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LightCycler® FastStart DNA Master HybProbe

Version 15

Content version: May 2011

Easy-to-use Hot Start Reaction Mix for PCR using HybProbe probes with the LightCycler® Carousel-Based System.

Cat. No. 03 003 248 001 Kit for 96 reactions
Cat. No. 12 239 272 001 Kit for 480 reactions

Store the kit at −15 to −25°C

www.roche-applied-science.com

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PROTOCOL

1. What this Product Does

Number of Tests

The kit is designed for:

- Cat. No. 03 003 248 001: 96 reactions, with a final volume of 20 µl each
- Cat. No. 12 239 272 001: 480 reactions, with a final volume of 20 µl each.

Kit Contents

Vial/Cap	Label	Contents/Function a) Cat. No. 03 003 248 001 b) Cat. No. 12 239 272 001
1a red cap	LightCycler® FastStart Enzyme	a) 3 vials 1a and 3 vials 1b, for 3 vials, 64 µl each LightCycler® FastStart DNA Master HybProbe, 10× conc. b) 15 vials 1a and 15 vials 1b, for 15 vials, 64 µl each LightCycler® FastStart DNA Master HybProbe, 10× conc.
1b colorless cap	LightCycler® FastStart Reaction Mix HybProbe, 10× conc.	▪ Ready-to use hot start PCR reaction mix (after pipetting 60 µl from vial 1b into one vial 1a). ▪ Contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and 10 mM $MgCl_2$.
2 blue cap	$MgCl_2$ stock solution, 25 mM	a) 1 vial, 1 ml b) 2 vials, 1 ml each ▪ To adjust $MgCl_2$ concentration in the reaction mix.
3 colorless cap	H_2O, PCR grade	a) 2 vials, 1 ml each b) 7 vials, 1 ml each ▪ To adjust the final 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
1a red cap	LightCycler® FastStart Enzyme	▪ Store at -15 to -25°C. ▪ **Avoid repeated freezing and thawing!**
1b colorless cap	LightCycler® FastStart Reaction Mix HybProbe, 10× conc.	

Vial	Label	Storage
1 red cap (after the addition of 1b to 1a)	LightCycler® FastStart DNA Master HybProbe, 10× conc.	▪ Store at -15 to -25°C for a maximum of three months. ▪ After thawing, store at +2 to +8°C for a maximum of one week. ▪ **Avoid repeated freezing and thawing!**
2 blue cap	$MgCl_2$ stock solution, 25 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 PCR reactions with the LightCycler® FastStart DNA Master HybProbe, using the LightCycler® Carousel-Based System:

- 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 includes Centrifuge Adapters that enable LightCycler® Capillaries to be centrifuged in a standard microcentrifuge 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 (20 µl) to the former LC Carousel Centrifuge, you need the LC Carousel Centrifuge 2.0 Rotor Set*.

- LightCycler® Color Compensation Set* (optional[#])
- LightCycler® Uracil-DNA Glycosylase* (optional[‡])
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile reaction (Eppendorf) tubes for preparing master mixes and dilutions and for synthesizing cDNA (if performing two-step RT-PCR)

[#] To perform color compensation, when using LightCycler® Red 640 and Cy5.5 labeled HybProbe probes 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® FastStart DNA Master HybProbe is a General Purpose Reagent, using HybProbe probes with the LightCycler® Carousel-Based System. This kit is ideally suited for hot start PCR applications in glass capillaries. In combination with the LightCycler® Carousel-Based System and suitable PCR primers and HybProbe probes, this kit enables very sensitive detection and quantification of defined DNA sequences. It can also be used to genotype single nucleotide polymorphisms (SNPs) and analyze mutations using Melting Curve analysis. Furthermore, this kit can be used to perform two-step RT-PCR, in combination with a reverse transcription kit for cDNA synthesis*.

In principle, LightCycler® FastStart DNA Master HybProbe can be used for the amplification and detection of any DNA or cDNA target. However, the amplification protocol must be optimized to the reaction conditions of the LightCycler® Carousel-Based System and specific PCR primers and HybProbe probes designed for each target.

