Roche Molecular Biochemicals
Technical Note No. LC 11/2000

LightCycler

Absolute Quantification with External Standards

Purpose of this Note

The easiest way to obtain an absolute value for an unknown concentration of a target is to use external standards. Therefore, this Technical Note:

- Gives detailed information on preparing external standards and generating a suitable standard curve,
- Recommends a PCR optimization strategy for achieving successful quantification results with the LightCycler instrument, and
- Provides some mathematical background for calculating the accuracy and the reproducibility of an individual system.

Note: The highly flexible LightCycler system offers a number of quantitative PCR methods to the user. For a comparison of this method to other quantification methods that can be performed in the LightCycler, see Roche Molecular Biochemicals Technical Note No. LC 10/2000: *Overview of LightCycler Quantification Methods.* For more information on the reproducibility of LightCycler amplification, see the article of G. Betzl et al. in *Biochemica* Nr.1/2000.

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1. Principles of Kinetic PCR

Introduction

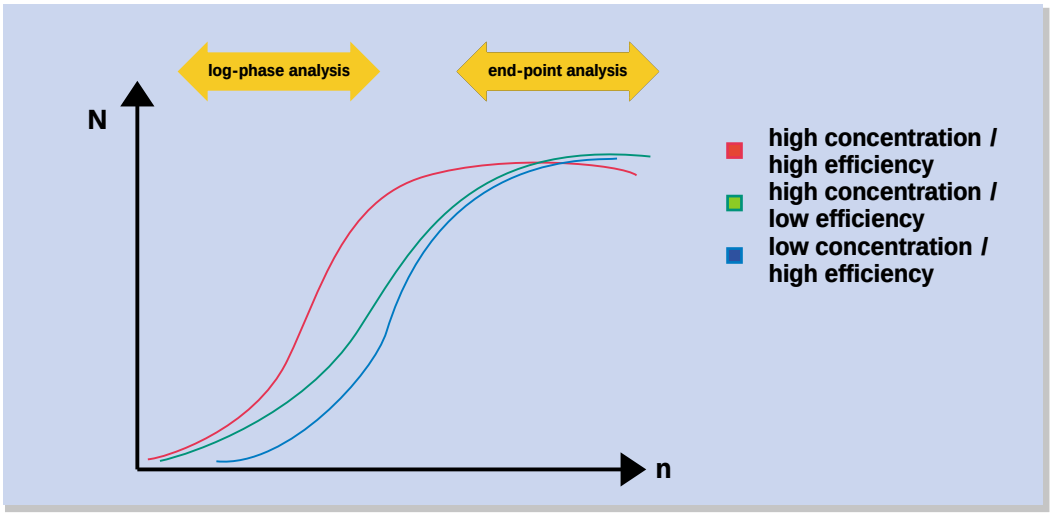
PCR has become an essential technique for quantitation due to its unsurpassed sensitivity and high dynamic range.
Before real time PCR was available, the copy number of a specific target had to be determined by conventional methods such as:

- Competitive PCR
- Limiting dilution PCR
- Radioactive methods

All these conventional methods are based on end-point analysis with its known limitations and disadvantages.

The real-time PCR detection capabilities of the LightCycler made kinetic quantification possible. This new method allows data analysis under conditions of constant amplification efficiency.
As explained below, kinetic quantification provides accurate results for external, internal or relative quantification methods.

Typical PCR



N: number of amplified molecules; n: number of amplification cycles

Figure 1 shows typical PCR amplification curves. Each curve has three segments:

- An early background phase,
- An exponential growth phase (or log linear phase), and
- A plateau phase.

The background phase lasts until the fluorescence signal from the PCR product is greater than the background fluorescence of the probe system. The exponential growth 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.
As shown in Figure 1, PCR amplification is template concentration dependent, but reactions with low starting copy number can reach the same plateau as reactions that started with higher template concentrations and/or different PCR efficiency.

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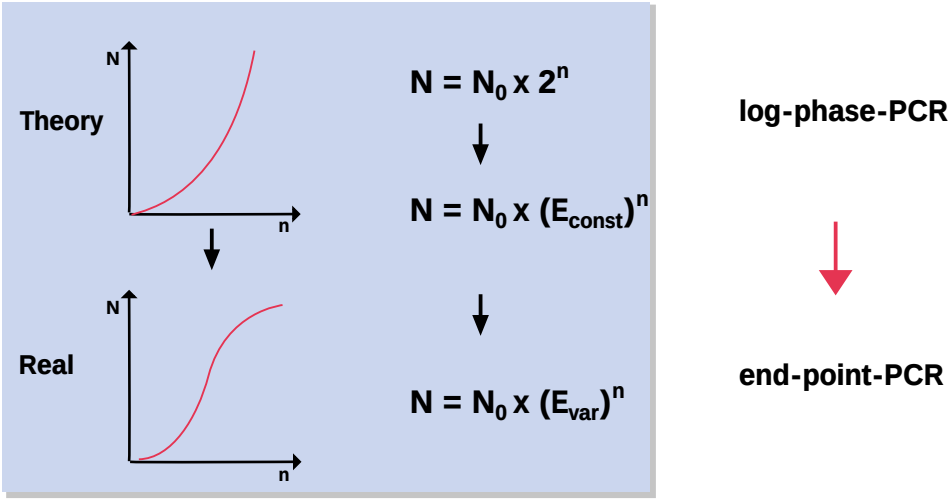
Theoretical Aspects



