For general laboratory use.

FastStart Essential DNA Green Master

Version: 08

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Ready-to-use hot start reaction mix for real-time PCR with the LightCycler® 96 System.

Cat. No. 06 402 712 001	1 kit 500 reactions of 20 µl final volume each
Cat. No. 06 924 204 001	1 kit 10 x 500 reactions of 20 µl final volume each

Store the kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Catalog Number	Content
1	green	FastStart Essential DNA Green Master, 2x conc.	▪ Ready-to-use hot start PCR mix. ▪ Contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye, and $MgCl_2$.	06 402 712 001	5 vials, 1 ml each
				06 924 204 001	10 vials, 5 ml each
2	colorless	FastStart Essential DNA Green Master, Water, PCR Grade	To adjust the final reaction volume.	06 402 712 001	5 vials, 1 ml each
				06 924 204 001	2 vials, 25 ml each

1.2. Storage and Stability

Storage Conditions (Product)

The kit is shipped on dry ice.
When stored at −15 to −25°C, the kit is stable through the expiry date printed on the label.
Once the kit is opened, store the kit components as described in the following table:

Vial / Bottle	Cap	Label	Storage
1	green	FastStart Essential DNA Green Master, 2x conc.	Store at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.*** After first thawing, the master may be stored for up to 4 weeks at +2 to +8°C. ⚠ ***Keep protected from light.***
2	colorless	FastStart Essential DNA Green Master, Water, PCR Grade	Store at −15 to −25°C.

Storage Conditions (Working Solution)

The PCR mix, that is, FastStart Essential DNA Green Master supplemented with primers and template is stable for up to 24 hours at +15 to +25°C.
⚠ ***Store the PCR mix protected from light.***

1.3. Additional Equipment and Reagent required

Instruments and consumables

- LightCycler® 96 Instrument*
  – Use with LightCycler® 8-Tube Strips (white)* or LightCycler® 480 Multiwell Plates 96 (white)* and LightCycler® 480 Sealing Foils*
- Standard swinging-bucket centrifuge containing a rotor for multiwell plates with suitable adaptor
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile 1.5 ml reaction tubes for preparing master mixes and dilutions

Reagents for the LightCycler® 96 Instrument*

- LightCycler® Uracil-DNA Glycosylase* (optional)

For details about prevention of carryover contamination, see section **Prevention of Carryover Contamination**.

1.4. Application

The FastStart Essential DNA Green Master is designed for research studies. When used with the LightCycler® 96 System, this kit is ideally suited for hot start PCR applications. In combination with the LightCycler® 96 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, and can be used with heat-labile Uracil-DNA Glycosylase to prevent carryover contamination during PCR.
In principle, the kit can be used for the amplification and detection of any DNA or cDNA target. However, each amplification protocol will need to be adapted to the reaction conditions of the LightCycler® 96 Instrument and specific PCR primers will need to be designed for each target.

1.5. Preparation Time

Typical Run Time

Variable, depending on the number of cycles and the annealing time. If the cycling program specifies 45 cycles with a 3-step protocol (10 second denaturation, 10 second annealing, 10 second elongation), a LightCycler® 96 PCR run will last approximately 70 minutes, including 10 minutes pre-incubation time, without melting.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Use any template DNA suitable for PCR in terms of purity, concentration, and absence of PCR inhibitors.
For reproducible isolation of nucleic acids, use:
- Either a MagNA Pure System together with a dedicated reagent kit (for automated isolation),
- or a High Pure Nucleic Acid Isolation Kit (for manual isolation).

- Use the RealTime ready Cell Lysis Kit* (for lysing cells prior to two-step real-time RT-PCR applications).
- Use up to 50 – 100 ng complex genomic DNA or up to 10^8 copies plasmid DNA for a reaction volume of 20 µl. For larger volumes, the amount of template can be increased equivalently.

⚠ ***Using too much 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 a maximum of 2 µl of that sample and applying a 10 minute pre-incubation at 95°C. This will result in lower crossing point (Cp), or quantification cycle (Cq) values with a decreased standard deviation.

Control Reactions

Always run a negative control with the samples. To prepare negative controls:
- Replace template DNA with Water, PCR Grade (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

Suitable concentrations of PCR primers range from 0.2 to 1 µM (final concentration in reaction). The recommended starting concentration is 0.5 µM each.

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

Mg^{2+} Concentration

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.

Prevention of Carryover Contamination

Uracil DNA N-Glycosylase (UNG) is suitable for preventing carryover contamination in PCR. This carryover 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.

⚠ ***To ensure optimal results in carryover prevention reactions with the FastStart Essential DNA Green Master, always use LightCycler® Uracil-DNA Glycosylase*.***

Follow the Instructions for Use for the enzyme. Since your target DNA template contains thymidine rather than uridine, it is not affected by this procedure.

The use of UNG may influence the melting temperature (Tm) in melting curve analysis.

2.2. Protocols

LightCycler® 96 Instrument Protocol

Program the LightCycler® 96 Instrument before preparing the reaction mixes.
A LightCycler® 96 Instrument protocol that uses FastStart Essential DNA Green Master contains the following programs:
- **Pre-Incubation** (Hold) for activation of FastStart Taq DNA polymerase and denaturation of the DNA
- **Amplification** of the target DNA
- **Melting Curve** for PCR product identification

For details on how to program the experimental protocol, see the LightCycler® 96 System Guides.

The following tables show the PCR parameters that must be programmed for a LightCycler® 96 System PCR run with the FastStart Essential DNA Green Master using the LightCycler® 8-Tube Strips or LightCycler® 480 Multiwell Plates. The hold times shown here are a robust protocol (standard protocol) and can be shortened depending on your assay design[1].

LightCycler® 96 Instrument Protocol

The following procedure is optimized for use with the LightCycler® 96 Instrument.

Run editor				
Detection format			Reaction volume [µl]	
Dyes 1: SYBR Green I			20	
Programs				
	Temp. [°C]	Ramp [°C/s]	Duration [s]	Acquisition mode
Pre-incubation	95	4.4	600[2]	None
3-Step amplification	No. of Cycles: 45			
	95	4.4	10[1][3]	None
	60 primer dependent[4]	2.2	10[1][3]	None
	72	4.4	10[1][3][5]	Single
Melting	95	4.4	10	None
	65	2.2	60	None
	97	0.1	1	5 readings / °C

[1] For well-established assays you may shorten the amplification times to: 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 10 to 20 seconds. Forty-five 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 (fast protocol).

[2] For some assays, a pre-incubation of 300 seconds is sufficient (fast protocol). However, if high polymerase activity is required in early cycles, a 600-second period is recommended, especially for higher reaction volumes and when working with unpurified cDNA samples as template. Do not use more than 2 µl unpurified cDNA sample.

[3] 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. This is especially recommended for higher reaction volumes.

[4] For initial experiments, set the target temperature (the primer annealing temperature) 5°C below the calculated primer Tm.

[5] Calculate the hold time for the PCR elongation step by dividing the amplicon length by 10, for example, a 150 bp amplicon requires 15 seconds elongation time. Do not exceed the hold time for elongation below 10 seconds.

Preparation of the PCR mix

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

⚠ ***Always wear gloves during handling.***

1. Thaw one vial of FastStart Essential DNA Green Master (Vial 1) and Water, PCR Grade (Vial 2).
   – Briefly spin vials in a microcentrifuge before opening to ensure recovery of all the contents.
   – Mix carefully by pipetting up and down and store on ice.

   ⚠ ***Keep the Master Mix protected from light.***

2. Prepare a 10x-concentrated 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 listed below:

Reagent	**Volume [µl]**
Water, PCR Grade (Vial 2)	3.0
PCR Primer, 10x conc.	2.0
Master Mix, 2x conc. (Vial 1)	10.0
Total Volume	**15.0**

ⓘ *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 plus sufficient additional reactions.*

4 Mix carefully by pipetting up and down. Do not vortex.
– Pipette 15 µl PCR mix into each reaction vessel of a LightCycler® 8-Tube Strip or LightCycler® 480 Multiwell Plate.
– Add 5 µl of the DNA template.
– Close the reaction vessels.

5 Place the LightCycler® 480 Multiwell Plate in a standard swinging-bucket centrifuge with suitable adapter.
– Balance it with a suitable counterweight, such as another LightCycler® 480 Multiwell Plate, or
– Place the 8-Tube Strips into a standard multiwell plate 96 (MWP) and balance them in the centrifuge.
– Centrifuge at 1,500 × *g* for 0.5 to 2 minutes.

