For general laboratory use.
FOR *IN VITRO* USE ONLY.

LightCycler RNA Amplification Kit Hybridization Probes

Kit for one-step RT-PCR using the LightCycler[1)] Instrument

Cat. No. 2 015 145
Kit for 96 reactions

Store the kit at −20°C!

Instruction Manual

Version 4, January 2003

Roche

1. Preface

1.1 Table of contents

1.2 Kit contents

Kit contents

Vial	Label	Content and use
1 red cap	LightCycler RT-PCR Enzyme Mix	• 2 × 20 µl • enzyme mix for RT-PCR
2 red cap	LightCycler RT-PCR Reaction Mix Hybridization Probes, 5× conc.	• 3 × 128 µl [5×] • reaction mix for RT-PCR • contains reaction buffer, dNTP mix (with dUTP instead of dTTP), and 15 mM $MgCl_2$
3 blue cap	$MgCl_2$ stock solution, 25 mM	• 1 ml • to adjust $MgCl_2$ concentration
4 color-less cap	H_2O, sterile, PCR grade	• 2 × 1 ml • to adjust the final reaction volume

Additional equipment and reagents required

Refer to the following table for a list of additional reagents and equipment required for a RT-PCR using the LightCycler Instrument:

Equipment and reagents	Cat. No.
• LightCycler Instrument • LightCycler Capillaries	2 011 468 1 909 339
• LightCycler Control Kit RNA (optional) • LightCycler Color Compensation Set (optional*)	2 158 841 2 158 850
• PCR template • Primers • Hybridization Probes	

* If you want to perform color compensation when using LightCycler Red 640 and LightCycler Red 705 labeled Hybridization Probes in multiplex dual color experiments (i.e. in one capillary).

Note: For two-step RT-PCR protocol using the LightCycler Instrument refer to:

- LightCycler DNA Master SYBR Green I (Cat. No. 2 015 099, 2 158 817), or
- LightCycler DNA Master Hybridization Probes (Cat. No. 2 015 102, 2 158 825), or
- LightCycler FastStart DNA Master SYBR Green I (Cat. No. 3 003 230, 2 239 264), or
- LightCycler FastStart DNA Master Hybridization Probes (Cat. No. 3 003 248, 2 239 272).

2. Introduction

2.1 Product overview

Kit description

The LightCycler RNA Amplification Kit Hybridization Probes is a specifically adapted product for one-step RT-PCR in glass capillaries using the LightCycler Instrument and Hybridization Probes as detection format.

Hybridization Probes consist of two, different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of PCR cycles. One probe is labeled at the 5'-end with a LightCycler Red fluorophore (LC – Red 640 or LC –Red 705), and to avoid extension, modified at the 3'-end by phosphorylation. The other probe is labeled at the 3'-end with fluorescein. Only after hybridization are the two probes in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, fluorescein, the donor fluorophore, is excited by the light source of the LightCycler Instrument, and part of the excitation energy is transferred to LightCycler Red, the acceptor fluorophore. The emitted fluorescence of the LightCycler Red fluorophore is measured.

LightCycler Red 640 and LightCycler Red 705 labeled Hybridization Probes can be used separately or in combination therefore allowing single or dual color detection. When performing single color detection, color compensation is not necessary. However, the use of a color compensation file is a prerequisite to perform dual color experiments with both LightCycler Red 640 and LightCycler Red 705 labeled Hybridization Probes in a single capillary. The color compensation can be performed during or after a run on the LightCycler Instrument.

Refer to the LightCycler Operator's Manual and to the pack insert of the LightCycler Color Compensation Set for further information on color compensation and the generation and use of a color compensation file.

The LightCycler RNA Amplification Kit Hybridization Probes provides convenience, high performance, reproducibility, and minimizes contamination risk. Only template RNA, primers, Hybridization Probes, and additional $MgCl_2$ (if necessary), have to be added.

Note: The described performance of the kit can be guaranteed for use on the LightCycler Instrument only.

Basic stages of the standard protocol

In principle, the LightCycler RNA Amplification Kit Hybridization Probes can be used for the amplification and detection of every RNA target. Each protocol needs to be adapted to the reaction conditions of the LightCycler Instrument and parameter specific Hybridization Probes need to be designed. Refer to the LightCycler Operator's Manual for general recommendations.

As an example for a standard protocol, the LightCycler RNA Amplification Kit Hybridization Probes is used in combination with the LightCycler Control Kit RNA. The latter kit provides a control RNA template and primers and differently labeled Hybridization Probe mixes (LC – Red 640 or LC – Red 705 labeled) for amplifying an *in vitro* transcribed cytokine RNA.

Amplify and monitor on-line by using a combined procedure on the LightCycler Instrument (stage 2 below). Interpret the results directly after completing the RT-PCR.

Stage	Description
1	Set-up the RT-PCR
2	RT-PCR using the LightCycler Instrument optional: additional melting curve analysis
3	Interpretation of results

Application

The LightCycler RNA Amplification Kit Hybridization Probes is suitable for amplification and detection of RNA using the LightCycler Instrument. Furthermore it can be used to genotype single nucleotide polymorphisms (SNPs) and analyze mutations.

Note: The amplicon size should not exceed 1 kb in length. For optimal results, select a product length ≤700 bp.

Assay time/ hands on time

Procedure (in combination with the LightCycler Control Kit RNA)	Time
Set-up the RT-PCR	15 min
LightCycler RT-PCR run	40 min
Total assay time	**55 min**

Number of tests

The kit is designed for 96 reactions with a final reaction volume of 20 µl each.

Quality control

The LightCycler RNA Amplification Kit Hybridization Probes is function tested using the LightCycler Control Kit RNA, according to the protocols described below.

Kit storage and stability

Store the unopened kit at −20°C through the expiration date printed on the label (12 months from date of manufacture).

The kit is shipped on dry ice.

3. Procedures and required materials

3.1 Before you begin

Introduction

As an example for a RT-PCR using the LightCycler Instrument, the LightCycler RNA Amplification Kit Hybridization Probes is combined with the LightCycler Control Kit RNA as described below:

A 322 bp fragment of *in vitro* transcribed cytokine RNA is amplified with specific primers. Amplification of target DNA is monitored using Hybridization Probes (labeled either with LC – Red 640 or LC – Red 705) that hybridize to an internal sequence of the amplified fragment.

Note: The protocol is designed for a final reaction volume of 20 µl. For volumes $<$20 µl, the reaction and cycle conditions have to be optimized.

Storage of kit solutions

Store the kit components as follows:

Vial	Label	Storage
1 red cap	LightCycler RT-PCR Enzyme Mix	• Store at −20°C. • **Avoid repeated freezing and thawing!**
2 red cap	LightCycler RT-PCR Reaction Mix Hybridization Probes, 10× conc.	• Store at −20°C. • **Avoid repeated freezing and thawing!**
3 blue cap	$MgCl_2$ stock solution, 25 mM	Store at −20°C.
4 color-less cap	H_2O, sterile, PCR-grade	Store at −20°C.

Additional reagents and equipment required

- LightCycler Instrument
- LightCycler Capillaries
- LightCycler Control Kit RNA
- Standard benchtop microcentrifuge containing a rotor for 2.0 ml reaction tubes.
- Optional*: LightCycler Color Compensation Set

* If you want to perform color compensation when using LightCycler Red 640 and LightCycler Red 705 labeled Hybridization Probes in multiplex dual color experiments (i.e. in one capillary).

Note: Centrifuge adapters that can be used in a standard microcentrifuge rotor are supplied with the LightCycler Instrument.

Sample material

Every sample material suitable for RT-PCR in terms of purity, concentration, and absence of inhibitors can be used. For reproducible nucleic acid preparations and RT-PCR set-up, use the MagNA Pure LC nucleic acid purification instrument (Cat. No. 2 236 931).
Note: Up to 500 ng total RNA or 100 ng mRNA can be used. Higher concentrations might result in inhibition of the reaction.

Refer to the LightCycler Operator's Manual for recommendations concerning sample material and nucleic acid purification.

For the standard protocol, *in vitro* transcribed cytokine RNA as provided in the LightCycler Control Kit RNA is used as starting sample material.

Primers

Use primers at a final concentration of 0.3–1 μM each. A recommended starting concentration is 0.5 μM each.
Refer to the LightCycler Operator's Manual for recommendations concerning primer design.

Hybridization Probes

Use the Hybridization Probes at a final concentration of 0.2 μM each. In some cases it might be advantageous to double the LightCycler Red- probe concentration to 0.4 μM. Refer to the LightCycler Operator's Manual for recommendations concerning Hybridization Probe design, detailed information about the dyes (Fluorescein, LightCycler Red 640 and LightCycler Red 705), and how to label the Hybridization Probes with the respective dyes.