N: number of amplified molecules; **N_0:** initial number of molecules; **n:** number of amplification cycles; **E:** amplification efficiency

Figure 2: Theoretical aspects of PCR amplification

In theory PCR is described by the formula $N = N_0 \times 2^n$. The optimum efficiency possible in PCR is two - every PCR product is replicated once every cycle.
In reality many PCR parameters influence the PCR efficiency, so the efficiency is constant, but often different from two: $N = N_0 \times (E_{const})^n$.
Note: Only a few detectable cycles of a PCR actually obey this equation and therefore allow a mathematically correct analysis.
Especially in the final phase, the exponential curve bends toward a plateau and efficiency becomes variable: $N = N_0 \times (E_{var})^n$. This plateau is caused by:

- Competition of re-annealing amplification product with primers
- Decrease of reagent concentration
- Product inhibition (*e.g.*, pyrophosphate accumulation)
- Enzyme instability

Real-Time Quantitative PCR

In contrast to analysis during end-point-PCR, analysis in the log-linear phase produces data that are much more accurate. Since amplification efficiency is constant throughout the analysis, the amount of starting material can be determined very precisely. An increase in signal during the log-linear phase corresponds directly to an increase in PCR product.
It is quite difficult to identify and measure the few detectable cycles of the log-linear phase by traditional methods such as agarose gel electrophoresis. However, real-time PCR monitoring (that is, measuring fluorescence intensity once per cycle during PCR on the LightCycler) offers a convenient way to identify and measure these log-linear cycles.

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Dynamic Range

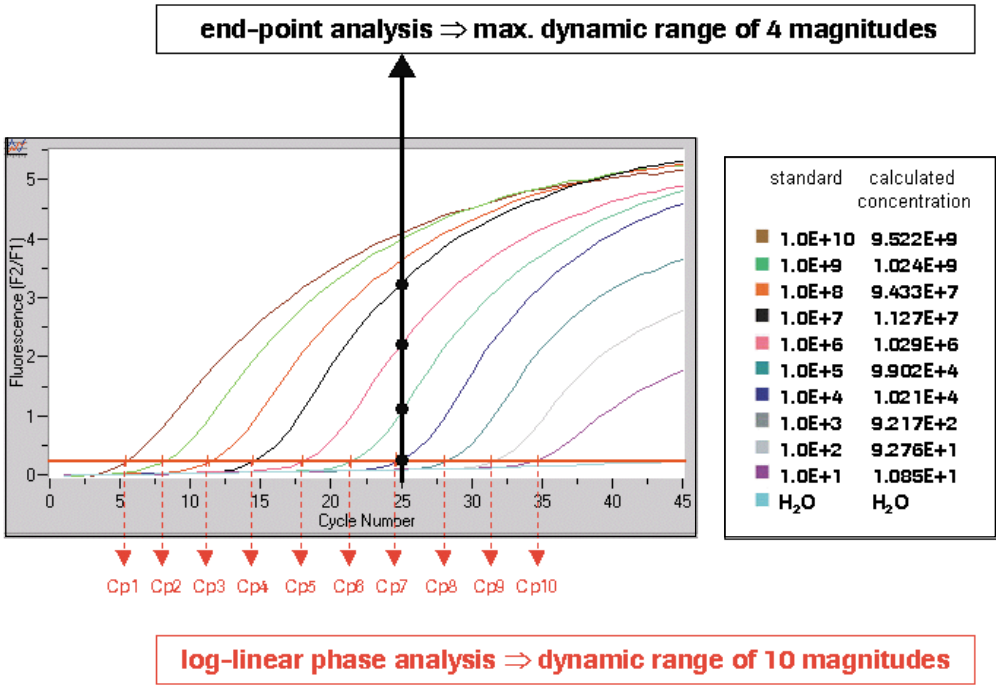

Figure 3: A comparison of the dynamic range achieved by end-point analysis versus log-phase analysis

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. Lower starting concentrations of the target, run in the same PCR, are not detectable at the time of analysis (after 25 cycles). Higher target amounts cannot be differentiated since they have already reached the plateau phase of PCR and accumulate at a similar rate. Figure 3 shows that significant variations in the amount of starting material cannot be detected by signals measured in the plateau phase. Measuring these variations requires very cumbersome methods.
In contrast, determination of the crossing points (Cp; see Figure 4), achieved with real-time fluorescence monitoring, can easily generate data during the log-linear phase of the PCR for calculating target concentration. Under optimized reaction conditions, such a Cp analysis allows accurate quantification over a dynamic range of at least 9-10 orders of magnitude.

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Accuracy of Quantification

Quantification by external standards is referred to as “absolute” quantification, because an actual number of DNA molecules can be obtained. But the accuracy of external standard quantification is entirely dependent on the accuracy of the standards.
The relationship between dilutions of the target and the crossing points of the amplification curves is highly reproducible. This is enough to guarantee a precise and reproducible quantification, but not necessarily an accurate quantification.
In fact, high accuracy is difficult to achieve. Since even commercial quality control labs have trouble calibrating standards, all absolute copy number claims should be treated with a healthy skepticism.
Final results are always reported relative to something, *e.g.* copies per cell, copies per A260 unit of starting nucleic acid, copies per gram of tissue, copies per milliliter of blood, etc.
Therefore, a suitable and consistent standard must be very carefully prepared, *e.g.* according to the recommendations outlined in Section 3 of this Note.

Limitation of Quantification

One limitation of this method is that it does not detect or compensate for PCR inhibitors that may be present in the sample material.
All quantitative PCR methods assume that the target and the sample amplify with identical efficiency. This method does not include a control function for variations in the PCR efficiency of unknowns. Hence, both template preparation and assay must be well characterized and optimize (see Section 4 of this note).
Note: To detect and compensate for PCR inhibitors, you can spike an internal positive control into the samples as described in Roche Molecular Biochemicals Technical Note No. LC 12/2000: *Absolute Quantification with External Standards and an Internal Control.*

2. The Standard Curve

Introduction

In the quantification method described in this Note, the LightCycler compares the amplification of target nucleic acids in an unknown sample against a standard curve prepared with known concentrations of the same target (homologous standards). The standard samples are amplified in separate capillaries but within the same LightCycler run (external standards).
The concentration chosen for the standard curve should match the expected concentration range of the target; typically a standard curve is prepared from at least five samples, which are prepared by serial dilution.

Example

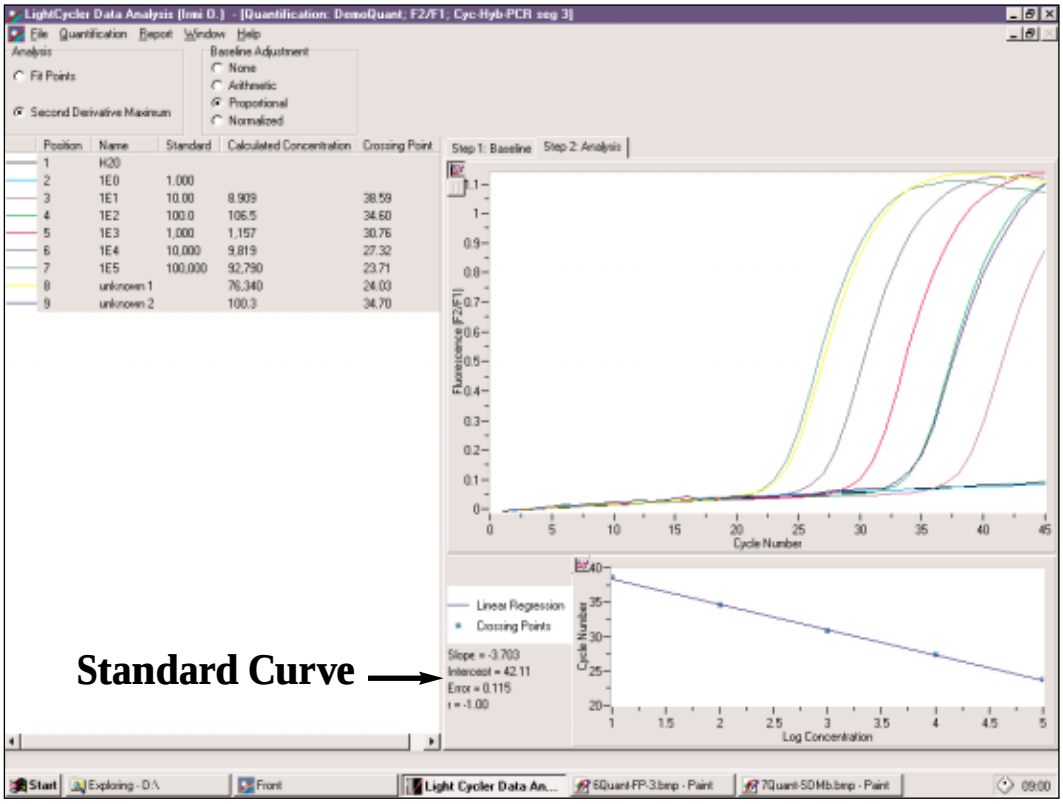

Figure 4 shows an example of a standard curve derived from β-globin data generated with the Hybridization Probe format.

The standard curve is the linear regression line through the data points on a plot of crossing point (threshold cycle) versus logarithm of standard sample concentration.
The values shown at the left of the standard curve in Figure 4 are derived from the calculated regression line:

- Slope = –3.703 (= –1/log E), where E = efficiency of the reaction
- Y-Intercept = 42.11 (= $\log N_{Cp}/\log E$; N_{Cp} = amount of PCR product at Cp)
- Error = 0.115 (mean squared error)
- r = –1.00 (regression coefficient)

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LightCycler 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, but are equally suitable for accurate quantification. In brief, here is how these two methods work:

- In the Fit Points Method, the data calculation is user-influenced:
  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 difficult standard curves.
  Note: Software versions 3.3 and above also offer the "Minimize Error" button for adjusting the crossing line to its optimal position.
- The Second Derivative Maximum Method offers automatic data calculation with no user influence (except selection/de-selection of standards). This method offers the advantages of speed and simplicity, especially for quick analysis of frequently repeated standard curves.
  Note: With the plausibility check introduced in software version 3.3, even complex fluorescence data can be handled by this method.

For both procedures the software performs all additional calculation steps necessary for generation of a standard curve. Then, the crossing points of unknown samples are determined from the curve and the corresponding concentrations are displayed in the appropriate column in the sample table.
Note: For further details, see the *LightCycler Operator's Manual*.

Import of an External Standard Curve

LightCycler software version 3.3 allows use of standard curves which have been generated in a previous run and stored as a file (*.xsc). This file can be loaded into runs without a standard curve, thus allowing quantitative analysis in those runs. This option is especially suitable for applications where the same parameter is analyzed in multiple samples.
Requirements for use of this method: One sample of known concentration (within the range covered by the standard curve) has to be included in each run. In addition, the selected signal channel and the analysis mode of the imported standard curve and the run file have to match.

Equal Efficiency Required for Accurate Analysis

Since the crossing point values of the unknowns will be converted to concentrations using the data derived from the standards, the amplification efficiency (E) of the standard and the target must be identical. Since the LightCycler software calculates the slope for each standard curve (see Figure 4), the PCR efficiency may be calculated from the following formula:

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

To check the efficiencies of the standards and target: Prepare serial dilutions of the target and the standard sample, amplify the dilutions in the LightCycler, and analyze the results as described above. Compare the efficiency values for the target and the standard.
Note: If homologous standards are used (as outlined in Section 3), identical amplification efficiencies can normally be achieved without difficulty. However, remember that even small differences in amplification efficiency can influence the final result substantially. This error can be calculated by substituting the difference in E values into the PCR equation: $\mathbf{N = N_0 \times E^n}$ (see Section 1). This is especially important if you use heterologous standards (*i.e.*, standards amplified with a different set of PCR primers).

3. Guidelines for Designing and Preparing External Standards

Introduction

Data obtained from the standards are used to plot a standard curve of crossing point (Cp) vs. log concentration. Care needs to be taken in designing, preparing, and calibrating standards as the absolute quantity of an unknown sample is based on the performance and consistency of the standard. In this section you find 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 (including having the same 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 as the target.
Purity	Use highly purified templates to ensure absence of nucleotides, primers and salt, which can interfere with PCR (*e.g.*, prepare nucleic acids with a High Pure kit).
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 (≥10^3 copies) in aliquots at –20°C (DNA) or at –70°C (RNA).

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3. Guidelines for Designing and Preparing External 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, the purity of the nucleic acid preparation can be determined:

Nucleic Acid	A_{260}/A_{280}*
DNA	= 1.8
RNA	= 2.0
*If actual value is lower, 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 pairs)
ssDNA	(number of base) × (330 daltons/base)
ssRNA	(number of base) × (340 daltons/base)

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

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

Example

For CycA plasmid that has a concentration of 152 ng/µl = 1.52×10^{-7} g/µl and a total size of 3,397 bp:

MW = 3,397 bp × 660 daltons/bp = 2.24×10^{6} daltons
1 mol = 2.24×10^{6} g
1 mol = 6×10^{23} molecules (= copies)

$$\frac{6 \times 10^{23}\ [copies/mol] \times 1.52 \times 10^{-7}\ [g/\mu l]}{2.24 \times 10^{6}\ [g/mol]} = 4 \times 10^{10}\ [copies/\mu l]$$

4. Optimizing PCR Conditions

Overview

After choosing a suitable standard, establish the optimal LightCycler PCR conditions for amplification and detection either in the SYBR Green I or the Hybridization Probe format. Follow the recommendations in this section to optimize all PCR parameters.

Note: For more information, see Roche Molecular Biochemicals Technical Notes No. LC 2–5/99 and LC 9/2000 *(Optimization Strategy)*, the *LightCycler Operator's Manual* and pack inserts of the respective LC Kits (for PCR or RT-PCR).

Reagents

Always use Roche ready-to-use reagents designed for the LightCycler.

$MgCl_2$ - Titration

In the first experiment, optimize the Mg^{2+} concentration by amplifying the target in the presence of different concentrations of $MgCl_2$. Use:

- 1–5 mM $MgCl_2$ for DNA assays
- 4–8 mM $MgCl_2$ for RNA assays

Template

Amplify at least two different template dilutions:

- High template concentration
- Medium/low template concentration

Note: Also include a "no template" control (NTC).

Amplicon Length

Amplicon length should not exceed 1000 bp. For best results, we recommend short amplicons.

Primers and Hybridization Probes

- For primer design guidelines, see Roche Molecular Biochemicals Technical Note No. LC 1/99.
- For Hybridization Probe design guidelines, see Technical Note LC 6/99.
- Always start with highly purified primers and probes (HPLC).
- Start with a 0.5 µM concentration of each primer; for optimization, test primer concentrations between 0.3 and 1.0 µM.
- Start with a 0.2 µM concentration of each Hybridization Probe; for optimization, test probe concentrations between 0.2 and 0.4 µM.
- Avoid duplex formation between primers and Hybridization Probe pairs.

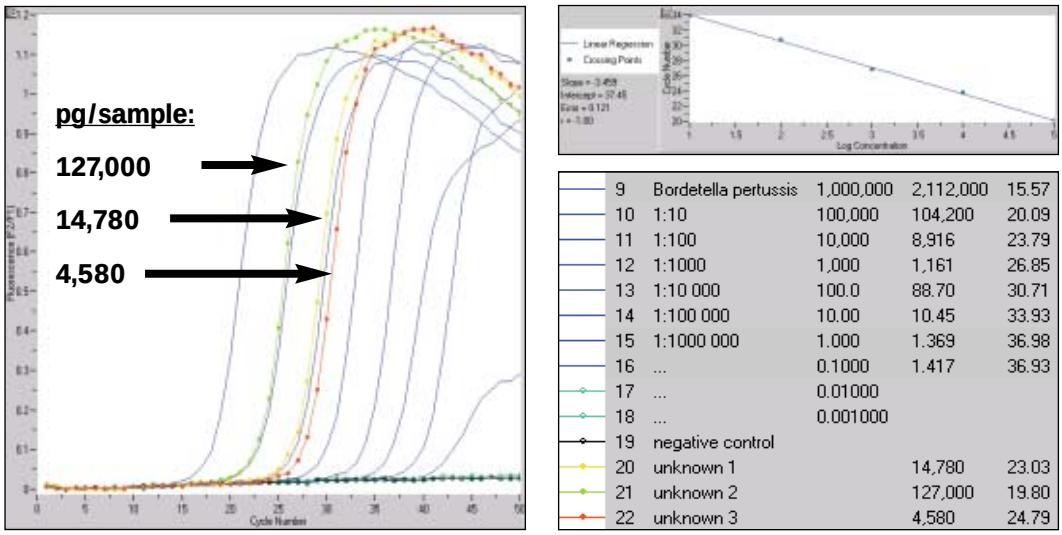
Further Optimization

For best results, also optimize the following parameters:

- Vary annealing temperature in 1°–2°C steps.
- Use a Hot Start method to reduce formation of primer dimers and improve sensitivity.

5. Application

Example



9	Bordetella pertussis	1,000,000	2,112,000	15.57
10	1:10	100,000	104,200	20.09
11	1:100	10,000	8,916	23.79
12	1:1000	1,000	1,161	26.85
13	1:10 000	100.0	88.70	30.71
14	1:100 000	10.00	10.45	33.93
15	1:1000 000	1.000	1.369	36.98
16	...	0.1000	1.417	36.93
17	...	0.01000		
18	...	0.001000		
19	negative control			
20	unknown 1		14,780	23.03
21	unknown 2		127,000	19.80
22	unknown 3		4,580	24.79

Figure 5 shows an example of absolute quantification with external standards.

Here, the LightCycler - FastStart DNA Master Hybridization Probes has been used in research applications to amplify a segment within the repetitive insertion sequence (IS481) of *Bordetella pertussis*.
Note: The primers and hybridization probes selected for this experiment recognize a species-specific region, which ensures strain-specific detection. In addition IS481 provides a very sensitive target, due to its high copy number.
The data above were gathered from the following samples:
- **Standards:** *Bordetella pertussis* DNA was isolated from cultured strains, using the High Pure PCR Template Preparation Kit. Eight DNA dilutions ranging from undiluted (1 µg/sample = approx. 5 x 10^8 organisms) to a 1:10^7 dilution (0.1 pg/sample = approx. 50 organisms) were used to generate a standard curve (samples 9 - 16). Two additional dilutions were added (sample 17-18) in order to determine the assay's lower limit of detection.
- **Control:** Sample 19 is the no template control (NTC).
- **Unknowns:** DNA prepared from nasopharyngeal specimens of *B. pertussis*-positive samples.

Note: The bacterium *Bordetella pertussis* is the causative agent of whooping cough, which is an infectious disease occurring worldwide with a high incidence among young, unvaccinated infants.

Result: The standard curve (Figure 5, upper right graph) was used to determine the amount of *B. pertussis* in the three unknown samples, namely 127,000 pg/sample (= 6 × 10^6 organisms), 14,780 pg/sample (= 7 × 10^5 organisms), and 4,580 pg/sample (= 2 × 10^5 organisms).
Note: Data obtained from Dr. Udo Reischl, University of Regensburg, Germany (Udo.Reischl@klinik.uni-regensburg.de).

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 the 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 determinants include pipetting precision (especially important for small volumes), PCR efficiency, and the instrumentation used for the analysis.
For very low copy numbers (≤ 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 thermal chamber with uniform temperature distribution to provide identical conditions for each PCR
  - A single optical unit for processing all data, to ensure identical measurement conditions
  - High 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, by setting the y-axis display of the fluorescence graph to F2/F1 or F3/F1. This 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), providing an internal "reference" for the displayed data.
  - Dual color evaluation, by using F2/back-F1 or F3/back-F1 (setting of the y-axis display of the fluorescence graph) for normalization.

Note: For further hints on optimization of software settings, see the *LightCycler Operator's Manual*.

- LightCycler ready-to-use reagents, to minimize sample and tube-to-tube variations.

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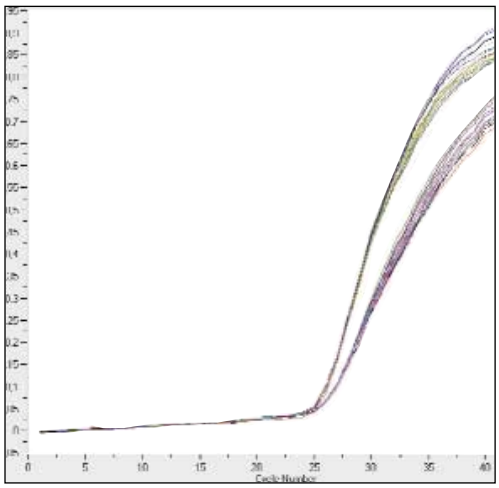
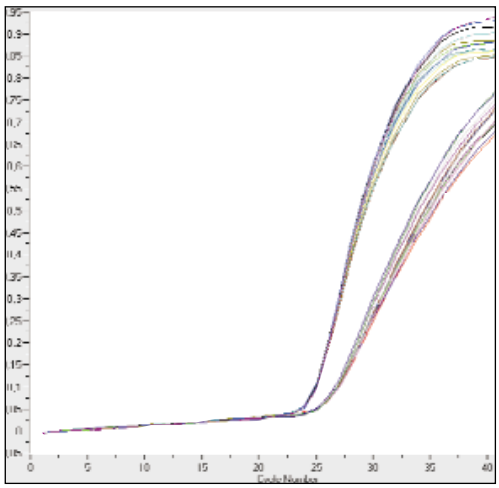
Examples of LightCycler Reproducibility

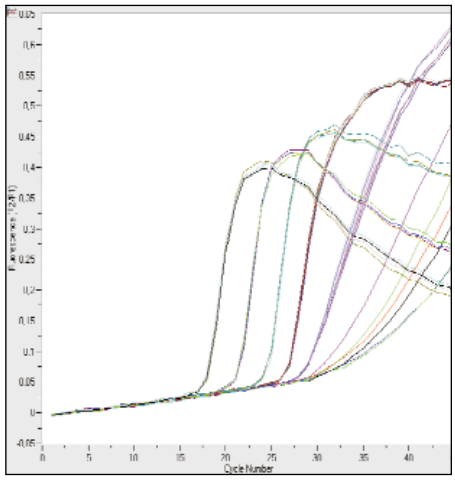


PCR on plasmid DNA, 130 bp
15 x 10,000 copies, 15 x 5,000 copies,
CV crossing point for 5,000 copies 0.3 %
CV crossing point for 10,000 copies 0.2 %



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 6 illustrates the use of a Hybridization Probe experiment to monitor the replication variance for samples with different initial copy numbers.
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 %
6 repl. 10^3 copies: CV 0.2 - 1.2 %
6 repl. 10^2 copies: CV 0.2 - 0.7 %
6 repl. 10^1 copies: CV 1.7 - 2.7 %

Figure 7 shows that reproducibility depends on the starting concentration of the template.
The same amplification system as described in Figure 6 was used, but with a broader range of starting concentrations (from 10^1 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.