6 Load the reaction vessels into the the LightCycler® 96 Instrument.

7 Start the PCR program described above.

If you use reaction volumes different from 20 µl, it may be advantageous to adapt the hold times of all amplification steps.

Two-Step RT-PCR

FastStart Essential DNA Green 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® 96 System. Subsequent amplification and online monitoring is performed according to the standard LightCycler® 96 System procedure, using the cDNA as the starting sample material.
The Transcriptor First Strand cDNA Synthesis Kit* is recommended for reverse transcription of RNA into cDNA. Synthesis of cDNA is performed according to the instructions provided with the kit.

⚠ ***For initial experiments, run undiluted, 1:10 diluted, and 1:100 diluted cDNA template in parallel to determine the optimum template amount. If you use undiluted cDNA as template, use a 10-minute pre-incubation.***

3. Results

Quantification analysis

The following amplification curves were obtained on the LightCycler® 96 Instrument using the FastStart Essential DNA Green Master. A reaction using primers specific for the target gene Cyp2C9 was performed. The intensity in relative fluorescence units (RFU) versus cycle number is displayed (see Fig. 1).

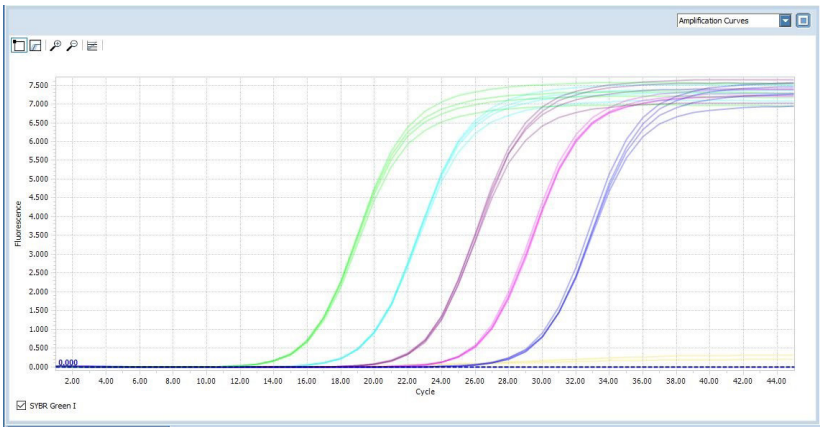

Fig. 1: The amplification diagram shows the result for the target gene Cyp2C9. The amplification curves shown were obtained from plasmid dilutions of 100 copies (far right), 1,000 copies, 10,000 copies, 100,000 copies, and 1,000,000 copies (far left) per well, including a no template control (yellow flat line). Singleplex qPCR with four replicates for each dilution was performed in a reaction volume of 20 µl per well.

Melting curve analysis

Specificity of the amplified PCR product was assessed by performing a melting curve analysis on the LightCycler® 96 Instrument. The resulting melting curves allow discrimination between primer-dimers and specific product (see Fig. 2). The specific product melts at a higher temperature than the primer-dimers (no primer-dimers visible for the Cyp2C9 example shown below). The melting curves display the specific amplification of the Cyp2C9 gene from plasmid DNA with 100 copies, 1,000 copies, 10,000 copies, 100,000 copies to 1,000,000 copies.

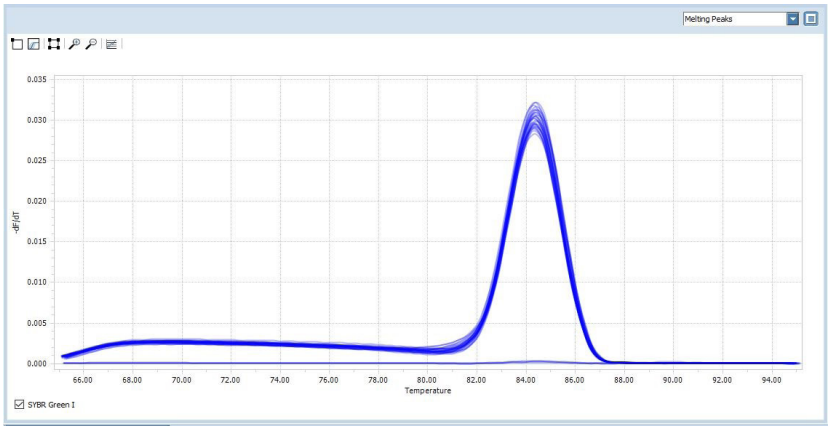

Fig. 2: Melting curve analysis of amplified samples with plasmid DNA dilutions of 100 copies, 1,000 copies, 10,000 copies, 100,000 copies, and 1,000,000 copies per well. As a no template control, template DNA was replaced by PCR-grade water (flat line).

4. Troubleshooting

Observation	Possible cause	Recommendation
Log-linear phase of amplification just starts as the amplification program ends.	Starting amount of nucleic acid is very low.	Use more starting DNA template.
		Improve PCR conditions, such as primer design.
		Repeat the run.
	Hold times in the amplification protocol are too short.	Optimize the run protocol by extending the hold times of annealing and elongation.
	The number of cycles is too low.	Increase the number of cycles in the amplification program.
No amplification detectable.	Wrong detection format.	Change the dye for the target.
	FastStart Taq DNA polymerase is not fully activated.	Make sure PCR included a pre-incubation step at 95°C for 5 to 10 minutes.
		Make sure denaturation time during cycles is 10 seconds.
	Pipetting errors or omitted reagents.	Check for missing reagents.
		Check for missing or defective dye.
	Zoom function in the graph is active and only a small part of the chart is visible.	Undo the zoom function.
	Measurements do not occur.	Check the cycling program of the experimental protocol. For Intercalating dyes, choose “Single” under Acquisition Mode (LightCycler® 96 Instrument) at the end of the elongation phase.
	Impure sample material inhibits reaction.	Do not use more than 5 µl of DNA per 20 µl PCR reaction mixture.
		Re-purify 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 protected from light.
		⚠ ***Avoid repeated freezing and thawing.***
	Wrong Optics Settings in the Run Settings folder.	Check the Optics Setting prior to each run.
	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.
Fluorescence intensity varies.	Varying volumes of master in different tubes.	Use one Master Mix for the different samples.
	Skin oils or dirt are present on the lid or sealing foil.	Always wear gloves.

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 to 100 ng of complex genomic DNA in a 20 µl reaction.
		Instead of SYBR Green I, use a sequence-specific probe-based detection format, such as hydrolysis probes which allows analysis of up to 500 ng DNA (for a 20 µl reaction volume).
	Dye bleached	Make sure the Master Mix is protected from light. ⚠ ***Avoid repeated freezing and thawing.***
Negative control samples give a positive signal.	Contamination, or presence of primer-dimers.	Remake all critical solutions.
		Pipette reagents on a clean bench.
		Use heat-labile UNG to eliminate carryover contamination.
		Redesign primer sequences.
Double melting peak appears for one product.	Two products of different length or GC-content are amplified, for example, 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 “touchdown” PCR ▪ using a probe-based detection format for better specificity.
Melting temperature of a product varies from experiment to experiment.	Variations in reaction mixture, such as salt concentration.	Check purity of template solution.
		Reduce variations in parameters such as 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.	Sequence of primers is inappropriate.	Redesign primers.
	Quality of primer is poor.	Purify primer more thoroughly.
Primer-dimer and product peaks are very close together.	Unusually high GC-content of the primers.	Redesign primers.
		Run melting curve with lower ramp rate.
Very broad primer-dimer peak with multiple peaks.	Heterogeneous primers with primer-dimer variations, for example, concatemers, loops.	Redesign primers.
One peak of the same height occurs in all samples.	Contamination in all samples.	Use fresh solutions.
High standard deviation of Cq values.	Impure, heterogeneous DNA template.	Increase pre-incubation time to 10 minutes.
		Use a maximum of 2 µl unpurified cDNA sample.
Baseline drift	Reaction vessels not sealed properly.	Be sure to seal the tubes or multiwell plates correctly.
	Cycler is loaded unbalanced.	If you use only two strips, be sure to load the 8-Tube Strips balanced in the outer rows.

5. Additional Information on this Product

5.1. Test Principle

Generation of PCR products can be detected by measurement of the SYBR Green I fluorescence signal.
SYBR Green I dye intercalates into the DNA helix (Zipper, H., et al., 2004). 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® 96 Instrument's optics match 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® 96 Systems 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' light emission 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 (Tm) of a particular DNA product (where the DNA is 50% double-stranded and 50% single-stranded). The most important factors that determine the Tm 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 nonspecific products are present, they will be shown as additional melting peaks. Checking the Tm of a PCR product can thus be compared with analyzing a PCR product by length in gel electrophoresis.

How this product works

FastStart Essential DNA Green Master is a ready-to-use reaction mix designed specifically for applying the SYBR Green I detection format in the LightCycler® 480 Multiwell Plates 96, or LightCycler® 8-Tube Strips on the LightCycler® 96 Instrument. It is used to perform hot start PCR. Hot start PCR has been shown to significantly improve the specificity and sensitivity of PCR (Chou, Q., et al., 1992, Kellogg, D.E., et al., 1994, Birch, D.E., 1996) by minimizing the formation of nonspecific 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 nonspecifically. The enzyme is completely activated (by removal of blocking groups) in a single pre-incubation step (95°C, 5 to 10 minutes) before cycling begins. Activation does not require the extra handling steps typical of other hot start techniques.

5.2. References

- Birch DE, Kolmodin L, Wong J, Zangenberg GA, Zoccoli MA, McKinney N, Young KKY. Simplified hot start PCR. Nature. 1996;381(6581):445-446.
- Chou Q, Russell M, Birch DE, Raymond J, Bloch W. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Research. 1992;7:1717-1723.
- Kellogg DE, Rybalkin I, Chen S, Mukhamedova N, Vlasik T, Siebert PD, Chenchika A. TaqStart antibody : hot start PCR facilitated by a neutralizing monoclonal antibody directed against Taq DNA polymerase. BioTechniques. 1994;16(6):1134-1137.
- Zipper H, Brunner H, Bernhagen J, Vitzthum F. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. Nucleic Acids Research. 2004;12.

5.3. Quality Control

The FastStart Essential DNA Green Master is function tested using the LightCycler® System.

6. Supplementary Information

6.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

Editorial changes.
Quality Control changed to LightCycler® System.
Regulatory Disclaimer has been changed to: “For general laboratory use”.
The LightCycler® Nano System has been phased out, therefore the LightCycler® Nano System information has been removed and the Chapter Results has been updated.

6.3. Ordering Information

Roche offers a large selection of reagents and systems for life science research. For a full overview of related products and manuals, please visit and bookmark our homepage lifescience.roche.com.

Product	Pack Size	Cat. No.
Consumables		
LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
LightCycler® 8-Tube Strips (white)	10 x 12 white strips and clear caps	06 612 601 001
LightCycler® 480 Multiwell Plate 96, white	5 x 10 plates	04 729 692 001
Instruments		
LightCycler® 96 Instrument	1 instrument	05 815 916 001
Reagents, kits		
FastStart Essential DNA Probes Master	1 kit, 500 reactions of 20 µl final volume each	06 402 682 001
	1 kit, 10 x 500 reactions of 20 µl final volume each	06 924 492 001
RealTime ready Cell Lysis Kit	1 kit, 50 lysis reactions with a final reaction volume of 40 µl each	06 366 821 001
	1 kit, 500 lysis reactions with a final reaction volume of 40 µl each	05 943 523 001
LightCycler® 480 RNA Master Hydrolysis Probes	1 kit, 5 x 100 reactions of 20 µl final volume each	04 991 885 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit, 50 reactions, including 10 control reactions	04 379 012 001
	1 kit, 100 reactions	04 896 866 001
	1 kit, 200 reactions	04 897 030 001
LightCycler® Uracil-DNA Glycosylase	50 µl, 100 U, (2 U/µl)	03 539 806 001

6.4. Trademarks

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All other product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For additional documentation such as certificates and safety data sheets, please visit:
documentation.roche.com.

6.6. Regulatory Disclaimer

For general laboratory use.

6.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.8. Contact and Support

If you have questions or experience problems with this or any Roche product for Life Science, please contact our Technical Support staff. Our scientists are committed to providing rapid and effective help.
Please also contact us if you have suggestions for enhancing Roche product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to the research community worldwide.
To ask questions, solve problems, suggest enhancements or report new applications,
please visit our **Online Technical Support** Site.

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