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Roche Applied Science

LightCycler® 480 Probes Master

Version October 2005

Ready-to-use hot start reaction mix for PCR with the LightCycler® 480 System

Cat. No. 04 707 494 001 Kit for 500 reactions

Store the kit at −15 to −25°C

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PROTOCOL

1. What this Product Does

Number of Tests

The kit is designed for 500 reactions with a final reaction volume of 20 µl each.

Kit Contents

Vial/Cap	Label	Contents/Function
1 red cap	LightCycler® 480 Probes Master; 2× conc.	• 5 vials, 1 ml each • 2× conc., ready-to-use hot-start PCR mix • contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), and 6.4 mM $MgCl_2$
2 colorless cap	LightCycler® 480 Probes Master; H_2O, PCR-grade	• 5 vials, 1 ml each • H_2O, PCR-grade to adjust the final reaction volume

Storage and Stability

Store the kit at −15 to −25°C through the expiration date printed on the label.

- The kit is shipped on dry ice.
- Once the kit is opened, store the kit components as described in the following table:

Vial	Label	Storage
1	LightCycler® 480 Probes Master; 2× conc.	• Store at −15 to −25°C. • **Avoid repeated freezing and thawing!**
2	LightCycler® 480 Probes Master; H_2O, PCR-grade	• Store at −15 to −25°C

Additional Equipment and Reagents Required

Additional reagents and equipment required to perform PCR with the LightCycler® 480 Probes Master include:

- LightCycler® 480 Instrument, 384-well*
- LightCycler® 480 Multiwell Plate 384* with LightCycler® 480 Sealing Foil*
- Standard swing-bucket centrifuge containing a rotor for multiwell plates with suitable adaptors.
- LightCycler® Uracil-DNA Glycosylase* (optional ‡)
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile 1.5 ml reaction tubes for preparing master mixes and dilutions

‡ for prevention of carry-over contamination; see Related Procedures section for details.

** available from Roche Applied Science*

Application

LightCycler® 480 Probes Master is designed for research studies on the LightCycler® 480 System. The LightCycler® 480 Probes Master is a ready-to-use hot-start reaction mix designed specifically for detecting DNA targets with hydrolysis probes during LightCycler® 480 System PCR. However, it may be used in other types of PCR on the LightCycler® 480 System. For best results, use this master mix with LightCycler® 480 Multiwell Plates.

The kit can also help prevent carryover contamination during PCR (when used with LightCycler® Uracil-DNA Glycosylase) or to perform the second step of a two-step RT-PCR.

Other assay formats may also be adapted to real-time PCR on the LightCycler® 480 Instrument. For example, probe formats that may be adapted to the LightCycler® 480 Instrument in addition to hydrolysis probes include FRET hybridization probes, Molecular Beacons and Scorpions. However, any fluorescent dyes used in a LightCycler® 480 analysis must be compatible with the optical unit of the LightCycler® 480 Instrument.

In principle, the LightCycler® 480 PCR Master Probes can be used to amplify and detect any DNA or cDNA target. However, you would need to adapt your detection protocol to the reaction conditions of the LightCycler® 480 Instrument, and design specific PCR primers and probes for each target. See the LightCycler® 480 Operator's Manual for general recommendations.

⚠ The amplicon should not be longer than 1,000 bp. For optimal results, select an amplicon of 500 bp or less.

⚠ The performance of the kit as described in this Instruction Manual is warranted only when it is used with the LightCycler® 480 System.

Assay Time

Variable, depending on the number of cycles and the annealing time.

For example, if the cycling program specifies 40 cycles and an annealing time of 20 seconds, a LightCycler® 480 PCR run will last about 44 minutes including 5 minutes preincubation time.

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, as long as it is sufficiently pure, concentrated and free of PCR inhibitors.

⚠ For reproducible isolation of nucleic acids use either:

- the MagNA Pure LC Instrument* or the MagNA Pure Compact Instrument* and a dedicated MagNA Pure nucleic acid isolation kit (for automated isolation), or
- a HIGH PURE nucleic acid isolation kit* (for manual isolation).

