For general laboratory use.
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Roche Applied Science

LightCycler® FastStart DNA Master HybProbe

Version September 2005

Ready-to-use hot start reaction mix for PCR using the LightCycler® 2.0 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

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PROTOCOL

1. What this Product Does

Number of Tests

The kit is designed for 96 or 480 reactions (depending on pack size) with a final reaction volume of 20 µl each.

Kit Contents

Vial/Cap	Label	Contents/Function a) Cat. No. 03 003 248 001 (96 reactions) b) Cat. No. 12 239 272 001 (480 reactions)
1a red cap	LightCycler® Fast-Start Enzyme	a) 1 × vial 1a, 3 × vial 1b for 3 × 64 µl LightCycler® FastStart DNA Master HybProbe (10× conc.) b) 5 × vial 1a, 15 × vial 1b for 15 × 64 µl LightCycler® FastStart DNA Master 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 $MgCl_2$
1b colorless cap	LightCycler® Fast-Start Reaction Mix HybProbe	
2 blue cap	$MgCl_2$ stock solution, 25 mM	a) 1 vial, 1 ml each b) 2 vials, 1 ml each • to adjust $MgCl_2$ concentration
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 through 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® Fast-Start Enzyme	• Store at -15 to -25°C. • **Avoid repeated freezing and thawing!**
1b colorless cap	LightCycler® Fast-Start Reaction Mix HybProbe	

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Vial	Label	Storage
1 red cap (after addition of 1a to 1b)	LightCycler® Fast-Start DNA Master HybProbe	• Store at −15 to −25°C for a maximum of three month. • After thawing store at +2 to +8°C for a maximum of 1 week. • **Avoid repeated freezing and thawing!**
2 blue cap	$MgCl_2$ stock solution	Store at −15 to −25°C
3 colorless cap	Water, 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® 2.0 System include:

- LightCycler® 2.0 System* (LightCycler® 2.0 Instrument* or LightCycler® 1.5 Instrument*)
- LightCycler® Capillaries*
- Standard benchtop microcentrifuge containing a rotor for 2.0 ml reaction tubes

The LightCycler® 2.0 System provides adapters that allow LightCycler® Capillaries to be centrifuged in a standard microcentifuge rotor.

or

- LC Carousel Centrifuge 2.0* for use with the LightCycler® 2.0 Carousel (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 Carousel 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

If you want to perform color compensation when using LightCycler® Red 640 and 705-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. Use LightCycler® Uracil-DNA Glycosylase in combination with LightCycler® FastStart Masters only.

** available from Roche Applied Science*

Application

LightCycler® FastStart DNA Master HybProbe is designed for research studies. When used with the LightCycler® 2.0 System, this kit is ideally suited for hot-start PCR applications. In combination with the LightCycler® 2.0 System and suitable primers and HybProbe probes, this kit allows very sensitive detection and quantification of defined DNA sequences. It can also be used to genotype single nucleotide polymorphisms (SNPs) and analyze mutations.

The kit can also be used to perform two-step RT-PCR. It can also be used with LightCycler® Uracil-DNA Glycosylase to prevent carry-over contamination during PCR. In principle, the LightCycler® FastStart DNA Master HybProbe can be used for the amplification and detection of any DNA or cDNA target. However, you would need to adapt your detection protocol to the reaction conditions of the LightCycler® Instruments, and design specific PCR primers and Hybprobe pairs for each target. See the LightCycler® Operator's Manual for general recommendations.

⚠ The amplicon size should not exceed 1 kb in length. For optimal results, select a product length of 700 bp or less.

⚠ The performance of the kit described in this Instruction Manual is warranted only when it is used with the LightCycler® 2.0 System.

Assay Time

Procedure	Time
Optional: dilution of template DNA	5 min
PCR Setup	15 min
LightCycler® 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:
  - either the MagNA Pure LC Instrument or the MagNA Pure Compact Instrument together with a dedicated nucleic acid isolation kit (for automated isolation) or
  - a High Pure nucleic acid isolation kit (for manual isolation).

For details see the Roche Applied Science Biochemicals catalog or home page, www.roche-applied-science.com.

- Use up to 50 ng complex genomic DNA or 10^1 – 10^{10} copies plasmid DNA

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.

Negative Control

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

Primers

Use PCR primers at a final concentration of 0.3 – 1 µM. The recommended starting concentration is 0.5 µM each.

Melting curve assays: 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 - 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 pair and will improve the subsequent melting curve analysis.

HybProbe Probes

Use the HybProbe probes at a final concentration of 0.2 µM each. In some cases it might be advantageous to double the concentration of the LightCycler® Red-labeled probe to 0.4 µM.

