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FastStart Essential DNA Probes Master

Version 02

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

Cat. No. 06 402 682 001

5 × 1 ml
(5 × 100 reactions, 20 µl each)

Store the kit at −15 to −25°C

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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	Use	Content
1 red cap	FastStart Essential DNA Probes Master; 2× conc.	▪ Ready-to-use hot start PCR mix ▪ Contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), and $MgCl_2$	▪ 5 vials, 1 ml each (2× conc.)
2 colorless cap	FastStart Essential DNA Probes Master; H_2O, PCR grade	▪ To adjust the final reaction volume	▪ 5 vials, 1 ml each

Storage and Stability

Store the kit at -15 to -25°C until the expiration date printed on the label.

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

Label	Storage
FastStart Essential DNA Probes Master; 2× conc.	▪ Store at -15 to -25°C. ▪ After first thawing, the master may be stored for up to 4 weeks at +2 to +8°C. ▪ Avoid repeated freezing and thawing.
FastStart Essential DNA Probes Master; H_2O, PCR grade	▪ Store at -15 to -25°C.

The complete PCR mix (*i.e.,* FastStart Essential DNA Probes 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!.

Additional Equipment and Reagents Required

Additional equipment and reagents required to perform reactions with the FastStart Essential DNA Probes Master using the LightCycler® Nano System include:

- LightCycler® Nano Instrument*
- LightCycler® 8-Tube Strips (clear)*
- Standard swing-bucket centrifuge containing a rotor for multiwell plates with suitable adaptor
- LightCycler® Uracil-DNA Glycosylase* (optional ‡)
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile 1.5 ml reaction tubes for preparing master mixes and dilutions

‡ for prevention of carryover contamination, see Related Procedures section for details.

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

Application

The FastStart Essential DNA Probes Master is designed for research studies on the LightCycler® Nano System. The FastStart Essential DNA Probes Master is a ready-to-use hot start reaction mix designed specifically for detecting DNA targets with hydrolysis probes during LightCycler® Nano System PCR, allowing very sensitive detection and quantification of defined DNA sequences as well as for endpoint genotyping analysis. The kit may also be used in other types of PCR on the LightCycler® Nano 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® Nano Instrument, and specific PCR primers and probes must be designed for each target.

⚠ The amplicon size should not exceed 200 bp in length.

⚠ The performance of the kit described in this Instruction Manual is tested and performance guaranteed on the LightCycler® Nano System.

Assay Time

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

For example, if the cycling program specifies 45 cycles and an annealing time of 30 seconds, a LightCycler® Nano PCR run will last about 56 minutes, including 10 minutes pre-incubation time.

2. How to Use this Product

2.1 Before You Begin

Sample Material

- Use any template DNA (*e.g.,* genomic or plasmid DNA, cDNA) suitable for PCR, as long as it is sufficiently pure, concentrated, and free of PCR inhibitors.

⚠ For reproducible isolation of nucleic acids use:

- the MagNA Pure LC Instrument*, or the MagNA Pure 96 Instrument*, or the MagNA Pure Compact Instrument*, and a dedicated MagNA Pure nucleic acid isolation kit (for automated isolation), or
- a High Pure Nucleic Acid Isolation Kit* (for manual isolation)
- RealTime ready Cell Lysis Kit* (for lysing cells prior to two-step real-time RT-PCR applications).

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

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

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

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.

Probes

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

The optimal probe concentration is the lowest concentration that results in the lowest 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 T_m of the probe should be only slightly higher than the T_m of the PCR primer, so the hybridization complex is stable. Furthermore, the probe sequence must account for mismatches in the DNA template, since these will also affect the annealing temperature.

$MgCl_2$

The reaction mix in this kit already contains an optimal concentration of $MgCl_2$, which works with nearly all primer combinations.

You do not need to adjust the $MgCl_2$ concentration to amplify different sequences.

2.2 Experimental Protocol

LightCycler® Nano Instrument Protocol

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

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

A LightCycler® Nano Instument 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® Nano System Guides.

The following table shows the PCR parameters that must be programmed for a LightCycler® Nano System PCR run with the FastStart Essential DNA Probes Master using a LightCycler® 8-Tube Strip. The hold times shown here are a robust protocol (standard protocol) and can be shortened depending on your assay design[1)].

Setup			
Run Settings			
Optics Settings			
Hydrolysis Probes		Normal Quality	
Profile			
Programs			
Temp. (°C)	Ramp (°C/s)	Hold (s)	Acquire
Hold			
95	4	600 [3]	
2-Step Amplification			
No. of Cycles: 45			
95	5	20 [1] [4]	
60 primer dependent [2]	4	40 [1] [4]	✓

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 10-30 seconds. 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 (fast protocol).

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

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.

⚠ Always wear gloves during handling.

❶ ▪ Thaw one vial of "FastStart Essential DNA Probes Master" (Vial 1, red cap) and Water, PCR grade (Vial 2, colorless cap).
▪ 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.

