Roche Molecular Biochemicals
Technical Note No. 4/99

LightCycler

LightCycler-RNA Amplification Kit SYBR Green I (96 rxn) Cat. No. 2 015 137

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

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

1. Adapting a Conventional RT-PCR Protocol to the LightCycler

Purpose of This Note

SYBR Green I dye provides a convenient, rapid way to detect and quantify any RT-PCR product, regardless of sequence. This note shows how to adapt RT-PCR protocols developed in a conventional thermal cycler so they can be used in the LightCycler with SYBR Green I dye. It also offers guidelines for programming and optimizing a LightCycler one-step RT-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 RNA with SYBR Green I. Covered topics include:

- Adapting one-step RT-PCRs to the LightCycler (below),
- Programming the LightCycler Experimental Protocol (Section 2), and
- Optimization strategy for analysis with SYBR Green I (Section 3).
- Hints for adapting two-step RT-PCRs to the LightCycler (Section 4)

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

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

Adapting a One-Step RT-PCR Protocol

The table below compares a reaction mix developed for a one-step RT-PCR in a conventional thermal cycler with a one-step RT-PCR mix for the LightCycler. Use the guidelines in the table to set up RT-PCR for your application.

Parameter	Conventional Thermal Cycler (One-Step)	LightCycler (One-Step)
Final Volume	50 µl	20 µl
Template RNA	• 1 pg – 1 µg total RNA • 1 pg – 1 µg mRNA	• 1 pg – 1 µg total RNA [1] • 1 pg – 1 µg mRNA [1] ***Note:*** Adjust template concentration so **2 – 8 µl** of RNA is added to the reaction. Do not use more than 8 µl DNA per 20 µl reaction.
Final Concentration of Primers (sequence-specific primers)	0.1 – 1.0 µM, each	0.3 – 1.0 µM, each [2] (For initial experiments: 0.5 µM)
Final Concentration of $MgCl_2$	1 – 5 mM	4 – 8 mM [2]
Amplicon Size	Up to 6 kb (with Titan One-Tube RT-PCR System)	100 – 1000 bp
Nucleotides	0.2 – 1.0 mM, each	Included in LightCycler-RNA Amplification Kit SYBR Green I [3]
RT-PCR Buffer	Included in Titan System	Included in LightCycler-RNA Amplification Kit SYBR Green I [3]
RT-PCR Enzyme Mix	Included in Titan System	Included in LightCycler-RNA Amplification Kit SYBR Green I [3]

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] For low RNA concentrations (<10 ng/µl), use 10 ng/µl MS2 or alternative RNA as carrier.

[2] Optimal concentration determined empirically.

[3] To each 20 µl (total volume) reaction, simply add 2 µl of the RT-PCR Reaction Mix and 0.4 µl of the RT-PCR Enzyme Mix from the LightCycler – RNA Amplification Kit SYBR Green I. The ready-to-use reagents already contain optimal concentrations of nucleotides, RT-PCR buffer, Taq DNA polymerase, and AMV Reverse Transcriptase.

2. Programming the LightCycler Experimental Protocol

Overview

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

- Program 1: Reverse transcription of the RNA template
- Program 2: Initial denaturation of cDNA
- Program 3: Amplification of target cDNA
- Program 4: Melting curve analysis (for product identification)
- Program 5: 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 reverse transcription of the RNA template as follows:

Parameter	Value
Display Mode	F1
Cycles	1
Analysis Mode	None
Target Temperature (°C)	55 [1]
Incubation Time (h : min : s)	10:00
Temperature Transition Rate (°C/s)	20
Acquisition Mode	None

[1] Always use 55°C as the temperature for reverse transcription. Higher temperatures may decrease the efficiency of the reaction. Lower temperatures may lead to production of additional, unwanted cDNA products.

Program 2: Denaturation

Set up the program for denaturation of the cDNA (and inactivation of the reverse transcriptase) 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 3: 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	10	Depends on amplicon [3]
Temp. Transition Rate (°C/s)	20	20	2 – 20 [4]
Acquisition Mode	None	None	Single

1 Use approx. 5 – 10 more cycles than 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 elongation, increase the incubation time (t) (in seconds) as the length of the amplicon (bp) increases, according to the following formula:

$$t = (bp \div 25)\ seconds$$

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

4 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 4: 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	15 – 2:00	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 5: 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 RNA. 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,
- Check for DNA contamination in the RNA template,
- Eliminate carry-over contamination with Uracil-DNA Glycosylase (UNG),
- Optimize the performance of the template and primers, and
- Perform a two-step RT-PCR.

Note: For more information on optimizing LightCycler runs, see Chapter C in the *LightCycler Operator's Manual.*

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...
Sample with high concentration of template RNA	• Efficiency of overall reaction [1] • T_m of product
Negative control (water in place of template)	Absence of contamination

[1] Evaluate the efficiency of the reaction as follows:
- Note the cycle number at which amplification begins (*i.e.,* fluorescence increases). Early in the run is preferable to late in the run.
- Note the intensity of the fluorescence obtained during amplification. You should typically see intensities up to 50 for a SYBR Green I analysis (when Display Mode is set to F1/1).
- When adapting a new protocol to the LightCycler, examine the reaction products by gel electrophoresis to get an idea of which product(s) you are monitoring in the LightCycler.

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 2 µl RT-PCR Reaction Mix SYBR Green I,0.4 µl RT-PCR Enzyme Mix, and 0.5 µM PCR primers.
2	Vary the $MgCl_2$ concentration in each assay. Test final $MgCl_2$ concentrations between 4 and 8 mM. ***Note:*** The high concentrations of $MgCl_2$ are required because the RT-PCR Reaction Mix contains dUTP (see next topic). ***Example:*** To *increase* the final concentration of the $MgCl_2$ in a 20 µl reaction mix by 1 mM (*e.g.,* from 3 mM to 4 mM), add 0.8 µl of the 25 mM $MgCl_2$ stock solution. In determining the final concentration of $MgCl_2$ in the reaction, remember that the RT-PCR Reaction Mix already contains 3 mM $MgCl_2$ (final concentration).
3	Test each concentration of $MgCl_2$ with two concentrations of template (*e.g.* 1 ng and 100 ng of total RNA).
4	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 T_m 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 3). 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.

Check for DNA Contamination in Template

To check for the presence of DNA in any RNA template sample, run a DNA control reaction containing just the RNA template, PCR primers, and 2 µl of LightCycler *DNA Master* SYBR Green I (Cat. No. 2 015 099). Use the same $MgCl_2$ concentration as for the RT-PCR.
Note: The DNA Master contains optimal concentrations of nucleotides,PCR reaction buffer, and Taq DNA polymerase and a basic concentration of $MgCl_2$. However, it does not contain reverse transcriptase, so the control will produce an amplified product only if the original sample contains a DNA contaminant that can be copied by the polymerase.

Optimize Performance of Template and Primers

The purity, concentration,and sequence of both template and primers greatly affect the efficiency and specificity of the amplification reaction. If amplification is still poor after you have optimized both the $MgCl_2$ concentration and the primer annealing temperature (see topics above), do the following:

- Check primer design.(For instance, does the reverse primer allow the reverse transcription reaction to be performed at 55°C? What is the primers' tendency to form primer dimers?) If necessary, design new primers.
- Check for purity and possible degradation of the template on electrophoretic gels. If necessary, prepare new template.
- Titrate the concentrations of primer and template in the reaction mix, to find the optimal concentration of each.
- Perform a two-step RT-PCR. (see below).

Perform a Two-Step RT-PCR

If no amplification or very poor amplification occurs in the initial LightCycler RT-PCR assay (one-step RT-PCR), try a two-step reaction:

- Perform the reverse transcription as established in your lab or use the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Cat. No. 1 483 188).
- Use aliquots of the cDNA product obtained in the first step of the two-step procedure as template for a LightCycler PCR with the LightCycler DNA Master SYBR Green I (Cat. No. 2 015 099).

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 cDNA denaturation step).

Note: UNG degrades chemically modified PCR products (containing uracil-DNA) from previous amplifications, but does not degrade primers or native DNA and RNA templates.
The RT-PCR Reaction Mix (from the LightCycler – RNA Amplification Kit 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.

4. Adapting a Two-Step RT-PCR Protocol to a One-Step LightCycler RT-PCR

Adapting a Two-Step RT-PCR Protocol

To adapt a two-step protocol to the one-step LightCycler RT-PCR format, use the following guidelines:

- For the LightCycler, use only RNA template concentrations that have given large amounts of amplified product in the two-step system.
- Do not try to amplify a sequence >1000 bp in the LightCycler.
- Use only sequence-specific primers in the LightCycler. Oligo(dT) and random hexamers should not be used as primers in the LightCycler.
- Use the LightCycler –RNA Amplification Kit SYBR Green I. The ready-to-use reagents already contain optimal concentrations of nucleotides, RT-PCR buffer, Taq DNA polymerase, and AMV Reverse Transcriptase.
- Determine the optimal concentration of $MgCl_2$ (*i.e.*, between 4 and 8 mM) experimentally, as outlined in Section 3 of this Note.
- Determine the optimal primer concentration for the LightCycler experimentally by performing a titration series with samples containing different concentrations of primer. Run these in parallel to determine which gives the highest product yield.

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