Roche Applied Science
Technical Note No. LC 13/2001

LightCycler

Relative Quantification

Purpose of this Note

The LightCycler provides great flexibility especially to the user interested in quantitative PCR. With the use of relative quantification methods the result is expressed as a relative ratio of the target of interest, to a reference target measured in the same sample material.

This Technical Note describes various approaches for relative quantification, gives information for the selection of suitable housekeeping genes, and offers some recommendations for PCR optimization to achieve successful quantification results with the LightCycler instrument. In addition the new LightCycler software for relative quantification is introduced and some mathematical background is provided for the calculation of efficiency corrected relative quantification values.

Note: For a comparison of this method to other quantification methods that can be performed in the LightCycler, see Technical Note No. LC 10/2000: *Overview of LightCycler Quantification Methods.*

Table of Contents

For Information on this Topic	See Page
Overview of Quantitative PCR Methods	2
Principle of Relative Quantification	4
• Introduction	4
• Selection of Suitable Housekeeping Genes	6
Relative Quantification with External Standards	8
• Concept and Requirements	8
• Application	10
Calibrator Normalized Relative Quantification	12
• Principle and Calculation	12
• The LightCycler Relative Quantification Software	16
• Applications	20
Optimizing PCR Conditions	27
Appendix	28

1. Overview of Quantitative PCR Methods

Introduction

Over the last years, PCR has become an essential technique for nucleic acid quantification due to its unsurpassable sensitivity and high dynamic range.
By the innovative introduction of real-time PCR detection, kinetic quantification was made possible. This technique allows data analysis in the phase of constant amplification efficiency (log-linear phase) and provides accurate results for external, internal or relative quantification methods.

Typical PCR

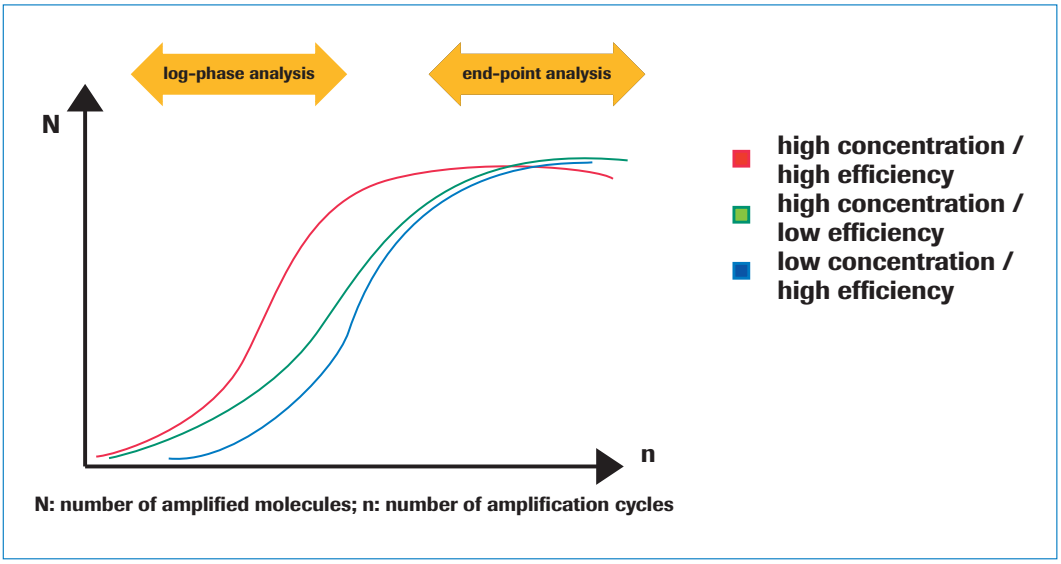
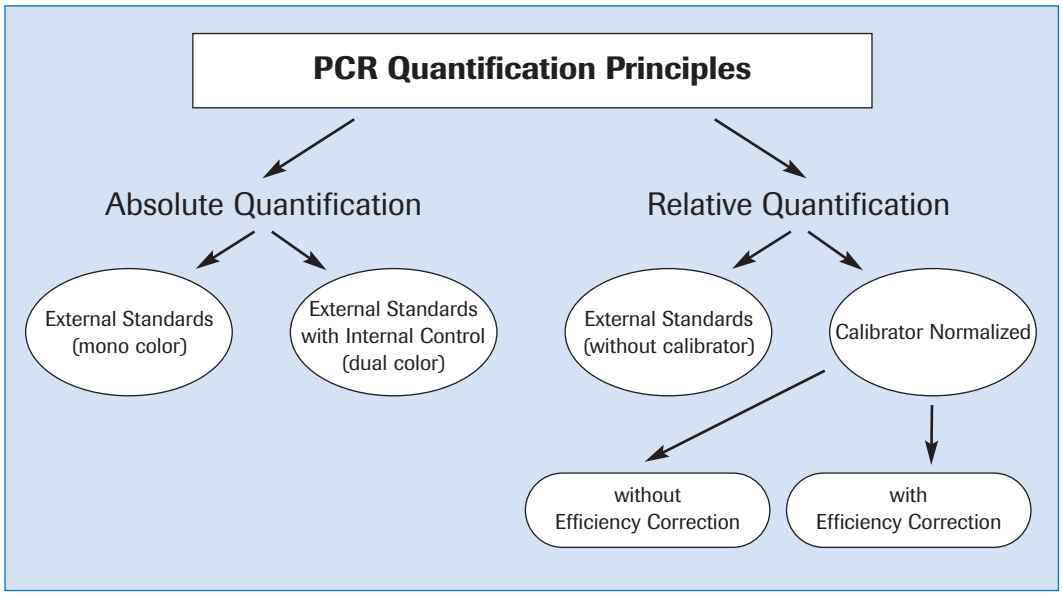


Figure 1: PCR and the Problem of Quantification
PCR amplification is template concentration dependent, but reactions with low starting copy number can reach the same plateau as reactions that started with higher template concentrations and/or a different PCR efficiency.
In contrast to analysis during end-point-PCR, analysis in the log-linear phase produces data that are much more accurate. Since amplification efficiency is constant, the amount of starting material can be determined very precisely.
Real-time PCR monitoring on the LightCycler, offers a convenient way to identify and measure these log-linear cycles.
For details regarding software settings for data analysis, see the *LightCycler Operator´s Manual.*

Continued on next page

Different Methods

Quantitative real-time PCR generally can be subdivided into two basic classes of concepts: The absolute quantification and the relative quantification.



Absolute Quantification

- **With external standards:** Serial dilutions of an external standard, with predefined known concentration, are used to create a standard curve. From this standard curve absolute values for the target concentration of unknown samples are determined.
  Note: See Technical Note No. LC11/2000

- **With external standards and an internal control:** This method also uses external standards and produces absolute values for unknown samples. In addition the amplification reaction is controlled, for e.g., PCR inhibition, due to co-amplification of an exogenous control target spiked into each capillary.
  Note: For details please refer to Technical Note No. LC12/2000.

Relative Quantification

- **With external standards:**
  The target concentration is expressed in relation to the concentration of a reference (housekeeping) gene. A standard curve is used to obtain the concentration of the target and the reference gene.

- **Calibrator Normalized:**
  The quantity of a target and a reference gene is a function of the PCR efficieny and the sample crossing point and does not require a standard curve in each LC analysis run for its determination. Results are expressed as the target/reference ratio of each sample normalized by the target/reference ratio of the calibrator. Thus in calibrator normalized relative quantification assays the accuracy is only influenced by different PCR efficiencies of target and reference. PCR efficiency differences can be corrected to achieve exact results or for an approximate calculation both PCR efficiencies are set to 2.
  Note: For more details please refer to chapter 4 of this Technical Note.

2. Principle of Relative Quantification

2.1 Introduction

Concept

As described in the previous chapter, different quantification methods have been developed, and according to the requirements of a particular application, the most suitable method needs to be selected.

- The easiest way to obtain a quantitative value for an unknown concentration of a target is to use external standards. A prerequisite for this type of absolute quantification is that the PCR efficiency of the standards is identical with the PCR efficiency of the samples. The advantage of this method is the high dynamic range ($10-10^{10}$ copies) and the result is directly calculated by the LightCycler analysis software. This method does not detect PCR inhibitors present in the sample material, but this limitation can be compensated by the use of an internal control. With this variation a qualitative control for factors influencing the PCR is implemented. However, the dynamic range is limited by the required dual color reaction set up. Typical application fields for both absolute quantification methods are usually found in virology and bacteriology.

- In many experimental studies with a quantitative approach an absolute value for the sample under investigation is not relevant. Various traditional techniques (e.g., Northern Blotting) express the target amount of an unknown sample relative to another gene transcript, the so-called housekeeping gene, which is assumed to be constant. The identical concept can be achieved with the LightCycler: The target concentration in each sample is calculated relative to this non-regulated reference and the result is expressed as a target/reference ratio. This method which uses an endogenous control as a reference has the advantage that it corrects for factors influencing the PCR. This type of quantitative analysis is a common and powerful tool for a highly sensitive determination of e.g., RNA expression. Reverse transcription PCR (RT-PCR) of gene expression is often performed using housekeeping genes as reference against which the expression level of a gene under investigation can be normalized. For study on the DNA level, e.g., determination of gene dosage values, single copy genes as non-regulated references in the same sample material are recommended. Preferentially a single copy gene located on the identical chromosome as the target sequence of interest is selected.

