LightCycler® 480 Real-Time PCR System: Innovative Solutions for Relative Quantification

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Introduction

Currently, analysis of gene expression is a critically important area of scientific research. Many genomic labs have found that quantitative real-time PCR is the gold standard for fast, sensitive determination of gene expression levels. However, not all quantitative real-time PCR techniques are appropriate for gene expression analysis.

There are many types and subtypes of quantitative real-time PCR methods, each of which is characterized by its requirements, its complexity, and its reliability. However, it is possible to group all these methods under two main analysis techniques - absolute and relative quantification (Figure 1). The technique you choose depends on the complexity of your analysis and the desired format of the final result:

- Absolute quantification allows you to quantify a single target sequence and express the final result as an absolute value (*e.g.*, viral load – copies/ml). Such analyses routinely occur in research areas like virology and microbiology.

- On the other hand, relative quantification compares the levels of two different target sequences in a single sample (*e.g.*, target gene of interest (GOI) and another gene) and expresses the final result as a ratio of these targets. For comparison purposes, the second gene is a reference gene [a constitutively expressed gene (housekeeping gene)] that is found in constant copy numbers under all tested conditions. This reference gene, which is also known as endogenous control, provides a basis for normalizing sample-to-sample differences. Such analyses are useful, for instance, in oncology research.

Gene expression studies usually try to determine the way a target gene changes its expression profile over time (*e.g.*, how much the expression changes in the course of a disease or treatment) relative to a defined starting point (*e.g.*, disease-free or untreated state). Since relative quantification allows users to easily compare the expression behavior of a target gene under at least two conditions (*e.g.*, disease-free / diseased or untreated / treated), relative quantification is the best technique for determining gene expression and gene dosage.

PCR-Efficiency: The Core of PCR Quantification

The reliability of all quantitative real-time PCR applications and, consequently, of all relative quantification calculations depends on the quality of the PCR.

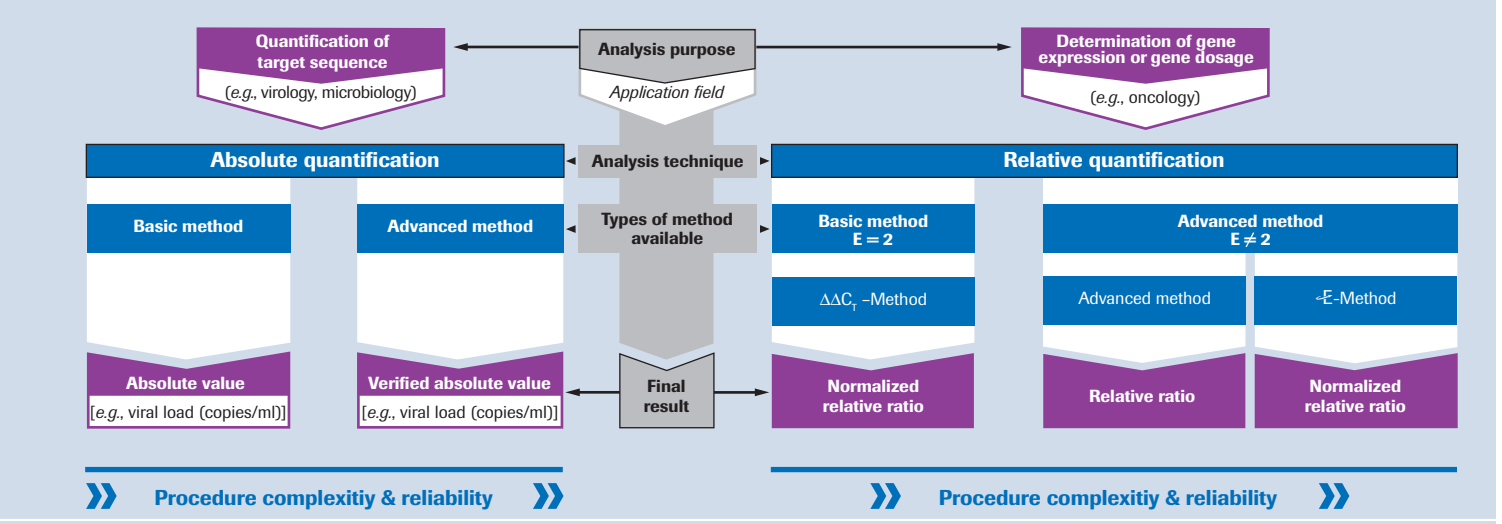


Figure 1: Overview of the different PCR quantification principles. For detailed information, please visit: www.lightcycler.com.