See the LightCycler® Operator's Manual for detailed information on designing the HybProbe probes and labeling them with various dyes. In addition, LightCycler® Probe Design Software 2.0 can help you design HybProbe probes.

$MgCl_2$

To ensure specific and efficient amplification with the LightCycler® 2.0 System, you must optimize the $MgCl_2$ concentration for each target. The LightCycler® FastStart DNA Master HybProbe contains a $MgCl_2$ concentration of 1 mM (final concentration). The optimum concentration for PCR with the LightCycler® 2.0 System may vary from 1 to 5 mM.

The table below shows the volume of the $MgCl_2$ stock solution (vial 2, blue cap) that you must add to a 20 µl reaction (final PCR volume) to increase the $MgCl_2$ concentration.

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

2.2 Procedure

LightCycler® 2.0 System Protocol

The following procedure is optimized for use with the LightCycler® 2.0 System.

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

A LightCycler® 2.0 System protocol that uses LightCycler® FastStart DNA Master HybProbe contains the following programs

- **Pre-Incubation** for activation of FastStart Taq DNA Polymerase and denaturation of the DNA
- **Amplification** of the target DNA
- **Melting curve** for amplicon analysis (Optional; only needed for SNP or mutation detection)
- **Cooling** the rotor and thermal chamber

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

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

⚠ 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® 2.0 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	HybProbe T_m - 5°C	30 s	none
		Melting	95°C slope = 0.1°C/sec [1)]	0 s	continuous
Cooling					
None	1		40°C	30 s	none

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

[3)] 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: $T_m = 2°C\ (A+T) + 4°C\ (G+C)$.

[4)] If high polymerase activity is needed in early cycles, you can sometimes improve results by extending the pre-incubation 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 prior to Version 3.5		
Display Mode	fluorescence channel F2 (for LightCycler® Red 640) or F3 (for LightCycler® Red 705)	
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 LightCycler® Red 705) • 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.	
LightCycler® Software Version 4.0		
Default Channel • during run • for analysis	• Depending on the LightCycler® Red dye used for labeling the HybProbe probe, choose Channel 610, 640, 670, or 705. • Depending on the LightCycler® Red 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.	

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Parameter	Setting
Fluorescence Gains	not required
"Max. Seek Pos"	Enter the number of sample positions the instrument should look for.
"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

❶	Thaw one vial of "Reaction Mix" (vial 1b, colorless cap). ⚠ 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. Recentrifuge to collect the reagent at the bottom of the tube, then put the vial back on ice. This treatment does not influence the performance in PCR.
❷	Briefly centrifuge one vial "Enzyme" (vial 1a, red cap) and the thawed vial of "Reaction Mix" (from Step 1).
❸	Pipet 60 µl from vial 1b (colorless cap) into vial 1a (red cap).
❹	Mix gently by pipetting up and down. ⚠ Do not vortex.
❺	Re-label vial 1a (red cap) with the new labels (vial 1: LightCycler® FastStart DNA Master HybProbe) that are provided with the kit. Place one on the top of the cap and one on the side of the vial.

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. For volumes < 20 µl, the reaction and cycle conditions must be optimized.

❶ 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 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.

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

❹ 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.
Water, PCR-grade (vial 3, colorless cap)	x µl	
$MgCl_2$ stock solution, (vial 2, blue cap)	y µl	Use concentration that is optimal for the target.
Primer mix [1], 10× conc.	2 µl	0.3 - 1.0 µM each (recommended conc. is 0.5 µM)
HybProbe mix [2], 10× conc.	2 µl	0.2 - 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.

❺
- Mix carefully by pipetting up and down. Do not vortex.
- Pipet 18 µl PCR mix into each precooled LightCycler® Capillary.
- Add 2 µl of the DNA template.
- Seal each capillary with a stopper.

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❻	• Place the 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.
❼	Transfer the capillaries into the sample carousel of the LightCycler® Instrument.
❽	Cycle the samples as described above.

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

[2)] If you want to peform dual color detection using LightCycler® Red 640- and Red 705-labeled HybProbe pairs simultaneously in one capillary, either use two separated HybProbe mixes (then you will have to add 2 µl each from both of the two mixes) or combine both HybProbe pair preparations in one mix (then you will have to add 2 µl only from this combined mix).

Color Compensation

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

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

Refer to the LightCycler® Operator's Manual and to the pack insert of the LightCycler® Color Compensation Set or LightCycler® Multiplex DNA Master HybProbe for further information on the generation and use of a color compensation file.
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-online.com), or the pack inserts of the LightCycler® Color Compensation Set and LightCycler® Multiplex DNA Master HybProbe.