RNA Level: mRNA Expression Studies	DNA Level: Gene Dosage Determination
↓	↓
$\frac{\text{Amount of Target}}{\text{Amount of Housekeeping Gene}}$	$\frac{\text{Amount of Target}}{\text{Amount of Single Copy Gene}}$

Continued on next page

Normalization to a Reference Gene

The concentration of the target in each sample is divided by the concentration of a reference in the same sample, thereby correcting the sample for differences in quality and quantity caused by:

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

Definitions

For maximum clarity in this Technical Note, we will use the following definitions when discussing relative quantification

Term	Definition
Sample	Material of interest (tissue, cells, blood etc.).
Target Nucleic Acid	Nucleic acid of interest (specific RNA or DNA sequence).
Reference Nucleic Acid • Housekeeping Gene • Single Copy Gene	A nucleic acid that is found at constant copy number in all samples (= endogenous control). The reference gene is used for normalization of sample to sample differences. For mRNA quantification. A gene that is expressed constitutively on an identical level in all samples to be analyzed. For gene dosage quantification at DNA level. A gene that is found in constant copy numbers in all samples.
Calibrator	A sample that is used for the normalization of final results (provided as purified NA in parameter-specific kits). The target/reference ratios of all samples are divided by the target/reference ratio of the calibrator. Therefore, a constant ratio of target to reference for the calibrator is required.
Relative Standards	Dilution series of target and reference nucleic acids that are used to determine the fit coefficients of the relative standard curve (efficiencies).
Fit Coefficients	Parameter values that describe mathematically the fitted "relative standard" curve. Coefficients are stored as *.cof files (lot-specific) and used for subsequent efficiency-corrected data analysis.

2.2 Selection of a Suitable Housekeeping Gene

General

The housekeeping genes are a large group of genes that code for proteins whose activities are essential for the maintenance of cell function. Detection of housekeeping gene mRNA is routinely used to control several variables that may affect RT-PCR. These endogenous controls are present in each experimental sample and therefore serve as perfect candidates for the normalization of the final result.

Note: For details please refer to Technical Note No. LC 15/2002

Prerequisites

Special care should be taken when choosing a housekeeping gene for relative quantification to normalize target gene expression. It is generally assumed that housekeeping genes which are essential for cell viability are constitutively expressed and the mRNA levels are similar in different cell types. However, the expression level of housekeeping genes may vary depending on the cell type analyzed or may show a differentiated expression pattern in various tissues (see example of figure 2).

Another point to be considered when choosing a reference gene for RT-PCR is that DNA may be co-amplified, even when the primers are carefully designed to bind to exon/intron boundaries to avoid amplification of contaminating traces of DNA. Processed intron-lacking pseudogenes give rise to the same or similar amplification products.

The copy numbers of the individual housekeeping gene chosen for relative quantification should be in a similar range than that of the target gene to make comparative quantification possible. In summary a suitable housekeeping gene needs to fulfill the following prerequisites:

- **No regulation of expression level in the system analysed**
  The housekeeping gene used for a certain approach should not be regulated in the type of tissue or sample material under investigation; a stable and constant reference is required for an accurate normalization. Similar tests as shown in the example below should be considered for any application with relative quantification.

- **RNA-specific detection**
  A pseudogene free amplification (specific primers) and/or detection specific for active target (specific Hybridization Probes) should be selected to avoid traces of DNA contributing to the result.

- **Similar expression level compared to the target analysed**

Continued on next page

2.2 Selection of a Suitable Housekeeping Gene, Cont.

Example

Features of human porphobilinogen deaminase (h-PBGD): PBGD is encoded by two distinct mRNA species expressed in a tissue-specific manner from a single gene. One transcript is only expressed in erythroid tissues, while the housekeeping transcript used in the Roche kit is expressed in all tissues.
PBGD belongs to the low abundance class of mRNA without pseudogenes known so far. The expression level of PBGD is relatively stable in a broad range of tissue.

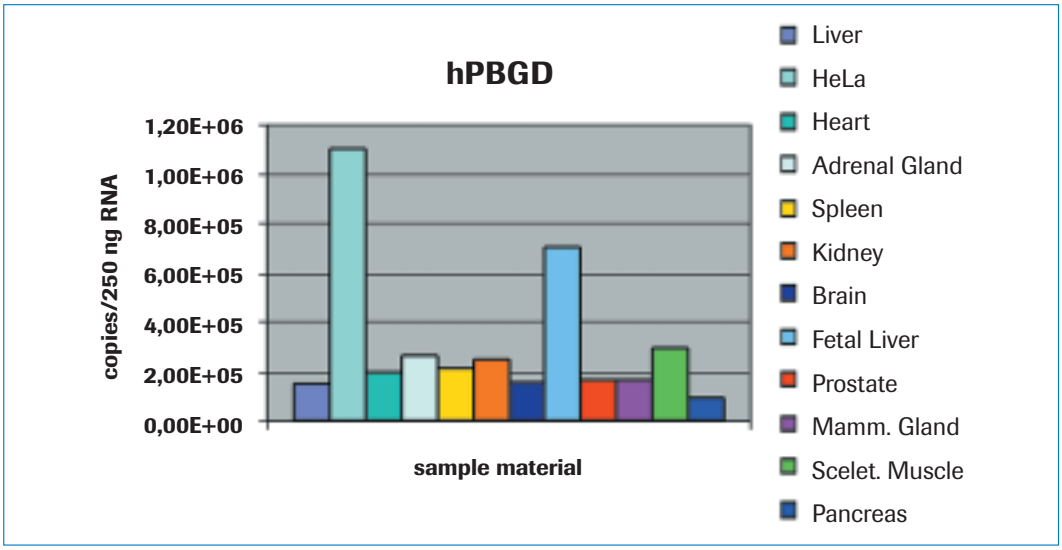


Figure 2 shows the various expression levels (measured in copy numbers per 250ng of total RNA) of human PBGD in different tissue samples and HeLa cells.

LightCycler – Housekeeping Gene Sets

Roche offers five different LightCycler Housekeeping Gene sets to provide suitable reference genes for an individual relative quantification approach. Detection mixes (primer/Hybridization Probes) and in vitro transcripts for the following housekeeping genes will be available:

With a low expression level
- **h-PBGD:** Phorphobilinogen deaminase
- **h-HPRT:** Hypoxanthine phosphoribosyltransferase

With a medium expression level
- **h-b2M:** β_2Microglobulin
- **h-G6PDH:** Glucose-6-phoshate dehydrogenase
- **h-ALAS:** 5-aminolevulinate-synthase

3. Relative Quantification with External Standards

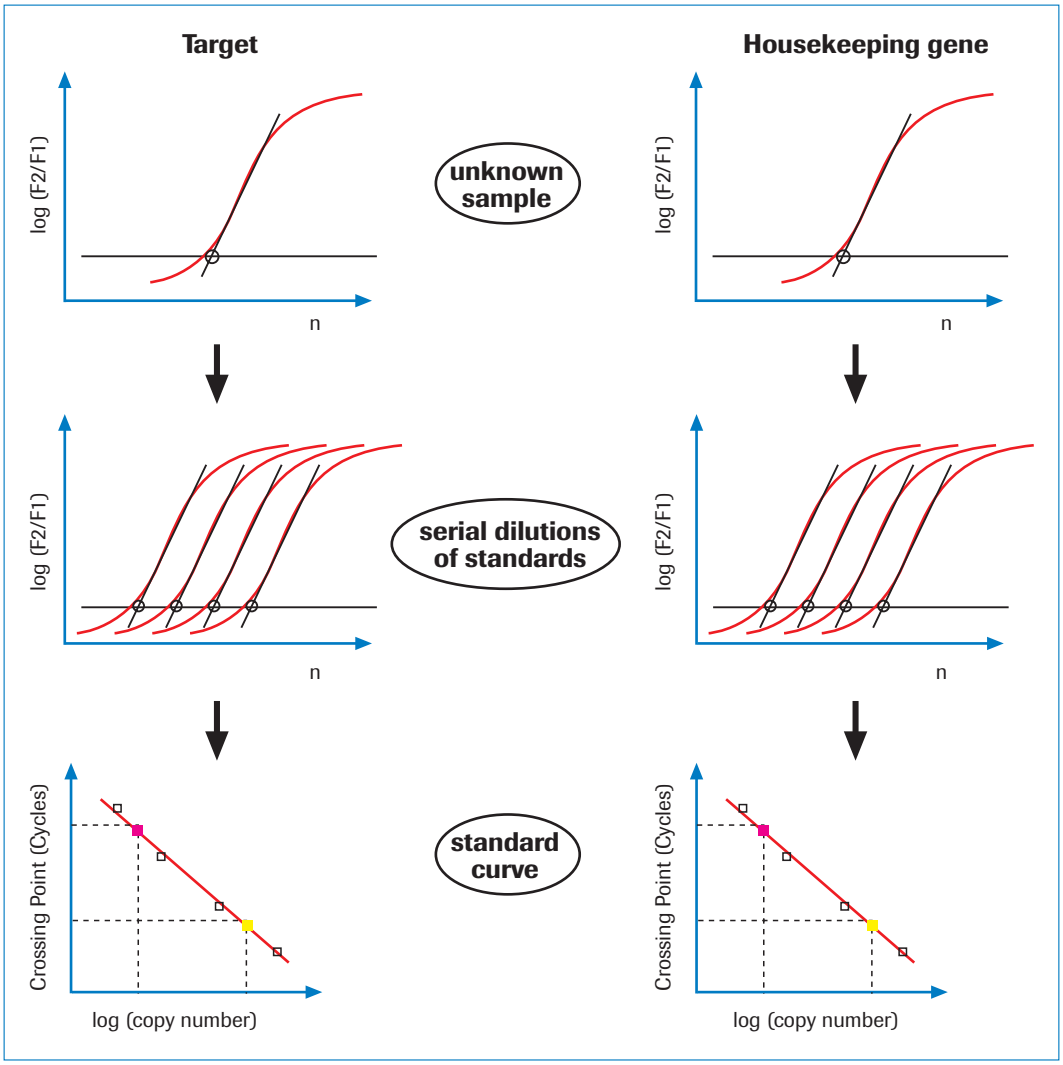
Introduction

This relative quantification approach first calculates the absolute value of the target under investigation and the value of the housekeeping gene in the same sample. To obtain the concentration of these two parameters an external standard curve is used. Then the absolute concentration of the target is divided by the absolute concentration of the housekeeping gene. The resulting target/reference ratio expresses the amount of target now normalized to the level of an endogenous reference gene within each unknown sample.

This method is probably the most common used for quantitative PCR in biological research for all applications where the determination of relative changes are important, e.g., comparison of transcript abundance in different tissue samples.

General Concept

Figure 3 illustrates the general quantification concept for the determination of the concentration of a target (T) and a housekeeping (R) gene using separate external standards, one for the target and one for the housekeeping gene.
The final result is expressed as the ratio T conc. : R conc.



Continued on next page

3. Relative Quantification with External Standards, Continued

General Concept (continued)

With this approach, serial dilutions of external standards with known copy number, one for the target PCR and one for the reference gene, are performed to generate standard curves for the two parameters. These respective curves are used for quantification of the target and the housekeeping gene in each sample. For each unknown the result is expressed as a relative ratio of the target divided by the housekeeping gene, thus normalizing each sample (see page 5).
Data analysis for the concentration of target and reference gene is automatically achieved with the LightCycler software. But the calculation of the ratio target/reference has to be done manually or using a spread sheet calculation programm such as MS Excel.

Roche Kit Concept

For all Roche LightCycler Quantification kits using the external standard method only one common standard curve needs to be included within each LightCycler run. The standard curve (ready-to-use standard dilutions are provided in the kits) is suitable to determine the concentration of both, the target and the housekeeping gene. This special kit design enables more unknown samples to be run within one rotor.

Requirements

The standard curve depends on the amplification efficiency of the standard samples. For accurate quantification with only one common standard curve per LightCycler analysis run, the amplification efficiencies of the target, the housekeeping gene, and the standards should be identical.

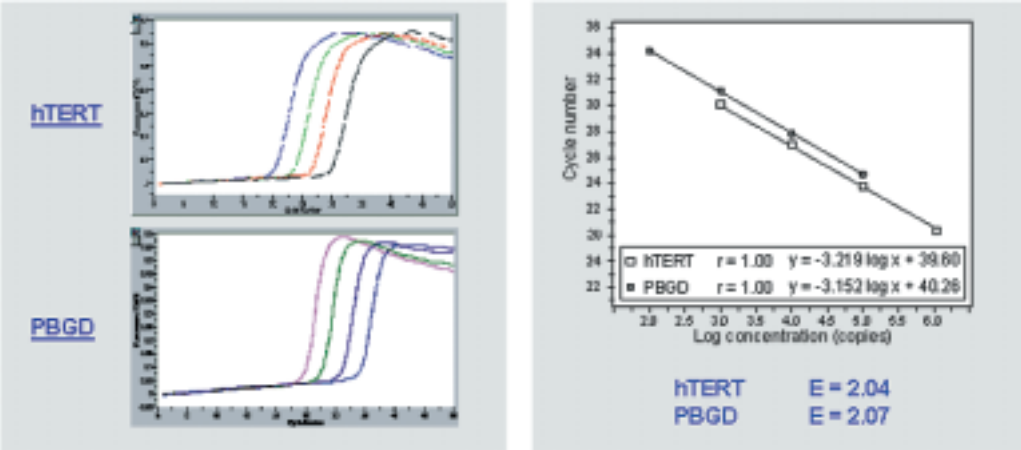
Note: For detailed information and guidelines on how to design external standards refer to Technical Note No. LC 11/2000.

Continued on next page

3. Relative Quantification with External Standards, Continued

Application Example

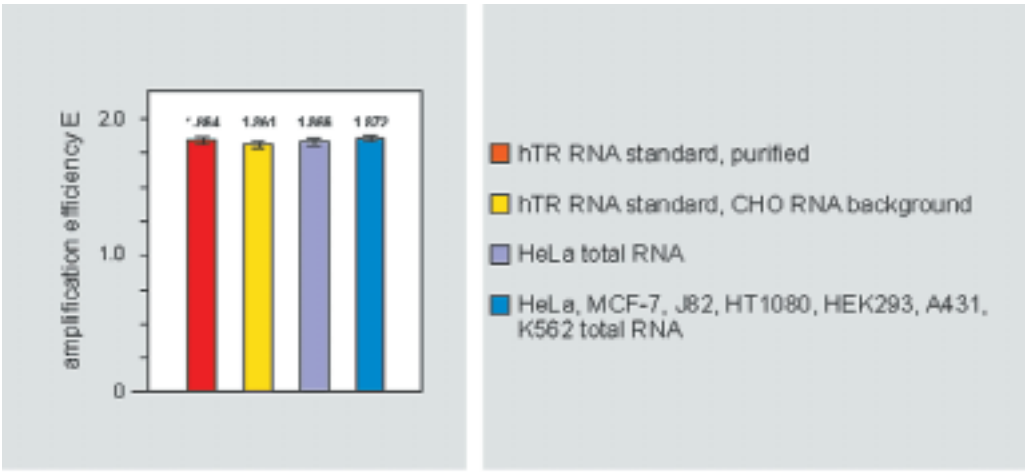
LightCycler *Telo TAGGG* Quantification Kit



Amplification efficiencies of hTERT and PBGD amplification were determined by serial 10-fold dilutions of purified hTERT RNA standard and total RNA from K562 cells respectively.

Figure 4: Experimental determination of target (hTERT) and reference (PBGD) efficiency by serial dilutions of standard RNA samples.
The resulting standard curves above show identical slopes, efficiencies ($E = 10^{-1/slope}$) for hTERT and PBGD. As long as amplification efficiencies differ not more than 0.05, one common standard curve can be used for the relative quantification of unknowns.

The highest sample purity is also a prerequisite to achieve identical PCR efficiencies in the samples and in the standards.



Serial dilutions of purified hTR RNA standard, hTR RNA standard spiked into 200 ng CHO RNA background, and 200 ng purified RNA from different cell lines were analysed for hTR expression in 5-fold replicates.

Figure 5 proves that the amplification efficiency is independent of the sample matrix, in case no inhibitory agents are present

Continued on next page

3. Relative Quantification with External Standards, Continued

Application Example, (continued)

In the LightCycler analysis software the concentrations of the target and the reference genes are listed. For the final calculation of the relative ratio for each sample programms such as MS Excel are recommended.

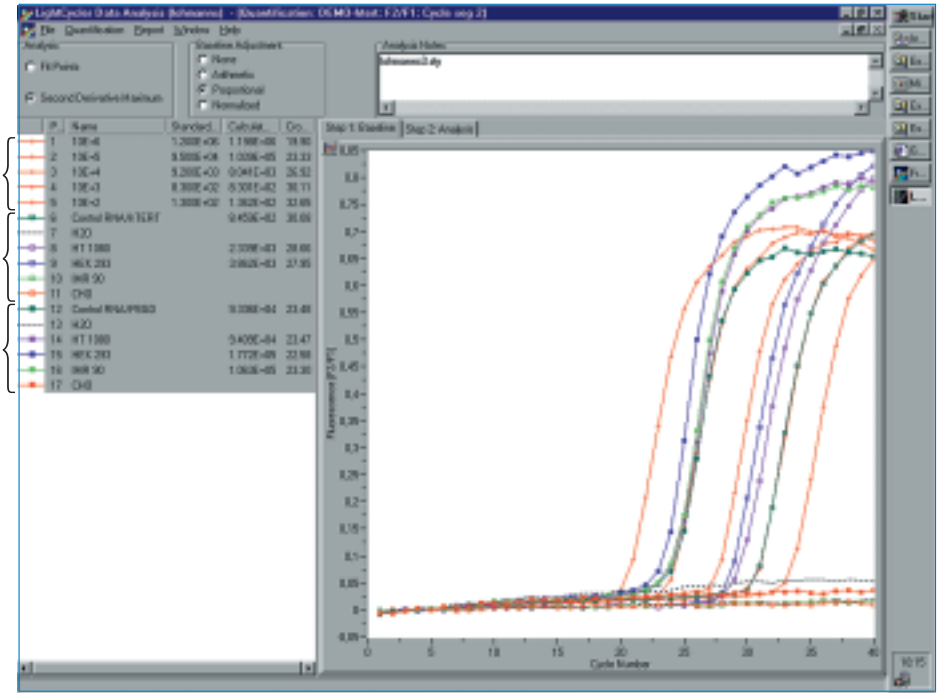
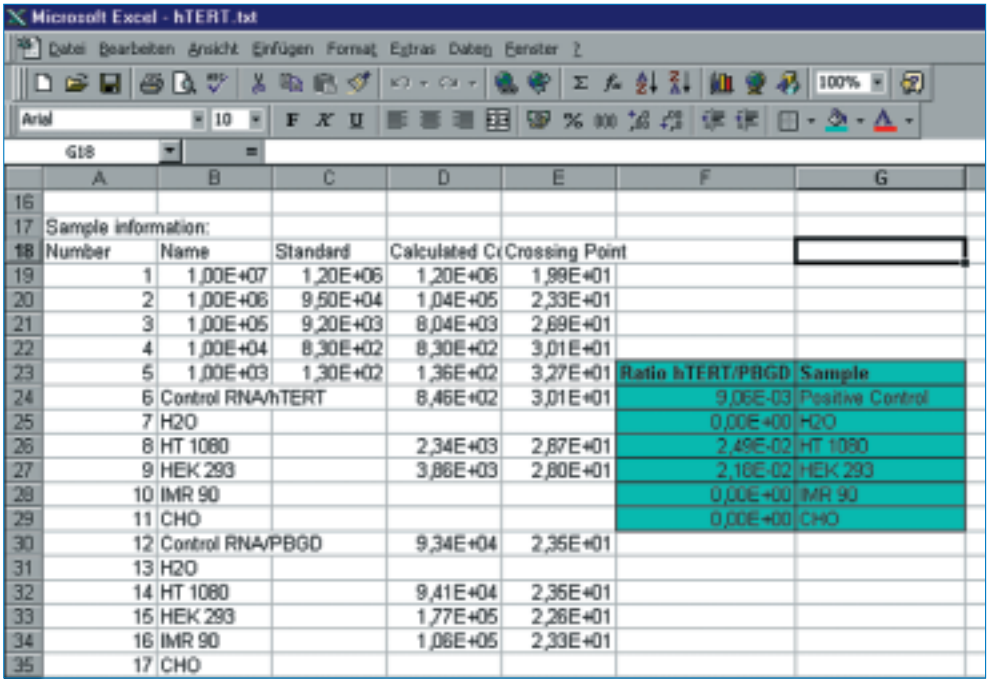


