Probe Multiplex OneStep RT-qPCR kit

Probe Multiplex OneStep RT-qPCR kit is one-step RT-qPCR kit that provides accurate real-time quantification of RNA targets in gene expression analysis, using dual-labeled probes. Kit is composed of unique reverse transcriptase and highly processive hot start tiTaq DNA Polymerase in easy to use format.

Probe Multiplex OneStep RT-qPCR kit

Kit Components

Component	Cat. No. E0819-01 100 reactions of 25 µl	Cat. No. E0819-02 500 reactions of 25 µl
4 x Buffer Mix *	650 µl	5 x 650 µl
Enzyme Mix	100 µl	500 µl
RNase-free Water	1500 µl	5 x 1500 µl

Storage:

Store at -20°C.

*Avoid repeated thawing and freezing (>4), as this may reduce assay sensitivity. Freeze the component in aliquots if they only be used intermittently.

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The kit provides:

- 4 x Buffer Mix 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 Enzyme Mix contains unique highly sensitive reverse transcriptase, hot start tiTaq 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.
- tiTaq 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 2-5 minutes.
- 4 x Buffer Mix contains dNTPs.
- 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.

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	-

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Protocol:

Component	Volume/reaction	Final concentration
4 x Buffer Mix	6.5 µl	1 x 4 mM $MgCl_2$
Forward Primer	Variable	0.2-0.4 µM
Reverse Primer	Variable	0.2- 0.4 µM
Probe	Variable	0.1-0.2 µM
Template RNA	Variable	1pg-500 ng
Enzyme Mix	1 µl	1 µl /reaction
RNase-free Water	To 25 µl	-
Total volume	25 µl	-

Notes:

1. Keep Enzyme Mix on ice, limit light exposure during handling to avoid loss of fluorescent signal intensity. Minimize thaw-freeze cycles of 4 x Buffer Mix.
2. Thaw and gently mix by pippeting 4 x Buffer Mix before use.
3. A reaction volume of 25 µl should be used with most real-time cyclers. Other reaction volumes may be used if recommended for a specific instrument.
4. Optimal amplicon length in real-time RT-PCR using probes is 70-150 bp.
5. To avoid amplification from genomic DNA design exon-exon primers.
6. Set up RT-PCR reactions on ice to minimize RNA template degradation.
7. 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 into cycler. Check if there are no bubbles left, if yes, spin again.
8. Place the samples in the cycler and start the program.
9. Reverse transcriptase works in a wide range of temperatures 35-55°C. The recommended temperature for reverse transcription is 50°C. For the individual experiment temperature might be changed.
10. Standard concentration of $MgCl_2$ in real-time RT-PCR reaction is 4 mM (as provided with the 1 x Buffer Mix in most cases this concentration will produce optimal results. However, if a higher $MgCl_2$ concentration is required, prepare a 25 mM $MgCl_2$ stock solution and add to the reaction.
11. A final primer concentration of 0.4 µM is usually optimal, but can be individually optimized in the 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 affect RT-PCR specificity. Optimal primer concentration depends on the individual reaction and the real-time PCR cycler used.
12. Optimal melting temperature (T_m) of primers should be near 60°C. The T_m of dual-labeled probes should be 8-10°C higher than the T_m of the primers.
13. Readjust the threshold value for analysis of every run.
14. Avoid G at the 5'-end of the dual-labeled probe, which causes quenching of fluorescence signal.

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Thermal Cycling Conditions:

Step	Temperature	Time	Number of Cycles
Reverse Transcription	50°C	15 min	1
Initial Denaturation	95°C	2 min	1
Denaturation Annealing/Extension/Data acquisition	95°C 60°C	10 s 40-60 s	40-45
Cooling	4°C	Indefinite	1

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