For general laboratory use.

LightCycler® RNA Master HybProbe

Version 11
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Easy-to-use Reaction Mix for One-Step RT-PCR, using the LightCycler® Carousel-Based System.

Cat. No. 03 018 954 001

Kit for 96 reactions

Store the kit at −15 to −25°C

www.roche-applied-science.com

PROTOCOL

1. What this Product Does

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

Kit Contents

Vial/Cap	Label	Contents / Function
1 red cap	LightCycler® RNA Master HybProbe, 2.7× conc.	• 3 vials, 250 µl each • Contains Tth DNA Polymerase, reaction buffer and dNTP mix (with dUTP instead of dTTP).
2 colorless cap	$Mn(OAc)_2$ stock solution, 50 mM	• 1 vial, 1 ml • To adjust $Mn(OAc)_2$ concentration.
3 colorless cap	H_2O, PCR grade	• 2 vials, 1 ml each • To adjust the reaction volume.

Storage and Stability

Store the kit at −15 to −25°C until the expiration date printed on the label.

The kit is shipped on dry ice.

Once the kit is opened, store the kit components as described in the following table:

Vial	Label	Storage
1 red cap	LightCycler® RNA Master HybProbe, 2.7× conc.	• Store at −15 to −25°C. • Avoid repeated freezing and thawing!
2 colorless cap	$Mn(OAc)_2$ stock solution, 50 mM	Store at −15 to −25°C.
3 colorless cap	H_2O, PCR grade	

Additional Equipment and Reagents Required

Additional reagents and equipment required to perform RT-PCR reactions with the LightCycler® RNA Master HybProbe, using the LightCycler® Carousel-Based System include:

- LightCycler® Carousel-Based System* (LightCycler® 2.0 Instrument*, LightCycler® 1.5 Instrument*, or an instrument version below)
- LightCycler® Capillaries*
- Standard benchtop microcentrifuge, containing a rotor for 2.0 ml reaction tubes

The LightCycler® Carousel-Based System provides Centrifuge Adapters that enable LightCycler® Capillaries to be centrifuged in a standard microcentifuge rotor.

or

- LC Carousel Centrifuge 2.0* for use with the LightCycler® 2.0 Sample Carousel (20 µl; optional)

⚠ If you use a LightCycler® Instrument version below 2.0, you need in addition the LC Carousel Centrifuge 2.0 Bucket 2.1*. To adapt the LightCycler® 2.0 Sample Carousel to the former LC Carousel Centrifuge, you need the LC Carousel Centrifuge 2.0 Rotor Set*.

- LightCycler® Color Compensation Set*# (optional)
- Uracil-DNA Glycosylase, heat-labile*‡ (optional)
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile reaction (Eppendorf) tubes for preparing master mixes and dilutions

If you want to perform color compensation when using LightCycler® Red 640 and Cy5.5-labeled HybProbe pairs in dual color experiments in the same capillary. See section Related Procedures for details.

‡ For prevention of carry-over contamination; see section Related Procedures for details.

** available from Roche Applied Science; see Ordering Information for details.*

Application

LightCycler® RNA Master HybProbe, a General Laboratory Reagent (GPR), is specifically adapted for one-step RT-PCR in glass capillaries. When combined with the LightCycler® Carousel-Based System, this kit uses a hot start RT-PCR protocol to provide very sensitive detection and quantification of defined RNA sequences (if suitable RT-PCR primers and HybProbe probes are supplied).

The kit is especially suitable for difficult RNA populations, as the elevated incubation temperature during the reverse transcription step will help to overcome secondary structures. The hot start feature will minimize mispriming during the initial phase of the reaction and therefore overall sensitivity of RT-PCR is increased. It can also be used to genotype single nucleotide polymorphisms (SNPs) and analyze mutations. Furthermore, it can be used with

Uracil DNA Glycosylase, heat-labile to prevent carry-over contamination during PCR.

In principle, the LightCycler® RNA Master HybProbe can be used for the amplification and detection of any RNA target. However, you would need to adapt each amplification protocol to the reaction conditions of the LightCycler® Carousel-Based System and design specific RT-PCR primers and HybProbe probes for each target. Refer to the LightCycler® Operator's Manual for recommendations.

⚠ The amplicon size should not exceed 750 bp in length. For optimal results, select a product length of 500 bp or less.

⚠ The performance of the kit described in this Instruction Manual is guaranteed only, when it is used with the LightCycler® Carousel-Based System.

Assay Time

Procedure	Time
RT-PCR Setup	15 min
Reverse Transcription	20 min
LightCycler® Carousel-Based System PCR run (incl. Melting Curve)	25 min
Total assay time	60 min

2. How to Use this Product

2.1 Before You Begin

Sample Material

- Use any template RNA (*e.g.,* total RNA or mRNA) suitable for RT-PCR in terms of purity, concentration and absence of inhibitors. For reproducible isolation of nucleic acids, use one of the following:
  - one of the MagNA Pure LC Instruments with a dedicated MagNA Pure LC reagent kit (for medium throughput automated isolation)
  - the MagNA Pure Compact Instrument with a dedicated MagNA Pure Compact reagent kit (for low throughput automated isolation)
  - a High Pure nucleic acid isolation kit (for manual isolation).