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

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

Component	Volume
Water, PCR grade (Vial 2, colorless cap)	3 µl
Primer-probe mix [1], 10× conc.	2 µl
Master Mix, 2× conc. (Vial 1, red cap)	10 µl
Total Volume	**15 µl**

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

⚠ To prepare the PCR mix for more than one reaction, multiply the amount in the "Volume" column above by z, where z = the number of reactions to be run + sufficient additional reactions.

❹ ▪ Mix carefully by pipetting up and down. Do not vortex.
▪ Pipet 15 µl PCR mix into each reaction vessel of a LightCycler® 8-Tube Strip (clear).
▪ Add 5 µl of the DNA template.
▪ Close the reaction vessels.

❺ ▪ For secure centrifugation, place the tube strips into a standard multiwell plate 96 (MWP) and balance them in the centrifuge.
▪ Centrifuge at 3,000 × *g* for a maximum of 30 seconds (in a standard swing-bucket centrifuge with suitable adapter).

❻ Load the reaction vessels into the LightCycler® Nano Instrument.

❼ Start the PCR program described above.

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

2.3 Related Procedures

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 in the Instruction for Use for the enzyme.

Two-Step RT-PCR

The 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® Nano System. Subsequent amplification and online monitoring is performed according to the LightCycler® Nano 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, we recommend running 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 curve is obtained by using the FastStart Essential DNA Probes Master in combination with the β-Actin RealTime ready Assay No. 1007903, targeting mouse β-Actin mRNA. The intensity in relative fluorescence units (RFU) versus cycle number is displayed (see Figure 1).

2-step protocol (95°C 10 minutes; 45 × 95°C 10 seconds, 60°C 10 seconds)

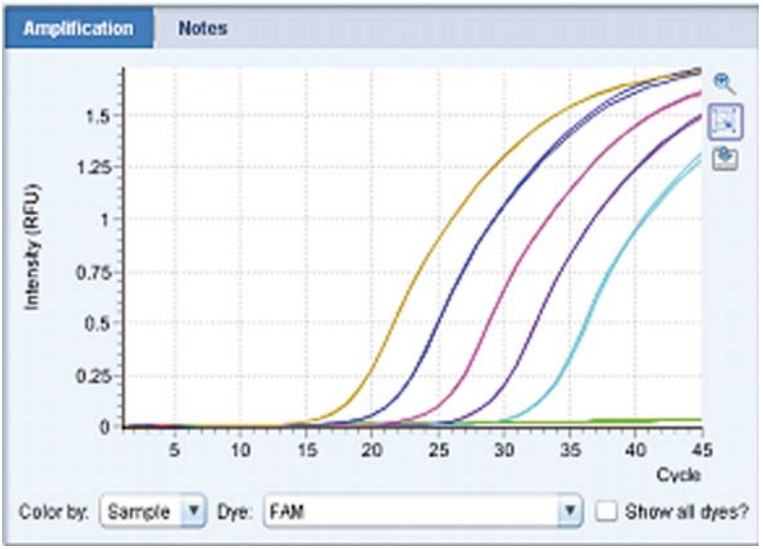

Fig. 1: Serially diluted samples containing cDNA derived from 50 ng, 5 ng, 500 pg, 50 pg, and 5 pg of total mouse RNA as starting template were amplified using the FastStart Essential DNA Probes Master. As negative control, template cDNA was replaced by PCR grade water.

4. Troubleshooting

	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 (*e.g.*, 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 under Samples - Targets.
	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 “Acquire” at the end of the annealing/elongation phase.
	Pipetting errors or omitted reagents.	Check for missing or defective reagents.
	Amplicon length is too long.	Do not design primers that produce amplicons >200 bp.
	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.)

	Cause	Recommendation
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 (*e.g.*, 30 seconds at 3,000 × *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 surface of the reaction vessel or lid.	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 (*e.g.,* Hydrolysis Probes, Normal Quality).
	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 and avoid repeated freezing and thawing.
Negative control sample gives a positive signal	Contamination	▪ Remake all critical solutions. ▪ Pipet reagents on a clean bench. ▪ Use UNG to eliminate carryover contamination.
High background	Fluorescence signals are very low, therefore the background seems relatively high.	Follow general strategies for optimizing PCR runs in the LightCycler® Nano System.
	Probe quality is poor.	Prepare a new probe solution.
High standard deviation of crossing point (Cp) values	Impure, heterogenous 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	Tubes not sealed properly.	Be sure to seal the tubes correctly.
	Cycler is loaded unbalanced.	If you use only two strips, be sure to load the 8-well strips balanced in row A and D.

5. Additional Information on this Product

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® 8-Tube Strips or single tubes on the LightCycler® Nano 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 (1, 2, 3, 4).

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 – 10 minutes) before cycling begins. Activation does not require the extra handling steps typical of other hot start techniques.

