



LightCycler

Overview of LightCycler Quantification Methods

1. General Introduction

Introduction

This Technical Note will introduce the reader to the quantification methods we currently recommend for the LightCycler. A detailed description of the various experimental approaches will follow in separate Technical Notes.

Note: Before reading this Note, the reader should be familiar with the display and analysis of quantification data on the LightCycler, as described in Section 5, Chapter B, page B57 of the LightCycler Operator's Manual (May 1999 version).

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Definitions

Discussions on quantification use many terms to describe standards. Unfortunately, these are often used in different ways. For maximum clarity, we will use the definitions in the table below when we discuss quantification methods.

Term	Definition
Standard	It has a known concentration and is used for quantification of an unknown
Control	It is not used to quantify the unknown. Instead, it helps monitor the quality of the amplification.
Housekeeping Gene	Gene that is expressed constitutively in the sample to be analyzed
External	The standard or control is amplified in a different capillary than the target
Internal	The standard or control is amplified in the same capillary as the target

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1. General Introduction, Continued

Definitions (continued)

Term	Definition
Heterologous	The standard or control has a different sequence than the target and is amplified with a different primer pair
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 as the target
Exogenous	The standard or control is added to the PCR mixture. It is either: <ul style="list-style-type: none">• RNA which is usually generated by <i>in vitro</i> transcription, or• 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., house-keeping genes
Calibrator	A sample that is used as a reference. Target concentrations from all samples are divided by the concentration of the calibrator to normalize the sample values. Such calculations can compensate for lot-to-lot and run-to-run variations.

2. Advantages of Kinetic Quantification

Kinetic Quantification vs. End-point Determination

The LightCycler offers kinetic quantification, a fast, accurate way for quantification by PCR. Real-time, kinetic quantification allows measurements to be made during the log-linear phase of a PCR.

Before kinetic measurement of PCR was possible, quantification data were acquired only in the plateau phase of the PCR (end-point determination). This section explains why this new kinetic quantification method is an improvement over conventional end-point methods.

Limitations of End-Point Method

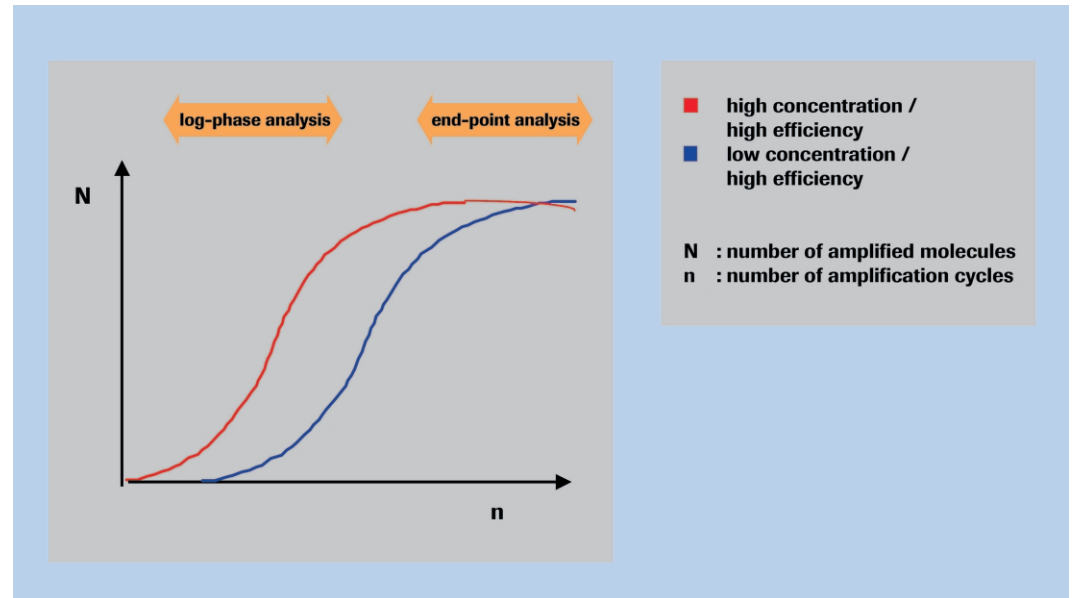


Fig. 1: PCR and the Problem of Quantification

Figure 1 shows that significant variations in the amount of starting material cannot be differentiated by signals measured in the plateau phase. These significant differences were easy to miss. Measuring them required very cumbersome methods.

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2. Advantages of Kinetic Quantification, Continued

Accuracy of Measurements during the Log-Linear Phase

In contrast to analysis in the plateau phase, analysis in the log-linear phase produces data that are much more accurate. Amplification is described as:

$$N = N_0 \times E_{\text{const.}}^n$$

(N: Number of amplified molecules; N_0 : Initial number of molecules; E: amplification efficiency; n: number of cycles).

Since amplification efficiency is constant, it is easy to determine the amount of starting material. An increase in signal during the log-linear phase corresponds directly to an increase in DNA. The log-linear phase in a run lasts only 2–5 cycles. As you can deduce from Figure 2, it is quite difficult to identify and measure these few cycles if you use conventional methods such as agarose gel electrophoresis. However, with the help of real-time PCR monitoring on the LightCycler, these log-linear cycles are easily identified and measured.

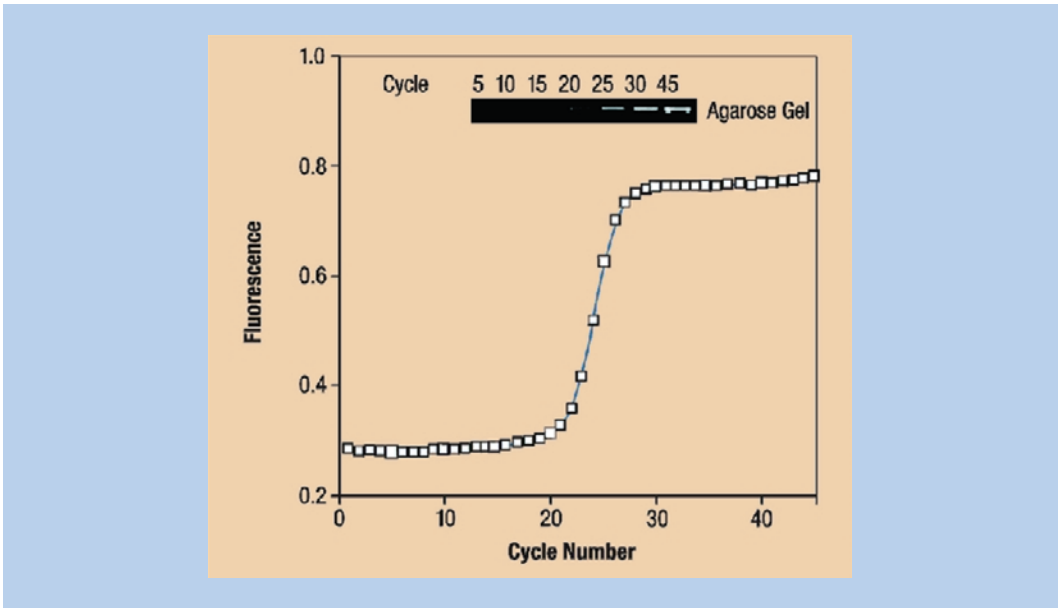


Fig. 2: Monitoring of PCR Reactions

3. Quantification Methods

Overview of Quantification Methods

In general, there are two types of quantification methods (see table below). Both are briefly described in the following sections of this Note.

Quantification	Description
Absolute quantification <ul style="list-style-type: none">• with external standards• with external standards and an internal positive control	Target concentration is expressed as an absolute value that is determined with the help of a standard.
Relative quantification	Target concentration is expressed in relation to the concentration of a housekeeping gene. A standard curve is used to obtain the concentration of the target and the housekeeping gene.

3.1. Absolute Quantification with External Standards

Principle

The easiest way to obtain an absolute value for an unknown concentration of target is to use external standards. In this quantification method, homologous standards and target samples are prepared in separate capillaries, then amplified during the same run. The standards are used to create a standard curve (Figure 3, graph on lower left). From this standard curve, the user can determine the concentration of a target sample (e.g., in Figure 3, the copy number for the target is represented by the yellow dot on the standard curve).

