

For general laboratory use.  
FOR *IN VITRO* USE ONLY.



*Roche Applied Science*

---

# LightCycler<sup>®</sup> FastStart DNA Master<sup>PLUS</sup> SYBR Green I

---

Version February 2005

Easy-to-use Hot Start Reaction Mix for PCR using the LightCycler<sup>®</sup> System

**Cat. No. 03 515 869 001**

Kit for 96 reactions

**Cat. No. 03 515 885 001**

Kit for 480 reactions

**Store the kit at –15 to –25°C**

⚠ Keep the reaction mix (vial 1b,  
green cap) away from light!

# Table of Contents

<b>1.</b>	<b>What this Product Does</b>	<b>3</b>
	Number of Tests	3
	Kit Contents	3
	Storage and Stability	3
	Additional Equipment and Reagents Required	4
	Application	5
	Assay Time	5
<b>2.</b>	<b>How To Use this Product</b>	<b>6</b>
2.1	Before You Begin	6
	Sample Material	6
	Negative Control	6
	Primers	6
	MgCl <sub>2</sub>	6
2.2	Experimental Protocol	7
	LightCycler® 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
<b>3.</b>	<b>Results</b>	<b>12</b>
	Quantification Analysis	12
	Melting Curve Analysis	13
<b>4.</b>	<b>Troubleshooting</b>	<b>14</b>
<b>5.</b>	<b>Additional Information on this Product</b>	<b>17</b>
	How this Product Works	17
	Test Principle	17
	References	18
	Quality Control	18
<b>6.</b>	<b>Supplementary Information</b>	<b>19</b>
6.1	Conventions	19
6.2	Ordering Information	19
6.3	Disclaimer of License	21
6.4	Trademarks	21

# 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  $\mu$ l each.

## Kit Contents

Vial/Cap	Label	Contents/Function
a) Cat. No. 03 515 869 001 (96 reactions) b) Cat. No. 03 515 885 001 (480 reactions)		
1a white cap	Enzyme	a) 1 $\times$ vial 1a, 3 $\times$ vial 1b for 3 $\times$ 128 $\mu$ l Master Mix (5 $\times$ conc)
1b green cap	Reaction Mix	b) 5 $\times$ vial 1a, 15 $\times$ vial 1b for 15 $\times$ 128 $\mu$ l Master Mix (5 $\times$ conc) <ul style="list-style-type: none"><li>• Ready-to-use hot-start PCR reaction mix (after pipetting 14 <math>\mu</math>l from vial 1a into one vial 1b).</li><li>• Contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye, and <math>MgCl_2</math>.</li></ul>
2 colorless cap	H <sub>2</sub> O, PCR- grade	a) 2 vials, 1 ml each b) 7 vials, 1 ml each <ul style="list-style-type: none"><li>• To adjust the final reaction volume</li></ul>

## Storage and Stability

Store the kit at  $-15$  to  $-25^{\circ}\text{C}$  through the expiration date printed on the label.

 Keep the Reaction Mix (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 white cap	Enzyme	<ul style="list-style-type: none"><li>• Store at <math>-15</math> to <math>-25^{\circ}\text{C}</math>.</li><li>• <b>Avoid repeated freezing and thawing!</b></li><li>• Keep vial 1b away from light</li></ul>
1b green cap	Reaction Mix	
1 green cap (after addition of 1a to 1b)	Master Mix	<ul style="list-style-type: none"><li>• Store at <math>-15</math> to <math>-25^{\circ}\text{C}</math> for a maximum of three month.</li><li>• After thawing store at <math>+2</math> to <math>+8^{\circ}\text{C}</math> for a maximum of 1 week.</li><li>• Avoid repeated freezing and thawing!</li><li>• Keep vial 1 away from light!</li></ul>
2 colorless cap	Water, PCR- grade	Store at $-15$ to $-25^{\circ}\text{C}$