Test Principle

Sequence-specific detection of PCR products relies on sequence-specific oligonucleotide probes that are coupled to fluorophores. These probes hybridize to their complementary sequence in target PCR products. 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 (5). This single probe contains two labels, a fluorescent reporter and a quencher, in close proximity to each other. When the probe is intact, the quencher dye is close enough to the reporter dye to suppress the reporter fluorescent signal (fluorescent quenching takes place via FRET). During PCR, the 5´-nuclease activity of the polymerase cleaves the hydrolysis probe, separating the reporter and quencher. The reporter dye is no longer quenched and emits a fluorescent signal when excited.

The LightCycler® Nano 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 either single- or dual-color detection. There is no need for color compensation/calibration runs.

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

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

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** (7), 1717-1723.

2 Kellogg, DE *et al.* (1994) TaqStart Antibody: hot-start PCR facilitated by a neutralizing monoclonal antibody directed against Taq DNA polymerase. *BioTechniques* **16** (6), 1134-1137.

3 Birch, DE *et al.* (1996) Simplified hot start PCR. *Nature* **381** (6581), 445-446.

4 PCR Manual, Roche Diagnostics (1999) 2nd edition. **2**, 52-58.

5 Holland, PM. *et al.* (1991) Detection of specific polymerase chain reaction product by utilizing the 5´->3´ exonuclease activity of Thermus aquaticus DNA polymerase. *Proc. Natl. Acad. Sci. USA.* **88** (16), 7276-7280.

Quality Control

The FastStart Essential DNA Probes Master is function tested using the LightCycler® Nano Instrument.

6. Supplementary Information

6.1 Conventions

Text Conventions

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 document, 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

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6.3 Ordering Information

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

- Real-Time PCR Systems (LightCycler® Carousel-Based System, LightCycler® Nano System, LightCycler® 480 System, LightCycler® 1536

System, Universal ProbeLibrary, and RealTime ready): http://www.lightcycler.com

- Automated Sample Preparation (MagNA Lyser Instrument, MagNA Pure Compact System, MagNA Pure LC, and MagNA Pure 96 System): http://www.magnapure.com
- DNA & RNA Preparation - Versatile Tools for Nucleic Acid Purification: http://www.roche-applied-science.com/napure
- RealTime ready Cell Lysis Kit: http://www.gene-expression.roche.com
- RealTime ready qPCR Assays with pre-tested UPL probes: http://www.realtimeready.roche.com

	Product	Pack Size	Cat. No.
Instrument and Software	LightCycler® Nano Instrument	1 instrument (including USB stick with software)	06 407 773 001
Accessories	LightCycler® 8-Tube Strips (clear)	120 strips – including caps	06 327 672 001
PCR Reagents	FastStart Essential DNA Green Master	1 kit (5 × 100 reactions, 20 µl each)	06 402 712 001
	FastStart Essential DNA Probes Master	1 kit (5 × 100 reactions, 20 µl each)	06 402 682 001
Starter Packs	LightCycler® Nano DNA Green Starter Pack	LightCycler® Nano Instrument, 8 packs of Fast Start Essential DNA Green Master, 4 packs of strips	06 444 199 001
	LightCycler® Nano DNA Probes Starter Pack	LightCycler® Nano Instrument, 8 packs of Fast Start Essential DNA Probes Master, 4 packs of strips	06 444 202 001
Value Packs	LightCycler® Nano DNA Green Value Pack S	2 packs of Fast Start Essential DNA Green Master, 1 pack of strips	06 444 229 001
	LightCycler® Nano DNA Green Value Pack L	4 packs of Fast Start Essential DNA Green Master, 2 packs of strips	06 444 172 001
	LightCycler® Nano DNA Probes Value Pack S	2 packs of Fast Start Essential DNA Probes Master, 1 pack of strips	06 444 164 001

	LightCycler® Nano DNA Probes Value Pack L	4 packs of Fast Start Essential DNA Probes Master, 2 packs of strips	06 444 156 001
Universal ProbeLibrary	Universal ProbeLibrary Set, Human	Library of 90 pre-tested detection probes	04 683 633 001
	Universal ProbeLibrary Set, Mouse	Library of 90 pre-tested detection probes	04 683 641 001
	Universal ProbeLibrary Set, Rat	Library of 90 pre-tested detection probes	04 683 650 001
	Universal ProbeLibrary Extension Set	Library of 75 pre-tested detection probes (probes #91 to #165)	04 869 877 001
RealTime ready Assays	RealTime ready Catalog Assay		05 532 957 001
	RealTime ready Designer Assay		05 583 055 001
Associated Kits and Reagents	LightCycler® 480 RNA Master Hydrolysis Probe	1 kit (5 × 100 reactions, 20 µl each)	04 991 885 001
	LightCycler® Uracil-DNA Glycosylase	100 U (50 ml)	03 539 806 001
	Transcriptor Reverse Transcriptase	250 U 500 U 2,000 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
	Transcriptor Universal cDNA Master	100 reactions	05 893 151 001

6.4 Disclaimer of License

NOTICE: This product may be subject to certain use restrictions. Before using this product please refer to the Online Technical Support page (http://technical-support.roche.com) and search under the product number or the product name, whether this product is subject to a license disclaimer containing use restrictions.

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