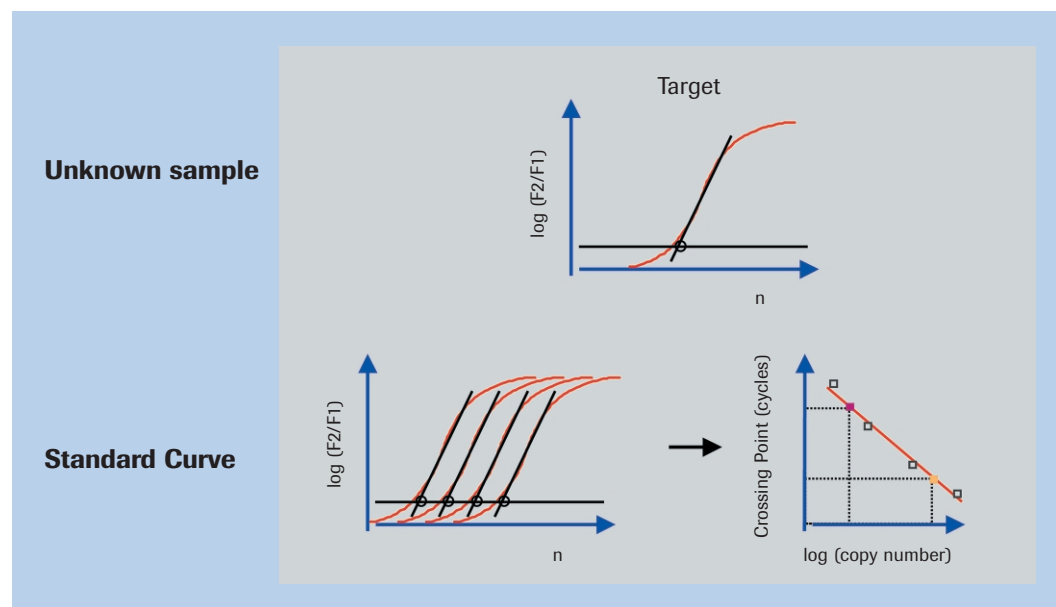


Fig. 3: Quantification Concepts
Quantification with External Standards

Quantification with external standards may be performed with either SYBR Green I or hybridization probes. For optimized reactions, the dynamic range of the method is up to 9 orders of magnitude.

Requirements

In order for this quantification method to be valid, the following conditions must be met:

- To ensure identical amplification efficiency of standards and unknown samples, the standard should be as similar to the target as possible. See Topic 4. in this Note for more details.
- This method does not detect or compensate for PCR inhibitors that may be present in the samples. Hence, both template preparation and assay must be well characterized and optimized.

Note: Alternatively, an internal positive control (as described in the following section) can be included.

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3.1. Absolute Quantification with External Standards,

Continued

Example

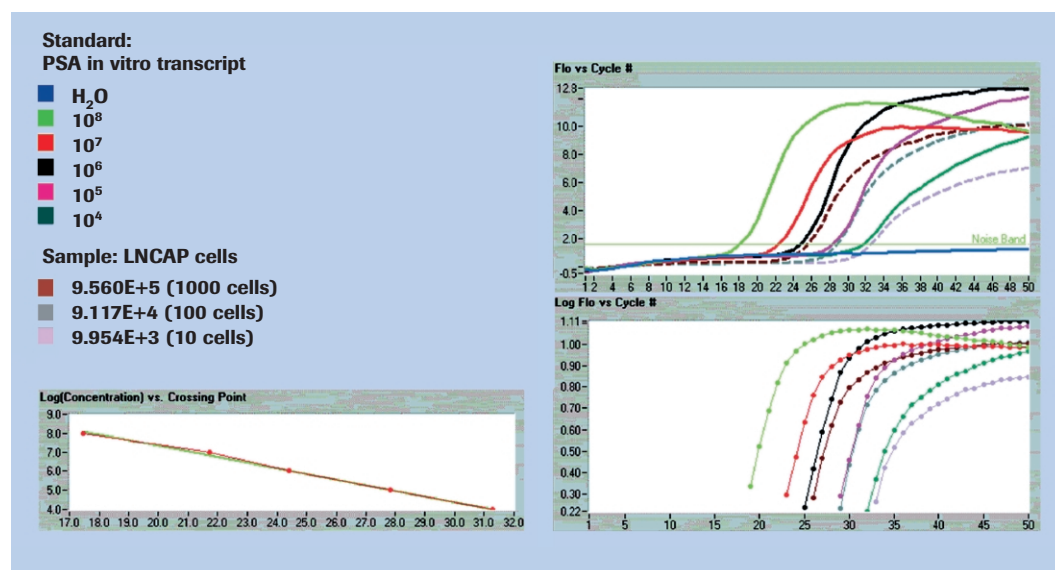


Fig. 4: LightCycler One Step RT-PCR
Quantification / Hybridization Probes

Figure 4 shows an example of absolute quantification with external standards. Here, a one-step RT-PCR (Figure 4, upper right graph) has been used to quantify expression of the prostate specific antigen (PSA) in LNCAP cells that were obtained from a human prostate cancer. The samples assayed were:

- Samples: Total RNA, isolated with the High Pure RNA Isolation Kit from samples containing between 10 and 1000 LNCAP cells
- Standards: PSA RNA obtained by transcription of a cloned cDNA

The amount of PSA transcript found in each cell sample was determined from the standard curve (Figure 4, lower left graph). The values obtained correspond to those described in the literature.

3.2. Absolute Quantification with External Standards and an Internal Control

Principle

This absolute quantification method also uses external standards and produces an absolute value for an unknown. However, in this method, each sample is spiked with a known amount of an internal control.

The sequences of the internal control and the target allow them to be co-amplified with the same pair of primers. However, simultaneous detection and differentiation of the target and the control requires two pairs of hybridization probes, one labeled with LightCycler Red 640 and the other with LightCycler Red 705.

Competition between Target and Control

When control and target sequences are co-amplified in one capillary, they both compete for Taq polymerase, nucleotides etc. If the concentration of the two sequences differ significantly, the more concentrated one will be amplified preferentially, resulting in almost no amplification of the less concentrated one. Therefore, the concentration of target and control must be carefully titrated against each other. Even when this is done, the dynamic range of the assay will be reduced to only 2–3 orders of magnitude.

Presence of Inhibitors

One advantage of this method is that the internal control can reveal the presence of possible inhibitors. For example, any shift in the crossing points obtained from the control indicates the presence of a PCR inhibitor.

Example

This example shows the quantification of TNF- α in total cell RNA using the following:

- An external standard, which is an *in vitro* RNA transcript of the wild type TNF- α sequence. The standard is detected with a pair of hybridization probes that is specific for TNF- α and is labeled with Fluorescein/LightCycler Red 640. This probe pair is also added to the samples to detect the TNF- α target.
- An internal control, which is an *in vitro* RNA transcript of an altered TNF- α sequence. The control is added to each sample at a concentration of 10^4 copies. The altered sequence in the control can be differentiated from the wild type sequence with another pair of hybridization probes. This second probe pair is labeled with Fluorescein/LightCycler Red 705.

In Figure 5, the standard curve (Samples 16–21) was used to determine the expression levels of TNF- α in the cells (Sample 22).

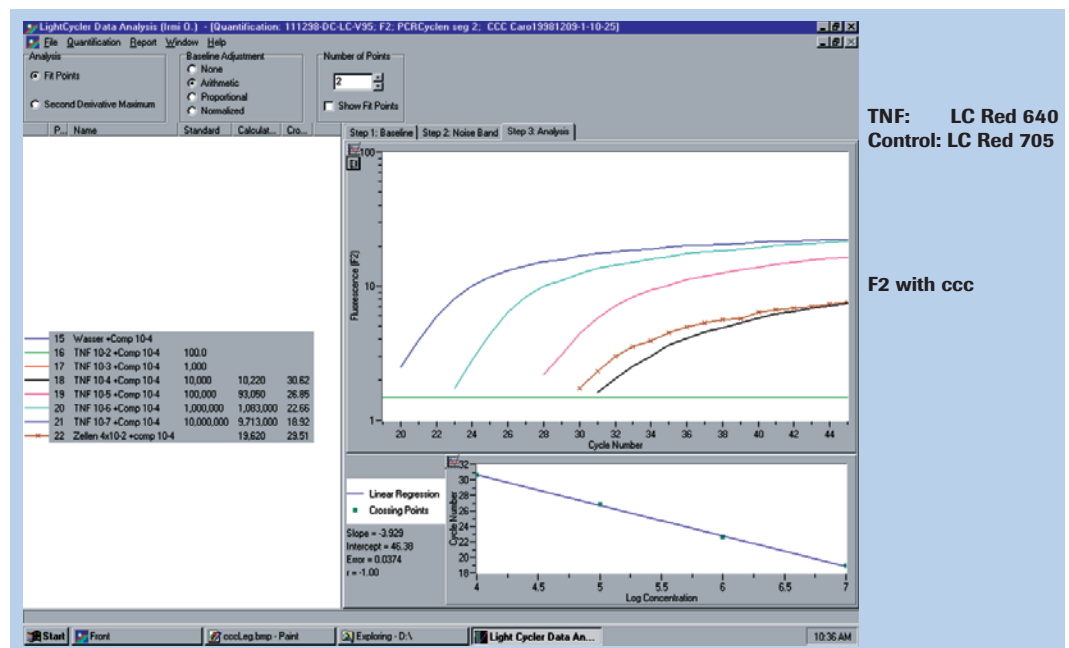


Fig. 5: Quantification with External Standard plus Internal Control: TNF- α

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3.2. Absolute Quantification with External Standards and an Internal Control, Continued

Example (continued)

In Figure 6, the fluorescence values of the control in the various samples are shown in channel 3 (F3).

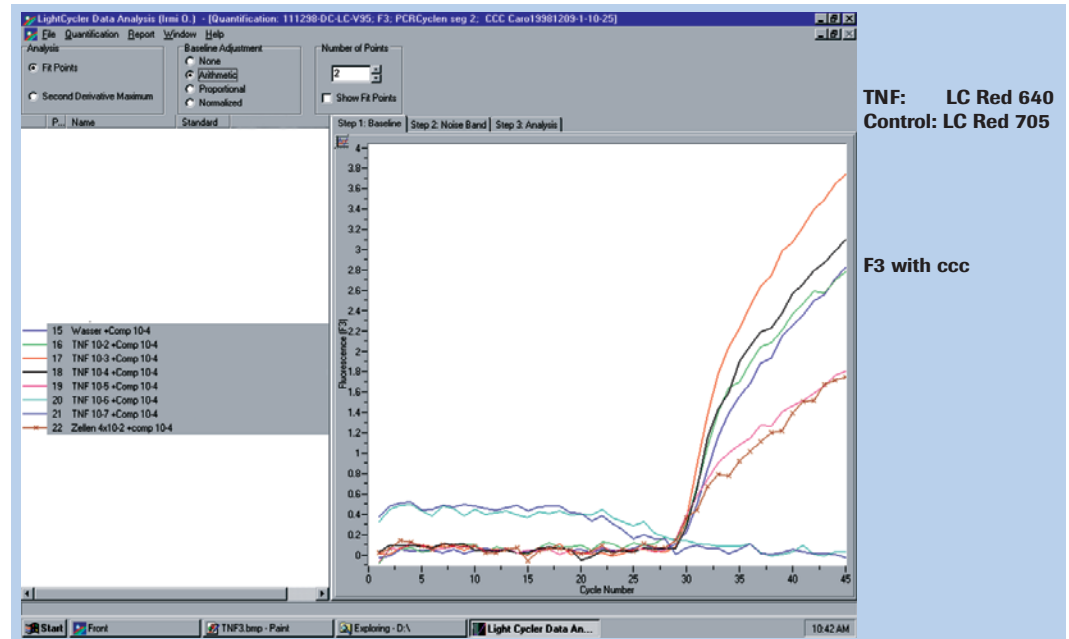


Fig. 6: Quantification with External Standard plus Internal Control: TNF α

The data clearly show that the crossing point is the same in all samples, i.e. the log-linear portion of the amplification curves overlap. This indicates that no inhibitory components were present in the samples. Consequently, the values obtained for the samples are reliable.

The graph in Figure 6 also shows the competitive effect between target and control in the late stages of the PCR. The greater the amount of target in the sample, the lower the fluorescence signal of the control in the plateau phase. In fact, the control produces no signal in samples 20 and 21.

Note: If you start with higher amounts of control, you can prevent this effect. Fortunately, however, the LightCycler does not need these late stages of PCR for quantification of target.

3.3. Quantification Relative to a Housekeeping Gene with External Standards

Principle

This quantification approach is an adaptation of quantification with external standards (see Topic 3.1.). In this approach, the level of an endogenous housekeeping gene in the sample is determined. The level of the housekeeping gene may:

- Be compared with the level of the target nucleic acid,
- Be used for normalization of the samples, and
- Act as an endogenous control that can compensate for variations in the sample volume, target recovery during nucleic acid extraction, and presence of inhibitors.

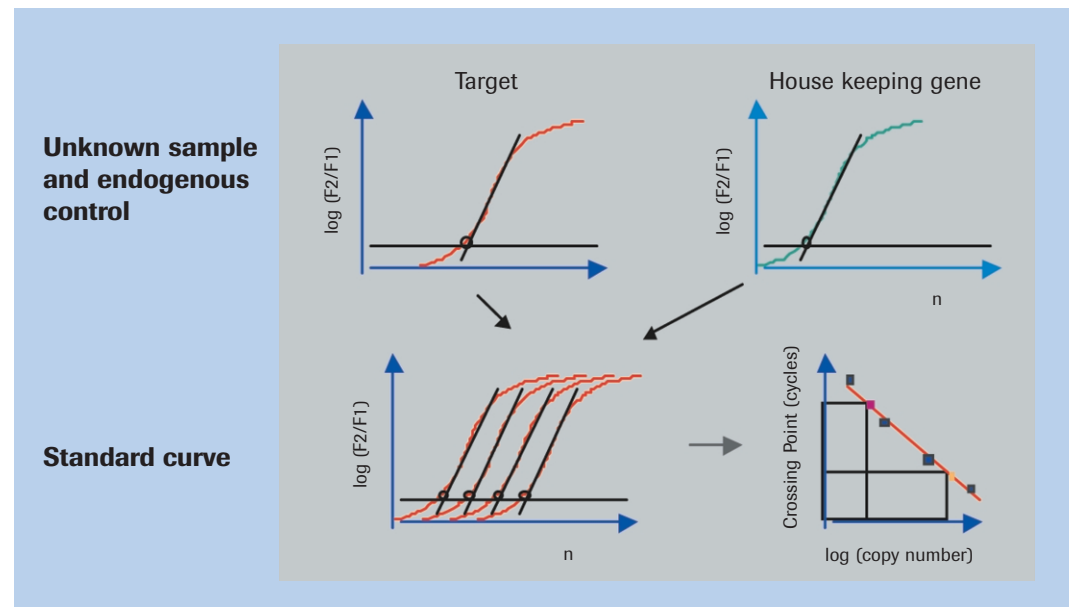


Fig. 7: Quantification Concepts.

Relative Quantification with External Standards.

In this method, external standards are used to generate a standard curve (Figure 7). That curve is used for quantification of both the target and the housekeeping gene in each sample. Then, the concentration of the target in each sample is divided by the concentration of the housekeeping gene in the same sample, thereby normalizing the samples.

This approach offers the advantage of a broad dynamic range coupled with the ability to correct for quantity and quality of the sample.

Requirements

The standard curve depends, in part, on the amplification efficiency of the standards. Therefore, the amplification efficiencies of the target, the housekeeping gene, and the standard should to be identical.

Application

This method may be used to determine the abundance of an mRNA transcript.

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3.3. Quantification Relative to a Housekeeping Gene with External Standards, Continued

Example

Figure 8 shows the quantification of fusion transcripts t(9;22) that have been reported to play a role in certain leukemias. These fusion transcripts are produced by translocation of segments from chromosomes 9 and 22.

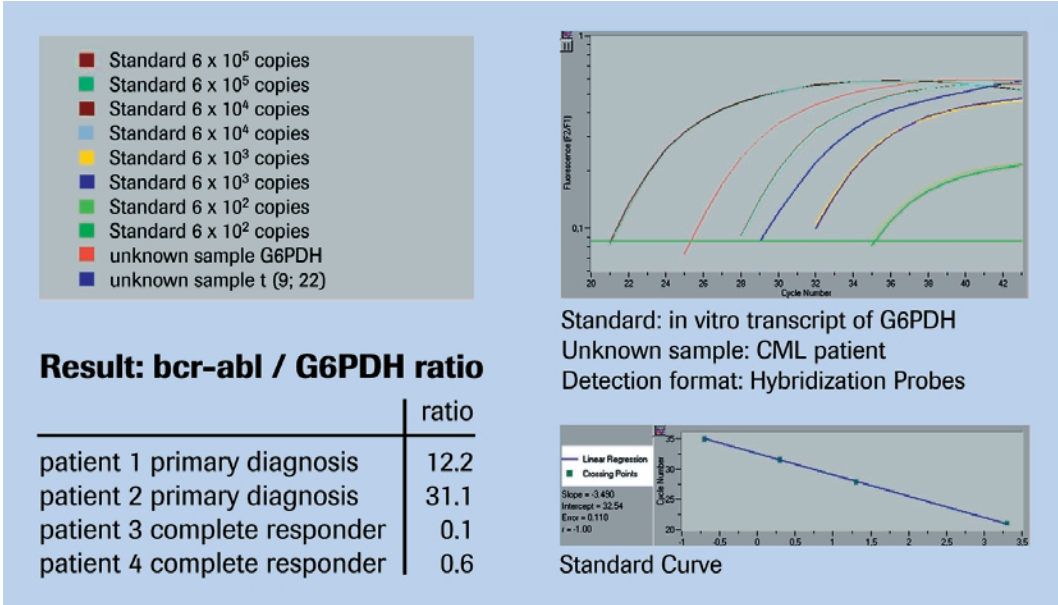


Fig. 8: Quantification of the Translocation t(9;22)

In this example, an external standard curve has been created with *in vitro* transcripts of a cloned glucose-6-phosphate dehydrogenase (G6PDH) gene. That gene is amplified with the same efficiency as the target and the housekeeping gene control. From each sample, one aliquot is used to determine the level of a bcr-abl fusion transcript and another aliquot is used to determine the level of G6PDH expression. Finally, the ratio of the fusion and G6PDH transcripts in each sample is calculated (table, lower left part of Figure 8).

Note: The ratio of the two genes may be studied further to determine whether it correlates to disease state.

4. Guidelines for Preparation of Standards and a Standard Curve

Guidelines for Preparation of a Standard Curve with External Standards

To create a reliable standard curve for quantification with external standards, please follow these guidelines:

- Use at least five points to create a standard curve, covering the expected concentration range of the target
- For medium and high concentrations, only a single determination is necessary for each dilution
- For quantification at the lower limit of sensitivity, you may use replicate determinations to improve accuracy

General Recommendations for External Standards

For accurate quantification, the amplification efficiency of the target must be the same as for the standard.

The main influences on amplification efficiency are the length of the fragment, the purity of the sample, the sequence amplified, and (for two-step RT-PCR only) the spacing between the RT primer and the PCR primers. Thus, ideally the standard should be homologous to the target.

The amplification efficiency is determined by running serial dilutions of standards on the Light-Cycler. The slope of the standard curve is converted to amplification efficiency E by the following algorithm:

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

The amplification efficiency of target and housekeeping gene should differ by no more than ± 0.05

Guidelines for Preparing Standards

Please follow the recommendations given below to prepare DNA standards for quantification:

Item	Recommendation
Sequence	The amplified sequence should be homologous to the target to ensure identical amplification efficiency at all dilutions. When this condition is met, a plot of Crossing Points vs. log (copy number) will have approximately the same slope for both standard and target.

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4. Guidelines for Preparation of Standards and a Standard Curve, Continued

Guidelines for Preparing Standards (continued)

Item	Recommendation
Source	<p>We recommend:</p> <p>DNA:</p> <ul style="list-style-type: none">• Linearized plasmid DNA• Purified PCR products <p>Note: Synthesize PCR products in the presence of dUTP to prevent carry-over contamination.</p> <p>RNA:</p> <ul style="list-style-type: none">• Synthetic, <i>in vitro</i> transcribed RNA. The RNA should have the same primer binding sites as the native RNA. The sequence should be as similar to the native sequence as possible. Avoid differences in length and GC content.
Purity	Use the High Pure PCR Product Purification Kit (for DNA) and the High Pure RNA Isolation Kit to prepare standards that are free of components that can interfere with PCR, e.g. nucleotides, primers, and salts.
Determination of Concentration	Measure absorbance at 260 nm according to standard procedures
Working Concentration	Adjust the working concentration of the standards so you have to add at least 2 µl of each standard to the final PCR mix. This minimizes variations due to pipetting.
Handling	<ul style="list-style-type: none">• Use siliconized tubes for diluting standards, control, and target nucleic acid.• Always prepare the dilution series shortly before use.• Dilute standards in a solution containing carrier nucleic acid, e.g., 10 ng MS2 RNA/µl.• Use aerosol-resistant pipette tips (Cat. No. 1667068, 1667076, 1667084, or 1667173).



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