QIAGEN Supplementary Protocol

Triplex and 4plex analysis using the QuantiTect® Virus +ROX Vial Kit and a 25 µl or 20 µl reaction volume

This protocol shows how to use the QuantiTect Virus +ROX Vial Kit to perform real-time PCR in a triplex or 4plex format with a 25 µl or 20 µl reaction volume. The protocol is intended for use with TaqMan® probes and the following real-time cyclers: Applied Biosystems® 7500, Applied Biosystems 7500 Fast, and instruments from Bio-Rad/MJ Research, Cepheid, Corbett, Roche, and Stratagene. When using an Applied Biosystems 7500 system, it is necessary to add ROX dye to the reaction mix. For details about the use of ROX dye, see "ROX passive reference dye" in the *QuantiTect Virus Handbook*.

IMPORTANT: Please consult the "Safety Information" and "Important Notes" sections in the *QuantiTect Virus Handbook* before beginning this procedure. For safety information on the additional chemicals mentioned in this protocol, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Equipment and reagents

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- QuantiTect Virus +ROX Vial Kit (cat. no. 211031, 211033, or 211035)
- Primers and probes: These should be purchased from an established oligonucleotide manufacturer. Primers should be of standard quality, and probes should be HPLC purified. Lyophilized primers and probes should be dissolved in TE buffer to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry (for details, see Appendix A in the *QuantiTect Virus Handbook*). Primer and probe stock solutions should be stored in aliquots at –20°C. Probe stock solutions should be protected from exposure to light.
- Nuclease-free (RNase/DNase free) consumables: Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive detection of viral nucleic acids. See Appendix C in the *QuantiTect Virus Handbook* for details about avoiding nucleases during PCR setup.
- Cooling device or ice
- Real-time thermal cycler
- PCR tubes or plates (use thin-walled PCR tubes or plates recommended by the manufacturer of your thermal cycler)

- Optional: Trizma® base and EDTA for preparing TE buffer for storing primers and probes (see Appendix A in the *QuantiTect Virus Handbook*). Use RNase/DNase-free water and plastic consumables to prepare TE buffer.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.** Please note that these cycling conditions differ from those described in the protocol for duplex assays (QIAGEN Supplementary Protocol PCR90). The cycling is optimized for PCR products between 60 and 150 bp. For PCR products >150 bp, different cycling conditions may improve results. For details, see Appendix A in the *QuantiTect Virus Handbook*.
- **Use the primer concentrations specified in this protocol.** Please note that these primer concentrations differ from those described in the protocol for duplex assays (QIAGEN Supplementary Protocol PCR90).
- We strongly recommend testing the performance of new primer–probe sets in individual assays before combining them in a multiplex assay.
- Read "Guidelines for effective multiplex assays" in the *QuantiTect Virus Handbook*. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established real-time, multiplex assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer-limiting concentrations again.
- **After reverse transcription, the PCR must start with an initial incubation step of 5 min at 95°C to activate HotStarTaq® *Plus* DNA Polymerase.**
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- For ease of use, we recommend preparing a 20x primer–probe mix for each of your targets containing target-specific primers and probe. See Appendix D in the *QuantiTect Virus Handbook*. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix E in the *QuantiTect Virus Handbook*.

Procedure

1. **Thaw QuantiTect Virus NR Master Mix, primer and probe solutions, RNase-free water, template nucleic acids (isolated viral nucleic acids), optional standards, and references. Mix the individual solutions.**

   Standards should be diluted in QuantiTect Nucleic Acid Dilution Buffer at an appropriate concentration to enable use of 2.5–5 µl per reaction.

2. **Prepare a reaction mix for the required number of reactions according to Table 1. It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.**

   Typically, reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep the reagents, samples, and controls on ice or in a cooling device.

   Note: For RT-PCR, QuantiTect Virus RT Mix should be taken from –15 to –30°C immediately before use, always kept on ice, and returned to –15 to –30°C immediately after use.

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

4. **Add template nucleic acids to the individual PCR tubes or wells and mix thoroughly.**

   Note: Ensure that the reaction mix and template are thoroughly mixed.

5. **Program the real-time cycler according to Table 2.**

Table 1. Reaction mix for triplex or 4plex analysis

	Volume per reaction		
Component	**25 µl***	**20 µl*†**	**Final concentration**
Reaction mix			
QuantiTect Virus NR Master Mix, 5x	5 µl	4 µl	1x
50x ROX Dye Solution‡	0.5 µl	0.4 µl	1x
20x primer–probe mix 1§	1.25 µl	1.0 µl	0.2 µM forward primer 1¶ 0.2 µM reverse primer 1¶ 0.2 µM probe 1**
20x primer–probe mix 2§	1.25 µl	1.0 µl	0.2 µM forward primer 2¶ 0.2 µM reverse primer 2¶ 0.2 µM probe 2**
20x primer–probe mix 3§	1.25 µl	1.0 µl	0.2 µM forward primer 3¶ 0.2 µM reverse primer 3¶ 0.2 µM probe 3**
For 4plex assays only: 20x primer–probe mix 4§	1.25 µl	1.0 µl	0.2 µM forward primer 4¶ 0.2 µM reverse primer 4¶ 0.2 µM probe 4**
For RT-PCR only: QuantiTect Virus RT Mix, 100x	0.25 µl	0.2 µl	1x
RNase-free water	Variable	Variable	–
Template DNA or RNA (added at step 4)	Variable	Variable	Maximum up to 50% of final reaction volume
Total volume	**25 µl***	**20 µl*†**	**–**

* If your real-time cycler requires a final reaction volume other than 25 µl or 20 µl, adjust the amount of master mix and all other reaction components accordingly.

† Refers to Applied Biosystems 7500 Fast Real-Time PCR System.

‡ For cyclers which do not require ROX dye, add RNase-free water instead.

§ For ease of use, we recommend preparing a 20x primer–probe mix for each of your targets containing target-specific primers and probe. See Appendix D in the *QuantiTect Virus Handbook*.

¶ A final primer concentration of 0.2 µM is optimal. Before adapting primer concentration, check the concentration of your primer solutions. In some cases, other primer concentrations between 0.1 µM and 0.3 µM may improve performance.

** A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 2. Cycling conditions for triplex or 4plex analysis

Step	Time	Temperature	Additional comments
For RT-PCR only: Reverse transcription	**20 min**	**50°C**	RNA is reverse transcribed into cDNA. Omit this step if you are analyzing DNA targets.
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions.**
Denaturation	**15 s**	**95°C**	
Annealing/extension	**75 s**	**60°C**	Combined annealing/extension step with fluorescence data collection, optimized for PCR products up to 150 bp. For PCR products >150 bp, different cycling conditions may improve results in some cases. For details, see Appendix A in the *QuantiTect Virus Handbook*.
Number of cycles	40–50		The number of cycles depends on the amount of template DNA or RNA.

6. **Place the PCR tubes or plate in the real-time cycler and start the PCR cycling program.**

7. **Perform data analysis.**

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

QIAGEN handbooks can be requested from QIAGEN Technical Service or your local QIAGEN distributor. Selected handbooks can be downloaded from www.qiagen.com/literature.

Material safety data sheets (MSDS) for any QIAGEN product can be downloaded from www.qiagen.com/Support/MSDS.aspx.

QuantiTect Virus Kits are intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

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Certain specific embodiments of the process of multiplex PCR may be covered by patents of third parties in certain countries and may require a license.

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