*Roche Molecular Biochemicals*
*Technical Note No. LC 12/2000*

LightCycler

# Absolute Quantification with External Standards and an Internal Control

## 1. General Introduction

**Purpose of this Note**

This Technical Note provides guidelines for quantifying target DNA or RNA with external standards and an internal control.
***Note:*** For a comparison of this method to other quantification methods that can be performed in the LightCycler system, 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.

**Overview of Method**

The LightCycler generates quantitative data by taking real-time measurements during the log-linear phase of PCR. This method is accurate and reproducible.
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 (external standards).
Since, in addition, each sample is spiked with a known amount of an internal control, this method can easily distinguish between truly negative results and suppression due to a PCR inhibitor.
***Notes:***
- This method requires dual color analysis (Hybridization Probe format).
- The internal control must be efficiently amplified under the same PCR conditions as the target.

**Contents of this Note**

The topics of this Note are arranged in the order that one would follow to develop an absolute quantification procedure. These topics include:

**Definitions**

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

| Term | Definition |
|---|---|
| Standard | It has a known concentration and is used for the quantification of unknowns. |
| External Standard | The standard is amplified in a different capillary than the target. |
| Homologous Standard | The standard is identical to or only differs slightly from the target in length and/or sequence and is amplified with the same primer pair as the target. |
| Control | Its concentration is not used for the quantification of unknowns and may not even be known. |
| Internal Control | The control is amplified in the same capillary as the target or the standard. |
| Exogenous Control | The control is added separately to the PCR mixture. |

**Acknowledgment**

This Technical Note was supported by Dr. Andreas Pardigol from BIOTECON Diagnostics GmbH, Potsdam, Germany. Dr. Pardigol generously contributed his expertise and his LightCycler data for detection of *Salmonella* in food samples (see Section 6, Application 1).

# 2. Guidelines for Designing External Standards

**Introduction**

In this method, standards are amplified during the same run as the unknowns, but in separate capillaries. Data obtained from the standards are used to plot a standard curve of crossing point (Cp) vs. log concentration.
In this section you find recommendations for preparing a suitable standard.
***Note:*** For more information on standards, see Roche Molecular Biochemicals Technical Notes No. LC 11/2000, *Absolute Quantification with External Standards*

**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.<br>***Note:*** A method for determining amplification efficiency is described on pages 4-5. |
| Sequence | • The amplified standard sequence should be homologous to the target (including having the same amplicon length and GC-content).<br>• 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.<br>• 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.<br>***Note:*** Hybridization Probes should be labeled with Fluorescein/LC Red 640. |
| 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.<br>• 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, control and target dilutions.<br>• Use aerosol-resistant, sterile pipette tips.<br>• Stabilize standards with carrier nucleic acid (*e.g.* MS2 RNA, 10 ng/µl).<br>• Store high concentrations of the stabilized standard ($\geq 10^3$ copies) in aliquots at –20°C (DNA) or at –70°C (RNA). |

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# 2. Guidelines for Designing External Standards,

Continued

**Setting Up the Standard Curve**

- Use at least five concentrations to generate a standard curve. The concentrations you choose for the standard curve should match the expected concentration range of the target.
- For optimized reactions, the dynamic range of this method is up to 9 orders of magnitude.

**Calculating the PCR Amplification Efficiency**

To determine the amplification efficiency of the target or the standard sample, do the following:

- Prepare serial dilutions of the sample and amplify in the LightCycler.
- Record the slope of the linear regression curve (crossing point vs. log concentration) generated during the amplification run.
- Convert the slope value into efficiency (E) by using the following formula

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

For an example of an efficiency determination, see Figure 1 below.
***Note:*** After they are determined, you should make sure the amplification efficiency of the target and the standards are identical.

The screenshot below shows the result of an amplification efficiency determination performed with serial dilutions of a standard.

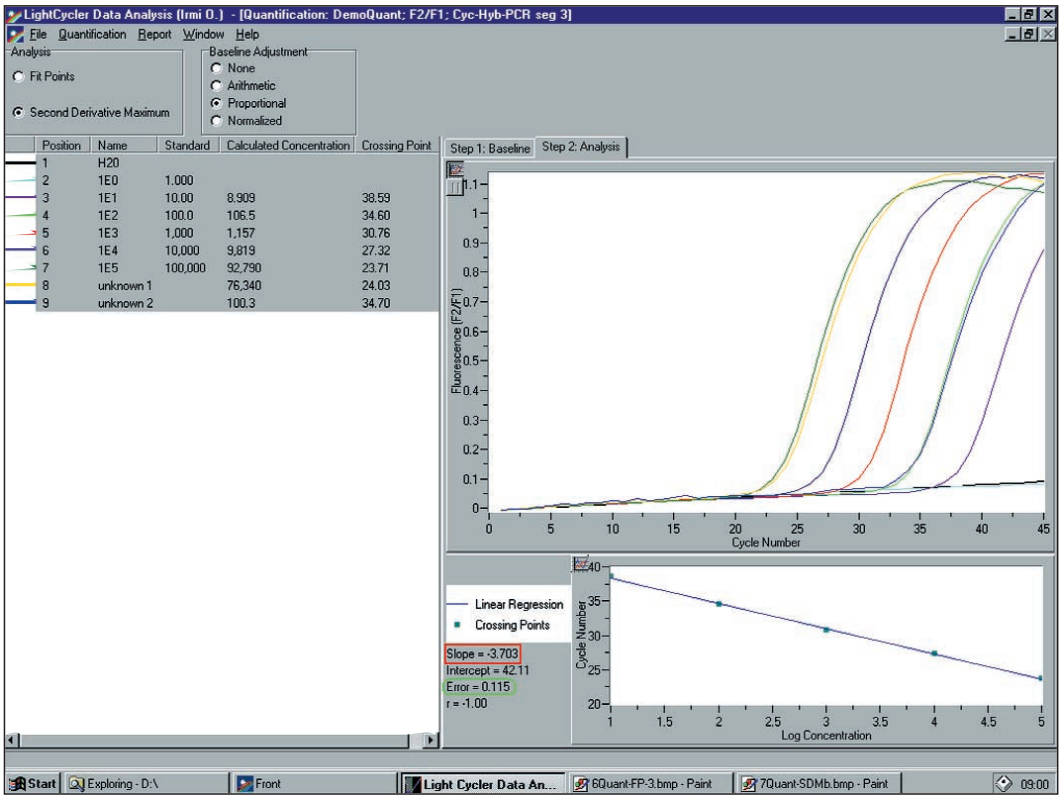


**Fig. 1: Example of Efficiency Determination**

**Experiment:** Serial dilutions of a standard were amplified and the run was analyzed with LightCycler software. The software settings were adjusted to achieve a low error rate (≤ 0.2). In this example, the Error = 0.115 (outlined in green on screenshot) and the slope of the linear regression curve was –3.703 (outlined in red). From the slope, the efficiency of the standard ($E_s$) can be calculated to be 1.86:

$$E_s = 10^{-1/slope} = 10^{-1/-3.703} = 10^{0.27} = 1.86$$

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# 2. Guidelines for Designing External Standards,

Continued

**Calculating the PCR Amplification Efficiency** (continued)

***Note:*** Depending on the analysis method used, the following parameters can be adjusted during the analysis to minimize the error:

- Fit Points Method: Adjust baseline, noise band, crossing line, and number of fit points. Also, if one dilution gives a result obviously different from the others, you can remove the data for that dilution from the analysis.
- Second Derivative Maximum Method: If one dilution gives a result obviously different from the others, you can remove the data for that dilution from the analysis.

For details regarding software settings, see the *LightCycler Operator's Manual.*

**Comparing the Efficiency of Standard and Target**

After using the same procedure as outlined in Figure 1 to determine the amplification efficiency of the target, you should compare the amplification efficiencies of the standard and the target.
If external standards are to be used to quantify target nucleic acid, the efficiencies must be identical. If you are using standards that are homologous to the target (as outlined on page 3), identical amplification efficiencies can normally be achieved without difficulty.

**Effect of Differences in Amplification Efficiency**

Especially when heterologous standards (i.e. standards amplified with a different set of PCR primers) are used, even a small difference in amplification efficiency can influence the final result substantially. The resulting error can be calculated by substituting E in the PCR equation:

$$\mathbf{N = N_0 \times E^n}$$

Where:
N = number of amplified molecules;
$N_0$ = initial number of molecules;
n = number of amplification cycles;
E = amplification efficiency = $10^{-1/\text{slope}}$.

# 3. Guidelines for Designing an Internal Control

**Introduction**

The Hybridization Probe format allows the LightCycler system to amplify and identify two target sequences in one capillary. This determination requires the use of two sets of Hybridization Probes that carry different labels (LC Red 640 and LC Red 705). The signals for the two amplicons are detected separately in fluorimeter channel 2 and channel 3, respectively.
One of these sequences can serve as an internal control that is co-amplified with the target sequence. Since factors that inhibit PCR will affect not only the target, but also the internal control fragment, the internal control:

- Can discriminate between a truly negative result and a false negative caused by inhibition of the PCR
- Serves as an indicator of PCR performance and therefore ensures accurate, reliable quantification

In this section you will find recommendations for designing a suitable internal control.

