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1-Tube Probe RT-qPCR Master Mix (2x)

REAL TIME PCR KIT

Kit Components:

- The **Probe Enzyme Mix** contains a unique highly sensitive reverse transcriptase, Hot Start Perpetual *Taq* DNA Polymerase, and RNase Inhibitor, respectively.
- **RT-PCR Probe Buffer (2x)** is a universal reaction buffer with dNTPs (dTTP is partially replaced with dUTP) that can be used with most real-time PCR cyclers available.
- **Thermolabile Uracil-N-Glycosylase** (UNG),
- **nuclease-free water**.

Probe 1-Tube RT-qPCR Master Mix (2x)

Component	**Cat. No.** E0812-01 25 reactions, 25 µl each, 625 µl [1x] final volume	**Cat. No.** E0812-02 100 reactions, 25 µl each, 2.5 ml [1x] final volume
1-Step Probe Enzyme Mix	25 µl	100 µl
RT-qPCR Probe Buffer (2x)	1 x 350 µl	2 x 0.7 ml
Thermolabile UNG (Uracil-N-Glycosylase) 1 U/µl	10 µl	30 µl
Water, nuclease free	1 x 0.5 ml	2 x 1.0 ml

Probe 1-Tube RT-qPCR Master Mix (2x) plus ROX Solution

Component	**Cat. No.** E0813-01 100 reactions, 25 µl each, 2.5 ml [1x] final volume	**Cat. No.** E0813-02 200 reactions, 25 µl each, 5 ml [1x] final volume
1-Step Probe Enzyme Mix	25 µl	100 µl
RT-qPCR Probe Buffer (2x)	1 x 350 µl	2 x 0.7 ml
ROX Solution, 25 µM	15 µl	60 µl
Thermolabile UNG (Uracil-N-Glycosylase) 1 U/µl	10 µl	30 µl
Water, nuclease free	1 x 1.25 ml	2 x 1 ml

Storage:
Store at -20˚C in the dark.

Probe OneStep RT-qPCR kit is a one-step RT-qPCR kit for providing accurate real-time quantification of RNA targets in gene expression analysis, using dual-labeled probes. The kit contains a unique reverse transcriptase and the highly processive "Hot Start" onTaq DNA Polymerase in an easy-to-use master mix format.

Description:

- 2 x RT-qPCR Probe Buffer is a universal solution for quantitative RT-qPCR one tube reaction using sequence-specific probes and can be used on most real-time PCR cyclers available.
- The Probe Enzyme Mix contains unique highly sensitive reverse transcriptase, hot start on*Taq* DNA Polymerase, and RNase Inhibitor .
- Reverse transcriptase works in a high range of temperatures from 35-55˚C without loss of specificity and sensitivity.
- Both cDNA synthesis and PCR are performed in a single tube using gene-specific primers and either total RNA or mRNA.
- onTaq DNA Polymerase is a modified „hot start" enzyme which provides very tight inhibition of the polymerase activity at moderate temperatures which is restored during the initial denaturation step at 95˚C for at least ten minutes.
- 2 x RT-qPCR Probe Buffer contains dUTP, which partially replaces dTTP. It allows the optional use of thermolabile uracil -N-glycosylase (UNG) to prevent carryover contamination between reactions. UNG removes uracil from any dU-containing contaminating amplicons at moderate temperatures and is inacivated during RT step at 50˚C.
- There are two variants of the kit: without ROX and with ROX Solution provided separately. The use of ROX passive reference dye is necessary for all real-time PCR cyclers from Applied Biosystems and optional for cyclers from Stratagene. ROX compensates for variations of fluorescent signal between wells due to slight differences in reaction volume and fluorescence fluctuations. ROX is not involved in PCR reaction and does not interfere with real-time PCR on any instrument. Refer to the table below to determine the recommended amount of ROX (25 µM) required for a specific PCR cycler.

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REAL TIME PCR PROTOCOL (1)

qPCR- Protocol

Recommended amounts of ROX for a specific real-time PCR cycler

Instrument	Amount of ROX per 25 µl reaction	Final ROX concentration
Applied Biosystems: 7300, 7900HT, StepOne, StepOnePlus, ABI PRISM 7000 and 7700	0.5 µl	500 nM
Applied Biosystems: 7500, ViiA 7 Stratagene: Mx3000P, Mx3005P, Mx4000	0.5 µl 10 x diluted (in water)	50 nM
PCR machines from other manufacturers: Bio-Rad, Roche, Corbett, Eppendorf, Cepheid, etc.	Not required	-

Preparation of PCR Reaction:

