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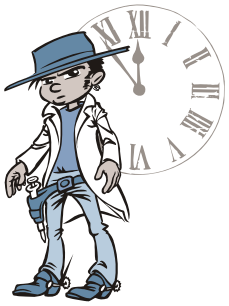
TRIPLE M

M³ Multiplex Master Mix Probe qPCR (2x)

REAL TIME PCR KIT FOR LABELED PROBES



Kit Components:

Probe qPCR Master Mix (2x)

Component	Cat. No. E0424-01 100 reactions, 25 µl each, 2.5 ml [1x] final volume	Cat. No. E0424-02 200 reactions, 25 µl each, 5 ml [1x] final volume	Cat. No. E0424-03 1.000 reactions, 25 µl each, 25 ml [1x] final volume
M³ Multiplex Probe qPCR Master Mix (2x) (MP qPCR Master Mix)	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

Storage:
Store at -20°C in the dark for long-term storage or at 4°C for up to 1 month.

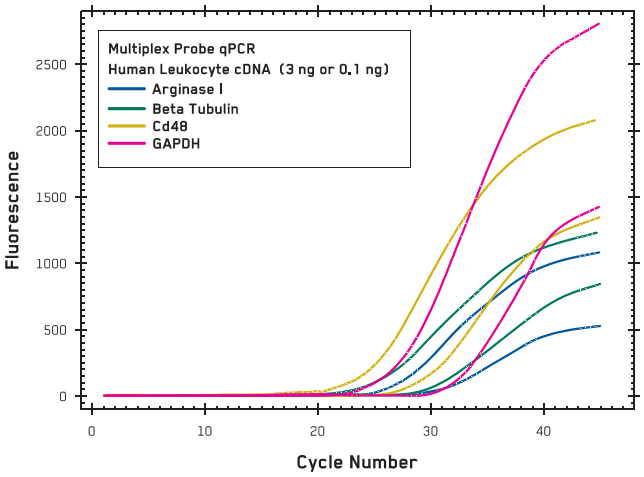


Figure 1: Probe-based real-time PCR plus UNG pretreatment with EURx M³ Multiplex Master Mix - Probe qPCR (= MP Probe qPCR Master Mix), specific primers and fluorescent labeled probes binding within the amplicon (FAM, HEX, Texas Red, Cy5). Reactions were performed by using human leukocyte cDNA (3 ng or 0.1 ng) as template. Measured PCR efficiency was greater than 85%.

Description:

- M³ Multiplex Master Mix - Probe qPCR (2x) is a universal solution for quantitative multiplex real-time PCR and two-step real-time RT-PCR and is compatible with most real-time PCR cyclers available.
- Depending on the real-time cycler used, up to 5 targets can be quantified simultaneously in the same well or tube.
- The master mix contains Perpetual *Taq* DNA Polymerase, optimized reaction buffer, and dNTPs (dTTP is partially replaced with dUTP).
- Perpetual *Taq* DNA Polymerase contains a recombinant *Taq* DNA Polymerase bound to anti-*Taq* monoclonal antibodies that block polymerase activity at moderate temperatures.
- The polymerase activity is restored during the initial denaturation step, when amplification reactions are heated at 95°C for at least two minutes.
- 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.
- The polymerase enables convenient reaction setup at room temperature.
- M³ Multiplex Master Mix - Probe qPCR contains dUTP, which partially replaces dTTP. It allows the optional use of an 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 required 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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M³ Multiplex Master Mix Probe qPCR (2x)

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	0.3-0.5 µl	300-500 nM
Applied Biosystems: 7500 Stratagene: Mx3000P, Mx3005P, Mx4000	0.3-0.5 µl 10 x diluted (in water)	30-50 nM
PCR machines from other manufacturers: Bio-Rad, Roche, Corbett, Eppendorf, Cepheid, etc.	Not required	-

Preparation of PCR Reaction:

Component	Volume/Reaction	Final Concentration
MP Probe qPCR Master Mix (2x)	12.5 µl	1 x
20 x primer-probe mix 1	1,25 µl	0.2 µM forward primer 1 0.2 µM reverse primer 1 0.2 µM probe 1
20 x primer-probe mix 1	1,25 µl	0.2 µM forward primer 2 0.2 µM reverse primer 2 0.2 µM probe 2
Optional: 20 x primer-probe mix 3 and 4	1,25 µl each	0.2 µM forward primer 3 and 4 0.2 µM reverse primer 3 and 4 0.2 µM probe 3 and 4
Template DNA	Variable	500 ng
Optional: ROX Solution, 25 µM	0.3-0.5 µl or 0.3-0.5 µl 10 x diluted	300-500 nM 30-50 nM
Optional: Thermolabile 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. **Minimize Light Exposure.** Minimize exposure of ROX to light during handling to avoid loss of fluorescent signal intensity.
2. **Recommended Reaction Volume.** 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.
3. **The optimal amplicon length** in real-time PCR using probes is 70-200 bp.
4. **Mix Before Use.** Thaw, gently vortex and briefly centrifuge all solutions.
5. **Setup at Room Temperature.** Set up PCR reactions at room temperature. Use of M³ Multiplex Master Mix - Probe qPCR (2x) allows room temperature reaction setup.
6. **Prepare a reaction master mix** by adding all the reaction components except template DNA.
7. **Mix and Dispense.** Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
8. **Add Template DNA.** 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.
9. **Remove Air Bubbles.** Centrifuge briefly to settle down the reaction components and remove bubbles. Bubbles interfere with fluorescent detection.
10. **Start.** Place the samples in the cycler and start the program.
11. **$MgCl_2$ Concentration.** The standard concentration of $MgCl_2$ in real-time PCR reactions is 3.0 mM (as provided with the 1 x Probe qPCR Master 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 (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.
12. **Primer Concentration.** A final primer concentration of 0.2 µM is usually optimal, but can be individually optimized in a range of 0.2 µM to 0.4 µM. The recommended starting concentration is 0.2 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.
13. **Probe Concentration.** A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and the purification method used, the optimal probe concentration may range between 0.1 µM and 0.4 µM, respectively.
14. **Optimal Melting Temperature.** The 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.
15. **Avoid G at the 5'-end** of the dual-labeled probe, which causes quenching of fluorescence signal.

M^3 Multiplex Master Mix
Probe qPCR (2x)
REALTIME PCR PROTOCOL (2)

qPCR- Protocol - Thermal Cycling Conditions

Thermal Cycling Conditions:

Step	Tempera-ture	Time	Number of Cycles
Optional: UNG pre-treatment	37˚C	2 min	1
Initial Denaturation	95˚C	5 min	1
Denaturation Annealing / Extension	95˚C 60˚C	15 s 90 s	40-50
Cooling	4˚C	Indefinite	1

Notes:

1. **2-step cycling protocol.** M3 Multiplex Master Mix - Probe qPCR has been developed for use in a two-step cycling protocol. The supplied protocol works well for most primers (even for primers with a Tm well below 60 ˚C).
2. **UNG Incubation Step (Optional).** An incubation step of 50˚C for 2 minutes must be added if uracil-N-glycosylase (UNG) is used to prevent carryover contamination. UNG degrades any dUMP-containing PCR products.
3. **UNG / Anti-*Taq* Antibody Heat Inactivation.** During the initial denaturation step UNG and antibodies that block *Taq* DNA Polymerase are inactivated.
4. **Agarose Gel Check During Assay Development.** It is recommended to check the PCR product specificity by gel electrophoresis when designing a new assay. Melting temperatures of the specific product and primer-dimers may overlap (see figure 2).

Figure 2: Agarose gel analysis of multiplex real-time PCR using EURx M^3 Multiplex Master Mix - qPCR Probe (as shown in figure 1).
Lane 1: EURx Perfect 100 bp DNA Ladder (Cat. No. E3134),
Lanes 2,3: Duplicate reactions from 3 ng of human leukocyte cDNA.
Bands:
104 bp and 108 bp (as one band) - CD48 and GAPDH, respectively,
131 bp - beta-tubulin,
149 bp - arginase I.
Bands appear in similar, comparable intensities, with defined, clear morphologies, with sharp, distinct boundaries, without odd migration behavior such as blurs or smears and without any sign of PCR artifact formation.