For life science research only. Not for use in diagnostic procedures.

LightCycler® FastStart DNA Master SYBR Green I

Version 18

Content version: May 2011

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

Cat. No. 03 003 230 001 Kit for 96 reactions
Cat. No. 12 239 264 001 Kit for 480 reactions

Store the kit at −15 to −25°C.

⚠ Keep LightCycler® FastStart Reaction Mix SYBR Green I (vial 1b, green cap) away from light!

Table of Contents

1. What this Product Does 3
Number of Tests 3
Kit Contents 3
Storage and Stability 3
Additional Equipment and Reagents Required 4
Application 5
Assay Time 5
2. How to Use this Product 6
2.1 Before You Begin 6
Sample Material 6
Primers 6
$MgCl_2$ 6
Negative Control 6
2.2 Experimental Protocol 7
LightCycler® Carousel-Based System Protocol 7
Fluorescence and Run Setup Parameters 8
Preparation of the Master Mix 9
Preparation of the PCR Mix 9
2.3 Related Procedures 11
Prevention of Carry-Over Contamination 11
Two-Step RT-PCR 11
3. Results 12
Quantification Analysis 12
Melting Curve Analysis 13
4. Troubleshooting 14
5. Additional Information on this Product 17
5.1 How this Product Works 17
Test Principle 17
5.2 Quality Control 18
5.3 References 19
5.4 Product Citations 19
6. Supplementary Information 20
6.1 Conventions 20
Text Conventions 20
Symbols 20
Abbreviations 20
6.2 Changes to Previous Version 20
6.3 Ordering Information 21
6.4 Disclaimer of License 22
6.5 Trademarks 22
6.6 Regulatory Disclaimer 22

PROTOCOL

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. 03 003 230 001 (96 reactions) b) Cat. No. 12 239 264 001 (480 reactions)
1a colorless cap	LightCycler® FastStart Enzyme	a) 1 vial 1a, 3 vials 1b for 3 vials, 64 µl each LightCycler® FastStart DNA Master SYBR Green I (10× conc.) b) 5 vials 1a, 15 vials 1b for 15 vials, 64 µl each LightCycler® FastStart DNA Master SYBR Green I (10× conc.) ▪ Ready-to-use hot start PCR reaction mix (after pipetting 10 µl from vial 1a into one vial 1b). ▪ Contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye and 10 mM $MgCl_2$.
1b green cap	LightCycler® FastStart Reaction Mix SYBR Green I, 10× conc.	
2 blue cap	$MgCl_2$ stock solution, 25 mM	a) 1 vial, 1 ml b) 2 vials, 1 ml each ▪ To adjust $MgCl_2$ concentration.
3 colorless cap	H_2O, PCR grade	a) 2 vials, 1 ml each b) 7 vials, 1 ml each ▪ To adjust the final reaction volume.

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

⚠ Keep the LightCycler® FastStart Reaction Mix SYBR Green I (vial 1b, green cap) away from light!

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 colorless cap	LightCycler® FastStart Enzyme	▪ Store at −15 to −25°C. ▪ **Avoid repeated freezing and thawing!** ▪ **Keep vial 1b away from light!**
1b green cap	LightCycler® FastStart Reaction Mix SYBR Green I, 10× conc.	
1 green cap (after addition of 1a to 1b)	LightCycler® FastStart DNA Master SYBR Green I, 10× conc.	▪ Store at −15 to −25°C for a maximum of three months. ▪ After thawing, store at +2 to +8°C for a maximum of one week. ▪ **Avoid repeated freezing and thawing!** ▪ **Keep vial 1 away from light!**
2 blue cap	$MgCl_2$ stock solution, 25 mM	Store at −15 to −25°C
3 colorless cap	H_2O, PCR grade	

Additional Equipment and Reagents Required

Additional reagents and equipment required to perform PCR reactions with the LightCycler® FastStart DNA Master SYBR Green I, using the LightCycler® Carousel-Based System include:

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

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

or

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

⚠ If you use a LightCycler® Instrument version below 2.0, you need in addition the LC Carousel Centrifuge 2.0 Bucket 2.1*. To adapt the LightCycler® 2.0 Sample Carousel (20 μl) to the former LC Carousel Centrifuge, you need the LC Carousel Centrifuge 2.0 Rotor Set*.