⚠ Although the optical filters of each detection channel of the LightCycler® Instruments 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® Instrument.
No universal color compensation set is available for dual-color applications using a different dye combination than LightCycler® Red 640/705 or multicolor applications on a LightCycler® 2.0 Instrument. Such assays must use a customized color compensation protocol. You must prepare a new color compensation object for each set of parameters.

Prevention of Carry-Over Contamination

Uracil DNA N-Glycosylase (UNG) is suitable for preventing carry-over contamination in PCR. This carry-over prevention technique involves incorporating deoxyuridine triphosphate (dUTP, a component of the Master Mix in this kit) 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.

⚠ Since your target DNA template contains thymidine rather than uridine, it is not affected by this procedure. When performing an additional melting curve analysis, the use of UNG lowers the respective melting temperature (T_m) by approx. 1°C.

⚠ If you use the LightCycler® FastStart DNA Master HybProbe, perform prevention of carry-over contamination with LightCycler® Uracil-DNA Glyocsylase*. Proceed as described in the package insert.

Two-Step RT-PCR

The 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® System. Subsequent amplification and online monitoring is performed according to the LightCycler® System standard procedure, using cDNA as starting sample material. One of the following reagents is required for reverse transcription of RNA into cDNA (see Ordering Information for details):

- Transcriptor Reverse Transcriptase
- Transcriptor First Strand cDNA Synthesis Kit
- 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 template in parallel to determine the optimum template amount.

3. Results

The following amplification curves were obtained by performing the procedure for single color detection and using LightCycler® Red 640 as acceptor fluorophore.

Displayed are the results in channel 2 and 3, with and without color compensation. Equivalent results (according to the table above) will be obtained using single color detection with LightCycler® Red 705 as acceptor fluorophore or dual color detection with LightCycler® Red 640 and Red 705 simultaneously.

The fluorescence values versus cycle number are displayed. 30 pg (approx. 10 genome equivalents) of human genomic DNA can be reproducibly detected by amplifying in the LightCycler® Instrument and using the HybProbe detection format. Three picograms (approx. 1 haploid genome equivalent) are sporadically detected due to statistical fluctuations.

