Quick-Start Protocol March 2016

QuantiTect® Multiplex RT-PCR NR Kit

The QuantiTect Multiplex RT-PCR NR Kit (cat. nos. 204843 and 204845) should be stored immediately upon receipt at –30 to –15°C in a constant-temperature freezer and protected from light. 2x QuantiTect Multiplex RT-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 RT-PCR Handbook*: **www.qiagen.com/HB-0156**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- This protocol is optimized for quantification of RNA targets for gene expression analysis 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 RT-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. Only heat-labile UNG should be used.
- We recommend preparing a 20x primer–probe mix for each target containing target-specific primers and probe (see Tables 1, 2 and 3).
- Set up all reactions on ice.
- After reverse transcription, the PCR must start with an initial incubation step of 15 min at 95°C to activate HotStarTaq® DNA Polymerase.
- For multiplex analyses, we strongly recommend using dual-labeled probes with nonfluorescent quenchers.

- For information on suitable combinations of dyes for multiplex PCR using various cyclers, refer to the *QuantiTect Multiplex RT-PCR Handbook*.
- Be sure to create a color compensation file for LightCycler 2.0 and LightCycler 480. For details, download QIAGEN® Supplementary Protocols PCR81and PCR82 at **www.qiagen.com/literature**.

1. Thaw 2x QuantiTect Multiplex RT-PCR NoROX Master Mix, template RNA, primer and probe solutions and RNase-free water. Mix the individual solutions, and place them on ice. QuantiTect Multiplex RT Mix should be taken from –30 to –15°C immediately before use, always kept on ice and returned to storage at –30 to –15°C immediately after use.
2. Prepare a reaction mix according to Table 1 (multiplex RT-PCR using the LightCycler 2.0), Table 2 (duplex RT-PCR using other real-time cyclers) or Table 3 (triplex or 4-plex RT-PCR using other real-time cyclers).

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiTect Multiplex RT-PCR NoROX Master Mix. For only a few targets, reactions may be improved by increasing the final Mg^{2+} concentration by 0.5–1 mM.
3. Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes, PCR capillaries or the wells of a PCR plate.
4. Add template RNA to the individual PCR tubes, capillaries or wells.
5. Program the real-time cycler according to Table 4. If performing UNG pretreatment, keep the samples on ice for at least 5 min.

   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).

   Note: If using the LightCycler 2.0 system, adjust **Seek Temperature** in the **Programs** tab to 50°C.
6. Place the PCR tubes, plates or capillaries 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 1. Reaction setup for multiplex RT-PCR on the LightCycler 2.0

Component	Volume/reaction
Reaction mix	
2x QuantiTect Multiplex RT-PCR NoROX Master Mix	10 µl
20x primer–probe mix 1*	1 µl
20x primer–probe mix 2*	1 µl
Only for triplex and 4-plex RT-PCR:	
20x primer–probe mix 3*	1 µl
Only for 4-plex RT-PCR:	
20x primer–probe mix 4*	1 µl
QuantiTect Multiplex RT Mix	0.2 µl
RNase-free water	Variable
Optional: Uracil-N-glycosylase, heat-labile	Variable (0.8 units/reaction)
Template RNA (added at step 4)	Variable (≤100 ng)
Total reaction volume	20 µl

* **IMPORTANT**: For duplex, triplex and 4-plex RT-PCR on LightCycler 2.0, 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.

Table 2. Reaction setup for duplex RT-PCR on other cyclers

Component	Volume/reaction
Reaction mix	
2x QuantiTect Multiplex RT-PCR NoROX Master Mix	25 µl
20x primer–probe mix 1*	2.5 µl
20x primer–probe mix 2*	2.5 µl
QuantiTect Multiplex RT Mix	0.5 µl
RNase-free water	Variable
Optional: Uracil-N-glycosylase, heat-labile	Variable (2 units/reaction)
Template RNA (added at step 4)	Variable (≤250 ng/reaction)
Total reaction volume	50 µl

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

Table 3. Reaction setup for triplex and 4-plex RT-PCR on other cyclers

Component	Volume/reaction
Reaction mix 2x QuantiTect Multiplex RT-PCR NoROX Master Mix	25 µl
20x primer–probe mix 1*	2.5 µl
20x primer–probe mix 2*	2.5 µl
20x primer–probe mix 3*	2.5 µl
Only for 4-plex RT-PCR: 20x primer–probe mix 4*	2.5 µl
QuantiTect Multiplex RT Mix	0.5 µl
RNase-free water	Variable
Optional: Uracil-N-glycosylase, heat-labile	Variable (2 units/reaction)
Template RNA (added at step 4)	Variable (≤250 ng)
Total reaction volume	50 µl

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

Table 4. Cycling conditions

Step	Time*	Temperature
Reverse transcription	20 min	50°C
PCR initial heat activation	15 min	95°C
2-step cycling: Denaturation	45 s	94°C
Annealing/extension Duplex RT-PCR	45 s	60°C
Triplex RT-PCR	75 s	60°C
4-plex RT-PCR	75 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 RNA and the expression level of the target gene.

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