Roche Applied Science
Technical Note No. LC 1/update 2002

LightCycler

Optimization of Reactions to Reduce Formation of Primer Dimers

Purpose of this Note

PCR is a powerful tool for amplification of nucleic acids. Non-specific amplification products such as primer dimers interfere with the reaction, leading to low level of specificity, loss of sensitivity and unsatisfactory quantification results.

This Technical Note describes several strategies for reducing the formation of primer dimers and increasing the stringency of LightCycler PCR.

Note: The reader of this note should be familiar with the display and analysis of data generated with the LightCycler Instrument, as described in the LightCycler Operator's Manual. For detailed information on optimization of PCR with the LightCycler System, see Roche Applied Science Technical Note No. LC 9/2001, *Optimization Strategy*.

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1. Identification of Primer Dimers

Introduction

Unspecific priming interferes with PCR. Even PCR reactions performed on block cycler instruments should be controlled for formation of unspecific by-products, such as primer dimers. The LightCycler System can perform rapid cycle real time PCR. However, for the fastest, most accurate amplification possible, optimized PCRs give the best results on the LightCycler Instrument. Optimization should include steps to reduce formation of primer dimers.

Primer Dimers are the product of nonspecific annealing and primer elongation events. These events take place as soon as PCR reagents are combined, especially if reagents are mixed at room temperature. Even if one primer is elongated by one false nucleotide, this primer may significantly enhance nonspecific amplification.

During PCR, formation of primer dimers competes with formation of specific PCR product, leading to reduced amplification efficiency and limited dynamic range. The result is a less successful PCR.

1.1 Visualization of Primer Dimers

Appearance of Primer Dimers

After PCR is performed in block cyclers, primer dimers appear as a smear of smaller amplicons (30 – 80 bp) that can be seen in high-resolution gel electrophoresis. In contrast, LightCycler PCR does not require gel electrophoresis since Melting Curve Analysis allows discrimination of primer and product peak in the presence of SYBR Green I (however, the content of the capillaries can also be loaded onto a gel).

Performed after amplification, melting curve analysis can identify both the desired PCR product and primer dimers by their unique melting peak behavior. Pure, homogenous PCR products usually produce a single, sharply defined melting curve with a narrow peak. Since they are smaller, primer dimers usually melt at a lower temperature than the product and have broader peaks (<80°C, depending on GC-content).

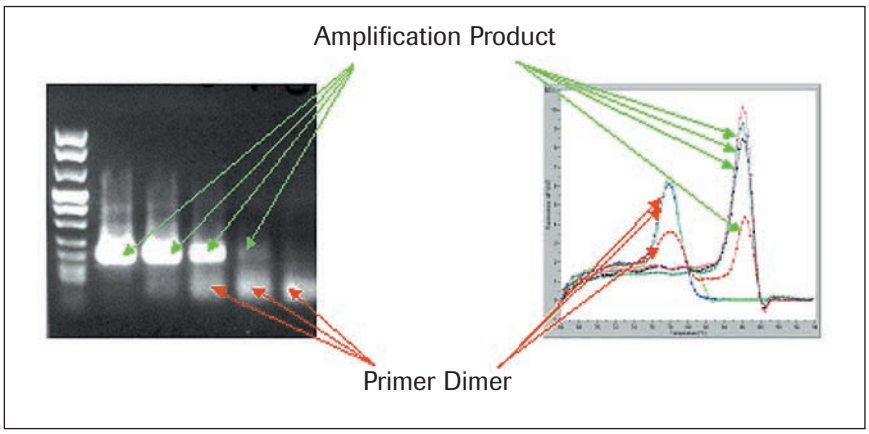

Figure 1: Identification of primer dimers in gel electrophoresis and LightCycler melting curve analysis.

1.2 Recommendations for Melting Curve Analysis

Melting Curve Analysis

Measurements during melting curve analysis are basically the inverse of those taken during LightCycler PCR. During PCR, fluorescence is initially low and increases during cycling. In contrast, at the beginning of a melting curve analysis, the reaction mix is at a low temperature, most nucleic acids are double-stranded and, consequently, the fluorescence signal is high. As the temperature steadily increases, the fluorescence will drop suddenly each time the temperature reaches the characteristic melting point (T_m) of a DNA fragment.

Melting curve analysis in the presence of SYBR Green I can resolve PCR products both by GC content and length, since both influence the T_m.

There are two general ways to acquire the data for a melting curve: continuously and stepwise.

