Roche Applied Science
Technical Note No. LC 10/update 2003

LightCycler

Overview of LightCycler Quantification Methods

Purpose of this Note

Over the last few years, real-time-PCR has become essential for nucleic acid quantification because it has unsurpassed sensitivity and broad dynamic range. Since rapid-cycle, real-time PCR sets the standard for accuracy and reproducibility, the LightCycler System is often used for quantification of unknowns. This Technical Note will introduce the reader to the different LightCycler quantification methods.

Note: Detailed descriptions of the experimental approaches described briefly in this Technical Note are available in other LightCycler Technical Notes (Nos. 11 – 13, and 15).

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1. General Introduction

Definitions

For maximum clarity in this note, we will use the following definitions when discussing quantification.

Term	Definition
Sample	Material of interest (blood, cells, tissue, etc.)
Target Nucleic Acid	Nucleic acid of interest (specific RNA or DNA sequence)
Control	It is used for verification of a result, not for the quantification of unknowns; its concentration may not even be known. • **Internal Control:** The control is amplified in the same capillary as the target or the standard; it may be endogenous or exogenous. • **Exogenous Control:** The control is added to the PCR mixture.
Standard	An accepted measure of comparison which is used for quantification of the unknown. • **External Standard:** It has a known concentration (*e.g.* copies or ng/µl) and is used to generate a standard curve for the quantification of unknowns. Serial dilutions of the standard are amplified in the same LightCycler run as the target, but not in the same capillary. • **Relative Standards:** Series of dilutions, containing target and/or reference nucleic acids in relative amounts, that are used to determine the fit coefficients (efficiencies) of a relative standard curve.
Heterologous	The standard or control has a different sequence than the target and is amplified with a different pair of primers.
Homologous	The standard or control differs only slightly by length and/or sequence from the target and is amplified with the same pair of primers.
Exogenous	The standard or control is added to the PCR mixture. It is either • RNA which is usually generated by in vitro transcription • DNA that is usually a cloned fragment, a cloned cDNA or a purified PCR product.
Endogenous	The standard or control occurs naturally in the sample, *e.g.* a reference nucleic acid.
Reference Nucleic Acid	An unregulated nucleic acid that is found at constant copy number in all samples. It is used to normalize sample-to-sample differences (also called endogenous control). • **Housekeeping Gene:** For mRNA quantification. A gene that is expressed constitutively and at the same level in all samples to be analyzed. • **Single Copy Gene:** For gene dosage quantification. Only one copy of the gene is found in all samples.
Calibrator	A sample that is used for the normalization of final results. The target/reference ratios of all samples are divided by the target/reference ratio of the calibrator. Therefore, for the calibrator a constant ratio of target to reference is required.
Fit Coefficient	Parameter that describes mathematically the best fit for the "relative standard" curve. Coefficients are stored as *.cof files (lot-specific) and used for efficiency-corrected data analysis.

Note: The term "internal standard" or "control gene" is frequently used in other publications as a synonym for endogenous reference genes, such as housekeeping genes or single copy genes (see definitions above).

2. Principles of Kinetic PCR

End-Point Determination vs. Kinetic Quantification

Before real-time PCR was available, conventional PCR methods were used for quantification of a specific target. These PCR methods, such as competitive PCR or limiting dilution PCR, are based on end-point analysis and determine the amount of PCR products after the run is completed (that is, during the plateau phase).

Such methods have well known limitations and disadvantages. For example, Figure 1 shows that, even though PCR amplification depends on template concentration, reactions with a low initial copy number can reach the same plateau as reactions that started with higher template concentrations and/or different PCR efficiency. Thus, measurements taken during the plateau phase cannot easily differentiate variations in amount of starting material or amplification efficiency. In addition, measuring great variations in starting template concentration can require running a multitude of end-point determination experiments.

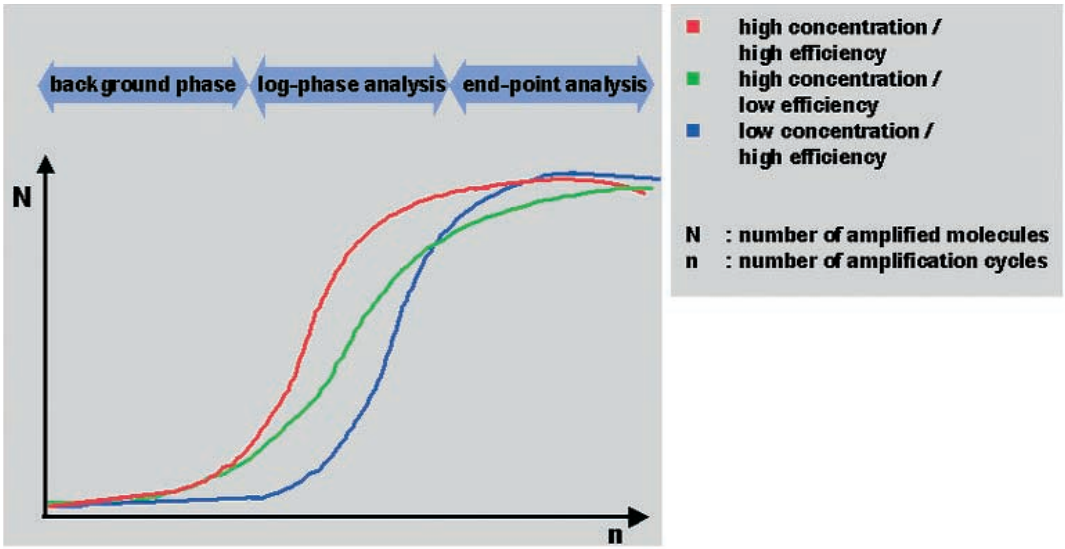

Figure 1: Typical amplification curves.

In contrast, real-time PCR provides accurate, kinetic quantification because it allows data analysis in the only phase (log-linear phase) where the amplification efficiency of each reaction is constant. Differences of efficiency can also easily be determined during this log-linear phase.

Note: Kinetic quantification, which is possible only with real-time PCR, allows the course of a PCR to be visualized as a curve similar to a population growth curve, *i.e.* one that contains an initial lag phase, an exponential (log-linear) phase, and a final plateau phase. The initial lag phase or background phase lasts until the fluorescence signal from the PCR product is greater than the background fluorescence of the probe system. The exponential log phase begins when sufficient product has accumulated to be detected above background and ends when the reaction enters the plateau phase and the reaction efficiency falls.

Theoretical Aspects

In theory, PCR is described by the formula:

$$N_n = N_0 \times 2^n$$

In this equation, PCR efficiency is assumed to be optimal and constant, *i.e.* every PCR product is replicated once every cycle. In reality many factors influence PCR, so the efficiency, although constant, may differ from optimal. Therefore, PCR amplification is more accurately expressed as:

$$N_n = N_0 \times (E_{const.})^n$$

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Theoretical Aspects, continued

Only a few detectable cycles of a PCR (during the log-linear phase) actually obey this equation and therefore allow mathematically correct analysis (Figure 2). For greatest accuracy, measurements must be taken during these cycles.

Note: In the final phase of PCR, the exponential curve bends toward a plateau, efficiency becomes variable, and amplification is best expressed as:

$$N_n = N_0 \times (E_{var})^n.$$

It is therefore very difficult to get accurate measurements during the final stages of PCR.

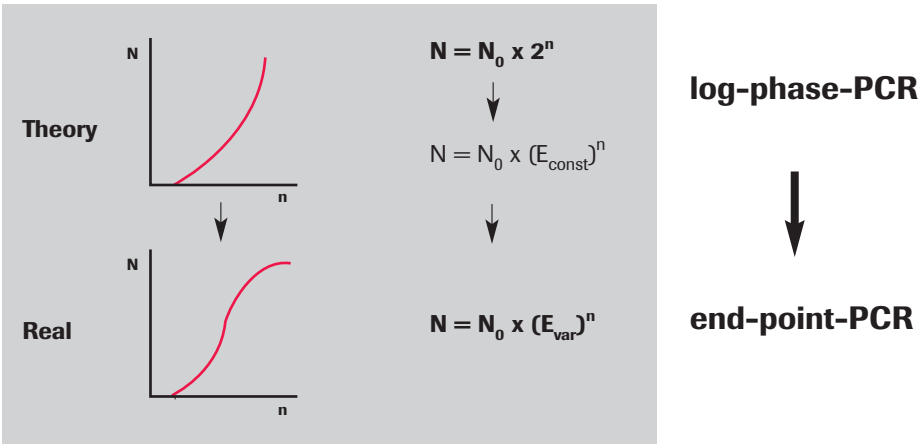

Figure 2: PCR amplification – theory vs. reality.
(N_n: number of molecules at n cycle; N_0: initial number of molecules;
E: amplification efficiency; n: number of cycles)

Monitoring the Log-linear Phase of PCR

Since the log-linear phase lasts very few cycles, without real-time PCR it is easy to miss (Figure 3). However, real-time PCR monitoring (measuring fluorescence intensity once per cycle during PCR on the LightCycler) offers a convenient way to identify and take measurements during these log-linear cycles. By monitoring the log-linear phase, it is possible to get accurate quantification, since an increase in signal during the log-linear phase corresponds directly to an increase in amplified DNA.

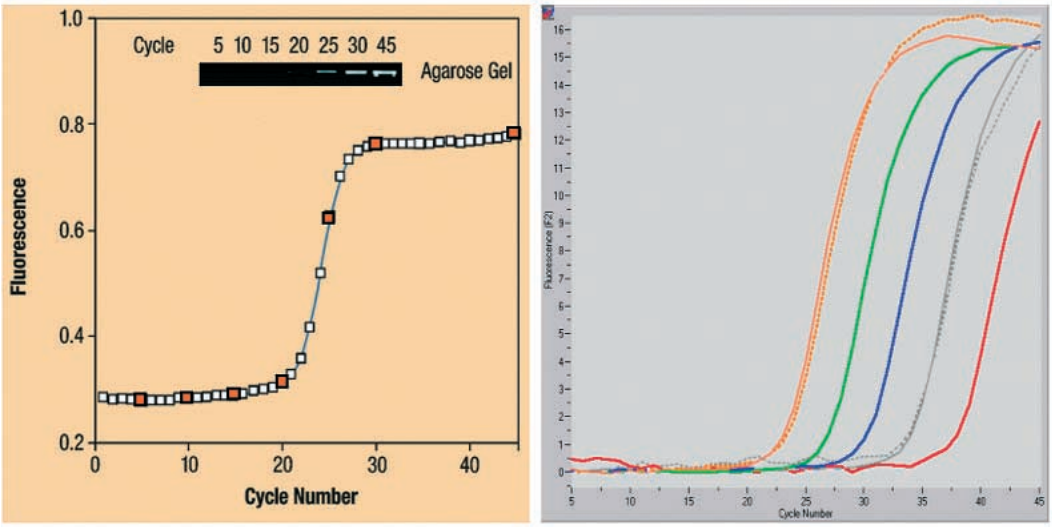

Figure 3: Duration of log-linear phase.

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Dynamic Range

A typical end-point analysis, routinely performed between cycle 20 and 30, allows differentiation of target amounts over a dynamic range of only 4 orders of magnitude (Figure 4). If starting concentrations of the target are too low, they will not be detectable at the time of analysis. If starting concentrations are too high, they cannot be differentiated since, at the time of analysis, they have already reached the plateau phase and are accumulating at a similar rate.

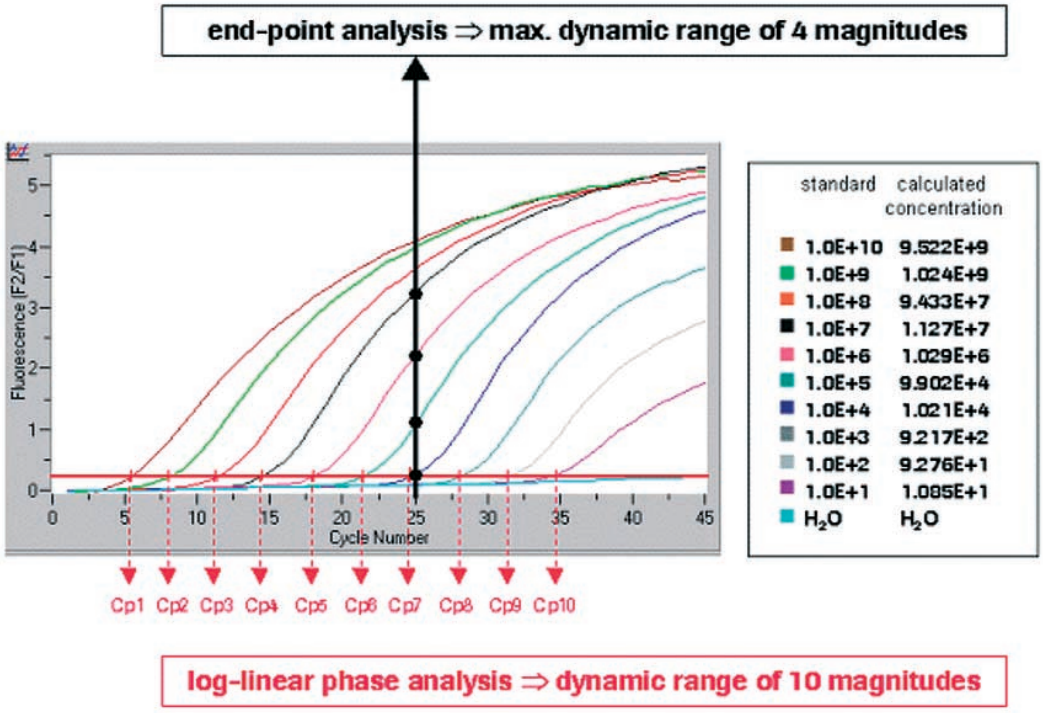


Figure 4: A comparison of the dynamic ranges possible in end-point analysis and log-phase analysis.

In contrast, real-time fluorescence monitoring allows the determination of crossing points (Cp; see Figure 4) in the exponential (log-linear) phase of PCR. This technique can easy differentiate starting concentrations over a range of 10 orders of magnitude.

Note: The Cp is the cycle which is assumed to represent the same amount of PCR product in every curve. At this crossing point, PCR amplification begins to become clearly positive above the background phase. Therefore, Cp is considered to be the point most reliably proportional to the initial concentration.

For visualization of PCR products, their number must exceed the detection limit of the reaction (at Cp, approximately 10^{11} to 10^{12} product molecules are present in the reaction). The Cp differs for each sample because different amounts of starting material need different numbers of cycles to reach this detection limit.

3. Overview of Quantitative PCR Methods

Quantification Methods, Overview

Quantitative real-time PCR can be subdivided into two basic types:

- **Absolute Quantification:** The target concentration is expressed as an absolute value (*e.g.*, copies, µg/µl, etc.)
- **Relative Quantification:** The target concentration is expressed as ratio of target vs. reference gene

The following sections give an overview of different quantification strategies.

Note: For experimental details and more information, please refer to Technical Notes Nos. 11 – 13, and 15.

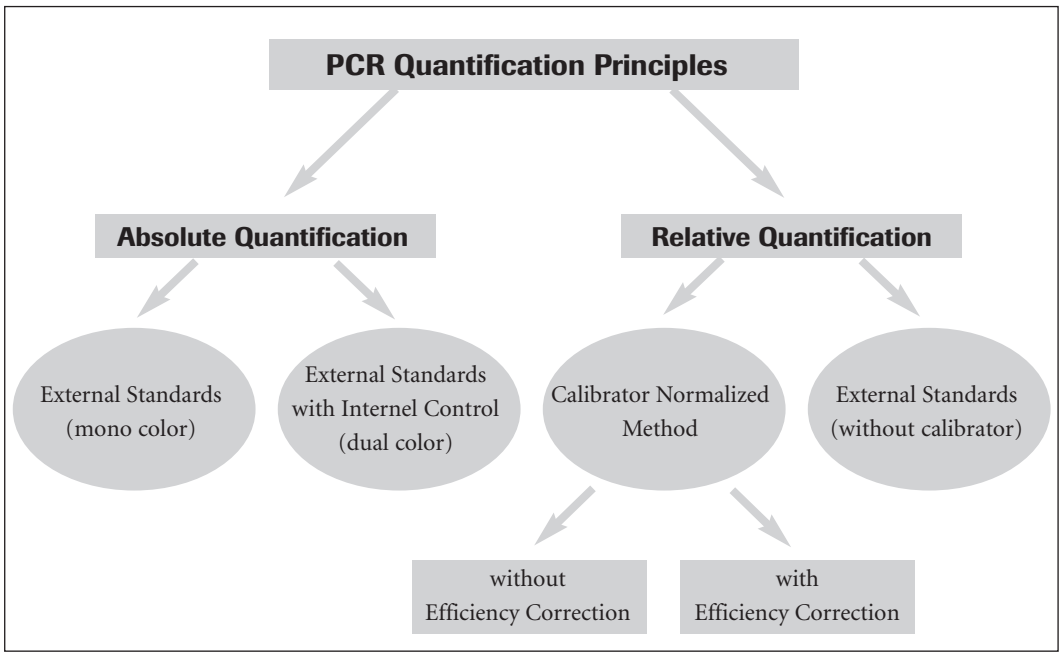


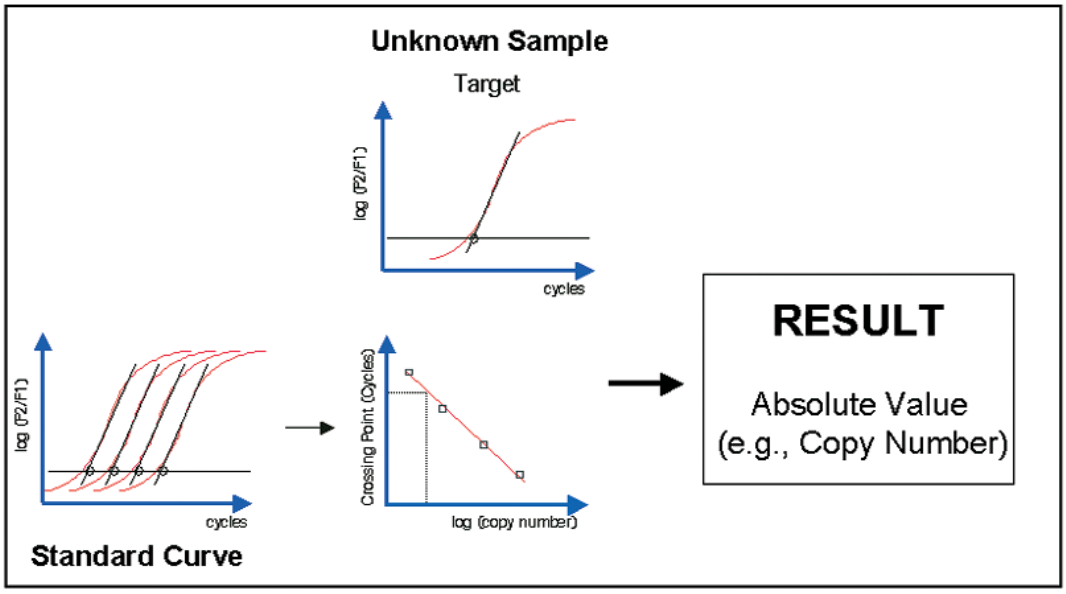
Figure 5: Overview of quantification methods.

3.1 Absolute Quantification with External Standards

Principle

Serial dilutions of an external standard with a predefined known concentration are used to create a standard curve. The determination of unknown sample concentration involves determination of the Cp value.

Note: For details, see Roche Applied Science Technical Note No. LC 11/update 2003.



Prerequisites

- External standards with defined concentrations of target NA
- Standards and samples must have identical PCR efficiency

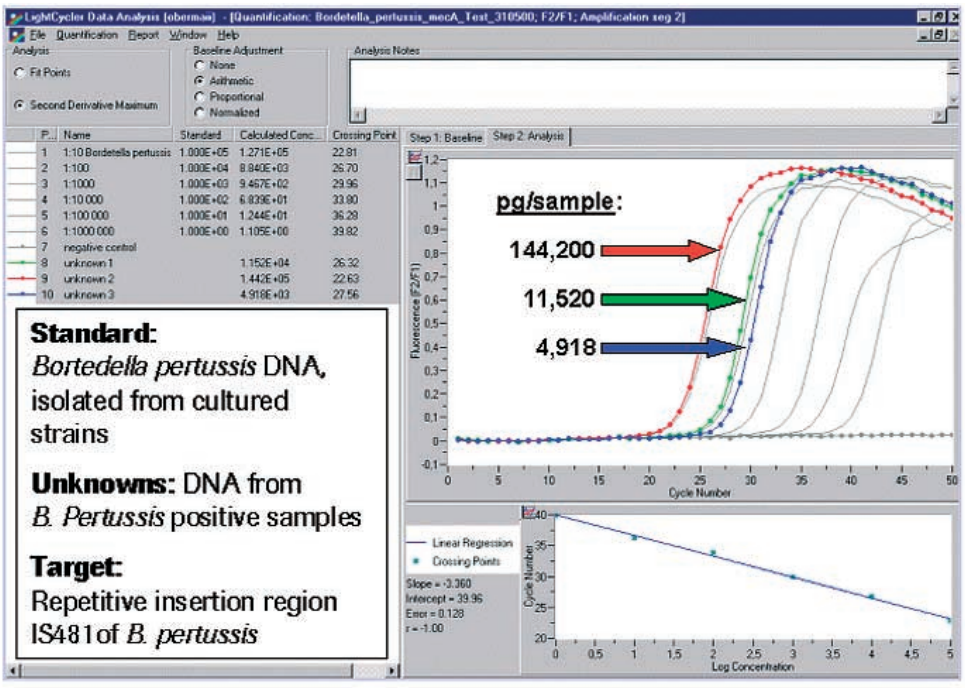
Advantages

- Broad dynamic range (10 – 10^{10} copies)
- Analysis with LightCycler software (LC SW)
- Can be determined with either Hybridization Probe (mono color) or SYBR Green I format

Limitations

- Effects of PCR inhibitors or other factors influencing PCR are unknown

Example



3.2 Absolute Quantification with External Standards and an Internal Control

Principle

Sike the method described on the previous page, this method also uses external standards and produces absolute values for unknown samples. However, for this PCR, each capillary is spiked with an exogenous control DNA, which is co-amplified with the target DNA. Thus, this method can detect PCR inhibition.

Note: For details, please see Roche Technical Note No. LC12/2000

Prerequisites

- External standards with defined concentrations of target NA
- PCR efficiency of standards and samples must be identical

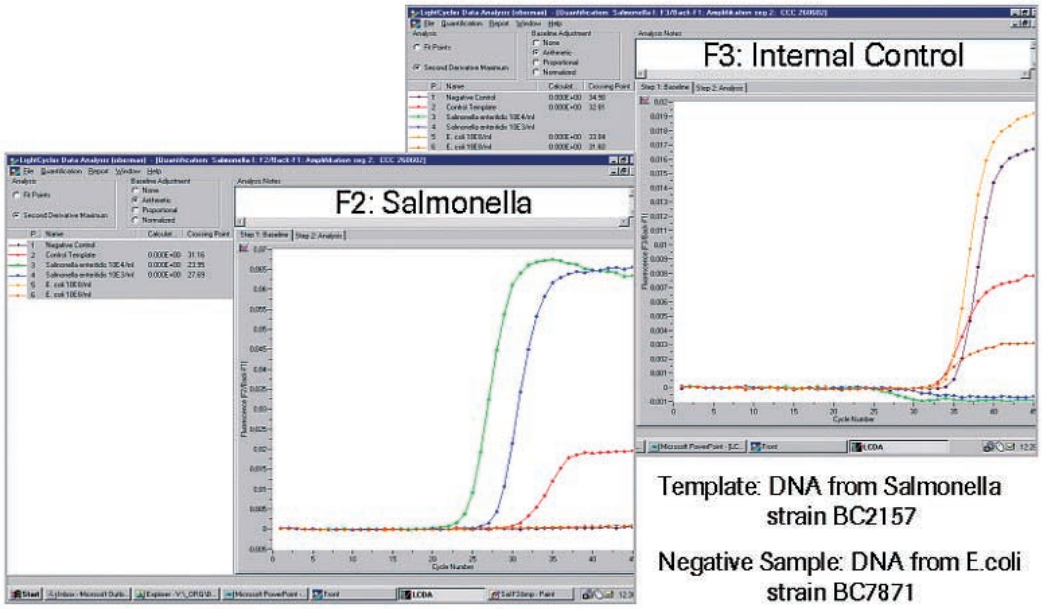
Advantages

- Analysis by LC SW
- Qualitative control for PCR inhibitors (reliable absolute values)

Limitations

- Limited dynamic range (approx. 3 – 5 magnitudes)
- Requires use of Hybridization Probe (dual color) format
- No quantitative correction for factors influencing PCR

Example

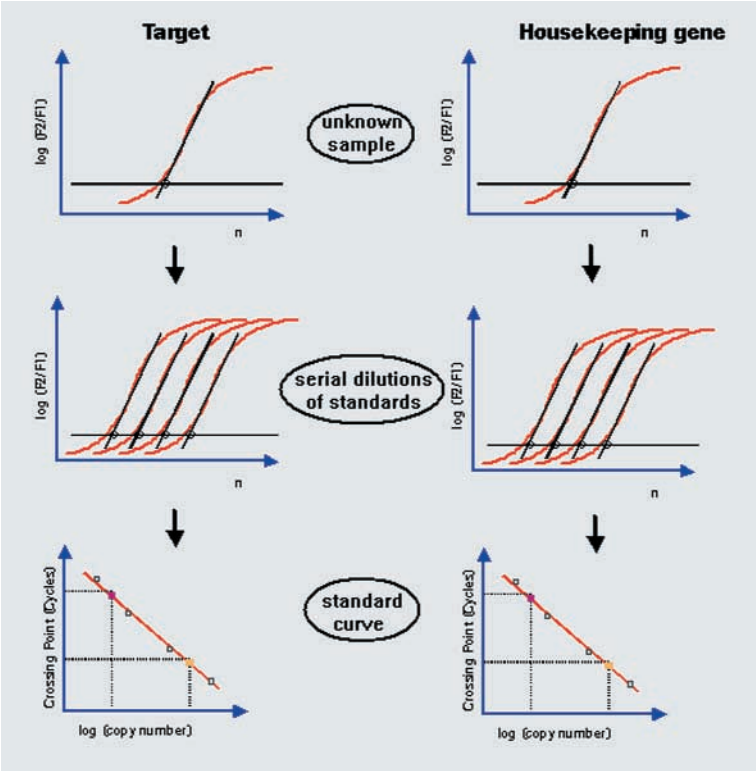


3.3 Relative Quantification with External Standards

Principle

The target concentration is expressed relative to the concentration of a reference (housekeeping) gene. A standard curve is used to obtain the concentration of the target and the reference gene.

Note: For details, see Roche Applied Science Technical Note No. LC13/2001



Prerequisites

- External standards with defined concentration of target and/or reference gene
- PCR efficiency of target/reference is identical in sample and standard
- Constitutive (unregulated) expression of reference gene in the system studied

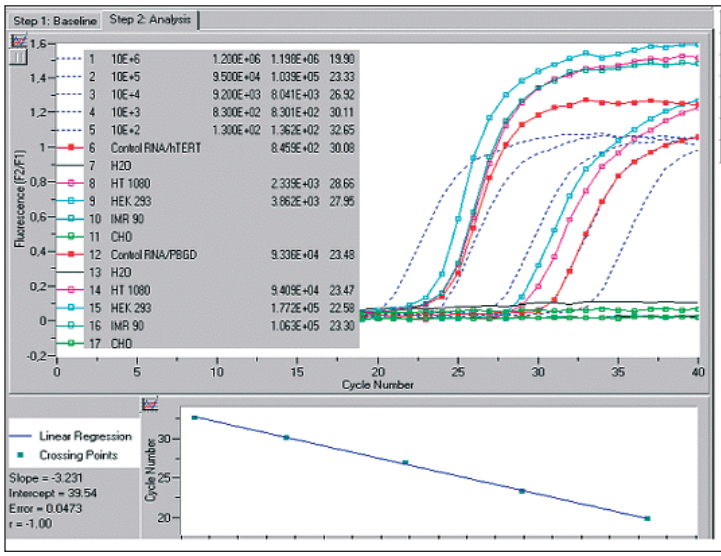
Advantages

- Broad dynamic range (approx. 9 – 10 orders of magnitude)
- Corrects for PCR inhibitors and other factors influencing PCR

Limitations

- Calculation of the target to reference ratio must be done manually or with spreadsheet
- No correction for run-to-run variations

Example



Ratio hTERT/PBGD	Sample
9,06E-03	Positive Control
0,00E+00	H2O
2,49E-02	HT 1080
2,18E-02	HEK 293
0,00E+00	IMR 90
0,00E+00	CHO

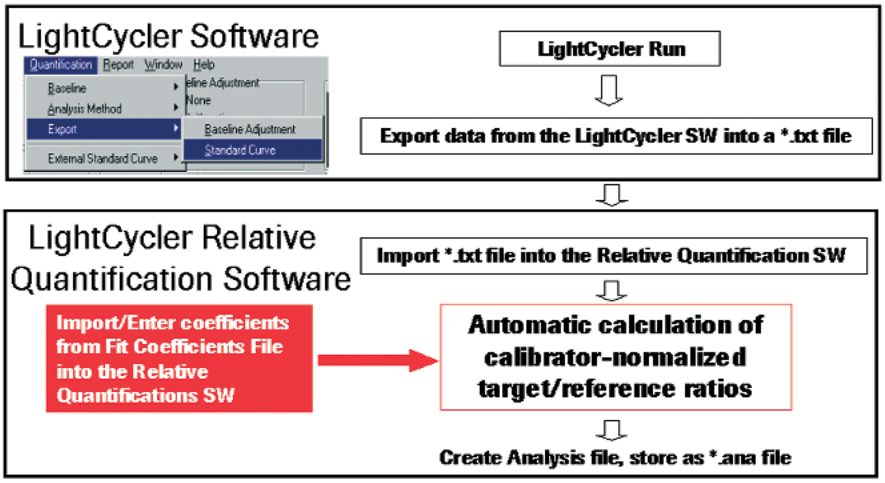
3.4 Calibrator Normalized Relative Quantification

Principle

This method does not require a standard curve to be run in each LC analysis. Instead, quantitation of a target and a reference gene is a function of PCR efficiency and the sample crossing point. Results are expressed as the target/reference ratio of each sample divided by the target/reference ratio of the calibrator. Thus in calibrator-normalized relative quantification assays the accuracy is influenced solely by differences in the PCR efficiencies of target and reference. For the most accurate results, the LightCycler Relative Quantification Software can correct for these differences; alternatively, for approximate results, both PCR efficiencies are assumed to be equal to 2.

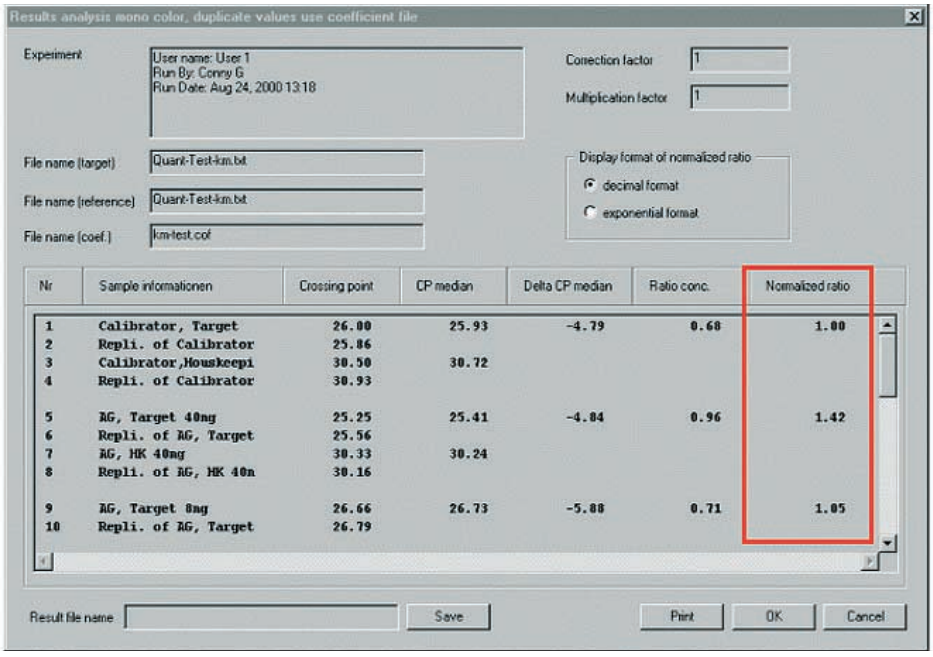
Advantages

- Software tool available for direct download of LightCycler data files:



- No need to include standard dilutions in sample analysis runs (one calibrator only)
- Relative quantification ratios may be calculated using either:
  - the default PCR efficiency (E = 2.00) or
  - an efficiency correction calculated by determining the actual PCR efficiencies of the target and the reference in separate runs
- Corrects for lot-to-lot differences of PCR components (calibrator normalization)

Example



3.5 Quantification Methods – Selection Guide

	Absolute quantification with external standards	Absolute quantification with external standards and internal control	Relative quantification with external standards	Relative quantification with calibrator normalization	Relative quantification with calibrator normalization and efficiency correction
Intended Use	Virology, microbiology	Virology, microbiology	mRNA quantification, gene dosage determination	mRNA quantification, gene dosage determination	mRNA quantification, gene dosage determination
Result	Absolute value	Absolute value	Relative ratio	Relative ratio	Relative ratio
Format	SYBR Green I or Hybridization Probes (mono color)	Hybridization Probes (dual color)	SYBR Green I or Hybridization Probes	SYBR Green I or Hybridization Probes	SYBR Green I or Hybridization Probes
Standard Curve	Yes	Yes	Yes	No	Relative Standards
Calibrator	No	No	No	Yes	Yes
Controls	Positive and negative	Positive and negative, Internal control	Positive and negative, Reference	Positive and negative, Reference, Calibrator	Positive and negative, Reference, Calibrator
Range (orders of magnitude)	10	3 – 5	Mono color: 10 Dual Color: 3 – 5	Mono color: 10 Dual Color: 3 – 5	Mono color: 10 Dual Color: 3 – 5
Prerequisites (E = efficiency)	E of sample = E of standard	E of sample = E of standard	E of sample = E of standard	E = 2	E of target and E of reference are constant
Correction for	No correction available	False negative	Quality of NA	Quality of NA, Run-to-run, variations	Quality of NA, Run-to-run, variations
Analyzed with	LightCycler Software	LightCycler Software	Spreadsheet, *e.g.* Microsoft Exel	LightCycler Relative Quantification Software	LightCycler Relative Quantification Software

4. Creating a Standard Curve

Introduction

Most quantification methods use a standard curve as an accepted measure of comparison for quantification of the unknown, even in relative quantification. In addition, relative quantification with calibrator normalization and efficiency correction uses the standard curve as a measure of amplification efficiency.

