LightCycler® 480
Relative Quantification Software

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Prologue

I. Revision History

Version	Revision Date
1.0	December 2005

Questions or comments regarding the contents of this manual can be directed to the address below or to your Roche representative.

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Every effort has been made to ensure that all the information contained in this Manual is correct at the time of printing. However, Roche Diagnostics GmbH reserves the right to make any changes necessary without notice as part of ongoing product development.

II. Contact Addresses

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III. Trademarks

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IV. Intended Use

The LightCycler® 480 Relative Quantification Software enables the user to perform precise relative PCR quantification, including the determination of target/reference ratio, calculation of the calibrator-normalized target/reference ratio and automated efficiency-corrected quantification.

The LightCycler® 480 Relative Quantification Software is intended for general laboratory use in combination with the LightCycler® 480 Instrument and LightCycler® 480 Basic Software.

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Parts of the Software used for the LightCycler® 480 System are licensed from Idaho Technology Inc., Salt Lake City, UT, USA.

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VII. Conventions Used in this Manual

Text Conventions

To impart information that is consistent and memorable, the following text conventions are used in this Operator´s Manual:

Numbered listing	Steps in a procedure that you must perform in the order listed.
Italic type, blue	Points to a different chapter in this Manual, which you should consult.
Italic type	Points to a software function or element.

Symbols

In this Manual, symbols are used as an optical signal to point out important things.

Symbol	Heading	Description
	IMPORTANT NOTE	Additional information about the current topic or procedure.
	INFORMATION NOTE	Information critical to the success of the procedure or use of the product.
▶▶▶		Table continued on next page.
■		End of table.

VIII. Warnings and Precautions

	The LightCycler® 480 Instrument PC and other PCs running LightCycler® 480 Software are not designed to be connected to a network. The connection to networks contains an inherent risk to be infected through viruses and worms as well as targeted attacks through malicious attackers through the network. In the event that you connect to a network, Roche shall not be liable for any kind of damages.
	Microsoft Office and Norton Antivirus software are tested not to interfere with LightCycler® 480 Software and LightCycler® 480 software modules. Any other additional software must not be installed on the LightCycler® 480 Instrument PC. Installation of any other additional software on the LightCycler® 480 Instrument PC presents the risk of interference with LightCycler® 480 Basic Software and LightCycler® 480 software modules, and could affect result security.
	**Anti-virus software is not provided.** Therefore, it is essential to take precautions to ensure that any software loaded onto the system is virus free.

LightCycler® 480 Relative Quantification Software

1. Overview

In many experimental studies with a quantitative approach, an absolute value for the sample under investigation is not relevant. In quantitative gene expression analysis it is common to calculate the expression level of a target gene transcript in an unknown sample relative to a reference gene transcript, which is assumed to be constant (*i.e.* is a housekeeping gene) and acts as an endogenous control.

By dividing the concentration of the target in each sample by the concentration of a reference in the same sample, this method corrects the sample for differences in quality and quantity caused by:

- Variations in initial sample amount
- Variations in nucleic acid recovery
- Possible RNA degradation of sample material
- Differences in sample and/or nucleic acid quality
- Variations in sample loading/pipetting errors
- Variations in cDNA synthesis efficiency

In combination with the LightCycler® 480 Relative Quantification Software, this concept for quantitative gene expression analysis can also be achieved with the LightCycler® 480 System. The LightCycler® 480 Relative Quantification Software not only allows you to determine the target/reference ratio - it extends and improves this concept by enabling

- the normalization of the target/reference ratios by a calibrator (allowing you to compare many different PCR experiments) and
- the correction of any differences in PCR efficiencies of target and reference genes (which is a prerequisite for accurate quantification).

By this means, LightCycler® 480 Relative Quantification Software meets the following demands of quantitative gene expression analysis:

- to quantitatively detect subtle changes in amounts of mRNA against a complex background
- to obtain reliable data that can be compared over a long period of time or between different experimental systems

In brief, in a LightCycler® 480 calibrator-normalized relative quantification assay the target concentration in each sample is calculated relative to a non-regulated reference. The result is expressed as the target/reference ratio of each sample normalized by the target/reference ratio of the calibrator. Because the quantity of a target and a reference gene is a function of the PCR efficiency and the sample crossing point, it does not require a standard curve in each analysis run for its determination. The accuracy of the result is only influenced by different PCR efficiencies of target and reference. PCR efficiency differences can be corrected by using relative standard curves to achieve exact results. (Alternatively, for an approximate calculation set both PCR efficiencies to 2.)

This type of quantitative analysis is not only suited for gene expression analysis, but can also be applied for studies on the DNA level, e.g., determination of gene dosage values. In this case, use a single copy gene as reference in the same sample material. Preferentially, select a single copy gene located on the identical chromosome as the target sequence of interest.

2. Installation of the LightCycler® 480 Relative Quantification Software

In order to assure the functionality and performance of the LightCycler® 480 Relative Quantification Software described in this manual, the PC system should fulfill the following minimum requirements:

- processor: Intel Pentium 4 (or equivalent), 3.0 GHz
- main memory: 512 MB RAM
- hard disk: 40 GB
- communication: LAN network card
- display: 1284 × 1024 resolution
- operating system: Windows XP Professional, service pack 1

To be able to run the LightCycler® 480 Relative Quantification Software you must have a valid software user license file from Roche Applied Science installed. Usually, the license file is generated and installed by a Roche specialist during installation of the complete system. In case a Roche specialist is not available for software installation, please follow the steps below to obtain the license file:

1. Determine the MAC address (alternatively physical or Ethernet address) of your data workstation:
   - Open the Windows Start menu and select "Run ...".
   - Type 'cmd" into the Open field. Press "Enter".
   - The Windows console opens. At the prompt, type the command 'ipconfig /all', then press enter.
   - The Windows IP Configuration parameters are listed. Find the line beginning with "Physical Address". Write down the number at the end of this line. (It has the following general format: ## - ## - ## - ## - ## - ##-; # being a number or character.)

2. Submit the MAC number together with the number appearing on your Software License Certificate (coming together with the software installation CD) to your local Roche office.

3. Your local Roche office will generate a software license and send it to you via email.

4. From your email client, save the email as plain text (text-only) file to a data carrier (*e.g.*, a USB memory stick). Assign a meaningful filename in combination with the file extension *.lic*. The License Manager software recognizes only files having the extension .lic!

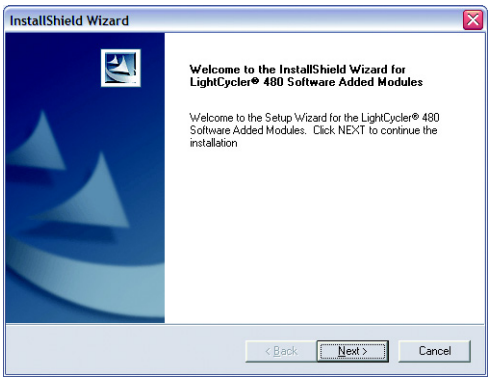
5. Transfer the file to the LightCycler® 480 data workstation (or any other PC on which LightCycler® 480 Basic Software is installed) and copy it to the following location: %programfiles%\Roche\RocheLM\files.

%programfiles% is an environmental variable of Windows XP which stands for the location of the default install directory for applications. The actual name of the install directory depends on the language version of your Windows XP Professional installation. To determine the name of the install directory, type "echo %programfiles%" at the cmd prompt. This will return the path of the install directory (e.g., for a standard installation in combination with an English version of Windows XP this is "C:\Program Files").

To install the LightCycler® Relative Quantification Software:

1. Insert the LightCycler® 480 Relative Quantification Software installation CD into the CD-ROM drive of the data station connected to your LightCycler® 480 Instrument.