LightCycler® FastStart DNA Master HybProbe can also be used with LightCycler® Uracil-DNA Glycosylase, to prevent carry-over contamination during PCR.

⚠ 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
Optional: dilution of template DNA	5 min
PCR Setup	15 min
LightCycler® Carousel-Based System PCR run (incl. Melting Curve)	45 min
Total assay time	**65 min**

2. How to Use this Product

2.1 Before You Begin

Sample Material

- Use any template DNA (*e.g.,* genomic or plasmid DNA, cDNA) suitable for 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 catalog or our homepage: www.roche-applied-science.com. See Ordering Information for selected products, recommended for the isolation of template DNA.

- Use up to 500 ng complex genomic DNA or 10^1 to 10^{10} copies plasmid DNA.

When using a non-purified cDNA sample after reverse transcription, especially if it contains high background concentrations of RNA and oligonucleotides, use 2 µl or less of that sample in the reaction.

Primers

Use 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", perform an asymmetric PCR. The "hook effect" does not influence final results of the real-time PCR, however, it occurs when the exponential rise in fluorescent signal reaches a maximum, then significantly drops in the later cycles. It is due to competition between binding of the HybProbe probes and amplicon reannealing.

To favor HybProbe probe annealing, perform asymmetric PCR using 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 (*i.e.,* titrate down from 0.5 to 0.2 µM). This favors synthesis of the strand binding 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.

Refer to the LightCycler® Instrument Operator's Manual and the Special Interest Site for the LightCycler® Real-Time PCR Systems (www.lightcycler.com) for detailed information on designing and labeling HybProbe probes with various dyes. In addition, the LightCycler® Probe Design Software 2.0 can design the best HybProbe probe-pair and primer combinations.

$MgCl_2$

To ensure specific and efficient amplification with the LightCycler® Carousel-Based System, the $MgCl_2$ concentration of the PCR reaction mix must be optimized for each target. The LightCycler® FastStart DNA Master HybProbe contains a $MgCl_2$ concentration of 1 mM (final concentration). The optimal $MgCl_2$ concentration for PCR with the LightCycler® Carousel-Based System may vary from 1 to 5 mM.

The table below gives the volumes of the $MgCl_2$ stock solution, 25 mM (vial 2, blue cap) that must be added to a 20 µl reaction (final PCR volume), to increase the $MgCl_2$ concentration to the indicated values.

To reach a final Mg^{2+} concentration (mM) of:	1	2	3	4	5
Add this amount of 25 mM $MgCl_2$ stock solution (µl)	0	0.8	1.6	2.4	3.2

The volume of H_2O, PCR grade in the PCR reaction must be reduced, accordingly.

Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template DNA with H_2O, PCR grade (vial 3, colorless cap).

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® FastStart DNA Master HybProbe, contains the following programs:

- **Pre-Incubation** for activation of the FastStart Taq DNA polymerase and denaturation of the template DNA
- **Amplification** of the target DNA
- **Melting Curve** for amplicon analysis (Optional: only required for SNP or mutation detection)
- **Cooling** of the rotor and the thermal chamber

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

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

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

Analysis Mode	Cycles	Segment	Target Temperature [1]	Hold Time	Acquisition Mode
Pre-Incubation					
None	1		95°C	10 min[4]	None
Amplification					
Quantification	45	Denaturation	95°C	10 s	None
		Annealing	primer dependent [2]	5 – 20 s [5]	Single
		Extension	72°C [3]	= amplicon [bp]/25 s [5]	None
Melting Curve (optional)					
Melting Curves	1	Denaturation	95°C	0 s	None
		Annealing	Probes T_m – 5°C	30 - 60 s	None
		Melting	95°C Ramp Rate = 0.1°C/sec	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] A 10 min pre-incubation time is recommended. However, depending on the individual assay, the pre-incubation time can be reduced to 5 min with no change in performance. In assays where high polymerase activity is required in the early cycles, in some cases, results can be improved by extending the pre-incubation time to 15 min.

[5] 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	30°C
	LightCycler® **Software Version 3.5**
Display Mode	
▪ during the run	▪ Fluorescence channel F2 (for LightCycler® Red 640) or F3 (for Cy5.5 {705})
▪ for analysis	▪ 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, or any calculated concentrations obtained.
	LightCycler® **Software Version 4.1**
Default Channel	
▪ during the run	▪ Depending on the red fluorophore dye used for labeling the HybProbe probe, choose Channel 610, 640, 670, or 705.
▪ for analysis	▪ 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". Channels 610 and 670 are only available on a LightCycler® 2.0 Instrument.
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 Master Mix

1. Thaw one vial of "Reaction Mix" (vial 1b, colorless cap).
   ⚠ A reversible precipitate may form in the LightCycler® FastStart Reaction Mix HybProbe (vial 1b, colorless cap) during storage. If a precipitate is visible, place the Reaction Mix at +37°C and mix gently from time to time, until the precipitate is completely dissolved. This treatment does not influence the performance in PCR.
2. Briefly centrifuge one vial "Enzyme" (vial 1a, red cap) and the thawed vial of "Reaction Mix" (from Step 1), then place the vials back on ice
3. Pipette 60 µl from vial 1b (colorless cap) into vial 1a (red cap).
4. Mix gently by pipetting up and down.
   ⚠ Do not vortex.
5. Re-label vial 1a (red cap) with the new label (vial 1: LightCycler® FastStart DNA Master HybProbe) provided with the kit.
6. Store on ice, or in the pre-cooled LightCycler® Centrifuge Adapters Cooling Block, until ready to use.

Preparation of the 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.

1. 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.
2. Prepare a 10x conc. solution of 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
4. In a 1.5 ml reaction tube on ice, prepare the 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)	x µl	
$MgCl_2$ stock solution, 25 mM (vial 2, blue cap)	y µl	Use concentration that is optimal for the target.
PCR Primer Mix, 10× conc.	2 µl	0.2 to 1.0 µM each (recommended conc. is 0.5 µM)
HybProbe Probe Mix, 10× conc.	2 µl	0.2 to 0.4 µM each
LightCycler® FastStart DNA Master HybProbe, 10× conc. (vial 1, red cap)	2 µl	1×
Total volume	**18 µl**	

	To prepare the 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.
5	▪ Mix gently by pipetting up and down. Do not vortex. ▪ Pipette 18 µl PCR mix into each pre-cooled LightCycler® Capillary. ▪ Add 2 µl of the DNA template. ▪ Seal each capillary with a stopper.
6	▪ 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.
7	Transfer the capillaries into the LightCycler® Sample Carousel and then into the LightCycler® Instrument.
8	Cycle the samples, as described above.

2.3 Related Procedures

Color Compensation

When using HybProbe probes that contain different red fluorophore labels in the same capillary, a (previously generated) color compensation file must be used to compensate for the crosstalk between the individual channels. A previously stored color compensation file can be activated during the LightCycler® Instrument run, or during data analysis, after the run.

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

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

No universal color compensation set is available for 6-channel applications on the LightCycler® 2.0 Instrument. All multi-color assays must use a specific color compensation protocol. A new color compensation object must be generated for each set of parameters.

For more information on the generation and use of a color compensation file, see the LightCycler® Instrument Operator's Manual, the Special Interest Site for the LightCycler® Real-Time PCR Systems (www.lightcycler.com), or the package inserts of the LightCycler® Color Compensation Set and the 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.

When using LightCycler® FastStart DNA Master HybProbe, perform prevention of carry-over contamination with LightCycler® Uracil-DNA Glycosylase*, prior to beginning real-time PCR. Proceed as described in the package insert and/or in the table below, to prevent carry-over contamination.

❶	Add 0.5 U LightCycler® Uracil-DNA Glycosylase to the master mix per 20 µl final reaction volume.
❷	Add template DNA and incubate the completed reaction mixture for 10 min at +40°C.
❸	Destroy any contaminating template and inactivate the UNG enzyme, by performing the initial denaturation step for 10 min at +95°C.

As the target DNA template contains thymidine rather than uridine, it is not affected by this procedure.

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

Two-Step RT-PCR

LightCycler® FastStart DNA Master HybProbe can also be used to perform two-step RT-PCR. In two-step RT-PCR, the reverse transcription of RNA into cDNA is separated from the other reaction steps and is performed outside the LightCycler® Carousel-Based System. Subsequent amplification and online monitoring is performed according to the standard LightCycler® Carousel-Based System procedure, using cDNA as starting sample material. One of the following reagents is required for reverse transcription of RNA into cDNA:

- Transcriptor Reverse Transcriptase*
- Transcriptor First Strand cDNA Synthesis Kit*
- Transcriptor High Fidelity cDNA Synthesis Kit*
- Transcriptor Universal cDNA Master*
- First Strand cDNA Synthesis Kit for RT-PCR (AMV)*

Synthesis of cDNA is performed according to the detailed instructions provided with the cDNA synthesis reagent.

⚠ Do not use more than 8 µl of undiluted cDNA template per 20 µl final reaction volume, because greater amounts may inhibit PCR. For initial experiments, we recommend running undiluted, 1:10 diluted and 1:100 diluted cDNA templates, in parallel to determine the optimal template amount.

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

3. Results

The following amplification curves were obtained using the LightCycler® FastStart DNA Master HybProbe, in combination with the LightCycler® Control Kit DNA. The single color detection protocol was performed, using LightCycler® Red 640 as the 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 the acceptor fluorophore, or dual color detection with LightCycler® Red 640- and Cy5.5-labeled HybProbe probes simultaneously.

The fluorescence values versus cycle number are displayed. Thirty picograms (approx. 10 genome equivalents) of human genomic DNA can be reproducibly detected by amplification in the LightCycler® Carousel-Based System Instrument using the detection format of the HybProbe probe. Three picograms (approx. 1 genome equivalent) are sporadically detected due to statistical fluctuations.

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

Fig. 1a–d: Serially diluted samples containing 30 ng, 3 ng, 300 pg, 30 pg, or 3 pg human genomic DNA as starting template were amplified using the LightCycler® FastStart DNA Master HybProbe. As a negative control, template DNA was replaced with PCR-grade water. LightCycler® Red 640 was used as the acceptor fluorophore.

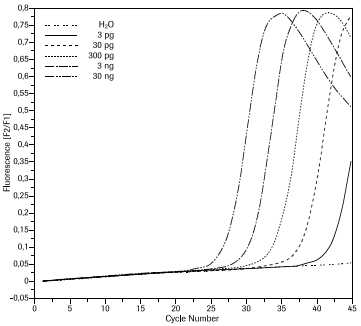


Fig. 1a: Channel F2 [640] [1)] (F2/F1) without color compensation

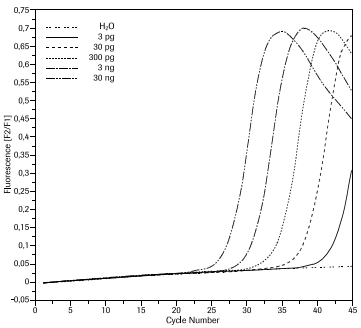


Fig. 1b: Channel F2 [640] [1)] (F2/F1) with color compensation

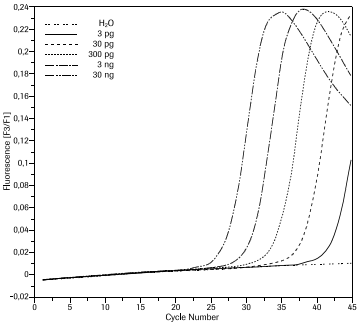


Fig. 1c: Channel F3 [705] [1)] (F3/F1) without color compensation

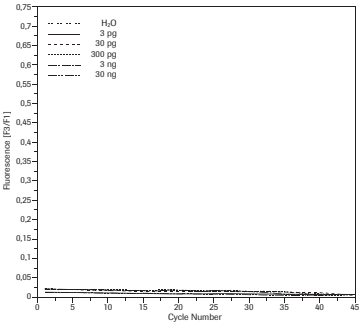


Fig. 1d: Channel F3 [705] [1)] (F3/F1) with color compensation

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 amount of nucleic acid	▪ Improve PCR conditions (*e.g.,* $MgCl_2$ concentration, primer and probe concentration or design). ▪ Use a 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.)
	FastStart Taq DNA polymerase is not fully activated.	▪ Ensure that the PCR programming includes a pre-incubation step at 95°C for 10 min. ▪ Ensure that the denaturation time during the amplification cycles is 10 s.
	Pipetting errors or omitted reagents	▪ Check for missing reagents. ▪ Titrate $MgCl_2$ concentration. ▪ Check for missing or defective dye. ▪ Always run a positive control with your samples.
	Measurements do not occur.	Check the amplification program. For the detection format of the HybProbe probe, choose “single” as the acquisition mode at the end of the annealing phase.
	Difficult template (*e.g.,* unusual GC-rich templates)	▪ Repeat PCR under the same conditions and add increasing amounts of DMSO (up to 10% of the final concentration). ▪ If the performance is still not satisfactory, optimize annealing temperature and $MgCl_2$ concentration, in combination with a titration of DMSO.
	Amplicon length is >1 kb.	Do not use amplicons >1 kb. Optimal results are obtained with amplicons of 500 bp or less.
	Impure sample material inhibits the reaction.	▪ Do not use more than 8 to 10 µl of DNA per 20 µl PCR reaction mixture. ▪ Dilute the sample 1:10 and repeat the analysis. ▪ Repurify the nucleic acids, to ensure removal of inhibitory agents.
	Unsuitable HybProbe probes	▪ Check sequence and location of the HybProbe probes. ▪ Check PCR product on an agarose gel.

	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 probe signals can be improved by using a two times higher concentration of the red fluorophore-labeled probe than of the fluoroscein-labeled probe.
Fluorescence intensity varies.	Pipetting errors	When using HybProbe probes and single color detection, pipetting errors can be diminished by interpreting results in the F2/F1 (640/530) or F3/F1 (705/530) mode.
	PCR Mix is still in the upper part of the capillary. Air bubble is trapped in capillary tip.	Repeat capillary centrifugation step.
	Skin oils on the surface of the capillary tip.	Always wear gloves when handling the capillaries.
Poor PCR efficiency	Reaction conditions are not optimized, leading to poor PCR efficiency.	▪ Titrate $MgCl_2$ concentration. ▪ Primer concentration should be in the range of 0.2 to 1.0 µM, probe concentration should be in the range of 0.2 to 0.4 µM. ▪ Check annealing temperature of primers and probes. ▪ Check experimental protocol. ▪ Always run a positive control along with the samples.
	Mutation analysis using HybProbe probes: The T_m of the hybrid between the mismatch strand and the HybProbe probes is lower than the annealing temperature. Therefore, the HybProbe probes can not bind and create a signal.	This will not affect the amplification efficiency. Ensure that the Melting Curve analysis starts at a temperature below the annealing temperature used for PCR. A clear signal will be displayed after Melting Curve analysis, enabling interpretation of data.
Negative control samples are positive.	Contamination	▪ Remake all critical solutions. ▪ Pipette reagents on a clean bench. ▪ Close the lid of the negative control reaction immediately after pipetting it. ▪ Use LightCycler® UNG for prevention of carry-over contamination.

	Possible cause	Recommendation
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 µM.
	Quality of HybProbe probes is poor.	Prepare new HybProbe probes.
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 the interpretation of the results. It can be avoided by performing an asymmetric PCR, favoring amplification of the DNA strand to which the HybProbe probes bind.
Melting peak is very broad and peaks can not be differentiated	°C to Average setting is too high.	Reduce the **°C to Average** (only applicable for LightCycler® Software versions prior to version 4.0).
Melting temperature of a product varies from experiment to experiment.	Variations in reaction mixture (*e.g.,* salt concentration)	▪ Check purity of the template. ▪ Reduce variations in parameters, such as $MgCl_2$ concentration, LightCycler® UNG and program settings.
No precise melting peak can be identified.	HybProbe probes are not homogeneous, or contain secondary structure.	Redesign HybProbe probes.
	Pseudogenes lead to multiple PCR products.	Check PCR products on an agarose gel.

5. Additional Information on this Product

5.1 How this Product Works

LightCycler® FastStart DNA Master HybProbe is a ready-to-use PCR reaction mix, designed specifically for real-time PCR assays using the detection format of the HybProbe probe with the LightCycler® Carousel-Based System. It is used to perform hot start PCR in 20 µl capillaries. Hot start PCR has been shown to significantly improve the specificity and sensitivity of PCR (1-4), by minimizing the formation of non-specific products at the beginning of the reaction.

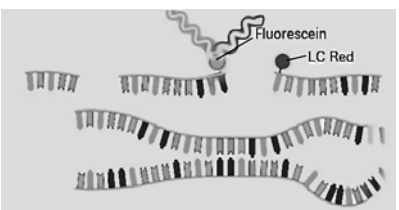
FastStart Taq DNA Polymerase is a chemically modified form of thermostable recombinant Taq DNA polymerase, that shows no activity up to +75°C. The enzyme is active only at high temperatures, where primers no longer bind non-specifically. The enzyme is completely activated (by removal of blocking groups) in a single pre-incubation step (+95°C, 10 minutes) before cycling begins. Activation does not require the extra handling steps typical of other hot start PCR techniques.

LightCycler® FastStart DNA Master HybProbe provides convenience, excellent performance and reproducibility, as well as minimal contamination risk. All that is required are template DNA, PCR primers, HybProbe probes and additional $MgCl_2$ (if necessary).

Test Principle

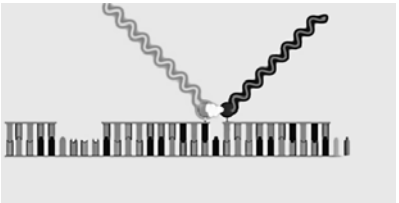
HybProbe probes consist of two different short oligonucleotides that bind to an internal sequence of the amplified fragment, during the annealing phase of the amplification cycle.

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

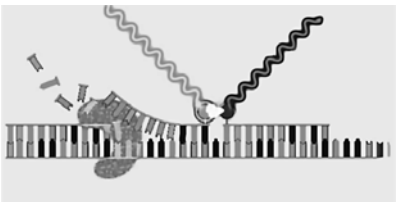


① The donor dye probe has a fluorescein label at its 3' end and the acceptor dye probe has a red fluorophore label [LightCycler® Red 610#, LightCycler® Red 640, Cy5 {670}#, or Cy5.5 {705}] at its 5' end (it is 3'-phosphorylated, so it can not be extended). Hybridization does not occur during the Denaturation 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.

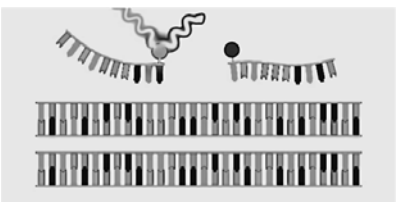
② During the Annealing phase, the probes hybridize to the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two fluorescent dyes close to each other. Fluorescein is excited by the light source of the LightCycler® Carousel-Based System, which causes it to emit green fluorescent light. The emitted energy excites the red fluorophore (acceptor dye) 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 for 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 using HybProbe probes to perform dual or multiple color experiments in a single capillary, a color compensation file must be used. Color compensation may be applied either during or after a run on the LightCycler® Carousel-Based System.

- See the LightCycler® Instrument 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 {670} can only be used on a LightCycler® 2.0 Instrument.

5.2 Quality Control

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

5.3 References

1 Chou, Q. *et al*. (1992). Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res*. **20**, 71717-71723.

2 Kellogg, DE. *et al*. (1994). TaqStart Antibody: "hot start" PCR facilitated by a neutralizing monoclonal antibody directed against Taq DNA polymerase. *BioTechniques* **16**, 1134-1137.

3 Birch, DE. *et al*. (1996). Simplified hot start PCR. *Nature* **381**, 445-446.

4 PCR Manual, Roche Diagnostics (1999). 2nd edition, **2**, 52-58.

5.4 Product Citations

5 Krüger, S. *et al*. (2005). Arg462Gln sequence variation in the prostate-cancer-susceptibility gene RNASEL and age of onset of hereditary non-polyposis colorectal cancer: a case-control study. *Lancet Oncol*. **6**, 566-572.

6 Rakvåg, TT. *et al*. (2005). The Val158Met polymorphism of the human catechol-O-methyltransferase (COMT) gene may influence morphine requirements in cancer pain patients. *Pain* **116**, 73-78.

7 Sturm, GJ. *et al*. (2005). 5-Oxo-6,8,11,14-eicosatetraenoic acid is a potent chemoattractant for human basophils. *J. Allergy Clin. Immunol.* **116**, 1014-1019.

8 Tomaso, H. *et al*. (2005). Antimicrobial susceptibilities of Austrian *Francisella tularensis holarctica* biovar II strains. *Int. J. Antimicrob. Agents* **26**, 279-284.

9 Sun, F. *et al*. (2005). Apoptosis and its pathway in early post-implantation embryos of diabetic rats. *Diabetes Res. Clin. Pract*. **67**, 110-118.

10 Curley, M. *et al*. (2004). Analysis of Maxi-K alpha subunit splice variants in human myometrium. *Reprod. Biol. Endocrinol*. **2**, 67.

11 De Clerck, E. & De Vos, P. (2004). Genotypic diversity among *Bacillus licheniformis* strains from various sources. *FEMS Microbiol. Lett*. **231**, 91-98.

12 Dufresne, PJ. *et al*. (2004). FRET hybridization probes for the rapid detection of disease resistance alleles in plants: detection of corky root resistance in lettuce. *Molecular Breeding* **13**, 323-332.

13 Duguay, Y. *et al*. (2004). A novel functional polymorphism in the uridine diphosphate- glucuronosyltransferase 2B7 promoter with significant impact on promoter activity. *Clin. Pharmacol. Ther*. **75**, 223-233.

14 Ulcar, R. *et al*. (2004). Cyclooxygenase inhibition in human monocytes increases endotoxin-induced TNFα without affecting cyclooxygenase-2 expression. *Eur. J. Pharmacol*. **501**, 9-17.

15 Zandieh-Doulabi, B. *et al*. (2004). Hyper and hypothyroidism change the expression and diurnal variation of thyroid hormone receptor isoforms in rat liver without major changes in their zonal distribution. *Mol. Cell. Endocrinol.* **219**, 69-75.

16 Bellete, B. *et al.* (2003). Influence of the quantity of nonspecific DNA and repeated freezing and thawing of samples on the quantification of DNA by the Light Cycler®. *J. Microbiol. Methods* **55**, 213-219.

17 de Castro, F. *et al.* (2003). Role of follicle-stimulating hormone receptor Ser680Asn polymorphism in the efficacy of follicle-stimulating hormone. *Fertil. Steril*. **80**, 571-576.

18 Gamberale, R. *et al.* (2003). *In vitro* susceptibility of $CD4^+$ and $CD8^+$ T cell subsets to fludarabine. *Biochem. Pharmacol*. **66**, 2185-2191.

19 Liu, YM. *et al.* (2003). Adiponectin Gene Expression in Subcutaneous Adipose Tissue of Obese Women in Response to Short-Term Very Low Calorie Diet and Refeeding. *J. Clin. Endocrinol. Metab.* **88**, 5881-5886.

20 Loeffler, J. *et al*. (2003). Automated RNA Extraction by MagNA Pure Followed by Rapid Quantification of Cytokine and Chemokine Gene Expression with Use of Fluorescence Resonance Energy Transfer. *Clin. Chem*. **49**, 955-958.

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:

Abbreviation	Meaning
SNP	single nucleotide polymorphism
T_m	melting temperature
UNG	Uracil-DNA Glycosylase

6.2 Changes to Previous Version

Update of License Disclaimer

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 Instrument, 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

	Product	Pack Size	Cat. No.
Instruments and Accessories	LightCycler® 2.0 Instrument	1 instrument plus accessories	03 531 414 001
	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
Software	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.
DNA Isolation Kits	MagNA Pure LC DNA Isolation Kit I	1 kit (192 isolations)	03 003 990 001
	MagNA Pure LC DNA Isolation Kit II (Tissue)	1 kit (192 isolations)	03 186 229 001
	MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi)	1 kit (192 isolations)	03 264 785 001
	MagNA Pure LC DNA Isolation Kit – Large Volume	1 kit (96 – 288 isolations)	03 310 515 001
Total Nucleic Acid Isolation Kits	MagNA Pure LC Total Nucleic Acid Isolation Kit	1 kit (192 isolations)	03 038 505 001
	MagNA Pure LC Total Nucleic Acid Isolation Kit – Large Volume	1 kit (192 isolations)	03 264 793 001
	MagNA Pure LC Total Nucleic Acid Isolation Kit – High Performance	1 kit (96 – 288 isolations)	05 323 738 001
	MagNA Pure Compact Nucleic Acid Isolation Kit I	1 kit (32 isolations)	03 730 964 001
	MagNA Pure Compact Nucleic Acid Isolation Kit I – Large Volume	1 kit (32 isolations)	03 730 972 001
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

	Product	Pack Size	Cat. No.
LightCycler® Kits for 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^{PLUS} HybProbe	1 kit (96 reactions) 1 kit (480 reactions) 1 kit (384 reactions, 100 µl)	03 515 575 001 03 515 567 001 03 752 178 001
	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) 1 kit (384 reactions, 100 µl)	03 515 869 001 03 515 885 001 03 752 186 001
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
	Transcriptor High Fidelity cDNA Synthesis Kit	1 kit (50 reactions, incl. 10 control reactions) 1 kit (100 reactions) 1 kit (200 reactions)	05 081 955 001 05 091 284 001 05 081 963 001
	Transcriptor Universal cDNA Master	1 kit (100 reactions)	05 893 151 001
	First Strand cDNA Synthesis Kit for RT-PCR (AMV)	1 kit (30 reactions, incl. 5 control reactions)	11 483 188 001

	Product	Pack Size	Cat. No.
Associated Kits and Reagents	High Pure PCR Template Preparation Kit	1 kit (100 isolations)	11 796 828 001
	High Pure Plasmid Isolation Kit	1 kit (50 isolations) 1 kit (250 isolations)	11 754 777 001 11 754 785 001
	High Pure RNA Isolation Kit	1 kit (50 isolations)	11 828 665 001
	High Pure FFPE RNA Micro Kit	1 kit (50 isolations)	04 823 125 001
	High Pure RNA Paraffin Kit	1 kit (100 isolations)	03 270 289 001
	LightCycler® Uracil-DNA Glycosylase	100 U (50 µl)	03 539 806 001
	LightCycler® Control Kit DNA	1 kit (50 reactions)	12 158 833 001
	LightCycler® Color Compensation Set	1 set (5 reactions)	12 158 850 001

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

6.4 Disclaimer of License

NOTICE: This product may be subject to certain use restrictions. Before using this product please refer to the Online Technical Support page (http://technical-support.roche.com) and search under the product number or the product name, whether this product is subject to a license disclaimer containing use restrictions.

6.5 Regulatory Disclaimer

For general laboratory use.

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