For general laboratory use.

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LightCycler® FastStart DNA Master HybProbe

Version: 16

Content version: December 2016

Easy-to-use hot start reaction mix for PCR using HybProbe Probes with the LightCycler® Carousel-Based System

Cat. No. 03 003 248 001	1 kit 96 reactions of 20 µl final volume each
Cat. No. 12 239 272 001	1 kit 480 reactions of 20 µl final volume each

Store the product at −15 to −25°C.

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1. General Information

1.1. Contents

Vial	Cap	Label	Function	Catalog Number	Content
1a	red	LightCycler® FastStart DNA Master HybProbe, LC FastStart Enzyme	▪ Ready-to-use hot start 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 10mM $MgCl_2$.	03 003 248 001	3 vials 1a and 3 vials 1b for 3 vials, 64 µl each LightCycler® FastStart DNA Master HybProbe, 10x conc.
1b	colorless	LightCycler® FastStart DNA Master HybProbe, LC FastStart Reaction Mix HybProbe, 10x conc.		12 239 272 001	15 vials 1a and 15 vials 1b for 15 vials, 64 µl each LightCycler® FastStart DNA Master HybProbe, 10x conc.
2	blue	LightCycler® FastStart DNA Master HybProbe, $MgCl_2$ stock solution, 25 mM	To adjust $MgCl_2$ concentration in the reaction mix.	03 003 248 001	1 vial, 1 ml
				12 239 272 001	2 vials, 1 ml each
3	colorless	LightCycler® FastStart DNA Master HybProbe, Water, PCR Grade	To adjust the final reaction volume.	03 003 248 001	2 vials, 1 ml each
				12 239 272 001	7 vials, 1 ml each

1.2. Storage and Stability

Storage Conditions (Product)

The product is shipped on dry ice.
When stored at −15 to −25°C, the kit is stable through the expiration date printed on the label.

Vial / Bottle	Cap	Label	Storage
1a	red	LC FastStart Enzyme	Store at −15 to −25°C ⚠ ***Avoid repeated freezing and thawing!***
1b	colorless	LC FastStart Reaction Mix HybProbe, 10x conc.	
1 after the addition of 1a to 1b	red	LC FastStart DNA Master HybProbe, 10x conc.	▪ Stored 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	$MgCl_2$ stock solution, 25 mM	Store at −15 to −25°C
3	colorless	Water, PCR Grade	

1.3. Additional Equipment and Reagents Required

Standard Laboratory Equipment

- Nuclease-free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile reaction tubes for preparing master mixes and dilutions

For PCR

- LightCycler® Carousel-Based System*
- 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 (optional)
- LightCycler® Uracil-DNA Glycosylase* (optional)

For prevention of carryover contamination; see section **Prevention of Carryover Contamination***. Use LightCycler® Uracil-DNA Glycosylase in combination with LightCycler® FastStart DNA Masters only.*

- LightCycler® Color Compensation Set* optional

1.4. Application

LightCycler® FastStart DNA Master HybProbe is an easy-to-use hot start reaction mix for sensitive PCR applications in LightCycler® Capillaries, using HybProbe probes as detection format. It is an ideal master mix for performing quantitative PCR as well as SNP and mutation detection, and can also be used in two-step RT-PCR.

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

1.5. Preparation Time

Assay Time

Procedure	Assay Time [min]
Optional: Dilution of template DNA	5
PCR Set-up	15
LightCycler® Carousel-Based System run incl. Melting Curve	45
Total assay time	**65**

2. How to Use this Product

2.1. Before you Begin

Sample Materials

- Use any template DNA *e.g.*, genomic or plasmid DNA, cDNA suitable for PCR in terms of purity, concentration and absence of inhibitors.
- 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.

If you are using a non-purified cDNA sample from reverse transcription, especially if it contains high background concentrations of RNA and oligonucleotides, you can improve your results by using 2 µl or less of that sample in the reaction.