## 1. What this Product Does, continued

---

### Additional Equipment and Reagents Required

Refer to the list below for additional reagents and equipment required to perform reactions with the LightCycler<sup>®</sup> FastStart DNA Master<sup>PLUS</sup> SYBR Green I using the LightCycler<sup>®</sup> System:

- LightCycler<sup>®</sup> System (LightCycler<sup>®</sup> 2.0 Instrument\*, LightCycler<sup>®</sup> 1.5 Instrument\*, or an instrument version below)
- LightCycler<sup>®</sup> Capillaries\*
- Standard benchtop microcentrifuge containing a rotor for 2.0 ml reaction tubes

Ⓢ The LightCycler<sup>®</sup> System provides adapters that allow LightCycler<sup>®</sup> Capillaries to be centrifuged in a standard microcentrifuge rotor.

or

- LightCycler<sup>®</sup> Carousel Centrifuge 2.0\* for use with the LightCycler<sup>®</sup> 2.0 Carousel (optional)

⚠ If you use a LightCycler<sup>®</sup> Instrument version below 2.0, you need in addition the LightCycler<sup>®</sup> Carousel Centrifuge 2.0 Bucket 2.1\*. To adapt the LightCycler<sup>®</sup> 2.0 Carousel to the former LightCycler<sup>®</sup> Carousel Centrifuge, you need the LightCycler<sup>®</sup> Carousel Centrifuge 2.0 Rotor Set\*.

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

Ⓢ <sup>†</sup> For prevention of carry-over contamination; see section Related Procedures for details. Use LightCycler<sup>®</sup> Uracil-DNA Glycosylase in combination with LightCycler<sup>®</sup> FastStart Masters only.

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

## 1. What this Product Does, continued

---

### Application

LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I is designed for research studies. When used with the LightCycler® System, this kit is ideally suited for hot-start PCR applications. In combination with the LightCycler® System and suitable PCR primers, this kit allows very sensitive detection and quantification of defined DNA sequences. The kit can also be used to perform two-step RT-PCR. It can be also be used with heat-labile Uracil-DNA Glycosylase to prevent carry-over contamination during PCR.

In principle, the kit can be used for the amplification and detection of any DNA or cDNA target. However, you would need to adapt each amplification protocol to the reaction conditions of the LightCycler® Instrument and design specific PCR primers 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.
- 🕒 LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I offers convenience and ease of use because the addition of MgCl<sub>2</sub> to the reaction mixture is not necessary, thus avoiding time-consuming optimization steps. The new buffer formulation results in increased PCR robustness.
- ⚠ The performance of the kit described in this Instruction Manual is guaranteed only when it is used with the LightCycler® System.

### Assay Time

Procedure	Time
Optional: Dilution of template DNA	5 min
PCR Set-up	15 min
LightCycler® PCR run (incl. Melting Curve)	45 min
<b>Total assay time</b>	<b>65 min</b>

## 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](http://www.roche-applied-science.com).

- Use up to 50 ng complex genomic DNA or  $10^1$  –  $10^{10}$  copies plasmid DNA
- ⚠ Using a too high amount of template DNA might 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  $\mu$ l (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 2, colorless cap).

**Primers** Use PCR primers at a final concentration of 0.3 – 1  $\mu$ M. The recommended starting concentration is 0.5  $\mu$ M each.

**MgCl<sub>2</sub>** All components in the Reaction Mix of the LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I are optimized for almost all primer combinations.

⚠ You do not need to add additional MgCl<sub>2</sub> to the mix to get efficient and specific PCR!

LightCycler® Protocol

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

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

A LightCycler® protocol that uses LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I contains the following programs:

- **Pre-Incubation** activation of FastStart DNA polymerase and denaturation of the DNA
- **Amplification** of the target DNA
- **Melting Curve** for PCR product identification
- **Cooling** the rotor and thermal chamber

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

⚠ <sup>1)</sup> Temperature Transition Rate/Slope is 20°C/sec, except where indicated

⚠ 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® PCR Run with the LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I.

Analysis Mode	Cycles	Segment	Target Temperature <sup>1)</sup>	Hold Time	Acquisition Mode
Pre-Incubation					
None	1		95°C	10 min <sup>4)</sup>	none
Amplification					
Quantification	45	Denaturation	95°C	10 s	none
		Annealing	primer dependent <sup>2)</sup>	0 – 10 s <sup>5)</sup>	none
		Extension	72°C <sup>3)</sup>	= amplicon [bp] / 25 s <sup>6)</sup>	single
Melting Curve					
Melting Curves	1	Denaturation	95°C	0 s	none
		Annealing	65°C	15 s	none
		Melting	95°C slope = 0.1°C/sec <sup>1)</sup>	0 s	continuous
Cooling					
None	1		40°C	30 s	none

<sup>2)</sup> 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:  
 $T_m = 2^{\circ}\text{C} (\text{A}+\text{T}) + 4^{\circ}\text{C} (\text{G}+\text{C})$ .



<sup>3)</sup> If the primer annealing temperature is low (<55°C), reduce the temperature transition rate/slope to 2 – 5°C/s.

<sup>4)</sup> If high polymerase activity is required in early cycles, you can sometimes improve results by extending the pre-incubation to 15 min.

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

<sup>6)</sup> 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	<table><tr><th>Fluorimeter</th><th>Gain Value</th></tr><tr><td>Channel 1 (F1)</td><td>3</td></tr><tr><td>Channel 2 (F2)</td><td>1</td></tr><tr><td>Channel 3 (F3)</td><td>1</td></tr></table>	Fluorimeter	Gain Value	Channel 1 (F1)	3	Channel 2 (F2)	1	Channel 3 (F3)	1
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 4.0									
Default Channel	fluorescence channel 530								
Fluorescence Gains	not required								
“Max. Seek Pos”	Enter the number of sample positions the instrument should look for.								
“Instrument Type”	<ul style="list-style-type: none"><li>• “6 Ch.”: for LightCycler® 2.0 Instrument (selected by default)</li><li>• “3 Ch.”: for</li><li>• LightCycler® 1.5 Instrument and instrument versions below</li></ul>								
“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.
2	Briefly centrifuge one vial “Enzyme” (vial 1a, white cap) and the thawed vial of “Reaction Mix” (from Step 1).
3	Pipet 14 µl from vial 1a (white cap) into vial 1b (green cap). ⌚ Each vial 1a contains enough enzyme 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 labels (vial 1: Master Mix) that are provided with the kit. Place one on the top of the cap and one on the side of the vial. ⚠ Always keep the Master Mix away from light!

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 LightCycler® Centrifuge Bucket.										
2	Prepare a 10× conc. solution of the PCR primers.										
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:										
<table><tr><th>Component</th><th>Volume</th></tr><tr><td>Water, PCR-grade (vial 2, colorless cap)</td><td>9 µl</td></tr><tr><td>PCR Primer 10× conc.</td><td>2 µl</td></tr><tr><td>Master Mix, 5× conc. (vial 1, green cap)</td><td>4 µl</td></tr><tr><td>Total volume</td><td>15 µl</td></tr></table>		Component	Volume	Water, PCR-grade (vial 2, colorless cap)	9 µl	PCR Primer 10× conc.	2 µl	Master Mix, 5× conc. (vial 1, green cap)	4 µl	Total volume	15 µl
Component	Volume										
Water, PCR-grade (vial 2, colorless cap)	9 µl										
PCR Primer 10× conc.	2 µl										
Master Mix, 5× conc. (vial 1, green cap)	4 µl										
Total volume	15 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.										