Figure 6 shows a typical quantification analysis screen on the LightCycler. Prediluted standards (*in-vitro* transcribed hTERT RNA) are used to determine the concentration of the target gene (hTERT) as well as the reference gene (PBGD) for five different samples and one positive control.



Microsoft Excel - hTERT.txt

	A	B	C	D	E	F	G
16							
17	Sample information:						
18	Number	Name	Standard	Calculated C(	Crossing Point		
19	1	1,00E+07	1,20E+06	1,20E+06	1,99E+01		
20	2	1,00E+06	9,50E+04	1,04E+05	2,33E+01		
21	3	1,00E+05	9,20E+03	8,04E+03	2,69E+01		
22	4	1,00E+04	8,30E+02	8,30E+02	3,01E+01		
23	5	1,00E+03	1,30E+02	1,36E+02	3,27E+01	Ratio hTERT/PBGD	Sample
24	6	Control RNA/hTERT		8,46E+02	3,01E+01	9,06E-03	Positive Control
25	7	H2O				0,00E+00	H2O
26	8	HT 1080		2,34E+03	2,87E+01	2,49E-02	HT 1080
27	9	HEK 293		3,86E+03	2,80E+01	2,10E-02	HEK 293
28	10	IMR 90				0,00E+00	IMR 90
29	11	CHO				0,00E+00	CHO
30	12	Control RNA/PBGD		9,34E+04	2,35E+01		
31	13	H2O					
32	14	HT 1080		9,41E+04	2,35E+01		
33	15	HEK 293		1,77E+05	2,26E+01		
34	16	IMR 90		1,06E+05	2,33E+01		
35	17	CHO					

Figure 7: Excel-sheet with calculation of relative ratios (hTERT/PBGD) for all six samples analysed in the example above. The standard curve data were exported with the help of the LightCycler software and the respective .txt file transfered to the calculation programm Excel.

4. Calibrator Normalizedd Relative Quantification

Overview

PCR amplification is described by the basic equation $\mathbf{N = N_o \times E^{Cp}}$.

N: number of molecules at a certain cycle
N_0: initial number of molecules
E: amplification efficiency
Cp: cycle number at detection threshold (crossing point)

The final ratio resulting from the calibrator normalized relative quantification is only a function of PCR efficiency, and of the determined crossing points. It does not require the knowledge of absolute copy numbers at the detection threshold.

In this quantification method the result is expressed as the target/reference ratio of each sample divided by the target/reference ratio of a so-called calibrator. The calculation of this calibrator normalized ratio does **not** require a standard curve in each LightCycler analysis run (see next page 13). The calibrator is typically a positive sample with a stable ratio of target to reference and is used to normalize all samples within one run, but in addition provides a constant calibration point between several LightCycler runs.

4.1 Principle

Data Acquisition

The relative amount of a target gene and a reference gene is determined for each sample and one calibrator, integrated in each LightCycler run.
For the calculation of the final result only the Cp-values obtained by the LightCycler analysis software are required.

Normalization to a Calibrator

1. The relative ratio of target to reference for each sample and for the calibrator is calculated in the first step. This corrects for sample to sample variations caused by differences in the initial quality and quantity of the nucleic acid – the basic concept of each relative quantification (see page 5).

2. The target/reference ratio of each sample is then divided by the target/reference ratio of the calibrator. This second step normalizes for different detection sensitivities of target and reference amplicons caused by different probe annealing, quantum yields of dye batches or FRET efficiency. Thus the normalization to a calibrator provides a constant calibrator point between PCR runs.

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

Continued on next page

Calculation

The calculation of the relative amount of any target or reference gene is based on the crossing point of a sample and the efficiency of the PCR reaction.
The calculations are made according to the general PCR equations:

$$N_T = N_{To} \times E_T^{CpT}$$
$$N_R = N_{Ro} \times E_R^{CpR}$$

At a certain fluorescence detection level (e.g. at Cp determined by the 2nd derivative maximum of each curve) the amplicon numbers of target and housekeeping gene are very unlikely to be identical due to differences in probe annealing, channel sensitivity, quantum yields/extinction coefficient of dye batches, and FRET efficiency:

$N_T \neq N_R \Rightarrow N_T/N_R \neq 1$, but constant for each sample and the calibrator.

To calculate the calibrator normalized relative ratio the relative amount target/reference of each sample $[N_{T(S)}/N_{R(S)}]$ is divided through the ratio target/reference of the calibrator $[N_{T(C)}/N_{R(C)}]$:

$$\text{Normalized Ratio} = \frac{\frac{N_{T(S)}}{N_{R(S)}}}{\frac{N_{T(C)}}{N_{R(C)}}} = \frac{\frac{N_{To(S)} \times E_T^{CpT(S)}}{N_{Ro(S)} \times E_R^{CpR(S)}}}{\frac{N_{To(C)} \times E_T^{CpT(C)}}{N_{Ro(C)} \times E_R^{CpR(C)}}} = \frac{K_{Sample}}{K_{Calibrator}} = 1 \quad (\text{for } K_{sample} = K_{calibrator}) \Rightarrow$$

$$\text{Normalized Ratio} = \frac{\frac{N_{To(S)}}{N_{Ro(S)}}}{\frac{N_{To(C)}}{N_{Ro(C)}}} = \frac{\frac{E_T^{CpT(C)}}{E_R^{CpR(C)}}}{\frac{E_T^{CpT(S)}}{E_R^{CpR(S)}}} = \frac{E_T^{CpT(C)}}{E_R^{CpR(C)}} \times \frac{E_R^{CpR(S)}}{E_T^{CpT(S)}} = E_T^{CpT(C) - CpT(S)} \times E_R^{CpR(S)-CpR(C)}$$

Abbreviations:

N_T/N_R	: Number of target/reference molecules at detection threshold Cp
N_{To} / N_{Ro}	: Initial number of target/reference molecules
C_{pT}/C_{pR}	: Cycle number at target/reference detection threshold (crossing point)
E_T/E_R	: Efficiency of target/reference amplification
T	: Target
R	: Reference
S	: Unknown sample
C	: Calibrator
K	: Constant

Basic prerequisite: Efficiencies of both target and housekeeping PCR do not vary from sample to sample.

Continued on next page

Importance of Efficiency

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

$N = No \times E^{Cp}$: According to this basic PCR equation, the generated copy number (N) at a certain cycle is a function of the initial copy number (N_0), the PCR efficiency (E) and the cycle number (Cp). In brief, the measured crossing point (Cp) is the cycle at which PCR amplification begins its exponential phase and is considered, the point that is most reliably proportional to the initial concentration. The efficiency of the PCR describes the kinetics during the reaction. Primers as well as Hybridization Probes for both parameter-specific components (target gene and reference gene) have individual PCR efficiencies, that are taken into account during the overall quantification. An **efficiency-corrected** calculation is also performed by the LightCycler Relative Quantification Software. This procedure allows maximum reproducibility and controls for factors influencing quantification.

- To achieve exact results, PCR efficiency differences can be easily corrected with the features of the LightCycler Relative Quantification Software by the use of so-called coefficient files (*.cof).
- An approximate calculation of data analysis can be performed without efficiency correction by setting both PCR efficiencies to 2.

Influence of Error

In most cases, a PCR reaction is not amplifying with the maximum efficiency E = 2, due to various factors, e.g. high GC-content. If the calculation of the final result is based on the equation $N = No \times \mathbf{2}^{Cp}$, and does not use the real PCR efficiency, the resulting error gets significantly higher with each cycle.

Detection Cycle (n) PCR efficiency (E)	10	20	30
2.00	–	–	–
1.97	16 %	35 %	57 %
1.95	29 %	66 %	113 %
1.90	67 %	179 %	365 %
1.80	187 %	722 %	2260 %
1.70	408 %	2480 %	13000 %

Error calculation: $(2^n/E^n - 1) \times 100$

Figure 8 lists the mathematical calculation of the expected systematical error caused by PCR efficiencies different from 2.00. E.g., when the difference in PCR efficiency is 0.05 between target and reference gene, a more than 2-fold difference in the final result will be calculated after 30 cycles. Depending on the required accuracy for each application the option for an efficiency correction should be considered.