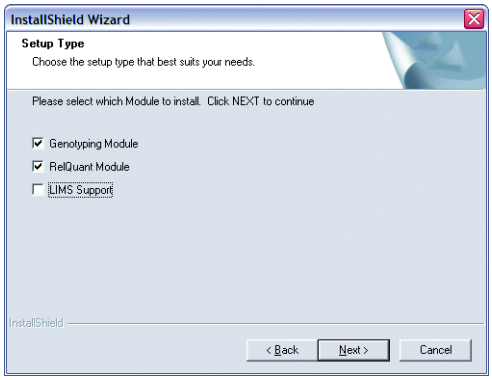
2. Open the software CD in Windows Explorer and double-click *AddedModules.exe* to start the installation program. The setup wizard Welcome window is displayed.



   Click *Next* to continue.

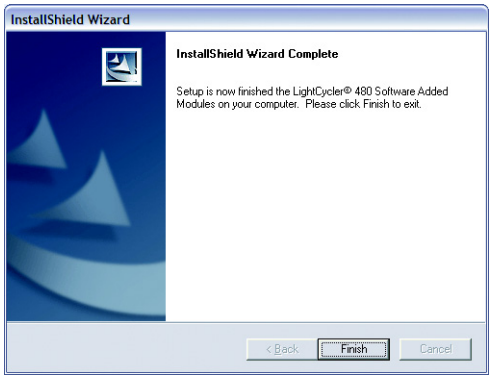
3. You are prompted to agree to the license conditions. Click *Yes*.

4. The next screen lets you select the additional LightCycler® 480 Software Module to install.



   Choose *RelQuant Module*.

5. When the installation is finished, the wizard displays the following window.



   Click *Finish* to complete the installation.

3. Performing Relative Quantification Analysis

3.1 Principle of Relative Quantification Analysis

A Relative Quantification analysis compares two ratios:

1) the ratio of a target DNA sequence to a reference DNA sequence in an unknown sample, and

2) the ratio of the same two sequences in a standard sample called a "calibrator".

The "target" is the nucleic acid of interest (specific RNA or DNA sequence), while the "reference" is a nucleic acid that is found at constant copy number in all samples and serves as endogenous control. The reference is used for normalization of sample-to-sample differences. The "calibrator" is typically a positive sample with a stable ratio of target-to-reference and is used to normalize all samples within one run, but in addition provides a constant calibration point between several LightCycler® 480 System runs.

While a calibrator corrects for differences in detection sensitivity between target and reference caused by differences in probe annealing, FRET efficiency, or dye extinction coefficients, it does not correct for differences in PCR efficiency between the target and reference gene!

The result is expressed as a normalized ratio, *i.e.* ratio (1) divided by ratio (2).

$$\text{Normalized Ratio} = \left(\frac{\text{conc. target}}{\text{conc.reference}}\right)_{sample} : \left(\frac{\text{conc. target}}{\text{conc.reference}}\right)_{calibrator}$$

A Relative Quantification analysis can be performed on an experiment that has an amplification program and that has the appropriate sample types. You can perform a Relative Quantification analysis on a single-color or on a dual-color experiment.

A Relative Quantification analysis is based on the assumption that the concentration of DNA at a sample's crossing point is the same for every sample containing the same target DNA. This is the DNA concentration necessary for the LightCycler® 480 Instrument to detect a signal above background noise.

Each sample may require a different number of cycles to reach the crossing point, depending on the initial concentration of DNA in the sample. At the end of the experiment, each sample's DNA concentration may vary, depending on how many cycles were completed by that sample after the crossing point was reached.

The analysis uses the sample's crossing point (expressed as a cycle number), the efficiency of the reaction, the number of cycles completed, and other values to determine how much the DNA concentration must have increased for each sample by the end of the amplification. The analysis uses these calculations to compare the samples and generate the ratios. The final ratio resulting from the calibrator normalized relative quantification is only a function of PCR efficiency and of the determined crossing points. It does not require the knowledge of absolute copy numbers at the detection threshold and thus the analysis does not determine the actual concentration of DNA in the samples. The calculation of the calibrator-normalized ratio does not require a standard curve in each LightCycler® 480 run.

The basic prerequisites for accurate relative quantification are:

- *the efficiencies of the relative standards and the unknown samples are identical*
- *the efficiencies of both target and reference PCR do not vary from sample to sample*
- *the reference gene is not regulated in the system under investigation*

When you perform the analysis, you provide a PCR efficiency value for the reaction by importing a standard curve, including standards in the experiment to determine the efficiency value, or simply specifying a fixed efficiency value. You can further specify crossing point ranges for calibrators. If a calibrator sample's crossing point is outside the specified range, then a message is displayed that the control failed.

3.2 PCR Efficiency Correction

Almost all variables influencing the final result, like variations in sample amount or probe annealing, are eliminated by the normalization to a reference and to a calibrator. Thus, the accuracy of this method, based on calibrator-normalized relative quantification, remains only influenced by different PCR efficiencies of target and reference gene.

According to the basic PCR equation, $N = N_0 \times E^{Cp}$, the generated copy number (N) at a certain cycle is a function of the initial copy number (N_0), the PCR efficiency (E) and the cycle number (Cp). In brief, the measured crossing point (Cp) is the cycle at which PCR amplification begins its exponential phase and is considered the point that is most reliably proportional to the initial concentration. The efficiency of the PCR describes the kinetics during the reaction. Primers as well as probes for both parameter-specific components (target gene and reference gene) have individual PCR efficiencies, that are taken into account during the overall quantification. An efficiency-corrected calculation is performed by the LightCycler® 480 Relative Quantification Software. This procedure allows maximum reproducibility and controls for factors influencing quantification.

- To achieve exact results, PCR efficiency differences can be easily corrected with the features of the LightCycler® 480 Relative Quantification Software by the use of relative standard curves.

- An approximate calculation of data analysis can be performed without efficiency correction by setting both PCR efficiencies to 2.

It is recommended to apply PCR efficiency correction for Relative Quantification, because
- *not every PCR assay is running with the optimal PCR efficiency of 2 and*
- *not every PCR assay is running with a constant PCR efficiency.*

Only by applying PCR efficiency correction, calculation errors due to differences in amplification of target and reference genes are significantly reduced!

The efficiency-corrected quantification performed automatically by the LightCycler® 480 Relative Quantification Software is based on relative standard curves describing the PCR efficiencies of the target and the reference gene. These standard curves can either be determined once and stored as an external standard curve which can be used for each analysis, or can be determined within the analysis run itself together with the CPs of target and reference gene.

Due to the principle of calibrator-normalized relative quantification it is not necessary to know the exact copy number of the relative standards. Only the relative dilution steps (1:10, 1:100, ...) of the used standards have to be entered; one dilution series for the target gene and one for the reference gene is required. We recommend to perform a LightCycler® 480 System run with *e.g.*, 15 samples of target standards and another 15 samples of reference standards covering a dynamic range of 4 orders of magnitude. The dilution series should be done with a typical nucleic acid, *e.g.,* total RNA or genomic DNA from the calibrator. In principle, any nucleic acid dilution with identical PCR efficiency to a typical sample can be used for creation of a relative standard curve.

3.3 Performing a Relative Quantification Experiment

The relative quantification experiment is an amplification experiment containing the target unknowns, calibrators, negatives, and relative standards (optionally). If you do not perform a separate reference experiment, then the relative quantification experiment must also include the reference unknowns, calibrators, negatives, and relative standards (optional).

Before you perform a Relative Quantification analysis, you must decide the following:

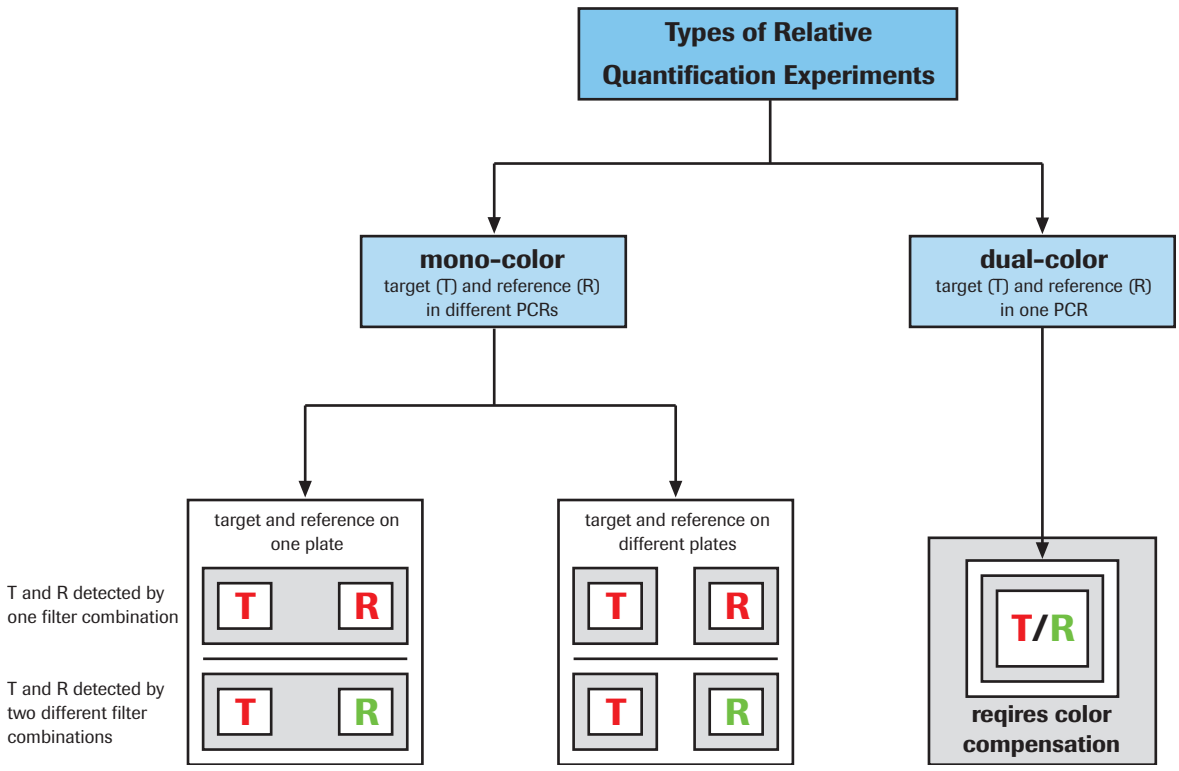
Whether you want to run a mono-color or a dual-color experiment:

- In a mono-color experiment, target and reference samples are amplified in separate reactions. For a mono-color experiment you have two different options how to handle the reference samples:
  - Target and reference samples are amplified in the same multiwell plate. In this case use the "In-Run" *Reference Sample Location* option (see below)
  - Target and reference samples are amplified in separate multiwell plates. In this case, use the "External" *Reference Sample Location* option (see below).

It is not required that target and reference are detected by the same filter combination.

- In a dual-color experiment, target and reference samples are amplified in the same reaction, which requires the detection by two different filter combinations. This setup requires to apply color compensation (with the exception of some specific filter combinations; see LightCycler® 480 Operator's Manual for details).

For dual-color experiments, only the "In-Run" Reference Sample Location option is available.



How to provide the efficiency value for the targets and for the references:

- By including relative standards in the current experiment to generate a relative standard curve
- By importing external relative standard curves
- By specifying an efficiency value of 2 (the only efficiency value that can be specified directly)

Using a fixed PCR efficiency value of 2 will not correct for any differences in PCR efficiency of target and reference gene. Use this option only for first approximations or if you are sure that the PCR efficiencies of both target and reference are equal to 2.

To perform a relative quantification experiment:

1. Perform an amplification experiment to amplify the target samples. If there is no separate reference experiment, include the reference samples in the experiment. If you do not want to use external relative standards (saved as described below), include relative standards for target (and reference) in the run. Follow the recommendations from section **To generate external relative standard curves** below.

2. Click *Sample Editor* in the *Module Bar*. First select the *General* tab to enter general sample information like sample name and replicates (see the LightCycler® 480 Operator's Manual for details).

3. Then select the Relative Quantification tab to enter Relative Quantification analysis specific sample information.

The definition of the Sample Type is obligatory, otherwise a Relative Quantification analysis cannot be calculated.

If this is an experiment using different filter combinations, make sure to select the appropriate filter combination for entering the sample information.

General		Abs Quant	Color Comp	Tm Calling	Genotyping	Rel Quant	
Pos	Sample Name	Filt. Comb.	Target Name	Sample Type	Conc.	Cp Low	Cp High
A1	P2 T	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A2	Repl. of P2 T	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A3	Repl. of P2 T	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A4	Repl. of P2 T	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A5	P4 T	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A6	Repl. of P4 T	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A7	Repl. of P4 T	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A8	Repl. of P4 T	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A9	C T	HybProbe Red 640	Target 483-640	Target Calibrator		0	0
A10	Repl. of C T	HybProbe Red 640	Target 483-640	Target Calibrator		0	0
A11	Repl. of C T	HybProbe Red 640	Target 483-640	Target Calibrator		0	0
A12	Sample 12	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A13	Sample 13	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A14	Sample 14	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A15	Sample 15	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A16	Sample 16	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A17	Sample 17	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A18	Sample 18	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A19	Sample 19	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A20	Sample 20	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A21	Sample 21	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A22	Sample 22	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A23	Sample 23	HybProbe Red 640	Target 483-640	Target Unknown		0	0
A24	Sample 24	HybProbe Red 640	Target 483-640	Target Unknown		0	0
B1	P2 R	HybProbe Red 640	Target 483-640	Reference Unknown		0	0
B2	Repl. of P2 R	HybProbe Red 640	Target 483-640	Reference Unknown		0	0
B3	Repl. of P2 R	HybProbe Red 640	Target 483-640	Reference Unknown		0	0
B4	Repl. of P2 R	HybProbe Red 640	Target 483-640	Reference Unknown		0	0
B5	P4 R	HybProbe Red 640	Target 483-640	Reference Unknown		0	0
B6	Repl. of P4 R	HybProbe Red 640	Target 483-640	Reference Unknown		0	0
B7	Repl. of P4 R	HybProbe Red 640	Target 483-640	Reference Unknown		0	0
B8	Repl. of P4 R	HybProbe Red 640	Target 483-640	Reference Unknown		0	0
B9	C R	HybProbe Red 640	Target 483-640	Reference Calibrator		0	0
B10	Repl. of C R	HybProbe Red 640	Target 483-640	Reference Calibrator		0	0
B11	Repl. of C R	HybProbe Red 640	Target 483-640	Reference Calibrator		0	0

To perform a relative quantification experiment:

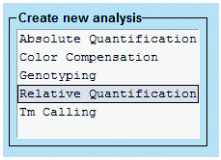
3

Column Name	Description	Valid Values
Target Name (optional)	Name of the (PCR) target (*e.g.,* the name of the gene amplified and detected) *The term "target" in this field is different to the sample type Target".*	Alphanumeric value (≤ 25 characters); default value is "Target xxx-yyy", where xxx and yyy are the excitation and emission wavelengths
Sample Type (obligatory)	Type of sample *Specify the reference sample types only if you did not perform a separate reference experiment.* *Always include target and reference negatives in your experiment. If at least one of the negatives is positive, the experiment has to be assessed as invalid.*	Target Unknown Target Calibrator Target Standard Target Negative Reference Unknown Reference Calibrator Reference Standard Reference Negative
Conc.	Concentration of a standard sample. *This field is active only when the sample type is Standard.*	Concentration value
Cp Low / Cp High (optional)	Low and high Cp values for samples of type Target Calibrator and Reference Calibrator. *If a calibrator sample's crossing point is outside the specified range, then a message is displayed that the control failed.*	Cp value

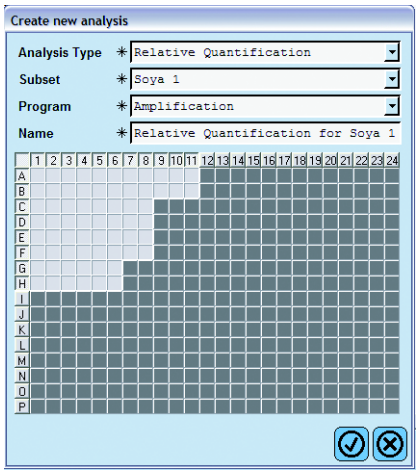
▶▶▶

To perform a relative quantification experiment:

4 Click *Analysis* in the *Module Bar*. In the *Create New Analysis* dialog box, select *Relative Quantification*.

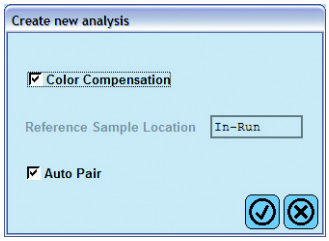


5 The *Create New Analysis* dialog opens. Select an analysis subset and an experimental program from the *Program* list (usually this is the *Amplification* program). If desired, you can change the analysis name (the default name is "*analysis type* for *subset name*"). Click *OK*.



6 A further dialog opens which lets you select color compensation, reference sample, and auto-pair options.

- Color Compensation: The Color Compensation option is only selectable if the experiment contains more then one filter combination.

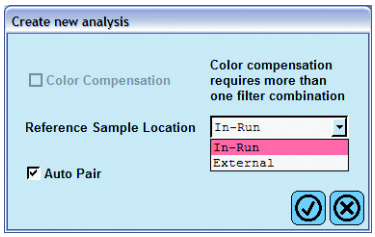


If you have selected Color Compensation, you can later choose the color compensation object from the Color Comp multiselect button (In use or In Database).

▶▶▶

To perform a relative quantification experiment:

6 ▶Reference Sample Location:

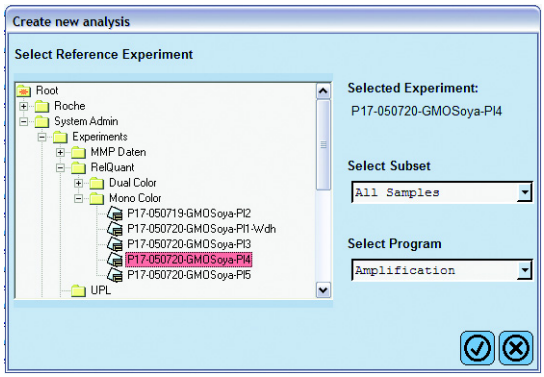


The *Reference Sample Location* option is only active if *Color Compensation* is not selected. If *Color Compensation* is selected, *Reference Sample Location* is automatically set to *In-Run*.
If the option is active, you can choose to use In-Run reference samples or to use the reference samples from an external experiment.

▶Auto Pair: If *Auto Pair* is selected, the software will automatically create result pairs based on pairing rules described in the *Pairing* section below.
▶When you made your choices, click OK.

The settings you make on the Create new analysis dialog are not editable after the Relative Quantification analysis has been created. If you want to apply different settings you have to create a new analysis.

7 If you choose to use an external reference experiment, the *Select Reference Experiment* dialog opens. Select the reference experiment you want to use in the tree pane. Then choose which subset and experimental program is to be used. Click *OK*.



The reference samples for the selected subset are then imported into the current experiment and added to the Reference tab (see below). The reference data cannot be changed after importing. If you change the external reference experiment after importing it into the Relative Quantification analysis, the software will not recognize this. You have to create a new Relative Quantification analysis to use the changed reference data.

8 The *Relative Quantification Analysis* screen opens.
See To perform the relative quantification analysis for details.

To generate external relative standard curves:

1 Perform an amplification experiment containing the relative standards for your target and reference gene. Apply the same experimental protocol you will use for your relative quantification experiment.

To create a statistically valid standard curve, consider the concentration range of samples to be analyzed and the required standard deviation. It is recommended to

- *include standard concentrations that cover at least 3-5 orders of magnitude in the range of the samples to be analyzed*
- *use a minimum of 4-5 dilution steps (e.g., 1:10 dilutions)*
- *use 3-6 replicates of each standard, to ensure a statistically valid result*

2 On the *Rel Quant* tab of the *Sample Editor*, enter the concentration for the dilution steps of target (T) and reference (R) standards.

General | Abs Quant | Color Comp | Tm Calling | Genotyping | Rel Quant

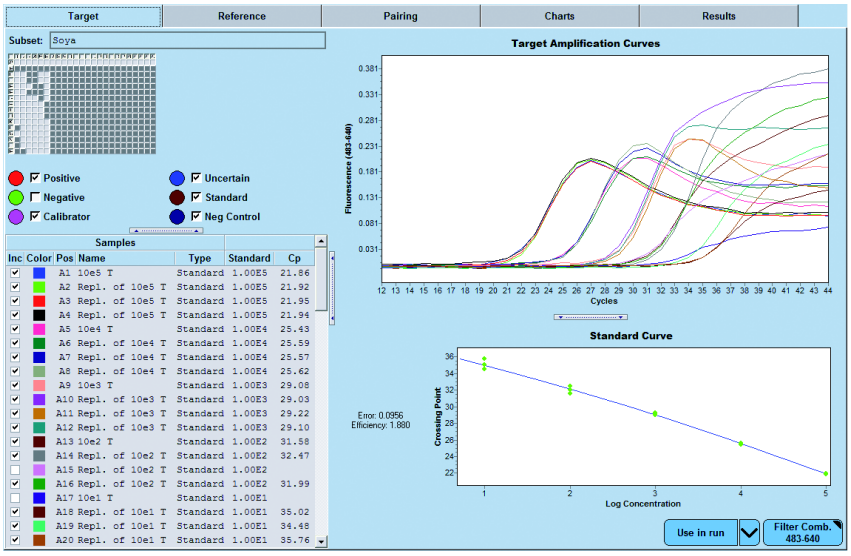
Pos	Sample Name	Filt. Comb.	Target Name	Sample Type	Conc.	Cp L...	Cp H
A1	10e5 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E5	0	0
A2	Repl. of 10e5 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E5	0	0
A3	Repl. of 10e5 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E5	0	0
A4	Repl. of 10e5 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E5	0	0
A5	10e4 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E4	0	0
A6	Repl. of 10e4 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E4	0	0
A7	Repl. of 10e4 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E4	0	0
A8	Repl. of 10e4 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E4	0	0
A9	10e3 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E3	0	0
A10	Repl. of 10e3 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E3	0	0
A11	Repl. of 10e3 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E3	0	0
A12	Repl. of 10e3 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E3	0	0
A13	10e2 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E2	0	0
A14	Repl. of 10e2 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E2	0	0
A15	Repl. of 10e2 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E2	0	0
A16	Repl. of 10e2 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E2	0	0
A17	10e1 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E1	0	0
A18	Repl. of 10e1 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E1	0	0
A19	Repl. of 10e1 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E1	0	0
A20	Repl. of 10e1 T	HybProbe Red 640 (483-640)	Target 483-640	Target Standard	1.00E1	0	0
A21	NTC T	HybProbe Red 640 (483-640)	Target 483-640	Target Negative		0	0
A22	Repl. of NTC T	HybProbe Red 640 (483-640)	Target 483-640	Target Negative		0	0
A23	Repl. of NTC T	HybProbe Red 640 (483-640)	Target 483-640	Target Negative		0	0
A24	Repl. of NTC T	HybProbe Red 640 (483-640)	Target 483-640	Target Negative		0	0
B1	10e5 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E5	0	0
B2	Repl. of 10e5 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E5	0	0
B3	Repl. of 10e5 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E5	0	0
B4	Repl. of 10e5 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E5	0	0
B5	10e4 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E4	0	0
B6	Repl. of 10e4 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E4	0	0
B7	Repl. of 10e4 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E4	0	0
B8	Repl. of 10e4 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E4	0	0
B9	10e3 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E3	0	0
B10	Repl. of 10e3 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E3	0	0
B11	Repl. of 10e3 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E3	0	0
B12	Repl. of 10e3 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E3	0	0
B13	10e2 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E2	0	0
B14	Repl. of 10e2 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E2	0	0
B15	Repl. of 10e2 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E2	0	0
B16	Repl. of 10e2 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E2	0	0
B17	10e1 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E1	0	0
B18	Repl. of 10e1 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E1	0	0
B19	Repl. of 10e1 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E1	0	0
B20	Repl. of 10e1 R	HybProbe Red 640 (483-640)	Target 483-640	Reference Standard	1.00E1	0	0
B21	NTC R	HybProbe Red 640 (483-640)	Target 483-640	Reference Negative		0	0
B22	Repl. of NTC R	HybProbe Red 640 (483-640)	Target 483-640	Reference Negative		0	0
B23	Repl. of NTC R	HybProbe Red 640 (483-640)	Target 483-640	Reference Negative		0	0
B24	Repl. of NTC R	HybProbe Red 640 (483-640)	Target 483-640	Reference Negative		0	0

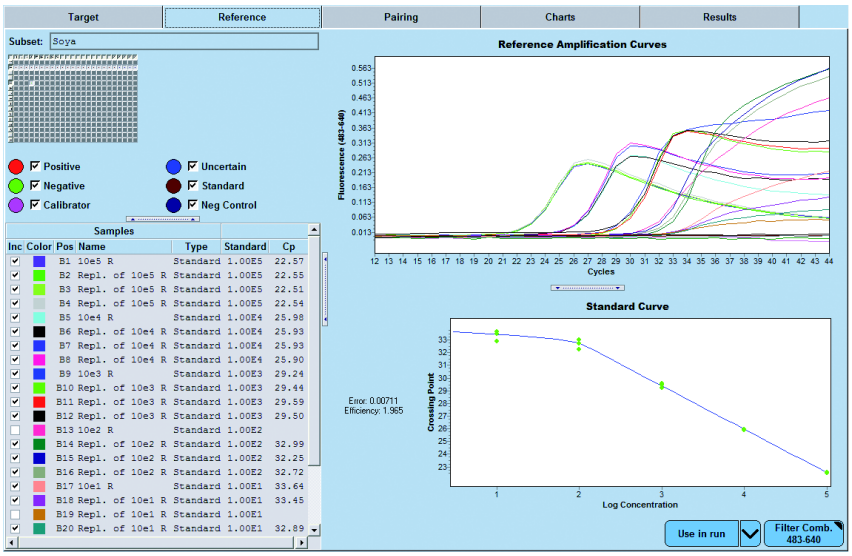
It is not necessary to know the absolute concentration (e.g., copy number) of the relative standards. You may also enter the dilution (e.g., 1 – 0.1 – 0.01).

To generate external relative standard curves:

3 Create a new *Relative Quantification* analysis. The Relative Quantification screen opens with the *Target* tab in the front.

4 On the *Target* tab and the *Reference* tab, enable those standards you want to include in the standard curve calculation. Select *Calculate* to calculate the standard curves. (It doesn't matter whether you use the *Calculate* button on the *Target* or *Reference* tab; both standard curves are calculated simultaneously.)

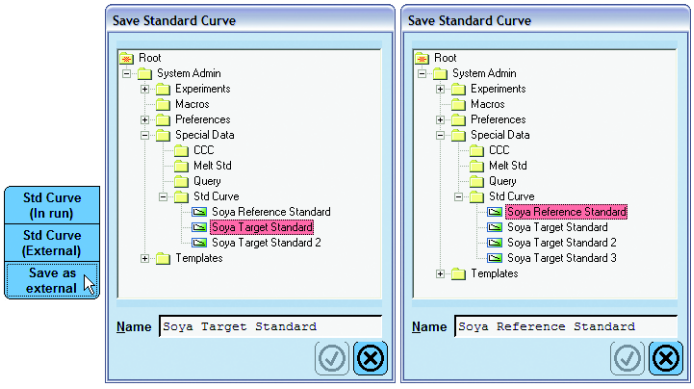




To generate external relative standard curves:

5. Set the Standard Curve multi-select button to *Use in run*.

6. Now you can save the standard curves as External Standard Curve: both on the *Target* tab and the *Reference* tab, select “Save as External”. Apply a meaningful name to the external standard curve and click *OK* to save it in the *StdCurve* directory.



To perform the relative quantification analysis:

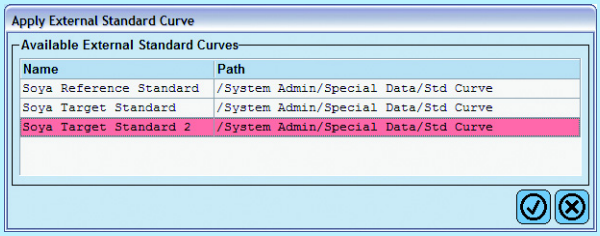
1. Use the *Target* tab to select target samples to be included in the analysis and to optionally select an external standard curve for defining a PCR efficiency different from 2.

   In the sample list of the Target tab only those samples are displayed that were specified in the Sample Editor to belong to the target group (i.e., target unknown, target calibrator, target standard, target negative).

2. Use *Filter Comb.* to select the filter combination used to detect the target.

3. If you specified target standards in the *Sample Editor*, the standards are used to generate a standard curve, displayed in the lower chart of the *Target* tab. You can use this standard curve, import an external standard curve, or specify an efficiency value of 2.
   Using the Standard Curve multi-select button, select one of the following:

Eff = 2	Select this option to use a fixed efficiency value of 2 in analysis calculations. No relative standard curve is needed.
Use in run	Use this option to use the standard curve generated from standards in the current experiment.
Use external …	Use this option to import a previously saved external standard curve. A dialog opens which lets you select a suited external standard curve:



The external standard curve must have been created from an experiment with the same channel settings and color compensation data (if any) as the current experiment. Further, you must have user rights to use the curve.

Changing the filter combination used for Relative Quantification analysis after loading an external standard curve makes a standard curve invalid. You will then be asked if you want to select a new standard curve. If you click OK, the standard curve selection dialog appears. If you select No or cancel the selection dialog, the "Eff=2" option is selected.

Select ***Save as external…*** if you would like to save the current standard curve as an external standard curve for use with other experiments. This option is only active if you use and calculated an in-run standard curve.

▶▶▶

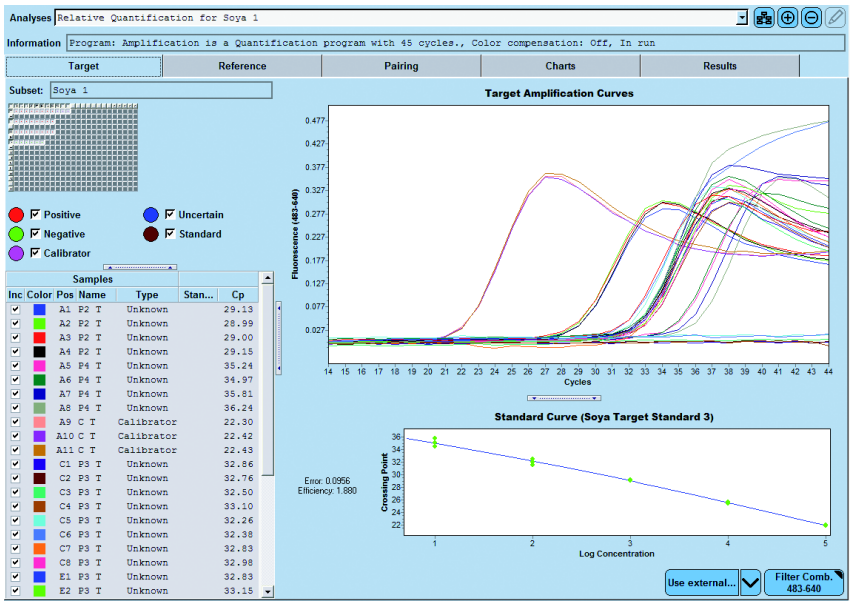
To perform the relative quantification analysis:

4. If you selected *Color Compensation* when creating the analysis, use the *Color Compensation* multi-select button to turn color compensation on or off and to select a color compensation object.

5. In the *Samples List*, select the check boxes of the target samples you want to make available for result sets. Double-click the check boxes to select or deselect them.

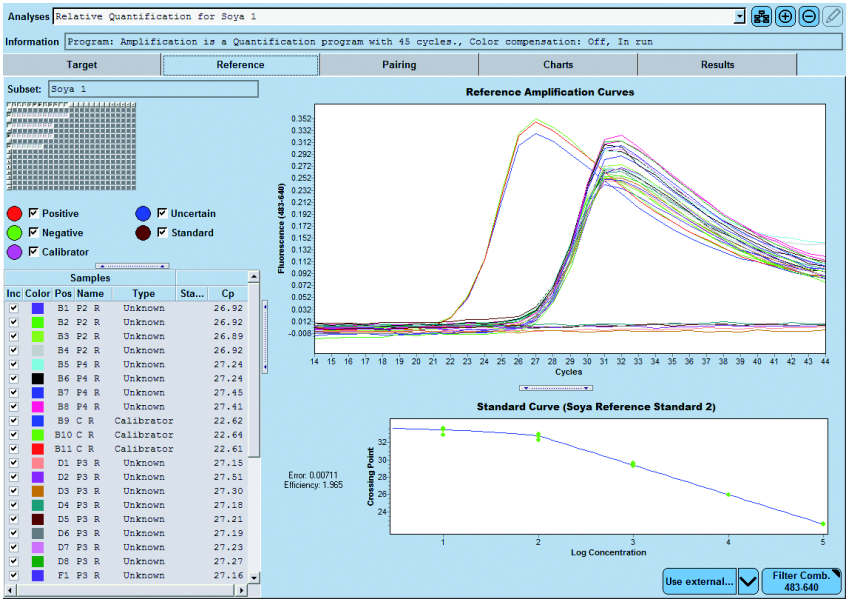
   The Sample List of both the Target and Reference tab displays only those samples matching the tab type (i.e., reference samples are inactive on the Target tab and vice versa).

   Below is an example of a *Target* tab using an external standard curve.

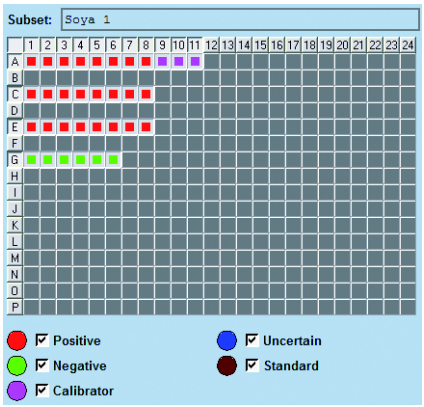


To perform the relative quantification analysis:

6 Now use the *Reference* tab (in the same way as the *Target* tab described above), to select reference samples to include in the analysis and to optionally select an external standard curve for defining a PCR efficiency different from 2. Below is an example of a *Reference* tab using an external standard curve.



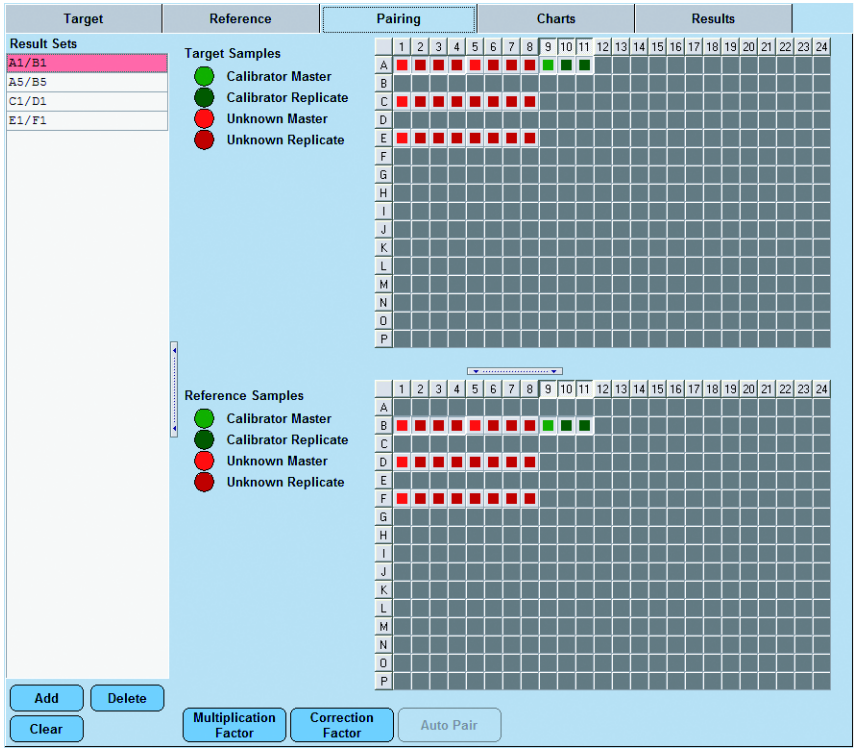
7 Both *Target* and *Reference* tab have a *Sample Selector*, which displays only those samples matching the tab type (*i.e.,* reference samples are inactive on the *Target* tab and vice versa). The sample classifications *Positive*, *Negative*, and *Uncertain* are based on CP calling.



When you select the *Positive*, *Negative*, *Uncertain*, *Calibrator*, or *Standard* check box, the wells with those classifications are selected in the *Sample Selector* and highlighted in the associated color. Selected samples are displayed in the *Amplification Curves* chart.

To pair the samples and create result sets:

1. Use the *Pairing* tab to manually form result sets consisting of an unknown target and reference and a calibrator target and reference (if you did not select Auto Pairing). The four samples together form a *Result Set*. You can further specify correction and multiplication factors for the sets.
The *Pairing* tab displays two Sample Selectors, the upper one for target samples, the lower one for reference samples. Only the unknown and calibrator wells in the subset are available for selection. All other wells are inactive.



The Auto Pair button is only enabled if no result sets are defined.

2. To create a new *Result Set*, perform the following steps in the *Pairing* tab:
   1. Select the unknown target (in the upper sample selector) and unknown reference (in the lower sample selector) that form a pair. Click *Add*.

      If this is a dual-color analysis, the target and reference samples do not have to be in the same wells.

      Optionally, together with the unknown target and unknown reference, respectively, select the target calibrator (in the upper sample selector) and reference calibrator (in the lower Sample Selector). In this case, these four samples form the result set. Note, that if you do not include a calibrator into the Result Sets no calibrator-normalized ratio is calculated.

   2. The new sets are added to the *Result Sets* list. A set is automatically assigned a default name (<first target well>/<first reference well>).
   3. (Optional) Click a result set name to activate the field, then edit the name.

▶▶▶

To pair the samples and create result sets:

3. To apply a correction factor to the result sets, select the *Correction Factor* button in the lower line of the tab, then type the new value. Valid values are 1-100. The correction factor is used to normalize results for lot-to-lot variations of a calibrator. The normalized ratio is divided by the correction factor.

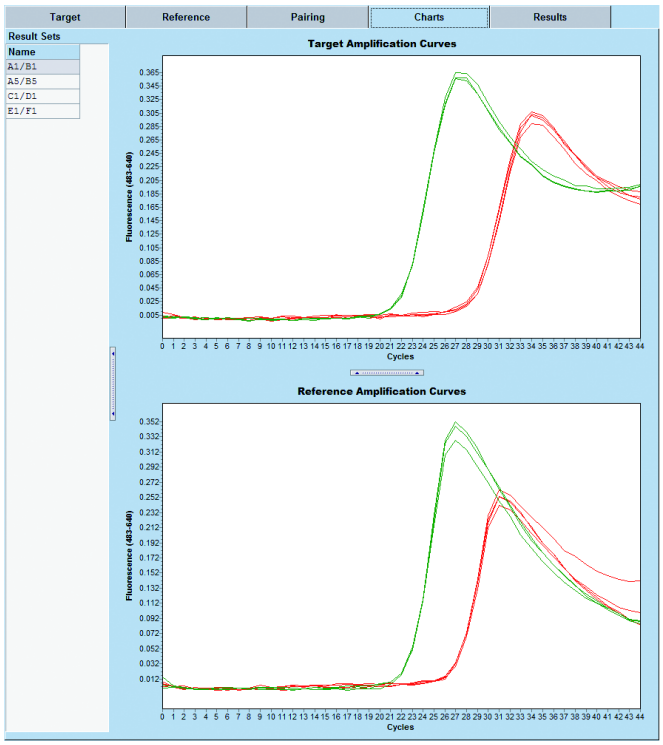
   Each new batch of a calibrator should be validated with a "master" calibrator.

4. To apply a multiplication factor to the result sets, select the *Multiplication Factor* button in the lower line of the tab, then type the new value. Valid values are 1-100. A multiplication factor might be used to adjust the final calibrator-normalized relative ratios to a reasonable value. The normalized ratio is multiplied by the multiplication factor. The multiplication factor thus only has "cosmetic" function for easier reading and interpretation of results.

   If you have defined more than one Target or Reference Calibrator, then the Correction Factor and Multiplication Factor buttons are invisible. Instead, a Correction Factor and Multiplication Factor column are added to the Pairings List. Each column has editable fields. You can assign factors to each Result Set by entering a value in the Correction Factor or Multiplication Factor field for that set.

5. Create other Results Sets, as needed.

6. To view graphs for the samples in a Result Set, click the *Chart* tab, then select a Result Set. The amplification curve (red) of the unknown target is displayed in the upper graph, while the amplification curve (red) of the unknown reference is displayed in the lower graph. If calibrator samples were paired together with unknowns, then their amplification curves (green) are displayed in addition.



If you selected the Auto Pair option when creating the analysis, the software will automatically create target/reference pairs, assign default names, and display the Result Sets in the list on the Pairing tab. If the experiment does not provide matchable samples, then no pairs are generated and a warning message will be displayed.

The Relative Quantification Module applies the following rules to auto pair target and reference samples:

- *If a target and reference sample have matching Sample IDs, these samples are paired. If Sample ID values are not defined, the following rules are applied.*
- *If one target calibrator and one reference calibrator (and their replicates) exist, these calibrators are added to every pairing.*

If you included several Target and Reference calibrators in the experiment, you must define them as replicates. Otherwise, the calibrators cannot be added to the pairings.

If the shape of the multiwell positions of the target samples matches the shape of the positions of the reference samples, and if the replicate groups with the targets and references are identical, the samples are paired. For example, if A1 and B2 are target master positions, and G1 is a reference master position, then auto-pairing of targets and references will be created if H2 is also a reference master position (generating result sets A1/G1 and B2/H2). This is independent of the positions of the targets and reference relative to each other.

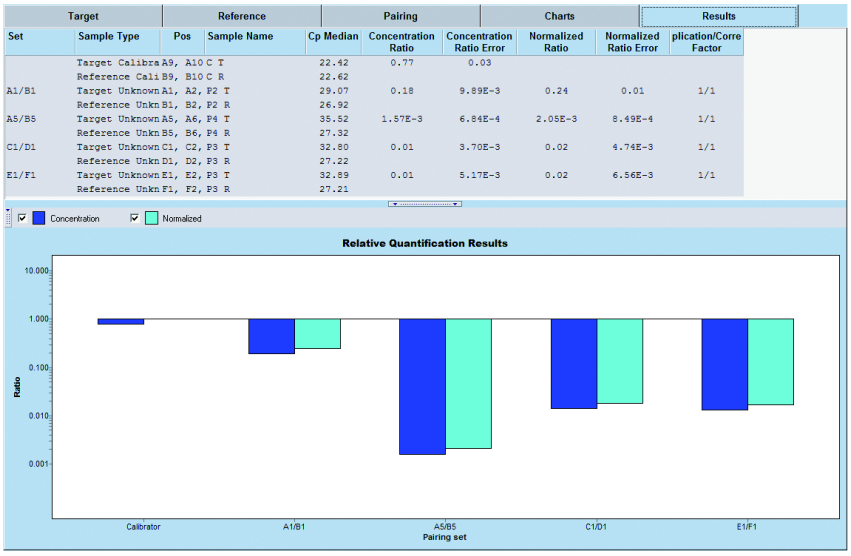
Auto-pairing is also applied to experiments that use external reference samples.

Auto-pairing cannot applied if the target experiment and the external reference experiment use different multiwell plate formats (i.e., 96-well vs. 384-well plates).

To view results on the Result tab:

1. Select the *Results* tab to view detailed results for each result set.

2. The *Results* tab is shown below.

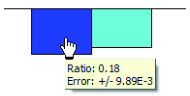


3. For each result set, the tab displays the *Sample Type*, position in the multiwell plate (*Pos*), *Sample Name*, the median of crossing points for sets of sample replicates (*Cp Median*), the concentration ratio of target to reference in the set (*Concentration Ratio*), and the normalized concentration ratio of the unknown result set to the calibrator (*Normalized Ratio*). If sample replicates were analyzed, the *Concentration Ratio Error* and the *Normalized Ratio Error* are displayed in addition.

   The error of a ratio value is the standard deviation of ratios computed from each combination of samples.

   - *For concentration ratio, the standard deviation is calculated from every combination of one target and one reference unknown.*
   - *For normalized ratio, the standard deviation is calculated from every combination of one target unknown, one reference unknown, one target calibrator, and one reference calibrator.*
   - *If there is only a single combination, the error is undefined.*

4. In the lower part of the *Results* tab the $\log_{10}$ of the *Concentration Ratio* and/or the *Normalized Ratio* of the result sets selected in the results table above is displayed as a column graph. Red bars indicate the respective error (if calculated).
   If you place the mouse pointer over a column, more detailed data are displayed:



   In case of changing the Rel Quant sample editor after having saved a Relative Quantification analysis, individual result sets (i.e. sets not containing an unknown target or reference sample anymore) are automatically excluded from the calculation.

4. Supplementary Information

4.1 Invalidating the Analysis

The following changes invalidate the Relative Quantification analysis and require to recalculate the analysis:

- Changing the target or reference standard curve or efficiencies:
  - by checking/un-checking samples to be included in calculations
  - by changing samples types (creating new standards or removing standards)
  - by changing standard concentrations
  - by changing the source of the efficiency value (external standard curve, internal standard curve, or efficiency = 2)
- Adding or deleting a pairing set
- Changing correction or multiplication factors
- Changing color compensation status (on/off/selected object)
- Applying a template
- Changing the currently selected channel of targets or references

Updating an external reference experiment after the experiment has been imported does neither update nor invalidate the analysis.

4.2 Relative Quantification Template

A Relative Quantification analysis template contains the following settings:

- Filter combinations of target and references

If you want to apply a template to an experiment that does not use the target or reference filter combinations defined in the template, a warning message will appear and the template will not be applied.

- Target/reference/calibrators pairing information (by position)

If the pairing information of the template is not consistent with the sample properties used in the current experiment, the template will not be applied.

- Target and reference experiment subsets

When a template is applied, the software checks whether the current experiment contains a subset with the same name and the same well positions as the subset in the template.
  - *If the current experiment does not contain a subset with the same name, the software will create the subset.*
  - *If the current experiment does contain a subset with the same name, but with different well positions as the subset in the template, the template will not be applied.*

- Standard curve information (or efficiency) of target and references
- Color compensation (none, smart, or current)
- Correction and multiplication factors
- External Reference Experiment or In-Run setting

If the template uses an external reference experiment, the software opens a dialog where you can select the reference experiment, program, and subset. If you cancel this dialog, the template cannot be applied.

4.3 Result Control Concept

The LightCycler® 480 Relative Quantification Software applies a control concept to assess whether a result is valid, uncertain, or invalid. Uncertain result values are displayed in brackets, while "invalid" or no value is displayed for invalid results.

See the table below for an overview over possible result states:

			Target positive			**Target uncertain**		**Target negative**
			all replicates positive	at least 1 replicate uncertain	at least 1 replicate negative	at least 1 replicate negative	all replicates uncertain	all replicates negative
		Display in 'Cp Median' column	median Cp	[median Cp]	blank	blank	[median Cp]	blank
Reference positive	all replicates positive	median Cp	ratio	[ratio]	'invalid'	'invalid'	[ratio]	'zero'
	at least 1 replicate uncertain	[median Cp]	[ratio]	[ratio]	'invalid'	'invalid'	[ratio]	[zero]
	at least 1 replicate negative	blank	'invalid'	'invalid'	'invalid'	'invalid'	'invalid'	'invalid'
Reference uncertain	at least 1 replicate negative	blank	'invalid'	'invalid'	'invalid'	'invalid'	'invalid'	'invalid'
	all replicates uncertain	[median Cp]	[ratio]	[ratio]	'invalid'	'invalid'	[ratio]	'(zero)'
Reference uncertain	all replicates negative	blank	'invalid'	'invalid'	'invalid'	'invalid'	'invalid'	'invalid'
			Display in 'Concentration Ratio' column					

If all selected calibrator replicates in a result set are positive, the normalized concentration ratio is calculated.

If at least one selected calibrator replicate in a result set is not positive, the normalized concentration ratio is invalid.

If the normalized concentration ratio is not invalid, and the sample concentration ratio is bracketed, then the normalized concentration ratio is bracketed.

4.4 LIMS for Relative Quantification

The LightCycler® 480 Relative Quantification Software provides a plug-in for the LightCycler® 480 LIMS/Bar-Code Module. When both the LightCycler® 480 LIMS/Bar-Code Module and the LightCycler® 480 Relative Quantification Software are licensed, the following values can be set from the LIMS interface for an experiment:

- Name of the external reference experiment, program, and subset.

The results returned from the Relative Quantification Software to the LIMS include the following:

- Result set name (this column is empty for calibrators)
- Sample Type, position, and sample name for samples in the results set. Values for sample type are Target Calibrator, Reference Calibrator, Target Unknown, and Reference Unknown.
- Cp call for each sample in the result set and the median Cp for the set.
- Concentration Ratio and error (Concentration ratio of the Calibrators or of the Target and Reference Unknowns in this result set.)
- Normalized Ratio and error (The normalized ratio of the Unknowns with the Calibrators for this result set.)
- Multiplication/Correction Factor (The multiplication and correction factors for the result set, expressed as a fraction.)

4.5 Unlicensed Features

The following requirements define the behavior of the LightCycler® 480 Software when a licensed relative quantification feature is used in the application and a license for the LightCycler® 480 Relative Quantification Software is not available:

- An experiment, macro or template that contains a Relative Quantification analysis cannot be imported or opened on a system where the LightCycler® 480 Relative Quantification Software has not been installed.
- An experiment that contains a Relative Quantification analysis can be imported into a system where the additional software module has been installed but is currently not licensed. In this case, the Relative Quantification analysis and the analysis-specific Sample Editor tab will be set to read-only.
- When a Macro that contains a Relative Quantification analysis is run on a system that does not have a current license, the Relative Quantification analysis will not be created.
- A Template for a Relative Quantification analysis cannot be applied on a system that does not have a current license for the LightCycler® 480 Relative Quantification Software.
- When a Report is generated from an experiment that contains a Relative Quantification analysis on a system where the LightCycler® 480 Relative Quantification Software has been installed but is not currently licensed, the report will contain the read-only information from the Relative Quantification analysis.

If you import an experiment from LightCycler® Software 4.0 that contains a relative quantification analysis into LightCycler® 480 Software, the analysis is discarded.

4.6 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, http://www.roche-applied-science.com, and visit our special interest site for

- the LightCycler® 480 System:
  http://www.roche-applied-science.com/lightcycler480
- the MagNA Pure System family for automated nucleic acid isolation:
  http://www.magnapure.com
- DNA & RNA preparation – Versatile Tools for Nucleic Acid Purification:
  http://www.roche-applied-science.com/napure

Instruments		
LightCycler® 480 Instrument, 96-well	1 instrument with data workstation and accessories	04 640 268 001
LightCycler® 480 Instrument, 384-well	1 instrument with data workstation and accessories	04 545 885 001
Software		
LightCycler® 480 Basic Software 1.0	1 software package	04 722 205 001
LightCycler® 480 LIMS/Bar-Code Module	1 software package	04 727 886 001
LightCycler® 480 Genotyping Software	1 software package	04 727 860 001
LightCycler® 480 Relative Quantification Software	1 software package	04 727 851 001
Accessories		
LightCycler® 480 Block Kit 96	96-well thermal block cycler unit, including block cycler cover, storage box and loading device	04 643 640 001
LightCycler® 480 Block Kit 384	384-well thermal block cycler unit, including block cycler cover, storage box and loading device	04 643 631 001
LightCycler® 480 Bar-Code Scanner	1 bar-code scanner	04 710 606 001

Disposables		
LightCycler® 480 Multiwell Plate 96	50 plates with 50 sealing foils	04 729 692 001
LightCycler® 480 Multiwell Plate 384	50 plates with 50 sealing foils	04 729 749 001
LightCycler® 480 Sealing Foil	5 × 10 foils	04 729 757 001
PCR Reagents		
LightCycler® 480 SYBR Green I Master	1 kit (5 × 100 reactions, 20 µl each)	04 707 516 001
LightCycler® 480 Probes Master	1 kit (5 × 100 reactions, 20 µl each)	04 707 494 001
LightCycler® 480 Genotyping Master	1 kit (4 × 96 reactions, 20 µl each)	04 707 524 001
LightCycler® 480 Control Kit	3 control experiments	04 710 924 001
Labeling Reagents		
SimpleProbe 519 Labeling Reagent	100 µmol	04 687 132 001
LightCycler® Fluorescein CPG	1 g 5 columns	03 138 178 001 03 113 906 001
LightCycler® Red 640-N-hydroxysuccinimide ester	1 vial	12 015 161 001
LightCycler® Red 610-N-hydroxysuccinimide ester	1 vial	03 561 488 001

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