Continuous Data Acquisition

In continuous mode, the instrument continuously measures the fluorescence of all samples as the temperature increases. It does this by reading the samples in sequence from the first to the last, then immediately starting again with the first. In most cases, a temperature transition rate of 0.1°C/s will produce good results in such analyses.

Note: The accuracy of the analysis will be inversely related to the number of capillaries analyzed, since it takes a fixed time to read each capillary. Thus, there will be more data points taken (and the analysis will be more accurate) when you analyze 10 capillaries than when you analyze 32. To increase the accuracy when analyzing 32 capillaries, reduce the temperature transition rate to, *e.g.*, 0.05°C/s.

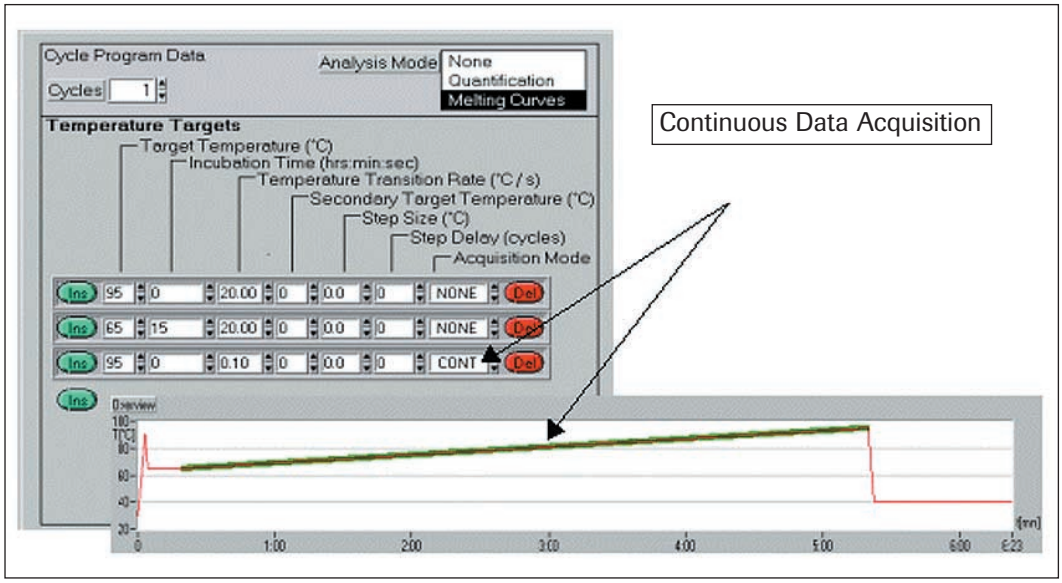

Figure 2: Data acquisition in continuous mode.

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Stepwise Data Acquisition

Data acquisition in stepwise mode is independent of the number of capillaries, since in this mode, the fluorescence in all capillaries is measured after each temperature transition. *E.g.*,the system will increase the temperature by 0.4°C, hold the new temperature until all samples are measured, then increase the temperature again. This mode will take longer than continuous data acquisition.

Note: Do not perform stepwise data acquisition at a temperature transition rate less than 0.3 - 0.4°C/s.

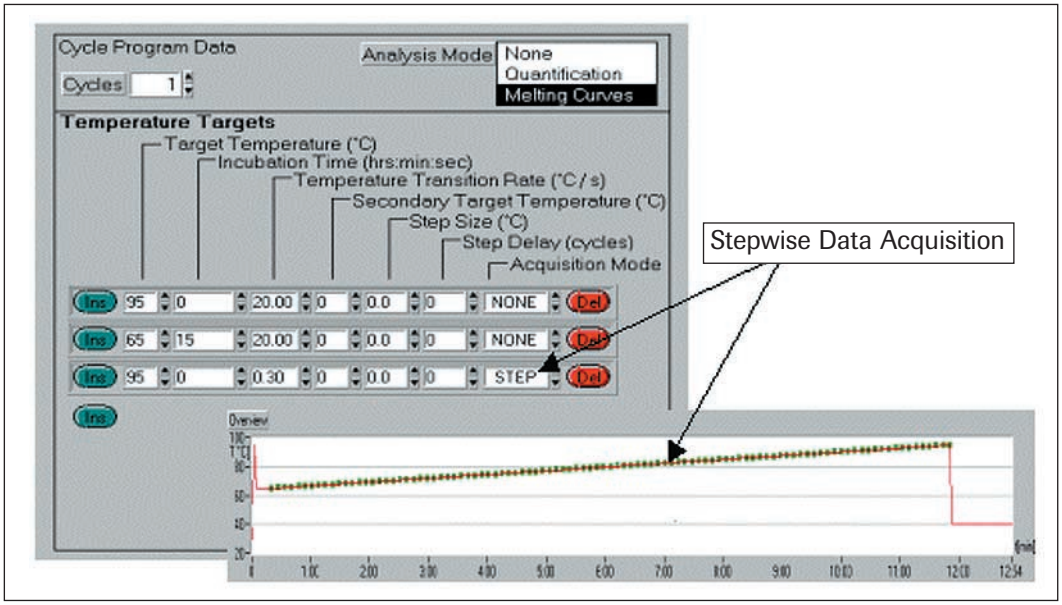

Figure 3: Data acquisition in stepwise mode.

