



LightCycler

## Optimization Strategy

### Introduction

The LightCycler system provides different detection formats for real-time PCR and various applications are easily established.

**Note:** For detailed information on setting up new LightCycler applications, refer to Technical Notes No. LC 1/99–6/99, as well as the pack inserts for the LightCycler PCR and RT-PCR Kits. In most cases, new applications give satisfactory results after a few optimization steps.

However, every successful PCR depends on many factors and some of them may not be optimal during these initial experiments. Then you will need to follow an optimization strategy to achieve a successful LightCycler PCR.

### Purpose of this Note

This LightCycler Technical Note gives guidelines for a step-by-step optimization procedure that will help you achieve the best possible results from real-time PCR, regardless of the assay format you use.

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# 1. Establishing a LightCycler Assay: Initial Optimization

## Introduction

The LightCycler system has a broad application range and provides two different detection formats for real-time PCR:

- SYBR Green I (for sequence-independent detection of DNA and RNA)
- Hybridization Probes (for sequence-specific detection of DNA and RNA)

**Note:** For all formats, optimized ready-to-use Roche LightCycler Kits are available (see Section 5).

This section gives:

- A brief summary of standard conditions and optimization steps to use when establishing a new LightCycler application (in any format), and
- Guidelines for determining whether results are satisfactory.

## Standard Conditions

In initial runs of any LightCycler application, use the following standard conditions:

- Primers: Start with 0.5  $\mu\text{M}$  for each primer.
- If using Hybridization Probes: Start with 0.2  $\mu\text{M}$  for each probe.

**Note:** Always use highly purified primers and Hybridization Probes (HPLC).

- Controls:
  - Always include a no template control (NTC) for each primer pair.
  - Include a positive control (if one is available).
  - Include a DNA contamination control (RT-minus control) in one-step RT-PCR applications.

**Note:** How to run a RT-minus control reaction see Section 3.1 and 3.3

## Step 1: Optimize Basic Parameters

When establishing a new LightCycler application always optimize the parameters in the table below.

Optimize this parameter...	By doing the following
MgCl <sub>2</sub> Concentration	<p>Amplify target in the presence of different MgCl<sub>2</sub> concentrations, using the following concentration ranges:</p> <ul style="list-style-type: none"> <li>• For DNA assays in the SYBR Green I and the Hybridization Probe format: 2–5 mM MgCl<sub>2</sub></li> <li>• For RNA assays in the SYBR Green I and in the Hybridization Probe format: 4–8 mM MgCl<sub>2</sub></li> </ul> <p><b>Analysis:</b> The optimal MgCl<sub>2</sub> concentration will have the lowest crossing point (C<sub>p</sub>), the highest fluorescence intensity and the steepest curve slope.</p> <p><b>Note:</b> The optimal MgCl<sub>2</sub> concentration must be determined for each primer/probe set. Therefore we strongly recommend including a MgCl<sub>2</sub> titration (as outlined above) in the first experiment.</p>
Template Concentration	<p>Amplify a series of DNA template dilutions. At a minimum, test at least two different dilutions:</p> <ul style="list-style-type: none"> <li>• High concentration</li> <li>• Medium/low concentration</li> </ul> <p><b>Analysis:</b> The crossing point (C<sub>p</sub>) for DNA working template dilutions should be 10–30. Use higher dilutions if initial C<sub>p</sub> &lt; 10; use higher concentrations if initial C<sub>p</sub> &gt; 30.</p> <p><b>Note:</b> For detailed information see Technical Notes No. LC 2/99 – 5/99, as well as the pack inserts for the LightCycler PCR and RT-PCR Kits.</p>

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# 1. Establishing a LightCycler Assay: Initial Optimization, Continued

## Step 2: Are Results Satisfactory?

After optimization of  $MgCl_2$  concentration and template concentration, determine whether fluorescence signals generated during the assay are satisfactory. That is:

For this format...	Is the increase in fluorescence...
SYBR Green I format	More than twofold above background signal?
Hybridization Probe format	More than 0.3-fold above background signal?

- If the answer to the above question is Yes, the assay is satisfactorily established. The assay performance can be improved by optimizing the further parameters mentioned below.
- If the answer to the above question is No, the fluorescence signals are very weak and results are unsatisfactory. We strongly recommend following the complete optimization strategy outlined in Sections 2 and 3 of this Note.

**Exception:** Performing mutation analysis assays, the PCR fluorescence signal can be low due to the low melting point ( $= T_m$ ) of the Hybridization Probes. Nevertheless, signals generated during a melting curve analysis can successfully be used to analyze the mutations.

## Further Optimization Parameters

**Note:** There are other steps that can be taken to improve assay performance. These include varying concentrations of primers (and Hybridization Probes, if applicable), varying annealing temperature, and using a Hot Start to reduce primer-dimer formation and improve sensitivity. These will be discussed in detail in later sections of this Note.

## 2. Establishing Optimal PCR Conditions for DNA Assays in the SYBR Green I Format

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### Introduction

The following optimization strategy is highly recommended if the results of the first experiments of a new LightCycler application are not satisfying and cannot be improved by optimization of basic parameters (see Section 1).

Regardless of the assay format that you will eventually use, we recommend that you begin optimization of your LightCycler assay by establishing optimal PCR conditions in the SYBR Green I format.

This section outlines a step by step optimization procedure for assays of a DNA (or cDNA template) in the SYBR Green I format. This optimization procedure will help you identify potential problems and develop a successful LightCycler PCR.

**Note:** Once you have optimized conditions for amplification of DNA/cDNA in the SYBR Green I format, you can easily adapt those conditions to other assay formats by performing minor additional steps. These steps are outlined in detail in Section 3 of this note.