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

‡ For prevention of carry-over contamination; see section Related Procedures for details. Use LightCycler® Uracil-DNA Glycosylase, in combination with LightCycler® FastStart DNA Masters only.

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

Application

LightCycler® FastStart DNA Master SYBR Green I is designed for life sience studies. When combined with the LightCycler® Carousel-Based System, this kit is ideally suited for hot start PCR applications. In combination with the LightCycler® Carousel-Based System and suitable PCR primers, this kit enables very sensitive detection and quantification of defined DNA sequences. Furthermore, the kit can be used to perform two-step RT-PCR, in combination with a reverse transcription kit for cDNA synthesis*.

In principle, the LightCycler® FastStart DNA Master SYBR Green I can be used for the amplification and detection of any DNA or cDNA target. However, you would need to optimize the detection protocol to the reaction conditions of the LightCycler® Carousel-Based System and design specific PCR primers for each target. Refer to the LightCycler® Operator's Manual for general recommendations.

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

⚠ 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® Carousel-Based System.

Assay Time

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

2. How to Use this Product

2.1 Before You Begin

Sample Material

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

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

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

Using a too high amount of template DNA may reduce the maximum fluorescence signal, by outcompeting the SYBR Green I dye.

⚠ 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.

Primers

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

$MgCl_2$

To ensure specific and efficient amplification with the LightCycler® Carousel-Based System, you must optimize the $MgCl_2$ concentration for each target. The LightCycler® FastStart DNA Master SYBR Green I contains a $MgCl_2$ concentration of 1 mM (final concentration). The optimal 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 (vial 2, blue cap) that you must add 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.

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).

2.2 Experimental Protocol

LightCycler® Carousel-Based System Protocol

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

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

A LightCycler® Carousel-Based System protocol that uses the LightCycler® FastStart DNA Master SYBR Green I, 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 PCR product identification/amplicon analysis
- **Cooling** the rotor and the thermal chamber

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

⚠ Set all other protocol parameters not listed in the table 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 SYBR Green I.

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]	0 – 10 s [5]	none
		Extension	72°C [3]	= (amplicon [bp]/25) s [6]	single
			Melting Curve		
Melting Curves	1	Denaturation	95°C	0 s	none
		Annealing	65°C	15 s	none
		Melting	95°C Ramp Rate = 0.1°C/sec[1]	0 s	continuous
			Cooling		
None	1		40°C	30 s	none

1) Temperature Transition Rate/Slope/Ramp Rate is 20°C/sec, except where indicated.
2) For initial experiments, set the target temperature (*i.e.,* the primer annealing temperature) 5°C below the calculated primer T_m. Calculate the primer T_m according to the following formula, based on the nucleotide content of the primer: T_m = 2°C (A+T) + 4°C (G+C).
3) If the primer annealing temperature is low (< +55°C), reduce the ramp rate to 2 to 5°C/s.

4) A 10 min pre-incubation time is recommended. However, depending on the individual assay, the pre-incubation time can be reduced to 5 min with no change in performance. In assays where high polymerase activity is required in the early cycles, in some cases, results can be improved by extending the pre-incubation time to 15 min.

5) For typical primers, choose an incubation time of 0 to 10 s for the annealing step. To increase the specificity of primer binding, use an incubation time of <5 s.

6) For greater precision in target quantification experiments, it can be advantageous (in some cases) to choose longer 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 F1	
Fluorescence Gains	**Fluorimeter**	**Gain Value**
	Channel 1 (F1)	3
	Channel 2 (F2)	1
	Channel 3 (F3)	1
LightCycler® **Software Version 3.5**		
Display Mode	Fluorescence channel 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 4.1**		
Default Channel	Fluorescence channel 530	
Fluorescence Gains	not required	
"Max. Seek Pos."	Enter the number of sample positions for which the Instrument should look.	
"Instrument Type"	▪ "6 Ch.": for LightCycler® 2.0 Instrument (selected by default) ▪ "3 Ch.": for LightCycler® 1.5 Instrument and instrument versions below	
"Capillary Size"	Select "20 µl" as the capillary size for the experiment. ⚠ For the "6 Ch." instrument type only.	