For further information, consult the Roche Applied Science Biochemicals catalog or our homepage: www.roche-applied-science.com. See Ordering Information for selected products, recommended for the isolation of template RNA.

- Use up to 500 ng total RNA or 100 ng mRNA.

⚠ Using a too high amount of RNA may result in inhibition of the reaction.

Q If the concentration of template RNA is lower than 10 µg/ml, the addition of unspecific carrier RNA (*e.g.,* MS2 RNA*) is recommended. To avoid loss of template RNA due to adsorption effects, the total RNA concentration of solutions (template plus carrier RNA) should not be lower than 10 µg/ml.

Primers

Use RT-PCR primers at a final concentration of 0.2 to 1 µM. The recommended starting concentration is 0.5 µM each.

⚠ If amplification curves show the "hook effect" (*i.e.,* after an exponential rise, the fluorescence signal reaches a maximum, then significantly drops in the later cycles), try using asymmetric PCR. To perform asymmetric PCR, use a higher concentration (0.5 to 1 µM) of the forward primer (i.e., the one priming the strand that binds the probes) and a lower concentration of the reverse primer (titrate down from 0.5 to 0.2 µM). This favors synthesis of the strand that binds the HybProbe probes and will improve the subsequent Melting Curve analysis.

HybProbe Probes

Use HybProbe probes at a final concentration of 0.2 µM each. In some cases it may be advantageous to double the concentration of the red fluorophore labeled probe to 0.4 µM.

Q See the LightCycler® Operator's Manual and the LightCycler® Online Resource Site (www.lightcycler.com) for detailed information on designing HybProbe probes and labeling HybProbe probes with various dyes. In addition, the LightCycler® Probe Design Software 2.0* can help you design HybProbe pairs.

$Mn(OAc)_2$

To ensure specific and efficient amplification with the LightCycler® Carousel-Based System, use $Mn(OAc)_2$ at a final concentration of 3.25 mM.

For most RNA targets tested so far, no titration of $Mn(OAc)_2$ was required. However, if necessary titrate $Mn(OAc)_2$ in a range from 2.5 to 4 mM, in steps of 0.25 mM (addition of 0.1 µl 50 mM $Mn(OAc)_2$ stock solution to a final volume of 20 µl, results in an increase of $Mn(OAc)_2$ concentration of 0.25 mM). The volume of water in the RT-PCR reaction must be reduced, accordingly.

Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template RNA with H_20, PCR grade (vial 3, colorless cap).

DNA Contamination Control

To test the template RNA for contamination with residual genomic DNA, perform PCR in combination with LightCycler® DNA Master HybProbe*, LightCycler® FastStart DNA Master HybProbe*, or LightCycler® FastStart DNA Master^{PLUS} HybProbe*. Because in this experimental setup the reverse transcription step is omitted, any PCR product generated is a signal for DNA contamination of the RNA template preparation.

2.2 Experimental Protocol

LightCycler® Carousel-Based System Protocol

The following procedure is optimized for use with the LightCycler® Carousel-Based System.

⚠ Program the LightCycler® Instrument before preparing the reaction mixes.

A LightCycler® Carousel-Based System protocol that uses the LightCycler® RNA Master HybProbe, contains the following programs:

- **Reverse Transcription** of template RNA
- **Denaturation** of cDNA/RNA hybrid
- **Amplification** of the cDNA
- **Melting Curve** for amplicon analysis (optional: only required for SNP or mutation detection)
- **Cooling** the rotor and the thermal chamber

For details on how to program the experimental protocol, see the LightCycler® Operator's Manual.

⚠ Set all other protocol parameters not listed in the tables below to '0'.

The following table shows the RT-PCR parameters that must be programmed for a LightCycler® Carousel-Based RT-PCR run with the LightCycler® RNA Master HybProbe.

Analysis Mode	Cycles	Segment	Target Temperature [1]	Hold Time	Acquisition Mode
Reverse Transcription					
None	1		61°C	20 min [4]	none
Initial Denaturation					
None	1		95°C	30 s [5]	none
Amplification					
Quantification	45	Denaturation	95°C	1–5 s [5]	none
		Annealing	primer dependent [2]	10–15 s [6]	single
		Extension	72°C [3]	= amplicon [bp]/25 s [6]	none
Melting Curve (optional)					
Melting Curves	1	Denaturation	95°C	0 s	none
		Annealing	HybProbe T_m - 5°C	30 s	none
		Melting	95°C Ramp Rate = 0.1°C/sec[1]	0 s	continuous
Cooling					
None	1		40°C	30 s	none

[1] Temperature Transition Rate/Slope/Ramp Rate is 20°C/sec, except where indicated.

[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: $T_m = 2°C\ (A+T) + 4°C\ (G+C)$.

[3] If the primer annealing temperature is low (< +55°C), reduce the ramp rate to 2 to 5°C/s.

[4] When amplifying GC-rich templates, or templates with a high degree of secondary structures, it is recommended to extend the reverse transcription incubation time to 30 mins, or longer.

[5] When amplifying GC-rich templates, or templates with a high degree of secondary structures, it is recommended to increase the Initial Denaturation incubation time up to 2 min and the Denaturation time in program 'Amplification' up to 5 s.

[6] For greater precision in target quantification experiments, it can be advantageous (in some cases) to choose longer annealing and extension times for the amplification cycles.

Fluorescence and Run Setup Parameters

Parameter	Setting	
All LightCycler® Software Versions		
Seek Temperature	61°C	
LightCycler® Software prior to Version 3.5		
Display Mode	Fluorescence channel F2 (for LightCycler® Red 640) or F3 (for Cy5.5)	
Fluorescence Gains	**Fluorimeter**	**Gain Value**
	Channel 1 (F1)	1
	Channel 2 (F2)	15
	Channel 3 (F3)	30
LightCycler® Software Version 3.5		
Display Mode • during run • for analysis	• Fluorescence channel F2 (for LightCycler® Red 640) or F3 (for Cy5.5) • For quantification analysis divide by Channel F1 for single color experiments; divide by "Back-F1" for dual color experiments (*e.g.,* F2/Back-F1). For melting curve analysis do not divide by Channel F1 or Back-F1.	
Fluorescence Gains	not required In data created with LightCycler® Software Version 3.5, all fluorescence values are normalized to a fluorescence gain of "1". This produces a different scale on the Y-axis than that obtained with previous LightCycler® Software versions. This difference does not affect the crossing points nor any calculated concentrations obtained.	

continued on next page

Parameter	Setting
	LightCycler® Software Version 4.1
Default Channel • during run • for analysis	• Depending on the red fluorophore dye used for labeling the HybProbe probe, choose Channel 610, 640, 670, or 705. • Depending on the red fluorophore dye used for labeling the HybProbe probe, choose Channel 610, 640, 670, or 705. For quantification analysis divide by channel 530 for single color experiments; divide by "Back 530" for dual color experiments (*e.g.,* 640/ Back 530). For automated T_m Calling analysis do not divide by channel 530 or "Back 530". Channel 610 and 670 are available on a LightCycler® 2.0 Instrument only.
Fluorescence Gains	not required
"Max. Seek Pos."	Enter the number of sample positions for which the instrument should look.
"Instrument Type"	"6 Ch.": for LightCycler® 2.0 Instrument (selected by default) "3 Ch.": for LightCycler® 1.5 Instrument and instrument versions below
"Capillary Size"	Select "20 µl" as the capillary size for the experiment. ⚠ For the "6 Ch." instrument type only.

Preparation of the RT-PCR Mix

Proceed as described below for a 20 µl standard reaction.

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

❶ Depending on the total number of reactions, place the required number of LightCycler® Capillaries in pre-cooled centrifuge adapters, or in a LightCycler® Sample Carousel in a pre-cooled LC Carousel Centrifuge Bucket.

❷ • Thaw the solutions and for maximal recovery of contents, briefly spin vials in a microcentrifuge before opening.
• Mix carefully by pipetting up and down and store on ice.

⚠ A reversible precipitate may form in the LightCycler® RNA Master HybProbe (vial 1, red cap) during storage. If a precipitate is visible, place the vial at room temperature (+15 to +25°C) and mix gently from time to time, until the precipitate is completely dissolved. This does not influence the performance in RT-PCR.

❸ Prepare a 10× conc. solution of RT-PCR primers and a 10x conc. solution of HybProbe probes.

If you are using the recommended final concentration of 0.5 µM for each primer, the 10× conc. solution would contain a 5 µM concentration of each primer.

❹ In a 1.5 ml reaction tube on ice, prepare the RT-PCR Mix for one 20 µl reaction, by adding the following components in the order mentioned below:

Component	Volume	Final conc.
H_2O, PCR grade (vial 3, colorless cap)	6.2 µl	–
$Mn(OAc)_2$ stock solution, 50 mM (vial 2, colorless cap)	1.3 µl	3.25 mM
RT-PCR Primer Mix, 10× conc. [1)]	2.0 µl	0.2 to 1.0 µM each (recommended conc. is 0.5 µM)
HybProbe Mix, 10× conc. [2)]	2.0 µl	0.2 to 0.4 µM each
LightCycler® RNA Master HybProbe (vial 1, red cap)	7.5 µl	1×
Total volume	**19 µl**	

To prepare the RT-PCR Mix for more than one reaction, multiply the amount in the "Volume" column above by *z*, where *z* = the number of reactions to be run + one additional reaction.

- Mix gently by pipetting up and down. Do not vortex.
- Pipette 19 µl RT-PCR mix into each pre-cooled LightCycler® Capillary.
- Add 1 µl of the RNA template.
- Seal each capillary with a stopper.

- Place the centrifuge adapters (containing the capillaries) into a standard benchtop microcentrifuge.

  ⚠ Place the centrifuge adapters in a balanced arrangement within the centrifuge.
- Centrifuge at 700× *g* for 5 s (3,000 rpm in a standard benchtop microcentrifuge).
- Alternatively, use the LC Carousel Centrifuge for spinning the capillaries.

❺ Transfer the capillaries into the LightCycler® Sample Carousel and then into the LightCycler® Instrument.

❻ Cycle the samples as described above.

[1] Due to possible primer/primer interactions generated during storage, it may be necessary to preheat the RT-PCR primer mix for 1 min at 95°C before starting the reaction, to achieve optimum sensitivity.

[2] If you want to perform dual color detection using LightCycler® Red 640- and Cy5.5-labeled HybProbe pairs simultaneously in one capillary, either use two separate HybProbe mixes (then you must add 2 µl each from both of the two mixes), or combine both HybProbe pair preparations in one mix. (You will then add 2 µl only, from this combined HybProbe mix).

2.3 Related Procedures

Color Compensation

If using acceptor HybProbe probes that contain different red fluorophore labels in the same capillary, you must compensate for the crosstalk between individual channels by using a (previously generated) color compensation file.

You can activate a previously stored color compensation file during the LightCycler® Instrument run or use it for data analysis after the run.

Although the optical filters of each detection channel of the LightCycler® Carousel-Based System Instrument are optimized for the different emission maxima of the fluorescent dyes, some residual crosstalk between the channels will occur unless you correct for it with a color compensation file.

⚠ Color Compensation is instrument-specific. Therefore, a color compensation file must be generated for each LightCycler® Carousel-Based System Instrument.

No universal color compensation set is available for 6-channel applications on a LightCycler® 2.0 Instrument. All multicolor assays must use a specific color compensation protocol. You must prepare a new color compensation object for each set of parameters.

For more information on the generation and use of a color compensation file, see the LightCycler® Operator's Manual, the LightCycler® Online Resource Site (www.lightcycler.com), or the package inserts of the LightCycler® Color Compensation Set and LightCycler® Multiplex DNA Master HybProbe.

Prevention of Carry-Over Contamination

Uracil-DNA Glycosylase, heat-labile (UNG, heat-labile) is suitable for preventing carry-over contamination in PCR. This carry-over prevention technique involves incorporating deoxyuridine triphosphate (dUTP, a component of the reaction mixes of all LightCycler® reagent kits) into amplification products, then pretreating later PCR mixtures with UNG. If a dUTP-containing contaminant is present in the later PCRs, it will be cleaved by a combination of the UNG and the high temperatures of the initial denaturation step; it will not serve as a PCR template.

Proceed as described in the package insert and/or in the table below to prevent carry-over contamination using UNG, heat-labile:

❶	Add 1 µl UNG, heat-labile to the master mix per 20 µl final reaction volume.
❷	Add template RNA and incubate the completed reaction mixture for 5 min at room temperature.
❸	Destroy any contaminating template and inactivate the UNG enzyme by performing the reverse transcription step at 61°C. ⚠ Do not perform an additional inactivation step at higher temperatures than 61°C, as the reverse transcriptase would be inactivated.

When performing Melting Curve analysis, the use of UNG may lower the melting temperature (T_m) by approx. 1°C.

3. Results

The following amplification curves were obtained using the LightCycler® RNA Master HybProbe, in combination with the LightCycler® Control Kit RNA, targeting *in vitro* transcribed cytokine RNA template. The single color detection protocol was performed using LightCycler® Red 640 as acceptor fluorophore. Displayed are the results in channel F2 [640][1)] and F3 [705][1)], with and without color compensation. Equivalent results will be obtained using single color detection with Cy5.5 as acceptor fluorophore, or dual color detection with LightCycler® Red 640- and Cy5.5-labeled HybProbe pairs simultaneously.

The fluorescence values versus cycle number are displayed. One hundred copies of the cytokine RNA can be reproducibly detected by amplification in the LightCycler® Carousel-Based System Instrument using the HybProbe detection.

1) Values in brackets refer to the LightCycler® Software 4.x (this includes LightCycler® Software 4.0, 4.05 and 4.1).

Fig. 1: Serially diluted samples containing 10^1 to 10^6 copies of cytokine RNA template from the LightCycler® Control Kit RNA were amplified using the LightCycler® RNA Master HybProbe in a LightCycler® Carousel-Based System Instrument. As a negative control, template RNA was replaced by PCR-grade water. LightCycler® Red 640 was used as the acceptor fluorophore.
Fig 1a and 1b display results in detection channel F2 [640][1)] without and with color compensation. Fig 1c and 1d display results in detection channel F3 [705][1)] without and with color compensation. Quantification analysis was performed using LightCycler® Software 3.5, applying arithmetic background subtraction.

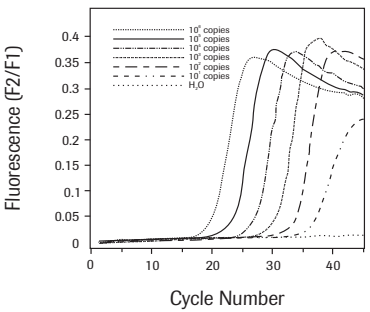


Fig. 1a: Channel F2 [640][1)] (F2/F1)without color compensation

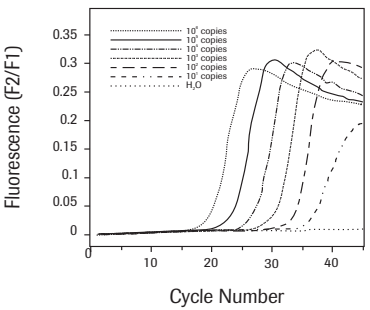


Fig. 1b: Channel F2 [640][1)] (F2/F1) with color compensation

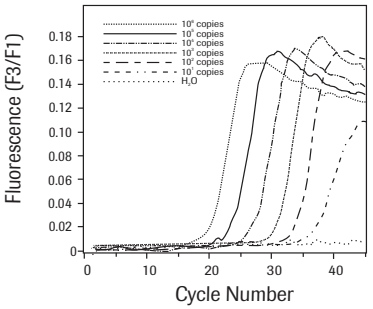


Fig. 1c: Channel F3 [705][2] (F2/F1) without color compensation

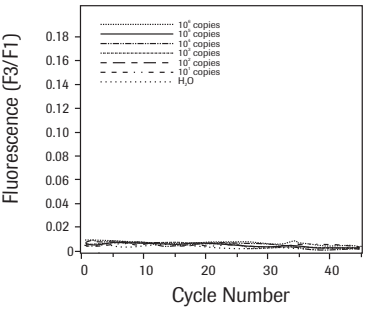


Fig. 1d: Channel F3 [705][2] (F2/F1) with color compensation

4. Troubleshooting

	Possible cause	Recommendation
Amplification reaches plateau phase before the program is complete.	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 amplification program.
Log-linear phase of amplification just starts as the amplification program finishes.	Very low starting amounts of nucleic acid	• Improve PCR conditions (*e.g.,* primer and probe concentration or design). • Use higher amount of starting material. • Repeat the run.
	The number of cycles is too low.	• Increase the number of cycles in the amplification program. • Use the **+10 cycles** button, to increase the number of cycles in the amplification program.
No amplification occurs.	Wrong channel has been chosen to display amplification online.	Change the channel setting on the programming screen. (The data obtained up to this point will be saved.)
	Pipetting errors or omitted reagents	• Check for missing reagents. • Check for missing or defective dye.
	Measurements do not occur.	Check the amplification program. For the HybProbe detection format, choose “single” as the acquisition mode at the end of the annealing phase.
	Amplicon length is >750 bp.	Do not use amplicons >750 bp. Amplification efficiency is reduced with amplicons of this size. Optimal results are obtained for amplicons up to 500 bp.
	Inhibitory effects of the sample material, due to insufficient purification.	• Do not use more than 8 to 10 ml of RNA per 20 ml RT-PCR reaction mixture. • Repurify the nucleic acids, to ensure removal of inhibitory agents.
	Unsuitable HybProbe probes	• Check sequence and location of the HybProbe probes. • Check RT-PCR product on an agarose gel.
	Unsuitable RT-PCR primers	• Check primer design (quality). • Check RT-PCR product on an agarose gel.
	RNA degradation, due to improper 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 can not 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 HybProbe probes. ⚠ Use an extra sample for this procedure, so the dyes in your experimental samples will not be bleached. LightCycler® Software versions 3.5 and higher do not require a gain setting.

	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 –15 to –25°C, protected from light. • Avoid repeated freezing and thawing. • Low HybProbe signals can be improved by using a two times higher concentration of the red fluorophore-labeled probe than of the fluorescein-labeled probe.
	Chosen gain settings are too low.	⚠ Optimize gain settings using the **Real Time Fluorimeter** function. Then repeat the run, using the optimal gain settings in the cycle programs.
	Reaction conditions are not optimized, leading to poor PCR efficiency.	• Primer concentration should be in the range of 0.2 to 1.0 mM, probe concentration should be in the range of 0.2 to 0.4 mM. • 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. • For most RNA targets tested so far, no titration of Mn(OAc)2 has been required. However, if necessary a titration of Mn(OAc)2 in a range from 2.5 to 4 mM in steps of 0.25 mM may be considered (0.1 ml Mn(OAc)2 stock solution, 50 mM corresponds to 0.25 mM Mn(OAc)2 in a final volume of 20 ml).
	Poor PCR efficiency, due to high GC content, or high degree of secondary structures of the RNA.	Extend the incubation time for Reverse Transcription to 30 min and for denaturation during cycling to 5 s.
	Poor PCR efficiency, due to unsuitable primers or probes.	• Check PCR product on agarose gel. • Redesign primers and probes.
Fluorescence intensity varies.	Pipetting errors	When using HybProbe probes and single color detection, pipetting errors can be diminished by interpreting the results in the F2/F1 or F3/F1 (640/530 or 705/530) mode.
	PCR mix is still in the upper part of the capillary. Air bubble is trapped in the capillary tip.	Repeat capillary centrifugation step.
	Skin oils on the surface of the capillary tip.	Always wear gloves when handling the capillaries.
Negative control samples are positive.	Contamination	• Remake all critical solutions. • Pipette reagents on a clean bench. • Close lid of the negative control reaction immediately after pipetting it. • Use heat-labile UNG for prevention of carry-over contamination.
High background	Very low fluorescence signals, therefore the background seems relatively high.	Follow general optimization strategies for PCR using the LightCycler® Carousel-Based System.
	HybProbe probe concentration is too high.	HybProbe probe concentration should be in the range of 0.2 to 0.4 mM.
	Insufficient quality of HybProbe probes	Prepare a new pair of HybProbe probes.
	Gain settings are too high.	• Reduce value of gain settings. • Use the **Real Time Fluorimeter** option to optimize the gain settings.
Amplification curve decreases after reaching a plateau in the later cycles.	"Hook effect": competition between binding of the HybProbe probes and reannealing of the PCR product.	This does not affect interpretation of the results. It can be avoided by performing an asymmetric PCR, favoring the amplification of the DNA strand to which the HybProbe probes bind.

5. Additional Information on this Product

5.1 How this Product Works

LightCycler® RNA Master HybProbe is an easy-to-use hot start reaction mix, specifically adapted for one-step RT-PCR in 20 µl glass capillaries, using the detection format of the HybProbe probe on the LightCycler® Carousel-Based System. Amplification and on-line monitoring of the template RNA is achieved by a combined procedure on the LightCycler® Carousel-Based System Instrument. The results are interpreted directly after completing the PCR and Melting Curve. The amplicon is detected by fluorescence using target-specific HybProbe probes (not provided by the kit).

The LightCycler® RNA Master HybProbe provides convenience, high performance, reproducibility and minimizes contamination risk. All you must supply is the template RNA, RT-PCR primers and HybProbe probes.

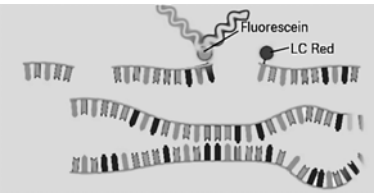
Test Principle

The hot start feature of the LightCycler® RNA Amplification Kit HybProbe is achieved by using Tth DNA Polymerase, in combination with Aptamers. Tth DNA Polymerase is a thermostable enzyme with RNA-dependent reverse transcriptase activity and DNA-dependent polymerase activity, allowing the combination of RT and PCR in a single-tube reaction. Aptamers are dedicated oligonucleotides that bind in the active center of the polymerase and prevent attachment to nucleic acid targets at temperatures below the optimal reaction temperature of the Tth enzyme. Therefore, no primer elongation occurs during setup of the reaction and the following heating phase prior to the RT step. At higher temperatures, the Aptamers are released from the enzyme and RT or DNA polymerization can be initiated. In addition, the recommended incubation temperature for reverse transcription with Tth (61°C) is helpful to overcome secondary structures of RNA. This results in highly specific and efficient cDNA synthesis, that leads to highly specific and sensitive PCR. Hot start with Aptamers is highly effective and very convenient, because it does not require additional incubation steps, pipetting steps, or an extension of reaction time. The hot start protocol with Aptamers does not interfere with other enzymatic processes, the online detection of amplification products, or subsequent handling steps.

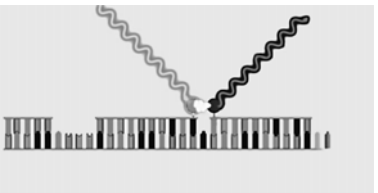
HybProbe probes consist of two different short oligonucleotides that bind to an internal sequence of the amplified fragment during the annealing phase of the amplification cycle.

The basic steps of DNA detection by HybProbe probes during real-time PCR on the LightCycler® Carousel-Based System are:

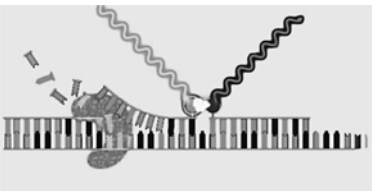
① The donor dye probe has a fluorescein label at its 3´-end and the acceptor dye probe has a red fluorophore label [LightCycler® Red 610#, LightCycler® Red 640, Cy5#, or Cy5.5] at its 5´-end (it is 3´-phosphorylated, so it can not be extended). Hybridization does not occur during the Denaturation phase of PCR. As the distance between the unbound dyes prevents energy transfer, no fluorescence will be detected from the red acceptor dye during this phase.



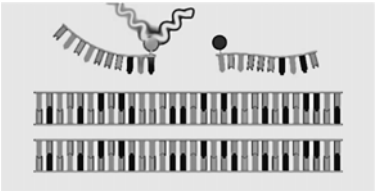
② During the annealing phase, the probes hybridize to the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two fluorescent dyes close to each other. Fluorescein is excited by the light source of the LightCycler® Carousel-Based System, which causes it to emit green fluorescent light. The emitted energy excites the red fluorophore by fluorescence resonance energy transfer (FRET). The red fluorescence emitted by the acceptor dye is measured at the end of each annealing step, when the fluorescence intensity is greatest



③ After annealing, an increase in temperature leads to elongation and displacement of the probes



④ At the end of the elongation step, the PCR product is double-stranded, while the displaced HybProbe probes are back in solution and too far apart to allow FRET to occur.



HybProbe probes that carry different red fluorophore labels can be used separately (for single color detection experiments) or combined (for dual or multiple color detection experiments). Color compensation is not necessary for single color detection experiments. However, if you are using HybProbe probes to perform dual or multiple color experiments in a single capillary, you must also use a color compensation file. Color compensation may be applied either during or after a run on the LightCycler® Carousel-Based System.

See the LightCycler® Operator's Manual and the package insert of the LightCycler® Color Compensation Set for more information on the generation and use of a color compensation file, or object.

#LightCycler® Red 610 and Cy5 can only be used on a LightCycler® 2.0 Instrument.

5.2 Quality Control

The LightCycler® RNA Master HybProbe is function tested using the LightCycler® Control Kit RNA with the LightCycler® Carousel-Based System.

5.3 Product Citations

1 Donckier, V. *et al.* (2004). Donor stem cell infusion after non-myeloablative conditioning for tolerance induction to HLA mismatched adult living-donor liver graft. *Transpl. Immunol.* **13**, 139-146.

2 Loi, P. *et al.* (2004). The Fate of Dendritic Cells in a Mouse Model of Liver Ischemia/Reperfusion Injury. *Transplant. Proc.* **36**, 1275-1279.

3 van Rijn, PA. *et al.* (2004). Detection of economically important viruses in boar semen by quantitative RealTime PCR™ technology. *J. Virol. Methods* **120**, 151-160.

4 Listvanova, S. *et al.* (2003). Optimal kinetics for quantification of antigen-induced cytokines in human peripheral blood mononuclear cells by real-time PCR and by ELISA. *J. Immunol. Methods* **281**, 27-35.

5 Nagy, J. *et al.* (2003). Inducible expression and pharmacology of recombinant NMDA receptors, composed of rat NR1a/NR2B subunits. *Neurochem. Int.* **43**, 19-29.

6 van der Linden, IFA. *et al.* (2003). Oral transmission of porcine reproductive and respiratory syndrome virus by muscle of experimentally infected pigs. *Vet. Microbiol.* **97**, 45-54.

7 Rabenau, HF. *et al.* (2002). Rapid detection of enterovirus infection by automated RNA extraction and real-time fluorescence PCR. *J. Clin. Virol.* **25**, 155-164.

8 Stordeur, P. *et al.* (2002). Analysis of spontaneous mRNA cytokine production in peripheral blood. *J. Immunol. Methods* **261**, 195-197.

9 Westerman, BA. *et al.* (2002). Quantitative Reverse Transcription-Polymerase Chain Reaction Measurement of *HASH1 (ASCL1)*, a Marker for Small Cell Lung Carcinomas with Neuroendocrine Features. *Clin. Cancer Res.* **8**, 1082-1086.

6. Supplementary Information

6.1 Conventions

Text Conventions To make information consistent and memorable, the following text conventions are used in this Instruction Manual:

Text Convention	Usage
Numbered stages labeled ①, ② etc.	Stages in a process that usually occur in the order listed.
Numbered instructions labeled ❶, ❷ etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Applied Science.

Symbols In this Instruction Manual, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
	Important Note: Information critical to the success of the procedure or use of the product.

Abbreviations In this Instruction Manual, the following abbreviations are used:

FRET	fluorescence resonance energy transfer
T_m	melting temperature
UNG	Uracil-DNA Glycosylase

6.2 Changes to Previous Version

License disclaimer - new information

6.3 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, www.roche-applied-science.com, and our Special Interest Sites, including:

- Real-time PCR Systems (LightCycler® Carousel-Based System, LightCycler® 480 System, LightCycler® 1536 System, RealTime ready qPCR assays and Universal ProbeLibrary):
  http://www.lightcycler.com
- Automated Sample Preparation (MagNA Lyser Instrument, MagNA Pure Compact System, MagNA Pure LC Systems and MagNA Pure 96 System): http://www. magnapure.com

Instruments and Accessories

Product	Pack Size	Cat. No.
LightCycler® 2.0 Instrument	1 instrument plus accessories	03 351 414 001
LightCycler® 1.5 Instrument	1 instrument plus accessories	04 484 495 001
LightCycler® Capillaries (20 µl)	1 pack (5 boxes, each with 96 capillaries and stoppers)	04 929 292 001
LC Carousel Centrifuge 2.0	1 centrifuge plus rotor and bucket	03 709 507 001 (115 V) 03 709 582 001 (230 V)
LC Carousel Centrifuge 2.0 Rotor Set	1 rotor + 2 buckets	03 724 697 001
LC Carousel Centrifuge 2.0 Bucket 2.1	1 bucket	03 724 689 001
MagNA Pure LC 2.0 Instrument	1 instrument plus accessories	05 197 686 001
MagNA Pure Compact Instrument	1 instrument plus accessories	03 731 146 001
LightCycler® Software 4.1	1 software package	04 898 915 001
LightCycler® Probe Design Software 2.0	1 software package	04 342 054 001

	Product	Pack Size	Cat. No.
RNA Isolation Kits	MagNA Pure LC RNA Isolation Kit – High Performance	1 kit (192 isolations)	03 542 394 001
	MagNA Pure LC RNA Isolation Kit III (Tissue)	1 kit (192 isolations)	03 330 591 001
	MagNA Pure LC mRNA HS Kit[1)]	1 kit (192 isolations)	03 267 393 001
	MagNA Pure Compact RNA Isolation Kit	1 kit (32 isolations)	04 802 993 001
	High Pure RNA Isolation Kit	1 kit (50 isolations)	11 828 665 001
	High Pure RNA Tissue Kit	1 kit (50 isolations)	12 033 674 001
	High Pure Viral RNA Kit	1 kit (100 isolations)	11 858 882 001
LightCycler® One-Step RT-PCR Kits	LightCycler® RNA Amplification Kit SYBR Green I	1 kit (96 reactions)	12 015 137 001
	LightCycler® RNA Amplification Kit HybProbe	1 kit (96 reactions)	12 015 145 001
	LightCycler® RNA Master SYBR Green I	1 kit (96 reactions)	03 064 760 001
cDNA Synthesis Reagents	Transcriptor Reverse Transcriptase	250 U 500 U 2,000 U	03 531 317 001 03 531 295 001 03 531 287 001
	Transcriptor First Strand cDNA Synthesis Kit	1 kit (50 reactions, incl. 10 control reactions) 1 kit (100 reactions) 1 kit (200 reactions)	04 379 012 001 04 896 866 001 04 897 030 001
	First Strand cDNA Synthesis Kit for RT-PCR (AMV)	1 kit (30 reactions)	11 483 188 001

	Product	Pack Size	Cat. No.
LightCycler® Reagent Kits for Two-Step RT-PCR	LightCycler® DNA Master HybProbe	1 kit (96 reactions) 1 kit (480 reactions)	12 015 102 001 12 158 825 001
	LightCycler® FastStart DNA Master HybProbe	1 kit (96 reactions) 1 kit (480 reactions)	03 003 248 001 12 239 272 001
	LightCycler® FastStart DNA Master^{PLUS} HybProbe	1 kit (96 reactions) 1 kit (480 reactions)	03 515 575 001 03 515 567 001
	LightCycler® DNA Master SYBR Green I	1 kit (96 reactions) 1 kit (480 reactions)	12 015 099 001 12 158 817 001
	LightCycler® FastStart DNA Master SYBR Green I	1 kit (96 reactions) 1 kit (480 reactions)	03 003 230 001 12 239 264 001
	LightCycler® FastStart DNA Master^{PLUS} SYBR Green I	1 kit (96 reactions) 1 kit (480 reactions)	03 515 869 001 03 515 885 001
Associated Kits and Reagents	LightCycler® Color Compensation Set	1 set (5 reactions)	12 158 850 001
	LightCycler® Control Kit RNA	1 kit (50 reactions)	12 158 841 001
	Uracil-DNA Glycosylase, heat-labile	100 U 500 U	11 775 367 001 11 775 375 001
	RNA, MS2	10 A_{260} U (500 µl)	10 165 948 001

[1)] the MagNA Pure LC mRNA HS Kit is only available for use with the MagNA Pure LC 1.0 Instrument (Cat. No. 12 236 931 001).

6.4 Disclaimer of License

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