For reproducible isolation of nucleic acids, we recommend:
- Either a MagNA Pure System together with a dedicated reagent kit for automated isolation,
- or a High Pure Nucleic Acid Isolation Kit for manual isolation.

Control Reactions

Always run a negative and a positive control with the samples.
To prepare a negative control, replace the template DNA with Water, PCR Grade (Vial 3).

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.

Probe

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 (free to download under www.lifescience.roche.com)can design the best HybProbe probe-pair and primer combinations.

Mg^{2+} Concentration

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) 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 water in the PCR reaction must be reduced accordingly.

General Considerations

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 multicolor applications using a different dye combination than LightCycler® Red 640 and Cy5.5. Such assays have to use a customized color compensation object. You have to 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® Instrument Operator's Manual, the Special Interest Site for the LightCycler® Real-Time PCR Systems www.lightcycler.com, or the Instructions for Use of the LightCycler® Color Compensation Set.

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*

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, Roche recommends running undiluted, 1:10 diluted and 1:100 diluted cDNA templates, in parallel to determine the optimal template amount.

2.2. Protocols

LightCycler® Carousel-Based System Protocol

The following procedure is optimized for use with all 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 DNA polymerase and denaturation of the 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.

LightCycler® Software Version 4.1		
Programs		
Setup	Setting	
Default Channel	**During the run**: Depending on the red fluophore 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 Tm Calling analysis, do not divide by Channel 530 or "Back 530". ⓘ *Channels 610 and 670 are only available on a LightCycler® 2.0 Instrument.*	
Seek Temperature	30°C	
Max Seek Pos.	Enter the total number of sample positions the instrument should look for.	
Instrument Type	"6 Ch." for LightCycler® 2.0 Instrument or "3 Ch." for LightCycler® 1.5 Instrument	
Capillary Size	Select "20 µl" as the capillary size for the experiment. available only for LightCycler® 2.0 Instrument (6 channels)	
Programs		
Program Name	Cycles	Analysis Mode
Pre-Incubation	1	None
Amplification	45	Quantification
Melting Curve optional	1	Melting Curve
Cooling	1	None

LightCycler® Software Version 4.1				
Programs				
Temperature Targets				
	Target [°C]	Hold [hh:mm:ss]	Ramp Rate[1] [°C/s]	Acquisition Mode [per °C]
Pre-Incubation	95	00:10:00[(2)]	20	None
Amplification	95	00:00:10	20	None
	primer dependent[(3)]	00:00:05 – 00:00:20[(4)]	20	Single
	72[(5)]	00:00:05 – 00:00:30 [(4,6)]	20	None
Melting Curve (optional)	95	00:00:00	20	None
	Probes Tm−5°C	00:00:30 (20 µl) 00:00:60 (100 µl)	20	None
	95	00:00:00	0.1[1]	Continuous
Cooling	40	00:00:30	20	None

(1) Temperature Transition Rate/Slope/Ramp Rate is 20°C/second, except where indicated.
(2) 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.
(3) For initial experiments, set the target temperature i.e., the primer annealing temperature 5°C below the calculated primer Tm . Calculate the primer Tm according to the following formula, based on the nucleotide content of the primer: Tm = 2°C A+T + 4°C G+C.
(4) 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.
(5) If the primer annealing temperature is low <+55°C, reduce the ramp rate to 2 to 5°C/second.
(6) Calculate the hold time for the PCR elongation step by dividing the amplicon length over 25 (*e.g.*, a 500 bp amplicon requires 20 seconds elongation time).

Preparation of the Master Mix

Prepare the 10x conc. Master Mix as described below.

1. Thaw one vial of “Reaction Mix” (Vial 1b).

   ⚠ ***A reversible precipitate may form in the LightCycler® FastStart Reaction Mix HybProbe Vial 1b 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 and the thawed vial of “Reaction Mix” from Step 1, then place the vials back on ice

3. Pipet 60 µl from Vial 1b into Vial 1a.

4. Mix gently by pipetting up and down.

   ⚠ ***Do not vortex.***

5. Re-label Vial 1a with the new label Vial 1: LightCycler® FastStart DNA Master HybProbe provided with the kit.

   ⚠ ***Always keep the Master Mix cool and away from light!***

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 precooled centrifuge adapters or in a LightCycler® Sample Carousel in a precooled 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 10x conc. solution would contain a 5 µM concentration of each primer

3. Thaw the LightCycler® FastStart DNA Master HybProbe, 10x conc. (Vial 1), mix gently and store on ice.

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

Component	Volume [µl]	Final conc.
Water, PCR Grade (Vial 3)	x	
$MgCl_2$ stock solution, 25 mM (Vial 2)	y	Use concentration that is optimal for the target
PCR Primer Mix, 10x conc.	2	0.2 to 1.0 µM each, recommended conc. is 0.5 µM
HybProbe Probe Mix, 10x conc.	2	0.2 to 0.4 µM each
LightCycler® FastStart DNA Master HybProbe, 10x conc. (Vial 1)	2	1x
Total volume	**18**	

   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.
   - Pipet 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 seconds 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. Other Parameters

Prevention of Carryover Contamination

Uracil-DNA Glycosylase (UNG) is suitable for preventing carryover contamination in PCR. This carryover 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.

⚠ ***If you use the LightCycler® FastStart DNA Master HybProbe, perform prevention of carryover contamination with LightCycler® Uracil-DNA Glycosylase, prior to beginning real-time PCR.***

Proceed as described in the Instructions for Use of LightCycler® Uracil-DNA Glycosylase and/or in the steps shown below, to prevent carryover contamination.

1. Add 0.5 U LightCycler® Uracil-DNA Glycosylase to the master mix per 20 µl final reaction volume.

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2. Add template DNA and incubate the completed reaction mixture for 10 min at +40°C.

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3. 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 Tm by approx. 1°C.

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 far left, 3 ng, 300 pg, 30 pg, or 3 pg far right human genomic DNA as starting template were amplified using the LightCycler® FastStart DNA Master HybProbe. As a negative control, template DNA was replaced with water, PCR Grade. LightCycler® Red 640 was used as the acceptor fluorophore.

