LightCycler

Optimization of Reactions to Reduce Formation of Primer Dimers

1. How to Reduce Primer Dimers in a LightCycler PCR

Introduction

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 only 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 a less successful PCR.
This application note describes several strategies for reducing the formation of primer dimers during LightCycler PCR.

Identifying Primer Dimers

To identify LightCycler PCR products generated in the presence of SYBR Green I, perform a melting curve analysis on the reaction. This analysis permits characterization of both the desired PCR products and primer dimers by their characteristic melting behavior.
Melting curve analysis is an inversion of the measurements 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 is at low temperature and the fluorescence signal is high. As the temperature steadily increases, the fluorescence will drop suddenly as the temperature reaches the characteristic melting point (T_m) of each DNA fragment.
Melting behavior is shown in the graphical data evaluation below [rate of change in fluorescence (–dF/dT) as a function of temperature]. Pure, homogeneous PCR products produce a single, sharply defined melting curve with a narrow peak. In contrast, primer dimers melt at relatively low temperatures and have broader peaks.

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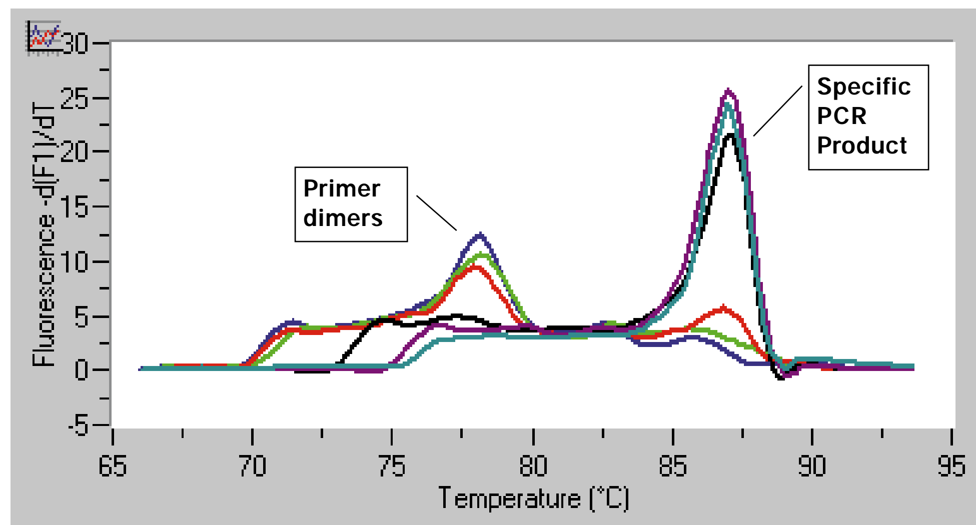
1. How to Reduce Primer Dimers in a LightCycler PCR *(Continued)*

Identifying Primer Dimers *(Continued)*



Strategies

Four main areas of a LightCycler PCR can be optimized:

- Workflow
- Primers
- Experimental Protocol
- Hot Start

They are explained in the following section.

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2. Optimization Strategies

Workflow

- Start the PCR as soon as the reaction mixture is prepared.
  Note: Any delay permits nonspecific annealing and primer elongation events.
- Keep all reagents chilled in the LightCycler cooling block. Do not allow them to stand at room temperature.

Primers

- The most important region for specific priming is the 3´ region of the primer; amplification starts here.
  In general, these ends should be free of secondary structures, repetitive sequences, palindromes, and highly degenerate sequences.
- The sequences of the two primers should not be complementary to each other, especially at their 3´ ends (so primer dimers will not form).
- Try to make the two primers equal in GC content.
- The GC content of the primers should range between 40% and 70%. Whenever possible, avoid an unbalanced distribution of G/C- and A/T-rich domains.
- Always use primers that are highly purified.

Experimental Protocol

When programming the Experimental Protocol of your PCR, try the following:

- To optimize the PCR reaction, always titrate the $MgCl_2$ concentration first. In general, the optimal $MgCl_2$ concentration ranges from 1–5 mM for PCR, and 3–7 mM for RT-PCR.
- Set the annealing temperature as high as your primers allow.
- Lower the annealing time to 1–5 seconds.
  Caution: This strategy can only be used for reactions performed in the presence of SYBR Green I!
- Avoid over-amplification by reducing the number of cycles, e.g. to 40.
- If the melting temperatures of the desired product and the primers are known, acquire the fluorescence signal during each cycle at a temperature just below the T_m of the product and above the T_m of the primer dimers.
  Note: This will eliminate detection of nonspecific fluorescence.

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Hot Start

To prevent nonspecific primer elongation, use the "Hot Start" method. In this method, at least one of the essential reaction components is separated from the reaction mixture (or blocked /inhibited) until the temperature in the tubes has exceeded the optimal primer annealing temperature.
For Hot Start with the LightCycler, we recommend using commercially available anti-Taq DNA polymerase antibodies (e.g., TaqStart Antibody from Clontech [Cat. No. 5400-1, 5400-2]).
This antibody keeps the polymerase inactive until the temperature rises above 70°C. The antibody is inactivated by the same heating step that denatures the target DNA.
To use the anti-Taq antibody in a LightCycler PCR, do the following:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>For each 20 µl reaction (final reaction volume), do one of the following:
<table>
<tr><th>IF...</th><th>THEN...</th></tr>
<tr><td>You use SYBR Green I for detection</td><td>Add 0.16 µl of the concentrated TaqStart Antibody solution to 2 µl LightCycler-DNA Master SYBR Green I per 20 µl final reaction volume.</td></tr>
<tr><td>You use Hybridization Probes for detection</td><td>Add 0.32 µl of the concentrated TaqStart Antibody solution to 2 µl LightCycler-DNA Master Hybridization Probes per 20 µl final reaction volume.</td></tr>
</table></td></tr>
<tr><td>2</td><td>Incubate the mixture for 5 min at room temperature.</td></tr>
<tr><td>3</td><td>Add primers, $MgCl_2$ (if required) H_2O, the Hybridization Probe pair, (if required), and the DNA template.
Note: The final volume is 20 µl.</td></tr>
<tr><td>4</td><td>Set the initial denaturation time for 1 min at 95 °C and start cycling as usual.</td></tr>
</table>

Order of Optimization

The strategies described above will reduce primer dimers in your LightCycler PCR. Use them in the following order:
1. Reduce delays in workflow.
2. Optimize primers.
3. Increase the annealing temperature, and, if possible, reduce annealing time.
4. Use Hot Start.
5. Reduce the number of PCR cycles.

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