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

LightCycler® FastStart DNA Master^{PLUS} SYBR Green I

Ready-to-use “Hot Start” reaction mix for PCR using the LightCycler System

Cat. No. 03 515 869 001
Kit for 96 reactions

Cat. No. 03 515 885 001
Kit for 480 reactions

Store at –15 to –25°C

Note: Protect the LightCycler Fast-Start Reaction Mix SYBR Green I (vial 1b, green cap) from light!

Instruction Manual

Version July 2004

Roche

1. Preface

1.1 Table of contents

1.2 Kit contents

Vial	Label	Function	Content	
			Cat. No. 03 515 869 001 (96 reactions)	**Cat. No. 03 515 885 001** (480 reactions)
1a white cap	Enzyme	• Ready-to-use hot start PCR reaction mix (after pipetting 14 µl from vial 1a into one vial 1b). • Contains FastStart Taq DNA Polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye, and dNTP mix (with dUTP instead of dTTP)	1 × vial 1a 3 × vial 1b for 3 × 128 µl Master Mix (5× conc)	5 × vial 1a 15 × vial1b for 15 × 128 µl Master Mix (5× conc)
1b green cap	Reaction Mix			
2 colorless cap	Water, PCR Grade	To adjust the final reaction volume	2 × 1 ml	7 × 1 ml

1.3 Kit storage and stability

The complete kit is stable through the expiration date printed on the label (12 months from date of manufacture) if stored properly at −15 to −25°C.

The kit is shipped on dry ice.

Store kit components as follows:

Vial	Label	Storage
1a white cap	Enzyme	• Store at −15 to −25°C. • Avoid repeated freezing and thawing! • **Keep vial 1b away from light!**
1b green cap	Reaction Mix	
1 green cap (after addition of 1a to 1b, see section 3.2)	Master Mix	• Store at −15 to −25°C for a maximum of three months. • After thawing, store at 2–8°C for a maximum of 1 week. • **Avoid repeated freezing and thawing!** • **Keep vial 1 away from light!**
2 colorless cap	Water, PCR grade	Store at −15 to −25°C.

2. Product overview

Product description

LightCycler FastStart DNA Master^{PLUS} 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^{PLUS} 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.

Notes:

- The reaction mix in this kit is optimized for a single $MgCl_2$ concentration, which works with nearly all primer combinations. You do not need to adjust the $MgCl_2$ concentration to amplify different sequences.
- The performance of the kit described in this Instruction Manual is guaranteed only when it is used with the LightCycler System.

Use of SYBR Green I for product detection and identification

Generation of PCR products can be detected by measurement of SYBR Green I fluorescence. SYBR Green I dye will emit a fluorescence signal (wavelength, 530 nm) only when bound to double-stranded DNA. Therefore, during PCR the increase in SYBR Green I fluorescence is directly proportional to the amount of double-stranded DNA generated.

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 of a particular DNA product (where the DNA is 50% double-stranded and 50% single-stranded). 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.

Application

The LightCycler FastStart DNA Master^{PLUS} SYBR Green I is designed for use in 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.

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

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.

Quality control

The LightCycler FastStart DNA Master^{PLUS} SYBR Green I is function tested using the LightCycler System.

3. Procedures and required material

3.1 Before you begin

Additional reagents and equipment required

Refer to the list below for additional reagents and equipment required for PCR with the LightCycler System.

- LightCycler System* (incl. LightCycler Instrument or LightCycler 2.0 Instrument)
- LightCycler Capillaries*
- Standard benchtop microcentrifuge containing a rotor for 2.0 ml reaction tubes
  Note: The LightCycler System provides adapters that allow LightCycler Capillaries to be centrifuged in a standard microcentifuge rotor.

or

- LightCycler Carousel Centrifuge* (optional; if you use the LightCycler 2.0 instrument you will need in addition LC Carousel Centrifuge 2.0 Rotor Set* and LC Carousel Centrifuge 2.0 Bucket*)

or

- LightCycler Carousel Centrifuge 2.0* (optional)
- PCR template DNA
- PCR primers

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

Assay time / hands on time

Procedure	Time
Optional: dilution of template DNA	5 min
PCR Set-up	15 min
LightCycler PCR run (incl. Melting Curve)	45 min
Total assay time	**65 min**

Sample material	Use any template DNA (*e.g.,* genomic or plasmid DNA, cDNA) that is 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 (Cat. No. 12 236 931 001) and a dedicated MagNA Pure LC reagent 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 the website: www.roche-applied-science.com. ***Note***: 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 0.2–1 µM. The recommended starting concentration is 0.5 µM each. ***Note***: The amplicon size should not exceed 1 kb in length. For optimal results, select a product length of 700 bp or less.
$MgCl_2$	All components in the Reaction Mix of the LightCycler FastStart DNA Master^{PLUS} SYBR Green I are optimized for almost all primer combinations. You do not need to add additional $MgCl_2$ to the mix to get efficient and specific PCR!
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).

3.2 LightCycler Protocol

Experimental Protocol

Program the LightCycler Experimental Protocol before preparing the reaction mixes.

Normally, a LightCycler protocol that uses the LightCycler FastStart DNA Master^{PLUS} SYBR Green I contains the following parts:

- **Pre-Incubation (activation of FastStart DNA polymerase and denaturation of the DNA)**
- **Amplification of the target DNA**
- **Melting curve analysis for product identification**
- **Cooling the rotor and thermal chamber**

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

The following table shows the PCR parameters that must be programmed for a normal LightCycler PCR Run with LightCycler FastStart DNA Master^{PLUS} SYBR Green I:

Program	Analysis Mode	Cycles	Segment	Target Temperature [1)]	Time	Fluorescence Acquisition Mode
Pre-Incubation	None	1	1	95°C	10 min [4)]	None
Amplification	Quantification	45	Denaturation	95°C	10 s	–
			Annealing	primer dependent [2)]	5-20 s [5)]	–
			Extension	72°C [3)]	Product [bp] /25 s [5)]	single
Melting Curve Analysis	Melting Curves	1	Denaturation	95°C	0 s	–
			Annealing	65°C	15 s	–
			Melting	95°C slope = 0.1°C/sec	0 s	cont
Cooling	None	1	1	40°C	30 s	–

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

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

[3)] For initial experiments, set the target temperature (*i.e.*, the primer annealing temperature) 5°C below the calculated primer T_m.

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

[5)] For greater precision in target quantification experiments, it can be advantageous (in some cases) to choose longer annealing and extension times for the amplification cycles.

Fluorescence parameters and setup parameters

Set the fluorescence parameters and run setup parameters as follows:

All LightCycler Software Versions
- Seek Temperature: 30°C

LightCycler Software Version 3.5 or earlier
- Display Mode: fluorescence channel F1

LightCycler Software Version 4.0
- Default Channel: Channel 530
- "Max. Seek Pos" field: Enter the number of sample positions the instrument shouldlook for.
- "Instrument Type" field: Choose the "6 Ch." Instrument type for a LightCycler 2.0 Instrument (selected by default). For LightCycler 1.1 and 1.2 Instruments choose the "3 Ch." Instrument type.
- For the "6 Ch." type a "Capillary Size" field will be displayed. Select "20 µl" as the capillary size for the experiment.

Preparation of the Master Mix

Prepare the 5× Master Mix as described below.

Step	Action
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 a total volume of 14 µl from vial 1a ("Enzyme", white cap) into vial 1b ("Reaction Mix", green cap). ***Note:*** Each vial 1a contains enough enzyme for three vials of Reaction Mix (vial 1b).
4	Mix thoroughly 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. ***Note***: Always keep the Master Mix away from light!

Preparation of the PCR master mix and starting the run

Prepare each 20 µl standard reaction as described below.

Notes:

- For reaction volumes < 20 µl, the reaction and cycle conditions must be optimized.
- Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries..

Step	Action
1	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 10x conc. solution of the PCR primers.
3	In a 1.5 ml reaction tube on ice, prepare one reaction by adding the following components in the order listed below, then mixing gently: **Component** / **Volume** Water, PCR grade (vial 2, colorless cap) — 9 µl PCR Primers, 10× conc. — 2 µl Master Mix, 5× conc. (vial 1) — 4 µl **Final volume** — **15 µl** ***Note:*** To prepare the Master 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 plus one additional reaction.
4	• Pipet 15 µl Master Mix into the precooled LightCycler Capillary. • Add 5 µl of the DNA template. ***Note***: Use up to 50 ng complex genomic DNA or 10^1 – 10^{10} copies plasmid DNA.
5	• Seal each capillary with a stopper and place the adapters, containing the capillaries, into a standard benchtop microcentrifuge. • Centrifuge at 700× g for 5 s (3000 rpm in a standard benchtop microcentrifuge)) or in the LC Carousel Centrifuge. ***Note***: Place the centrifuge adapters in a balanced arrangement within the centrifuge.
6	Place the LightCycler Carousel with capillaries into the LightCycler Instrument, then start your protocol.

3.3 Related procedures

Two-step RT-PCR

The LightCycler FastStart DNA Master^{PLUS} SYBR Green I can also be used to perform a two step RT-PCR protocol.

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 recommended for reverse transcription of RNA into cDNA (see Ordering Information for details):

- Transcriptor Reverse Transcriptase
- Transcriptor First Strand cDNA Synthesis Kit
- First Strand cDNA Synthesis Kit for RT-PCR (AMV)

Synthesis of cDNA is performed according to the detailed instructions provided with the cDNA synthesis reagent.

Note: Do not use more than 8 µl of undiluted cDNA template per 20 µl final reaction volume because greater amounts may inhibit the 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.

Prevention of carry-over contamination

Uracil DNA N-Glycosylase (UNG) can prevent carry-over contamination in PCR. This carry-over prevention technique involves incorporating deoxyuridine triphosphate (dUTP) into the amplification products, then pretreating all subsequently prepared PCR mixtures with UNG. If there are carry-over amplicons in the PCR mixture, UNG cleaves the DNA at any site where a deoxyuridylate residue has been incorporated. Subsequently, the resulting abasic sites are hydrolyzed by the high temperatures of the initial denaturation step and cannot serve as PCR templates.

Since normal DNA template contains thymidine, not uridine, it is not affected by this procedure.

Note: Such decontamination can be achieved with the LightCycler reagents in this kit, since they contain dUTP instead of dTTP.

4. Results

4.1 Typical results

The following amplification and melting curves were obtained in combination with the LightCycler Control Kit DNA using ten-fold dilution of the contained human genomic DNA.

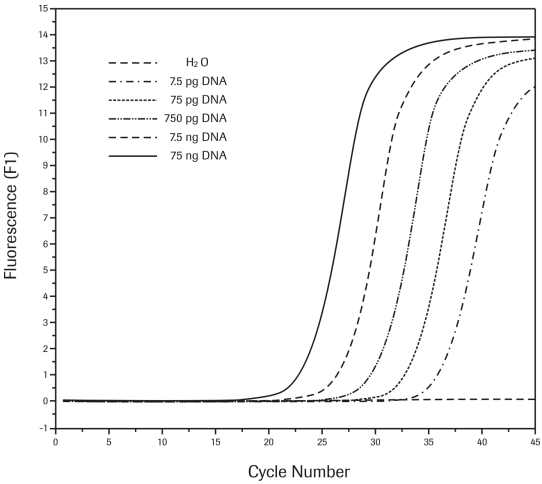

Fig. 1: Amplification curves in the quantification module of the LightCycler Software 3.5 (channel F1, arithmetic baseline adjustment).