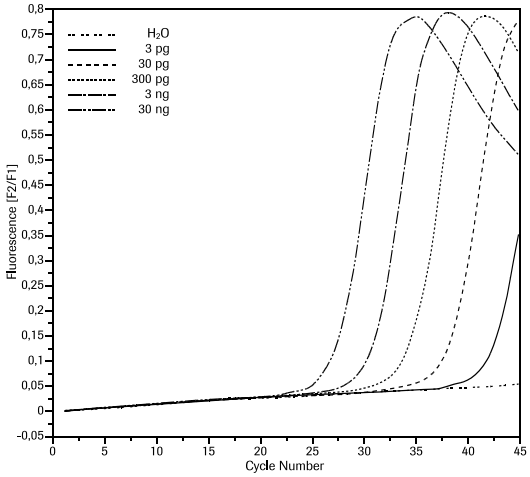


Fig. 1a: Channel F2 [640](1) (F2/F1). with color compensation

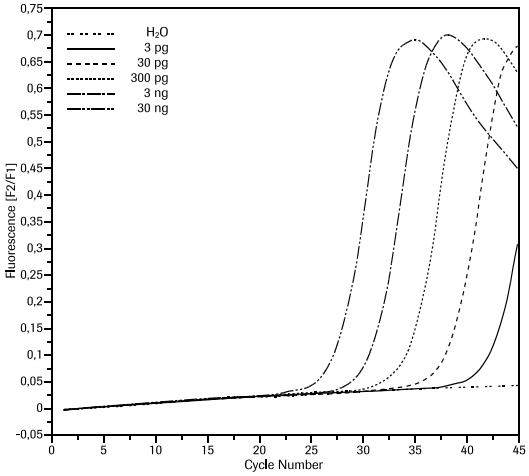


Fig. 1b: Channel F2 [640](1) (F2/F1) with color compensation

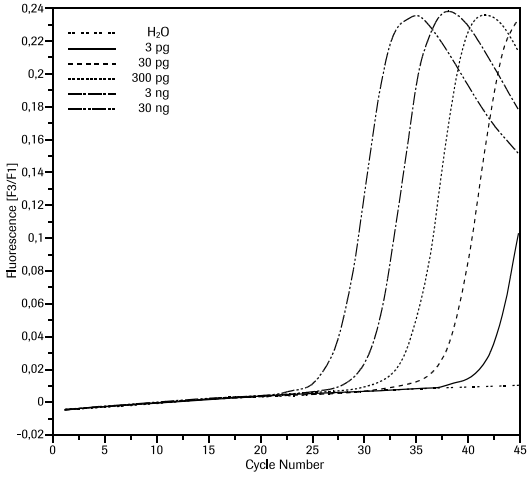


Fig. 1c: Channel F3 [705](1) (F3/F1) with color compensation

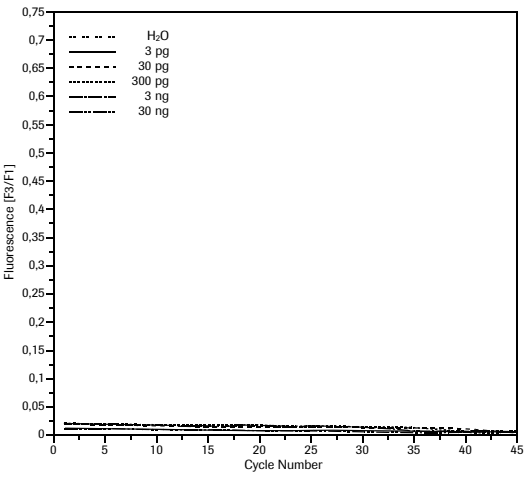


Fig. 1d: Channel F3 [705](1) (F3/F1) with color compensation

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 protocol.
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 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 sequence.	Repeat PCR under the same conditions and add increasing amounts of DMSO up to 10 % of the final concentration.
		If performance is still not satisfactory, optimize annealing temperature an $MgCl_2$ 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 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 varies.	Pipetting errors.	When using HybProbe probes and single-color detection, pipetting errors can be diminished through interpreting the 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 the capillary tip.	Repeat capillary centrifugation step.
	Skin oils on the surface of the capillary tip.	Always wear gloves when handling the capillaries.
Fluorescence intensity is too low.	Low concentration or deterioration of dyes in 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 fluorescein-labeled probe.
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 Tm 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.
		Pipet reagents on a clean bench.
		Close the lid of the negative control reaction immediately after pipetting it.
		Use LightCycler UNG to eliminate carry-over contamination.
High background	Very low fluorescence signals, therefore the background seems relatively high.	Follow general optimization strategies for PCR using LightCycler® Carousel-Based System PCR.
	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 a new solution of HybProbe probes.
Amplification curve decreases after reaching a plateau in the later cycles	“Hook effect”: Competition between binding of the HybProbe probes and re-annealing 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.

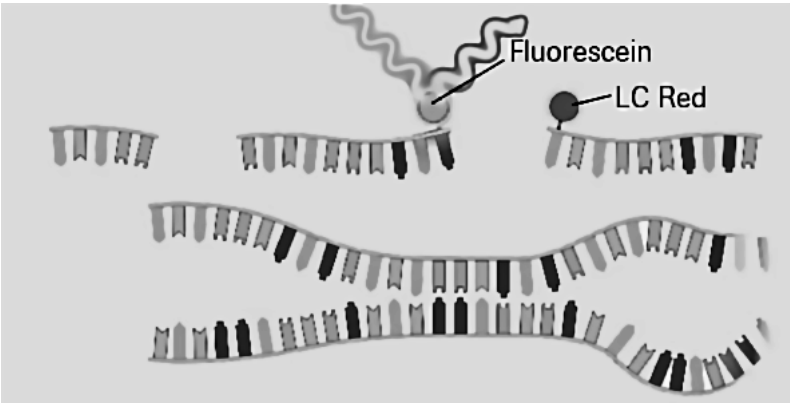
	Possible cause	Recommendation
Melting peak is very broad and peaks can not be differentiated.	°C to Average setting is too high.	Reduce the **°C to Average** only applicable for LightCycler® Software versions prior to version 4.0
Melting temperature of a product varies from experiment to experiment.	Variations in reaction mixture *e.g.,* salt concentration.	Check purity of template solution.
		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

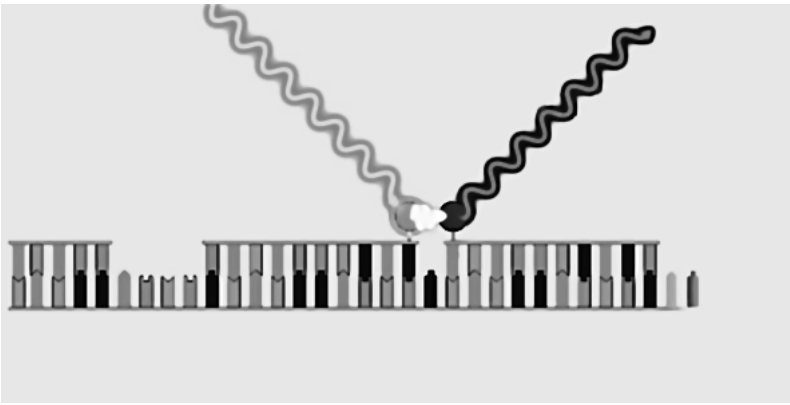
5.1. Test Principle

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:

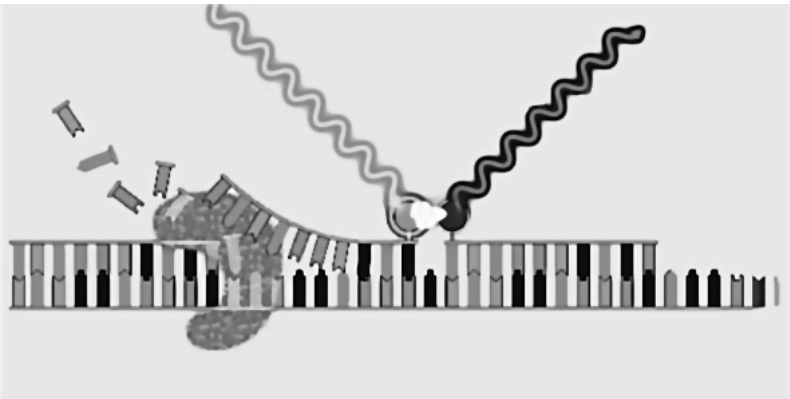
① The donor dye probe has a fluorescein label at its 3' end and the acceptor dye probe has a red fluorophore label [LightCycler® Red 610[1], LightCycler® Red 640, Cy5 {670}[1], or Cy5.5 {705}] at its 5' end it is 3'-phosphorylated, so it can not be extended. Hybridization does not occur during the Denaturation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



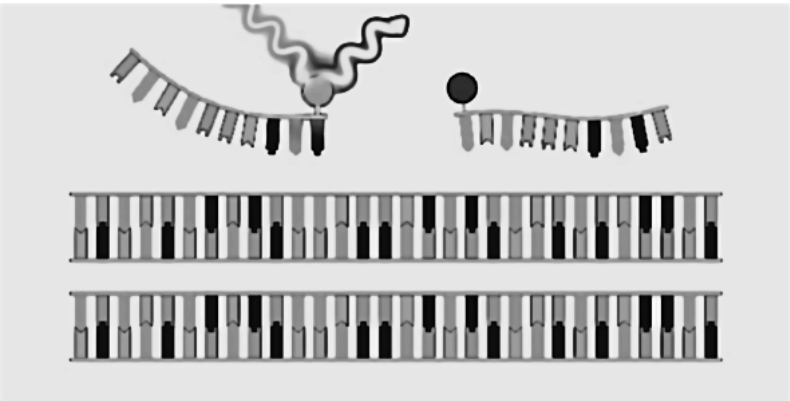
② During the Annealing phase, the probes hybridize to the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two fluorescent dyes close to each other. Fluorescein is excited by the light source of the LightCycler® Carousel-Based System, which causes it to emit green fluorescent 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 Instructions for Use of the LightCycler® Color Compensation Set for more information on the generation and use of a color compensation file or object.