$MgCl_2$

For specific and efficient amplification using the LightCycler Instrument, it is essential to optimize the target-specific $MgCl_2$ concentration. The LightCycler RNA Amplification Kit Hybridization Probes contains a basic $MgCl_2$ concentration of 3 mM (final concentration).The optimal concentration for RT-PCR with the LightCycler Instrument may vary from 3 to 7 mM.
The table below gives the volumes of the $MgCl_2$ stock solution (vial 3, blue cap) which give the designated $MgCl_2$ concentration, when added to a 20 μl final RT-PCR volume.

To reach a final Mg^{2+} concentration (mM) of:	3	4	5	6	7
Add this amount of 25 mM $MgCl_2$ stock solution (μl)	0	0.8	1.6	2.4	3.2

Negative control

Always run a negative control with the samples. To prepare a negative control, replace the template RNA with PCR-grade water (vial 4, colorless cap).

DNA contamination control

The RNA preparation can be tested for DNA contamination when the template RNA is used in combination with the LightCycler DNA Master Hybridization Probes (Cat. No. 2 015 102, 2 158 825) or LightCycler FastStart DNA Master Hybridization Probes (Cat. No. 3 003 248, 2 239 272).

Capillary handling

Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries.

3.2 Procedure

General remarks

The following procedure is adapted to use with the LightCycler Control Kit RNA, and serves as an example for the application of the LightCycler RNA Amplification Kit Hybridization Probes.

Thawing the solutions

Thaw the following reagents, mix gently, and store on ice:

From the...	Thaw the...
LightCycler RNA Amplification Kit Hybridization Probes	• LightCycler RT-PCR Enzyme Mix (vial 1, red cap) • LightCycler RT-PCR Reaction Mix Hybridization Probes (vial 2, red cap) • $MgCl_2$ stock solution (vial 3, blue cap)
LightCycler Control Kit RNA	• Cytokine RNA (vial 1–4, purple cap) • Cytokine Hybridization Probe Mix, LC-Red 640 labeled (vial 6, yellow cap), and/or: Cytokine Hybridization Probe Mix, LC–Red 705 labeled (vial 7, yellow cap), • Cytokine Primer Mix (vial 5, white cap) • H_2O sterile, PCR-grade (vial 8, colorless)

Note: A reversible precipitate may form in the LightCycler RT-PCR Reaction Mix Hybridization Probes (vial 2, red cap) during storage, which does not influence the perfomance in RT-PCR.

If a precipitate is visible proceed as described below:

Step	Action
1	Place the RT-PCR Reaction Mix at room temperature.
2	Mix gently from time to time until the precipitate is completely dissolved.
3	Place on ice.

Note: Define the experimental protocol before preparing the master mix.

3.3 Experimental Protocol

General remarks

The following experimental protocol is adapted to use with the LightCycler Control Kit RNA, and serves as an example for the application of the LightCycler RNA Amplification Kit Hybridization Probes.

The following table gives a guideline for optimization of the cycle parameters:

Parameter	Value
Denaturation temperature (°C)	95
Denaturation time (s)	0
Annealing temperature (°C)	Primer and Hybridization Probes dependent
Annealing time (s)	15
Elongation temperature (°C)	72
Elongation time (s)	length of product [bp]/25

***Note*:** The recommended temperature to perform the reverse transcription reaction is 55°C.

Experimental Protocol

The described protocol consists of four programs:

- Program 1: Reverse transcribtion of template RNA
- Program 2: Denaturation of the cDNA/RNA hybrid
- Program 3: Amplification of cDNA
- Program 4: Cooling the rotor and thermal chamber

and the subsequent setting of the fluorescence parameters, followed by defining the adjustments for the reverse transcription reaction.

Program 1 Reverse Transcription

Set the values for reverse transcription of the cytokine RNA template as follows:

Cycle Program Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	***Segment 1***
Target Temperature (°C)	55
Incubation time (h:min:s)	10:00*
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0.0
Step Delay (Cycles)	0
Acquisition Mode	None

* When amplifying GC-rich templates or templates with a high degree of secondary structures, it is recommended to extend the incubation time to 30 min.

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Program 2 Denaturation

Set the values for denaturation of the cDNA/ RNA hybrid as follows:

Cycle Program Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	***Segment 1***
Target Temperature (°C)	95
Incubation time (h:min:s)	30
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0.0
Step Delay (Cycles)	0
Acquisition Mode	None

Program 3 Amplification

Set the values for amplifiying the target DNA as follows:

Cycle Program Data	Value		
Cycles	45		
Analysis Mode	Quantification		
Temperature Targets	***Segment1***	***Segment 2***	***Segment 3***
Target Temperature (°C)	95	50	72
Incubation time (h:min:s)	0**	15	13
TemperatureTransition Rate (°C/s)	20.0	20.0	2.0
Secondary Target Temperature (°C)	0	0	0
Step Size (°C)	0.0	0.0	0.0
Step Delay (Cycles)	0	0	0
Acquisition Mode	None	Single	None

** When amplifying GC-rich templates or templates with a high degree of secondary structures, it is recommended to extend the incubation time to 5 s.

Program 4 Cooling

Set the values for cooling the rotor and thermal chamber at the end of the protocol as follows:

Cycle Program Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	***Segment 1***
Target Temperature (°C)	40
Incubation time (h:min:s)	30
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0.0
Step Delay (Cycles)	0
Acquisition Mode	None

Setting the fluorescence parameters

Set the fluorescence parameters as follows:

Display Mode:

- for single color detection during the run:
  Fluorescence channel 2 (F2) when using LightCycler Red 640 as acceptor fluorophore, or fluorescence channel 3 (F3) when using LightCycler Red 705 as acceptor fluorophore.
- for single color detection after the run:
  Fluorescence channel 2/ fluorescence channel 1 (F2/F1) when using LightCycler Red 640 as acceptor fluorophore, or fluorescence channel 3/fluorescence channel 1 (F3/F1) when using LightCycler Red 705 as acceptor fluorophore.
- for dual color detection during and after the run:
  When using both, LightCycler Red 640 and LightCycler Red 705 labeled Hybridization Probes, choose either fluorescence channel 2 (F2) or fluorescence channel 3 (F3).

Set the fluorescence gains as follows:

Fluorimeter Gain	Value
Channel 1 (F 1)	1
Channel 2 (F 2)	15
Channel 3 (F 3)	30

Note: With LightCycler Software V.3.5 and higher, no gain setting is required.

Define the adjustments for the RT reaction

Refer to the following table to define the adjustments for the RT reaction:

Step	Action
1	Click on **Edit Samples** button.
2	Enter sample information.
3	Set temperature to 55°C.
4	Click on **Done** button.

Refer to the LightCycler Operator's Manual for further recommendations concerning the one-step RT-PCR.

Color compensation (optional)

When performing a dual color detection experiment using both LightCycler Red 640 and LightCycler Red 705 labeled Hybridization Probes in a single capillary, a previously stored color compensation file can be activated or deactivated during the run of the LightCyclerInstrument.

Step	Action
1	Select the data file in the **Programming Screen.**
2	Click on **Choose CCC File** and select the appropriate file.
3	Click on the **Use Color Compensation** button to enable color compensation during on-line display of data.
4	Click on the **Use Color Compensation** button again to return to the raw data.

Alternative: A stored color compensation file can be used for data analysis after the run on the LightCycler Instrument (see section 3.5).

3.4 Preparation of the master mix

Procedure

Depending on the total number of reactions place LightCycler Capillaries in precooled centrifuge adapters.

Prepare a master mix by multiplying the amount in the "Volume" column by the number of reactions to be cycled, plus one additional reaction.

