement repeats
Scan.SpectralCompensation -> 0	type of the color compensation (off, standard, select)
Scan.FromCol -> 1	begin of the scanned area of the plate
Scan.ToCol -> 12	end of the scanned area of the plate
Scan.ColRangeType -> 1	select scanned area from layout (-1) or manually (0)
Layout	
Layout.Code -> Blue.470.520.11.3	color module the following gene is measured with
Layout.Infos[A1].Gene through Layout.Infos[H12].Gene	gene name of each well of the plate
Layout.Infos[A1].Concentration through Layout.Infos[H12].Concentration	standard concentration of each well of the plate
Units -> ng	concentration unit
Samples[A1].Name through Samples[H12].Name	sample name for all wells
Samples[A1].Typ through Samples[H12].Typ	sample type for all wells
Samples[A1].SubTyp through Samples[H12].SubTyp	sample sub type for all wells

Samples[A1].Comment through Samples[H12].Comment	comments for each well
Samples[A1].Active through Samples[H12].Active	Is this well activated?
Samples[A1].Marked through Samples[H12].Marked	Is this well marked in the explorer?
Groups[A1] -> 0 through Groups[H12]	To which group does the well belong?
GroupNames -> Group 1 through GroupNames -> Group 12	names of the 12 groups possible
LOCK -> 0	template locked? Yes (-1), No (0)
Calculate Ct	
CtAnalysis.Thresholds -> 0,794712458619839	threshold value the Ct values were calculated with
CtAnalysis.Smooth -> -1	smoothing On(-1), Off(0)
CtAnalysis.SmoothMode -> 5	number of points used for smoothing
CtAnalysis.Log -> 0	logarithmic chart On(-1), Off(0)
CtAnalysis.BaseLineRange[0].Min -> 3	manually set lower boundary of the base line (cycle number)
CtAnalysis.BaseLineRange[0].Max -> 15	manually set upper boundary of the base line (cycle number)
CtAnalysis.BaseLineRange[1].Min -> 5	automatically set lower boundary of the base line (cycle number)
CtAnalysis.BaseLineRange[1].Max -> -1	calculate automatically upper boundary of the base line range Yes (-1), No (0)
CtAnalysis.AutoBaseLine -> -1	automatic base line determination On (-1) or Off (0)
CtAnalysis.AutoThreshold -> 0	auto threshold On(-1), Off(0)
CtAnalysis.Filter -> -1	filter On(-1), Off(0)
CtAnalysis.FilterOptions -> 2	filter strength slight(0), medium(1) or strong(2)
CtAnalysis.FilterSmooth -> 0	smooth the filter data, Yes (-1), No (0)
Calculate Tm	
TmAnalysis.GOI.Color ->	melting curve GOI
TmAnalysis.GOI.Gene ->	melting curve of gene
TmAnalysis.Threshold -> 0	threshold value for determination of melting temperature
TmAnalysis.Smooth -> -1	smooth melting curve , Yes (-1), No (0)
TmAnalysis.SmoothMode -> 3	number of points used for smoothing
TmAnalysis.Log -> 0	logarithmic chart On(-1), Off(0)
TmAnalysis.BaseLineRange.Min -> 1	manually set lower boundary of the base line (cycle number)
TmAnalysis.BaseLineRange.Max -> 5	manually set upper boundary of the base line (cycle number)
TmAnalysis.AutoThreshold -> 0	auto threshold for melting curve, Yes (-1), No (0)

TmAnalysis.FlipCurve -> 0	flip curve horizontally, On(-1), Off(0)
TmAnalysis.Scaling -> 1	All curves start at 100%(0), Maximum initial fluorescence = 100%(1)
Absolute Quantification	
AbsQuantAnalyzes.Smooth -> -1	smoothing On(-1), Off(0)
AbsQuantAnalyzes.SmoothMode -> 5	number of points used for smoothing
AbsQuantAnalyzes.Log -> 0	logarithmic chart On(-1), Off(0)
AbsQuantAnalyzes.BaseLineRange[0].Min -> 3	manually set lower boundary of the base line (cycle number)
AbsQuantAnalyzes.BaseLineRange[0].Max -> 15	manually set upper boundary of the base line (cycle number)
AbsQuantAnalyzes.BaseLineRange[1].Min -> 5	automatically set lower boundary of the base line (cycle number)
AbsQuantAnalyzes.BaseLineRange[1].Max -> -1	calculate automatically upper boundary of the base line range Yes (-1), No (0)
AbsQuantAnalyzes.AutoBaseLine -> -1	automatic base line determination On (-1) or Off (0)
AbsQuantAnalyzes.AutoThreshold -> 0	auto threshold On(-1), Off(0)
AbsQuantAnalyzes.Filter -> -1	filter On(-1), Off(0)
AbsQuantAnalyzes.FilterOptions -> 1	filter strength slight(0), medium(1) or strong(2)
AbsQuantAnalyzes.FilterSmooth -> 0	smooth the filter data, Yes (-1), No (0)
AbsQuantAnalysis.Description -> Quantitation 1	title of this analysis
AbsQuantAnalysis.Group -> 0	valid for group
AbsQuantAnalysis.REF -> 0	passive reference used? Yes (-1), No (0)
AbsQuantAnalysis.GOI.Color -> FAM	dye of GOI
AbsQuantAnalysis.GOI.Gene ->	name of GOI
AbsQuantAnalysis.Threshold -> 1,13570592788773	threshold value the Ct values were calculated with
AbsQuantAnalysis.FitData.Count -> 4	number of standards
AbsQuantAnalysis.FitData.M -> -3,67716352286466	slope of the standard curve
AbsQuantAnalysis.FitData.N -> 28,0638862770259	intercept of the standard curve
AbsQuantAnalysis.FitData.R2 -> 0,999236478923372	correlation coefficient of the linear curve fit
AbsQuantAnalysis.FitData.Extern -> 0	Was the standard curve imported, Yes (-1), No (0)
MIQE.TypeInfo -> 1	MIQE documentation for DNA(0) or RNA(1)
LOCK -> 0	template or project locked? Yes (-1), No (0)
Relative Quantification	
RelQuantAnalyzes.GOISmooth -> -1	smoothing GOI, On(-1), Off(0)
RelQuantAnalyzes.GOISmoothMode -> 5	number of points used for smoothing, GOI

RelQuantAnalyzes.GOILog -> 0	logarithmic chart GOI On(-1), Off(0)
RelQuantAnalyzes.GOIBaseLineRange[0].Min -> 3	manually set lower boundary of the base line (cycle number GOI)
RelQuantAnalyzes.GOIBaseLineRange[0].Max -> 15	manually set upper boundary of the base line (cycle number GOI)
RelQuantAnalyzes.GOIBaseLineRange[1].Min -> 5	automatically set lower boundary of the base line (cycle number GOI)
RelQuantAnalyzes.GOIBaseLineRange[1].Max -> -1	calculate automatically upper boundary of the base line range Yes (-1), No (0)
RelQuantAnalyzes.GOIAutoBaseLine -> -1	automatic base line determination On (-1) or Off (0)
RelQuantAnalyzes.GORSmooth -> -1	smoothing reference gene, On(-1), Off(0)
RelQuantAnalyzes.GORSmoothMode -> 5	number of points used for smoothing of reference gene
RelQuantAnalyzes.GORLog -> 0	logarithmic chart GOI On(-1), Off(0), reference gene
RelQuantAnalyzes.GORBaseLineRange[0].Min -> 3	manually set lower boundary of the base line (cycle number reference gene)
RelQuantAnalyzes.GORBaseLineRange[0].Max -> 15	manually set upper boundary of the base line (cycle number reference gene)
RelQuantAnalyzes.GORBaseLineRange[1].Min -> 5	automatically set lower boundary of the base line (cycle number reference gene)
RelQuantAnalyzes.GORBaseLineRange[1].Max -> -1	automatically set upper boundary of the base line (cycle number reference gene)
RelQuantAnalyzes.GORAutoBaseLine -> -1	automatic base line determination On (-1) or Off (0), reference gene
RelQuantAnalyzes.GOIAutoThreshold -> 0	auto threshold On(-1), Off(0), GOI
RelQuantAnalyzes.GORAutoThreshold -> 0	auto threshold On(-1), Off(0), Referenzgen
RelQuantAnalyzes.GOIFilter -> -1	filter On(-1), Off(0), GOI
RelQuantAnalyzes.GOIFilterOptions -> 1	filter strength GOI, slight(0), medium(1) or strong(2)
RelQuantAnalyzes.GOIFilterSmooth -> 0	smooth the filter data GOI, Yes (-1), No (0)
RelQuantAnalyzes.GORFilter -> -1	filter On(-1), Off(0), reference gene
RelQuantAnalyzes.GORFilterOptions -> 1	filter strength reference gene, slight(0), medium(1) or strong(2)
RelQuantAnalyzes.GORFilterSmooth -> 0	smooth the filter data of reference gene, Yes (-1), No (0)
RelQuantAnalysis.Description -> RelQ	title of this analysis
RelQuantAnalysis.Group -> 0	valid for group
RelQuantAnalysis.REF -> 0	passive reference used? Yes (-1), No (0)
RelQuantAnalysis.GOI.Color -> FAM	dye of GOI
RelQuantAnalysis.GOI.Gene ->	name of GOI

RelQuantAnalysis.GOIThreshold -> 0,794712458619839	threshold value the Ct values of the GOI were calculated with
RelQuantAnalysis.GOR.Color ->	dye of reference gene
RelQuantAnalysis.GOR.Gene ->	name of the reference gene
RelQuantAnalysis.GORThreshold -> 50	threshold value the Ct values of the reference gene were calculated with
RelQuantAnalysis.GOIFitData.Count -> 4	number of standards, GOI
RelQuantAnalysis.GOIFitData.M -> -3,62354563433653	slope of the standard curve, GOI
RelQuantAnalysis.GOIFitData.N -> 27,5354442047509	intercept of the standard curve, GOI
RelQuantAnalysis.GOIFitData.R2 -> 0,999100282770293	correlation coefficient of the linear curve fit, GOI
RelQuantAnalysis.GOIFitData.Extern -> 0	Was the standard curve for GOI imported, Yes (-1), No (0)
RelQuantAnalysis.GORFitData.Count -> 0	number of standards, reference gene
RelQuantAnalysis.GORFitData.M -> 1	slope of the standard curve, reference gene
RelQuantAnalysis.GORFitData.N -> 0	intercept of the standard curve, reference gene
RelQuantAnalysis.GORFitData.R2 -> 0	correlation coefficient of the linear curve fit, reference gene
RelQuantAnalysis.GORFitData.Extern -> 0	Was the standard curve for reference gene imported, Yes (-1), No (0)
DeltaDeltaCt Analysis	
DeltaDeltaCtAnalyzes.GOISmooth -> -1	smoothing GOI, On(-1), Off(0)
DeltaDeltaCtAnalyzes.GOISmoothMode -> 5	number of points used for smoothing, GOI
DeltaDeltaCtAnalyzes.GOILog -> 0	logarithmic chart GOI On(-1), Off(0)
DeltaDeltaCtAnalyzes.GOIBaseLineRange[0].Min -> 3	manually set lower boundary of the base line (cycle number GOI)
DeltaDeltaCtAnalyzes.GOIBaseLineRange[0].Max -> 15	manually set upper boundary of the base line (cycle number GOI)
DeltaDeltaCtAnalyzes.GOIBaseLineRange[1].Min -> 5	automatically set lower boundary of the base line (cycle number GOI)
DeltaDeltaCtAnalyzes.GOIBaseLineRange[1].Max -> -1	calculate automatically upper boundary of the base line range Yes (-1), No (0)
DeltaDeltaCtAnalyzes.GOIAutoBaseLine -> -1	automatic base line determination On (-1) or Off (0)
DeltaDeltaCtAnalyzes.GORSmooth -> -1	smoothing reference gene, On(-1), Off(0)
DeltaDeltaCtAnalyzes.GORSmoothMode -> 5	number of points used for smoothing of reference gene
DeltaDeltaCtAnalyzes.GORLog -> 0	logarithmic chart GOI On(-1), Off(0), reference gene
DeltaDeltaCtAnalyzes.GORBaseLineRange[0].Min -> 3	manually set lower boundary of the base line (cycle number reference gene)

DeltaDeltaCtAnalyzes.GORBaseLineRange[0].Max -> 15	manually set upper boundary of the base line (cycle number reference gene)
DeltaDeltaCtAnalyzes.GORBaseLineRange[1].Min -> 5	automatically set lower boundary of the base line (cycle number reference gene)
DeltaDeltaCtAnalyzes.GORBaseLineRange[1].Max -> -1	automatically set upper boundary of the base line (cycle number reference gene)
DeltaDeltaCtAnalyzes.GORAutoBaseLine -> -1	automatic base line determination On (-1) or Off (0), reference gene
DeltaDeltaCtAnalyzes.GOIAutoThreshold -> 0	auto threshold On(-1), Off(0), GOI
DeltaDeltaCtAnalyzes.GORAutoThreshold -> 0	auto threshold On(-1), Off(0), Referenzgen
DeltaDeltaCtAnalyzes.GOIFilter -> -1	filter On(-1), Off(0), GOI
DeltaDeltaCtAnalyzes.GOIFilterOptions -> 1	filter strength GOI, slight(0), medium(-1) or strong(2)
DeltaDeltaCtAnalyzes.GOIFilterSmooth -> 0	smooth the filter data GOI, Yes (-1), No (0)
DeltaDeltaCtAnalyzes.GORFilter -> -1	filter On(-1), Off(0), reference gene
DeltaDeltaCtAnalyzes.GORFilterOptions -> 1	filter strength reference gene, slight(0), medium(1) or strong(2)
DeltaDeltaCtAnalyzes.GORFilterSmooth -> 0	smooth the filter data of reference gene, Yes (-1), No (0)
DeltaDeltaCtAnalysis.Description -> ddCt	title of this analysis
DeltaDeltaCtAnalysis.Group -> 0	valid for group
DeltaDeltaCtAnalysis.GOI.Color -> FAM	dye of GOI
DeltaDeltaCtAnalysis.GOI.Gene ->	name of GOI
DeltaDeltaCtAnalysis.GOIThreshold -> 0,794712458619839	threshold value the Ct values of the GOI were calculated with
DeltaDeltaCtAnalysis.GOR.Color ->	dye of reference gene
DeltaDeltaCtAnalysis.GOR.Gene ->	name of the reference gene
DeltaDeltaCtAnalysis.GORThreshold -> 50	threshold value the Ct values of the reference gene were calculated with
DeltaDeltaCtAnalysis.EfficiencyCalc -> 0	PCR efficiency calculation according to Livak (0) or Pfaffl (1)
DeltaDeltaCtAnalysis.EfficiencyType -> 0	if Pfaffl: calculate efficiency from standards (0) or entered values (1)
DeltaDeltaCtAnalysis.GOIEfficiency -> 1	PCR efficiency of the GOI
DeltaDeltaCtAnalysis.GOREfficiency -> 1	PCR efficiency of the reference gene
DeltaDeltaCtAnalysis.GOIFitData.Count -> 4	number of standards, GOI
DeltaDeltaCtAnalysis.GOIFitData.M -> -3,62354563433653	slope of the standard curve, GOI
DeltaDeltaCtAnalysis.GOIFitData.N -> 27,5354442047509	intercept of the standard curve, GOI
DeltaDeltaCtAnalysis.GOIFitData.R2 -> 0,999100282770293	correlation coefficient of the linear curve fit, GOI
DeltaDeltaCtAnalysis.GORFitData.Count -> 0	number of standards, reference gene

DeltaDeltaCtAnalysis.GORFitData.M -> 1	slope of the standard curve, reference gene
DeltaDeltaCtAnalysis.GORFitData.N -> 0	intercept of the standard curve, reference gene
DeltaDeltaCtAnalysis.GORFitData.R2 -> 0	correlation coefficient of the linear curve fit, reference gene
DeltaDeltaCtAnalysis.ValFitData.Count -> 0	number of standards used for validation
DeltaDeltaCtAnalysis.ValFitData.M -> 1	slope of the validation curve
DeltaDeltaCtAnalysis.ValFitData.N -> 0	intercept of the validation curve
DeltaDeltaCtAnalysis.ValFitData.R2 -> 0	correlation coefficient of the linear curve fit, validation
Melt Curve Analysis	
MeltCurveAnalyzes.Smooth -> -1	smoothing GOI, On(-1), Off(0)
MeltCurveAnalyzes.SmoothMode -> 3	number of points used for smoothing
MeltCurveAnalyzes.Log -> 0	logarithmic chart On(-1), Off(0)
MeltCurveAnalyzes.BaseLineRange.Min -> 1	manually set lower boundary of the base line
MeltCurveAnalyzes.BaseLineRange.Max -> 5	manually set upper boundary of the base line
MeltCurveAnalyzes.AutoThreshold -> 0	auto threshold On(-1), Off(0), GOI
MeltCurveAnalyzes.FlipCurve -> -1	flip curve horizontally, On(-1), Off(0)
MeltCurveAnalyzes.Scaling -> 0	All curves start at 100%(0), Maximum initial fluorescence = 100%(1)
MeltCurveAnalysis.Description -> melt	title of this analysis
MeltCurveAnalysis.Group -> 0	valid for group
MeltCurveAnalysis.GOI.Color -> FAM	dye of GOI
MeltCurveAnalysis.GOI.Gene ->	name of GOI
MeltCurveAnalysis.Threshold -> 0	threshold value above which the melt temperatures are determined
Genotyping	
GenoTypingAnalyzes.GOISmooth -> -1	smoothing GOI, On(-1), Off(0)
GenoTypingAnalyzes.GOISmoothMode -> 5	number of points used for smoothing, GOI
GenoTypingAnalyzes.GOILog -> 0	logarithmic chart GOI On(-1), Off(0)
GenoTypingAnalyzes.GOIBaseLineRange[0].Min -> 3	manually set lower boundary of the base line (cycle number GOI)
GenoTypingAnalyzes.GOIBaseLineRange[0].Max -> 15	manually set upper boundary of the base line (cycle number GOI)
GenoTypingAnalyzes.GOIBaseLineRange[1].Min -> 5	automatically set lower boundary of the base line (cycle number GOI)
GenoTypingAnalyzes.GOIBaseLineRange[1].Max -> -1	automatically set upper boundary of the base line (cycle number GOI)
GenoTypingAnalyzes.GOIAutoBaseLine -> -1	automatic base line determination On (-1) or Off (0)
GenoTypingAnalyzes.GORSmooth -> -1	smoothing reference gene, On(-1), Off(0)

GenoTypingAnalyzes.GORSmoothMode -> 5	number of points used for smoothing of reference gene
GenoTypingAnalyzes.GORLog -> 0	logarithmic chart GOI On(-1), Off(0), reference gene
GenoTypingAnalyzes.GORBaseLineRange[0].Min -> 3	manually set lower boundary of the base line (cycle number reference gene)
GenoTypingAnalyzes.GORBaseLineRange[0].Max -> 15	manually set upper boundary of the base line (cycle number reference gene)
GenoTypingAnalyzes.GORBaseLineRange[1].Min -> 5	automatically set lower boundary of the base line (cycle number reference gene)
GenoTypingAnalyzes.GORBaseLineRange[1].Max -> -1	calculate automatically upper boundary of the base line range Yes (-1), No (0)
GenoTypingAnalyzes.GORAutoBaseLine -> -1	automatic base line determination On (-1) or Off (0), reference gene
GenoTypingAnalyzes.GOIAutoThreshold -> 0	auto threshold On(-1), Off(0), GOI
GenoTypingAnalyzes.GORAutoThreshold -> 0	auto threshold On(-1), Off(0), Referenzgen
GenoTypingAnalyzes.GOIFilter -> -1	filter On(-1), Off(0), GOI
GenoTypingAnalyzes.GOIFilterOptions -> 1	filter strength GOI, slight(0), medium(1) or strong(2)
GenoTypingAnalyzes.GOIFilterSmooth -> 0	smooth the filter data GOI, Yes (-1), No (0)
GenoTypingAnalyzes.GORFilter -> -1	filter On(-1), Off(0), reference gene
GenoTypingAnalyzes.GORFilterOptions -> 1	filter strength reference gene, slight(0), medium(1) or strong(2)
GenoTypingAnalyzes.GORFilterSmooth -> 0	smooth the filter data of reference gene, Yes (-1), No (0)
GenoTypingAnalyzes.SPType -> 1	genotyping based on Ct (1) or dRn (0)
GenoTypingAnalyzes.EPLast -> -1	end point is the last cycle (1) or enter cycle manually
GenoTypingAnalyzes.EPCycle -> -1	manually entered end point cycle
GenoTypingAnalyzes.InfoText -> wild type	text 1
GenoTypingAnalyzes.InfoText -> mutant	text 2
GenoTypingAnalyzes.InfoText -> heterozygote	text 3
GenoTypingAnalyzes.InfoText -> error	text 4
GenoTypingAnalysis.Description -> Geno	title of this analysis
GenoTypingAnalysis.Group -> 0	valid for group
GenoTypingAnalysis.REF -> 0	passive reference used? Yes (-1), No (0)
GenoTypingAnalysis.GOI.Color -> FAM	dye of GOI
GenoTypingAnalysis.GOI.Gene ->	name of GOI
GenoTypingAnalysis.GOIThreshold -> 0,794712458619839	threshold value the Ct values of the GOI were calculated with
GenoTypingAnalysis.GOR.Color ->	dye of reference gene

GenoTypingAnalysis.GOR.Gene ->	name of the reference gene
GenoTypingAnalysis.GORThreshold -> 50	threshold value the Ct values of the reference gene were calculated with
GenoTypingAnalysis.SPCutOff0 -> 0	cut off value 1, Ct
GenoTypingAnalysis.SPCutOff1 -> 0,794712458619839	cut off value 2, Ct
GenoTypingAnalysis.EPCutOff0 -> 49	cut off value 1, Cycle
GenoTypingAnalysis.EPCutOff1 -> 51	cut off value 2, cycle
End Point Analysis	
EndPointAnalyzes.Smooth -> -1	smoothing GOI, On(-1), Off(0)
EndPointAnalyzes.SmoothMode -> 5	number of points used for smoothing, GOI
EndPointAnalyzes.Log -> 0	logarithmic chart GOI On(-1), Off(0)
EndPointAnalyzes.BaseLineRange[0].Min -> 3	manually set lower boundary of the base line (cycle number GOI)
EndPointAnalyzes.BaseLineRange[0].Max -> 15	manually set upper boundary of the base line (cycle number GOI)
EndPointAnalyzes.BaseLineRange[1].Min -> 5	automatically set lower boundary of the base line (cycle number GOI)
EndPointAnalyzes.BaseLineRange[1].Max -> 25	automatically set upper boundary of the base line (cycle number GOI)
EndPointAnalyzes.AutoBaseLine -> -1	automatic base line determination On (-1) or Off (0)
EndPointAnalysis.Description -> Endp	title of this analysis
EndPointAnalysis.Group -> 0	valid for group
EndPointAnalysis.GOI.Color -> FAM	dye of GOI
EndPointAnalysis.GOI.Gene ->	name of GOI
EndPointAnalysis.GOICutOff -> 2040,83542831301	cut off value of GOI
EndPointAnalysis.IPC.Color ->	Dye of the internal positive control (IPC)
EndPointAnalysis.IPC.Gene ->	gene name of the internal positive control
EndPointAnalysis.IPCCutOff -> 0	cut off value of the internal positive control
EndPointAnalysis.OptionCycles -> -1	end point analysis based on end point intensities, Yes (-1), No (0)
EndPointAnalysis.OptionLastCycles -> 2	number of last cycles used for calculation
EndPointAnalysis.OptionFromCycle -> 38	start cycle end point range
EndPointAnalysis.OptionToCycle -> 40	end cycle end point range
EndPointAnalysis.OptionCutOff -> -1	end point analysis based on NTC intensities, Yes (-1), No (0)
EndPointAnalysis.OptionCutOffNTC -> 10	factor applied to the NTC intensities for threshold calculation
EndPointAnalysis.OptionCutOffNTC_IPC -> 2	confidence intervals: (0)95%, (1)99%, (2)99.5%, (3)99.7%, (4)99.9%

EndPointAnalysis.OptionCutOffInput -> 0	use cut off values from table Yes(-1), No (0)
MIQE.TypeInfo -> 1	MIQE documentation for DNA(0) or RNA(1)
LOCK -> 0	template or project locked? Yes (-1), No (0)

15 Appendix E – Available Color Modules and Dyes to be detected

Following color modules are available:

Description / order number	Dyes (examples)
Color module 1, order number: 844-00520-0	FAM, SYBR Green, Alexa488
Color module 2, order number: 844-00521-0	JOE, HEX, VIC, YakimaYellow
Color module 3, order number: 844-00522-0	TAMRA, DFO, Alexa546, NED
Color module 4, order number: 844-00523-0	ROX, TexasRed, Cy3.5
Color module 5, order number: 844-00524-0	Cy5, Alexa633, Quasar670
Color module 6, order number: 844-00525-0	Cy5.5, LightCycler Red
FRET module 1, order number: 844-00526-0	FAM (Donor) / TAMRA (Akzeptor)
FRET module 2, order number: 844-00527-0	FAM (Donor) / Cy5 (Akzeptor)
FRET module 3, order number: 844-00528-0	FAM (Donor) / Cy5.5 (Akzeptor)
FRET module 4, order number: 844-00529-0	JOE (Donor) / Cy5 (Akzeptor)
FRET module 5, order number: 844-00531-0	FAM (Donor) / ROX (Akzeptor)
Color module Protein 1, order number: 844-00530-0	SYPRO Orange