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

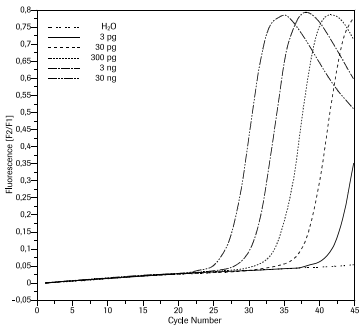

Fig. 1a: Channel 2 (F2/F1) without color compensation

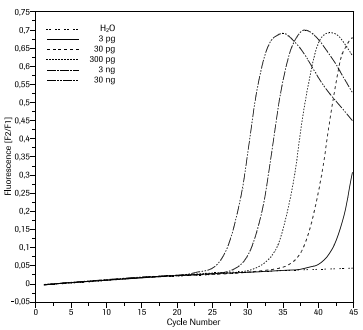

Fig. 1b: Channel 2 (F2/F1) with color compensation

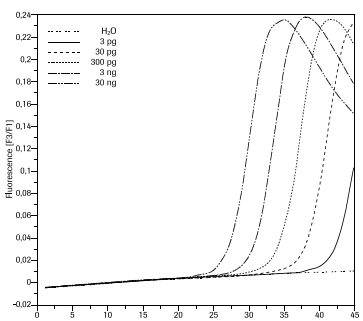

Fig. 1c: Channel 3 (F3/F1) without color compensation

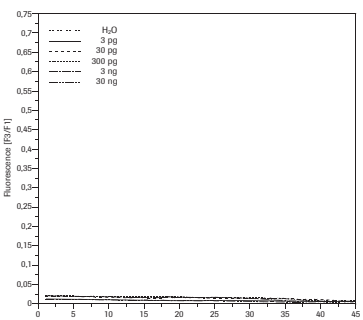

Fig. 1d: Channel 3 (F3/F1) with color compensation

	Possible cause	Recommendation
Fluorescence curves reach maximum long before cycling is complete.	Starting amount of nucleic acid is very high.	Stop the program by clicking on the End Program button. The next cycle program will start automatically.
	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.	Number of cycles is too low.	• Use the **Add 10 Cycles** button to increase number of cycles in the program. • Increase the number of cycles in the protocol. • Use more starting material. • Optimize the PCR conditions (*e.g.*, primer/probe design, protocol).
No amplification occurs.	Using wrong channel to display amplification on screen.	Change the channel setting on the programming screen. (Data obtained up to this point will be saved.)
	Measurements do not occur at the right time.	Check the cycle programs. For HybProbe detection format, choose "single" as the acquisition mode at the end of the annealing phase.
	Impure sample material inhibits reaction.	• Dilute sample 1:10 and repeat the analysis. • Repurify the nucleic acids to ensure removal of inhibitory agents.
	Pipetting errors or omitted reagents.	Replace missing or defective reagents.
	Difficult template (e.g., unusual GC-rich sequence).	• Repeat PCR under same conditions and add increasing amounts of DMSO (up to 10% of the final concentration). • If performance is still not satisfactory, optimize annealing temperature and $MgCl_2$ concentration, while also titrating the DMSO concentration.
	Amplicon length is >1kb.	Do not use amplicons >1 kb. Optimal results are obtained with amplicons of 700 bp or less.
	Unsuitable HybProbe pair.	Check sequence and binding site of the HybProbe pair.
Fluorescence intensity varies.	Pipetting errors.	When using HybProbe probes for single color detection, you can minimize the effects of pipetting errors by viewing the results in the F2/F1 or F3/F1 mode.
	Some of the reagent is still in the upper part of the capillary, or an air bubble is trapped in the capillary tip.	Repeat capillary centrifugation step.
	Skin oils or dirt are 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 the reaction mixtures; dyes not stored properly.	• Keep the dye-labeled reagents away from light. • Store the reagents at -15 to -25°C, and avoid repeated freezing and thawing. • After thawing, store the Master Mix at 2°C to 8°C for a maximum of one week. • Improve low HybProbe signals by making the concentration of the LightCycler® Red-labeled probe twice as high as the concentration of the fluorescein-labeled probe.
	Chosen gain settings are too low (only if using Light Cycler software version 3.3).	Use the **Real Time Fluorimeter** to find suitable gain settings. Change gain settings in the protocol, then repeat the run. LightCycler® Software versions >3.3 do not require gain settings.
	Reaction conditions are not optimized, leading to poor PCR efficiency.	• Titrate $MgCl_2$ concentration. • Primer concentration should be between 0.3 and 1.0 μM; probe concentration should be between 0.2 and 0.4 μM. • Check annealing temperature of primers and probes. • Check experimental protocol. • Always run a positive control along with your samples.

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	Possible cause	Recommendation
	Mutation analysis using HybProbe probes: The melting temperature of the hybrid between the mismatch strand and the HybProbe pair is lower than the annealing temperature.	Therefore, the HybProbe pair can't bind and create a signal. This will not affect amplification efficiency. Ensure that the melting curve starts at a temperature below the annealing temperature used for PCR. Then, you will get a clear signal after melting curve analysis and will be able to interpret the data.
Fluorescence intensity overflows (only if using LightCycler® software version 3.3).	Unsuitable gain settings.	Gain settings cannot be changed during or after a run, so you must repeat the run. Before repeating the run, use the **Real Time Fluorimeter** option to find suitable gain settings. Use an extra sample for this procedure, so the dyes in your experimental sample will not be bleached. LightCycler® Software versions >3.3, do not require gain settings.
Negative control give a positive signal.	Contamination.	• Remake all critical solutions. • Pipet reagents on a clean bench. • Close lid of the negative control reaction tube immediately after pipetting it. • Use heat-labile UNG to eliminate carry-over contamination.
High background.	Fluorescence signals are very low, therefore the background seems relatively high.	Follow general optimization strategies for LightCycler® PCR.
	HybProbe concentration is too high.	HybProbe concentration should be between 0.2 and 0.4 µM.
	Quality of HybProbe probes is poor.	Prepare a new solution of HybProbe probes.
Amplification curve decreases in late cycles after reaching a plateau.	"Hook effect": competition between binding of the HybProbe pair and reannealing of the PCR product.	This does not affect interpretation of the results. You can avoid it by performing an asymmetric PCR, which favors amplification of the DNA strand that the HybProbe pair bind.
Melting peak is very broad and peaks cannot be differentiated.	**°C to Average** setting is too high.	Reduce the value of **°C to Average**. Applicable for LightCycler® Software 3.0 to 3.5 only.
Melting temperature of a product varies from experiment to experiment.	Variations in reaction mixture.	• Check reagent purity. • Reduce variations in parameters such as $MgCl_2$, heat-labile UNG, and program settings.
No precise melting peak can be identified.	• HybProbe pairs are not homogeneous, or contain secondary structures. • Pseudogenes lead to multiple PCR products.	• Redesign HybProbe pairs. • Check PCR products on an agarose gel.

5. Additional Information on this Product

How this Product Works

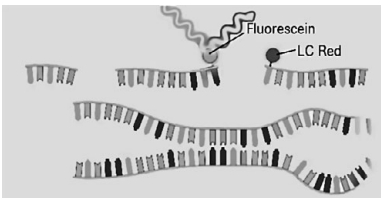
The LightCycler® FastStart DNA Master HybProbe is a ready-to-use reaction mix designed specifically for the HybProbe detection format using the LightCycler® System. It is used to perform hot-start PCR in 20 µl glass capillaries. Hot-start PCR has been shown to significantly improve the specificity and sensitivity of PCR (1, 2, 3, 4) by minimizing the formation of non-specific amplification 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.

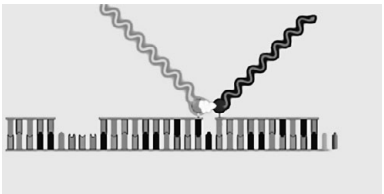
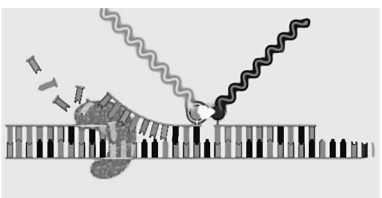
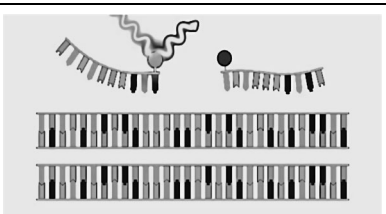
The LightCycler® FastStart DNA Master HybProbe provides convenience, excellent performance, reproducibility and minimal contamination risk. All you have to supply is template DNA, PCR primers, HybProbe pairs and additional $MgCl_2$ (if necessary).

Test Principle

HybProbe probes consist of two different short labeled 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® System are:

①	 The donor dye probe has a fluorescein label at its 3´-end, and the acceptor dye probe has a LightCycler® Red label (LightCycler® Red 610#, 640, 670#, or 705) at its 5´-end (it is also 3´-phosphorylated, so it cannot be extended). Hybridization does not occur during the denaturation phase of PCR. Since the distance between the unbound dyes prevents energy transfer, no fluorescence will be detected from the red acceptor dye during this phase.

continued on next page

②		The probes hybridize to the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two fluorescent dyes into close proximity. Fluorescein is excited by the light source of the LightCycler® Instrument, which causes it to emit green fluorescent light. The emitted energy excites LightCycler® Red by fluorescence resonance energy transfer (FRET). The red fluorescence emitted by the second probe 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 this step, the PCR product is double-stranded, the displaced HybProbe probes are back in solution and too far apart to allow FRET to occur.

HybProbe probes which carry different LightCycler® Red 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 experiment. However, if you are using HybProbe pairs 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® Instrument.

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

LightCycler® Red 610 and LightCycler® Red 670 can be used on a LightCycler® 2.0 Instrument only.

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Quality Control

The LightCycler® FastStart DNA Master HybProbe is function tested using the LightCycler® 2.0 System.

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.

6.2 Changes to Previous Version

- Editorial changes only.

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:

- The LightCycler® 2.0 System family for real-time, online PCR: http://www.lightcycler-online.com

Instrument 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 (8 boxes, each with 96 capillaries and stoppers)	11 909 339 001
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
LC Carousel Centrifuge 2.0	1 centrifuge plus rotor and bucket	03 709 507 001 (115 V) 03 709 582 001 (230 V)

	Product	Pack Size	Cat. No.
Software	LightCycler® Software 4.0	1 software package	03 604 012 001
	LightCycler® Probe Design Software 2.0	1 software package	04 342 054 001
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)	03 515 575 001 03 515 567 001
	LightCycler® FastStart DNA Master^{PLUS} HybProbe, 100 µl Reactions	1 kit (384 reactions)	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)	03 515 869 001 03 515 885 001
	LightCycler® FastStart DNA Master^{PLUS} SYBR Green I, 100 µl Reactions	1 kit (384 reactions)	03 752 186 001
Associated Kits and Reagents	LightCycler® Color Compensation Set	1 set (5 reactions)	12 158 850 001
	LightCycler® Uracil-DNA Glycosylase	100 U (50 µl)	03 539 806 001
	LightCycler® TaqMan Master	1 kit (96 reactions)	04 535 286 001
	LightCycler® Multiplex DNA Master HybProbe	1 kit (96 reactions)	04 340 019 001

6.4 Disclaimer of License

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