Preparation of the Master Mix

1. Thaw one vial of "Reaction Mix" (vial 1b, green cap) and shield it from light.

   ⚠ A reversible precipitate may form in the LightCycler® FastStart Reaction Mix SYBR Green I (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, colorless cap) and the thawed vial of "Reaction Mix" (from Step 1), then place the vials back on ice.

3. Pipette 10 µl from vial 1a (colorless cap) into vial 1b (green cap).

   Each vial 1a contains enough enzyme solution for three vials of "Reaction Mix" (vial 1b).

4. Mix gently by pipetting up and down.

   ⚠ Do not vortex.

5. Re-label vial 1b (green cap) with the new label (vial 1: LightCycler® FastStart DNA Master SYBR Green I) provided with the kit.

   ⚠ Always keep the Master Mix 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 pre-cooled centrifuge adapters or in a LightCycler® Sample Carousel in a pre-cooled LC Carousel Centrifuge Bucket.

2. Prepare a 10× conc. solution of the PCR primers.

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

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 mentioned below:

Component	Volume	Final conc.
H_2O, PCR grade (vial 3, colorless cap)	x µl	
$MgCl_2$ stock solution, 25 mM (vial 2, blue cap)	y µl	Use concentration that is optimal for the target.
PCR Primer Mix, 10× conc.	2 µl	0.2 to 1.0 µM each (recommended conc. is 0.5 µM)
LightCycler® FastStart DNA Master SYBR Green I, 10× conc. (vial 1, green 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.

❹
- Mix gently by pipetting up and down. Do not vortex.
- Pipette 18 µl PCR mix into each pre-cooled LightCycler® Capillary.
- Add 2 µl of the DNA template
- Seal each capillary with a stopper.

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

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

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

❼ Cycle the samples as described above.

2.3 Related Procedures

Prevention of Carry-Over Contamination

Uracil-DNA 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 reaction mixes of all LightCycler® System 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 SYBR Green I, perform prevention of carry-over contamination with LightCycler® Uracil-DNA Glyocsylase*. Proceed as described in the package insert and/or in the table below to prevent carry-over contamination.

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

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

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

Two-Step RT-PCR

LightCycler® FastStart DNA Master SYBR Green I 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 the cDNA as the 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*
- 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 optimal template amount.

3. Results

Quantification Analysis

The following amplification curves were obtained using the LightCycler® FastStart DNA Master SYBR Green I, in combination with the LightCycler® Control Kit DNA, targeting human β-globin gene. The fluorescence values versus cycle number are displayed. Thirty picograms (approx. 10 genome equivalents) of human genomic DNA can be reproducibly detected by amplifying in the LightCycler® Carousel-Based System and using the SYBR Green I detection format. Three picograms (approx. 1 haploid genome equivalent) are sporadically detected, due to statistical fluctuations.

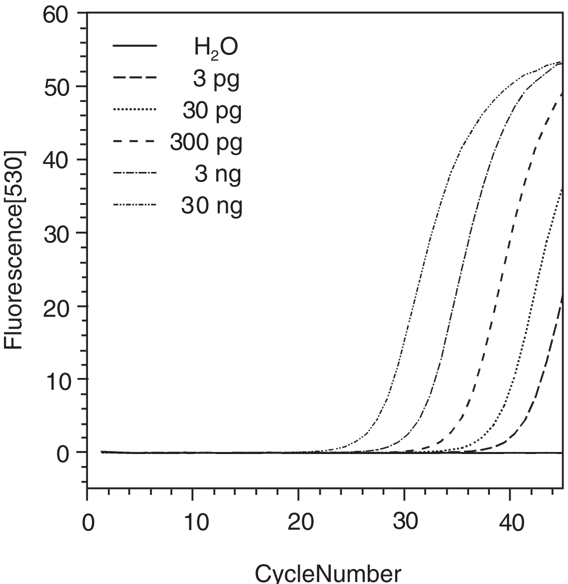

Fig. 1: Serially diluted samples containing 30 ng, 3 ng, 300 pg, 30 pg, or 3 pg human genomic DNA as starting template were amplified using the LightCycler® FastStart DNA Master SYBR Green I. As a negative control, template DNA was replaced by PCR-grade water.