The external standard curve is a plot of the cycle number at the crossing point (Y-axis) versus the log of initial template amount (X-axis). The standards are either different well-characterized concentrations of the same target (homologous standards) or serial dilutions with defined dilution steps (*e.g.* 1:10, 1:100 etc.) representing relative concentrations (*e.g.* 0.1, 0.01 etc.).

For methods using external standards, the standard samples are amplified in separate capillaries but within the same LightCycler run. The concentrations chosen for the standard curve should match the expected concentration range of the target. For high accuracy of results, it is highly recommended to choose an appropriate statistical base.

Note: For additional information, see Technical Note No. 11.

Example

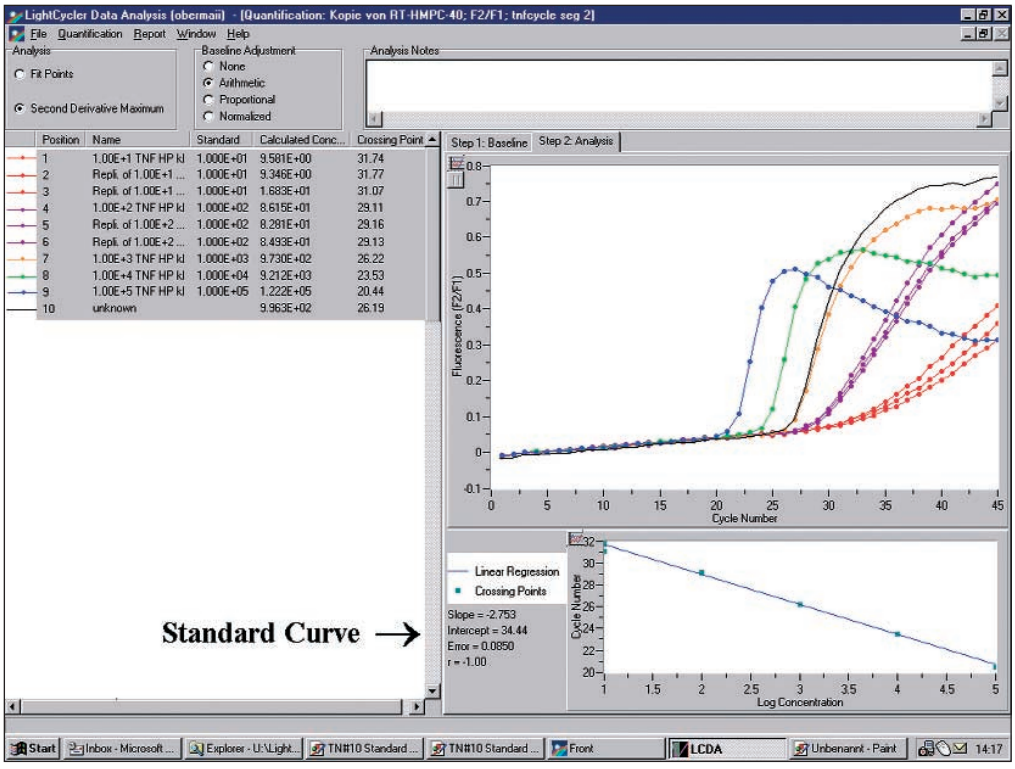


Figure 6 shows an example of a standard curve derived from data of the TNFα system using the Hybridization Probe format.

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Standard Curve Parameters

The standard curve is the linear regression line through the data points on a plot of crossing point (i.e., threshold detection cycle) versus the logarithm of concentration of standard sample. Given the Cp-cycle number of any unknown, you can calculate its concentration from the standard curve.
In a LightCycler run, these parameters are displayed beside the standard curve and are used to calculate the data for the regression line:

Parameter	Mathematical Description	Impact on PCR
Slope	–1/log E	Represents the overall efficiency of PCR
Y-Intercept	$\log N_n/\log E$	
Error	$\sum (\Delta x)^2/n$ (mean squared error) Δx = vertical distance between data point and regression line n = number of data points	Gives information about tube-to-tube variations, *e.g.* pipetting errors • Allowable value: ≤ 0.1
r	Regression Coefficient	Describes the suitability of the linear fit. May indicate systematic errors, *e.g.* accumulated error in dilution series • Allowable value: = –1.00

Slope

The slope defines the distance between data points on the regression line, *e.g.* for a 1-to-10 dilution under optimal conditions (E = 2), the slope is equal to 3.3 Cp. The slope can be converted into efficiency with the following formula:

$$E = 10^{-1/slope}$$

The table below shows some slopes and the corresponding efficiencies :

Slope	E
– 3.20	2.05
– 3.30	2.00
– 3.40	1.97
– 3.50	1.93
– 3.60	1.89
– 3.70	1.86
– 3.80	1.83
– 3.90	1.80
– 4.00	1.77

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LightCyler Methods to Generate a Standard Curve

The LightCycler software provides two analysis algorithms for generating a standard curve: the **Fit Points Method** and **the Second Derivative Maximum Method**. The two methods differ in the way the threshold cycle (where the fluorescence signal first exceeds the level of background noise) is determined. In brief, here is how these two methods work:

- **Fit Points Method** (allows user input):
The user determines the baseline adjustment, noise band setting, crossing line setting and choice of fit points. This manual selection of parameters allows optimization of standard curves in difficult systems.

Note: Software version 3.3 (and later versions, >3.3) also offer a "Minimize Error" button for adjusting the crossing line to its optimal position.

- **Second Derivative Maximum Method** (for automatic calculation):
This method allows no user input (except selection/de-selection of standards), but it offers the advantages of speed and simplicity, especially for quick analysis of frequently repeated standard curves.

Note: With the plausibility check introduced in software versions >3.3, this method can be used to analyze even complex fluorescence data.

For both procedures the software performs all calculation steps necessary for generation of a standard curve. Then, it determines the crossing points of unknown samples and displays the concentrations of the unknowns (calculated from the curve) in the appropriate column of the sample table.

Import of an External Standard Curve

LightCycler software versions > 3.3 allow reuse of a standard curve which has been generated in a previous run and stored as a *.xsc file. This file can be loaded into runs which do not have a standard curve, thus allowing quantitative analysis of those runs. This option is especially suitable for applications where the same parameter is analyzed in multiple samples.

Note: For more information, see Technical Note No. 11.

Requirements

Crossing point data from the standards will be used to convert crossing point data from the unknowns into concentrations of unknowns. For this procedure to work, the amplification efficiencies of the standard and the sample must be identical.

It is possible to check the efficiencies of the standard and the sample by preparing serial dilutions of a typical sample and of the standard, amplifying the dilutions in the LightCycler, and then analyzing the results. The slopes from these runs can be converted into efficiencies. The efficiency values of the sample and the standard can then be compared.

Note: The LightCycler software reports the value of the slope for each standard curve (as in Figure 6). This slope is mathematically correlated to the PCR efficiency by the following formula:

$$E = 10^{-1/slope}$$

If homologous standards are used (as outlined on the next page), achieving identical amplification efficiencies is usually not difficult. However, be aware that even small differences in amplification efficiency can influence the final result substantially. Keep this in mind especially when using heterologous standards (*i.e.,* standards amplified with a different set of PCR primers).