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

- Use up to 500 ng complex genomic DNA or 10^1 – 10^{10} copies plasmid DNA.

🔍 If you are using an unpurified cDNA product from a reverse transcription reaction, especially if it contains high background concentrations of RNA and oligonucleotides, you can improve your results by using 2 µl (or less) of that sample in the reaction.

Negative Control

Always run a negative control with the samples. To prepare a negative control:

- replace the template DNA with PCR-grade water (vial 2). (This will reveal, whether a contamination proplem exists.)
- (in a 2-step RT-PCR setup) omit addition of reverse transcriptase to the cDNA synthesis reaction (this will indicate whether DNA in RNA samples causes false-positive results).

Primers

Suitable concentrations of PCR primers range from 0.3 to 1 µM (final concentration in reaction). The recommended starting concentration is 0.5 µM each.

⚠ Optimize the primer concentration first, then determine the probe optimization using the optimized primer concentrations.

🔍 The optimal primer concentration is the lowest concentration that results in the lowest CP and an adequate fluorescence for a given target concentration.

Probes

Suitable concentrations of hydrolysis probes range from 0.05 to 0.2 µM (final concentration in reaction).

🔍 The optimal probe concentration is the lowest concentration that results in the lowest CP and an adequate fluorescence for a given target concentration.

⚠ For a digestible hybridization complex to form correctly, the hydrolysis probe must anneal to the target before primer extension. The T_m of the probe should be only slightly higher than the T_m of the PCR primer, so the hybridization complex is stable. Furthermore, the probe sequence must account for mismatches in the DNA template, since these will also affect the annealing temperature.

$MgCl_2$

The reaction mix in this kit already contains an optimal concentration of $MgCl_2$, which works with nearly all primer combinations. You do not need to adjust the $MgCl_2$ concentration to amplify different sequences.

LightCycler® 480 Instrument Protocol

The following procedure is optimized for use with the LightCycler® 480 System.

⚠ Program the LightCycler® 480 Instrument before preparing the reaction mixes.

A LightCycler® 480 Instument protocol that uses LightCycler® 480 Probes Master should contain the following programs:

- **Pre-Incubation** for activation of FastStart Taq DNA Polymerase and denaturation of the DNA
- **Amplification** of the target DNA
- **Cooling** of the thermal block

For details on how to program the experimental protocol, see the LightCycler® 480 Operator´s Manual.

The following table shows the PCR parameters that must be programmed for a LightCycler® 480 System PCR Run with the LightCycler® 480 PCR Probes Master and hydrolysis probes.

Setup				
Detection Format		**Block Type**		**Reaction Volume**
Mono Color hydrolysis probes or Multi Color hydrolysis probes		384		3 – 20 µl
Programs				
Program Name		**Cycles**		**Analysis Mode**
Pre-Incubation		1		None
Amplification		45 [1]		Quantification
Cooling		1		None
Temperature Targets				
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
Pre-Incubation				
95	None	00:05:00 [3]	4.8	–
Amplification				
95	None	00:00:10	4.8	–
primer dependent [2]	None	00:00:15 – 00:00:30 [4]	2.5	–
72	Single	00:00:01	4.8	–
Cooling				
40	None	00:00:10	2	–

[1] 45 cycles are suitable for most assays. If the assay is optimized and has steep amplification curves and early crossing points (even when target concentrations are low), 40 cycles should be sufficient. Reducing the number of cycles will reduce the time required for the assay!

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

[3] If high polymerase activity is required in early cycles, you can sometimes improve results by extending the pre-incubation to 10 min.

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

Preparation of the PCR Mix

Thaw the LightCycler® 480 Probes Master (2× conc.) (vial 1) and water (vial 2). Follow the procedure below to prepare one 20 µl standard reaction.

⚠ Do not touch the surface of the the LightCycler® 480 Multiwell Plate when handling it.

❶
- Thaw the solutions and, to ensure recovery of all the contents, briefly spin vials in a microcentrifuge before opening.
- Mix carefully by pipetting up and down and store on ice.