continued on next page

## 2.2 Experimental Protocol, continued

- 
- ④
    - Mix carefully by pipetting up and down. Do not vortex.
    - Pipet 15  $\mu$ l PCR mix into each precooled LightCycler<sup>®</sup> Capillary.
    - Add 5  $\mu$ l of the DNA template.
    - Seal each capillary with a stopper.
  - ⑤
    - Place the adapters (containing the capillaries) into a standard benchtop microcentrifuge.
    - ⚠ Place the centrifuge adapters in a balanced arrangement within the centrifuge.
    - Centrifuge at  $700 \times g$  for 5 s (3000 rpm in a standard benchtop microcentrifuge).
    - Alternatively, use the LightCycler<sup>®</sup> Carousel Centrifuge for spinning the capillaries.
  - ⑥ Transfer the capillaries into the sample carousel of the LightCycler<sup>®</sup> Instrument.
  - ⑦ Cycle the samples as described above.
-

### 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 reaction mixes of all LightCycler® reagent kits) into amplification products, then pretreating later PCR mixtures with UNG. If a dUTP-containing contaminant is present in the later PCRs, it will be cleaved by a combination of the UNG and the high temperatures of the initial denaturation step; it will not serve as a PCR template.

- ⚠ If you use the LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I, perform prevention of carry-over contamination with LightCycler® Uracil-DNA Glycosylase\*. Proceed as described in the package insert.
- Ⓢ Since your target DNA template contains thymidine rather than uridine, it is not affected by this procedure.
- Ⓢ The use of UNG lowers the melting temperature ( $T_m$ ) in melting curve analysis by approx. 1°C.

**Two-step RT-PCR** LightCycler® FastStart DNA Master<sup>PLUS</sup> 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® System. Subsequent amplification and online monitoring is performed according to the standard LightCycler® 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.

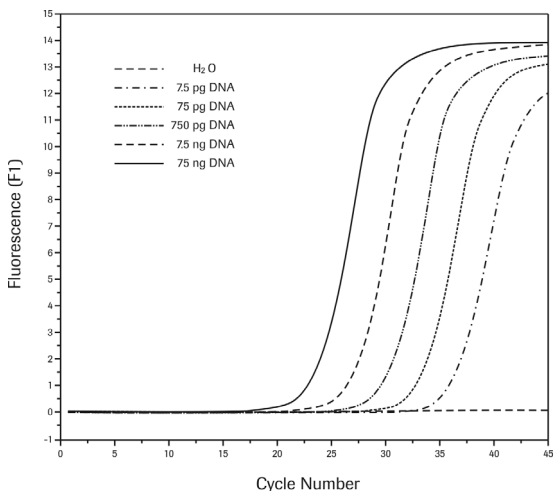
- ⚠ Do not use more than 8 µl of undiluted cDNA template per 20 µl final reaction volume because greater amounts may inhibit PCR. For initial experiments, we recommend running undiluted, 1 : 10 diluted, and 1 : 100 diluted cDNA template in parallel to determine the optimum template amount.

### 3. Results

---

#### Quantification Analysis

The following amplification curves were obtained using the LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I in combination with the LightCycler® Control Kit DNA targeting human  $\beta$ -globin gene. The fluorescence values versus cycle number are displayed.

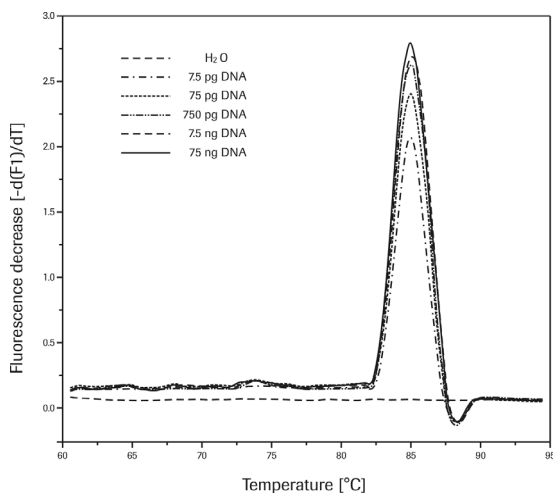


**Fig. 1:** Serially diluted samples containing 75 ng, 7.5 ng, 750 pg, 75 pg, or 7.5 pg human genomic DNA as starting template were amplified using the LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I. As a negative control, template DNA was replaced by PCR-grade water. Arithmetic background subtraction was applied and the fluorescence channel was set to F1/1.

