EURx® MOLECULAR BIOLOGY PRODUCTS

Probe qPCR Master Mix (2x)

Kit Components

Component	Cat. No. E0420-01 100 reactions of 25 µl	Cat. No. E0420-02 200 reactions of 25 µl	Cat. No. E0420-03 1000 reactions of 25 µl
Probe qPCR Master Mix (2x)	1 x 1.25 ml	2 x 1.25 ml	10 x 1.25 ml
UNG (uracil-N-glycosylase) 1 U/µl	30 µl	55 µl	270 µl
Water, nuclease free	1 x 1.25 ml	2 x 1.25 ml	10 x 1.25 ml

Probe qPCR Master Mix (2x), plus ROX Solution

Kit Components

Component	Cat. No. E0421-01 100 reactions of 25 µl	Cat. No. E0421-02 200 reactions of 25 µl	Cat. No. E0421-03 1000 reactions of 25 µl
Probe qPCR Master Mix (2x)	1 x 1.25 ml	2 x 1.25 ml	10 x 1.25 ml
ROX Solution, 25 µM	55 µl	110 µl	530 µl
UNG (uracil-N-glycosylase) 1 U/µl	30 µl	55 µl	270 µl
Water, nuclease free	1 x 1.25 ml	2 x 1.25 ml	10 x 1.25 ml

Storage

Store at -20°C in the dark for long-term storage or at 4°C for up to 2 weeks.

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Description

- Probe qPCR Master Mix (2x) is a universal solution for quantitative real-time PCR and two-step real time RT-PCR using sequence-specific probes and can be used on most real-time PCR cyclers available.
- The master mix contains onTaq DNA Polymerase, optimized reaction buffer, dNTPs (dTTP is partially replaced with dUTP).
- onTaq DNA Polymerase is a modified „hot start" enzyme that is blocked at moderate temperatures and allows room temperature reaction setup. The polymerase activity is restored during 15 min initial denaturation step at 95°C.
- Use of the "hot start" enzyme prevents extension of misprimed products and primer-dimers during reaction setup leading to higher specificity and sensitivity of PCR reactions.
- Probe qPCR Master Mix (2x) contains dUTP, which partially replaces dTTP. It allows the optional use of a uracil-N-glycosylase (UNG) to prevent carryover contamination between reactions. UNG removes uracil from any dU-containing contaminating amplicons, leaving abasic sites and making DNA molecules susceptible to hydrolysis during the initial denaturation step.
- 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	Amount of ROX per	Final ROX concentration
Applied Biosystems: 7300, 7900HT, StepOne, StepOnePlus, ABI PRISM	0.5 µl	50 µl	500 nM
Applied Biosystems: 7500, ViiA 7, Stratagene: Mx3000P, Mx3005P,	0.5 µl 10 x diluted (in water)	50 µl 10 x diluted (in water)	50 nM
PCR machines from other manufacturers: Bio-Rad, Roche, Corbett, Eppendorf, Cepheid, etc.	Not required	Not required	-

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Protocol

Preparation of PCR Reaction:

Component	Volume/reaction	Final concentration
Probe qPCR Master Mix (2x)	12.5 µl	1 x 4 mM $MgCl_2$
Forward Primer	Variable	0.5 µM
Reverse Primer	Variable	0.5 µM
Probe	Variable	0.2 µM
Template DNA	Variable	≤500 ng
Optional: ROX Solution, 25 µM	0.5 µl or 0.5 µl 10 x diluted	500 nM 50 nM
Optional: UNG (uracil-N-glycosylase) 1 U/µl	0.25 µl	0.25 U/reaction
Water, nuclease free	To 25 µl	-
Total volume	25 µl	-

Notes:

1. 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.
2. Optimal amplicon length in real-time PCR using probes is 70-150 bp.
3. Thaw, gently vortex and briefly centrifuge all solutions.
4. Set up PCR reactions at room temperature. Use of Probe qPCR Master Mix (2x) allows room temperature reaction setup.
5. Prepare a reaction master mix by adding all the reaction components except template DNA.
6. Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
7. Add template DNA/cDNA (≤500 ng/reaction) to the individual PCR tubes or wells containing the reaction mix. For two-step RT-PCR, the volume of cDNA added should not exceed 10% of the final PCR volume.
8. Centrifuge briefly to settle down the reaction components and remove bubbles. Bubbles interfere with fluorescent detection.
9. Place the samples in the cycler and start the program.
10. $MgCl_2$ concentration provided with the 1 x Probe qPCR Master Mix is 4 mM. 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 a reaction.
11. A final primer concentration of 0.4-0.5 µM is usually optimal, but can be individually optimized in range of 0.4 µM to 1 µM. The recommended starting concentration is 0.5 µM. Raising primer concentration may increase PCR efficiency, but negatively affect 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. Avoid G at the 5'-end of the dual-labeled probe, which causes quenching of fluorescence signal.
14. Readjust the threshold value for analysis of every run.

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Thermal Cycling Conditions:

2-step cycling

Step	Temperature	Time	Number of Cycles
Optional: UNG pre-treatment	50°C	2 min	1
Initial Denaturation	95°C	15 min	1
Denaturation Annealing/Extension	94°C 60°C	15 s 60 s	35-50
Cooling	4°C	Indefinite	1

3-step cycling

Step	Temperature	Time	Number of Cycles
Optional: UNG pre-treatment	50°C	2 min	1
Initial Denaturation	95°C	15 min	1
Denaturation Annealing/ Extension	94°C 50–60°C 72°C	15 s 30 s 30 s	35-50
Cooling	4°C	Indefinite	1

Notes:

1. The incubation step of 50°C for 2 minutes must be added if a uracil-N-glycosylase is used to prevent carryover contamination. UNG degrades any dUMP-containing PCR products.
2. During the initial denaturation step onTaq DNA Polymerase is activated and UNG is inactivated. onTaq DNA polymerase requires 15 min incubation at 95°C to be activated.
3. UNG activity may be partially restored at temperatures lower than 50°C due to refolding. After completing the PCR, cool reactions to 4°C and load directly on a gel or store frozen.
4. It is recommended to check the PCR product specificity by gel electrophoresis when designing a new assay.

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