Principles, Workflows and Advantages of the New LightCycler Relative Quantification Software

Gregor Sagner and Cornelia Goldstein, Roche Molecular Biochemicals, Penzberg/Germany*
**corresponding author: gregor.sagner@roche.com*

Accuracy of PCR quantification methods depends on correction of variations caused by sample quality, PCR components or PCR efficiencies. The LightCycler Relative Quantification Software provides a tool for fully automated calculation of calibrator-normalized and PCR efficiency-corrected relative quantification of mRNA expression levels or gene dosage values. No manual calculation or use of a spread sheet program like MS Excel is required. Relative ratios calculated by using a specific efficiency-correction algorithm display a copy number-independent, improved accuracy compared to alternative methods. No standard dilutions are required in sample analysis runs. Long term comparability of calibrator-normalized data is provided independent of batch-variations.

Introduction

The LightCycler provides a platform for kinetic on-line PCR quantification by allowing measurements during the log-linear phase of a PCR reaction. PCR on-line quantification principles can generally be subdivided into two basic concepts:

I. Absolute Quantification:
This method is based on the use of a standard dilution series with known concentrations. The target concentration is expressed as an absolute value. Absolute quantification is used to determine absolute numbers of infectious particles such as viruses and bacteria in body fluids or tissues.

II. Relative (Comparative) Quantification:
The target concentration is expressed relative to the concentration of a reference gene from the same sample material omitting the need for a standard with known concentrations. The method is recommended for determining mRNA expression levels or gene dosis values from tissues or cell cultures.

Here we describe the advantages of using the LightCycler Relative Quantification Software to determine exact relative nucleic acid values. The software's calculation method is based on PCR efficiency-corrected relative quantification normalized to a calibrator. Features and Benefits are summarized on the right.

Relative Quantification Analysis

For analysis, a calibrator provided with the parameter-specific LightCycler Quantification Kits is included into each LightCycler run. In contrast, customer-specific self-made assays require the definition of a calibrator before relative quantification analysis is performed. The analysis workflow is outlined in Figure 1. It is not necessary to include standards in the LightCycler analysis runs when using stored fit coefficient files for analysis. The two alternative workflows for creating a fit coefficients file are outlined in Figure 2. For definitions of terms used in this article please see page 17.

The LightCycler Relative Quantification Software

Features

- Highly reproducible results by correction of experimental differences of PCR components
- No need to include standards in sample analysis runs
- Option to calculate calibrator-normalized ratios using default PCR efficiency (E = 2.00) or using predetermined target- and reference-specific PCR efficiencies
- Single, duplicate and triplicate determinations possible
- Usable for mono-color and dual-color quantifications
- Comparability of calibrator-normalized results over long time periods independent of calibrator batch variations

Benefits

- Time saving and reduced labor costs (no standards are required in sample analysis runs)
- Results are unambiguous and comparable between runs, individual samples and reagent batches
- No expert knowledge is required for calculation of relative expression or gene dosis values

Results

To demonstrate the improved accuracy of quantification using the LightCycler Relative Quantification Software in comparison to alternative relative quantification methods, the expression level of the target gene

cyclophilin A (CycA) relative to the expression level of the housekeeping gene porphobilinogen deaminase (PBGD) was calculated. Total RNA from human adrenal gland tissue was used as a sample and total RNA from HeLa cells was used as a calibrator.

To define fit coefficients for the Relative Standard curves HeLa RNA was diluted in 5-fold dilution steps covering the expected detection range of CycA and PBGD in human tissue RNA. Cp-values for CycA and PBGD were determined in triplicates for each dilution step in a LightCycler run. Cp-values were plotted against log concentration and two different regression fits were performed:

- A linear fit was performed using LightCycler Data Analysis (LCDA) Software. The slope of the regression line was converted into PCR efficency ($E = 10^{-1}$/slope) and this value was used for subsequent analysis.
- A non-linear fit was performed by exporting LCDA data into the coefficients module of the Relative Quantification Software. The automatically calculated fit coefficients were stored in a file for subsequent analysis.

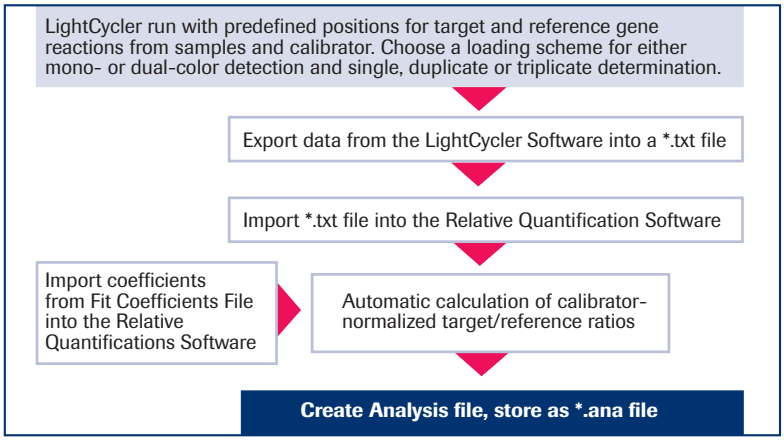

Figure 1: Workflow – Data analysis

Table 1: Accuracy of calculated relative ratios using different efficiency correction methods

Adrenal gland RNA	Without efficiency correction	Efficiency correction with linear fit function	Efficiency correction with non-linear fit function
40 ng	1.03	1.18	1.44
8 ng	2.21	1.79	1.01
1.6 ng	6.00	4.17	1.17
Mean value	3.08	2.38	1.21
Standard deviation	2.5967	1.5799	0.2173
Coefficient of variation	84.3 %	66.4 %	18.0 %

To provide a measure for accuracy of different analysis methods sample RNAs from human adrenal gland were diluted in 5-fold dilution steps (40 ng, 8 ng and 1.6 ng). Triplicate determinations were performed for each dilution step and each RNA sample. For each sample RNA, the calibrator-normalized CycA/PBGD ratio is theoretically expected to be constant over the different dilution steps determined.

We compared the calculated ratios of three different calculation methods in respect to the expected result (Table 1):

- Calibrator-normalized relative quantification without efficiency correction.
- Calibrator-normalized relative quantification using efficiency correction based on a linear regression fit.
- Calibrator-normalized relative quantification based on a non-linear regression fit performed automatically by the Relative Quantification Software.

Results are shown in Table 1. It was shown that consistency of relative ratios determined for different

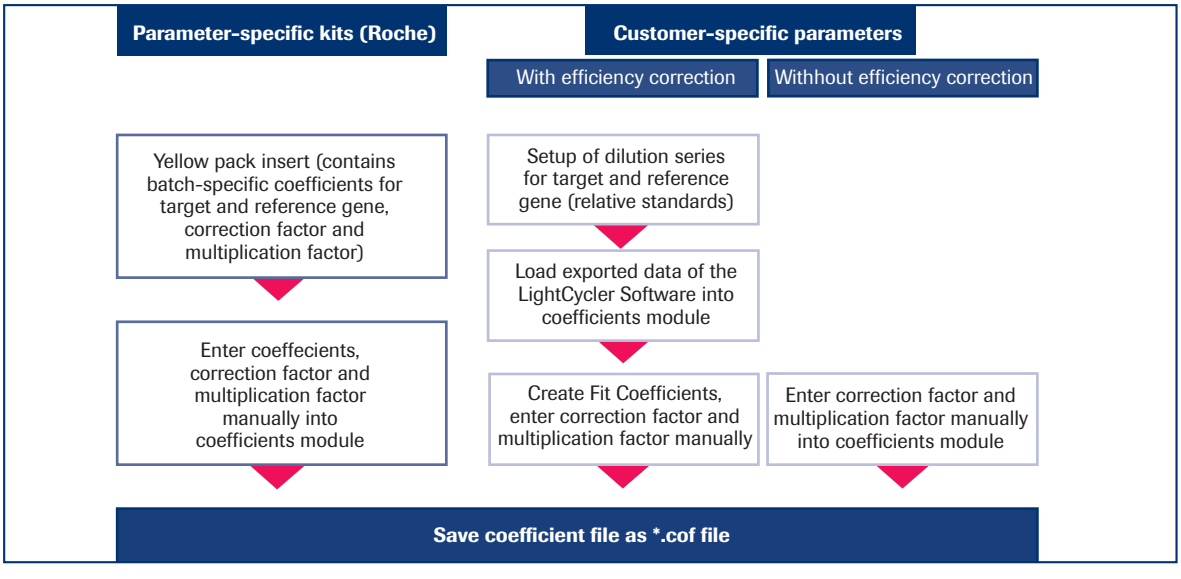

Figure 2: Workflow – Creation of a fit coefficients file

Terms and definitions used to describe the concept of the relative quantification method

- **Target Nucleic Acid:** Nucleic acid of interest (specific RNA or DNA sequence).
- **Reference Nucleic Acid:** A nucleic acid that is found at constant copy number in all samples. This can either be a single copy gene (for gene dosis quantification) or a housekeeping gene (for mRNA quantification). It is used for normalization of sample differences (nucleic acid quality, recovery etc.).
- **Housekeeping Gene**: A gene that is expressed constitutively on identical levels in all samples to be analyzed.
- **Sample**: Material of interest (tissue, cells, blood etc.).
- **Calibrator**: A sample that is used for normalization of final results. In principal any sample can be defined as the calibrator. In the parameter-specific kits, the calibrator is provided as purified nucleic acid. The target/reference ratios of all samples are divided by the target/reference ratio of the calibrator. This normalizes different detection sensitivities for target and reference amplicons.
- **Relative Standards**: Dilution series of target and reference nucleic acids that are used to determine the fit coefficients that describe the function "logarithm of relative copy number versus Cp-value". It is not necessary to know absolute copy numbers of target and reference standards.
- **Fit Coefficients**: Numerical values that describe the fitted Relative Standard curves. The kit- and batch-specific coefficients of the parameter-specific LC Quantification kits are provided with the product information (yellow pack insert). Fit coefficients are manually transferred into the coefficients module of the software and stored in a file. The calculations of all experiments subsequently performed with the kit use this file for accurate quantification. To determine fit coefficients in customer-specific self-made assays, relative standard dilutions using a typical nucleic acid have to be performed on the LightCycler. Resulting LCDA data are exported into the coefficients module of the software. Coefficients are then calculated automatically and stored in a file. The two alternative workflows for creating a fit coefficients file are outlined in Figure 2.
- **Correction Factor**: Target/reference ratios of all samples are referenced to the target/reference ratio of the calibrator. Thus it is highly important for comparability and consistence of data from different calibrator batches to ensure that calibrators are normalized to a master calibrator. Thus, a batch-specific correction factor is defined and provided in the product information (yellow pack insert).
- **Multiplication Factor**: To correct for experimental differences of the calibrator, a multiplication factor might be used to adjust the final calibrator-normalized relative ratios to a reasonable value. The multiplication factor thus only has "cosmetic" function for easier reading and interpretation of results.

dilutions was significantly improved when using Relative Quantification Software based calculation in comparison to non-efficiency corrected calculation or to efficiency correction based on a linear regression fit. For the adrenal gland RNA, the coefficient of variation (CV) of ratios calculated for different dilutions was shown to be 18.0 % using Relative Quantification Software based calculation whereas the two alternative methods displayed CV values of 66.4 % and 84.3 %, respectively.

Summary

Kinetic on-line PCR quantification is a method to measure amplicon generation in the exponential phase of a PCR. Thus it is the method of choice for determining target quantities over a wide dynamic range (up to 10 orders of magnitude depending on the parameter). Due to the wide measuring range and the exponential nature of the PCR process, even minor variations of initial sample quality, PCR components or PCR efficiency result in significant errors in template copy number calculation. To correct for these errors, a fully automated calculation tool was developed. The Relative Quantification Software provides highly accurate and convenient calculation of relative mRNA expression levels or gene dosis values.

http://biochem.roche.com/lightcycler

Order INFO

Product	Pack Size	Cat. No.
LightCycler Relative Quantification Software	1 CD + manual	3 158 527
LightCycler – CK20 Quantification Kit	1 kit (96 reactions)	3 118 835
LightCycler – HER2/neu DNA Quantification Kit	1 kit (32 reactions)	3 113 922
LightCycler – HER2/neu RNA Quantification Kit*	1 kit (96 reactions)	3 051 200
LightCycler – t(14;18) Quantification Kit (mbr)*	1 kit (96 reactions)	3 062 651
LightCycler – inv(16) Quantification Kit*	1 kit (96 reactions)	3 051 226
LightCycler – t(8;21) Quantification Kit*	1 kit (96 reactions)	3 051 218
LightCycler – DPD mRNA Quantification Kit	1 kit (96 reactions)	3 136 957
LightCycler – TP mRNA Quantification Kit	1 kit (96 reactions)	3 136 965
LightCycler – TS mRNA Quantification Kit	1 kit (96 reactions)	3 137 104

* will be available soon