For general laboratory use.

# FastStart Essential DNA Probes 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. 06 402 682 001** | 1 kit<br>500 reactions of 20 µl final volume each |
| **Cat. No. 06 924 492 001** | 1 kit<br>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 | red | FastStart Essential DNA Probes Master, 2x conc. | ▪ Ready-to-use hot start PCR mix.<br>▪ Contains FastStart Taq DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), and $MgCl_2$. | 06 402 682 001 | 5 vials, 1 ml each |
| | | | | 06 924 492 001 | 10 vials, 5 ml each |
| 2 | colorless | FastStart Essential DNA Probes Master, Water, PCR Grade | To adjust the final reaction volume. | 06 402 682 001 | 5 vials, 1 ml each |
| | | | | 06 924 492 001 | 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 | red | FastStart Essential DNA Probes Master, 2x conc. | Store at −15 to −25°C.<br>⚠ ***Avoid repeated freezing and thawing.***<br>After first thawing, the master may be stored for up to 4 weeks at +2 to +8°C.<br>⚠ ***Keep protected from light.*** |
| 2 | colorless | FastStart Essential DNA Probes Master, Water, PCR Grade | Store at −15 to −25°C. |

## 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)

*i* *For details about prevention of carryover contamination, see the* **“Prevention of Carryover Contamination”** *section.*

## 1.4. Application

The FastStart Essential DNA Probes Master is designed for research studies on the LightCycler® 96 System. The FastStart Essential DNA Probes Master is a ready-to-use hot start reaction mix designed for detecting DNA targets with hydrolysis probes. It allows very sensitive detection and quantification of defined DNA sequences as well as endpoint genotyping analysis. The kit may also be used in other types of PCR on the LightCycler® 96 System.
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.
In principle, the FastStart Essential DNA Probes Master can be used to amplify and detect any DNA or cDNA target. However, the detection protocol must be adapted to the reaction conditions of the LightCycler® 96 Instrument, and specific PCR primers and probes must be designed for each target.

## 1.5. Preparation Time

### Typical Run Time

Variable, depending on the number of cycles and the annealing time. For example, if the cycling program specifies 45 cycles with 10 seconds denaturation and 30 seconds annealing, a LightCycler® 96 PCR run will last approximately 70 minutes, including 10 minutes pre-incubation time.

# 2. How to Use this Product

## 2.1. Before you Begin

### Sample Materials

Use any template RNA (DNA) suitable for RT-qPCR (qPCR) in terms of purity, concentration, and absence of RT-PCR (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 500 ng complex genomic DNA or 1 to $10^{10}$ copies plasmid DNA for a reaction volume of 20 µl. For larger volumes, the amount of template can be increased equivalently.

*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 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 0.3 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.*

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

## Probe

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 Cq 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 Tm of the probe should be only slightly higher than the Tm 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.***

## $Mg^{2+}$ 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) 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 optimal results in carryover prevention reactions with the FastStart Essential DNA Probes Master, always use LightCycler® Uracil-DNA Glycosylase*. Follow the Instructions for Use for the enzyme.***

# 2.2. Protocols

### LightCycler® 96 Instrument Protocols

Program the LightCycler® 96 Instrument before preparing the reaction mixes.
A LightCycler® 96 Instrument protocol that uses FastStart Essential DNA Probes Master should contain the following programs:

- **Pre-Incubation** (Hold) for activation of FastStart Taq DNA polymerase and denaturation of the DNA.
- **Amplification** of the target DNA.

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

The following table shows the PCR parameters that must be programmed for a LightCycler® 96 System PCR run with the FastStart Essential DNA Probes Master using 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] | |
| FAM | | | 20 | |
| **Programs** | | | | |
| | Temp. [°C] | Ramp [°C/s] | Duration [s] | Acquisition mode |
| Pre-incubation | 95 | 4.4 | 600[3] | None |
| 2-Step amplification | No. of Cycles: 45 | | | |
| | 95 | 4.4 | 10[1][4] | None |
| | 60<br>primer dependent[2] | 2.2 | 30[1][4] | Single |

[1] For well-established assays (with amplicon size not exceeding 200 bp in length), you may shorten the amplification times to: 95°C for 10 seconds, and 60°C for 10 to 30 seconds. Fourty-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 initial experiments, set the target temperature (the primer annealing temperature) 5°C below the calculated primer Tm.

[3] 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.

[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. This is especially recommended for higher reaction volumes.

## Preparation of the PCR mix

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

1. Thaw one vial of FastStart Essential DNA Probes 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.

2. Prepare a 10x-concentrated solution that contains PCR primers and hydrolysis probe.

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:

| **Component** | **Volume [µl]** |
|---|---|
| Water, PCR Grade (Vial 2) | 3.0 |
| Primer-probe mix[1], 10x conc. | 2.0 |
| Master Mix, 2x conc. (Vial 1) | 10.0 |
| **Total Volume** | **15.0 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 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-TubeStrip 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 a 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 LightCycler® 96 Instrument.

7 Start the PCR program described above.

*If you use reaction volumes different from 20 µl, it might be advantageous to adapt the hold times.*

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

## Two-Step RT-PCR

FastStart Essential DNA 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® 96 System. Subsequent amplification and online monitoring is performed according to the LightCycler® 96 System standard procedure, using cDNA as 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 600-second pre-incubation.***

# 3. Results

## Quantification analysis

The following amplification curves were obtained on the LightCycler® 96 Instrument using the FastStart Essential DNA Probes Master. A reaction using primers and a FAM-labeled probe specific for the target gene HSPA2 was performed.

The intensity in relative fluorescence units (RFU) versus cycle number is displayed (see Fig. 1).

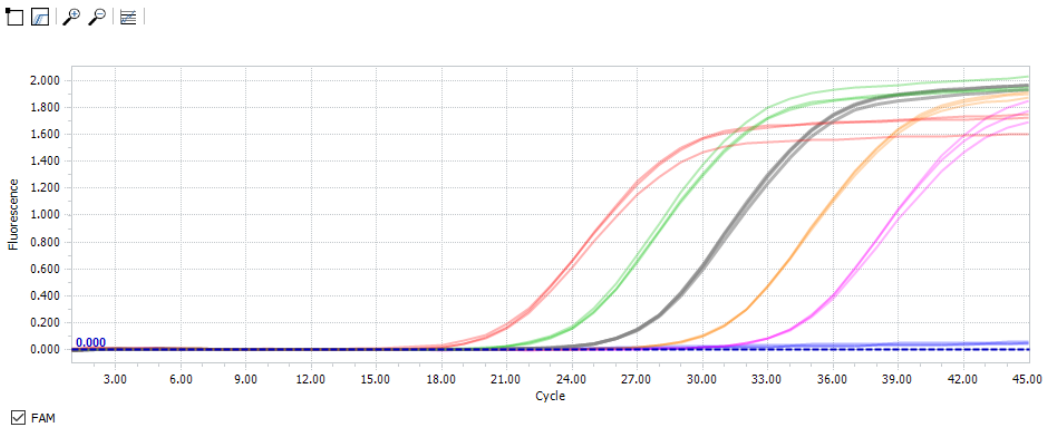


**Fig. 1**: The amplification diagram shows the result for the target gene HSPA2. The amplification curves shown were obtained from human genomic DNA from human blood 1,000 ng (far left) to 0.1 ng (far right) per well, including a no template control (blue flat line). Singleplex qPCR with three replicates for each dilution was performed in a reaction volume of 20 µl per well.

# 4. Troubleshooting

| Observation | Possible cause | Recommendation |
|---|---|---|
| Log-linear phase of amplification just starts as the cycling program ends. | Starting amount of nucleic acid is very low. | Improve PCR conditions, such as primer design. |
| | | Use more starting DNA template. |
| | | Repeat the run. |
| | Hold times in the cycling protocol are too short. | Optimize the run protocol by extending the hold times for annealing and elongation. |
| | The number of cycles is too low. | Increase the number of cycles in the cycle program. |
| No amplification detectable. | Wrong detection format. | Change the dye for the target. |
| | 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 protocol includes an initial pre-incubation step (95°C for 10 minutes). |
| | | Make sure denaturation time during amplification is 10 seconds. |
| | 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 Hydrolysis Probes, choose “Single” under Acquisition Mode (LightCycler® 96 Instrument) at the end of the annealing/elongation phase. |
| | Pipetting errors or omitted reagents. | Check for missing or defective reagents. |
| | Difficult template, for example, unusually 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.) |
| | Amplicon length is too long. | Do not design primers that produce amplicons >200 bp. |
| Fluorescence intensity varies. | Some of the reagent is still in the upper part of the reaction vessel, or an air bubble is trapped in the reaction vessel. | Repeat centrifugation, but allow sufficient centrifugation time, for example, 0.5 to 2 minutes at 1,500 × *g*, for all reagents to reach the bottom of the reaction vessel and/or to expel air bubbles. |
| | Skin oils or dirt are present on the lid or sealing foil. | Always wear gloves. |

| | | |
|---|---|---|
| Fluorescence intensity is very low. | Wrong Optics Settings in the Run Settings folder. | Check Optics Settings for proper selection prior to each run. |
| | Poor PCR efficiency (reaction conditions not optimized). | Check concentrations of reagents and probes. |
| | | Optimize protocol. |
| | | Always run a positive control along with your samples. |
| | 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. |
| | | ⚠ ***Avoid repeated freezing and thawing.*** |
| Negative control sample gives a positive signal. | Contamination | Remake all critical solutions. |
| | | Pipette 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. |
| | Probe quality is poor. | Prepare a new probe solution. |
| High standard deviation of Cq values. | Impure, heterogeneous DNA template. | Make sure PCR included a pre-incubation step at 95°C for 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 plate 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

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. Hydrolysis probe chemistry uses the so-called FRET principle. Fluorescence Resonance Energy Transfer (FRET) is based on the transfer of energy from one fluorophore (the donor or reporter) 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 (Holland, PM. et al., 1991). 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® 96 Instrument is factory calibrated for the following commonly used reporter dyes for hydrolysis probes: FAM, VIC, HEX, Yellow 555, LightCycler® Red 610, Texas Red, and Cy5. These labeled hydrolysis probes can be used separately or in combination, which permits up to four-color (LightCycler® 96 Instrument) detection. There is no need for color compensation/calibration runs.

*For multicolor hydrolysis probe assays, use dark quencher dyes, that is, dye molecules which efficiently quench the fluorescence of a FRET reporter dye without emitting fluorescence themselves. For example, use BHQ-2 (quenching range 550 to 650 nm) for all hydrolysis probe reporter dyes listed above.*

*Color compensation is automatically performed (all analysis data are color compensated).*

### How this product works

FastStart Essential DNA Probes Master is a ready-to-use reaction mix specifically developed for the hydrolysis probe detection format in LightCycler® 480 Multiwell Plates 96, or 8-Tube Strips on the LightCycler® 96 Instrument. It contains FastStart Taq DNA Polymerase for hot start PCR, which significantly improves the specificity and sensitivity of PCR by minimizing the formation of nonspecific amplification products (Chou, Q., et al., 1992, Kellogg, D.E., et al., 1994, Birch, D.E., 1996).
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, 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.
- Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing 5′ → 3′ the exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A. 1991;16:7276-7280.
- 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.


## 5.3. Quality Control

The FastStart Essential DNA Probes 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”.
Chapter Results has been included.

## 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® 8-Tube Strips (white) | 10 x 12 white strips and clear caps | 06 612 601 001 |
| LightCycler® 480 Sealing Foil | 50 foils | 04 729 757 001 |
| 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 | | |
| LightCycler® 480 RNA Master Hydrolysis Probes | 1 kit, 5 x 100 reactions of 20 µl final volume each | 04 991 885 001 |
| FastStart Essential DNA Green Master | 1 kit, 500 reactions of 20 µl final volume each | 06 402 712 001 |
| | 1 kit, 10 x 500 reactions of 20 µl final volume each | 06 924 204 001 |
| 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 |
| 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 |

## 6.4. Trademarks

FASTSTART, MAGNA PURE and LIGHTCYCLER are trademarks of Roche.
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.

Visit **documentation.roche.com**, to download or request copies of the following Materials:
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- Information Material

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