Continued on next page

Correction for Differences in Efficiency

The efficiency-corrected quantification performed automatically by the Relative Quantification Software is based on relative standard curves describing the PCR efficiencies of the target and the reference gene. These standard curves are determined once and stored as a coefficient (*.cof) file which can be used for each analysis.
Note: For more details refer to page 18 – 19.

Requirements for Using the Calibrator Normalized Quantification

The following information is needed to perform a calibrator normalized relative quantification assay:

Cp-value of target and reference gene for samples and calibrator
⇒ obtained by the LightCycler quantification analysis software

E: PCR-efficiency of target and reference gene
⇒ obtained by a LightCycler run with relative standards
⇒ for Roche parameter-specific kits from pack inserts or Internet
⇒ without efficiency-correction (E = 2)

Scope for Error Corrections

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

Calibrator:
- Compensates for constant differences between detection of target and reference gene (e.g. Probe annealing, FRET efficiency)
- Provides a constant calibration point between PCR runs

Efficiency differences between target and reference gene caused by:
- Primer annealing
- Efficiency of cDNA synthesis
- Amplicon sequence specificities, GC-content, fragment length

Continued on next page

4.2 LightCycler Relative Quantification Software

Features

The LightCycler Relative Quantification Software is an additional module, that significantly expands the range of possible applications of the LightCycler system.

- The software is designed for direct download of LightCycler data files.
- The software provides efficiency-corrected, calibrator normalized relative quantification results.
- No manual calculation is necessary.
- The sample setup in the LightCycler rotor has to follow a predefined loading scheme (several optional loading schemes possible: mono color, dual color, single/duplicate/triplicate measurement).
- For data analysis LightCycler version 3.3 or higher must be used.
- The software is released together with parameter-specific kits based on relative quantification.

Note: For detailed information regarding the LightCycler Relative Quantification Software (Cat. No.: 3 158 527), refer to the Manual.

Data Analysis

As demonstrated with the formula mentioned above, the relative quantity of a target gene is a function of PCR efficiency and the crossing point. For the calculation of a relative target amount only the respective Cp values of the target and the reference gene for each sample and a calibrator need to be determined with the LightCycler analysis software.

Perform a LightCycler run with calibrator and samples (all defined as "unknowns" in the sample edit sheet) and analyse the data as described in the *LightCycler Operator's Manual.*
Use the Second Derivative Maximum Method with the Arithmetic (default) baseline adjustment for determination of the crossing point. It is recommended that the crossing point of each sample is checked individually to correspond to the appropriate curve. Once the crossing points have been determined, export the data into a *.txt file.

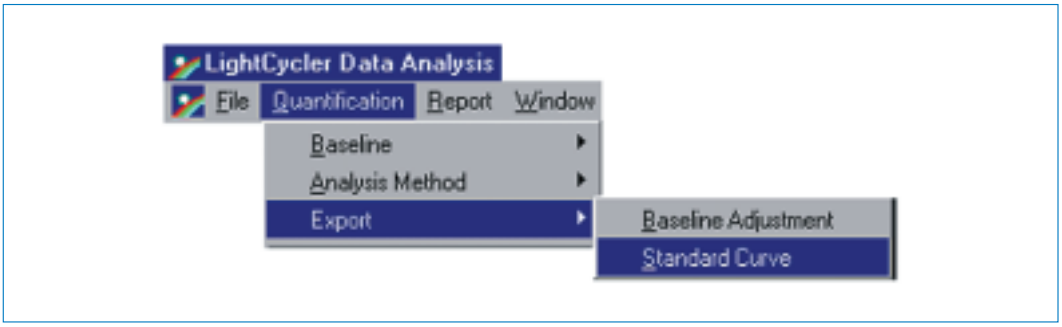


Figure 9 shows the path in the LightCycler quantification analysis software to create a *.txt file of the respective Cp value for export.

Continued on next page

4.2 LightCycler Relative Quantification Software,

Continued

Data Analysis
(continued)

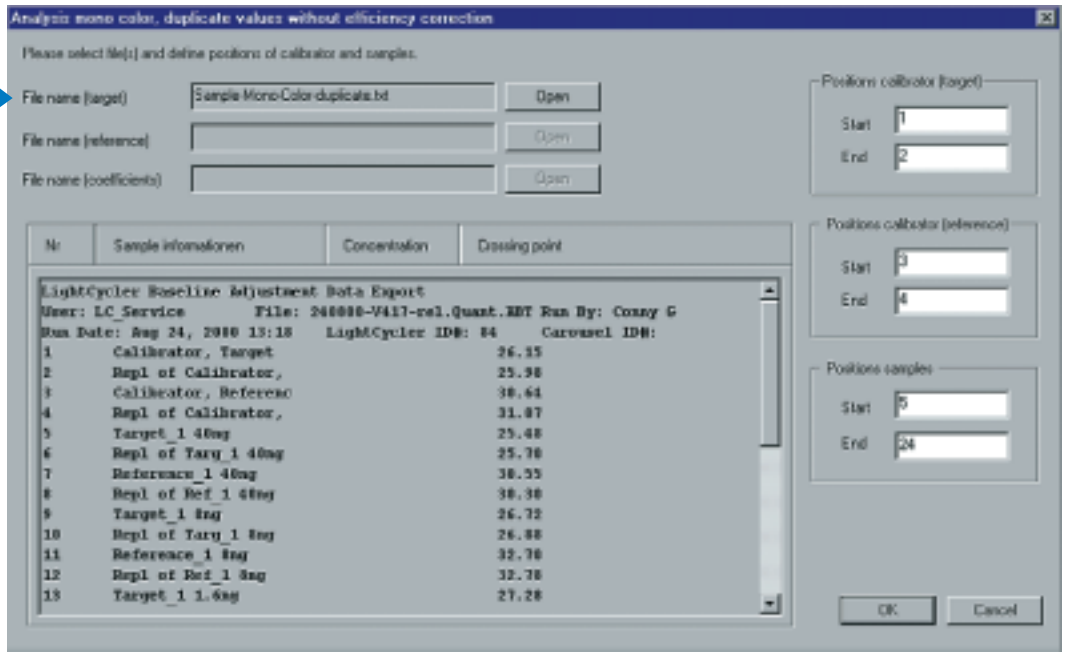


Figure 10: Open the exported *.txt file with the LightCycler Relative Quantification Software, and enter the calibrator (target/reference) and sample rotor positions of the respective LightCycler run.

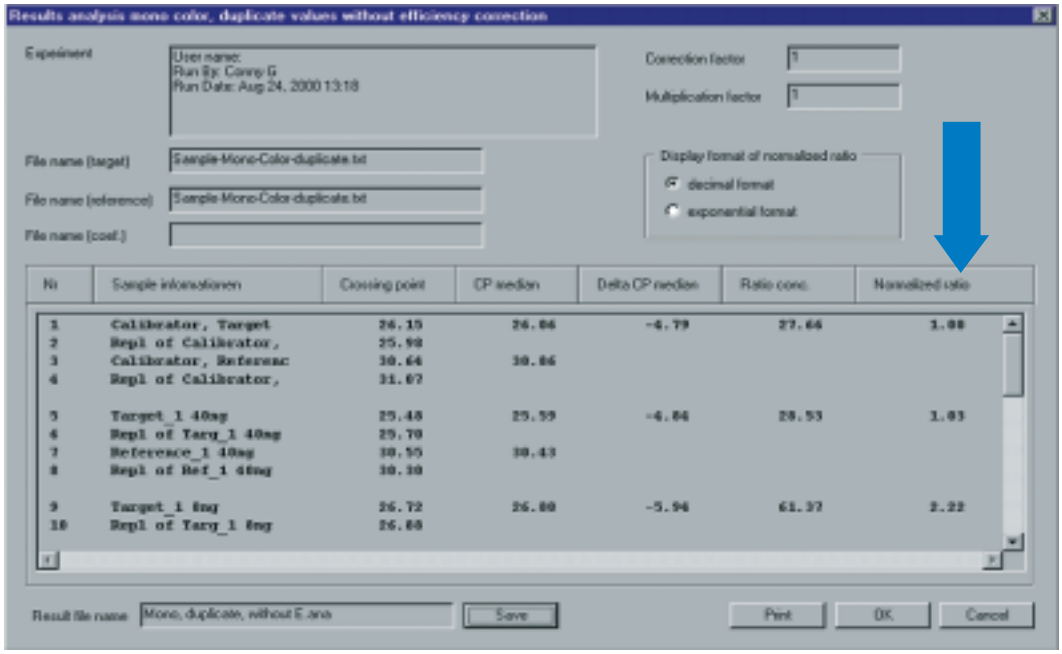


Figure 11: After verifying the data with "OK" the software will automatically provide the normalized ratio as final result.

Continued on next page

4.2 LightCycler Relative Quantification Software,

Continued

Data Analysis
(continued)

Figure 12 shows a screen shot of the front page of the Relative Quantification Software. In the analysis mode one of the two options, with/without efficiency correction, can be selected.

Without Efficiency Correction

The option “without efficiency correction” uses the assumption that the efficiency of the target and reference gene is identical and equals 2. The result is calculated according to the formula $N = N_0 \times 2^{Cp}$.

With Efficiency Correction

To achieve results with maximum accuracy different PCR efficiencies can be corrected by selecting the "use coefficient file" for data analysis. For this option a so-called coefficient file, which defines the efficiency of target and reference, needs to be created. The Relative Quantification Software allows two possibilities to define such a *.cof file:

- For Roche parameter-specific LightCycler kits several coeffient factors provided in the pack inserts can be entered manually or the lot-specific *.cof files can be downloaded directly from the Roche internet page. *(http://biochem.roche.com/lightcycler/lc_support/rqc_download.htm)*
- For user-specific assays the efficiencies for the target and the reference gene have to be determined in advance as precise as possible. Therefore a separate LightCycler run with relative standard dilutions for the target and the reference need to be performed. It is highly recommend to use replicates and multiple dilution steps as accuracy is always limited by statistics. Again these data are exported as *.txt file and the Relative Quantification SW will automatically create a corresponding *.cof file.

The final value of the normalized ratio for each sample is then calculated with efficiency correction and displayed in the result screen.

Continued on next page

4.2 LightCycler Relative Quantification Software,

Continued

Create a Coefficient File

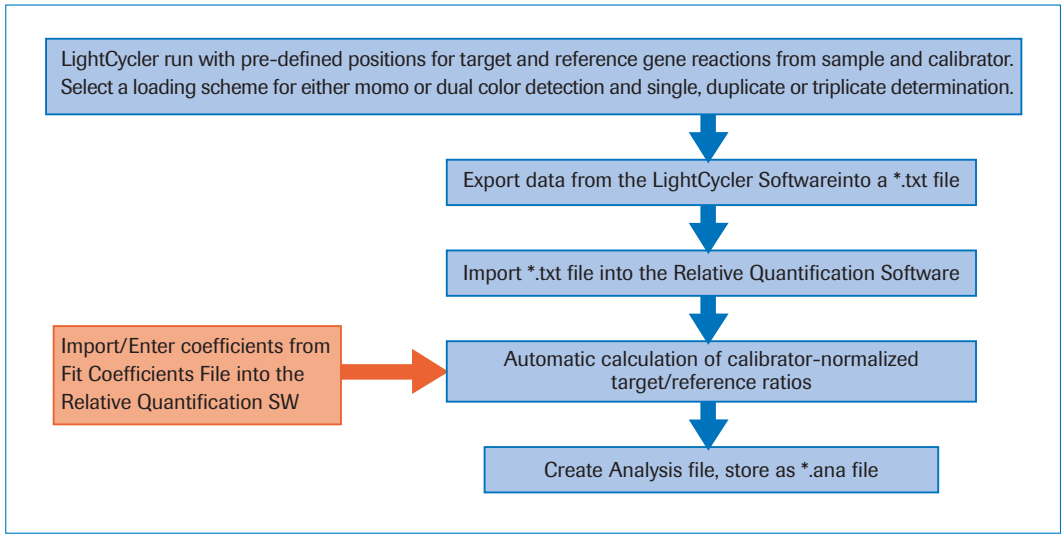
As mentioned above, for user-specific applications the efficiency for the target and the reference gene needs to be determined and stored in a coefficient file. The fit coefficients, describing the kinetics during PCR, are created from dilution series. Due to the principle of calibrator normalized relative quantification it is not necessary to know the exact copy number of these standards. Only the relative dilution steps (1:10, 1:100, ...) of the used standards have to be entered; one dilution set for the target gene and one for the reference gene is required. We recommend to perform a LightCycler run with e.g., 15 capillaries of target standards and another 15 capillaries of reference standards covering a dynamic range of 4 orders of magnitudes. Then these LightCycler data are transfered into the Relative Quantification Software to create a corresponding *.cof file.
The dilution series should be done with a typical nucleic acid, e.g., total RNA or genomic DNA from the calibrator. In principle, any nucleic acid dilution with identical PCR efficiency to a typical sample can be used for creation of a relative standard curve.

- Dilution series of target and reference gene ("relative standards") are set up to cover the respective dynamic range of detection.
- The function "log concentration vs. Cp" is displayed and a curve (instead of a regression line) is fitted through the data points to minimize the fit error. This polynomial fit function provides a method to compensate for the observed "low concentration shift" of the Cp values.
- Relative concentrations of target and reference gene of each sample and calibrator are calculated automatically from these curves.

Note: For customized applications please refer to Technical Note No. LC16/2002.

Workflow

Figure 13 gives an overview of the required steps for data analysis with the Relative Quantification Software:



Reference

For step-to-step instructions on the LightCycler Relative Quantification Software, refer to the manual accompanying the software (Cat. No.: 3 158 527).

4.3 Application 1: CK20 mRNA Quantification

Example 1

LightCycler – CK20 mRNA Quantification Kit

Background Information

The human Cytokeratin 20 (CK20) belongs to the epithelial subgroup of the intermediate filament protein family that is involved in cytoskeletal structure. Like other cytokeratins, the CK20 gene expression varies according to the tissue type. Expression studies suggest CK20 is restricted to gastrointestinal epithelium, urothelium, and Merkel cell carcinomas of the skin.
Many subjects suffering from cancer experience tumor recurrence despite a diagnosis of metastasis-free margins. It is believed that many of these recurrences occur due to the presence of micrometastases that are currently undetectable by conventional methods. PCR-based methods are being investigated as a possible means of micrometastases detection. For these purposes, many genes are being evaluated for their suitability, with the prime candidates being those with restricted expression. CK20 has been examined as a marker of micrometastasis, primarily for colorectal and urothelial cancer, when detected in lymph nodes, blood or bone marrow. In some studies, CK20 detection has been correlated with increased recurrence risk and tumor stage.
Due to technical variations between studies and a lack of quantitative assessment, the reasons for these discrepancies are poorly understood. As a result there is an important need to continue further studies on CK20 to involve standardized techniques with an ability to discriminate expression levels in a quantitative RT-PCR experiment.

CK 20 Kit Concept

The quantification of CK20 mRNA is performed in a two-step procedure. In the first step, cDNA is prepared from RNA by reverse transcription with random hexamers serving as primers. For every sample only one cDNA reaction is required as template for amplification and detection of CK20 (target) and PBGD (reference) by a LightCycler PCR.
The LightCycler – CK20 calibrator RNA, provided in the kit, contains a stabilized fraction of a total RNA purified from an immortalized cell line constitutively expressing CK20. It must be included with each RT-PCR LightCycler run. It serves to compensate for the constant differences between the detection of the target (CK20) and reference gene (PBGD), and provides a constant calibration point between PCR runs.
By using the Relative Quantification Software for the LightCycler Instrument, the amount of mRNA encoding for CK20 is expressed as a relative ratio to a reference gene (PBGD) in a sample relative to the CK20:PBGD ratio in a calibrator. See scheme in figure 14 below.

Note: To guarantee a correct analysis of an experiment by the Relative Quantification Software, it is essential to fulfill some requirements for the loading of the LightCycler carousel. Please follow the workload scheme mentioned in the LightCycler Relative Quantification Software manual prior to performing this assay.

Continued on next page

4.3 Application 1: CK20 mRNA Quantification

Continued

CK20 Kit Concept (continued)

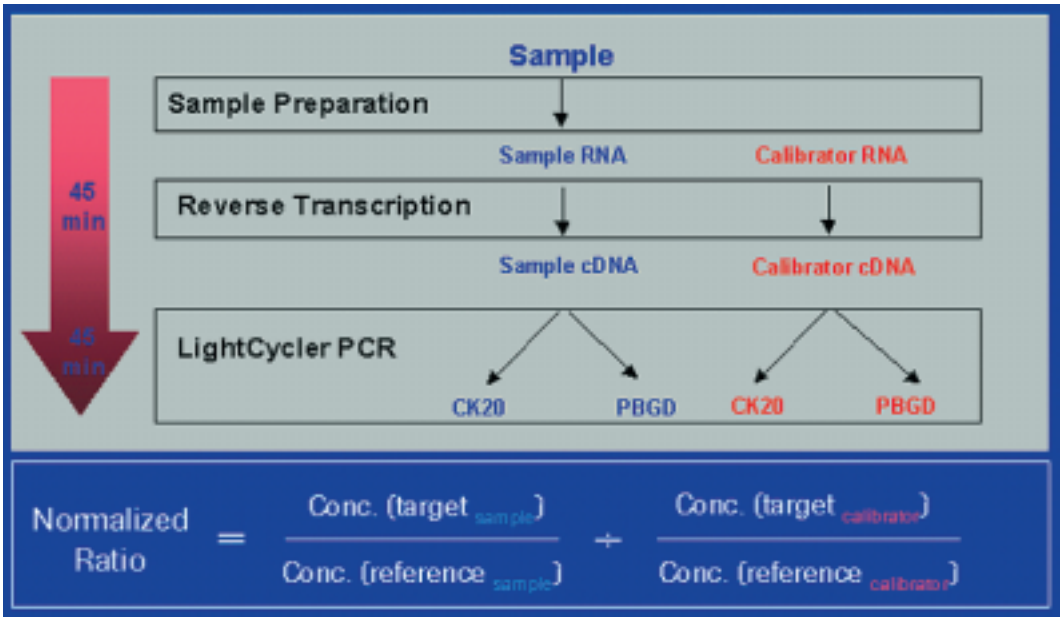


Figure 14: Scheme of the LightCycler – CK20 Quantification Kit concept.

Reaction Setup

Research samples of primary tumors (S1) and lymph nodes (S2) were collected. Sample RNA was extracted from the frozen tissue by using the TriPure reagent or HighPure RNA Purification Kit from Roche.
The LightCycler run was performed with the samples and the calibrator, provided in the kit. As the CK20 kit concept follows a mono color approach, the target PCRs and reference PCRs were setup in separate capillaries, according to one of the loading schemes described in the pack insert.