#### Melting Curve Analysis

Specificity of the amplified PCR product was assessed by performing a melting curve analysis. The resulting melting curves allow discrimination between primer-dimers and specific product. The specific  $\beta$ -globin product melts at a higher temperature than the primer-dimers. The melting curves display the specific amplification of the  $\beta$ -globin gene when starting from 75 ng, 7.5 ng, 750 pg, 75 pg, or 7.5 pg human genomic DNA.

⚠ Smaller reaction volumes may result in melting temperature variations.




**Fig. 2:** Melting curve analysis of amplified samples containing 75 ng, 7.5 ng, 750 pg, 75 pg, or 7.5 pg human genomic DNA as starting template. As a negative control, template DNA was replaced PCR-grade water.

## 4. Troubleshooting

**Amplification reaches plateau phase before the program is complete.**

**Log-linear phase of amplification just starts as the amplification program finishes.**


**No amplification occurs.**

Possible cause	Recommendation
Starting amount of nucleic acid is very high.	Stop the program by clicking on the <b>End Program</b> button. The next cycle program will start automatically.
The number of cycles is too high.	Reduce the number of cycles in the cycle program.
Starting amount of nucleic acid is very low.	<ul style="list-style-type: none"> <li>• Improve PCR conditions (e.g., <math>MgCl_2</math> concentration, primer and probe design).</li> <li>• Use more starting material</li> <li>• Repeat the run.</li> </ul>
The number of cycles is too low.	Increase the number of cycles in the cycle program.
Using wrong channel to display amplification onscreen.	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.	<ul style="list-style-type: none"> <li>• Make sure PCR included a pre-incubation step at 95°C for 10 min.</li> <li>• Make sure denaturation time during cycles is 10 s.</li> </ul>
Pipetting errors or omitted reagents.	<ul style="list-style-type: none"> <li>• Check for missing reagents.</li> <li>• Titrate <math>MgCl_2</math> concentration.</li> <li>• Check for missing or defective dye.</li> </ul>
Chosen gain settings are too low.	Optimize gain setting using the <b>Real Time Fluorimeter</b> function. Then repeat the run, using the optimal gain settings in the cycle programs.  LightCycler® software versions 3.5 and higher do not require a gain setting.
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 changing to suitable values.
Measurements do not occur.	Check the cycle programs. 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 reaction.	<ul style="list-style-type: none"> <li>• Do not use more than 8 – 10 ml of DNA per 20 ml PCR reaction mixture.</li> <li>• Repurify the nucleic acids to ensure removal of inhibitory agents</li> </ul>

*continued on next page*

#### 4. Troubleshooting, continued

**Fluorescence intensity is too high and reaches overflow.**

Possible cause	Recommendation
Gain settings are too high.	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. 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.**

Deterioration of dye in reaction mixtures; dyes not stored properly	<ul style="list-style-type: none"> <li>• Store the dye containing reagents at <math>-15</math> to <math>-25^{\circ}\text{C}</math>, and keep them away from light.</li> <li>• Avoid repeated freezing and thawing.</li> <li>• After thawing, store the LightCycler® DNA Master SYBR Green I at <math>+2</math> to <math>+8^{\circ}\text{C}</math> for a maximum of 1 weeks and keep it away from light.</li> </ul>
Reaction conditions are not optimized, leading to poor PCR efficiency	<ul style="list-style-type: none"> <li>• Titrate <math>\text{MgCl}_2</math> concentration.</li> <li>• Primer concentration should be between 0.3 and 1.0 mM</li> <li>• Check annealing temperature of primers.</li> <li>• Check experimental protocol.</li> <li>• Always run a positive control along with your samples.</li> </ul>

**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 dye, producing a high background signal. There is not enough dye left to monitor the increase of fluorescence signal during amplification.	<ul style="list-style-type: none"> <li>• Do not use more than 50 ng of complex genomic DNA in a 20 ml reaction.</li> <li>• Use the HybProbe format (which allows analysis of up to 500 ng DNA) instead of SYBR Green I.</li> </ul>
Dye bleached.	Make sure the reagents containing the dye are stored away from light. Avoid repeated freezing and thawing.

*continued on next page*

#### 4. Troubleshooting, continued

**Negative control samples give a positive signal.**

Possible cause

Contamination, or presence of primer-dimers.

Recommendation

- Remake all critical solutions.
- Pipet 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 cannot be differentiated.**

°C to Average setting is too high.

Reduce the value of **°C to Average** (only applicable for LightCycler® software version 3.5).

**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 HybProbes 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<sub>2</sub>, heat-labile UNG, Hot Start antibody, and program settings.

**Only a primer-dimer peak appears, with no specific PCR product peak seen; or very high primer-dimer peaks.**

Primer-dimers have out-competed specific PCR product for available primers.

- 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<sub>2</sub>.
- Increase annealing temperature in order to enhance stringency.

Quality of primer is poor.

- Purify primer more thoroughly.
- Use a Hot Start method.

Sequence of primer is inappropriate.

Redesign primer.

**Primer-dimer and product peaks are very close together.**

Unusually high GC-content of the primers.

- Redesign primers.
- Run melting curve at lowest ramping 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 version 3.5).

**Very broad primer-dimer peak with multiple peaks.**

Heterogeneous primers with primer-dimer variations (e.g., concatemers, loops)

- Redesign primers.
- Use hot-start method.

**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

---

### How this Product Works

LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I is a ready-to-use reaction mix designed specifically for the SYBR Green I detection format in LightCycler® Instruments. 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 modified form of thermostable recombinant Taq DNA polymerase that is inactive at room temperature and below. 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.

The LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I provides convenience, excellent performance and reproducibility, as well as minimizing contamination risk. All you have to supply is template DNA and primers.

⚠ The reaction mix in this kit is optimized for a single MgCl<sub>2</sub> concentration, which works with nearly all primer combinations. You do not need to adjust the MgCl<sub>2</sub> concentration to amplify different sequences.

### Test Principle

Generation of PCR products can be detected by measurement of the SYBR Green I fluorescence signal. SYBR Green I binds to the minor groove of the DNA double helix. In solution, the unbound 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. Since 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® System are:

- ① At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis.
- ② After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation.

- ③ During elongation, more and more 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 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.

## References

- 1 Chou, Q. *et al* (1992) Prevention of pre-PCR mis-priming and primer dimerization improves low- copy-number amplifications. *Nucleic Acid Res.* **20**, 71717 - 1723.
- 2 Kellogg, D.E. *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, D.E. *et al* (1996). Simplified hot start PCR. *Nature* **381**, 445-446.
- 4 PCR Manual, (1999) Roche Diagnostics, 2nd edition **2**, 52 - 58.
- 5 Wikman, H. *et al* (2005). Caveolins as tumour markers in lung cancer detected by combined use of cDNA and tissue microarrays. *The Journal of Pathology* **203**, 584 - 593.
- 6 Ellis, R.C. *et al* (2004). Cathepsin B mRNA and protein expression following contusion spinal cord injury in rats. *Journal of Neurochemistry* **88**, 689.
- 7 Klein, J.D. *et al* (2004). Upregulation of Urea Transporter UT-A2 and Water Channels AQP2 and AQP3 in Mice Lacking Urea Transporter UT-B. *J Am Soc Nephrol* **15**, 1161 - 7.
- 8 Paux, E. *et al* (2004). Identification of genes preferentially expressed during wood formation in *Eucalyptus*. *Plant Molecular Biology* **55**, 263-80.
- 9 Da Silva, Patr. S. *et al* (2004). Cloning and gene silencing of LAT2, the L-3,4-dihydroxyphenylalanine (L-DOPA) transporter, in pig renal LLC-PK1 epithelial cells. *FASEB J.* **18**, 1489 - 98.

## Quality Control

The LightCycler® FastStart DNA Master<sup>PLUS</sup> SYBR Green I is function tested using the LightCycler® 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 ①, ②, 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 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](http://www.roche-applied-science.com), and our Special Interest Sites including:

- The LightCycler® 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 ml)	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
LightCycler® Carousel Centrifuge 2.0	1 centrifuge plus rotor and bucket	03 709 507 001 (115 V) 03 709 582 001 (230 V)

## 6. Supplementary Information, continued

	Product	Pack Size	Cat. No.
<b>LightCycler® Kits for PCR</b>	LightCycler® DNA Master HybProbe	1 kit (96 reactions) 1 kit (480 reactions)	12 015 102 001 12 158 825 001
	LightCycler® FastStart DNA Master HybProbe	1 kit (96 reactions) 1 kit (480 reactions)	03 003 248 001 12 239 272 001
	LightCycler® FastStart DNA MasterPLUS HybProbe	1 kit (96 reactions) 1 kit (480 reactions)	03 515 575 001 03 515 567 001
	LightCycler® FastStart DNA MasterPLUS HybProbe, 100 ml 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 MasterPLUS SYBR Green I, 100 ml Reactions	1 kit (384 reactions)	03 752 186 001
<b>cDNA Synthesis Reagents</b>	Transcriptor Reverse Transcriptase	250 U 500 U 2000 U	03 531 317 001 03 531 295 001 03 531 287 001
	Transcriptor First Strand cDNA Synthesis Kit	1 kit	04 379 012 001
	First Strand cDNA Synthesis Kit for RT-PCR (AMV)	1 kit (30 reactions)	11 483 188 001
	LightCycler® Uracil-DNA Glycosylase	100 U (50 ml)	03 539 806 001

## 6.3 Disclaimer of License

---

LIMITED LICENSE: A license under U.S. Patents 4,683,202, 4,683,195, and 4,965,188 or their foreign counterparts, owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd ("Roche"), has an up-front fee component and a running-royalty component. The purchase price of this product includes limited, nontransferable rights under the running-royalty component to use only this amount of the product to practice the Polymerase Chain Reaction ("PCR") and related processes described in said patents, including homogeneous PCR methods described in U.S. Patent Nos. 5,994,056 and 6,171,785 and their foreign counterparts, solely for the research and development activities of the purchaser when this product is used in conjunction with a thermal cycler whose use is covered by the up-front fee component. Rights to the up-front fee component must be obtained by the end user in order to have a complete license to use this product in the PCR process. These rights under the up-front fee component may be purchased from Applied Biosystems or obtained by purchasing an Authorized Thermal Cycler. No right to perform or offer commercial services of any kind using PCR, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted by implication or estoppel. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404 or the Licensing Department, Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

DISCLAIMER OF LICENSE: No rights for any other application, including any in vitro diagnostic application, are conveyed expressly, by implication or by estoppel under patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd covering the 5' nuclease detection technology.

The technology used for the LightCycler® System is licensed from Idaho Technology Inc., Salt Lake City, UT, USA.

Purchase of this product includes a limited non-transferable end-user license to the purchaser under the SYBR Green I Technology owned by Idaho Technology under U.S. Patents 6,569,627 and foreign counterparts to use this product for any purpose.

## 6.4 Trademarks

LightCycler, LC, FastStart, HybProbe, MagNA Pure, and High Pure are Trademarks of a Member of the Roche Group.

SYBR is a trademark of Molecular Probes, Inc., Eugene, OR, USA. SYBR Green I is manufactured by Molecular Probes, Inc., and is provided under license from Molecular Probes, Inc., for direct research use for PCR, where the dye is present during the PCR.





---

## 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](http://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](http://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.

---

## Inspiring Discovery



## Diagnostics

Roche Diagnostics GmbH  
Roche Applied Science  
68298 Mannheim  
Germany