For general laboratory use.

LightCycler® FastStart DNA Master HybProbe

Version: 17

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Easy-to-use hot start reaction mix for PCR using HybProbe Probes with the LightCycler® PRO or the LightCycler® Carousel-Based Systems.

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 kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	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 10 mM $MgCl_2$.	03 003 248 001	3 vials 1a and 3 vials 1b, for 3 × 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 × 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 kit is shipped on dry ice.
When stored at −15 to −25°C, the kit is stable through the expiry 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 1b to 1a	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 Reagent required

Standard laboratory equipment

- Nuclease-free pipette tips
- 1.5 mL RNase-free microcentrifuge tubes to prepare master mixes and dilutions
- To minimize risk of RNase contamination, autoclave all vessels

For qPCR

- Real-Time PCR systems such as the LightCycler® PRO or LightCycler® Carousel-Based Systems*
- LightCycler® 480 Multiwell Plate 96, white*
- LightCycler® 480 Multiwell Plate 384, white*
- LightCycler® 480 Multiwell Plate 96, white, 4 bar codes*
- LightCycler® 480 Multiwell Plate 384, white, 4 bar codes*
- Sealing Foil Applicator*
- LightCycler® 480 Sealing Foil*
- LightCycler® 8-Tube Strips (white)*
- LightCycler® 8-Tube Strip Adapter Plate*
- LightCycler® Capillaries*
- LightCycler® Color Compensation Set* (optional)
- Centrifuge with swinging bucket rotor for multiwell plates
- LightCycler® Uracil-DNA Glycosylase* (optional)

For details about prevention of carryover contamination, see Section, **Prevention of Carryover Contamination**.

Centrifugation of 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 microcentrifuge rotor.

or

- LC Carousel Centrifuge 2.0 for use with the LightCycler® 2.0 Sample Carousel (optional)

1.4. Application

LightCycler® FastStart DNA Master HybProbe is an easy-to-use hot start reaction mix for sensitive PCR applications 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

Typical Run Time

The LightCycler® FastStart DNA Master HybProbe can be used for multiplex qPCR protocols. For example, a duplex protocol using 45 cycles requires less than 75 minutes when using the LightCycler® PRO System.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Use any template DNA suitable for qPCR, or 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.

For reproducible isolation of nucleic acids, use:
- 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 appropriate positive and negative controls with the samples.
- To check for the presence of contamination, prepare and include a negative control by replacing 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, that is, the one priming the strand that binds the probes, and a lower concentration of the reverse primer, that is, 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.

Mg^{2+} Concentration

To ensure specific and efficient amplification with the LightCycler® 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® 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 for the LightCycler® Carousel-Based System

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 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 or the Instructions for Use of the LightCycler® Color Compensation Set.

Color Compensation for the LightCycler® PRO Instrument

The LightCycler® PRO Instrument is factory calibrated for commonly used reporter dyes for hybridization probes. There is no need for color compensation runs.

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® System. Subsequent amplification and online monitoring is performed according to a LightCycler® 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, run undiluted, 1:10 diluted and 1:100 diluted cDNA templates, in parallel to determine the optimal template amount.***

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 UNG during incubation at +40°C and will not serve as a PCR template. The high temperatures of the initial denaturation step will heat-inactivate the UNG to prevent degradation of the newly synthesized dUTP-containing PCR products.

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

To ensure optimal results in carryover prevention reactions with the LightCycler® FastStart DNA Master HybProbe, always use LightCycler® Uracil-DNA Glycosylase*. Follow the Instructions for Use for the enzyme.

When performing Melting curve analysis, the use of UNG may lower the melting temperature Tm by approximately 1°C.

2.2. Protocols

LightCycler® PRO and LightCycler® Carousel-Based System protocols

The following procedure is optimized for use with the corresponding LightCycler® System you are using.

Program the LightCycler® Instrument before preparing the reaction mixes.

For details on how to program the experimental protocol, see the LightCycler® PRO System User Assistance or the LightCycler® Instrument Operator's Manual.

