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Roche Applied Science

LightCycler® 480 SYBR Green I Master

Version October 2005

Easy-to-use hot-start reaction mix for PCR using the LightCycler® 480 System

Cat. No. 04 707 516 001

Kit for 5 × 100 reactions (20 µl each)

Store the kit at −15 to −25°C

⚠ Keep LightCycler® 480 SYBR Green I Master (vial 1, green cap) away from light!

Table of Contents

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 green cap	Master	• 5 vials, 1 ml each • Ready-to-use hot-start PCR reaction mix • Contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye, and $MgCl_2$
2 colorless cap	H_2O, PCR-grade	• 5 vials, 1 ml each • 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.

⚠ Keep the Master (vial 1, 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
1 green cap	Master	• Store at −15 to −25°C. • **Avoid repeated freezing and thawing!** • **Keep vial 1 away from light!**
2	Water, PCR-grade	• Store at −15 to −25°C

Additional Equipment and Reagents Required

Additional reagents and equipment required to perform reactions with the LightCycler® 480 SYBR Green I Master using the LightCycler® 480 System 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 reaction (Eppendorf) tubes for preparing master mixes and dilutions

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

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

Application LightCycler® 480 SYBR Green I Master is designed for research studies. When used with the LightCycler® 480 System, this kit is ideally suited for hot-start PCR applications. In combination with the LightCycler® 480 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 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® 480 Instrument and design specific PCR primers for each target. See the LightCycler® 480 Operator's Manual for general recommendations.

⚠ The amplicon size should not exceed 750 bp in length. For optimal results, select a product length of 500 bp or less.

The ready-to-use LightCycler® 480 SYBR Green I Master offers convenience and ease of use because

- no additional pipetting steps to combine enzyme and reaction buffer are necessary,
- the addition of $MgCl_2$ to the reaction mixture is not necessary, thus avoiding time-consuming optimization steps.

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

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, http://www.roche-applied-science.com.
- Use up to 50 – 100 ng complex genomic DNA or up to 10^8 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 µl (or less) of that sample in the reaction.

Negative Control

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

- replace the template DNA with PCR-grade water (vial 2; this will reveal whether a contamination problem 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

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

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

$MgCl_2$

The composition of the LightCycler® 480 SYBR Green I Master is optimized for almost all primer combinations.

🔍 You do not need to add additional $MgCl_2$ to the mix to get efficient and specific PCR!

LightCycler® 480 Instrument Protocol

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

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

A LightCycler® 480 Instrument protocol that uses LightCycler® 480 SYBR Green I Master contains the following programs:

- **Pre-Incubation** for activation of FastStart Taq DNA polymerase and denaturation of the DNA
- **Amplification** of the target DNA
- **Melting Curve** for PCR product identification
- **Cooling** the multiwell plate

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 SYBR Green I Master.

Setup				
Detection Format		**Block Type**		**Reaction Volume**
SYBR Green		384		3 – 20 µl
Programs				
Program Name		**Cycles**		**Analysis Mode**
Pre-Incubation		1		None
Amplification		45 [1]		Quantification
Melting Curve		1		Melting Curves
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	00:00:05 – 00:00:20 [4]	2.5	–
72	Single	00:00:05 – 00:00:20 [4] [5]	4.8	–
Melting Curve				
95	None	00:00:05		–
65	None	00:01:00		–
97	Continuous	–	–	5 – 10 [6]
Cooling				
40	None	00:00:10	2.0	–

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.
5) Calculate the hold time for the PCR elongation step by dividing the amplicon length over 25 (*e.g.,* a 500 bp amplicon requires 20 s elongation time).
6) Melting temperatures that are calculated based on experiments using either the lowest or highest value of the recommended range might differ by approx. 0.5°C.

Preparation of the PCR Mix

Follow the procedure below to prepare one 20 µl standard reaction.

⚠ Do not touch the surface of the LightCycler® 480 Multiwell Plate and Multiwell Sealing Foil when handling them. Always wear gloves during handling.

❶ Thaw one vial of "LightCycler® 480 SYBR Green I Master" (vial 1, green cap) and Water, PCR-grade.

⚠ Keep the Master mix away from light.

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

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 mentioned below:

Component	Volume
Water, PCR-grade (vial 2, colorless cap)	3 µl
PCR Primer, 10× conc.	2 µl
Master Mix, 2× conc. (vial 1, green cap)	10 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 + sufficient 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 the Multiwell Plate with LightCycler® 480 Multiwell Sealing Foil.

❺
- Place the Multiwell Plate in the centrifuge and balance it with a suitable counterweight (*e.g.*, another Multiwell Plate).
- Centrifuge at 1500 × *g* for 2 min (3000 rpm in a standard swing-bucket centrifuge containing a rotor for multiwell plates with suitable adaptors).

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

❼ Start the PCR program described above.

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

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 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.

⚠ Use only LightCycler® Uracil-DNA Glycosylase* in combination with the LightCycler® 480 SYBR Green I Master.

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

The use of UNG might influence the melting temperature (T_m) in melting curve analysis.

Two-step RT-PCR

LightCycler® 480 SYBR Green I Master 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® 480 System. Subsequent amplification and online monitoring is performed according to the standard LightCycler® 480 System procedure, using the cDNA as the starting sample material. Transcriptor First Strand cDNA Synthesis Kit* is recommended for reverse transcription of RNA into cDNA. Synthesis of cDNA is performed according to the detailed instructions provided with the kit.

⚠ 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® 480 SYBR Green I Master in combination with the LightCycler® h-G6PDH Housekeeping Gene Set*, targeting human glucose-6-phosphate dehydrogenase (G6PDH) mRNA. The fluorescence values versus cycle number are displayed.

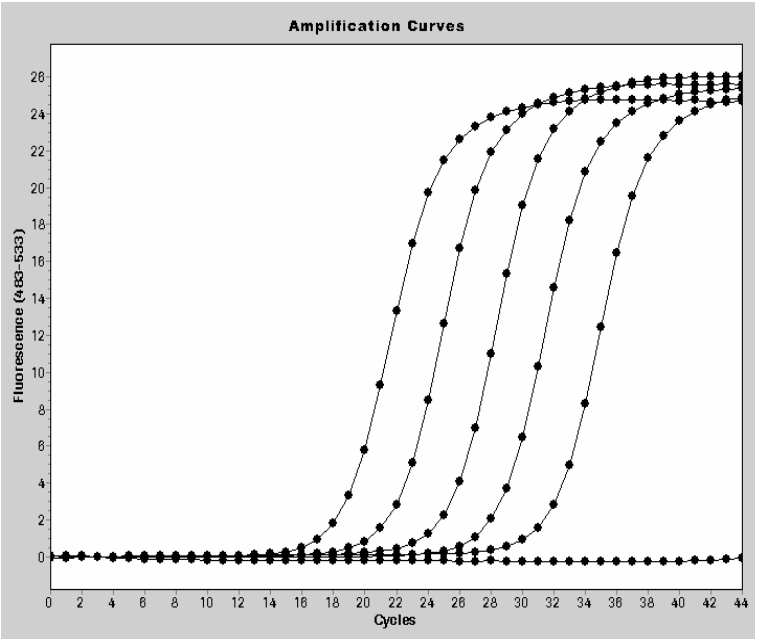

Fig. 1: Serially diluted samples containing cDNA derived from 5×10^5, 5×10^4, 5×10^3, 5×10^2 copies of *in vitro* transcript as starting template were amplified using the LightCycler® 480 SYBR Green I Master. As a negative control, template cDNA was replaced by PCR-grade water.

continued on next page

Melting Curve Analysis

Specificity of the amplified PCR product was assessed by performing a melting curve analysis on the LightCycler® 480 Instrument. The resulting melting curves allow discrimination between primer-dimers and specific product. The specific G6PDH product melts at a higher temperature than the primer-dimers. The melting curves display the specific amplification of the G6PDH RNA when starting from cDNA derived from 5×10^5, 5×10^4, 5×10^3, 5×10^2 copies of *in vitro* transcript.

Smaller reaction volumes may result in melting temperature variations.

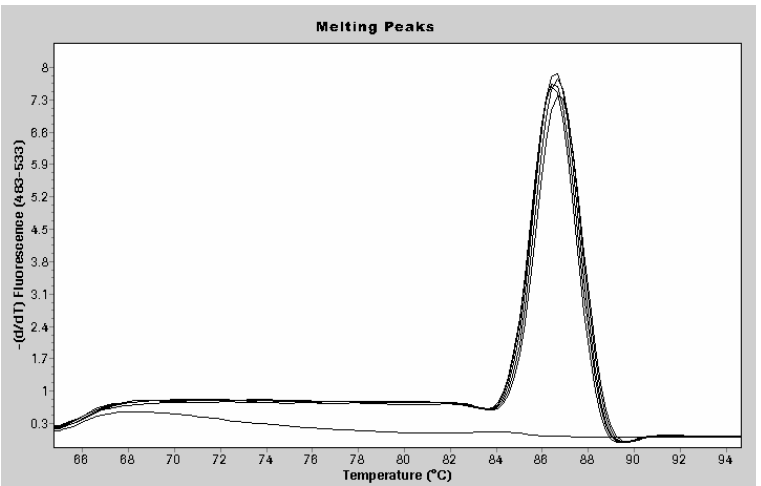

Fig. 2: Melting curve analysis of amplified samples with cDNA derived from 5×10^5, 5×10^4, 5×10^3, 5×10^2 copies of *in vitro* transcript as starting template. As a negative control, template DNA was replaced by PCR-grade water.

	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 start automatically.
	The number of cycles is too high.	Reduce the number of cycles in the program *Amplification*.
Log-linear phase of amplification just starts as the amplification program finishes.	Starting amount of nucleic acid is very low.	• Improve PCR conditions (*e.g.,* primer design). • Use more starting DNA template. • Repeat the run.
	The number of cycles is too low.	Increase the number of cycles in the cycle program.
No amplification occurs.	Using wrong filter combination to display amplification on screen.	Change the filter combination on the Run screen. (The data obtained up to this point will be saved.)
	FastStart Taq DNA polymerase is not fully activated.	• Make sure PCR included a pre-incubation step at 95°C for 5 – 10 min. • Make sure denaturation time during cycles is 10 s.
	Pipetting errors or omitted reagents.	• Check for missing reagents. • Check for missing or defective dye.
	Scale of axes on graph are unsuitable for analysis.	Change the values for the x- and y-axis: right-click on the chart and select Chart Preferences from the context menu. Change the maximum and/or minimum axis values appropriately.
	Measurements do not occur.	Check the temperature targets of the experimental protocol. For SYBR Green I detection format, choose “Single” as the acquisition mode at the end of the elongation phase.
	Amplicon length is >900 bp.	Do not use amplicons >900 bp. Optimal results are obtained with amplicons of 500 bp or less
	Impure sample material inhibits reaction.	• Do not use more than 5 µl of DNA per 20 µl PCR reaction mixture. • Repurify the nucleic acids to ensure removal of inhibitory agents
Fluorescence intensity is too low.	Deterioration of dye in reaction mixtures; dyes not stored properly.	• Store the Master Mix at −15 to −25°C, and keep it away from light. • Avoid repeated freezing and thawing.
	Reaction conditions are not optimized, leading to poor PCR efficiency.	• 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.

	Cause	Recommendation
Fluorescence intensity varies.	Skin oils on the surface of the multiwell sealing foil.	Always wear gloves when handling the multiwell plate and the sealing foil.
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.	• Do not use more than 50–100 ng of complex genomic DNA in a 20 µl reaction. • Instead of SYBR Green I use a sequence-specific probe-based detection format (*e.g.,* hydrolysis probes) which allows analysis of up to 500 ng DNA.
	Dye bleached.	Make sure the Master Mix is kept away from light. Avoid repeated freezing and thawing.
Negative control samples give a positive signal.	Contamination, or presence of primer-dimers.	• Remake all critical solutions. • Pipet reagents on a clean bench. • Use heat-labile UNG to eliminate carry-over contamination.

Double melting peak appears for one product.	Two products of different length or GC-content are amplified (*e.g.,* due to pseudogenes or misprimming).	• Check products on an agarose gel.
		• Elevate the reaction stringency by:
		  • redesigning the primers
		  • checking the annealing temperature
		  • performing a "touch-down" PCR
		  • using a probe-based detection format 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 heat-labile UNG, primer preparation, 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 outcompeted 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. • Increase annealing temperature in order to enhance stringency.
	Quality of primer is poor.	Purify primer more thoroughly.
	Sequence of primers is inappropriate.	Redesign primers.
Primer-dimer and product peaks are very close together.	Unusually high GC-content of the primers.	• Redesign primers. • Run melting curve at high acquisition/°C rate (>10 acquisitions/°C).

	Cause	Recommendation
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.	Use fresh solutions.

5. Additional Information on this Product

How this Product Works

LightCycler® 480 SYBR Green I Master is a ready-to-use reaction mix designed specifically for applying the SYBR Green I detection format on the LightCycler® 480 Instrument. It is used to perform hot-start PCR in 384 Multiwell plates. 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 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

Generation of PCR products can be detected by measurement of the SYBR Green I fluorescence signal. SYBR Green I intercalates into the DNA helix (5). 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® 480 Instrument's 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® 480 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 97°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, 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 Roche Diagnostics (1999). PCR Manual, 2nd edition. pp 52-58.
5 Zipper, H et al (2004). Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nuc. Acid Res.* **32**, e103.

Quality Control The LightCycler® 480 SYBR Green I Master is function tested using the LightCycler® 480 Instrument.

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

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 for:

- The LightCycler® 480 System: http://www.roche-applied-science.com/lightcycler480
- The MagNA Pure System family for automated nucleic acid isolation: http://www.magnapure.com
- DNA & RNA preparation - Versatile Tools for Nucleic Acid Purification: http://www.roche-applied-science.com/napure

	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 Probes Master	1 kit (5 × 100 reactions, 20 µl each)	04 707 494 001
	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
	LightCycler® h-G6PDH Housekeeping Gene Set	1 set (96 reactions)	03 261 883 001
	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

6.4 Disclaimer of License

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

The product is covered in-part by US 5,871,908, co-exclusively licensed from Evotec OAI AG.

Parts of the Software used for the LightCycler® 480 System are licensed from Idaho Technology Inc., Salt Lake City, UT, 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.

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