Quick-Start Protocol December 2016

QuantiNova® Multiplex RT-PCR Kit

The QuantiNova Multiplex RT-PCR Kit (cat. nos. 208552, 208554, 208556) should be stored immediately upon receipt at –15 to –30°C in a constant-temperature freezer and protected from light. QuantiNova Multiplex RT-PCR Master Mix can also be stored protected from light at 2–8°C for up to 12 months, depending on the expiration date.

Further information

- *QuantiNova Multiplex RT-PCR Kit Handbook*: **www.qiagen.com/HB-2313**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- This protocol is optimized for quantification of RNA targets in a multiplex format (up to 5-plex), using TaqMan® probes with any real-time cycler and condition for fluorescence normalization. ROX™ dye is required for various cyclers at the following concentrations:

  No requirement for ROX dye: Rotor-Gene®, Bio-Rad® CFX, Roche® LightCycler® 480 and Agilent® Technologies Mx instruments.

  Low concentration of ROX dye: Applied Biosystems® 7500, ViiA®7 and QuantStudio™ Real-Time PCR Systems.

  High concentration of ROX dye: ABI PRISM® 7000, Applied Biosystems 7300, 7900 and StepOne™ Real-Time PCR Systems.
- QuantiNova ROX Reference Dye should be used as a 20x concentrated solution for a 1x reaction when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, use the dye as a 200x concentrate.
- The reference dye in QuantiNova Yellow Template Dilution Buffer allows tracking of pipetted samples in the qRT-PCR. When template is added to the blue QuantiNova Multiplex RT-PCR Master Mix, the color changes from blue to green.

It is provided as a 100x concentrate and should be diluted (using water) to obtain a 1x final concentration within the sample*. The buffer does not affect sample stability or qPCR.

- For the highest efficiency in real-time RT-PCR using TaqMan probes, amplicons should ideally be 60–150 bp in length.
- Before performing multiplex analysis, choose suitable combinations of reporter dyes and quenchers that are compatible with multiplex analysis using the detection optics of your real-time cycler. We strongly recommend using dual-labeled probes with non-fluorescent quenchers.
- Always start with the cycling conditions and primer concentrations specified in this protocol.
- The RT-PCR must start with an initial reverse transcription step of 10 min at 50°C to transcribe the RNA into cDNA, followed by an incubation step of 2 min at 95°C to activate the QuantiNova DNA Polymerase and to inactivate the HotStaRT-Script Reverse Transcriptase.
- For ease of use, we recommend preparing a 20x primer–probe mix consisting of 16 µM forward primer, 16 µM reverse primer and 5 µM probe in TE buffer for each target.
- For information on suitable combinations of dyes for multiplex PCR using various cyclers, please refer to the *QuantiNova Multiplex RT-PCR Handbook*.
- The QuantiNova Internal Control RNA (QN IC RNA) is optionally used to test successful reverse transcription/amplification and to report instrument or chemistry failures, or the presence of inhibitors. It is detected as a 200 bp internal control (IC) in the yellow (Rotor-Gene Q) or in the VIC®/HEX dye channel (other real-time PCR instruments) when using the QuantiNova IC Probe Assay (cat. no. 205813). Alternatively, it is detected in the red (Rotor-Gene Q) or in the Cy®5 dye channel (other real-time PCR instruments) when using the QuantiNova IC Probe Assay Red 650 (cat. no. 205824). Before use, add 180 µl (or 900 µl) of RNase-free water to 20 µl (or 100 µl) of QN IC RNA provided in the kit and mix thoroughly by vortexing.

* Example: Add 0.5 µl Yellow Template Dilution Buffer to a 50 µl sample, which can be used as template in various PCR runs, regardless of the volume added to each reaction. Yellow Template Dilution Buffer can be pre-diluted using water. In this example, add 5 µl of 1:10 pre-diluted Yellow Template Dilution Buffer.

1. Thaw 4x QuantiNova Multiplex RT-PCR Master Mix, QuantiNova Yellow Template Dilution Buffer, template RNA, primers, probes, QN ROX Reference Dye (if required), and RNase-free water. Mix the individual solutions. Put QuantiNova Multiplex Reverse Transcription Mix on ice.
2. Prepare a reaction mix according to Table 1. Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

Table 1. Reaction mix setup

Component	96-well block, Rotor-Gene	384-well block	Final concentration
4x Multiplex RT-PCR Master Mix	5 µl	2.5 µl	1x
QN ROX Reference Dye (AB instruments only)	1 µl/0.1 µl*	0.5 µl/0.05 µl*	1x
100x Multiplex Reverse Transcription Mix	0.2 µl	0.1 µl	1x
20x primer–probe mix† (for each of up to 5 targets)	1 µl	0.5 µl	0.8 µM forward primer 0.8 µM reverse primer 0.25 µM TaqMan probe
QuantiNova IC Probe Assay 10x (optional)	2 µl	1 µl	1x
QN IC RNA (optional)	1 µl	1 µl	1x
RNase-Free Water	Variable	Variable	–
Template RNA (added at step 4)	Variable	Variable	≤800 ng/reaction
Total reaction volume	**20 µl**	**10 µl**	**–**

*Results in a 1:20 dilution for high ROX dye cyclers (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900 and StepOne Real-Time PCR Systems) and a 1:200 dilution for low-ROX dye cyclers (i.e., Applied Biosystems 7500 and ViiA7 Real-Time PCR Systems) in the final 1x reaction.

† A 20x primer–probe mix consists of 16 µM forward primer, 16 µM reverse primer, and 5 µM probe in TE buffer for each target. Primers and probes can either be pre-mixed and added simultaneously, or primer-probe mixes for each target can be added separately. If concentration of primer-probe mix(es) differ, the respective added volume needs to be adjusted, to achieve a final concentration of 0.8 µM for each primer and 0.25 µM for each probe.

3. Mix the reaction thoroughly and dispense appropriate volumes into PCR tubes or wells of a PCR plate.
4. Add template RNA (≤800 ng/reaction) to the individual PCR tubes or wells containing the reaction mix.
5. Program the real-time cycler according to Table 2.

Note: Data acquisition should be performed during the combined annealing/extension step.

Table 2. Cycling conditions

Step	Time	Temperature	Ramp rate
Reverse transcription step	10 min	50°C	Maximal/fast mode
PCR initial heat activation	2 min	95°C	Maximal/fast mode
2-step cycling			
Denaturation	5 s	95°C	Maximal/fast mode
Combined annealing/extension	30 s	60°C	Maximal/fast mode
Number of cycles	**40***		

* The number of cycles depends on the amount of template RNA.

6. Place the PCR tubes or plates 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.

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