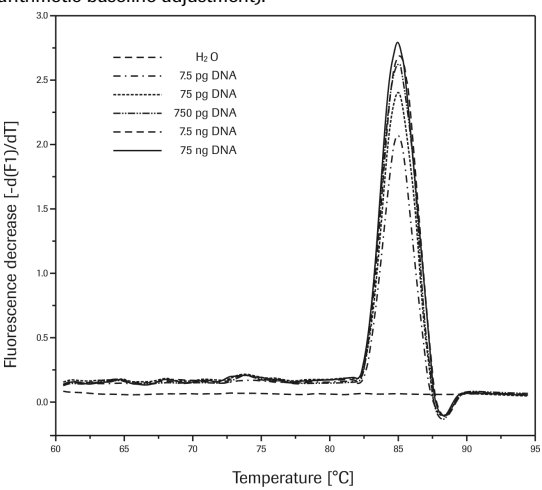

Fig. 2: Melting curve analysis in the melting curve module of the LightCycler Software 3.5.

5. Appendix

5.1 Troubleshooting

Problem	Cause	Recommendation
Fluorescence curves reach maximum long before cycling is complete.	Starting amount of nucleic acid is very high.	Stop the program by clicking the **End Program** button. The next cycle program will start automatically.
	Number of cycles is too high.	Reduce the number of cycles in the protocol.
Log-linear phase of amplification just starts as the amplification program finishes.	Number of cycles is too low.	• While the cycling program is in progress, use the **Add 10 Cycles** button to increase the number of cycles. • Increase the number of cycles in the protocol. • Use more starting material. • Optimize the PCR conditions (*e.g.*, primer/probe design, protocol).
No amplification occurs.	Using wrong channel to display amplification on screen.	Change the channel setting to channel 1 on the programming screen. (Data from all channels is always saved by the instrument.)
	Measurements do not occur at the right time.	For SYBR Green I detection, choose “single” as the acquisition mode at the end of the extension phase.
	Impure sample material inhibits the reaction.	• Dilute your sample 1:10 and repeat the analysis. • Purify the nucleic acids from your sample material to ensure removal of inhibitory agents.
	FastStart DNA Polymerase is not fully activated.	• Make sure PCR includes a 10 min pre-incubation step at 95°C. • Make sure the denaturation time during the cycles is about 10 s.
	Pipetting errors or omitted reagents.	Replace missing or defective reagents.
	Difficult, *e.g.* GC-rich, template.	• Repeat PCR under same conditions, but add increasing amounts of DMSO (up to 10% of the final volume). • If performance is still not satisfactoy, optimize the annealing temperature while titrating the DMSO concentration.
	Amplicon length is >1 kb.	Do not use amplicons >1 kb. Optimal results are obtained with amplicons of 700 bp or less.

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Problem	Cause	Recommendation
Fluorescence intensity is very low.	Low concentration or deterioration of dye in the reaction mixtures; dyes were not stored properly.	• Keep the dye-containing reagents away from light. • Store the reagents at −15 to −25°C, and avoid repeated freezing and thawing. • After thawing, store the Master Mix at 2–8°C for a maximum of one week, while keeping it away from light.
	Reaction conditions are not optimized, leading to poor PCR efficiency.	Use the reagent concentrations and the LightCycler protocol described in section 3 of this manual.
	For LightCycler Software version 3.3 only: Gain settings are too low.	Use the **Real Time Fluorimeter** to find suitable gain settings. Change gain settings in the protocol, then repeat the run. ***Note***: For LightCycler Software version 3.5 or above, no gain setting is required.
Fluorescence intensity is too high and reaches overflow (only applies to LightCycler Software version 3.3).	Gain settings are too high.	Gain settings cannot be corrected during or after a run, so you must repeat the run. Before repeating the run, use the **Real Time Fluorimeter** to find suitable gain settings. ***Note***: For LightCycler Software version 3.5 or above, no gain setting is required.
Fluorescence intensity varies.	Some of the reagent is still in the upper part of the capillary, or an air bubble is trapped in the capillary tip.	Repeat capillary centrifugation step.
	Skin oils or dirt are on the surface of the capillary tip.	Always wear gloves when handling the capillaries.
Negative control gives a positive signal.	Contamination.	• Remake all critical solutions. • Pipet reagents on a clean bench. • Close the lid of the negative control reaction immediately after pipetting it. • Use UNG to eliminate carry-over contamination.
High fluorescence background.	Starting amount of DNA is too high; DNA captures dye, producing a high background signal. There is not enough dye left to monitor increase of signal during amplification.	• Do not use more than 50 ng of double-stranded DNA in a 20 µl reaction. • Use HybProbe Probes (which allow analysis of up to 500 ng DNA) instead of SYBR Green I.
Melting peak is very broad and peaks cannot be differentiated.	**°C to Average** setting is too high.	Decrease value of **°C to Average**.
Melting temperature of a product varies from experiment to experiment.	Variations in reaction mixture.	Check the purity of reagents.

continued on next page

5.1 Troubleshooting, continued

Problem	Cause	Recommendation
More than one melting peak appears for one product.	Products of different length or GC content have been amplified (*e.g.*, due to pseudogenes or mispriming).	• Check products on an agarose gel. • Raise the reaction stringency by: – redesigning primers – using a higher annealing temperature – performing a "touch-down PCR" – reducing enzyme activity by shortening pre-incubation to 5 min. • Use HybProbe Probes instead of SYBR Green I for more specific detection.
PCR results in primer-dimers, but no specific product.	Quality of primers is poor.	Purify primers more thoroughly.
	Suboptimal conditions for PCR.	• Use the conditions recommended in chapter 3 of this manual. • Raise stringency by: – redesigning primers – using higher annealing temperatures – performing a "touch-down PCR."

5.2 References

1 Chou, Q *et al* (1992) *Nucleic Acid Res.* **20**,1717-1723.
2 Kellogg, D.E. *et al* (1994) *BioTechniques* **16**,1134-1137.
3 Birch, D.E. *et al* (1996) *Nature* **381**,445-446.
4 PCR Manual, Roche Diagnostics (1999) 2nd edition (1999) 2, 52-58.

5.3 Ordering information

For a complete overview of related products, please visit and bookmark our Special Interest Sites for:

- LightCycler Reagents:
  http://www.roche-applied-science.com/lightcycler-online/lc_sys/reagent_ind.htm
- the MagNA Pure Family for automated nucleic acid isolation:
  http://www.roche-applied-science.com/sis/magnapure/
- manual Nucleic Acid Isolation and Purification:
  http://www.roche-applied-science.com/napure

Product	Pack size	Cat. No.
Instruments		
LightCycler 2.0 Instrument	1 instrument plus accessories	03 531 414 201
LightCycler Capillaries (20 µl)	1 pack (8× 96 capillaries)	11 909 339 001
LightCycler Carousel Centrifuge	1 centrifuge plus rotor and bucket	03 030 512 001 (115 V) 12 189 682 001 (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
LightCycler Carousel Centrifuge 2.0	1 centrifuge plus rotor and bucket	03 709 507 001 (115 V)/ 03 709 582 001 (230 V)
Software		
LightCycler Probe Design Software 2.0	1 package	04 342 054 001
cDNA Synthesis Reagents		
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	11 483 188 001
Associated Reagents and Kits		
LightCycler DNA Master SYBR Green I	1 kit (96 reactions) 1 kit (480 reactions)	12 015 099 001 12 158 817 001
LightCycler FastStart DNA Master SYBR Green I	1 kit (96 reactions) 1 kit (480 reactions)	03 003 230 001 12 239 264 001
LightCycler FastStart DNA Master^{PLUS} SYBR Green I, 100 µl Reactions	1 kit (384 reactions)	03 752 168 001
LightCycler Control Kit DNA	1 kit (50 reactions)	12 158 833 001
LightCycler Uracil-DNA Glycosylase	100 U (50 µl)	03 539 806 001

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