

QuantiTect® Multiplex PCR NoROX Kit

The QuantiTect Multiplex PCR NoROX Kit (cat. nos. 204741, 204743 and 204745) should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light. 2x QuantiTect Multiplex PCR NoROX Master Mix can also be stored protected from light at 2 – 8°C for up to 6 months, depending on the expiration date.

Further information

- *QuantiTect Multiplex PCR Handbook*: www.qiagen.com/HB-0881
- Safety Data Sheets: www.qiagen.com/safety
- Technical assistance: support.qiagen.com

Notes before starting

- This protocol is optimized for quantification of gDNA or cDNA targets in a multiplex format, using TaqMan®/hydrolysis probes with real-time cyclers that do not require ROX passive reference dye (i.e., LightCycler® 2.0, LightCycler 480 and cyclers from Bio-Rad, Cepheid, Eppendorf and Agilent/Stratagene). Using this protocol, multiplex PCR is carried out without any ROX dye in the reaction.
- 2x QuantiTect Multiplex PCR NoROX Master Mix contains dUTP, which allows the use of a uracil-N-glycosylase (UNG) pretreatment of the reaction if contamination with carried-over PCR products is suspected.
- We recommend preparing a 20x primer–probe mix for each target containing target-specific primers and probe (see Tables 1 and 2).
- For information on suitable combinations of dyes for multiplex PCR using various cyclers, please refer to the *QuantiTect Multiplex PCR Handbook*.
- For multiplex analyses, we strongly recommend using dual-labeled probes with nonfluorescent quenchers.
- The PCR must start with an initial incubation step of 15 min at 95°C to activate HotStarTaq® DNA Polymerase.



- Be sure to create a color compensation file for LightCycler 2.0 and LightCycler 480. For details, download QIAGEN® Supplementary Protocol PCR81 and PCR82 at www.qiagen.com/literature.
1. Thaw 2x QuantiTect Multiplex PCR NoROX Master Mix, template DNA or cDNA, primer and probe solutions and RNase-free water. Mix the individual solutions, and place them on ice.
 2. Prepare a reaction mix according to Table 1 (duplex PCR using cyclers other than LightCycler 2.0) or Table 2 (duplex, triplex or 4-plex PCR using the LightCycler 2.0, or triplex or 4-plex PCR using other cyclers).

Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiTect Multiplex PCR NoROX Master Mix. For a very limited number of targets, reactions may be improved by increasing the final Mg^{2+} concentration by 0.5–1 mM.

Table 1. Reaction setup for duplex PCR on cyclers other than LightCycler 2.0

Component	Volume/reaction
Reaction mix	
2x QuantiTect Multiplex PCR NoROX Master Mix	25 μ l
20x primer–probe mix 1*	2.5 μ l
20x primer–probe mix 2*	2.5 μ l
RNase-free water	Variable
Optional: Uracil-N-glycosylase†	Variable (0.5 units/reaction)
Template gDNA or cDNA (added at step 4)	Variable (\leq 500 ng/reaction)
Total reaction volume	50 μ l‡

* **IMPORTANT:** For duplex PCR using cyclers other than LightCycler 2.0 that do not require ROX passive reference dye, a 20x primer-probe mix consists of 8 μ M forward primer, 8 μ M reverse primer and 4 μ M probe in TE buffer, resulting in a final concentration of 0.4 μ M forward and reverse primer and 0.2 μ M probe.

† The activity of uracil-N-glycosylase may differ between suppliers. Depending on your supplier, the optimal concentration may be 0.25–1 units per 50 μ l reaction.

‡ If your real-time cycler requires a final reaction volume other than 50 μ l, adjust the amount of 2x QuantiTect Multiplex PCR NoROX Master Mix and all other reaction components accordingly. If using 384-well plates on the LightCycler 480, use a reaction volume of 10 μ l.

Table 2. Reaction setup for duplex, triplex and 4-plex PCR on the LightCycler 2.0 and triplex and 4-plex PCR on other cyclers

Component	Volume/reaction	
	Duplex, triplex and 4-plex PCR on LightCycler 2.0	Triplex and 4-plex PCR on other cyclers*
Reaction mix		
2x QuantiTect Multiplex PCR NoROX Master Mix	10 µl	25 µl
20x primer–probe mix 1 [†]	1 µl	2.5 µl
20x primer–probe mix 2 [†]	1 µl	2.5 µl
Only for triplex PCR:		
20x primer–probe mix 3 [†]	1 µl	2.5 µl
Only for 4-plex PCR:		
20x primer–probe mix 4 [†]	1 µl	2.5 µl
RNase-free water	Variable	Variable
Optional: Uracil-N-glycosylase [‡]	Variable (0.2 units/reaction)	Variable (0.5 units/reaction)
Template gDNA or cDNA (added at step 4)	Variable (≤200 ng)	Variable (≤500 ng)
Total reaction volume	20 µl [§]	50 µl [¶]

* LightCycler 480 and other real-time cyclers that do not require ROX passive reference dye (i.e., from Bio-Rad, Cepheid, Eppendorf and Agilent/Stratagene).

[†] **IMPORTANT:** For duplex, triplex and 4-plex PCR using LightCycler 2.0 and triplex and 4-plex PCR using other cyclers, a 20x primer-probe mix consists of 4 µM forward primer, 4 µM reverse primer and 4 µM probe in TE buffer, resulting in a final concentration of 0.2 µM forward primer, reverse primer and probe.

[‡] The activity of uracil-N-glycosylase may differ between suppliers. Depending on your supplier, the optimal concentration may be 0.25–1 units per 50 µl reaction.

[§] If using capillaries for 100 µl reaction volumes, adjust the amount of 2x QuantiTect Multiplex PCR NoROX Master Mix and all other reaction components accordingly.

[¶] If your real-time cycler requires a final reaction volume other than 50 µl, adjust the amount of 2x QuantiTect Multiplex PCR NoROX Master Mix and all other reaction components accordingly. If using 384-well plates on the LightCycler 480, use a reaction volume of 10 µl.

3. Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.

4. Add template gDNA or cDNA to the individual PCR tubes or wells.

Note: For two-step RT-PCR, the volume of cDNA (from the undiluted reverse-transcription reaction) added as template should not exceed 10% of the final PCR volume.

5. Program the real-time cycler according to Table 3.

Note: Check the real-time cycler's user manual for correct instrument setup for multiplex analysis (e.g., setting up detection of multiple dyes from the same well). Be sure to activate the detector for each reporter dye used. Depending on your instrument, it may also be necessary to perform a calibration procedure for each of the reporter dyes before they are used for the first time.

6. Place the PCR tubes or plate in the real-time cycler, and start the cycling program.
7. Perform data analysis.

Note: Before performing data analysis, select the analysis settings for each probe (i.e., baseline settings and threshold values). Optimal analysis settings are a prerequisite for accurate quantification data.

Table 3. Cycling conditions

Step	Time*	Temperature
Optional: UNG pretreatment (if UNG included in reaction mix)	2 min	50°C
PCR initial heat activation	15 min	95°C
2-step cycling:		
Denaturation	60 s	94°C
Annealing/extension		
Duplex PCR	60 s	60°C
Triplex and 4-plex PCR	90 s	60°C
Number of cycles	40–50†	

* If using a LightCycler 2.0, set ramp at 20°C/s.

† The number of cycles depends on the amount of template gDNA or cDNA and, for cDNA targets, the expression level of the target gene.



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