Component	Volume/Reaction	Final Concentration
RT-qPCR Probe Buffer (2x)	12.5 µl	1 x 3 mM $MgCl_2$
Forward Primer	Variable	0.4 µM
Reverse Primer	Variable	0.4 µM
Probe	Variable	0.1 - 0.2 µM
Template RNA	Variable	1 µg - 500 ng
Optional: ROX Solution, 25 µM	0.5 µl or 0.5 µl 10 x diluted	500 nM 50 nM
Optional: Thermolabile UNG (uracil-N-glycosylase) 1 U/µl	0.25 µl	0.25 U / reaction
Probe Enzyme Mix	1 µl	1 µl / reaction
Water, nuclease free	To 25 µl	-
Total volume	25 µl	-

Notes:

1. **Minimize freeze-thaw cycles** of RT-qPCR Probe Buffer. Always keep Master Probe Enzyme Mix, fluorescent probes and ROX solution on ice and minimize light exposure during handling to avoid losses in fluorescent signal intensity.
2. **Concentration Differences.** Completely thaw and gently mix RT-qPCR Probe Buffer (2x) before use.
3. **Reaction Volume.** A reaction volume of 25 µl is recommended for most real-time cyclers. Other reaction volumes may be used if recommended for a specific instrument.
4. **Amplicon Length.** The optimal amplicon length in real-time PCR using Probes is 70-150 bp.
5. **Exon-Exon Primers.** To avoid amplification from contaminating genomic DNA, design exon-exon primers. For primers designed for *H.sapiens* RNA, free online software is available: http://primerdepot.nci.nih.gov/
6. **Setup On Ice.** Set up RT- PCR reactions on ice to minimize any RNA template degradation.
7. **Prepare a reaction master mix** by adding all the reaction components except template RNA. The RNA template (<500 ng/reaction) should be added to the individual PCR tubes or wells containing the whole reaction mix. Centrifuge briefly before placing the reactions into the cycler. Check if there remain no residual air bubbles. In case of any remaining air bubbles, repeat the centrifugation step. Air bubbles interfere with fluorescent detection.
8. **Start.** Place the samples in the cycler and start the program.
9. **RT Temperature Range**. The reverse transcriptase works in a broad range of temperatures between 35˚C and 55˚C. The recommended starting temperature for reverse transcription is 50 ˚C. For individual experimental requirements, the RT incubation temperature temperature might be changed.
10. **$MgCl_2$.** The standard concentration of $MgCl_2$ in real-time PCR reactions is 3.0 mM (as provided with the RT-qPCR Probe buffer). In most cases this concentration will yield optimal results. However, if higher $MgCl_2$ concentrations are required, prepare a 25 mM $MgCl_2$ stock solution (or request us to ship an aliquot along with your order) and add an appropriate amount to the reaction. Adding 1 µl of a 25 mM $MgCl_2$ solution to a total reaction volume of 25 µl will add 25 nmol $MgCl_2$ and thus increase total $MgCl_2$ reaction concentration in 1.0 mM.
11. **Primer Concentration.** A final primer concentration of 0.4 µM is usually optimal, but can be individually optimized in a range of 0.1 µM to 1 µM. The recommended starting concentration is 0.4 µM. Raising primer concentration may increase PCR efficiency, but negatively affects PCR specificity. The optimal primer concentration depends on the individual reaction and the real-time PCR cycler used.
12. **Melting Temperature.** Optimal melting temperature (Tm) of primers should be near 60˚C. The Tm of dual-labeled probes should be 8-10˚C higher than the Tm of the primers.
13. **Readjust the threshold value** for analysis of every run.
14. **Well Factors.** When using a Bio-Rad iCycler iQ or MyiQ instruments collect well factors at the beginning of each experiment. Use an external well factor plate according to the manufacturer's recommendations. Well factors are used to compensate for any excitation or pipetting variations.

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REALTIME PCR PROTOCOL (2)

RT-qPCR- Protocol - Thermal Cycling Conditions

Thermal Cycling Conditions:

Step	Tempera-ture	Time	Number of Cycles
Reverse Transcription	50˚C	20 min	1
Initial Denaturation	95˚C	15 min	1
Denaturation, Annealing, Extension, Data acquisition	94˚C 60˚C	15 s 60 s	40-45
Cooling	4˚C	Infinite	1

Notes:

1. **Thermolabile Uracil-N-Glycosylase (UNG).** During the reverse transcription step at 50˚C, thermolabile uracil-N-glycosylase might be used for protection of carryover contamination (e.g. by aerosol formation) with Uracil-labelled qPCR products from previously performed analyses. Don't use UNG from *E.coli*, which may degrade any newly synthesized cDNA.
2. **QC by Agarose Gel Electrophoresis.** Always check the PCR product specificity by gel electrophoresis when designing a new assay. Melting temperatures of the specific product and primer-dimers may overlap.

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