Protocol for use with the LightCycler® PRO System (Multiwell Plate 96 or 384)

The LightCycler® PRO System protocol contains the following programs:

- **Pre-incubation** for activation of DNA polymerase and denaturation of the DNA
- **Amplification** of the target DNA
- **Melting curve** for SNP or mutation detection
- **Cooling** of the thermal cycler

Setup	
Thermal cycler type	Reaction volume [µL]
96 (384)	10 – 100 (5 – 20)
Detection format	
Select dyes used in your assays. If the dye is not predefined, use appropriate filter combination in the user-defined detection format.	
Programs	
Program name	Cycles
Pre-incubation	1
Amplification	45(1)
Melting curve	1
Cooling	1

Temperature targets					
	Target [°C]	Acquisition mode	Duration [s]	Ramp rate[1] [°C/s]	Reading
Pre-incubation	95	None	600[2]	4.4 (4.8)	–
3-step amplification	95	None	10	4.4 (4.8)	–
	primer dependent[3]	Single	5 – 20[4]	2.2 (2.5)	–
	72	None	5 – 30[4][5]	4.4 (4.8)	–
Melting curve (optional)	95	None	10	4.4 (4.8)	–
	40	None	60 – 120	2.2 (2.5)	–
	95[6]	Continuous	1	0.2 (0.2)	1 – 5
Cooling (automatically added)	40	None	30	2.2 (2.5)	–

[1] Forty-five cycles is suitable for most assays. If the assay shows steep amplification curves and early crossing points, 40 cycles should be sufficient. Reducing the number of cycles will improve uniformity of the product and reduce the time required for the assay.

[2] For some assays, a pre-incubation of 300 seconds is sufficient (fast protocol). However, if high polymerase activity is required in early cycles, a 600-second period is recommended, especially for higher reaction volumes and when working with unpurified cDNA samples as template. Do not use more than 2 µL unpurified cDNA sample.

[3] For initial experiments, set the target temperature, that is the primer annealing temperature, 5°C below the calculated primer Tm. Annealing usually occurs at +60°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] Calculate the duration time for the PCR elongation step by dividing the amplicon length over 25, for example, a 500 bp amplicon requires 20 second elongation time.

[6] You can shorten the time required for the melting curve run by setting the target temperature to less than 95°C. The target temperature must be at least +5°C higher than the highest Tm you want to determine.

Color Compensation protocol for the LightCycler® PRO Instrument

The LightCycler® PRO Instrument does not require the creation of a color compensation object.

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. Pipette 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.***

6. Store on ice until ready to use.

Setup of the qPCR reaction for the LightCycler® PRO Instrument

Follow the procedure below to prepare one 20 µL standard reaction.
Always wear gloves during handling.

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

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. 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 listed below:
   – To prepare the PCR mix for more than one reaction, multiply the amount in the "Volume" column below by z, where z = the number of reactions to be run plus sufficient additional reactions.

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	**15**	

4. Mix carefully by pipetting up and down; do not vortex.

5. Pipette 15 µL qPCR Mix into a multiwell plate.
   – Add 5 µL of the DNA template.
   – Seal multiwell plate with a LightCycler® 480 Sealing Foil.

6. Place the multiwell plate into the centrifuge with a swinging-bucket rotor and suitable adapter, and balance it with a suitable counterweight, such as another multiwell plate.
   – Centrifuge at 1,500 × *g* for 2 minutes.

7. Load the multiwell plate into the LightCycler® PRO Instrument.

8. Start the PCR program described above.
   – If you use reaction volumes other than 20 µL, be sure to adapt the right volume in the running protocol. To start, we recommend using the same hold times as for the 20 µL volume.

Protocol for use with the LightCycler® Carousel-Based System

The 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

⚠ ***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, for example, 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			
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	
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[5]	Single
	72	00:00:05 – 00:00:30 [4, 6]	20	None
Melting curve (optional)	95	00:00:00	20	None
	40°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 minute pre-incubation time is recommended. However, depending on the individual assay, the pre-incubation time can be reduced to 5 minutes 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 minutes.
(3) For initial experiments, set the target temperature, that is, 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, for example, a 500 bp amplicon requires 20 seconds elongation time.