Melting Curve Analysis

Specificity of the amplified PCR product was assessed by performing a Melting Curve analysis. The resulting melting curves enable discrimination between primer-dimers and specific PCR product. The specific β-globin product melts at a higher temperature than the primer-dimers. The melting curves display the specific amplification of the β-globin gene when starting from 30 ng, 3 ng, 300 pg, 30 pg, or 3 pg human genomic DNA.

⚠ Smaller reaction volumes may result in melting temperature variations.

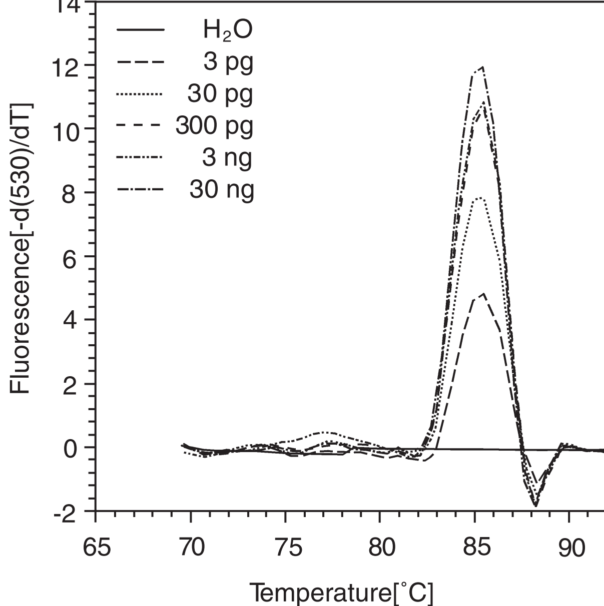

Fig. 2: Melting Curve analysis of amplified samples containing 30 ng, 3 ng, 300 pg, 30 pg, or 3 pg human genomic DNA as starting template. As a negative control, template DNA was replaced by PCR-grade water.

4. Troubleshooting

	Possible cause	Recommendation
Amplification reaches plateau phase before the program is complete.	Very high starting amount of nucleic acid	The program can be finished by clicking on the **End Program** button. The next cycle program will start automatically.
	The number of cycles is too high.	Reduce the number of cycles in the amplification program.
Log-linear phase of amplification just starts as the amplification program finishes.	Very low starting amount of nucleic acid	▪ Improve PCR conditions (*e.g.,* $MgCl_2$ concentration, primer concentration or design). ▪ Use higher amount of starting template. ▪ Repeat the run.
	The number of cycles is too low.	▪ Increase the number of cycles in the amplification program. ▪ Use the **+10 cycles** button, to increase the number of cycles in the amplification program.
No amplification occurs.	Wrong channel has been chosen to display amplification online.	Change the channel setting on the programming screen. (The data obtained up to this point will be saved.)
	FastStart Taq DNA polymerase is not fully activated.	▪ Ensure that the PCR programming includes a pre-incubation step at 95°C for 10 min. ▪ Ensure that the denaturation time during the amplification cycles is 10 s.
	Pipetting errors or omitted reagents	▪ Check for missing reagents. ▪ Titrate $MgCl_2$ concentration. ▪ Check for defective SYBR Green I dye.
	Chosen gain settings are too low.	⚠ Optimize gain settings using the **Real Time Fluorimeter** function. Then repeat the run, using the optimal gain settings in the cycle programs.
	Scale of axes on graph are unsuitable for analysis.	Change the values for the x- and y-axis by double-clicking on the maximum and/or minimum values, then change to more suitable values.
	Measurements do not occur.	Check the amplification program. For SYBR Green I detection format, choose "single" as the acquisition mode at the end of the elongation phase.
	Amplicon length is >1 kb.	Do not use amplicons >1 kb. Optimal results are obtained with amplicons of 700 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.