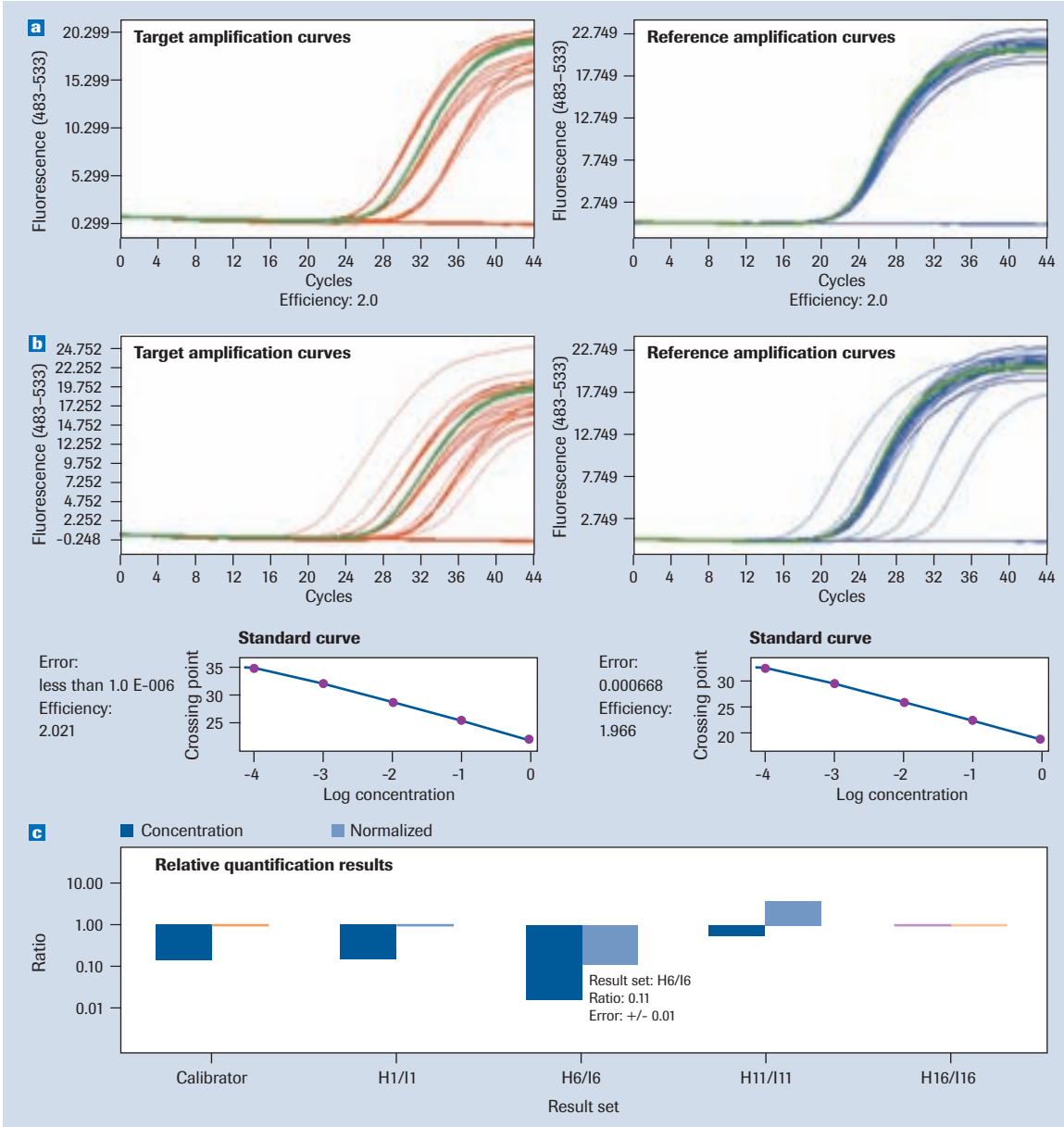
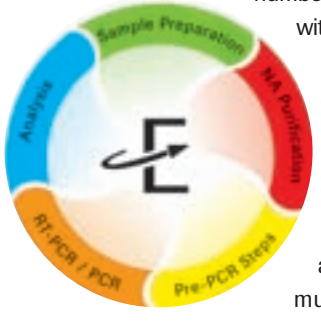


Figure 2: Two different relative quantification analyses of the same run with the LightCycler® 480 System. RNA was reversely transcribed using the Transcriptor Reverse Transcriptase. Target cDNA sequences were amplified with the LightCycler® 480 Probes Master and detected with Universal ProbeLibrary Probes. This figure shows a typical target and reference run, with unknowns (in red or blue) and calibrator samples (in green), which are analyzed via either **(a)** the $\Delta\Delta C_T$-Method or **(b)** the $\mathcal{E}$-Method. Whereas the data in (a) are based on an assumption that the efficiency (E) = 2, the $\mathcal{E}$-Method data in (b) are based on the true efficiency of each reaction, which is derived from serial dilutions of the target (light red, E = 2.021) and reference genes (light blue, E = 1.966). The final results from (b) are automatically calculated from the Cp values of the target (unknowns and calibrator) and the reference (unknowns and calibrator); these results are depicted in **(c)**.

PCR amplification is driven by enzymatic catalysis. Just like every enzymatic process, indeed every biocatalytic process, a given PCR may vary in quality. For calculation purposes, the overall quality of a PCR may be expressed as a single number, the "efficiency" (E) of the PCR. The highest quality PCRs run at an efficiency of two (E = 2), which means that the number of target molecules doubles with every PCR cycle.



Like an ancient clockwork that is driven and influenced by numerous small gears, a PCR depends on numerous factors. For a PCR to achieve maximum efficiency, every single factor affecting the process (*e.g.*, sample preparation, nucleic acid (NA) purification, pre-PCR steps) must occur optimally. Not surprisingly, most PCRs run at an efficiency less than two (E < 2).

Recall that relative quantification assays involve a comparison of two PCRs (*e.g.*, amplification of a target gene and a housekeeping gene in the same sample). However, two different PCRs may not have identical efficiencies. In such cases, an analysis based on comparison of these PCRs may not be entirely accurate.

In addition, not all amplification reactions have the same efficiency throughout the entire process. They may not follow a linear regression as described by the formula: ($N_n = N_0 \times 2n$). This may also affect the final result.

Obviously, since these efficiency considerations affect the accuracy of an assay, you have to take them into account when selecting the best relative quantification method for a given experimental system

Two Relative Quantification Methods

The novel plate-based LightCycler® 480 Real-Time PCR System (96-/384-well format) offers several software-based methods for precise relative quantification (*e.g.*, $\Delta\Delta C_T$-Method, $\mathcal{E}$-Method), each of which handles the problem of efficiency differently. The design of the LightCycler® 480 Relative Quantification Software is very flexible, thus simplifying the fast-tracking of research.

$\Delta\Delta C_T$-Method

The $\Delta\Delta C_T$-Method was the method used in early relative quantification experiments; today, it can still provide fast, easy analysis of gene expression. However, this method provides reliable quantitative data only if certain assumptions inherent in the method are met, (*i.e.*, the efficiency of the PCR assays for both target and reference genes must be optimal and/or identical). For the $\Delta\Delta C_T$-Calculation, the efficiency of both PCRs is assumed to be 2, which represents a doubling of molecules in each cycle (Figure 2a).

If target and reference PCR efficiencies are neither identical nor optimal, the $\Delta\Delta C_T$-Method may produce incorrect gene expression data. In such cases, methods that are based on true efficiency values (*e.g.*, the Roche Applied Science $\mathcal{E}$-Method) can provide more accurate data.

The $\mathcal{E}$-Method

The $\mathcal{E}$ (Efficiency)-Method from Roche Applied Science can produce more accurate relative quantification data because it can compensate for differences in target and reference gene amplification efficiency either within an experiment or between experiments.

The $\mathcal{E}$-Method analyzes the amplification efficiency of target and reference genes by using so-called relative standards (Figure 2b and Figure 2c). These standards are serial dilutions of a single sample (*e.g.*, undiluted, 1:10, 1:100, *etc.*); their concentrations are expressed in relative units (*e.g.*, 1, 0.1, 0.01, *etc.*). By using such dilutions to generate a standard curve, the $\mathcal{E}$-Method avoids the time-consuming preparation of artificial or cloned standards and the determination of their absolute values. In addition, since the relative standards contain normal sample material, they are very likely to have PCR efficiencies that are similar to those of all the unknown samples.

Furthermore, the $\mathcal{E}$-Method can normalize for run-to-run differences (*e.g.*, those caused by variations in reagent chemistry). For such normalization, one sample (*e.g.*, one of the relative standards) is designated an inter-assay calibrator for the target, and for the reference gene. These inter-assay calibrators are then used repeatedly for all runs during the study, thus guaranteeing a common reference point and allowing all experiments within the series to be compared.

Conclusions

The new LightCycler® 480 System includes sophisticated software algorithms that generate data for absolute and relative quantification. For gene expression and gene dosage studies the highly flexible LightCycler® 480 Relative Quantification Software provides the user with more than one choice for relative quantification. The $\Delta\Delta C_T$-Method or the Roche Applied Science $\mathcal{E}$-Method can both generate reliable gene expression data, depending on the PCR efficiency of a particular experimental system. ■

Additional information about the LightCycler® 480 Real-Time PCR System and the gene expression approaches described above is available on www.lightcycler.com or the Special Interest Page (Integrated Solutions) "Gene Expression Analysis".

Order INFO

Product	Pack Size	Cat. No.
LightCycler® 480 Instrument	1 instrument (96 well) 1 instrument (384 well)	04 640 268 001 04 545 885 001
LightCycler® 480 Relative Quantification Software	1 software package	04 727 851 001
LightCycler® 480 Multiwell Plate 96	5 x 10 plates	04 729 692 001
LightCycler® 480 Multiwell Plate 384	5 x 10 plates	04 729 749 001
LightCycler® 480 Probes Master	5 x 1 ml (2x conc.) 10 x 5 ml (2x conc.)	04 707 494 001 04 887 301 001
Universal ProbeLibrary Sets*	1 set (*e.g.*, human, mouse, rat)	
Transcriptor Reverse Transcriptase	250 U (25 reactions) 500 U (50 reactions) 2,000 U (200 reactions)	03 531 317 001 03 531 295 001 03 531 287 001

* For detailed information, see www.universalprobelibrary.com.