Strategy to Identify Primer Dimers

To identify primer dimers:

- Since primer dimers are only visible in the presence of SYBR Green I, begin the optimization process by performing a PCR with this dye.
- Always run a no template control (NTC = water instead of sample material) for each primer pair. During optimization, always use the highest $MgCl_2$ concentration for the NTC.
- Include a positive and a negative control, if available.
- When analyzing the melting peaks, try to distinguish between the primer dimer peak (broader peak, <80°C), the product peak and any contaminant peaks.
- Use gel electrophoresis to help identify the different peaks as additional tool.

2. Guidelines for Reducing Primer Dimers

Template

Nucleic acid preparations of various sample material might contain low amounts of template but in some cases high amounts of inhibitors, too. We recommend template preparation with products (*e.g.,* MagNA Pure LC System or Roche High Pure Kits) that produce high quality, high purity nucleic acids.

It is not possible to monitor a Reverse Transcription reaction directly. Thus, even if the final application will be a one-step RT-PCR we recommend establishing optimal PCR conditions, using cDNA as template.

Note: For cDNA synthesis, use the First Strand cDNA Synthesis Kit for RT-PCR (AMV) or Expand Reverse Transcriptase. For GC-rich templates choose *C. therm.* Polymerase. After completing the RT reaction, always denature the cDNA (5 min at 95°C).

Primer Design

Accurate primer design should ensure that the primers bind specifically at the desired target sequence, and that the formation of primer dimers is minimized.

Follow these guidelines for best results:

- The most important region of the primer is the 3´ region, because amplification starts there. In general these 3´ ends should be free of secondary structure, repetitive sequences, palindromes, and highly degenerate sequences.
- Forward and reverse primers sequences should not be complementary to each other, especially at their 3´ ends.
- Forward and reverse primers should have equal GC content, ideally between 40% and 70%.
- Whenever possible, avoid an unbalanced distribution of G/C and A/T-rich domains.
- We recommend highly purifying (*e.g.,* by HPLC) the primers, since shorter primer fragments (by-products of manufacturing) will increase the formation of primer dimers.
- The binding sites should not have extensive secondary structure.

Note: Commercial software is available for selection of primer sequences. For example, the Roche LightCycler Probe Design Software (Cat. No. 3 139 174) can be used not only for selection of Hybridization Probes but also for identification of primers. This software also allows analysis of established primer sets and even creation of Hybridization Probes for existing primer sets.

Some housekeeping genes are known to have pseudogenes. In these cases, the same primer pair can amplify both the housekeeping gene and the pseudogene. Amplification of pseudogenes cannot be eliminated by increasing the stringency of the reaction. The only solution for the pseudogene problem is redesigning the primers so they recognize a different priming site or switching to one of the Roche Housekeeping Gene Sets for convenient pseudogene-free quantification. (For additional information on housekeeping genes, see Technical Note No. 15/2002.)

PCR Setup

The LightCycler System provides different detection formats, mainly:

- SYBR Green I (for sequence-independent detection)
- Hybridization Probes (for sequence-specific detection)

Note: Since detection of primer dimers is not possible with Hybridization Probes, we strongly recommend optimizing the PCR first with SYBR Green I (to eliminate primer dimers) before performing Hybridization Probe experiments.

The easiest way to eliminate primer dimers is to use a Hot Start, *e.g.,* with Roche LightCycler FastStart DNA Master. For existing protocols, it may be easier to use LightCycler DNA Master together with LightCycler Taq Block-Antibody for a Hot Start.

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PCR Setup, continued

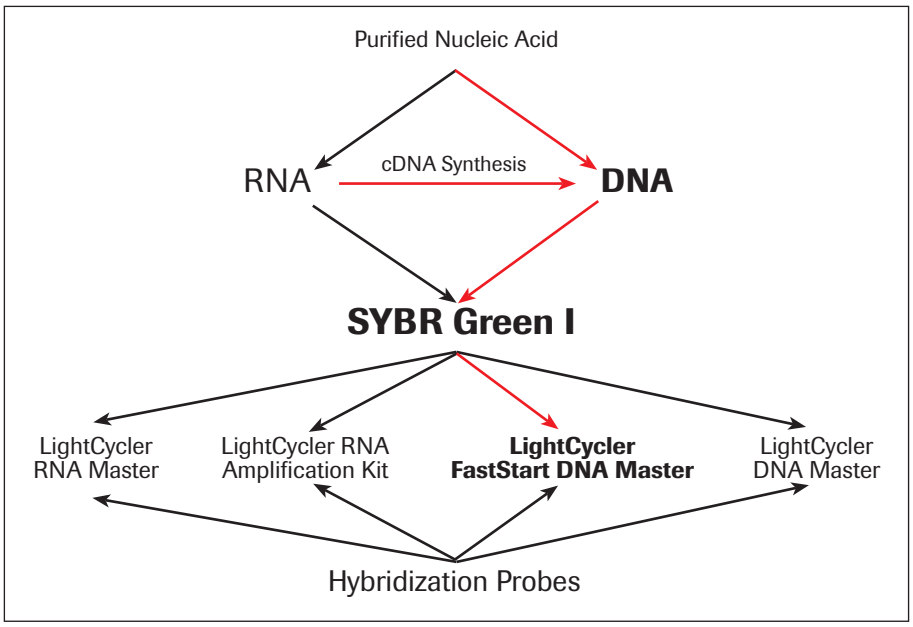

Figure 4: Selection of current assays.

Since $MgCl_2$ concentration influences the outcome of PCR, initial experiments to optimize PCR on the LightCycler Instrument should always include a $MgCl_2$ titration (DNA/cDNA, 1–5mM; RNA, 4–8mM). For additional information, refer to Technical Note 9/2000 and pack inserts.

High primer concentrations may promote mispriming and accumulation of non-specific products. If this happens, you can lower the initial concentration of primers from 0.5 µM to 0.3 µM.

Note: Low primer concentration may lead to lower product yields.

Experimental Protocol

Always define the experimental protocol before preparing the reagents. Set the annealing temperature as high as your primer allows for maximum stringency:

- If a higher annealing temperature causes reduced amplification yields, try touch down strategies to enhance both stringency and yield.
- If the primer annealing temperature is low (< 55°C), you may reduce the temperature transition rate to 2–5°C/s.

Optimal annealing time for SYBR Green I assays is between 1 and 5 seconds, and might be long for Hybridization Probe format (*e.g.,* 10 seconds).
Avoid over-amplification by reducing the number of cycles, *e.g.,* to 40-45.

Workflow

Keep all reagents chilled in the LightCycler Cooling Block, because suboptimal primers can form primer dimers at temperatures just above 4°C. Do not allow the reagents to stand at room temperature, even when working with a Hot Start technique.
Start the PCR as soon as the reaction mixture is prepared.

3. Data Acquisition at an Elevated Temperature

Introduction

Sometimes even after optimization, a PCR will lead to the formation of primer dimers, *e.g.*, due to unsophisticated primer design. If this occurs, the experimental system is not well suited for accurate quantification purposes and should be redesigned.

One way to reduce the influence of primer dimers in a SYBR Green I assay is to measure fluorescence at an elevated temperature. To do this, you must first know the exact T_ms of the product and the primer dimers. Both peaks must also be clearly separable by melting curve analysis.

Note: Try data acquisition at an elevated temperature only if reoptimization and/or primer redesign does not eliminate primer dimers in SYBR Green I assays.

Experimental Protocol

- To the amplification program, add a fourth step at a temperature higher than the T_m of the primer dimer, yet lower than the T_m of the product.
- Hold the temperature for 1 second and acquire data a single time.

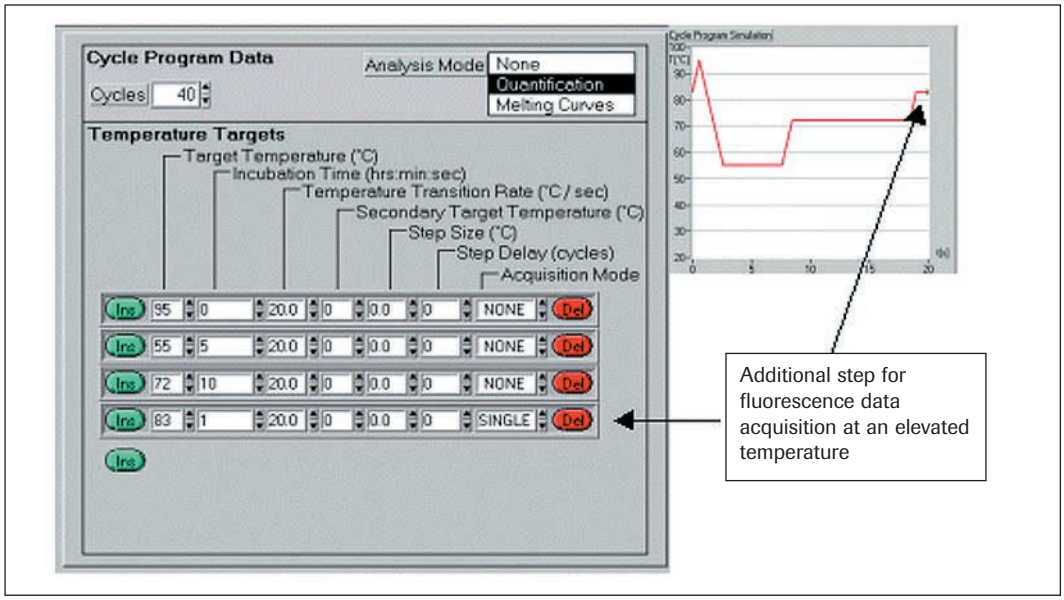

Figure 5: Data acquisition at an elevated temperature.

Effect of Using Elevated Temperatures

Measurement at an elevated temperature is only a "cosmetic" fix for detection that does not prevent formation of primer dimers. Since the primer dimers are still present in the reaction, melting curve analysis or gel electrophoresis will still show their formation.

However, when the fluorescence is measured at the elevated temperature, any increase in the fluorescence signal represents only an increase in specific product, since all primer dimers in the reaction are already single-stranded at this temperature and cannot contribute to the signal.

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Effect of Using Elevated Temperatures, continued

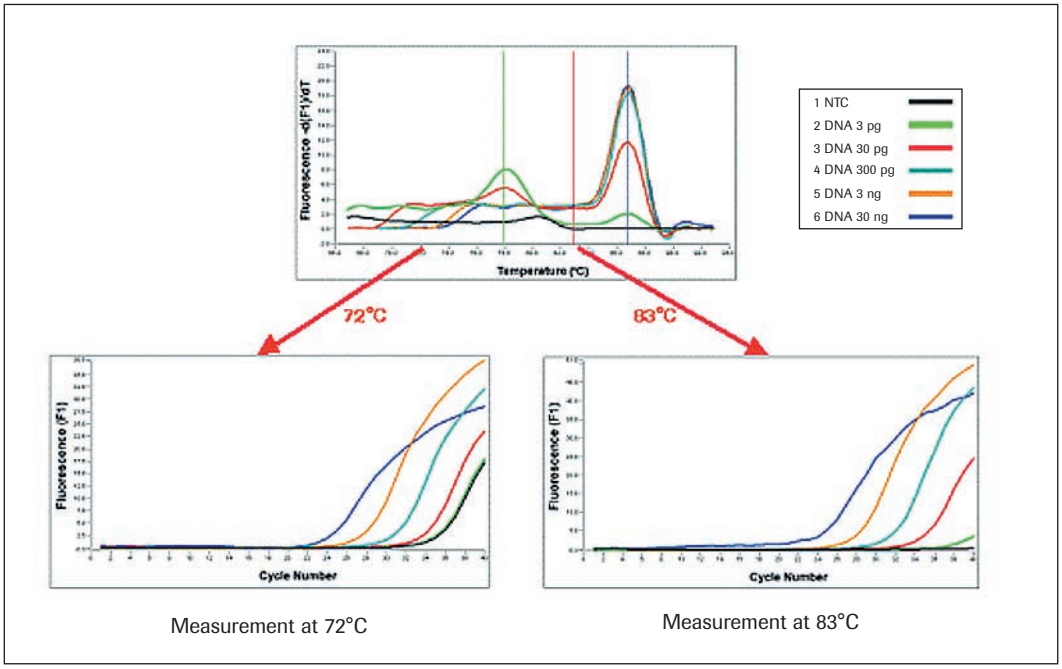

Figure 6: Effect of measurement at an elevated temperature.

Note: Be aware that formation of primer dimers always competes with the main amplification reaction and still can influence quantitative PCR, even if they are not detectable.

Similarly, when you work with Hybridization Probes, primer dimers still influence the specific amplification reaction even though they are not visible. Thus, we strongly recommend that you initially optimize new PCR systems with a SYBR Green I reaction, even if you are planning eventually to run a Hybridization Probe assay.

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