Note: The error resulting from different efficiencies can be calculated by substituting different values of E in the PCR equation:

$$N = N_0 \times E^n.$$

5. Guidelines for Designing and Preparing Standards

Introduction

Data obtained from the standards are used to plot a standard curve of crossing point (Cp) vs. log concentration. Be careful when designing, preparing, and calibrating standards, since the absolute quantity of an unknown sample is based on the performance and consistency of the standard.

This section gives recommendations for preparing a suitable standard.

Requirements for a Standard

For successful and accurate quantification, the external standard should meet all the criteria listed in the following table.

Property	Criterion To Be Met
Amplification Efficiency	The amplification efficiency of the standards and the target must be identical.
Sequence	• The amplified standard sequence should be homologous to the target. (identical amplicon length and GC-content). • The standards should have the same primer and Hybridization Probe binding sites as the target sequence to ensure equal amplification and detection efficiency.
Source	• For PCR: Preferably linearized plasmid DNA carrying the cloned target sequence, or purified PCR products, or reference DNA (*e.g.*, genomic DNA) isolated from biological material. • For RT-PCR: Synthetic, usually *in vitro* transcribed RNA or total RNA/mRNA containing the target sequence (reference RNA).
Detection	Detectable with same Hybridization Probe pair (preferably labeled with Fluorescein/LC Red 640) as the target.
Purity	Use highly purified templates to ensure absence of contaminants (nucleotides, primers and salt) which can interfere with PCR. (Prepare nucleic acids with a High Pure kit or use the MagNAPure LC System).
Concentration	• Determine the concentration by measuring absorbance at 260 nm according to standard procedures. • To minimize pipetting errors, we recommend adjusting the volume of each standard so at least 2 µl of each standard is added to the LightCycler capillary (*e.g.*, 10^9–10^0 copies/2 µl).
Handling	• Use siliconized tubes for standard and target dilutions. • Use aerosol-resistant, sterile pipette tips. • Stabilize standards with carrier nucleic acid (*e.g.*, MS2 RNA, 10 ng/µl). • Store high concentrations of the stabilized standard ($\geq 10^5$ copies) in aliquots at –20°C (DNA) or at –70°C (RNA).

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5. Guidelines for Designing and Preparing Standards,

continued

Estimation of the Standard Concentration

(1) Determine the concentration of the standard nucleic acid by measuring the absorbance at 260 nm according to standard procedures.
For spectrophotometric equivalents see table below:

	Nucleic Acid	Concentration
1 A_{260} unit	double-stranded DNA	50 µg/ml
	single-stranded DNA	33 µg/ml
	single-stranded RNA	40 µg/ml

Optionally, you may determine the purity of the nucleic acid preparation:

Nucleic Acid	A_{260} / A_{280}*
DNA	= 1.8
RNA	= 2.0
* lower values indicate that the preparation contains contaminants (*e.g.*, protein or phenol)	

(2) If required, calculate the copy number of the standard. Use the following mathematical correlation and formulas as a guideline:

For average molecular weight of	Use this calculation
dsDNA	(number of base pairs) × (660 daltons/base pair)
ssDNA	(number of bases) × (330 daltons/base)
ssRNA	(number of bases) × (340 daltons/base)

1 mol = molecular weight (MW) [g]
1 mol = 6×10^{23} molecules (= copies)

$$\frac{\mathbf{6 \times 10^{23}\ [copies/mol]} \times \mathbf{concentration\ [g/\mu l]}}{\mathbf{MW\ [g/mol]}} = \mathbf{amount\ [copies/\mu l]}$$

Example

Concentration of CycA plasmid = 152 ng/µl = 1.52×10^{-7} g/µl
Plasmid containing insert is 3,397 bp long

MW = 3,397 bp × 660 daltons/bp = 2,242,020 daltons
1 mol = 2,242,020 g
1 mol = 6×10^{23} molecules (= copies)

$$\frac{6 \times 10^{23}\ [copies/mol] \times 1.52 \times 10^{-7}\ [g/\mu l]}{2{,}242{,}020\ [g/mol]} = 4 \times 10^{10}\ [copies/\mu l]$$

6. Reproducibility

Introduction

Reproducibility (as measured by the standard deviation or the coefficient of variation of an assay) is a key parameter to consider in quantitative PCR. For any given assay system, reproducibility determines the minimum difference in initial target concentration that the assay can distinguish.

Note: Due to the exponential nature of PCR amplification, the imprecision of the PCR assay is unavoidably larger than that observed in classical clinical chemistry or immunological assays.

Factors Influencing Reproducibility

Reproducibility of PCR depends on almost every aspect of the assay. All steps of the analysis process, from sample preparation through amplification and final detection of the PCR product, will influence reproducibility. Other critical factors include pipetting precision (especially important for small volumes), PCR efficiency, and the instrument used for the analysis.

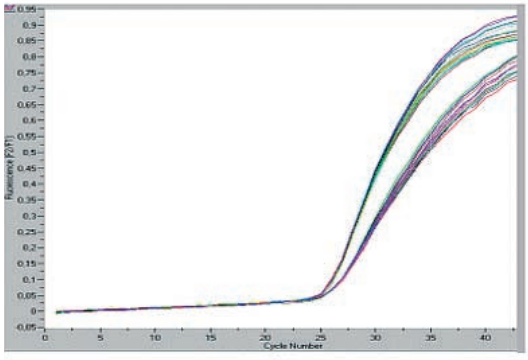
For very low copy numbers ($\leq$ 50 copies), even Poissonian statistics (which defines the probability of having at least one copy in a given sample) affects reproducibility.

Advantages of the LightCycler System

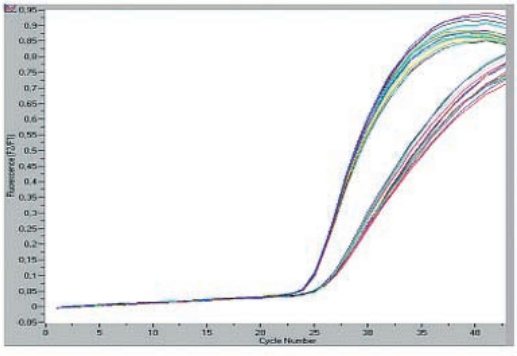
The LightCycler system has several unique features that guarantee highly reproducible measurement, including:

- Mechanical features of the instrument:
  - A single circular thermal chamber with uniform temperature distribution to provide identical conditions for each PCR
  - A single optical unit for processing all data, to ensure identical highly sensitive measurements
  - Precision sample positioning and adjustment, to ensure optimal data sampling
- Software tools that ensure precision analysis:
  - Proportional baseline adjustment, which can remove some of the tube-to-tube variations caused by pipetting
  - Signal normalization for single color Hybridization Probe experiments (Setting the y-axis display of the fluorescence graph to F2/F1 or F3/F1 will cause the signal of the reporter dye (*e.g.,* LC Red 640, measured in channel 2) to be divided by the signal of the donor dye (Fluorescein, measured in channel 1), thus providing an internal "reference" for the displayed data (and eliminating effects of pipetting errors and positioning errors).
  - In dual color evaluation, using F2/back-F1 or F3/back-F1 (setting of the y-axis display of the fluorescence graph) normalizes results (and eliminates the effects of pipetting errors).
- LightCycler ready-to-use reagents, to minimize sample and tube-to-tube variations.