**Requirements for an Internal Control**

For best results, the internal control should meet all the criteria listed in the following table.

| Property | Criterion To Be Met |
|---|---|
| Sequence | • We recommend that target and internal control share the same primer binding site. This avoids interference of two primer pairs in a multiplex reaction.<br>• The target and control should contain small amplicon differences. A small deletion, insertion or mutation, present only in the control, makes it possible to differentiate the two amplified products.<br>• The amplification efficiencies of the target and control do not have to be identical. |
| Source | • For PCR: Preferably linearized plasmid DNA carrying the cloned target sequence, or purified PCR products.<br>• For RT-PCR: Synthetic, usually *in vitro* transcribed RNA. |
| Detection | • Use a Hybridization Probe pair that is specific for the sequence variations of the internal control.<br>• Label the probe pair with Fluorescein and LC Red 705. |
| 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.<br>• To minimize pipetting errors, we recommend adjusting the volume of the control so at least 2 µl of the control is added to the LightCycler capillary.<br>• Add the same concentration of internal control fragment to each sample. |
| Handling | • Use siliconized tubes for standard, control and target dilutions.<br>• Use aerosol-resistant, sterile pipette tips.<br>• Stabilize control with carrier nucleic acid (*e.g.* MS2 RNA, 10 ng/µl).<br>• Store high concentrations of the stabilized control ($\geq 10^3$ copies) in aliquots at –20°C (DNA) or at –70°C (RNA). |

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# 3. Guidelines for Designing an Internal Control,

Continued

**Requirements for an Internal Control** (continued)

Frequently, procedures are used to create controls that introduce slight modifications into the amplicon sequence of the target.

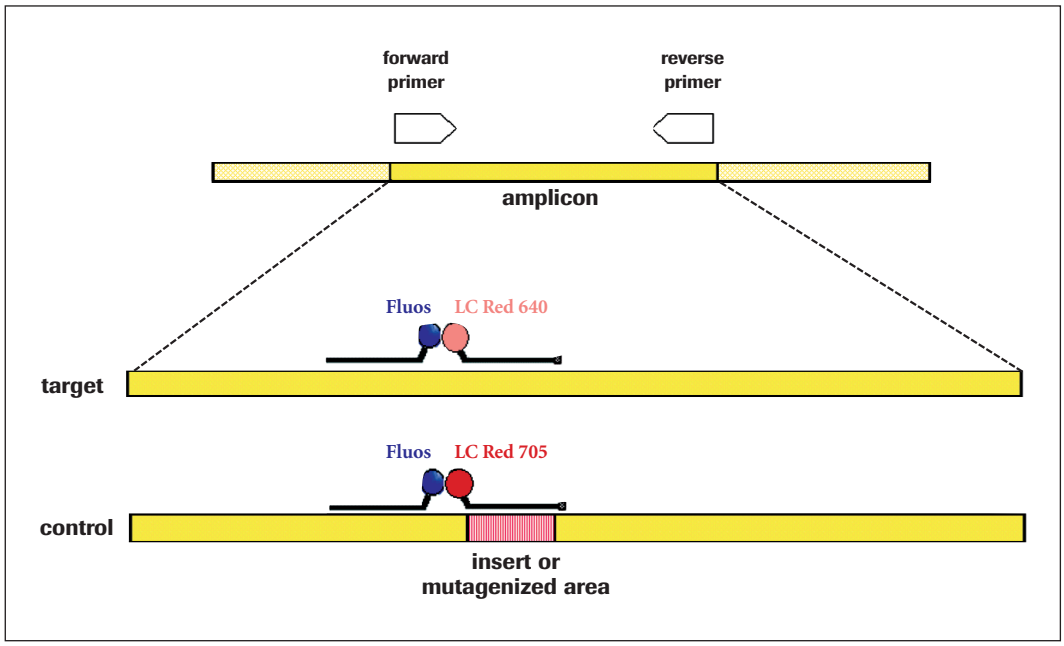


**Fig. 2: Construction of an Internal Control**

**Examples of Control Construction**

Various methods have been developed for generating mutations in cloned genes. Three of these methods are summarized in the table below.
***Note:*** For details of the methods, see *Molecular Cloning*, Chapter 15, or *Current Protocols in Molecular Biology*, Supplement 8.

| Method | Description |
|---|---|
| Site-directed mutagenesis | Use mutagenesis primers to modify only the binding site for the LC Red 705-labeled Hybridization Probe.<br>***Note:*** The binding site for the Fluorescein-labeled Hybridization Probe is unchanged and recognizes the same probe as the standard/target. |
| Insertion | Insert an additional binding site (for the LC Red 705-labeled Hybridization Probe) into the site that recognizes the LC Red 640-labeled Hybridization Probe. (For example, insert a 15 bp oligonucleotide.)<br>***Note:*** The binding site for the Fluorescein-labeled Hybridization Probe is unchanged and recognizes the same probe as the standard/target. |
| Exchange of amplicon sequence | Replace the original amplicon sequence with a stuffer fragment.<br>***Note:*** The binding sites for both Hybridization Probes (Fluorescein- and LC Red 705-labeled) will be changed. |

**Special Considerations**

The internal control is mainly used to indicate inhibitory effects during PCR. However, it could also be used as a standard to monitor recovery during sample preparation.
***Note:*** Add the control to the sample preparation after the initial lysis of the biological sample material.

# 4. Optimizing PCR Conditions for the Target

**Overview**

After choosing standards and a control, establish optimal LightCycler PCR conditions for amplification and detection (with Fluorescein- and LC Red 640- labeled probes) of the target alone. 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 LC Kit 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 to reduce formation of primer dimers and improve sensitivity.

# 5. Setting Up Dual Color Reactions for Target and Control

**Single Color Analysis for the Internal Control**

After optimizing PCR parameters for the target, run a dilution series of the internal control under "target" PCR conditions. Analyze the results in channel 3 (for LC Red 705-labeled Hybridization Probes). This will determine the sensitivity of the control system in a single color analysis.

**Dual Color Analysis**

After you determine the sensitivity of the control system, you can combine both target and control in one capillary and run as a dual color reaction. Add the same concentration of control to all samples and standards. Monitor the target data in channel 2 (F2) and the control data in channel 3 (F3).

For details, see the *LightCycler Operator´s Manual,* or the pack insert of the *LightCycler Color Compensation Set.*

***Notes:***

- Remember to load and activate a ccc-file for complete crosstalk color compensation in channel 2 and 3.
- Successful simultaneous detection of two targets requires co-amplification of both DNA sequences. Due to the competition of control and target, the dynamic range of concentrations that can be detected is reduced to approx. 3 magnitudes, in most cases.
- An increase in the amount of target/standard causes a decrease in the amplification of the internal control and vice versa. If one of the sequences is present at a significantly higher concentration (by a factor of 10–100), it will outcompete the other one, and the lower concentration sequence will produce no fluorescence signals.
- It may be advantageous, especially when the relative concentration of control to target is not known, to use a titration experiment to determine the optimal amount of the internal control. (For an example of such a titration experiment, see Figure 5 on page 11.)

**Amount of Control to Use**

Use low amounts of internal control to:

- Identify false negatives by detecting PCR inhibition, and
- Ensure reliable quantification of low target concentrations (near the sensitivity limit of the assay).

Use medium to high amounts of internal control if you need to ensure reliable quantification of medium or high target concentrations.

***Note:*** For an illustration of when to use different amounts of control, see Figures 3 and 4 on page 10.

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# 5. Setting Up Dual Color Reactions for Target and Control, Continued

**Amount of Control to Use** (continued)

The example below illustrates the use of a low amount of internal control.

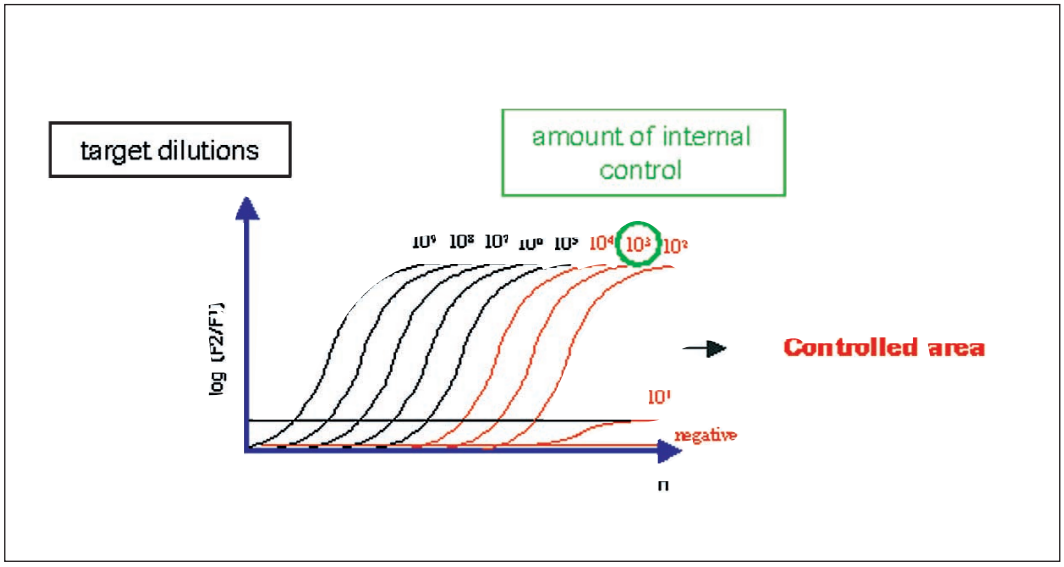


**Fig. 3: Low Amount of Control**

**Experiment:** The detection limit (lowest concentration to give a positive result) for the target was determined ($10^2$ copies in this example). Then, we added control to all target samples and standards at a concentration ($10^3$ copies) that was approx. tenfold higher than this detection limit. The addition of $10^3$ copies of internal control allowed reliable quantification of target concentrations (red lines on graph) as high as $10^4$ copies (in rare cases $10^5$) and as low as 0 (negative control).

The example below illustrates the use of a higher amount of internal control.

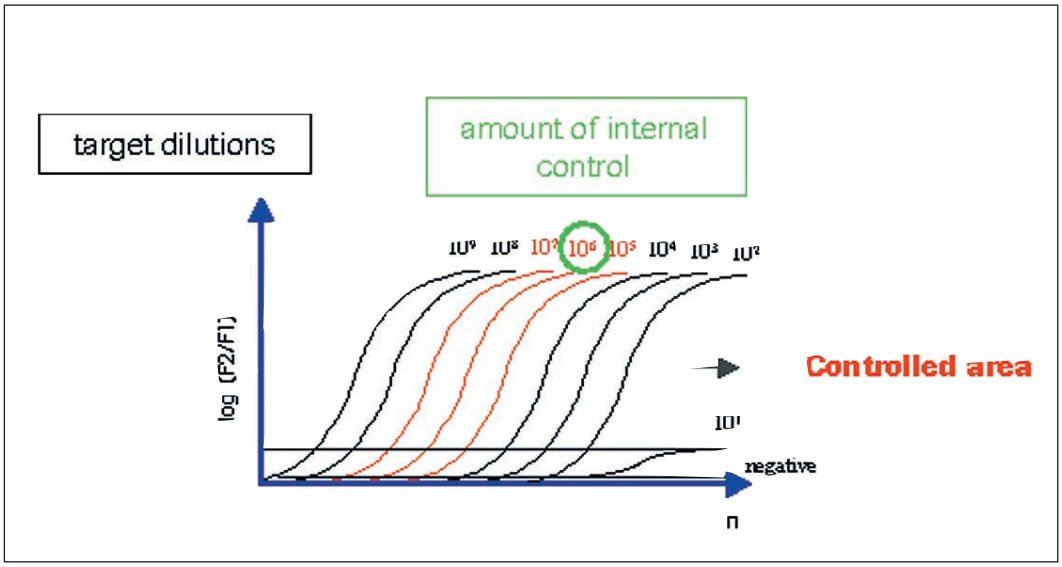


**Fig. 4: High Amount of Control**

The addition of $10^6$ copies of internal control allowed reliable quantification of target concentrations (red lines on graph) as high as $10^7$ copies and as low as $10^5$ copies.

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# 5. Setting Up Dual Color Reactions for Target and Control, Continued

**Amount of Control to Use** (continued)

Three sets of PCR samples were prepared, each with an identical dilution series of a standard ($10^3$–$10^9$ copies). Each set of standards was spiked with a different amount of internal control (set a, $10^5$ copies/capillary; set b, $10^4$ copies; set c, $10^3$ copies). The goal was accurate quantification of low target concentrations without affecting the sensitivity limit of the assay.

The results were analyzed in:

- Channel 2 (F2), to determine whether sensitivity was reduced.
  **Result:** Sensitivity for the standard was not reduced in either b) or c).
- Channel 3 (F3), to determine the control concentration that gives the widest range of measurable results.
  **Result:** The control results were measurable over a range of at least three magnitudes in both a) and b).