Analysis Method

Data analysis is done, as described in the *LightCycler Operator's Manual*. The Second Derivative Maximum Method has to be used for the determination of the crossing points.

- Once the crossing points have been determined, export the respective data to generate a .txt file and download the information in the LightCycler Relative Quantification Software, which will automatically calculate the quantification ratio.
- The LightCycler-CK20 Quantification Kit contains a yellow *"Important Note"* insert, stating the lot-specific Fit Coefficients and Correction Factor, that must be entered prior to analysis. Alternatively, the resulting coefficient file can be imported directly from the Internet LightCycler Homepage into the Relative Quantification Software. *(http://biochem.roche.com/lightcycler/lc_support/rqc_download.htm)*

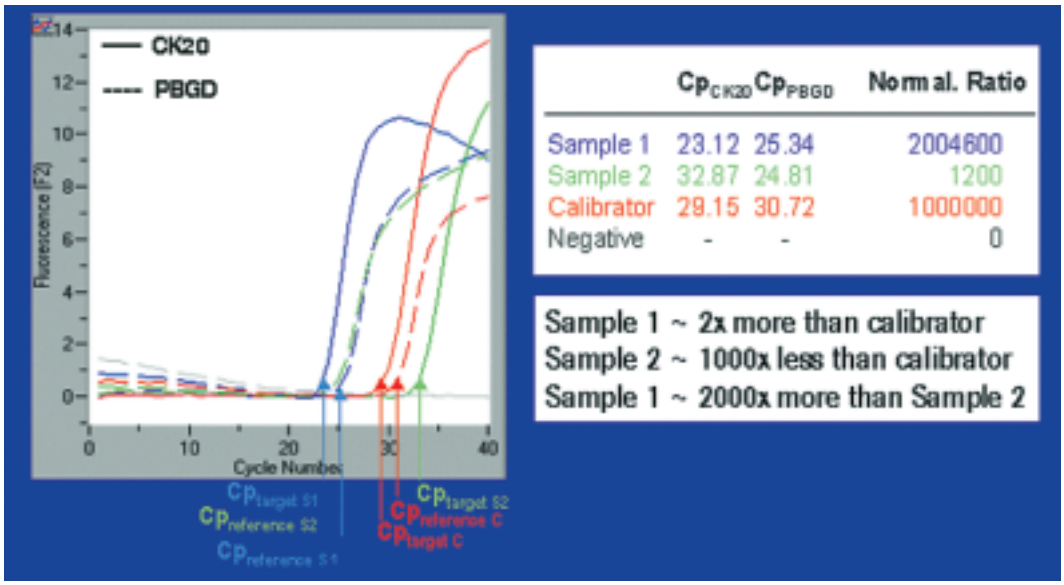
Continued on next page

4.3 Application 1: CK20 mRNA Quantification,

Continued

Calculation of Results

In the first step, the relative amount of target (CK20) is calculated as a ratio of target (T) concentration to reference (R) concentration (PBGD). In the second step, this ratio of target to reference (T:R) of the sample is divided by the T:R ratio of the calibrator, that is run in parallel with the samples.



Interpretation of Results

Figure 15 visualizes a typical result for the CK20 Quantification kit:
It shows the determination of crossing points for the target CK20 and the housekeeping gene PBGD of two different samples (S1, S2) and the calibrator (C)). The final result is expresses as the normalized ratio. The CK20 level of the sample S2 is significantly lower than that from sample S1. In the CK20 kit, the ratio of T:R in the calibrator RNA is set to a value of 1,000,000 for easier reading of the results. Therefore, a sample with 2,000,000 has 2 times more CK20 mRNA expression than the provided calibrator. A typical CK20-negative sample material results in normalized ratios of approx. 1000, which is 1000 times lower than the calibrator.

Due to statistical reasons, CK20 quantification of very low copy numbers becomes less reliable. Therefore, it is better to treat results in this range qualitatively or perform replicate analysis to increase reproducibility.
Additionally, the dynamic range of quantification must be considered when interpreting results. Although the dynamic range of quantification is large, not all samples can take advantage of this range, particularly if the crossing point of the reference gene is in a later cycle.

For example, a relative ratio of 100 will roughly correspond to a sample with a target crossing point that is 12 cycles later than that of the reference. If the reference crossing point was 22 (therefore target = 34), then this would be detectable, however, if the reference crossing point was 30 (target 42), this would be difficult to detect and considered negative. This also has implications for the quantification reliability of relative ratio amounts. The relative ratio of 100 is reliable when the reference crossing point is 22, but is less reliable when the reference crossing point is 30.

4.4 Application 2: HER2/neu DNA Quantification

Example 2

LightCycler – HER2/neu DNA Quantification Kit

Background Information

The human epidermal growth factor receptor-2 (HER2, also referred to as HER2/neu or c-erbB2) gene is a proto-oncogene mapped on chromosome 17q21. It encodes a 185 kDa transmembrane cell surface glycoprotein, which is often called HER2/neu protein or receptor. HER2/neu is a member of the tyrosine kinase family with a high degree of homology with the human epidermal growth factor receptor (EGF-R).
HER2/neu gene amplification and/or overexpression is frequently observed in subsets of a wide range of human cancers (e.g., breast cancer). The LightCycler HER2/neu DNA Quantification Kit is a convenient PCR method for the determination of the DNA encoding for the human HER2/neu.

HER2/neu Kit Concept

Using the LightCycler Instrument as a closed-tube, rapid PCR amplification and real-time fluorescence detection system, quantitative measurements can be performed with minimal risk of cross contamination. Normalization against the calibrator DNA, provided with the kit, corrects for differences in HER2/neu values, resulting from the combined variation in the quantity and quality of DNA sample and the efficiency of PCR.
The LightCycler HER2/neu DNA Quantification Kit allows the simultaneous quantitative detection of DNA encoding human HER2/neu, in DNA preparations from cell cultures and other biological samples, relative to a reference gene, by dual color detection. The use of a previously stored color compensation file is a prerequisite in this dual color experiment (i.e., both Light Cycler Red 640 and LightCycler Red 705 -labeled Hybridization Probes are used in a single capillary). A single copy gene located on the identical chromosome as the HER2/neu gene (chromosome 17) was selected as a reference, to avoid incorrect quantification in case of chromosomal aberration.

Note: The described performance of the kit is guaranteed only for use on the LightCycler Instrument and in conjunction with the LightCycler Relative Quantification Software. Please read the LightCycler Relative Quantification Software manual and pack insert thoroughly prior to performing this assay.

Continued on next page

4.4 Application 2: HER2/neu DNA Quantification,

Continued

HER2/neu Kit Concept (continued)

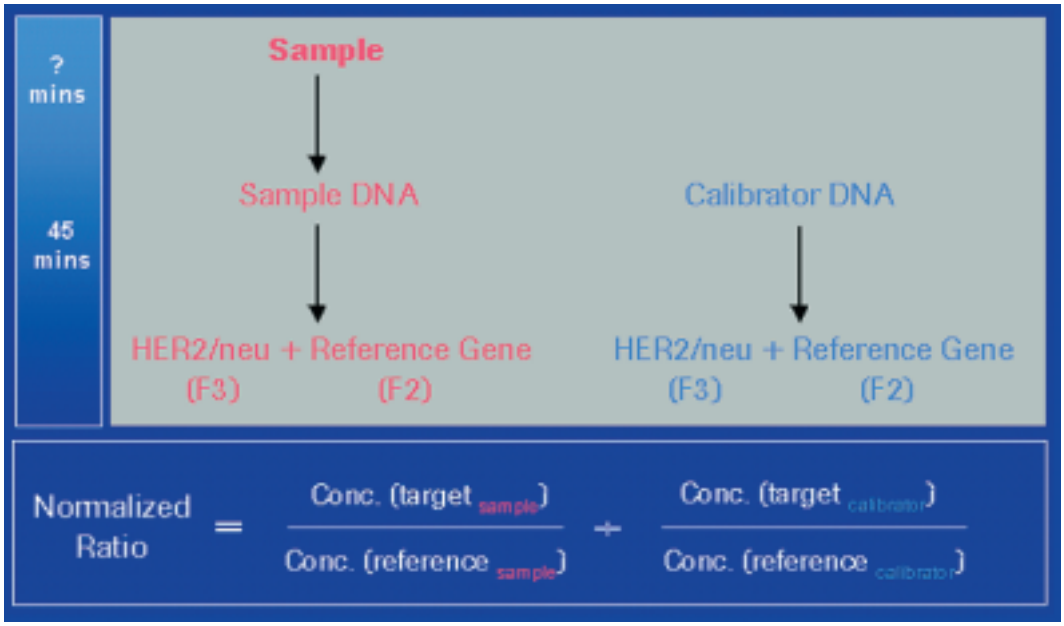


Figure 16: Scheme of the LightCycler – HER2/neu DNA Quantification Kit concept.

Reaction Setup

Purified DNA from cell cultures, scientific biopsy material, or other biological samples (e.g., frozen, formalin-fixed paraffin-embedded tissue) can be used as template to perform the LightCycler run together with the calibrator DNA provided in the kit. The LightCycler HER2/neu DNA Quantification Kit allows the simultaneous quantitative detection of HER2/neu and the reference gene by dual color detection, within one capillary. It is essential to follow a special loading scheme, as described in the pack insert.

Analysis Method

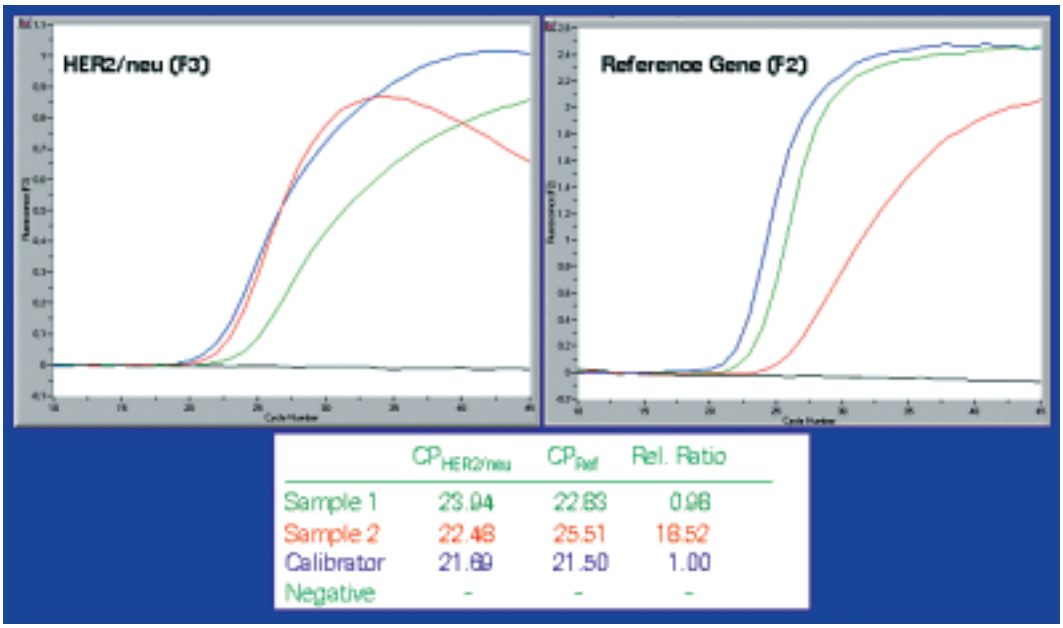
The calculation of the HER2/neu DNA amount is based on the resulting crossing point of one particular sample and the efficiency of the PCR. The actual calculation is performed by the Light-Cycler Relative Quantification Software. Prior to analysis the crossing point data (F2 for the reference and F3 for HER2/neu) from the LightCycler software (Second Derivative Maximum method) need to be imported together with the lot-specific fit coefficient file (download from the internet or enter manually from yellow *"Important Note"* insert). See also chapter 4.3. page 21.

Note: Refer to the instructions accompanying the LightCycler Relative Quantification Software for more information.

Continued on next page

4.4 Application 2: HER2/neu DNA Quantification,

Continued



Typical Result

Figure 17 shows typical results regarding the specificity and sensitivity of HER2/neu detection, and relative quantification of HER2/neu DNA, relative to a reference gene. Fluorescence data for HER2/neu is shown in channel 3 (F3) and for the reference gene in channel 2 (F2), respectively. Sample 1 (ratio of 0.98) is negative for HER2/neu DNA over-amplification, sample 2 (ratio of 18.52) positive.

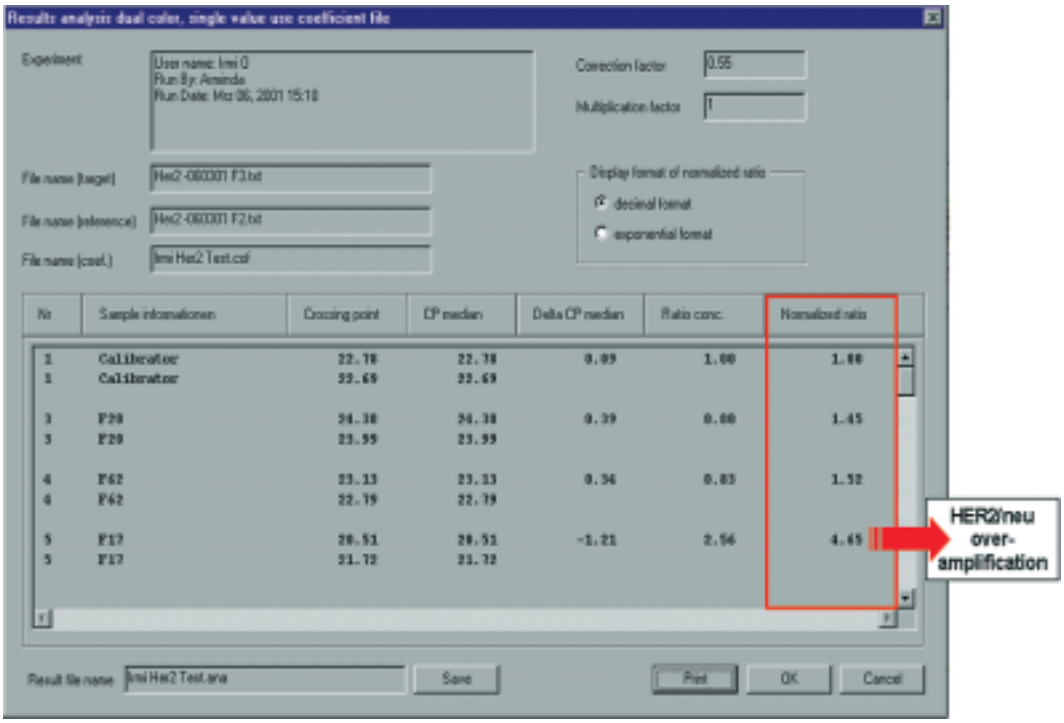


Figure 18 The final result is expressed as normalized ratio (T:R in the sample, relative to the ratio of T:R in the calibrator DNA), by the LightCycler Relative Quantification Software.

Continued on next page

4.4 Application 2: HER2/neu DNA Quantification,

Continued

Interpretation of Results

The ratio of T:R in the LightCycler calibrator, provided with the kit has a value of 1.00 to simulate the conditions in a normal cell (stabilized solution of plasmid DNA containing cloned fragments of the HER2/neu gene and the reference gene).

Ratio of < 2.00: sample is negative for HER2/neu DNA over-amplification.
Ratio of ≥ 2.00: sample is positive for HER2/neu DNA over-amplification.

Note: In biological samples, cells with amplified HER2/neu DNA are dispersed in a background of non-amplified cells, thereby leading to a decreased sensitivity (i.e., positive samples may have values lower than 2.00). In many cases this can be avoided by briefly removing the non-tumorous cells, for example, by macrodissection with a scalpel.

Due to statistical reasons, HER2/neu detection and quantification becomes less reliable at low-copy numbers, i.e. results obtained from sample material with crossing points greater than 35 for either the target (HER2/neu) and/or the reference gene.

5. Optimizing PCR Conditions

Overview

At the beginning the PCR conditions need to be established. Start with the target reaction set up and optimize all PCR parameters with consideration of the recommendations listed below. After the PCR parameters of the target have been selected, adapt the reference gene amplification to these "target conditions".

- For assay development always use Roche LC ready-to-use reagents.

For further details see Technical Note No. LC 2-5/99 and LC 9/2000 Optimization Strategy, in addition pack inserts of the LC Kits for PCR or RT-PCR and the LightCycler Operator´s Manual

$MgCl_2$ – Titration

Include a $MgCl_2$ – titration in the first experiment:

- 1 – 5 mM $MgCl_2$ for DNA assays
- 4 – 8 mM $MgCl_2$ for RNA assays (with the exception of the LightCycler RNA Masters ⇒ $Mn(OAc)_2$ dependent)

Template

Start with at least two different dilutions:

- high concentration
- medium/low concentration

always add some controls:

- no template control (NTC)
- RT-minus control
- positive control.

Primers and Hybridization Probes

For the design of primers and Hybridization Probes follow the guidelines in Technical Notes No. LC 1/99 and LC 6/99 or the LightCycler Operator´s Manual.

- amplicon length should not exceed 1000 bp; for best results short amplicons are recommended.
- always use highly purified primers and probes (HPLC)
- start with standard concentrations: Primers 0.5 µM each
  Hybridization Probes 0.2 µM each
- avoid duplex formation between primers and Hybridization Probe pairs

Further Optimization

An assay performance can be improved by the following parameters:

- annealing temperature: optimize in 1 – 2 °C steps
- primer concentration: in the range of 0.3 – 1.0 µM each
  Hybridization Probe concentration: in the range of 0.2 – 0.4 µM each
- use Hotstart to reduce formation of primer dimers and improve sensitivity

6. Appendix

Further Readings

Beyser K, Reiser A, Gross C, Möller C, Tabiti K and Rüschoff J (2001)
Real-time Quantification of HER2/neu Gene Amplification by LightCycler Polymerase Chain Reaction (PCR) – a New Research Tool.
Biochemica **2**: 15 – 18.

Soong R and Tabiti K (2001) *Relative Quantification of Cytokeratin 20 on the LightCycler Instrument.* Biochemica **2**: 19 – 22.

Sagner G and Goldstein C (2001) *Principles, Workflows and Advantages of the New LightCycler Relative Quantification Software.* Biochemica **3**: 15 – 17.

Soong R et al (2001) *Quantitative Reverse Transcription-Polymerase Chain Reaction Detection of Cytokeratin 20 in Noncolorectal Lymph Nodes.* Clinical Cancer Research, **7**: 3423 – 3429.

01.02-**3 274 381**

Roche Diagnostics GmbH
Roche Applied Science
68298 Mannheim
Germany