Examples of LightCycler Reproducibility



PCR on plasmid DNA, 130 bp
15 x 10,000 copies, 15 x 5,000 copies,
CV crossing point for 5,000 copies 0.3%
CV crossing point for 10,000 copies 0.2%



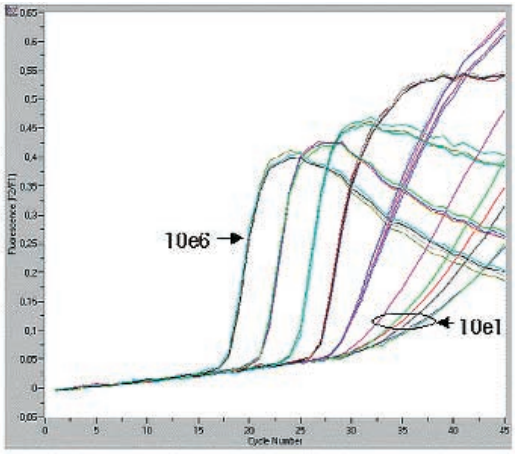
PCR on plasmid DNA, 130 bp
15 x 25,000 copies, 15 x 5,000 copies,
CV crossing point for 5,000 copies 0.4%
CV crossing point for 25,000 copies 0.2%

Figure 7 illustrates the use of a Hybridization Probe experiment to monitor the replication variance for samples with different initial copy numbers.

Continued on next page

Examples of LightCycler Reproducibility, continued

The target sequence was human TNFα-cDNA, cloned into a plasmid. For amplification, different copy numbers of the linear plasmid were used as template. The first experiment measured a two-fold difference in copy number (5,000 and 10,000 initial copies in different capillaries). The second experiment measured a fivefold difference in starting copy number (5,000 and 25,000 initial copies in different capillaries). Fifteen replicates of each sample were assayed.
Result: The LightCycler assay can distinguish 5,000, 10,000, and 25,000 initial copies with a high degree of confidence, as indicated by the low CV calculated for the crossing point data (approx. 0.3% for each initial copy number).



Experiment repeated 3 times
plasmid/PCR/130 bp/ Hybridization Probes
4 repl. 10^6 copies: CV 0.7 – 1.2%
4 repl. 10^5 copies: CV 0.3 – 0.6%
4 repl. 10^4 copies: CV 0.2 – 0.4%
4 repl. 10^3 copies: CV 0.2 – 1.2%
4 repl. 10^2 copies: CV 0.2 – 0.7%
4 repl. 10^1 copies: CV 1.7 – 2.7%

Figure 8 shows that reproducibility depends on the starting concentration of the template. The same amplification system as described in Figure 7 was used, but with a broader range of starting concentration (from 1^1 to 10^6 copies).
Result: The CV is low down to 100 copies. Below 100 copies the CV of the crossing point (see table above) goes up as a result of particle distribution.

Calculation of the Coefficient of Variation

For reproducibility experiments, the coefficient of variation (CV) is usually used as an indicator of relative precision and reproducibility. The CV of a set of data is determined by dividing the standard deviation (SD) by the arithmetic mean of the measured values:

CV [%] = SD/mean value x 100.

Note:
- The SD and arithmetic mean of replicate samples are automatically calculated and displayed by the LightCycler Software.
- CVs may be calculated for crossing point data as well as for calculated concentrations. However, because concentration and crossing point have a log-linear relationship, the expected CVs for crossing points are lower than those for concentrations. When comparing different quantitative methods, remember the different behavior of these two parameters.

Guidelines for High Precision Quantitative Analysis

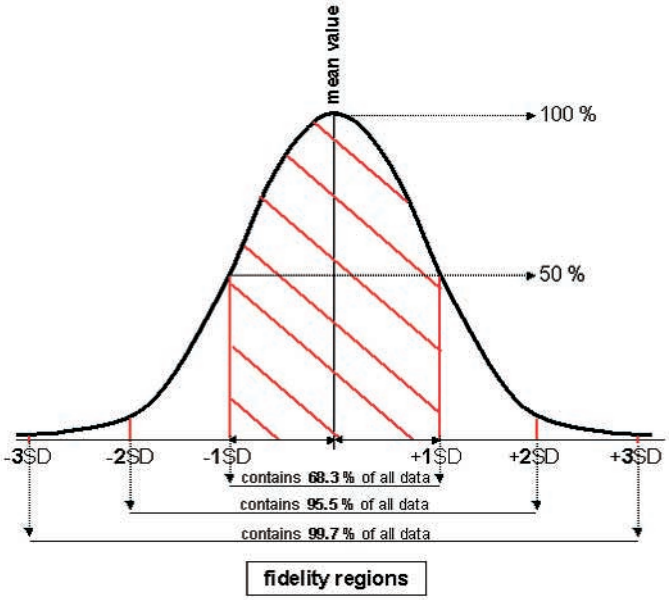
The CV of a quantitative analysis depends upon copy number (see Figure 8) and the efficiency of the PCR. Therefore, if high precision is important, always:
- Optimize PCR conditions before performing the quantitative analysis.
- Use templates that have high initial copy numbers.
- If you must work near the detection limit of the system, use replicate samples.

Continued on next page

Quantification Fidelity Regions

For most experiments it is possible to calculate the minimum difference in initial copy number that an assay can distinguish. For this calculation, we assume that the mean and the standard deviation of a sample are typical for the experimental population. Then, for each calculated mean sample value, we may define a "fidelity region" within which a given percentage of results (obtained from replicates of that sample) will fall. If that percentage is high (*e.g.* 95%), we may conclude that any sample that gives a value outside the fidelity region in fact contains a different number of initial copies (see Figure 9).

Note: The width of this region increases with imprecision and high fidelity levels.



$$SD = \sqrt{\frac{\sum_{i=1}^{n} (\bar{x} - x_i)^2}{n - 1}}$$

SD = standard deviation

x_i = single data point; $\bar{x}$ = mean value; n = amount of data points

Figure 9: Typical Gaussian distribution as an illustration of fidelity regions around a sample mean. Note that the +/– 2 SD fidelity region contains 95.5% of the data and the +/– 3 SD region contains 99.7% of the data. Thus a result that lies outside these fidelity regions is unlikely to originate from the sample with this mean value.

Example: For a LightCycler PCR with 15 replicate determinations of a 5,000 copy sample, assayed with Hybridization Probes (see Figure 7), the data are:

mean value (copies)	CV [%] (copies)	standard deviation (copies)	3SD (copies)
5009	6.2%	311	933

Conclusion:
Statistically, 99.7% of all concentration values for that sample will lie within a fidelity region bounded by 4,076 copies (5009 – 3SD) and 5,942 copies (5009 + 3SD). Any values outside that region must come from a different sample with a different initial copy number.

Note: This example underlines the great precision of the LightCycler instrument.

7. Appendix

Further Readings

Betzl G et al (2000) *Reproducibility of PCR on the LightCycler System.* Biochemica **1**/2000.

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Ferre F (1997) *Gene Quantification*: Methods and Applications. Birkhauser, Boston.

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Reischl U et al (2000) *Rapid and Specific Detection of Bordetella pertussis in Clinical Specimens.* Preprint from Rapid Cycle Real-Time PCR, Methods and Applications. Springer 2000.

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