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### Reagent for Best Results

For best results, use this Roche LightCycler Kit:  
LightCycler – DNA Master SYBR Green I,  
Cat. No. 2 015 099 (for 96 rxn) or Cat. No. 2 158 817 (for 480 rxn)

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### Use DNA/cDNA as a Template

- Always use DNA/cDNA as starting material.
- Real-time PCR with the LightCycler is a powerful tool to monitor amplification, but it is not possible to monitor an RT reaction directly. Even if the final application will be a one-step RT-PCR we recommend establishing optimal PCR conditions with a cDNA as template.

**Note:** For cDNA synthesis reagents see Section 5.

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### Controls To Use

- Always include a no template control (NTC) for each primer pair.
  - Include a positive control (if one is available).
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## 2. Establishing Optimal PCR Conditions for DNA Assays in the SYBR Green I Format, Continued

### Parameters to Optimize

To get the best PCR results with a DNA/cDNA template in the SYBR Green I format, optimize the parameters in the table below.

**Note:** A MgCl<sub>2</sub> titration should always be the first experiment.

Optimize this parameter...	By doing the following
MgCl <sub>2</sub> Concentration	<p>Amplify target in the presence of different MgCl<sub>2</sub> concentrations, (between 1 and 5 mM MgCl<sub>2</sub>).</p> <p><b>Note:</b> The 10x conc. LightCycler – DNA Master SYBR Green I contains 10 mM MgCl<sub>2</sub>.</p> <p><b>Analysis:</b> The optimal MgCl<sub>2</sub> concentration will have the lowest crossing point (Cp), the highest fluorescence intensity and the steepest curve slope. Most primer-template sets require 2 – 4 mM MgCl<sub>2</sub>.</p> <p><b>Tip:</b> To identify a primer dimer peak, run the NTC in the presence of the highest MgCl<sub>2</sub> concentration used in this MgCl<sub>2</sub> optimization assay.</p> <p><b>Note:</b> Figure 1 illustrates the effect of MgCl<sub>2</sub> concentration (see Section 2.1)</p>
Template Concentration	<p>Amplify a series of DNA template dilutions to determine the one that gives optimal amplification. At a minimum, test 2–3 template dilutions (high/medium/low) within these ranges:</p> <ul style="list-style-type: none"> <li>• Genomic DNA: 50 ng – 5 pg</li> <li>• Plasmid DNA: approx. 10<sup>6</sup> copies</li> <li>• cDNA: 2 µl of original reverse transcription product; then 1:10 and 1:100 dilutions of the original</li> </ul> <p><b>Analysis:</b> The Cp for DNA working template dilutions should be 10–30. Use higher dilutions if initial Cp &lt; 10; use higher concentrations if initial Cp &gt; 30</p> <p><b>Note:</b> Figure 2 illustrates the effect of template concentration (see Section 2.1)</p>
PCR Inhibitors	<p>Minimize the effects of possible inhibitors by diluting the sample.</p> <p><b>Exception:</b> Some template preparations might contain low amounts of template but high concentrations of inhibitor. In such cases, dilution cannot solve the problem since diluted samples will have inadequate sensitivity. In this case, we recommend repurification of the template, e.g. with a Roche High Pure kit.</p> <p><b>Note:</b> Figure 3 shows an example for the presence of amplification inhibitors (see Section 2.1)</p>
Primer Concentration	<ul style="list-style-type: none"> <li>• For initial experiments, use a standard concentration of 0.5 µM for each primer. This concentration will work for most amplifications.</li> <li>• Vary primer concentration between 0.3 and 1.0 µM to see if these concentrations give better results than the standard primer concentration.</li> </ul> <p><b>Note:</b> Low primer concentrations may be exhausted before the reaction is completed, resulting in lower yields of desired product. High primer concentrations may promote mispriming and accumulation of non-specific product.</p>

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## 2. Establishing Optimal PCR Conditions for DNA Assays in the SYBR Green I Format, Continued

### Parameters to Optimize (continued)

Optimize this parameter...	By doing the following
Primer Purity	Use highly purified PCR primers to avoid potential problems (e.g. diminished sensitivity due to primer dimer formation). <b>Note:</b> Figure 4 illustrates the effect of impure primers (see Section 2.1)
Annealing Temperature	<ul style="list-style-type: none"> <li>For initial experiments, set the primer annealing temperature 5°C below the calculated primer <math>T_m</math>.</li> </ul> <b>Note:</b> See Section 5 for information on calculating $T_m$ . <ul style="list-style-type: none"> <li>Vary the annealing temperature in 1°–2°C steps.</li> </ul> <b>Note:</b> The actual annealing temperature of primers during PCR has to be determined empirically. It may differ from the $T_m$ calculated from the primer sequence. <b>Note:</b> Figure 5 shows an example with an additional product peak due to secondary priming site (see Section 2.1)
Hot Start	If primer dimers form and the sensitivity of the assay is low, try a "Hot Start" method by using either: <ul style="list-style-type: none"> <li>LightCycler – FastStart DNA Master SYBR Green I, Cat. No. 3 003 230 (96 rxn) or Cat. No. 2 239 264 (480 rxn), in place of the LightCycler – DNA Master SYBR Green I, or</li> <li>Commercially available anti-Taq DNA polymerase antibodies (e.g. TaqStart Antibody from Clontech, Cat. No. 5400-1 or 5400-2).</li> </ul> <b>Note:</b> See Section 5 for information on primer design. <b>Note:</b> Figure 6 shows an example of prevention of primer dimer formation by using a "Hot Start" method (see Section 2.1)