Proceed as described below for a 20 µl standard reaction, when performing single or dual color detection.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>In a 1.5 ml reaction tube on ice, add the following components in the order mentioned below:
<table>
<tr><th>Component</th><th colspan="3">Volume</th><th>Final conc.</th></tr>
<tr><td></td><td>A</td><td>B</td><td>C</td><td></td></tr>
<tr><td>H_2O, sterile, PCR grade (vial 8, colorless cap)</td><td>8.2 µl</td><td>8.2 µl</td><td>6.2 µl</td><td></td></tr>
<tr><td>$MgCl_2$ stock solution (vial 3, blue cap)</td><td>2.4 µl</td><td>2.4 µl</td><td>2.4 µl</td><td>6 mM</td></tr>
<tr><td>LightCycler RT-PCR Reaction Mix Hybridization Probe (vial 2, red cap)</td><td>4 µl</td><td>4 µl</td><td>4 µl</td><td>1×</td></tr>
<tr><td>Cytokine Hybridization Probe Mix, LC-Red 640 labeled (vial 6, yellow cap)</td><td>2 µl</td><td>-</td><td>2 µl</td><td rowspan="2">0.2 µM each</td></tr>
<tr><td>Cytokine Hybridization Probe Mix, LC-Red 705 labeled (vial 7, yellow cap)</td><td>-</td><td>2 µl</td><td>2 µl</td></tr>
<tr><td>Cytokine Primer Mix (vial 5, white cap)</td><td>2 µl</td><td>2 µl</td><td>2 µl</td><td>0.5 µM each</td></tr>
<tr><td>LightCycler RT-PCR Enzyme Mix, (vial 1, red cap)</td><td>0.4 µl</td><td>0.4 µl</td><td>0.4 µl</td><td>1×</td></tr>
<tr><td>Total volume</td><td>19 µl</td><td>19 µl</td><td>19 µl</td><td></td></tr>
</table>
A: Single color detection using LC- Red 640
B: Single color detection using LC- Red 705
C: Dual color detection using LC- Red 640 and LC-Red 705</td></tr>
<tr><td>2</td><td>• Mix gently.
• Pipet 19 µl master mix into the precooled LightCycler Capillary.
• Add 1 µl of the Cytokine RNA template (vial 1-4).</td></tr>
<tr><td>3</td><td>• Seal each capillary with a stopper and place the adapters, containing the capillary, into a standard benchtop microcentrifuge.
• Centrifuge at 700 × g for 5 s (3000 rpm in a standard benchtop microcentrifuge).
<u>Note:</u> Place the centrifuge adapters in a balanced arrangement within the centrifuge.</td></tr>
<tr><td>4</td><td>Place the capillaries in the rotor of the LightCycler Intrument.</td></tr>
<tr><td>5</td><td>Cycle the samples as described in section 3.3.</td></tr>
</table>

3.5 Typical results

Introduction

Data analysis includes only one step since the sequence-specific Hybridization Probes require no additional melting curve analysis.

Color Compensation

The use of a previously generated color compensation file is a prerequisite for the analysis of a dual color experiment with both LightCycler Red 640 and LightCycler Red 705 labeled Hybridization Probes in a single capillary. Optionally, color compensation can be done when the detection is performed with a single color (LC-Red 640 or LC-Red 705).

Refer to the LightCycler Operator's Manual and to the pack insert of the LightCycler Color Compensation Set for further information on the generation and use of a color compensation file.

Proceed as described below to use the stored color compensation file for data analysis after a run on the LightCycler Instrument:

Step	Action
1	Select the data file in the **LightCycler Data Analysis** module of the LightCycler Software.
2	Click on the **Select a Program** button and select the program to be analyzed.
3	Under the **Color Compensation** menu, select **Load Calibration Data** and highlight the stored 'CCC' color compensation file. Alternative: Click on the **Select CCC Data** button and choose **Import CCC File**.
4	To display the color compensated data: Click on the **Color Compensation** button. Alternative: Select **Enable** under the **Color Compensation** pull-down menu.
5	To return to the raw data: Click on the **Color Compensation** button again. Alternative: Select **Disable** under the **Color Compensation** pull down menu.

Refer to the following table to get an overview how color compensation effects the obtained fluorescence signals:

	Signals visible in			
	Channel 2 (F2)		Channel 3 (F3)	
	Without	With	Without	With
	color compensation			
Single color detection				
LightCycler Red 640	+ (Fig. 1a)	+ (Fig. 1b)	+ (Fig. 1c)	– (Fig. 1d)
LightCycler Red 705	(+)	–	+	+
Dual color detection				
LightCycler Red 640 and LightCycler - Red 705	+ (uncompensated)	+ (compensated)	+ (uncompensated)	+ (compensated)

\+ : signal
(+) : weak signal
– : no signal

Refer to the LightCyler Operators Manual for further information.

Quantification program

The following amplification curves were obtained by performing the procedure for single color detection and using LightCycler Red 640 as acceptor fluorophore. Displayed are the results in channel 2 and 3, with and without color compensation.

Equivalent results (according to the table above) will be obtained using single color detection with LightCycler Red 705 as acceptor fluorophore or dual color detection with LC-Red 640 and LC Red-705 simultaneously.

The fluorescence values versus cycle number are displayed. 100 copies of the Cytokine RNA can be reproducibly detected by amplification in the LightCycler Instrument and using Hybridization Probes as detection format.

Fig. 1a: Channel 2 (F2/F1) without color compensation

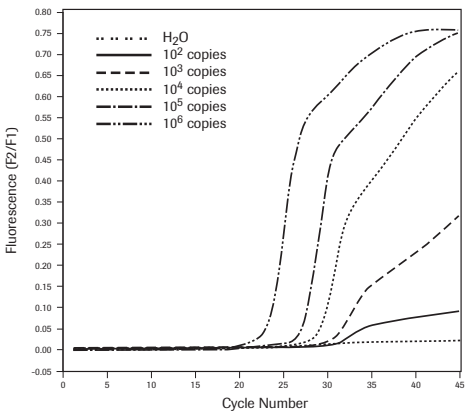

Fig. 1b: Channel 2 (F2/F1) with color compensation

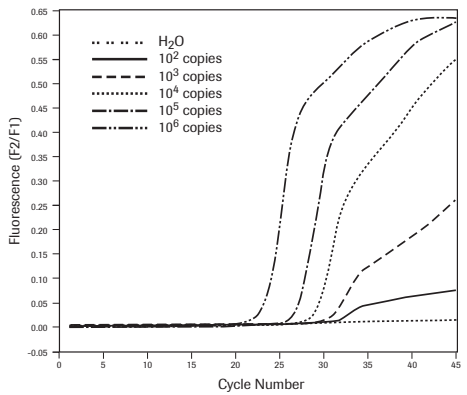

Fig. 1a and 1b: Amplify serially diluted samples containing 10^2 - 10^6 copies of cytokine RNA. As a negative control, the template RNA was replaced with PCR-grade water. LightCycler Red 640 was used as acceptor fluorophore.

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Fig. 1c: Channel 3 (F3/F1) without color compensation

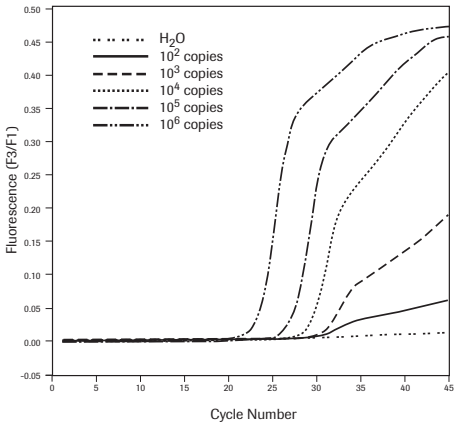

Fig. 1d: Channel 3 (F3/F1) with color compensation

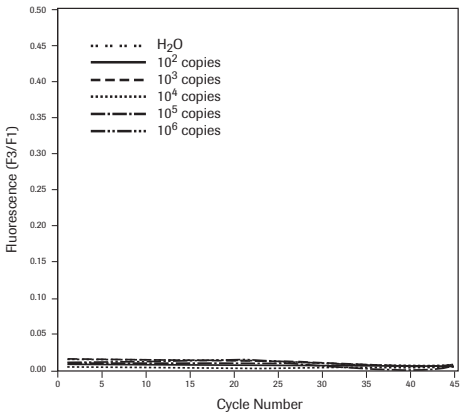

Fig. 1c and 1d: Amplify serially diluted samples containing 10^2 - 10^6 copies of cytokine RNA. As a negative control, the template RNA was replaced with PCR-grade water. LightCycler Red 640 was used as acceptor fluorophore. Arithmetic background subtraction was selected.

3.6 Related Procedures

3.6.1 Melting curve analysis for genotyping single nucleotide polymorphisms (SNPs) and analyzing mutations

General remarks

The melting curve analysis is an additional cycle that has to be performed after the amplification cycles are completed and the amplicon is formed. It has to be added to the Experimental protocol following Program 3: Amplification (see section 3.3). The following table gives a guideline for genotyping SNPs and analyzing mutations using the melting curve program.

Set the values for the melting curve analysis as follows:

Cycle Program Data	**Value**		
Cycles	1		
Analysis Mode	Melting Curve		
Temperature Targets	***Segment 1***	***Segment 2***	***Segment 3***
Target Temperature (°C)	95	annealing temperature −5	95
Incubation time (h:min:s)	0	30–60	0
Temp. Transition Rate (°C/s)	20.0	20.0	0.1
Secondary Target Temperature (°C)	0	0	0
Step Size (°C)	0.0	0.0	0.0
Step Delay (Cycles)	0	0	0
Acquisition Mode	None	None	Cont.