❷ Prepare a 10× conc. solution that contains PCR primers and hydrolysis probe.

❸ In a 1.5 ml reaction tube on ice, prepare the PCR mix for one 20 µl reaction by adding the following components in the order listed below:

Component	Volume
Water, PCR-grade (vial 2)	3 µl
Primer-probe mix [1], 10× conc.	2 µl
LightCycler® 480 Probes Master, 2× conc. (vial 1)	10 µl
Total volume	**15 µl**

[1] Due to possible primer/primer interactions that occur during storage it may be necessary to preheat the PCR primer-probe mix for 1 min at 95°C before starting the reaction. This extra step will ensure optimum sensitivity.

⚠ 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 + two additional reactions.

❹
- Mix carefully by pipetting up and down. Do not vortex.
- Pipet 15 µl PCR mix into each well of the LightCycler® 480 Multiwell Plate.
- Add 5 µl of the DNA template.
- Seal Multiwell Plate with LightCycler® 480 Sealing Foil.

❺
- Place the Multiwell Plate in the centrifuge and balance it with a suitable counterweight (*e.g.*, another Multiwell Plate).
- Centrifuge for 2 min at 1500 × *g* in a standard swing-bucket centrifuge, containing a rotor for multiwell plates with suitable adaptors.

❻ Load the Multiwell Plate into the LightCycler® 480 Instrument.

❼ Start the PCR program described above.

⚠ If you use a reaction volume < 20 µl, the reaction and cycle conditions must be reoptimized.

Color Compensation

For information on generating and using a color compensation file, see the LightCycler® 480 Operator's Manual or the LightCycler® 480 Online Resource Site (**www.roche-applied-science.com/lightcycler480**).

Prevention of Carryover Contamination

Uracil DNA N-Glycosylase (UNG) can help prevent carryover contamination in PCR. The prevention technique involves incorporating deoxyuridine triphosphate (dUTP, a component of the Master Mix in this kit) into amplification products, then pretreating later PCR mixtures with UNG. If a dUTP-containing contaminant is present in the later PCRs, it will be cleaved by a combination of the UNG and the high temperatures of the initial denaturation step; it will not serve as a PCR template.

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

⚠ To ensure optimum results in carryover prevention reactions with the LightCycler® 480 Probes Master, always use LightCycler® Uracil-DNA Glycosylase*. Follow the instructions in the package insert for the enzyme.

Two-Step RT-PCR

The LightCycler® 480 Probes Master can also be used to perform the second step of a two-step RT-PCR.

In two-step RT-PCR, the first step, reverse transcription of RNA into cDNA, is performed outside the LightCycler® 480 System. Subsequent amplification and online monitoring is performed according to the LightCycler® 480 System standard procedure, using cDNA as starting sample material.

One of the following reagents is required for reverse transcription of RNA into cDNA (see Ordering Information for details):

- Transcriptor Reverse Transcriptase
- Transcriptor First Strand cDNA Synthesis Kit

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

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

3. Results

The following amplification curves were obtained by using the LightCycler® 480 Probes Master. The fluorescence values versus cycle number are displayed.

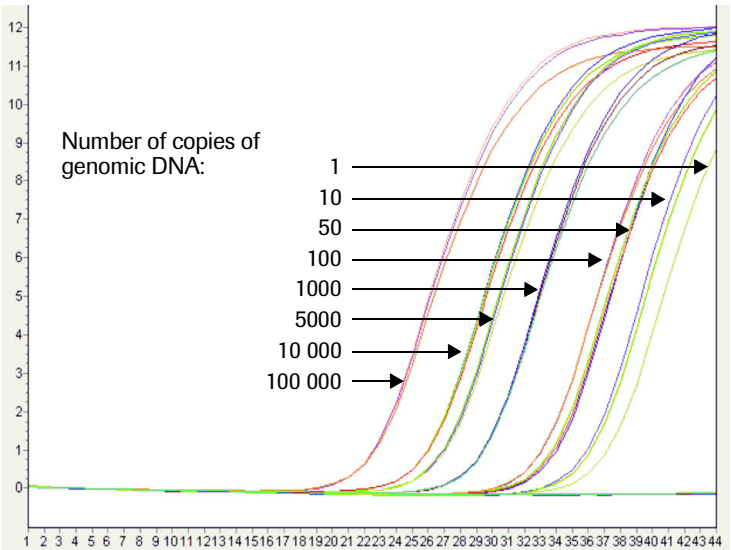

Fig. 1: Amplification curves

Serial tenfold and twofold dilutions of human genomic DNA as template, a specific set of primers and a Fam/Tamra-labeled hydrolysis probes that recognizes a 196-bp fragment of the Cytochrome P450 2C9 gene were used.

	Cause	Recommendation
Amplification curves reach plateau phase before cycling is complete.	Starting amount of nucleic acid is very high.	Stop the cycling program by clicking the *End Program* button. The next cycle program will continue automatically.
	The number of cycles is too high.	Reduce the number of cycles in the cycling program.
Log-linear phase of amplification just starts as the cycling program ends.	The number of cycles is too low.	• While cycling is still going on, use the *Add 10 Cycles* button to increase the number of cycles. • Increase the number of cycles in the cycling program. • Use more starting material. • Optimize PCR conditions (primer/probe design, protocol).
No amplification detectable	Wrong filter combination was used to display amplification on screen.	Select appropriate filter combination for your assay on the analysis screen and start again.
	Wrong detection format was chosen for experimental protocol.	Select appropriate detection format for your assay and start again.
	Impure sample material inhibits reaction.	• Try a 1:10 dilution of your sample. • Purify the nucleic acids from your sample material to ensure removal of inhibitory agents.
	FastStart DNA Polymerase is not sufficiently activated.	• Make sure PCR includes an initial 5 min pre-incubation at 95°C. • Make sure denaturation time during amplification is at least 10 s.
	Pipetting errors or omitted reagents.	Check for missing or defective reagents.
	Amplicon length is $>$1 kb.	Do not design primers that produce amplicons $>$1 kb, which are inefficiently amplified. Optimal results are obtained with amplicons $<$500 bp.
	Difficult template, *e.g.,* unusual GC-rich sequence.	• Optimize temperatures and times used for the amplification cycles. • Optimize primer/probe sequences. • Repeat PCR but add increasing amounts of DMSO. (Use as much as 10% DMSO in the reaction).

continued on next page

	Cause	Recommendation
Fluorescence intensity varies.	Some of the reagent is still in the upper part of the microwell, or an air bubble is trapped in microwell.	Repeat centrifugation, but allow sufficient centrifugation time (*e.g.*, 2 min at 1500 × *g*) for all reagent to reach the bottom of the microwell and/or to expel air bubbles.
	Skin oils or dirt on the surface of the microwell.	Always wear gloves when handling the Multiwell Plate.
Fluorescence intensity is very low.	Low concentration or deterioration of dyes in the reaction mixtures because dye was not stored properly.	• Keep dye-labeled reagents away from light. • Store the reagents at −15 to −25°C and avoid repeated freezing and thawing.
	Poor PCR efficiency (reaction conditions not optimized).	• Check concentrations of reagents and probes. • Optimize protocol.
	Chosen imaging time is too low.	• Choose adequate Roche Detection Format in combination with "dynamic" detection mode or • Increase imaging time when using "manual" detection mode For details see LightCycler® 480 Operator's Manual
Negative control sample gives a positive signal.	Contamination	• Remake all critical solutions. • Pipet reagents on a clean bench. • Use UNG to eliminate carryover contamination.
High background	Fluorescence signals are very low, therefore the background seems relatively high.	Follow general strategies for optimizing PCR runs in the LightCycler® 480 System.
	Probe quality is poor.	Prepare a new probe solution.

5. Additional Information on this Product

How this Product Works

Hot-start PCR has been shown to significantly improve the specificity and sensitivity of PCR (1, 2, 3, 4) by minimizing the formation of nonspecific amplification products at the beginning of the reaction. FastStart Taq DNA Polymerase is specifically designed for hot-start PCR.

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.

Test Principle

Sequence-specific detection of PCR products relies on sequence-specific oligonucleotide probes that are coupled to fluorophores. These probes hybridize to their complementary sequence in target PCR products. Probe chemistries that are suitable for use in the LightCycler® 480 Instrument include single-labeled probes, hybridization probes, and hydrolysis probes. Hybridization and hydrolysis probe chemistries use the so-called FRET principle. Fluorescence Resonance Energy Transfer (FRET) is based on the transfer of energy from one fluorophore (the donor or reproter) to another adjacent fluorophore (the acceptor or quencher).

Hydrolysis probe assays can technically be described as homogeneous 5´-nuclease assays, since a single 3´-non-extendable probe, which is cleaved during PCR amplification, is used to detect the accumulation of a specific target DNA sequence (5). This single probe contains two labels, a fluorescent reporter and a quencher, in close proximity to each other. When the probe is intact, the quencher dye is close enough to the reporter dye to suppress the reporter fluorescent signal (fluorescent quenching takes place via FRET). During PCR, the 5´-nuclease activity of the polymerase cleaves the hydrolysis probe, separating the reporter and quencher. The reporter dye is no longer quenched and emits a fluorescent signal when excited.

The LightCycler® 480 Instrument can detect hydrolysis probes that are labeled with the reporter dyes LightCycler® Red 610, LightCycler® Red 640, LightCycler® Cyan 500, FAM or HEX. These labeled hydrolysis probes can be used separately or in combination, which permits either single- or multicolor detection.

⚠ For multicolor hydrolysis probe assays it is recommended to use dark quencher dyes (*i.e.*, dye molecules which efficiently quench the fluorescence of a FRET reporter dye without emitting fluorescence themselves). Roche Applied Science recommends to use BHQ-2 (quenching range 550-650 nm) for all hydrolysis probe reporter dyes listed above.

References

1 Chou, Q. et al. (1992). Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Research* **20**, 1717-1723.
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. (1996). Simplified hot start PCR. *Nature* **381**, 445-446.
4 PCR Manual, Roche Diagnostics (1999) 2nd edition (1999) **2**, 52-58.
5 Holland, P.M. et al (1991). Detection of specific polymerase chain reaction product by utilizing the 5´->3´ exonuclease activity of Thermus aquaticus DNA polymerase. *Proc. Natl. Acad. Sci. USA*. **88**, 7276-7280.

Quality Control

The LightCycler® 480 Probes Master is function tested using the LightCycler® 480 System.

6. Supplementary Information

6.1 Conventions

Text Conventions

To make information consistent and memorable, the following text conventions are used in this Instruction Manual:

Text Convention	Usage
Numbered stages labeled ①, ②, etc.	Stages in a process that usually occur in the order listed.
Numbered instructions labeled ❶, ❷, etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Applied Science.

Symbols

In this Instruction Manual, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
	Important Note: Information critical to the success of the procedure or use of the product.

6.2 Changes to Previous Version

- Editorial changes.

6.3 Ordering Information

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

- The LightCycler® 480 System family for real-time, online PCR:
- www.roche-applied-science.com/lightcycler480

	Product	Pack Size	Cat. No.
Instrument and Accessories	LightCycler® 480 Instrument, 384-well	1 instrument with data workstation and accessories	04 545 885 001
	LightCycler® 480 Multiwell Plate 384	50 plates and foils	04 729 749 001
	LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
LightCycler® 480 Kits for PCR	LightCycler® 480 SYBR Green I Master	1 kit (5 × 100 reactions, 20 µl each)	04 707 516 001
	LightCycler® 480 Genotyping Master	1 kit (4 × 96 reactions, 20 µl each)	04 707 524 001
	LightCycler® 480 Control Kit	3 runs	04 710 924 001
Associated Kits and Reagents	LightCycler® Uracil-DNA Glycosylase	100 U (50 µl)	03 539 806 001
	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

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Further information on purchasing licenses to practice real-time PCR processes may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

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