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## 2. Establishing Optimal PCR Conditions for DNA Assays in the SYBR Green I Format, Continued

### Programming the Run

Use the following program settings for the LightCycler optimization experiments:

For this step...	Use
Initial Denaturation Amplification	<ul style="list-style-type: none"> <li>• 2 min at 95 °C</li> <li>• Denaturing: 0 s at 95°C If the template is GC rich increase up to 5 s</li> <li>• Annealing: 5 s at annealing temperature (depends on primers, see "Parameters to Optimize" above)</li> <li>• Elongation temperature: 72 °C</li> <li>• Elongation time (s) = amplicon length (bp) ÷ 25</li> <li>• Ramp rate: Reduce to 2–5 °C if annealing temperature is low (&lt;55°C)</li> <li>• Acquisition mode: single (at the end of elongation)</li> </ul>
Melting Curve	<ul style="list-style-type: none"> <li>• Annealing: 30 s at 65°C</li> <li>• Ramp rate 0.1°C/s with Acquisition mode: cont., or Ramp rate 0.3°C/s with Acquisition mode: step</li> </ul>
Gain Settings	<ul style="list-style-type: none"> <li>• F1 = 5</li> <li>• F2 = 15</li> <li>• F3 = 30</li> </ul>

**Note:** For detailed information about programming, see Technical Note No. LC 2/99, the pack insert for LightCycler – DNA Master SYBR Green I and the LightCycler Operator's Manual.

### Analysis of Results

Analyze the results by:

- **Quantification Analysis**, to determine the optimal MgCl<sub>2</sub> concentration and the optimal template working concentrations.
- **Melting Curve Analysis**, to identify specific PCR products and primer dimers (by differences in T<sub>m</sub>).  
**Note:** Product T<sub>m</sub> is a function of GC content and length. Primer dimers will always have a lower T<sub>m</sub> (generally between 75° and 82°C) than specific PCR products. These dimers are most likely to form in the NTC and in samples with low template concentrations.
- **Agarose Gel Electrophoresis**, to correlate product length with melting peaks and identify product and primer dimer bands.  
**Note:** Electrophoresis separates PCR products only by length, whereas Melting Curve Analysis resolves by GC content **and** length.  
**Tip:** When optimizing PCR conditions for DNA assays in the SYBR Green I format we recommend loading all samples from the first LC run onto an agarose gel containing EtBr. Start gel electrophoresis immediately after the LC run (or store samples at 4°C until they can be analyzed).

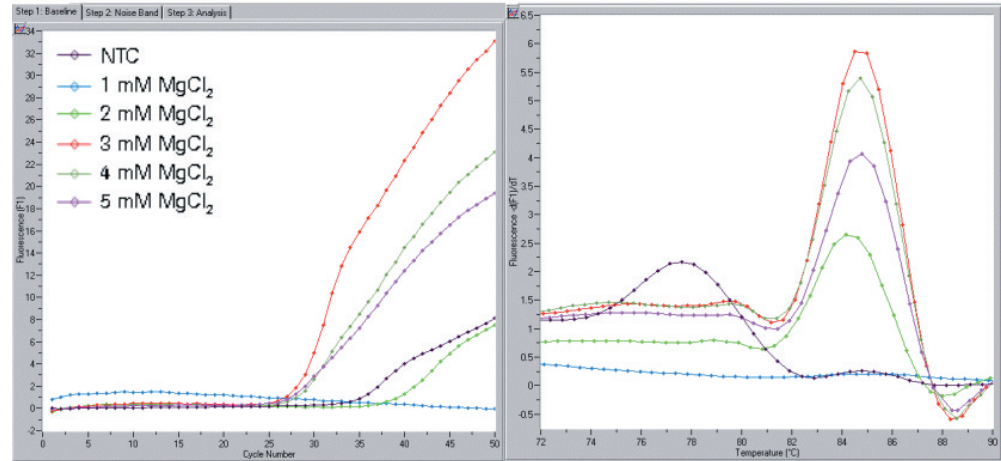
## 2.1 Examples of Optimization Effects

### Introduction

The following examples will illustrate some results and effects that are frequently seen during optimization.

### Example 1: MgCl<sub>2</sub>

MgCl<sub>2</sub> concentration greatly affects amplification efficiency. In the example below MgCl<sub>2</sub> was varied between 1 and 5 mM.

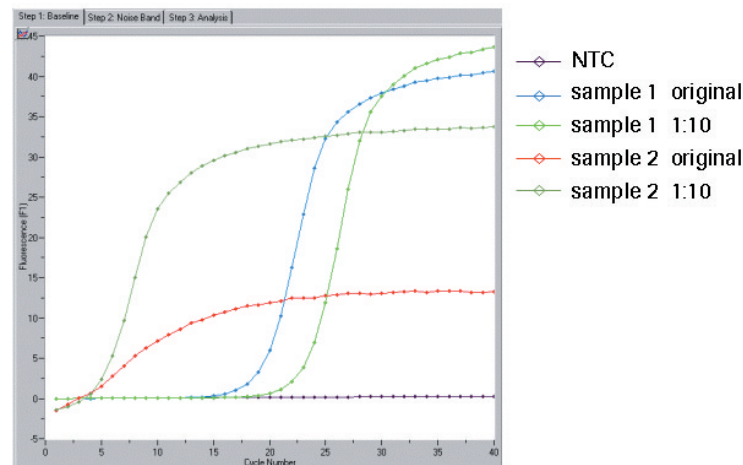


**Figure 1:** Effect of MgCl<sub>2</sub> Concentration.

**Result:** In this example a mid-range MgCl<sub>2</sub> concentration (3 mM) gives the best result with respect to crossing point (Cp), signal intensity and slope of the curve (left panel). As illustrated by the melting curve data (right panel), only primer dimers are formed in the NTC (in the presence of 5 mM MgCl<sub>2</sub>).

### Example 2: Template

A high template concentration can lead to an early reaction plateau and results that are difficult to interpret as in the example below.



**Figure 2:** Effect of Template Concentration.

**Result:** Sample 1 shows a C<sub>p</sub> of 18; its 1:10 dilution shows a C<sub>p</sub> shift, as expected. In contrast, sample 2 shows an early C<sub>p</sub> (<5), a flat curve and a very early plateau; in addition, its 1:10 dilution shows no C<sub>p</sub> shift. These results show that the concentrations of both the original sample 2 and its dilution were too high.

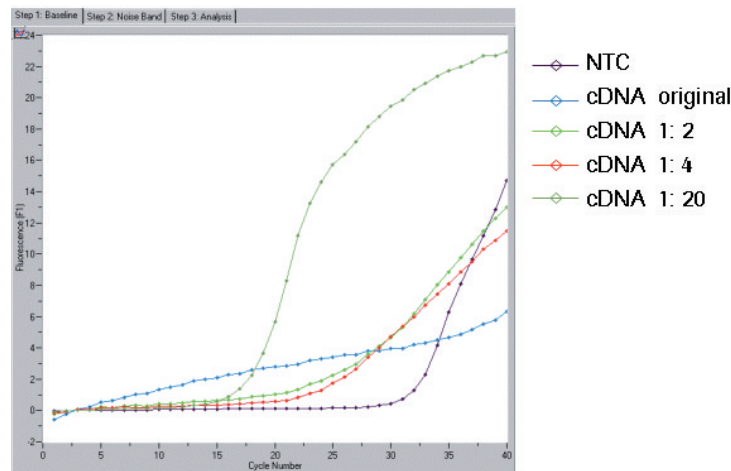
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## 2.1 Examples of Optimization Effects, Continued

### Example 3: Inhibitors

Some sample preparations contain compounds that can inhibit PCR, *e.g.* ethanol or anticoagulants (citrate, EDTA and heparin) in blood preparations. The example below shows that even some cDNA preparations can contain inhibiting factors.

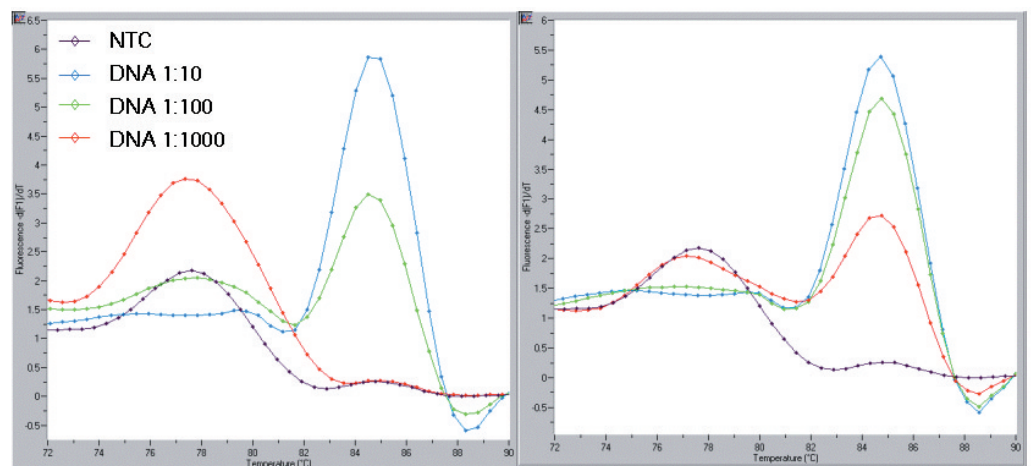


**Figure 3:** Presence of Amplification Inhibitors

**Result:** The undiluted template shows a very flat curve and almost no amplification. The 1:2 and the 1:4 dilutions perform slightly better but still show flat curves without reaching a plateau. Only the 1:20 dilution is amplified correctly (since the inhibiting factors in the sample material have been diluted).

### Example 4: Primer Purity

Impure primers can lead to diminished sensitivity because they promote primer dimer formation. The example below shows amplification with the same pair of primers before and after the primers were HPLC purified.



**Figure 4:** Effect of Impure Primers.

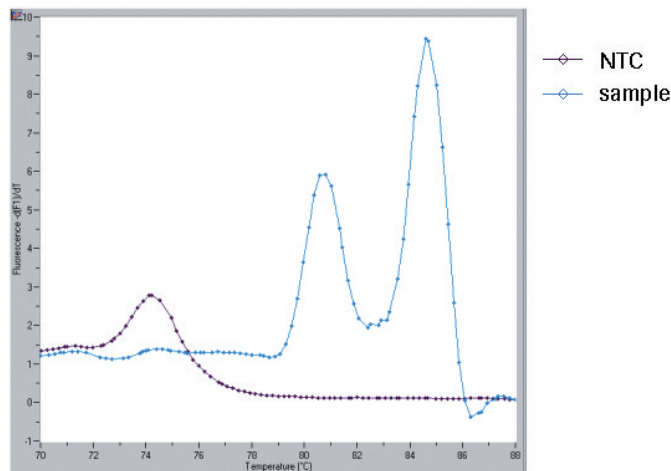
**Result:** Impure primers cause significant primer dimer formation (1:1000 template dilution in the left panel). By contrast, the same primers, after purification promote formation of specific PCR product, even when template concentration is low (1:1000 template dilution in the right panel).

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## 2.1 Examples of Optimization Effects, Continued

### Example 5: Additional Products

Melting Curve Analysis allows differentiation of specific PCR product from non-specific products, such as primer dimers. The example below shows formation of an unusual double product peak in addition to the primer dimer peak.



**Figure 5:** Additional Product Peak Due to Secondary Priming Site.

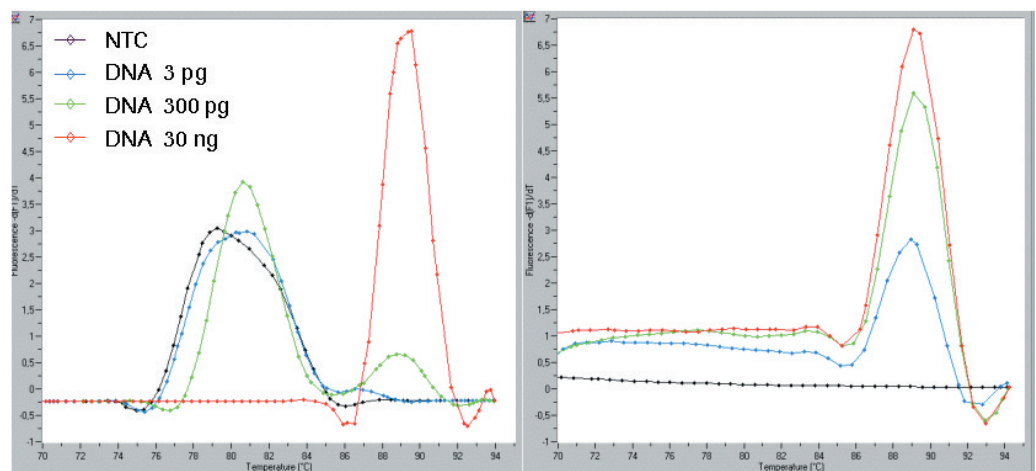
**Conclusion:** Additional product peaks are caused by mispriming or priming at a secondary site. These secondary priming events can be reduced by increasing the stringency of the PCR or by using a new set of PCR primers.

**Note:** Some housekeeping genes (e.g. GAPDH, HPRT and  $\beta$ -Actin) are known to have pseudogenes that contain altered internal sequences but can be amplified by the same primer pair. Amplification of pseudogenes cannot be eliminated by higher stringency. However, redesigning the primers so they recognize a different priming region may help.

**Tip:** Use an electrophoretic gel to analyze PCR products showing unusual or unexpected peak formation. Melting Curve Analysis resolves PCR products by GC content **and** length, whereas electrophoresis separates only by length.

### Example 6: Hot Start

Suboptimal primers can form primer dimers during reaction setup at room temperature (and even at 4°C). Primer dimer formation leads to decreased sensitivity. Nonspecific primer elongation and the resulting primer dimers can be avoided by using a "Hot Start" method as shown below.



without Hot Start

with FastStart

**Figure 6:** Prevention of Primer Dimer Formation by Using a Hot Start Method.

### 3. Adaptation of Optimal PCR Conditions to Other Formats

#### Introduction and Overview

Once PCR conditions are optimized for amplification of DNA/cDNA in the SYBR Green I format, adaptation of these conditions to another format requires only minor additional steps. For each format, the table below lists the additional parameters and the section of this note that describe the adaptation.

This assay format..	Requires adaptation of these parameters	See Section
DNA assays, SYBR Green I format	No further optimization required	2
RNA assays, SYBR Green I format	<ul style="list-style-type: none"> <li>• MgCl<sub>2</sub> concentration</li> <li>• Reverse transcription</li> </ul>	3.1
DNA assays, Hybridization Probe format	<ul style="list-style-type: none"> <li>• MgCl<sub>2</sub> concentration</li> <li>• Hybridization Probes</li> </ul>	3.2
RNA assays, Hybridization Probe format	<ul style="list-style-type: none"> <li>• MgCl<sub>2</sub> concentration</li> <li>• Reverse transcription</li> <li>• Hybridization Probes</li> </ul>	3.3

### 3.1 Adaptations for RNA Assays in the SYBR Green I Format

#### Introduction

After optimal PCR conditions for a cDNA template are established (Section 2), adaptation of the conditions to one-step RT-PCR requires two additional optimization steps as well as several other reaction modifications. This section explains the additional steps needed for optimization of one-step RT-PCR.

#### Reagent

For best results, use this Roche LightCycler Kit:  
LightCycler – RNA Amplification Kit SYBR Green I, Cat. No. 2 015 137 (for 96 rxn)

#### Parameters to Optimize

To adapt the optimal PCR conditions determined in Section 2 to one-step RT-PCR, optimize the parameters in the table below.

Optimize this parameter...	By doing the following
MgCl <sub>2</sub> Concentration	<p>Amplify target in the presence of different MgCl<sub>2</sub> concentrations, (between 4 and 8 mM MgCl<sub>2</sub>).</p> <p><b>Note:</b> The 10x conc. LightCycler – RNA Amplification Kit SYBR Green I contains 30 mM MgCl<sub>2</sub>.</p>
Reverse Transcription	<p>Test a series of RNA template dilutions for optimal RT-PCR amplification. At a minimum, test at least two different template dilutions within the following ranges:</p> <ul style="list-style-type: none"> <li>• 1 pg – 1 µg total RNA</li> <li>• 1 pg – 1 µg mRNA</li> </ul> <p><b>Note:</b> For low template concentrations (&lt;10 ng/µl), add 10 ng/µl MS2 or alternative RNA as carrier.</p>

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## Controls To Use

- Always include a no template control (NTC) for each primer pair.
- Run a positive control in each experiment (if one is available).
- Include a DNA contamination control (RT-minus control) to check for possible DNA contamination in the template preparation.  
**Note:** For the RT-minus control reaction, in which the reverse transcriptase is absent, do one of the following:
  - Use the LightCycler – DNA Master SYBR Green I instead of the LightCycler – RNA Amplification Kit SYBR Green I, or
  - Use 1–1.5 U Taq DNA polymerase/20 µl reaction in place of the LightCycler – RT-PCR Enzyme Mix.

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## Programming the Run

Use the following program parameters for the LightCycler run:

For this step...	Use the following settings
Reverse Transcription	10 min at 55°C
Amplification	Same conditions that gave optimal results in Section 2 with cDNA as template.
Melting Curve	Same conditions that gave optimal results in Section 2 with cDNA as template.
Seek Process	At 55°C. <b>Note:</b> Select the proper temperature in the left corner box of the "Edit Sample" screen (Loading Screen).

**Note:** For detailed information about programming, see Technical Note No. LC 4/99, the pack insert for LightCycler – RNA Amplification Kit SYBR Green I and the LightCycler Operator's Manual

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## Troubleshooting the Reaction

If you are not satisfied with the sensitivity of the assay, try the following:

- If the template is GC rich:
    - Increase reverse transcription time to 30 min at 55°C
  - Use carrier RNA (e.g. 10 ng/µl MS2 RNA or alternative RNA) for all RNA dilutions.
-

## 3.2 Adaptations for DNA Assays in the Hybridization Probe Format

### Introduction

After optimal PCR conditions for a DNA template are established (Section 2), adaptation of the conditions to the Hybridization Probe format requires two additional optimization steps as well as several other reaction modifications. This section explains the additional steps needed for DNA assays in the Hybridization Probe format.

### Reagents

For best results, use these Roche LightCycler Kits:

- For regular PCR:  
LightCycler – DNA Master Hybridization Probes,  
Cat. No. 2 015 102 (for 96 rxn) or Cat. No. 2 158 825 (for 480 rxn)
- For Hot Start applications:  
LightCycler – FastStart DNA Master Hybridization Probes,  
Cat. No. 3 003 248 (for 96 rxn), Cat. No. 2 239 272 (for 480 rxn)

### Parameters to Optimize

To adapt the optimal PCR conditions determined in Section 2 to assay of DNA with Hybridization Probes, optimize the parameters in the table below.

Optimize this parameter...	By doing the following
MgCl <sub>2</sub> Concentration	Increase the MgCl <sub>2</sub> concentration from the SYBR Green I optimum (determined in Section 2) in 0.5–1 mM steps. <b>Note:</b> Do not increase the concentration higher than 2 mM over the SYBR Green I optimum.
Hybridization Probe Concentration	<ul style="list-style-type: none"> <li>• For initial experiments, use 0.2 μM for each Hybridization Probe.</li> <li>• Try concentrations up to 0.4 μM (for one or both Hybridization Probes) to improve signal intensity.</li> </ul> <b>Note:</b> For selection of Hybridization Probe Sequences, follow guidelines in Technical Note No. LC 6/99.

### Controls To Use

- Always include a no template control (NTC) for each primer pair.
- Run a positive control in each experiment (if one is available).

### Programming the Run

Use the following program parameters for the LightCycler run:

For this step...	Use the following settings
Amplification	Basically the same conditions that gave optimal results in Section 2 with DNA as template, except: <ul style="list-style-type: none"> <li>• Use an annealing time of 5–15 seconds.</li> <li>• Acquire signal at the end of annealing instead of elongation.</li> </ul>
Melting Curve	<ul style="list-style-type: none"> <li>• Annealing: 30 s – 2 min at 45°C</li> <li>• Ramp rate: 0.1°C/s</li> <li>• Acquisition mode: cont.</li> </ul> <b>Note:</b> Melting curves only required for mutation analysis.
Gain Settings	<ul style="list-style-type: none"> <li>• F1 = 1</li> <li>• F2 = 15</li> <li>• F3 = 30</li> </ul>

**Note:** For detailed information about programming, see Technical Note No. LC 3/99, the pack inserts for LightCycler – DNA Master Hybridization Probes and LightCycler – FastStart DNA Master Hybridization Probes.

### Problems with Hybridization Probes

If optimized conditions developed with SYBR Green I do not give satisfactory results in the presence of Hybridization Probes, the most probable cause is the Hybridization Probes themselves. For strategies to solve these problems see Section 4 of this note.

## 3.3 Adaptation for RNA Assays in the Hybridization Probe Format

### Introduction

After optimal PCR conditions for a cDNA template are established (Section 2), adaptation of the conditions to one-step RT-PCR in the Hybridization Probe format requires three additional optimization steps as well as several other reaction modifications. This section explains the additional steps needed for one-step RT-PCR assays in the Hybridization Probe format.

### Reagent

For best results, use this Roche LightCycler Kit:  
LightCycler – RNA Amplification Kit Hybridization Probes  
Cat. No. 2 015 145 (for 96 rxns)

### Parameters to Optimize

To adapt the optimal PCR conditions determined in Section 2 to assay of RNA (one-step RT-PCR) with Hybridization Probes, optimize the parameters in the table below.

Optimize this parameter...	By doing the following
MgCl <sub>2</sub> Concentration	Amplify target in the presence of different MgCl <sub>2</sub> concentrations, (between 4 and 8 mM MgCl <sub>2</sub> ). <b>Note:</b> The 10x conc. LightCycler – RNA Amplification Kit Hybridization Probes contains 30 mM MgCl <sub>2</sub> .
Reverse Transcription	Test a series of RNA template dilutions for optimal RT-PCR amplification. At a minimum, test at least two different template dilutions within the following ranges: <ul style="list-style-type: none"> <li>• 1 pg – 1 µg total RNA</li> <li>• 1 pg – 1 µg mRNA</li> </ul> <b>Note:</b> For low template concentrations (<10 ng/µl), add 10 ng/µl MS2 or alternative RNA as carrier.
Hybridization Probe Concentration	<ul style="list-style-type: none"> <li>• For initial experiments, use 0.2 µM for each Hybridization Probe.</li> <li>• Try concentrations up to 0.4 µM (for one or both Hybridization Probes) to improve signal intensity.</li> </ul> <b>Note:</b> For selection of Hybridization Probe Sequences, follow guidelines in Technical Note No. LC 6/99.

### Controls To Use

- Always include a no template control (NTC) for each primer pair.
- Run a positive control in each experiment (if one is available).
- Include a DNA contamination control (RT-minus control) to check for possible DNA contamination in the template preparation.  
**Note:** For the RT-minus control reaction, in which the reverse transcriptase is absent, do one of the following:
  - Use the LightCycler – DNA Master Hybridization Probes instead of the LightCycler – RNA Amplification Kit Hybridization Probes, or
  - Use 1–1.5 U Taq DNA polymerase/20 µl reaction in place of the LightCycler – RT-PCR Enzyme Mix.

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## 3.3 Adaptation for RNA Assays in the Hybridization Probe Format, Continued

### Programming the Run

Use the following program parameters for the LightCycler run:

For this step...	Use the following settings
Reverse Transcription	10 min at 55°C
Amplification	Basically the same conditions that gave optimal results in Section 2 with cDNA as template, except: <ul style="list-style-type: none"><li>• Use an annealing time of 5–15 seconds.</li><li>• Acquire signal at the end of annealing instead of elongation.</li></ul>
Gain Settings	<ul style="list-style-type: none"><li>• F1 = 1</li><li>• F2 = 15</li><li>• F3 = 30</li></ul>
Seek Process	At 55°C. <b>Note:</b> Select the proper temperature in the left corner box of the "Edit Sample" screen (Loading Screen).

**Note:** For detailed information about programming, see Technical Note No. LC 5/99 and the pack insert for LightCycler – RNA Amplification Kit Hybridization Probes.

### Troubleshooting the Reaction

If you are not satisfied with the sensitivity of the assay, try the following:

- If the template is GC rich:
  - Increase reverse transcription time to 30 min at 55°C
- Use carrier RNA (e.g. 10 ng/μl MS2 RNA or alternative RNA) for all RNA dilutions.

### Problems with Hybridization Probes

If optimized conditions developed with SYBR Green I do not give satisfactory results in the presence of Hybridization Probes, the most probable cause is the Hybridization Probes themselves. For strategies to solve these problems, see Section 4 of this note.

## 4. Appendix: Hybridization Probe Evaluation

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### Introduction

After you adapt your optimized PCR conditions to the Hybridization Probes format, your results may still not be comparable to those obtained with the SYBR Green I format. If this occurs, there is strong evidence that the Hybridization Probes are the cause.

This section explains how to prevent the two most common problems encountered with Hybridization Probes:

- Formation of primer-probe dimers due to suboptimal Hybridization Probe design, and
  - Hybridization Probe elongation due to incomplete or missing phosphorylation.
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### Preventing Primer-Probe Dimer Formation

Hybridization Probe sequences that can hybridize with the 3' termini of PCR primers can cause primer elongation, thus leading to amplification of primer-probe dimers. During PCR, amplification of primer-probe dimers competes with amplification of specific product, leading to reduced amplification efficiency.

To prevent primer-probe dimer formation, avoid complementarity of primers and probes by following the guidelines in Technical Note No. LC 6/99.

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### Preventing Probe Elongation

Because Hybridization Probes have a higher melting temperature than the primers, these probes could also serve as primers for de novo synthesis during amplification.

To prevent elongation of the probes during PCR, always phosphorylate the 3' end of the acceptor probe (*i.e.* the one that is labeled with a LightCycler RED dye at its 5' end).

If phosphorylation is not done or incomplete, the acceptor probe will serve as a PCR primer. The amplification product will appear in the PCR as an additional product, shorter than the desired product.

**Note:** The donor probe cannot be elongated since the 3' end of that probe is blocked by the fluorescein label.

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## 4. Appendix: Hybridization Probe Evaluation, Continued

### Testing for Hybridization Probe Problems

To test for problems with Hybridization Probes, do the following:

1. Compare the results of two LightCycler reactions performed in the SYBR Green I format. In one reaction, omit Hybridization Probes; in the second reaction, include both Hybridization Probes. Use the same primers in both reactions.
2. Set up two reactions, each of which contains only one of the Hybridization Probes and the respective reverse primer (*i.e.* the Hybridization Probes with + strand sequence together with the reverse primer or the Hybridization Probes with – strand sequence together with the forward primer).

For all four reactions listed in 1 and 2 above, use the following:

Format	SYBR Green I
Template	Use optimal DNA or cDNA dilution <ul style="list-style-type: none"> <li>• with both primers, without Hybridization Probes</li> <li>• with both primers and with both Hybridization Probes</li> <li>• with LC RED probe and the respective reverse primer</li> <li>• with Fluorescein probe and the respective reverse primer</li> </ul>
Controls	<ul style="list-style-type: none"> <li>• NTC</li> <li>• NTC with both Hybridization Probes</li> </ul>
Programming	<ul style="list-style-type: none"> <li>• Use optimized protocol for SYBR Green I</li> <li>• Display Mode: F1 / 1</li> </ul>
Gain Settings	<ul style="list-style-type: none"> <li>• <b>F1 = 3</b></li> <li>• F2 = 15</li> <li>• F3 = 30</li> </ul> <p><b>Note:</b> Gain reduction for F1 is necessary when detecting SYBR Green I in the presence of Hybridization Probes.</p>
Analysis of results	For each reaction, analyze the results by: <ul style="list-style-type: none"> <li>• <b>Melting Curve Analysis</b>, to identify products. Pay special attention to the appearance of "extra" peaks in addition to the expected product peak.</li> <li>• <b>Agarose Gel Electrophoresis</b>, to correlate product bands with melting peaks.</li> <li>• <b>Quantification Analysis</b>, to compare crossing points and signal intensities.</li> </ul>

## 5. Supplementary Information

### Other LightCycler Technical Notes

For more information on the LightCycler System, please see these earlier Technical Notes in the LightCycler Technical Note series:

No.	Title
LC 1/99	Optimization of Reactions to Reduce Formation of Primer Dimers
LC 2/99	Adaptation Protocol for Sequence-Independent Detection of DNA with SYBR Green I
LC 3/99	Adaptation Protocol for Sequence-Specific Detection of DNA with Hybridization Probes
LC 4/99	Adaptation Protocol for Sequence-Independent Detection of RNA with SYBR Green I
LC 5/99	Adaptation Protocol for Sequence-Specific Detection of RNA with Hybridization Probes
LC 6/99	Selection of Hybridization Probe Sequences for Use with the LightCycler
LC 7/99	Preparing LightCycler Data for Printing or Use in Other Programs
LC 8/99	Decrease of Fluorescent Signal in the Plateau Phase of a LightCycler PCR – Hook Effect

### LightCycler Reagents

For best results with LightCycler PCR, use the following Roche products:

- For sequence-independent detection of DNA:
  - LightCycler – DNA Master SYBR Green I,  
Cat. No. 2 015 099 (96 rxn) or Cat. No. 2 158 817 (480 rxn)
  - LightCycler – FastStart DNA Master SYBR Green I,  
Cat. No. 3 003 230 (96 rxn) or Cat. No. 2 239 264 (480 rxn)
- For sequence-independent detection of RNA:
  - LightCycler – RNA Amplification Kit SYBR Green I,  
Cat. No. 2 015 137 (96 rxn)
- For sequence-specific detection of DNA:
  - LightCycler – DNA Master Hybridization Probes,  
Cat. No. 2 015 102 (96 rxn) or Cat. No. 2 158 825 (480 rxn)
  - LightCycler – FastStart DNA Master Hybridization Probes,  
Cat. No. 3 003 248 (96 rxn) or Cat. No. 2 239 272 (480 rxn)
- For sequence-specific detection of RNA:
  - LightCycler – RNA Amplification Kit Hybridization Probes,  
Cat. No. 2 015 145 (96 rxn)

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## 5. Supplementary Information, Continued

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### Products for cDNA Synthesis

To produce cDNA templates, we recommend use of:

- First Strand cDNA Synthesis Kit for RT-PCR (AMV), Cat. No. 1 483 188,
- Expand Reverse Transcriptase, Cat. No. 1 785 826, or
- C. therm. Polymerase for Reverse Transcription in Two-Step RT-PCR, Cat. No. 2 016 311 used for GC-rich templates with specific or oligo (dT) priming.

**Note:** After completing the RT reaction, always denature the cDNA (5 min, 95 °C).

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### Design of Primers

For design of primers:

- Refer to Technical Note No. LC 1/99 and the LightCycler Operator's Manual for detailed information.
- Use primer design software, *e.g.*
  - OLIGO, from Molecular Biology Insights, Inc., Cascade, CO, USA (<http://www.olygo.net>), or
  - Medprobe, Oslo, Norway (<http://www.medprobe.com>)

**Notes:** 1. Do not let the amplicon length be >1000 bp.

For best results, we recommend amplicons < 500 bp.

2. Always use highly purified primers (HPLC).

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### Design of Hybridization Probes

For detailed information on design of Hybridization Probes refer to Technical Note No. LC 6/99.

**Notes:** 1. Avoid complementarity of PCR primers and Hybridization Probes.

2. Always use highly purified Hybridization Probes (HPLC).

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### Calculation of Melting Temperature ( $T_m$ )

To calculate  $T_m$  use:

- Programs for  $T_m$  calculation, *e.g.*
  - The  $T_m$  Utility from Idaho Tech., Inc., Salt Lake City, UT, USA (<http://www.idahotec.com>)
  - A JAVA Script available from TIB MOLBIOL, Berlin, Germany ([www.TIB-MOLBIOL.de/oligo\\_ag.html](http://www.TIB-MOLBIOL.de/oligo_ag.html)), or
- On-line oligonucleotide  $T_m$  calculators *e.g.*
  - (<http://alces.med.umn.edu/rawtm.html>)

**Note:** For calculation of the melting temperature ( $T_m$ ) of primers and Hybridization Probes, we strongly recommend using a program that can calculate  $T_m$ s thermodynamically, *i.e.* a program that takes into account all neighboring bases and not just the GC content.

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