Refer to the LightCycler Operator's Manual for further recommendations concerning genotyping SNPs and analyzing mutations.

3.6.2 Carry-over prevention using UNG

Introduction

The heat-labile Uracil-DNA Glycosylase (UNG) is suitable for preventing carry-over contamination between PCRs. This technique relies on the incorporation of deoxyuridine triphosphate (dUTP) during all amplification reactions and the pretreatment of all successive PCR mixtures with the heat-labile UNG. The UNG cleaves DNA at any site where a deoxyuridylate residue has been incorporated. Subsequently, the resulting abasic sites are hydrolized due to the high temperatures during the initial denaturation step, and can not serve as PCR templates any longer. The heat-labile UNG is inactivated at the same time.

UNG is inactive on RNA and native DNA.

Note: Decontamination can be achieved with all the provided LightCycler reagents, since dTTP is replaced with dUTP.

Refer to the LightCycler Operator's Manual for further informations.

Additional reagent required

Uracil-DNA Glycosylase, heat-labile
(Cat. No. 1 775 367)

Procedure

Prior to the thermocycling procedure, add heat-labile Uracil-DNA Glycosylase (UNG) to the master mix.

Step	Action
1	Add 1 µl heat-labile UNG to the master mix per 20 µl final reaction volume.
2	Add template RNA and incubate the completed reaction mixture for 5 min at room temperature.
3	Destroy any contaminating template and inactivate the UNG enzyme by performing the RT at 55°C. **Note**: Do not perform an additional inactivation step at higher temperatures (>55°C) since the RT enzyme will be inactivated.

Note: When performing an additional melting curve analysis, the use of UNG lowers the respective melting temperature (T_M) by approx. 1°C.

4. Appendix

4.1 Trouble shooting

Problem	Possible cause	Recommendation
Precipitate in RT-PCR reaction buffer.	Concentrated compounds in the RT-PCR reaction buffer in combination with storage conditions.	Place the RT-PCR reaction mix at room temperature. Mix gently from time to time until the precipitate is completely dissolved and place on ice.
Amplification reaches plateau phase before the program is finalized.	Very high starting amount of nucleic acid.	The program can be finished by clicking on the **End Program** button. The next cycle program will start automatically.
	The number of cycles is too high.	Reduce the number of cycles in the cycle program.
Log-linear phase of amplification just starts when the amplification program finishes.	Very low starting amounts of nucleic acid.	• Increase number of cycles by 10 in the corresponding cycle program. • Improve PCR conditions (e.g. $MgCl_2$ concentration, primer and probe design). • Use higher amount of starting material. • Repeat the run.
No amplification occurs.	Wrong channel has been chosen to detect amplification online.	Check the channel chosen in the programming screen and change.(The data obtained up to this point will be saved.)
	Pipetting errors or omitted reagents.	• Check for missing reagents. • Titrate $MgCl_2$ concentration. • Check for missing or defective dye.
	Measurements do not occur	Check the cycle programs. For Hybridization Probe detection format, choose “single” as acquisition mode at the end of the annealing phase.
	Amplicon length is >1 kb.	Do not use amplicons >1 kb. Amplification efficiency is reduced with amplicons of this size. Optimal results are obtained for amplicons ≤ 700 bp.
	Inhibitory effects of the sample material due to insufficient purification.	• Do not use more than 8-10 µl of RNA per 20 µl RT-PCR reaction mixture. • Repurify the nucleic acids to ensure removal of inhibitory agents.
	Unsuitable Hybridization Probes.	Check sequence and location of the Hybridization Probes.
	RNA degradation due to unproper storage or isolation.	• Check RNA quality on a gel. • Check RNA with an established primer pair if available.
Fluorescence intensity is too high and reaches overflow.	Unsuitable gain settings.	Gain settings cannot be changed during or after a run. Before repeating the run, use the **Real Time Fluorimeter** option to find suitable gain settings. The background fluorescence at measuring temperature should not exceed 20 for Hybridization Probes. ***Note*:** Avoid bleaching of dyes by using an extra sample for this procedure.

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Problem	Possible cause	Recommendation
Fluorescence intensity is too low.	Low concentration or deterioration of dyes in the reaction mixtures due to unsuitable storage conditions.	Store the dye containing reagents at –20°C, protected from light. Avoid repeated freezing and thawing. Low Hybridization Probe signals can be improved by using a two times higher concentration of the LC-Red labeled probe than of the fluorescein-labeled probe.
	Chosen gain are too low.	Optimize gain setting using the **Real Time Fluorimeter** function. Change the gain settings in the cycle programs appropriately and repeat the run.
	Poor PCR efficiency due to non optimized reaction conditions.	• Titrate $MgCl_2$ concentration • Primer concentration should be in the range of 0.3 – 1.0 µM, probe concentration should be in the range of 0.2–0.4 µM • Check annealing temperature of primers and probes. • Check experimental protocol • Always run a positive control along with your samples. • Increase amount of RNA template up to 500 ng total RNA or 100 ng mRNA.
	Poor PCR efficiency due to high GC content or high degree of secondary structures of the RNA.	• Extend the incubation time for the Reverse Transcription (program 1) to 30 min, and for the denaturation during cycling (program 3) to 5 s.
Fluorescence intensity varies	Pipetting errors	When using Hybridization Probes and single color detection, pipetting errors can be diminished by interpreting the results in the F2/F1 or F3/F1 mode.
	• Prepared PCR mix is still in the upper vessel of the capillary. • Air bubble is trapped in the capillary tip.	Repeat centrifugation step.
	• Skin oils on the surface of the capillary tip	Always wear gloves when handling the capillaries.
Negative control samples are positive.	Contamination	• Exchange all critical solutions. • Pipet reagents on a clean bench. • Close lid of the negative control reaction tube immediately after pipetting. • Use heat-labile UNG for decontamination of carry-over cross contamination.

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4.1 Trouble shooting, continued

Problem	Possible cause	Recommendation
High background	Very low fluorescence signals, therefore the background seems relatively high.	Follow general optimization. strategies for LightCycler PCR.
	Hybridization Probe concentration is too high.	Hybridization Probe concentration should be in the range of 0.2–0.4 µM.
	Insufficient quality of Hybridization Probes.	Prepare a new pair of Hybridization Probes.
	Gain settings are too high.	Reduce value of gain setting. Use the **Real Time Fluorimeter** option to optimize the gain settings.
Amplification curve decreases after reaching the plateau in late cycles.	"Hook effect": competition between binding of the Hybridization Probes pair and re-annealing of the PCR product.	This does not affect interpretation of the results. It can be avoided by performing an asymmetric PCR by favoring the amplification of the DNA strand that the Hybridization Probes bind to.

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4.2 Related products

Product	Pack size	Cat. No.
LightCycler Instrument	1 instrument plus accessories	2 011 468
LightCycler Capillaries	1 pack (8 boxes, each with 96 capillaries and stoppers)	1 909 339
LightCycler Centrifuge Adapters	1 set (32 adapters in an aluminum cooling block)	1 909 312
LightCycler Sample Carousel	1 carousel	1 909 282
LightCycler DNA Master SYBR[3)] Green I	1 kit (96 reactions) 1 kit (480 reactions)	2 015 099 2 158 817
LightCycler FastStart DNA Master SYBR[3)] Green I	1 kit (96 reactions) 1 kit (480 reactions)	3 003 230 2 239 264
LightCycler DNA Master Hybridization Probes	1 kit (96 reactions) 1 kit (480 reactions)	2 015 102 2 158 825
LightCycler FastStart DNA Master Hybridization Probes	1 kit (96 reactions) 1 kit (480 reactions)	3 003 248 2 239 272
LightCycler Control Kit DNA	1 kit (50 control reactions)	2 158 833
LightCycler RNA Amplification Kit SYBR Green I	1 kit (96 reactions)	2 015 137
LightCycler RNA Master SYBR Green I	1 kit (96 reactions)	3 064 760
LightCycler RNA Master Hybridization Probes	1 kit (96 reactions)	3 018 954
LightCycler Control Kit RNA	1 kit (50 control reactions)	2 158 841
LightCycler Red 705-Phosphoramidite	1 vial for synthesis of 10 oligo-nucleotides labeled at the 5'-end (0.2 μmol scale)	2 157 594
LightCycler Red 640-NHS ester	1 vial for labeling a minimum of 5 × 50 nmol oligonucleotides	2 015 161
LightCycler Fluorescein CPG	5 columns 1 g	3 113 906 3 138 178
LightCycler Color Compensation Set	1 set for 5 calibration runs	2 158 850
Uracil-DNA-Glycosylase, heat-labile	100 units	1 775 367

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