Quick-Start Protocol March 2016

# QuantiTect® Multiplex RT-PCR Kit

The QuantiTect Multiplex RT-PCR Kit (cat. nos. 204643 and 204645) 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 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 from Applied Biosystems. Using this protocol, duplex, triplex or 4-plex RT-PCR is carried out in the presence of ROX passive reference dye, which is included in 2x QuantiTect Multiplex RT-PCR Master Mix.
- 2x QuantiTect Multiplex RT-PCR Master Mix contains dUTP, which allows the use of a uracil-N-glycosylase (UNG) pretreatment of the reaction. 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 and 2).
- For information on suitable combinations of reporter dyes for multiplex RT-PCR on various cyclers, please refer to the *QuantiTect Multiplex RT-PCR Handbook*.
- For multiplex analyses, we strongly recommend using dual-labeled probes with nonfluorescent quenchers.
- 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.

1. Thaw 2x QuantiTect Multiplex RT-PCR 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 for duplex RT-PCR and according to Table 2 for triplex and 4-plex RT-PCR.

   **Note**: We strongly recommend starting with the optimized $Mg^{2+}$ concentration provided by 2x QuantiTect Multiplex RT-PCR 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.
3. Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.

**Table 1. Reaction setup for duplex RT-PCR on Applied Biosystems® cyclers**

| Component | Volume/reaction |
|---|---|
| **Reaction mix** | |
| 2x QuantiTect Multiplex RT-PCR 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 on Applied Biosystems cyclers, 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.

† If your real-time cycler requires a final reaction volume other than 50 µl, adjust the amount of 2x QuantiTect Multiplex RT-PCR Master Mix and all other reaction components accordingly. If using 384-well plates on the ABI PRISM® 7900, use a reaction volume of 20 µl.

**Table 2. Reaction setup for triplex and 4-plex RT-PCR on Applied Biosystems cyclers**

| Component | Volume/reaction |
|---|---|
| **Reaction mix** | |
| 2x QuantiTect Multiplex RT-PCR 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/reaction) |
| **Total reaction volume** | 50 µl† |

* **IMPORTANT**: For triplex and 4-plex RT-PCR on Applied Biosystems 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.

† If your real-time cycler requires a final reaction volume other than 50 µl, adjust the amount of 2x QuantiTect Multiplex RT-PCR Master Mix and all other reaction components accordingly. If using 384-well plates on the ABI PRISM 7900, use a reaction volume of 20 µl.

4. Add template RNA (≤250 ng/50 µl reaction) to the individual PCR tubes or wells.

   **Note**: Keep the tubes or plate on ice until the real-time cycler is programmed.

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

**Note**: If using the Applied Biosystems 7500, it is necessary to adjust the preset threshold value to a lower value. Use a value of 0.01 as a starting point.

**Table 3. 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* | |

* The number of cycles depends on the amount of template RNA and the expression level of the target gene.

Scan QR code for handbook.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.