	Possible cause	Recommendation
Fluorescence intensity is too high and reaches overflow.	Unsuitable gain settings	Gain settings cannot be changed during or after a run. Before repeating the run, use the **Real Time Fluorimeter** option to find suitable gain settings. For SYBR Green I, the background fluorescence at measuring temperature should not exceed 10. ⚠ Use an extra sample for this procedure, so the dyes in your experimental samples will not be bleached. LightCycler® Software versions 3.5 and higher do not require a gain setting.
Fluorescence intensity is too low.	Low concentration or deterioration of SYBR Green I dye in the reaction mixtures, due to unsuitable storage conditions.	▪ Store the SYBR Green I dye containing reagents at −15 to −25°C and keep them away from light. ▪ Avoid repeated freezing and thawing. ▪ After thawing, store the LightCycler® FastStart DNA Master SYBR Green I at +2 to +8°C for a maximum of one week and keep it away from light.
	Reaction conditions are not optimized, leading to poor PCR efficiency.	▪ Titrate $MgCl_2$ concentration. ▪ Primer concentration should be between 0.2 and 1.0 μM ▪ Check annealing temperature of primers. ▪ Check experimental protocol. ▪ Always run a positive control along with your samples.
Fluorescence intensity varies.	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.
Amplification curve reaches plateau at a lower signal level than the other samples.	Starting amount of genomic DNA is too high; DNA captures SYBR Green I dye, leading to a high background signal. Insufficient amounts of SYBR Green I dye are left to monitor the increase of fluorescence signal during amplification.	▪ Do not use more than 50 ng of complex genomic DNA in a 20 μl reaction. ▪ Use the format of the HybProbe (which enables analysis of up to 500 ng DNA) instead of SYBR Green I.
	SYBR Green I dye bleached.	Ensure the reagents containing the SYBR Green I dye are stored away from light. Avoid repeated freezing and thawing.
Negative control samples are positive.	Contamination, or presence of primer-dimers.	▪ Remake all critical solutions. ▪ Pipette reagents on a clean bench. ▪ Close lid of the negative control reaction immediately after pipetting it. ▪ Use LightCycler® UNG to eliminate carry-over contamination.
Melting peak is very broad and peaks can not be differentiated.	°C to Average setting is too high.	Reduce the value of **°C to Average** (only applicable for LightCycler® Software versions prior to version 4.0).

	Possible cause	Recommendation
Double melting peak appears for one product.	Two products of different length or GC content have been amplified (*e.g.,* due to pseudogenes or mispriming)	▪ Check products on an agarose gel ▪ Elevate the reaction stringency by: – redesigning the primers, – checking the annealing temperature, – performing a “touch-down” PCR, or – using HybProbe Probes for better specificity.
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 $MgCl_2$, heat-labile UNG, primer preparation and program settings.
Only a primer-dimer peak appears, with no specific PCR product peak; or very high primer-dimer peaks.	Primer-dimers have out-competed amplification of specific PCR product.	▪ Keep all samples at +2 to +8°C until the run is started. ▪ Keep the time between preparing the reaction mixture and starting the run as short as possible. ▪ Increase starting amount of DNA template. ▪ Titrate $MgCl_2$. ▪ Increase annealing temperature, in order to enhance stringency.
	Quality of primers are poor.	Purify primers more thoroughly.
	Sequence of primers are inappropriate.	Redesign primers.
Primer-dimer and product peaks are very close together.	Unusually high GC-content of PCR primers.	▪ Redesign primers. ▪ Run melting curve at the lowest ramp rate (0.1 °C/sec with continuous measurement) ▪ Expand scale of the x-axis. ▪ Reduce the value of **°C to Average** (only applicable for LightCycler® Software versions prior to version 4.0).
Very broad primer-dimer peak with multiple peaks	Heterogeneous primers with primer-dimer variations (*e.g.,* concatemers, loops)	Redesign primers.
One peak of the same height occurs in all samples.	Contamination in all samples.	▪ Close capillaries during centrifugation step. ▪ Use fresh solutions.

5. Additional Information on this Product

5.1 How this Product Works

LightCycler® FastStart DNA Master SYBR Green I is a ready-to-use PCR reaction mix, designed specifically for real-time PCR assays using the SYBR Green I detection format on the LightCycler® Carousel-Based 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 – 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 techniques.

LightCycler® FastStart DNA Master SYBR Green I provides convenience, excellent performance and reproducibility, as well as minimizing contamination risk. All you must supply is template DNA, PCR primers and additional $MgCl_2$ (if necessary).

Test Principle

Generation of PCR products can be detected by measurement of the SYBR Green I fluorescence signal. SYBR Green I intercalates into the DNA double helix. In solution, the unbound SYBR Green I dye exhibits very little fluorescence; however, fluorescence (wavelength, 530 nm) is greatly enhanced upon DNA-binding. Therefore, during PCR, the increase in SYBR Green I fluorescence is directly proportional to the amount of double-stranded DNA generated.

As SYBR Green I dye is very stable (only 6% of the activity is lost during 30 amplification cycles) and the LightCycler® Instruments' optical filter set matches the wavelengths of excitation and emission, it is the reagent of choice when measuring total DNA.

The basic steps of DNA detection by SYBR Green I, during real-time PCR on the LightCycler® Carousel-Based System are:

①	At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers and the SYBR Green I dye. The unbound SYBR Green I dye molecules weakly fluoresce, producing a minimal background fluorescence signal, which is subtracted during computer analysis.
②	After annealing of the primers, a few SYBR Green I dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green I dye molecules to emit light upon excitation.
③	During elongation, more and more SYBR Green I dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the SYBR Green I dye molecules are released and the fluorescence signal falls.
④	Fluorescence measurement at the end of the elongation step of every PCR cycle is performed, to monitor the increasing amount of amplified DNA.

To prove that only your desired PCR product has been amplified, you may perform a Melting Curve analysis after PCR. In Melting Curve analysis, the reaction mixture is slowly heated to +95°C, which causes melting of double-stranded DNA and a corresponding decrease of SYBR Green I fluorescence. The Instrument continuously monitors this fluorescence decrease and displays it as melting peaks. Each melting peak represents the characteristic melting temperature (T_m) of a particular DNA product (where the DNA is 50% double-stranded and 50% single-stranded). The most important factors that determine the T_m of dsDNA are the length and the GC-content of that fragment. If PCR generated only one amplicon, Melting Curve analysis will show only one melting peak. If primer-dimers or other non-specific products are present, they will be shown as additional melting peaks. Checking the T_m of a PCR product can thus be compared with analyzing a PCR product by length in gel electrophoresis.

5.2 Quality Control

The LightCycler® FastStart DNA Master SYBR Green I is function tested using the LightCycler® Carousel-Based System.

5.3 References

1 Chou, Q. *et al*. (1992). Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res*. **20**, 1717-1723.

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

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

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

5.4 Product Citations

5 Becker, J. *et al*. (2004). MOR1 receptor mRNA expression in human brains of drug-related fatalities — a real-time PCR quantification. *Forensic Sci. Int.* **140**, 13-20.

6 Beuret, C. (2004). Simultaneous detection of enteric viruses by multiplex real-time RT-PCR. *J. Virol. Methods* **115**, 1-8.

7 Eleaume, H. and Jabbouri S. (2004). Comparison of two standardization methods in real-time quantitative RT-PCR to follow *Staphylococcus aureus* genes expression during in vitro growth. *J. Microbiol. Methods* **59**, 363-70.

8 Fu, D. *et al*. (2004). Permanent occlusion of the middle cerebral artery upregulates expression of cytokines and neuronal nitric oxide synthase in the spinal cord and urinary bladder in the adult rat. *Neuroscience* **125**, 819-31.

9 Nomura, K. *et al*. (2004). Caffeine suppresses the expression of the Bcl-2 mRNA in BeWo cell culture and rat placenta. *J. Nutr. Biochem*. **15**, 342-349.

10 Peinnequin, A. *et al*. (2004). Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. *BMC Immunol*. **5**, 3.

11 van der Ploeg, JR. et al. (2004). Quantitative detection of *Porphyromonas gingivalis fimA* genotypes in dental plaque. *FEMS Microbiol*. Lett. **232**, 31-37.

12 Moeller, F. *et al.* (2003). New tools for quantifying and visualizing adoptively transferred cells in recipient mice. *J. Immunol. Methods* **282**, 73-82.

13 Drummond, AE. *et al.* (2002). Expression and Localization of Activin Receptors, Smads, and β glycan to the Postnatal Rat Ovary. *Endocrinology* **143**, 1423-1433.

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 symbol)	Information Note: Additional information about the current topic or procedure.
⚠	Important Note: Information critical to the success of the procedure or use of the product.

Abbreviations

In this Instruction Manual, the following abbreviations are used:

Abbreviation	Meaning
dsDNA	double-stranded DNA
T_m	melting temperature
UNG	Uracil-DNA Glycosylase

6.2 Changes to Previous Version

Update of License Disclaimer

6.3 Ordering Information

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

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

	Product	Pack Size	Cat. No.
Instrument and Accessories	LightCycler® 2.0 Instrument	1 instrument plus accessories	03 531 414 001
	LightCycler® 1.5 Instrument	1 instrument plus accessories	04 484 495 001
	LightCycler® Capillaries (20 µl)	1 pack (5 boxes, each with 96 capillaries and stoppers)	04 929 292 001
	LightCycler® Software 4.1	1 software package	04 898 915 001
	LC Carousel Centrifuge 2.0	1 centrifuge plus rotor and bucket	03 709 507 001 (115 V) 03 709 582 001 (230 V)
	LC Carousel Centrifuge 2.0 Rotor Set	1 rotor + 2 buckets	03 724 697 001
	LC Carousel Centrifuge 2.0 Bucket 2.1	1 bucket	03 724 689 001
	MagNA Pure LC 2.0 Instrument	1 instrument plus accessories	05 197 686 001
	MagNA Pure Compact Instrument	1 instrument plus accessories	03 731 146 001
DNA Isolation Kits	MagNA Pure LC DNA Isolation Kit I	1 kit (192 isolations)	03 003 990 001
	MagNA Pure LC DNA Isolation Kit II (Tissue)	1 kit (192 isolations)	03 186 229 001
	MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi)	1 kit (192 isolations)	03 264 785 001
	MagNA Pure LC DNA Isolation Kit - Large Volume	1 kit (96 - 288 isolations)	03 310 515 001
Total Nucleic Acid Isolation Kits	MagNA Pure LC Total Nucleic Acid Isolation Kit	1 kit (192 isolations)	03 038 505 001
	MagNA Pure LC Total Nucleic Acid Isolation Kit - Large Volume	1 kit (192 isolations)	03 264 793 001

Product	Pack Size	Cat. No.
MagNA Pure LC Total Nucleic Acid Isolation Kit - High Performance	1 kit (96 – 288 isolations)	05 323 738 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	1 kit (32 isolations)	03 730 964 001
MagNA Pure Compact Nucleic Acid Isolation Kit I - Large Volume	1 kit (32 isolations)	03 730 972 001

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

6.4 Disclaimer of License

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

6.5 Trademarks

LIGHTCYCLER, LC, FASTSTART, HYBPROBE, REALTIME READY, MAGNA LYSER, MAGNA PURE and HIGH PURE are trademarks of Roche.

SYBR is a registered trademark of Molecular Probes, Inc.

Exiqon and ProbeLibrary are registered trademarks of Exiqon A/S, Vedbaek, Denmark.

Other brands or product names are trademarks of their respective holders.

6.6 Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

Contact and Support

If you have questions or experience problems with this or any Roche Applied Science (RAS) product, please contact our Technical Support staff. Our scientists commit themselves to providing rapid and effective help.

We also want you to contact us if you have suggestions for enhancing RAS product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to RAS and the world-wide research community.

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

www.roche-applied-science.com/support

To call, write, fax, or email us, visit the Roche Applied Science home page, www.roche-applied-science.com, and select your home country.
Country-specific contact information will be displayed.
On the Roche Applied Science home page select **Printed Materials** to find:

- in-depth Technical Manuals
- Lab FAQS: Protocols and references for life science research
- our quarterly Biochemica Newsletter
- Material Safety Data Sheets
- Pack Inserts and Product Instructions

or to request hard copies of printed materials.

0212.12242770001 ⑱

Roche Diagnostics GmbH
Roche Applied Science
68298 Mannheim
Germany