(1) LightCycler® Red 610 and Cy5 {670} can only be used on a LightCycler® 2.0 Instrument.

How this Product Works

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 carryover 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.***

Hot start PCR has been shown to significantly improve the specificity and sensitivity of PCR by minimizing the formation of non-specific amplification products at the beginning of the reaction (Chou Q. et al., 1992; Kellogg, D.E. et al., 1994; Birch, D.E. et al., 1996). 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 techniques.

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

5.2. References

- Birch DE, Kolmodin L, Wong J, Zangenberg GA, Zoccoli MA, McKinney N, Young KKY - Simplified hot start PCR (1996) *Nature* **381** (6581), 445-446
- Chou Q, Russell M, Birch DE, Raymond J, Bloch W - Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications (1992) *Nucleic Acids Research* 7, 1717-1723
- Kellogg DE, Rybalkin I, Chen S, Mukhamedova N, Vlasik T, Siebert PD, Chenchika A - TaqStart antibody : hot start PCR facilitated by a neutralizing monoclonal antibody directed against Taq DNA polymerase (1994) *BioTechniques* **16** (6), 1134-1137

5.3. Quality Control

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

6. Supplementary Information

6.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

Layout changes.
Editorial changes.

6.3. Ordering Information

Roche 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 homepage lifescience.roche.com.

Product	Pack Size	Cat. No.
Accessories general (hardware)		
LightCycler® Software 4.1	1 software package	04 898 915 001
LC Carousel Centrifuge 2.0	1 centrifuge plus rotor and rotor bucket (115 V)	03 709 507 001
	1 centrifuge plus rotor and rotor bucket (230 V)	03 709 582 001
Consumables		
LightCycler® Capillaries (20 µl)	5 x 96 capillaries, containing 5 boxes, each with 96 capillaries and stoppers, 5 boxes, each with 96 capillaries and stoppers	04 929 292 001
Instruments		
LightCycler® 2.0 Instrument	1 instrument	03 531 414 001
Reagents , kits		
LightCycler® FastStart DNA Master^{PLUS} HybProbe	1 kit, 96 reactions of 20 µl final volume each	03 515 575 001
	1 kit, 480 reactions of 20 µl final volume each	03 515 567 001
	1 kit, 1,920 reactions of 20 µl or 384 reactions of 100 µl final volume each	03 752 178 001
LightCycler® Uracil-DNA Glycosylase	50 µl, 100 U, (2 U/µl)	03 539 806 001
LightCycler® Color Compensation Set	1 set, 4 vials, 5 calibration runs	12 158 850 001
Transcriptor Reverse Transcriptase	250 U, 25 reactions of 20 µl final volume	03 531 317 001
	500 U, 50 reactions of 20 µl final volume	03 531 295 001
	2,000 U, 4 x 500 U, 200 reactions of 20 µl final volume	03 531 287 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit, 50 reactions, including 10 control reactions	04 379 012 001
	1 kit, 100 reactions	04 896 866 001
	1 kit, 200 reactions	04 897 030 001

6.4. Trademarks

FASTSTART, HIGH PURE, HYBPROBE, LIGHTCYCLER and MAGNA PURE are trademarks of Roche.
All third party product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For patent license limitations for individual products please refer to: http://technical-support.roche.com.

6.6. Regulatory Disclaimer

For general laboratory use.

6.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.8. Contact and Support

If you have questions or experience problems with this or any Roche product for Life Science, please contact our Technical Support staff. Our scientists are committed to providing rapid and effective help.
Please also contact us if you have suggestions for enhancing Roche product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to the research community worldwide.

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support** Site.

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