QIAGEN Supplementary Protocol

QuantiTect® Probe RT-PCR Kit research protocol for S-OIV

This protocol is for use in swine-origin influenza A (H1N1) virus (S-OIV) research applications using sequences available from the World Health Organization (WHO) (www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/). Real-time one-step RT-PCR is performed on the Applied Biosystems® 7500 or other real-time PCR cycler using the QuantiTect Probe RT-PCR Kit in combination with degenerate primers and probe.

IMPORTANT: Please consult the "Safety Information" and "Important Notes" sections in the *QuantiTect Probe RT-PCR 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.

The QuantiTect Probe RT-PCR Kit is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

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 Probe RT-PCR Kit (cat. no. 204443 or 204445)
- 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, visit www.qiagen.com/resources/info/Guidelines_RTPCR/Assay_Probe.aspx). 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.
- Cooling device or ice
- Applied Biosystems 7500 Real-Time PCR System or other real-time PCR cycler
- PCR tubes or plates (use thin-walled PCR tubes or plates recommended by the manufacturer of the cycler)

- Optional: Trizma® base and EDTA for preparing TE buffer for storing primers and probes (see www.qiagen.com/resources/info/Guidelines_RTPCR/Assay_Probe.aspx). 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.
- After reverse transcription, the PCR step of the RT-PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq® DNA Polymerase.
- Set up all reactions on ice to avoid premature cDNA synthesis.
- Always readjust the threshold value for analysis of every run.
- 2x QuantiTect Probe 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.
- When 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.

Procedure

1. **Thaw 2x QuantiTect Probe RT-PCR Master Mix (if stored at –20°C), template RNA, primer and probe solutions, and RNase-free water. Mix the individual solutions, and place them on ice. QuantiTect RT Mix should be taken from –20°C immediately before use, always kept on ice, and returned to storage at –20°C immediately after use.**
2. **Prepare a reaction mix according to Table 1.**

   Keep samples on ice while preparing the reaction mix.

   Note: We strongly recommend starting with an initial Mg^{2+} concentration of 4 mM as provided by 2x QuantiTect Probe RT-PCR Master Mix. For a few targets, reactions may be improved by using Mg^{2+} concentrations of up to 6 mM.

Table 1. Reaction setup

Component	Volume per 25 µl reaction	Final concentration
2x QuantiTect Probe RT-PCR Master Mix*	12.5 µl	1x
Forward primer	Variable	0.8 µM
Reverse primer	Variable	0.8 µM
Probe	Variable	0.2 µM†
QuantiTect RT Mix	0.25 µl	–
Template RNA (added at step 4)	5 µl	–
RNase-free water	Variable	–
Optional: Uracil-N-glycosylase, heat-labile	Variable	2 units/reaction
Total volume per reaction	**25 µl**	**–**

* Provides a final concentration of 4 mM $MgCl_2$.

† A final probe concentration of 0.2 µM gives satisfactory results when using the InfA, SW InfA, and RNase P primer–probe sets. **For the SW H1 primer–probe set, use a final probe concentration of 0.4 µM.**

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or plates.**

   Keep the PCR tubes or plates on ice.

4. **Add template RNA (5 µl) to the individual PCR tubes or wells containing the reaction mix.**

5. **Program your real-time cycler according to the program outlined in Table 2.**

   For optional UNG treatment, leave the samples for at least 5 min on ice.

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

Table 2. Cycling conditions

Step	Time	Temperature	Additional comments
Reverse transcription	**30 min**	**50°C**	Temperatures up to 55°C can be used to eliminate secondary structure in the template RNA.
PCR initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
2-step cycling:			
Denaturation	15 s	95°C	
Combined annealing/extension	60 s	52°C	Perform fluorescence data collection
Number of cycles	45		

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

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