Setup of the qPCR reaction for the LightCycler® Carousel-Based System

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. Pipette 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.***

6. Store on ice, or in the precooled 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.

   ℹ *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:
– To prepare the PCR mix for more than one reaction, multiply the amount in the "Volume" column by z, where z = the number of reactions to be run plus one additional reaction.

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**	

5 Mix gently by pipetting up and down. Do not vortex.
– Pipette 18 µL PCR mix into each precooled 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.

3. Results

Results on the LightCycler® PRO Instrument

The following results were obtained using the LightCycler® FastStart DNA Master HybProbe on the LightCycler® PRO Instrument. A reaction using primers specific for the target gene **JAK2** was performed (see Fig. 1).

Red 640 channel (621 - 636)

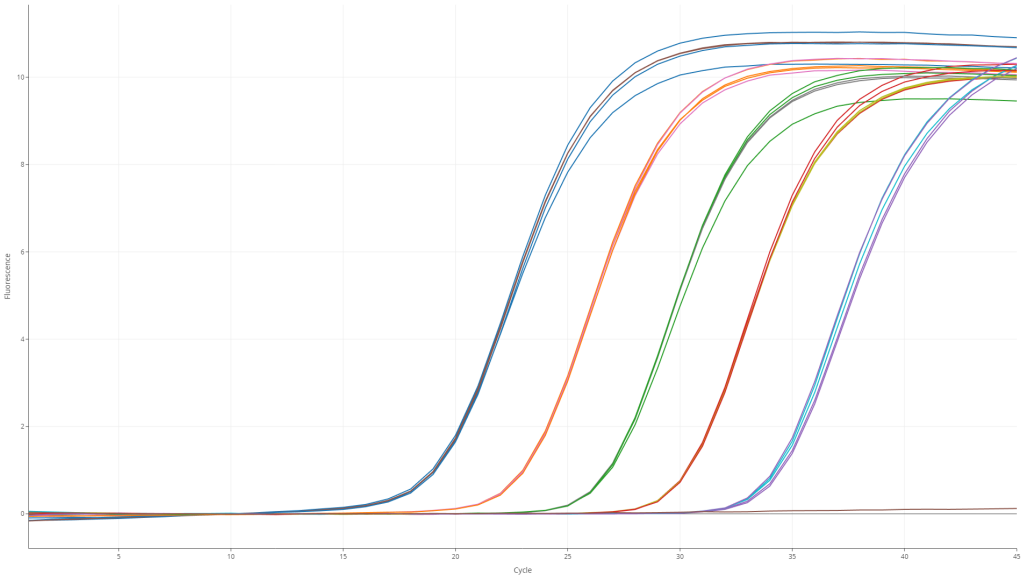


Fig. 1: The amplification diagram shows the result for an amplification of a 267 bp fragment of the **JAK2 exon 12** gene detected with a Red 640-labeled HybProbe probe. The amplification curves shown were obtained from a plasmid dilution series of 100 copies (far right), 1,000 copies, 10,000 copies, 100,000 copies, and 1,000,000 copies (far left) per well, including a no template control (flat line). Singleplex qPCR with five replicates for each dilution was performed in a reaction volume of 20 µL per well.

Melting curve analysis

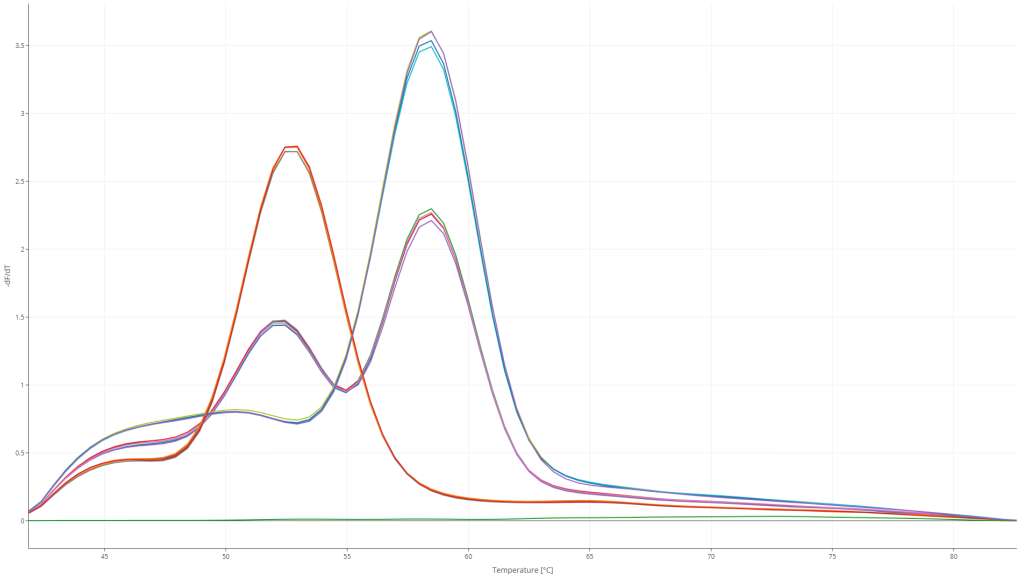


Fig. 2: Melting curve analysis of three different samples (plasmid concentration of 10,000 copies with six replicates). A 267 bp fragment from **JAK2 gene exon 12** is amplified and detected with a Red 640-labeled HybProbe specific for the **539Leu** mutation. The peak at approximately +53°C (far left) represents the wild type, the peak at approximately +58°C (far right) represents the mutation**,** and the double-peak visualizes the heterozygous samples. As a no template control, template DNA was replaced by PCR-grade water (flat line).

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 (LightCycler® Carousel-Based System) or the **Terminate program** button (LightCycler® PRO System). 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, for example, $MgCl_2$ 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 (LightCycler® Carousel-Based System) or the **Add 5 cycles** button (for details see User Assistance of the LightCycler® PRO System) 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 minutes.
		Ensure that the denaturation time during the amplification cycles is 10 seconds.
	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, such as 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 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 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.

Fluorescence intensity varies.	Pipetting errors.	When using HybProbe probes and single-color detection on the LightCycler® Carousel-Based System, pipetting errors can be diminished through interpreting the results in the 640/530 or 705/530 mode.
	PCR mix is still in the upper part of the capillary. Air bubble is trapped in the capillary tip.	Repeat capillary centrifugation step.
	Skin oils on the surface of the capillary tip.	Always wear gloves when handling the capillaries.
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 cannot 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 to eliminate carryover contamination.
High background	Very low fluorescence signals, therefore the background seems relatively high.	Follow general optimization strategies for PCR using LightCycler® 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.
Melting temperature of a product varies from experiment to experiment.	Variations in reaction mixture, such as 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.

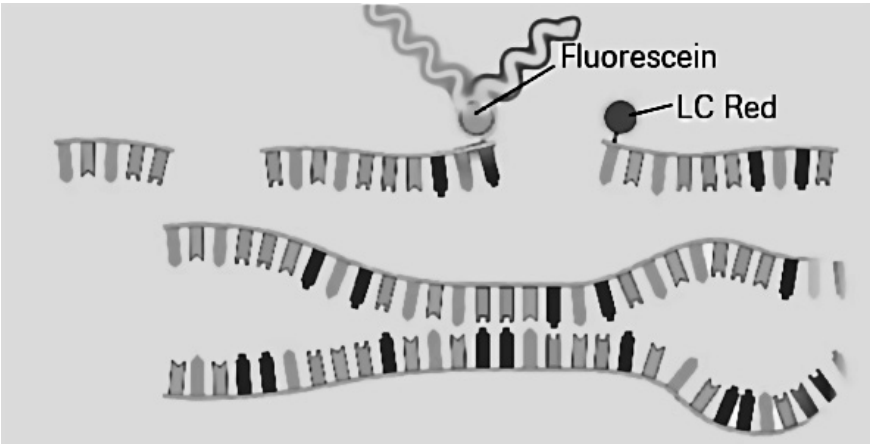
5. Additional Information on this Product

5.1. Test Principle

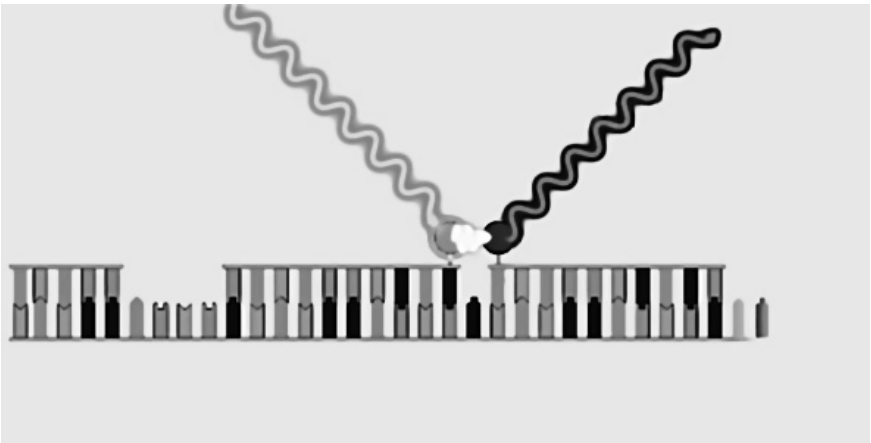
HybProbe probes consist of two different short oligonucleotides that bind to an internal sequence of the amplified fragment, during the annealing phase of the amplification cycle.

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

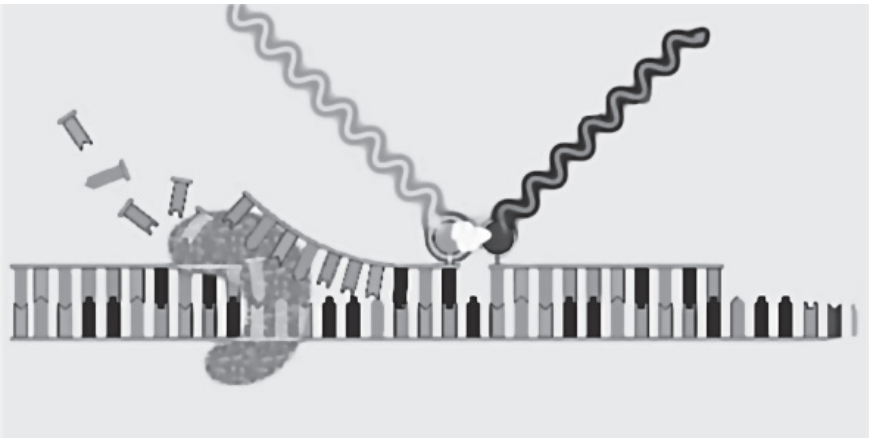
① The donor dye probe has a fluorescein label at its 3' end and the acceptor dye probe has a red fluorophore label (Red 610, Red 640, Cy5, or Cy5.5) at its 5' end it is 3'-phosphorylated, so it cannot be extended. Hybridization does not occur during the Denaturation phase of PCR. As the distance between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



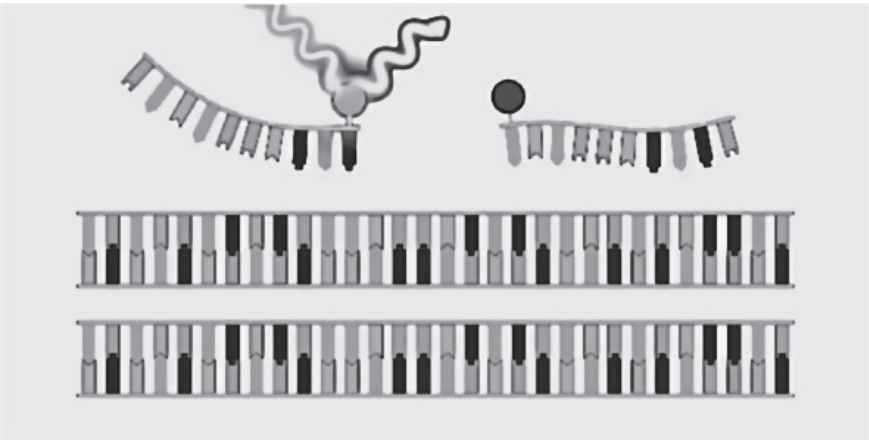
② During the Annealing phase, the probes hybridize to the amplified DNA fragment in a head-to-tail arrangement, thereby bringing the two fluorescent dyes close to each other. Fluorescein is excited by the light source of the LightCycler® System, which causes it to emit green fluorescent light. The emitted 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.

- The LightCycler® PRO Instrument does not require the creation of a color compensation object.
- For the LightCycler® Carousel-Based System, 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.

How this product works

This kit is ideally suited for hot start PCR applications. Using 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® 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 nonspecific 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 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. Quality Control

The LightCycler® FastStart DNA Master HybProbe is function tested using the LightCycler® 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	
ℹ *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

Editorial changes.
Information about the LightCycler® PRO System has been added.
List of additional reagents and equipment has been updated.
Quality Control has been changed to LightCycler® System.

6.3. Ordering Information

Roche offers a large selection of reagents and systems for life science research. For a full overview of related products and manuals, please visit and bookmark our homepage lifescience.roche.com.

Product	Pack Size	Cat. No.
Accessories general (hardware)		
Thermal Cycler Assembly 96	1 piece	09 742 565 001
Thermal Cycler Assembly 384	1 piece	09 742 581 001
Sealing Foil Applicator	1 piece	10 018 607 001
Consumables		
LightCycler® Capillaries (20 µl)	5 × 96 capillaries, containing 5 boxes, each with 96 capillaries and stoppers	04 929 292 001
LightCycler® 480 Multiwell Plate 96	5 x 10 plates without sealing foils	05 220 319 001
LightCycler® 480 Multiwell Plate 384	5 x 10 plates without sealing foils	05 217 555 001
LightCycler® 8-Tube Strips (white)	10 x 12 white strips and clear caps	06 612 601 001
LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
LightCycler® 480 Multiwell Plate 96, white	5 x 10 plates	04 729 692 001
MagNA Pure 96 Internal Control Tube	150 tubes	06 374 905 001
LightCycler® 480 Multiwell Plate 384, white	5 x 10 plates	04 729 749 001
Instruments		
LightCycler® PRO Instrument	1 instrument, 96-well version	09 541 713 001
	1 instrument, 384-well version	09 582 487 001
MagNA Pure 24 Instrument	Instrument with built-in control unit, software and accessories	07 290 519 001
MagNA Pure 96 Instrument	1 instrument	06 541 089 001
Reagents, kits		
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	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
High Pure Viral Nucleic Acid Kit	1 kit, up to 100 isolations	11 858 874 001
MagNA Pure 96 DNA and Viral NA Large Volume Kit	Kit for up to 3 x 96 isolations	06 374 891 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	For up to 3 x 192 isolations	06 543 588 001
MagNA Pure 24 Total NA Isolation Kit	Kit for up to 96 isolations (200 µL)	07 658 036 001

6.4. Trademarks

MAGNA PURE, LIGHTCYCLER and FASTSTART are trademarks of Roche.
All other product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For additional documentation such as certificates and safety data sheets, please visit:
documentation.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.

Visit **documentation.roche.com**, to download or request copies of the following Materials:
- Instructions for Use
- Safety Data Sheets
- Certificates of Analysis
- Information Material

To call, write, fax, or email us, visit **lifescience.roche.com** and select your home country to display country-specific contact information.

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany