For life science research only.
Not for use in diagnostic procedures.

Print Protocol | Print Changes to Previous Version | Print

FastStart Universal Probe Master (Rox)

Version: 08
Content version: October 2017

2x concentrated, ready-to-use hot start master mix for qPCR and qRT-PCR using the hydrolysis probe detection format on real-time PCR instruments (except the LightCycler® Instruments)

Cat. No. 04 913 949 001	2 x 1.25 ml 100 reactions of 50 µl final volume each
Cat. No. 04 913 957 001	10 x 1.25 ml 500 reactions of 50 µl final volume each
Cat. No. 04 914 058 001	10 x 5 ml 2,000 reactions of 50 µl final volume each

Store at −15 to −25°C

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function	Catalog Number	Content
1	colorless	FastStart Universal Probe Master (Rox)	Ready-to-use 2x master mix	04 913 949 001	2 vials, 1.25 ml each
				04 913 957 001	10 vials, 1.25 ml each
				04 914 058 001	10 vials, 5 ml each

1.2. Storage and Stability

Storage Conditions (Product)

When stored at −15 to −25°C, this product is stable through the expiration date printed on the label.

Vial / Bottle	Cap	Label	Storage
1	colorless	FastStart Universal Probe Master (Rox)	For short-term storage (up to 3 months), store product at +2 to +8°C. ⚠ ***Keep the FastStart Universal Probe Master (ROX) away from light.*** ⚠ ***Avoid repeated freezing and thawing.*** ⚠ ***The complete PCR mix (i.e., FastStart Universal Probe Master supplemented with primers, probe, and template) is stable for up to 24 hours at +15 to +25°C. Keep the PCR mix away from light!***

1.3. Additional Equipment and Reagents Required

Standard Laboratory Equipment

- Nuclease free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile reaction tubes for preparing PCR mixes and dilutions
- Standard benchtop microcentrifuge

For cDNA Synthesis

- Transcriptor First Strand cDNA Synthesis Kit*

For Real-Time PCR

- PCR reaction vessels (*e.g.,* transparent PCR tubes or PCR microplates)
- Sequence-specific primers
- Hydrolysis probe (*e.g.,* from the Universal ProbeLibrary*)
- Water, PCR Grade

For Prevention of Carryover Contamination (optional)

- LightCycler® Uracil-DNA Glycosylase*

1.4. Application

The FastStart Universal Probe Master (ROX) is a ready-to-use, 2x concentrated master mix that contains all the reagents (except primers, probe, and template) needed for running quantitative, real-time DNA detection assays, including qPCR and 2-step qRT-PCR, in the hydrolysis probe detection format. It contains a special ROX reference dye which makes it suitable for all real-time instruments on which a ROX reference dye is needed for quantitative analysis.
In combination with a real-time PCR instrument, suitable PCR primers, and hydrolysis probe, FastStart Universal Probe Master (ROX) allows very sensitive detection and quantification of defined DNA sequences.

⚠ ***Do not use this product on the LightCycler® Instruments.***

2. How to Use this Product

2.1. Before you Begin

Sample Materials

- Use any template DNA (*e.g.*, genomic or plasmid DNA, cDNA) suitable for PCR in terms of purity, concentration, and absence of inhibitors.
- Use up to 250 ng complex genomic DNA or 50 ng cDNA.

For reproducible isolation of nucleic acids, we recommend:
- Either a MagNA Pure System together with a dedicated nucleic acid isolation kit (for automated isolation)
- or a High Pure nucleic acid isolation kit (for manual isolation).

⚠ ***Store the template DNA either in water, PCR Grade or 5 – 10 mM Tris-HCl (pH 7.5 – 8.0). Avoid dissolving the template in TE buffer because EDTA chelates Mg^{2+}.***

Control Reactions

To detect DNA contamination, always include a negative control in each run. To prepare this control, replace template DNA with water, PCR Grade.

Primers

Use PCR primers at a final concentration of 0.3 – 1.0 µM. The recommended starting concentration is 0.9 µM each.

⚠ ***Always use equimolar primer concentrations.***
⚠ ***If you are using probes from the Universal ProbeLibrary*, use at least 200 nM of each primer.***

ⓘ *The design of the PCR primers determines amplicon length, melting temperature, amplification efficiency, and yield. Primer design may also depend on the choice of PCR program (2-step versus 3-step protocol).*

Several programs for primer design are freely available or provided by the suppliers of real-time PCR instruments (e.g., PrimerExpress). Alternatively, such programs are available to the public on the web for free. For example, use the free online ProbeFinder software (www.universalprobelibrary.com) to design primers that may be paired with probes from the Universal ProbeLibrary*.

Probe

The probe concentration should be significantly lower than the primer concentration. As a starting point, we recommend using 250 nM probe. However, suitable concentrations range from 100 nM to 300 nM.

⚠ ***To ensure a specific and sensitive assay, the probe must anneal to the DNA at a significantly higher temperature than the primers. Therefore, the Tm of the probe should be 68 – 70°C and the Tm of the primers about 58 – 60°C.***
⚠ ***For maximum assay sensitivity, avoid placing a terminal G at the 5' end of the probe because the fluorescent signal (arising after cleavage of the probe) is adversely affected by such a terminal G.***

To ensure that the fluorescent reporter dye within the unreacted probe is quenched, the length of the probe should not exceed 28 nucleotides.
If you use probes from the Universal ProbeLibrary, start with a probe concentration of 100 nM and keep the probe concentration lower than the primer concentration. Set the annealing temperature to 60°C.

General Considerations

The optimal reaction conditions (concentration of template DNA and PCR primers, incubation temperatures and times, cycle number) depend on the specific template/primer system and must be determined individually.

Reaction Volume

Various reaction volumes of the FastStart Universal Probe Master (ROX) can be used. Please refer to recommendations from the supplier of the instrument for suitable volumes and tubes/plates.

ROX Reference Dye

In principle, real-time PCR instruments except the LightCycler® Instruments offer three different modes:
- Detection of released signal in relationship to a reference dye (usually ROX).
- Detection of released signal in relationship to the quencher dye of the probe (*e.g.,* TAMRA).
- Detection of released signal alone.

The choice of mode depends on the instrument (*e.g.,* whether a channel for detecting the reference dye is available) and on the light source of the instrument (halogen versus laser).
The FastStart Universal Probe Master (ROX) is supplemented with ROX reference dye and is proven to run on Applied Biosystems (ABI) instruments (PRISM 7000 Sequence Detection System, the ABI 7300 Real-Time PCR System, the ABI 7500 Real-Time PCR System, the ABI 7700 Real-Time PCR System, and the ABI PRISM 7900HT Sequence Detection System) as well as on the Stratagene Mx3000P QPCR System without the need to adjust the ROX concentration.

If you do not want to use the reference channel of your real-time PCR instrument or the instrument is not equipped with a reference channel, use the FastStart TaqMan® Probe Master (without ROX).*

If you use the Bio-Rad iCycler iQ5 Real-Time PCR Detection System, use the FastStart TaqMan® Probe Master (without ROX) and apply the External Well Factor Plate procedure for determining the well factors. For details on how to perform the External Well Factor Plate procedure, consult the Bio-Rad iCycler iQ5 Real-Time PCR Detection System Instruction Manual.*

Two-Step RT-PCR

FastStart Universal Probe Master (ROX) 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 real-time PCR instrument. Subsequent amplification and online monitoring is performed according to the standard real-time PCR 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.

2.2. Protocols

Preparation of PCR Master Mix

For each 50 µl reaction, prepare the following reaction mix:

1 – Thaw the solutions and, for maximum recovery of the contents, briefly spin vials in a microcentrifuge before opening.
– Mix solutions carefully by pipetting them up and down, then store on ice.

⚠ ***If you are using the 5 ml vials of the FastStart Universal Probe Master (ROX), put the vial on a roller incubator for 1 min.***

2 Prepare 100x conc. solutions of the PCR primers and the hydrolysis probe.

3 In a 1.5 ml reaction tube on ice, prepare the PCR mix for one 50 µl reaction by adding the following components in the order listed below.

Reagent	Volume[1]	Final conc.
FastStart Universal Probe Master (ROX)	25 µl	1x
Hydrolysis probe (25 µM)	0.5 µl	250 nM
Forward primer (90 µM)	0.5 µl	900 nM
Reverse primer (90 µM)	0.5 µl	900 nM
Water, PCR Grade	18.5 µl	
Total Volume	**45 µl**	

4 –Mix the solution carefully by pipetting up and down. Do not vortex.
– Pipet 45 µl PCR mix into each PCR reaction vessel or well of a PCR microplate (depending on your real-time PCR instrument).

5 –Add 5 µl of template DNA (up to 250 ng total DNA or 50 ng cDNA) .

In initial experiments to determine the optimum amount of cDNA template, we recommend running undiluted, 1:10 diluted, and 1:100 diluted cDNA template in parallel.

– Mix carefully by pipetting up and down.

6 According to the instructions supplied with your instrument, prepare the tubes or microplates for PCR (*e.g.,* seal tubes with optical tube caps or the plate with self-adhesive foil).

[1] To prepare the PCR mix for more than one reaction, multiply the amounts in the “Volume” column by z, where z = the number of reactions to be run + one additional reaction.

Performing PCR

There are several different ways to program the PCR. Either two-step or three-step PCR programs will provide suitable experimental results. The amplicon should be short (approx. 150 bp) and the annealing/elongation temperature should be +60°C (*e.g.,* a typical PCR protocol is 40 cycles of +95°C/15 s, followed by +60°C/1 min).

For best results, be sure the instrument is calibrated correctly.The table below shows an example of a standard PCR protocol.

If you want to perform qPCR in 20 µl reactions on an instrument equipped with a FastPlate (e.g., the ABI 7500 Fast Real-Time PCR System or ABI PRISM 7900HT Fast Sequence Detection System), apply the Hold Time given in brackets. This will reduce cycling time to about 1 hour.

1. Following the Operator's Manual of your instrument supplier, program the instrument with the following parameters:

Cycles	Analysis Mode	Target Temperature	Hold Time	Remarks
1 (optional)	None	50°C	2 min	Only if UNG has been added for carryover prevention.
1	None	95°C	10 min	Activation of FastStart Taq DNA Polymerase
40	None	95°C	15 s (10 s[(1)])	Amplification and real-time analysis
	Quantification	60°C	1 min (30 s[(1)])	

2. Place your tubes or plate in the instrument and start the reaction.

3. At the end of the reaction, follow instrument instructions for quantification/analysis.

(1) Hold Time when applying a fast PCR protocol

2.3. Other Parameters

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 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.

dUTP is a component of the FastStart Universal Probe Master (ROX).

Perform prevention of carryover contamination with LightCycler® Uracil-DNA Glycosylase*. Add 1.25 – 2.5 U per 50 µl PCR reaction. Proceed as described in the Instructions for Use.

3. Troubleshooting

Observation	Possible cause	Recommendation
No amplification detectable and no band in gel analysis	Error in PCR program (*e.g.,* activation step omitted).	Adjust PCR program.
	Pipetting errors (*e.g.,* DNA not added).	Repeat experiment; check pipetting steps carefully.
	Amplicon too long.	Redesign primers.
	Inhibitory effects of impurities.	Repeat isolation of template.
	Bad primer design.	Redesign primer.
No or low amplification detectable but strong band in gel analysis	PCR is working but the probe is poorly designed.	Redesign probe.
Fluorescence varies within a run	Instrument not correctly calibrated.	Recalibrate instrument.
	Variations in pipetting.	Monitor the channel in which ROX is detected.
High background in the negative (no template) control	Contamination	Remake or replace critical solutions (*e.g.,* water).
		Clean lab bench.
		Use UNG to prevent carryover contamination.

4. Additional Information on this Product

4.1. Test Principle

The FastStart Universal Probe Master (ROX) can be used for the amplification and detection of any DNA or cDNA target, including those that are GC-rich or GC-poor. However, you would need to adapt your detection protocol to the reaction conditions of the particular real-time PCR instrument in use, and design a specific hydrolysis probe and PCR primers for each target. See the Operator's Manual of your real-time PCR instrument for general recommendations.

⚠ ***The mix is designed for optimal amplification of targets up to 500 bp long. Do not use the mix to amplify longer targets.***

FastStart Universal Probe Master (ROX) offers convenience and ease-of-use because
– adjustment of ROX concentration is not required in order to achieve the same performance on different real-time PCR instruments which need ROX for quantitative analysis.
– the addition of $MgCl_2$ to the reaction mixture is not necessary, thus avoiding time-consuming optimization steps.

The mix contains dUTP so that it may be used with LightCycler® Uracil-DNA Glycosylase to prevent false positives arising from carryover contamination (i.e., contamination with amplified DNA).*

The FastStart Universal Probe Master (ROX) is fully compatible with the real-time PCR probes of the Universal ProbeLibrary.*

FastStart Taq DNA Polymerase

The FastStart Universal Probe Master (ROX) contains the FastStart Taq DNA Polymerase for hot start PCR to improve specificity and sensitivity of the PCR by minimizing the formation of nonspecific amplification products (Chou, Q. et al., 1992; Kellogg, D.E. et al., 1994). This enzyme delivers excellent results thanks to its special enzyme design and optimized buffer system.
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, 10 min) before cycling begins. Activation does not require the extra handling steps typical of other hot start techniques.

Detection of PCR Products

Real-time DNA detection assays based on the hydrolysis probe format (also known as 5'-nuclease assays) require a single, signal-generating probe that contains two labels, a fluorescent reporter dye at the 5' end and a (fluorescent or dark) quencher label at or near the 3' end (Holland, P. M. et al., 1991). When the probe is intact, the fluorescent signal is almost completely suppressed by the quenching label. When the probe is hybridized to its target sequence, it is cleaved by the 5'→3' exonuclease activity of the FastStart Taq DNA Polymerase, which "unquenches" the fluorescent reporter dye. During each PCR cycle, more of the released fluorescent dye accumulates, boosting the fluorescent signal.

If you use the hydrolysis probe format for detection, you cannot perform a subsequent melting curve analysis. For melting curve analysis, we recommend using the FastStart Universal SYBR Green Master.*

4.2. References

- Chou Q, Russell M, Birch DE, Raymond J, Bloch W - Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications (1992) *Nucleic Acids Research* **7**, 1717-1723
- 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 (1991) *Proc Natl Acad Sci U S A* **16**, 7276-7280
- 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 (1994) *BioTechniques* **16** (6), 1134-1137

4.3. Quality Control

Each lot is tested for performance in qPCR using three templates: a GC-rich template, a GC-poor template, and a long template (about 440 bp).

5. Supplementary Information

5.1. 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.

5.2. Changes to previous version

In the previous version the section **Probe** was missing. This section is now included again.
Editorial changes.

5.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents , kits		
LightCycler® Uracil-DNA Glycosylase	50 µl, 100 U, (2 U/µl)	03 539 806 001
Universal ProbeLibrary Extension Set, Probes #91 to #165	1 set, 75 Universal ProbeLibrary probes, 125 µl, 10 µM, each.	04 869 877 001
Universal ProbeLibrary Set, Human	1 set, 90 Universal ProbeLibrary probes, 125 µl, 10 µM, each	04 683 633 001
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
High Pure PCR Template Preparation Kit	1 kit, up to 100 purifications	11 796 828 001
FastStart SYBR Green Master	5 ml, 4 x 1.25 ml, 200 reactions of 50 µl final volume each	04 673 484 001
	50 ml, 10 x 5 ml, 2,000 reactions of 50 µl final volume each	04 673 492 001
FastStart TaqMan® Probe Master	2 x 1.25 ml, 100 reactions of 50 µl final volume each	04 673 409 001
	10 x 1.25 ml, 500 reactions of 50 µl final volume each	04 673 417 001
	10 x 5 ml, 2,000 reactions of 50 µl final volume each	04 673 433 001
FastStart Universal SYBR Green Master (Rox)	4 x 1.25 ml, 200 reactions of 50 µl final volume each	04 913 850 001
	10 x 5 ml, 2,000 reactions of 50 µl final volume each	04 913 914 001

5.4. Trademarks

FASTSTART, HIGH PURE, LIGHTCYCLER, MAGNA PURE and TAQMAN are trademarks of Roche.
SYBR is a trademark of Thermo Fisher Scientific Inc..
All third party product names and trademarks are the property of their respective owners.

5.5. License Disclaimer

For patent license limitations for individual products please refer to: **List of LifeScience products**

5.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

5.7. Safety Data Sheet

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

5.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support** Site.

To call, write, fax, or email us, visit **sigma-aldrich.com** and select your home country. Country-specific contact information will be displayed.

1017.05015839001®

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany