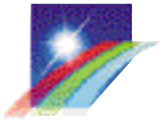
Roche Molecular Biochemicals
Technical Note No. 2/99



LightCycler

LightCycler-DNA Master SYBR Green I (96 rxn) Cat. No. 2 015 099
LightCycler DNA Master SYBR Green I (480 rxn) Cat. No. 2 158 817

Adaptation Protocol for Sequence-Independent Detection of DNA with SYBR Green I

Guidelines for adapting conventional PCR protocols to protocols that work in the LightCycler

1. Adapting a Conventional PCR Protocol to the LightCycler

Purpose of This Note

SYBR Green I dye provides a convenient, rapid way to detect and quantify any PCR product, regardless of sequence. This note shows how to adapt a PCR protocol developed in a conventional thermal cycler so it can be used in the LightCycler with SYBR Green I dye. It also offers guidelines for programming and optimizing a LightCycler PCR analysis with SYBR Green I.

How to Use This Note

Use this note to get a quick overview of the reagents and reaction conditions necessary for sequence-independent analysis of DNA with SYBR Green I. Covered topics include:

- Adapting a PCR to the LightCycler (below),
- Programming the LightCycler Experimental Protocol (Section 2),and
- Optimization strategy for the SYBR Green I reaction (Section 3).

Note: For details on the function of SYBR Green I, the preparation of master reaction mixes, and the programming and execution of a LightCycler experiment, see the *LightCycler Operator's Manual* and the pack insert for LightCycler – DNA Master SYBR Green I.

1. Adapting a Conventional PCR Protocol to the LightCycler, Continued

Adapting a PCR Protocol

The table below compares a reaction mix developed for a conventional thermal cycler with a corresponding reaction mix that is suitable for the LightCycler. Use the guidelines in the table to set up a SYBR Green I reaction for your application.

Parameter	Conventional Thermal Cycler	LightCycler
Final Volume	50 or 100 µl	20 µl
Template DNA	0.1 – 1.0 µg/100 µl	• Up to 50 ng complex genomic DNA • 10^1 – 10^{10} copies plasmid DNA ***Note:*** Adjust template concentration so **2 – 10 µl** of DNA is added to the reaction. Do not use more than 10 µl DNA per 20 µl reaction.
Final Concentration of Primers	0.1 – 1.0 µM, each primer	0.3 – 1.0 µM, each primer
Final Concentration of $MgCl_2$	1 – 3 mM	1 – 5 mM [1]
Amplicon Size	• Up to 3 kb genomic DNA • Up to 15 kb lambda DNA (with Taq DNA polymerase)	100 – 1000 bp
Nucleotides	0.2 mM, each nucleotide	Included in LightCycler-DNA Master SYBR Green I [2]
Taq PCR Buffer	Included with Taq polymerase	Included in LightCycler-DNA Master SYBR Green I [2]
Taq DNA Polymerase	1 – 5 units/100 µl	Included in LightCycler-DNA Master SYBR Green I [2]

Note: If required data are not available, use the same concentration of these components that you used in the conventional thermal cycler adjusted for the smaller LightCycler volume.

[1] Optimal concentration determined empirically.

[2] Simply add 2 µl LightCycler-DNA Master SYBR Green I to each 20 µl (total volume) reaction mixture. The ready-to-use reagent already contains an optimal concentration of nucleotides, Taq DNA polymerase, and Taq PCR buffer.

2. Programming the LightCycler Experimental Protocol

Overview

A typical LightCycler protocol for sequence-independent detection and analysis of DNA with SYBR Green I contains four cycle programs:

- Program 1: Initial denaturation of template DNA
- Program 2: Amplification of target DNA
- Program 3: Melting curve analysis (for product identification)
- Program 4: Cooling of the instrument

This section gives the settings that need to be programmed into the LightCycler to perform each of these.

Note: The settings given in the tables below are for a typical LightCycler experiment. The values in the unshaded (white) table cells are constant for most LightCycler runs. However, some amplification parameters (colored table cells) depend greatly on the identity of the template and primers. Therefore, these parameters need to be adjusted for each specific template-primer combination. For these values, use the guidelines given in the tables to determine the setting that will give optimal results with your primers and template.

Program 1: Denaturation

Set up the program for denaturation of the DNA template as follows:

Parameter	Value
Display Mode	F1
Cycles	1
Analysis Mode	None
Target Temperature (°C)	95
Incubation Time (h : min : s)	30
Temperature Transition Rate (°C/s)	20
Acquisition Mode	None

2. Programming the LightCycler Experimental Protocol, Continued

Program 2: Amplification

Set up the amplification program as follows:

Parameter	Value		
Display Mode	F1		
Cycles	40 – 45 [1]		
Analysis Mode	Quantification		
	Segment 1 Denaturation	**Segment 2 Annealing**	**Segment 3 Elongation**
Target Temperature (°C)	95	Depends on primer [2]	72
Incubation Time (h : min : s)	0	0 – 10 [3]	Depends on amplicon [4]
Temp. Transition Rate (°C/s)	20	20	2 – 20 [5]
Acquisition Mode	None	None	Single

[1] Use approx. 5 – 10 more cycles that you would in a conventional thermal cycler.

[2] For initial experiments, set the target temperature (*i.e.,* the primer annealing temperature) 5 °C *below* the calculated primer T_m. Calculate the primer T_m according to the following formula, based on the nucleotide content of the primer:

$$T_m = 2\ °C\ (A+T) + 4\ °C\ (G+C)$$

Note: See Section 3 of this Note for guidelines on determining the actual primer annealing temperature (target temperature) experimentally.

[3] For typical primers, choose an incubation time of 0 – 10 s for the annealing step. To increase the specificity of primer binding, use an incubation time of < 5 s.

[4] For elongation, increase the incubation time (t) (in seconds) as the length of the amplicon (bp) increases, according to the following formula:

$$t = (bp \div 25)\ \text{seconds}$$

Example: For a 100 bp amplicon, use an incubation time of 4 s.

[5] For elongation of most amplicons, choose a temperature transition rate of 20 °C/s.
Exception: If the primer annealing temperature is low (< 55 °C), reduce the transition rate to 2 – 5 °C/s.

2. Programming the LightCycler Experimental Protocol, Continued

Program 3: Melting Curve

Set up the program for melting curve analysis as follows:

Parameter	Value		
Display Mode	F1		
Cycles	1		
Analysis Mode	Melting Curves		
	Segment 1	**Segment 2**	**Segment 3**
Target Temperature (°C)	95	Depends on primer [1]	95
Incubation Time (h : min : s)	0	30 – 2:0	0
Temp. Transition Rate (°C/s)	20	20	0.1
Acquisition Mode	None	None	Cont.

[1] For Segment 2, make the target temperature approx. 5 – 10 °C *higher* than the primer annealing temperature during amplification (Program 2, Segment 2, target temperature).

Program 4: Cooling

Set up the program for cooling the instrument (at the end of the run) as follows:

Parameter	Value
Display Mode	F1
Cycles	1
Analysis Mode	None
Target Temperature (°C)	40
Incubation Time (h : min : s)	30
Temperature Transition Rate (°C/s)	20
Acquisition Mode	None

3. Optimization Strategy for Analysis with SYBR Green I

Overview

This section provides hints and experimental procedures you can use to get the best results from SYBR Green I analysis of DNA. In this section, you will learn how to:

- Include informative samples and controls in the run,
- Optimize the $MgCl_2$ concentration in the reaction mix,
- Determine primer annealing temperature experimentally,
- Reduce the amount of primer-dimers in the reaction,
- Optimize Performance of Template, Primers,and
- Eliminate carry-over contamination with Uracil-DNA Glycosylase (UNG),

Note: For more information on optimizing LightCycler runs, see Chapter C in the *LightCycler Operator's Manual* and other Technical Notes available from Roche Molecular Biochemicals.

Include Informative Samples and Controls

Include the following samples and controls in your run to obtain information that will help you judge the efficiency and accuracy of the reaction:

Note: Prepare these samples and controls with the same reaction mix that you are using for your experimental (unknown) samples.

Use this sample or control...	To determine...
Positive control (DNA template of known performance)	Whether reaction mix and primers work
Negative control (water in place of template)	Identity of primer-dimer melting peak

Optimize $MgCl_2$ Concentration

To determine the optimal $MgCl_2$ concentration for amplification of a new template, always perform a magnesium titration series that includes samples with varying amounts of $MgCl_2$, as follows:

Step	Action
1	Set up a series of SYBR Green I assays. Each should contain the same amount of template, primers (*e.g.*, 0.5 µM), and LightCycler DNA Master SYBR Green I (2 µl).
2	Vary the $MgCl_2$ concentration in each assay. Test final $MgCl_2$ concentrations between 1 and 5 mM. ***Example:*** The LightCycler – DNA Master SYBR Green I, when diluted 1:10, produces a final concentration of 1 mM $MgCl_2$ in the reaction mix. To make a reaction mix with a final concentration of 2 mM, add the normal amount (2 µl) of LightCycler – DNA Master SYBR Green I plus 0.8 µl of the 25 mM $MgCl_2$ stock solution (for a total reaction volume of 20 µl).
3	Determine the $MgCl_2$ concentration that performs best in the SYBR Green I assay.

3. Optimization Strategy for Analysis with SYBR Green I, Continued

Determine Primer Annealing Temperature Experimentally

The actual annealing temperature of primers during PCR may be much higher or lower than the Tm calculated from the sequence of the primers.
To determine the actual primer annealing temperature, vary the target temperature in the annealing segment (Segment 2) of the amplification program (Program 2). For each trial, we recommend raising or lowering the temperature by 2 – 3°C and repeating the experiment. Repeat as necessary until the optimal temperature is found.
Note: If a given annealing temperature produces significant nonspecific background, try a higher annealing temperature.

Reduce Primer-Dimer Formation

To reduce the amount of primer-dimers formed during a run, try the following:
- Set the primer annealing temperature to its optimum value (determined experimentally as described above) and reduce the annealing time (Program 2, Segment 2, incubation time) to 1 – 5 s.
- Include an anti-Taq polymerase antibody in the reaction mix and perform a hot start PCR (see Chapter C in the LightCycler Operator's Manual for details).
- Follow the hints given in Roche Molecular Biochemicals Technical Note LC 1/99.

Optimize Performance of Template, Primers, and Hybridization Probes

The purity, concentration,and sequence of template and primers greatly affect the efficiency and specificity of the amplification reaction. If amplification is still poor even after you have optimized both the $MgCl_2$ concentration and the primer annealing temperature (see topics above), do the following:
- Check primer design. If necessary, design new primers.
- Check purity of template. If necessary repurify or prepare new template.
- In separate series of reactions, titrate the concentrations of template and primers in the reaction mix, to find the optimal concentration of each.

Eliminate Carry-over Contamination

To eliminate carry-over contamination from previous amplifications, add 1 unit Uracil-DNA Glycosylase (UNG, Cat. No. 1 775 367) to the (20 µl) reaction mix and include a UNG incubation step (5 min, room temperature) in the experimental protocol (before the initial denaturation step).
Note: UNG degrades chemically modified PCR products (containing uracil-DNA) from previous amplifications, but does not degrade native DNA templates or primers. The LightCycler – DNA Master SYBR Green I includes dUTP in place of dTTP and therefore produces uracil-containing PCR products that can be degraded by UNG in subsequent PCR runs.

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