**Conclusion:** The optimal amount of internal control for further experiments was b), which meets both requirements.

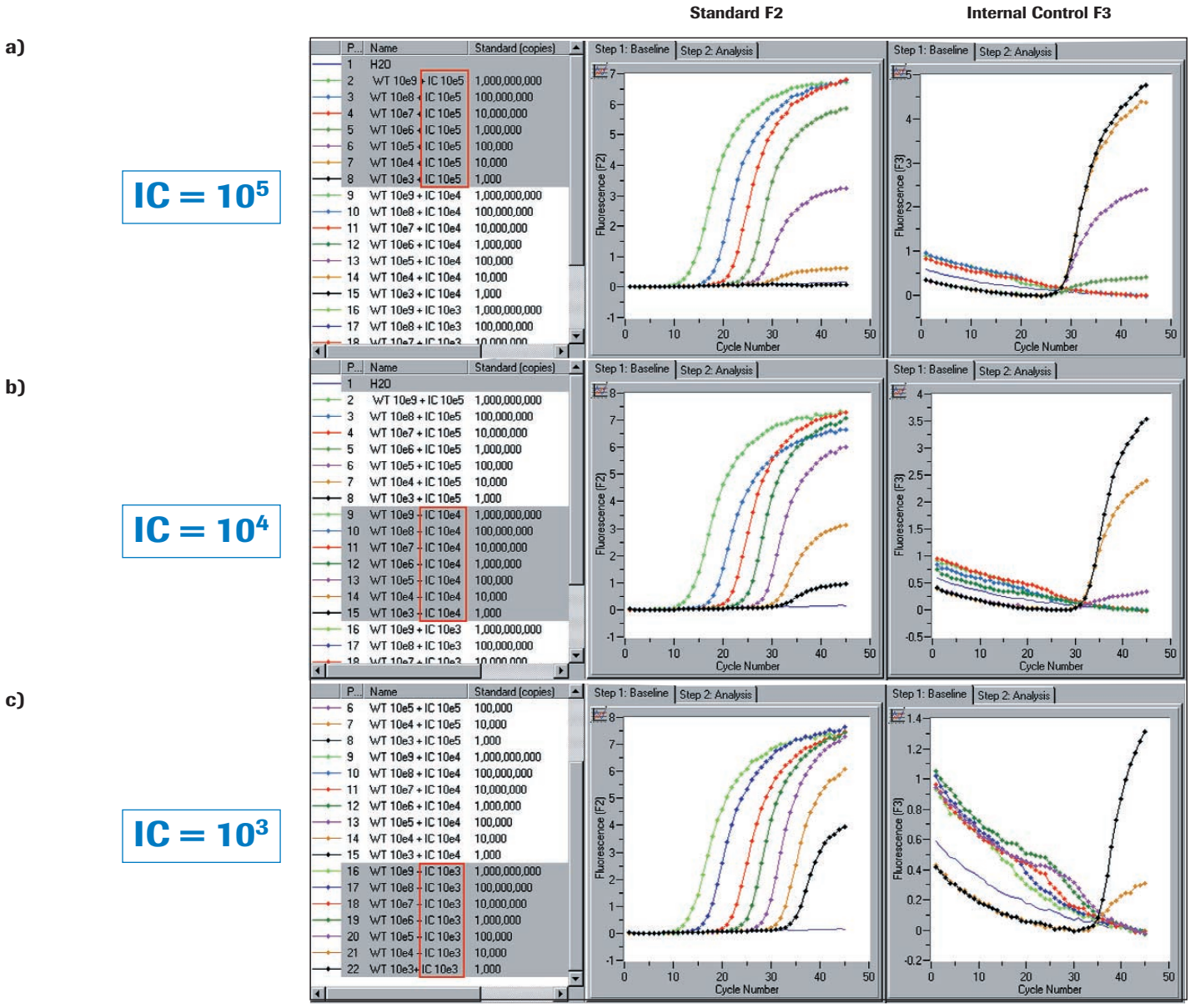


**Fig. 5: Experimental Determination of Control Concentration**

# 6. Dual Color Analysis: Applications

**Overview**

The examples in this section illustrate three different methods for constructing an internal control (described in Section 3) and the different applications for the control (described in Section 5). The examples are:

| | Target | Application | Method for Constructing Control |
|---|---|---|---|
| 1 | Salmonella (DNA) | Identification of false negatives | Site-directed mutagenesis |
| 2 | PSA (mRNA) | Reliable quantification for lower target amounts; detection of inhibitors | Insertion |
| 3 | TNF-α (cDNA) | Reliable quantification for medium target amounts; detection of inhibitors | Exchange of amplicon sequence |

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**Application 1**

Different amounts of *Salmonella* genomic DNA were amplified in the presence of an internal control. In this application, the internal control distinguishes true negative (i.e., *Salmonella*-free) food samples from false negatives (due to PCR inhibition) in a non-quantitative assay. For this test system, a low amount of internal control was selected since the only purpose of the control is to identify false negatives.

***Note:*** Data obtained from BIOTECON Diagnostics GmbH, Potsdam, Germany (apardigol@Analyticon-ag.com).

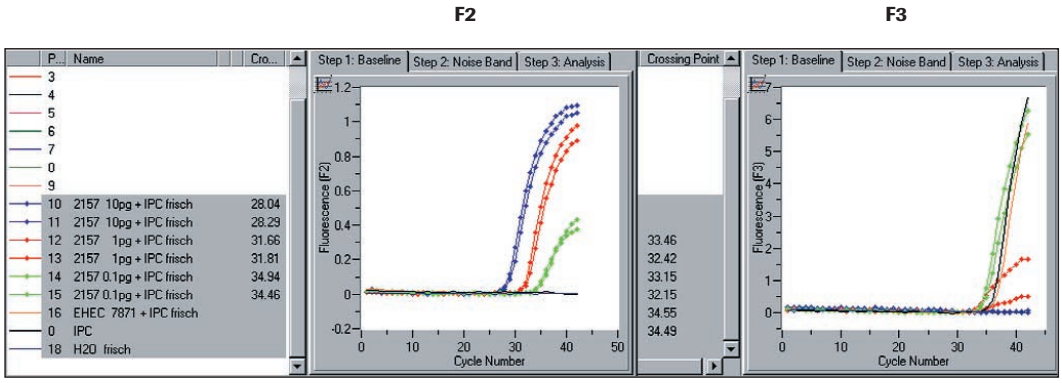


**Fig. 6: Application 1 *Salmonella***

| | |
|---|---|
| Control | **IPC** (= internal positive control): plasmid DNA constructed with mutagenesis primers; detected in channel 3 with Hybridization Probes that were specific for the mutagenized region and labeled with Fluorescein/LC Red 705. |
| Samples | Purified genomic DNA isolated from *Salmonella* strain BC2157; detected (in channel 2) with *Salmonella*-specific Hybridization Probes that were labeled with Fluorescein/LC Red 640.<br>**Amounts:** Samples contain 10 pg, 1 pg, or 0.1 pg DNA; assayed in duplicate. |
| Negative control | DNA isolated from *E. coli* (EHEC) strain BC7871 without any *Salmonella* contamination.<br>**Amount** of DNA: 1 ng/capillary. |
| Analysis in F2: target | Fluorescence values of the target Salmonella (LC Red 640) are visible in channel F2. Conclusions:<br>• Positive signal ⇒ presence of Salmonella in the food sample (positions 10–15).<br>• No fluorescence signal from negative control (position 16). |
| Analysis in F3: control | Fluorescence values of the internal control (LC Red 705) are visible in channel F3. Conclusions:<br>• Positive signal (same crossing point) ⇒ no inhibitory component in the samples (10–15) and proof of a true negative sample (16).<br>• Differences in fluorescence signal levels seen during late stages of PCR are due to competition between target and control. |

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**Application 2**

Abundance of prostate specific antigen (PSA) mRNA in LNCAP cells was quantified in a 1-step RT-PCR. A low concentration of internal control was used and monitored in channel 3 (F3); this allows accurate quantification of very low target amounts (at the detection limit of the assay). To minimize effects of competition between target and control, PCR was run at various sample dilutions.

**Results:** The Cp of the internal control was identical in all quantified samples. (See F3 data below.) This proves that PSA concentrations (target values in F2) determined for the sample material were reliable.

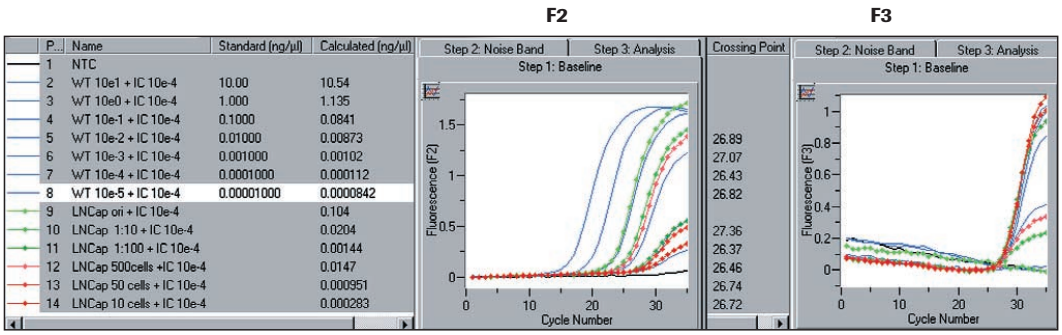


**Fig. 7: Application 2 PSA**

| | |
|---|---|
| Standard | *In vitro* RNA transcript containing a 137bp fragment of the target sequence (PSA); detected (in channel 2) with PSA-specific Hybridization Probes that were labeled with Fluorescein/LC Red 640.<br>**Amounts** used: 2 µl standards containing $10^{1}$–$10^{-5}$ ng RNA. |
| Control | *In vitro* RNA transcript that includes the 137bp fragment of the PSA sequence plus a 14bp insertion next to the binding site for the Fluorescein-labeled Hybridization Probe; detected (in channel 3) with an insert-specific Hybridization Probe that was labeled with LC Red 705.<br>***Note:*** The same Fluorescein-labeled Hybridization Probe was used for both control and target RNA.<br>**Amount** added to each standard and each target: 2 µl control containing $10^{-4}$ ng RNA. |
| Samples | Total RNA isolated (with a High Pure kit) from LNCAP cells (a hormone-sensitive prostate cancer cell line); detected (in channel 2) with PSA-specific Hybridization Probes that were labeled with Fluorescein/LC Red 640.<br>**Amounts:** 2 µl samples containing 100 ng, 10 ng, or 1 ng RNA or containing RNA isolated from 500, 50, or from 10 cells. |
| Analysis in F2: target | Fluorescence values of PSA target (LC Red 640) are visible in F2.<br>• The standards (position 2–7) are used to quantify the concentration of PSA in the cells (position 9–14). |
| Analysis in F3: control | Fluorescence values of the internal control (LC Red 705) are visible in F3. Conclusions:<br>• The control Cp is the same in all samples (approx. 27) ⇒ There are no inhibitory components in the cell material.<br>• Differences in fluorescence signal levels seen during late stages of PCR are due to competition between target and control. Therefore, analysis is only possible for the diluted samples.<br>**Example:** In position 9, 100 ng target RNA out-competes the control completely ⇒ No analysis is possible in this sample. |

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**Application 3**

A cDNA for TNF-α (tumor necrosis factor alpha) was transcribed from total RNA of stimulated cells with the 1st Strand cDNA Synthesis Kit for RT-PCR and a TNF-α specific reverse primer. Then, the synthesized cDNA was quantified in the LightCycler. In this assay, a medium amount of internal control (monitored in channel 3) was used to ensure accurate quantification of the expected target values. Since the detectable concentration range is only approx. 3 magnitudes, samples containing very high target concentrations were diluted for the run. False negatives were not a problem in this TNF-α detection.

**Results:** The Cp of the internal control was identical in all quantified samples (see F3 data below). This proves that the TNF-α concentrations (target values in F2) determined for the sample material were reliable.

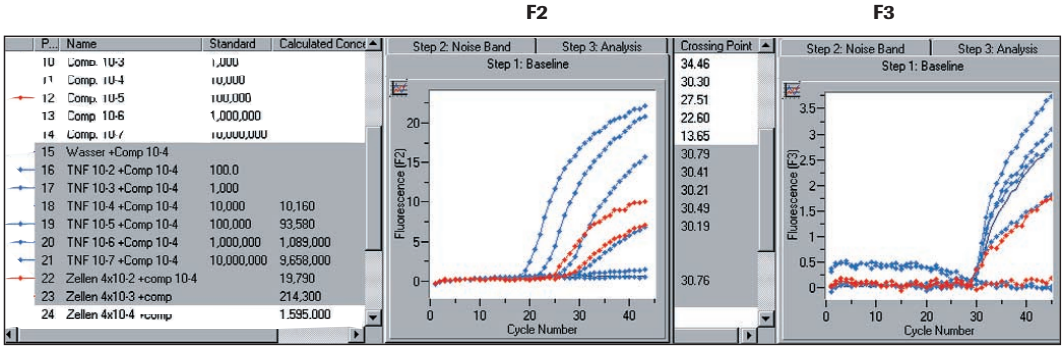


**Fig. 8: Application 3 TNF-α**

| | |
|---|---|
| Standard | *In vitro* RNA transcript containing a 385bp fragment of the original target (TNF-α) sequence; detected (in channel 2) with TNF-specific Hybridization Probes that were labeled with Fluorescein/LC Red 640;<br>**Amounts** used: 2 µl standards containing $10^7$–$10^2$ copies. |
| Control | *In vitro* RNA transcript that includes both TNF-specific primer binding sites flanking a stuffer fragment; detected (in channel 3) with control-specific Hybridization Probes that were labeled with Fluorescein/LC Red 705.<br>**Amount** added to each standard and each target: 2 µl control containing $10^4$ copies. |
| Samples | Total RNA isolated (with a High Pure kit) from 2 x $10^5$ stimulated cells; detected (in channel 2) with TNF-specific Hybridization Probes that were labeled with Fluorescein/LC Red 640.<br>**Amounts:** 2 µl samples containing cDNA from either 4 x $10^2$ cells or 4 x $10^3$ cells. |
| Analysis in F2: target | Fluorescence values of the TNF-α target (LC Red 640) are visible in F2. Results:<br>• The standards (positions 16–21) are used to quantify the concentration of TNF-α in the cells (positions 22–23) ⇒ Assayed concentration of TNF-α was approx. 20,000 copies in 4 x $10^2$ and 200,000 in 4 x $10^3$ cells. |
| Analysis in F3: control | Fluorescence values of the internal control (LC Red 705) are visible in F3. Conclusions:<br>• The control Cp is the same in all samples (approx. 30) ⇒ There are no inhibitory components in the cell material.<br>• Differences in fluorescence signal levels seen during late stages of PCR are due to competition between target and control. The sample concentration in position 23 (4 x $10^3$/2 µl) out-competes the control completely. ⇒ Accurate quantification analysis is only possible for the sample in position 22 (4 x $10^2$/2 µl). |

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**Detection of PCR Inhibition**

- In the application 1 in this section, only samples which were negative for the target PCR (detected in channel 2) require an internal control. Since factors that inhibit PCR will affect not only the target, but also the internal control fragment, a negative result also for the control PCR (detected in channel 3) would identify an inhibitor in the respective sample material.

- In the examples 2 and 3 in this section, the reliability of the quantification of a target needs to be controlled. If an inhibitor were present in one of the unknown samples, the analysis of the internal control data (in channel 3) would detect it. The Cp of that sample would be shifted towards higher cycle numbers, *i.e.* its Cp would be higher (usually $>$ 1 cycle) than the Cp of the control in all the standard samples.

**What To Do If a Sample Contains an Inhibitor**

Theoretically, to eliminate the effects of the inhibitor, you could dilute the sample containing the inhibitor and repeat the PCR with the higher dilution. However, we recommend that you repeat the sample preparation step, *e.g.* with our Roche High Pure kits, to eliminate the inhibitor.

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