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6. Reproducibility, Continued

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 × 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 points data as well 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.
Note: 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 7) and on the efficiency of the PCR. Therefore, if high precision is important, always:
- Optimize PCR conditions (as outlined in Section 4) 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.

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 the respective population parameters. 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 8).
Note: The width of this region increases with imprecision and high fidelity levels.

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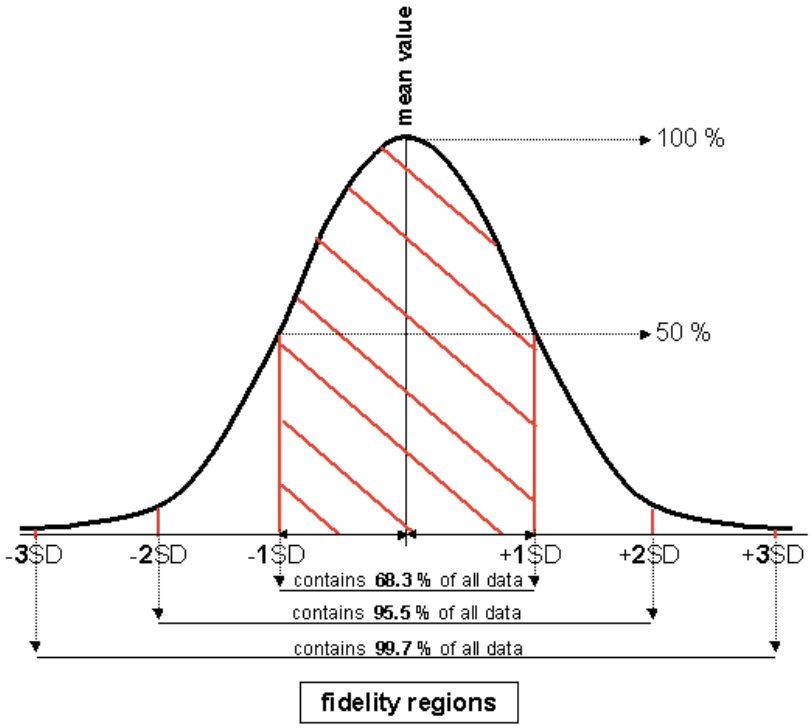
Quantification Fidelity Regions, continued



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

SD = standard deviation

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

Figure 8: Typical Gaussian distribution as an illustration of various fidelity regions. Note that the +/- 2 SD fidelity region contains 95.5% and the +/- 3 SD region contains 99.7% of the data. Thus a value that lies outside these regions is unlikely to originate from the sample with the given mean.

Example: For a LightCycler PCR with 15 replicate determinations of a 5,000 copy sample, assayed with Hybridization Probes (see Figure 6), 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 high precision of the LightCycler instrument.

7. Detection Formats

Quantification with SYBR Green I

SYBR Green I is a dye that apparently binds in the minor groove of dsDNA. When it binds, its fluorescence increases over a hundredfold. This dye binds to any dsDNA; thus specific product, non-specific products and primer-dimers are detected equally well.
For accurate quantification with the SYBR Green I format, the assay must exclude signals derived from non-specific products and/or primer-dimers. There are basically two ways to avoid non-specific detection:

- Optimizing the PCR reaction (see Section 4) will usually reduce primer-dimers and non-specific products to a level that affects only assay of very low copy numbers.
  Example: Use of Hot Start techniques, *e.g.* with the LightCycler - FastStart DNA Master Kits, is extremely helpful in reducing primer-dimers.
- Perform a melting curve determination after each PCR. The melting curve data will contain distinct signals from the desired product and the primer-dimers. Once the melting point of the product has been identified, the LightCycler can be programmed to acquire fluorescence above the melting temperature of the primer-dimers, but below the melting temperature of the product (see Figure 9 below). Note that using this technique does not reduce the primer-dimers in the PCR, but only excludes them from the analysis. There is strong evidence that high amounts of primer-dimers can influence amplification. Therefore, we strongly recommend optimization of the reaction before quantification.

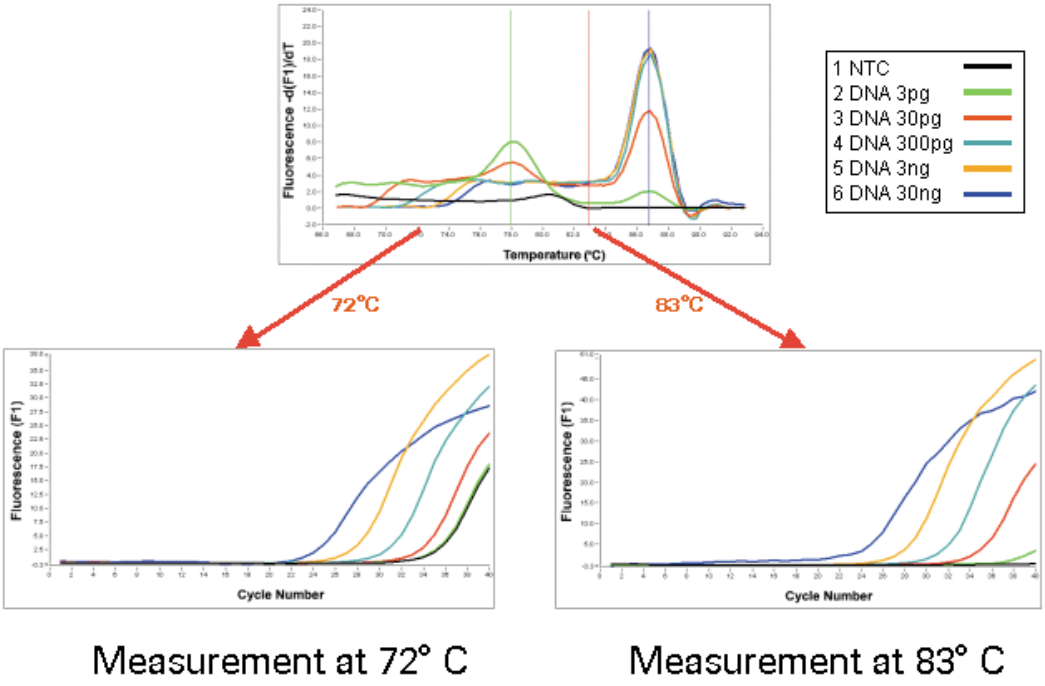


Figure 9 illustrates the acquisition of SYBR Green I signal at an elevated temperature to avoid detection of primer-dimer fluorescence.

Melting curve analysis (upper graph) of β-globin shows that the primer-dimer fraction is already denatured at temperatures above 83°C. Measuring the PCR at 83°C rather than at the elongation temperature (72°C) increases the specificity, because the contribution of primer-dimer signal is minimized.

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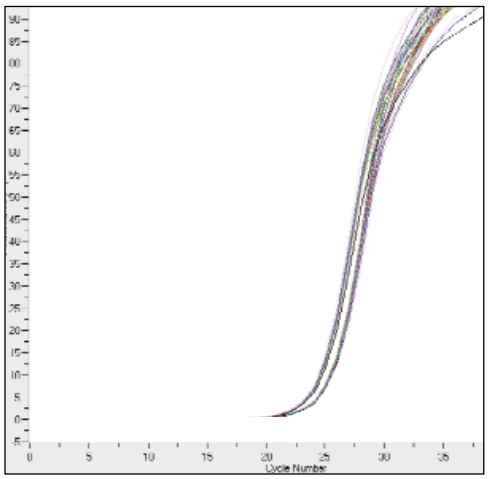
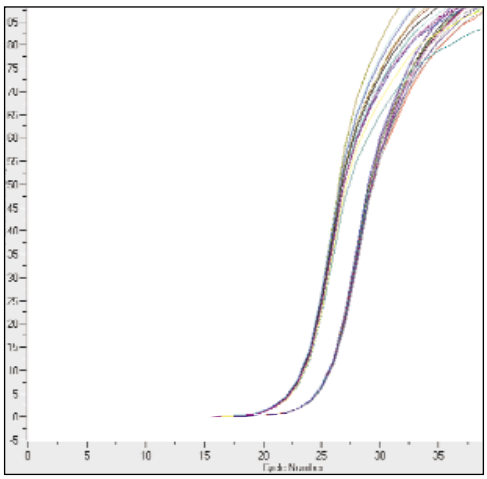
Reproducibility with SYBR Green I



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 0.4 %



PCR on plasmid DNA, 130 bp
15 x 25,000 copies, 15 x 5,000 copies,
CV crossing point for 5,000 copies 0.3 %
CV crossing point for 25,000 copies 0.5 %

Figure 10 illustrates the use of SYBR Green I for differentiation of samples that have twofold and fivefold differences in initial copy numbers.

Result: Again 5,000, 10,000, and 25,000 copies can be distinguished with high confidence, as indicated by the low CVs (approx. 0.4%).

Note: Detection with SYBR Green I dye and Hybridization Probes (see Figure 6) give equally reproducible results. These experiments prove that the reproducibility of the LightCycler system does not depend on the fluorescence format used.

Quantification with Hybridization Probes

For sequence specific analysis, choose the Hybridization Probe format. In this format, two probes are designed so they can only hybridize side-by-side on the PCR product, thus inducing a fluorescence resonance energy transfer (FRET). The resulting fluorescence signal can then be monitored in the LightCycler.

Note: Optimization of the amplification reaction is again important, since only high performance PCRs guarantee high efficiency and sensitivity.

8. Appendix

Further Readings

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Acknowledgment

This Technical Note was supported by Dr. Udo Reischl, Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany. Dr. Reischl generously contributed his expertise and his LightCycler data for the quantification of *Bordetella pertussis* (see Section 5, Application).

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