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QIAprep& Viral RNA UM Kit Handbook

For fast and convenient sample preparation and
detection of RNA viruses from universal transport
media

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Kit Contents

QIAprep& Viral RNA UM Kit	(600)	(2400)
Catalog no.	221415	221417
Viral RNA UM Prep Buffer	1.2 ml	4 x 1.2 ml
Viral RNA Master Mix, 4x	2 x 1.5 ml	8 x 1.5 ml
RNA IC Template + Assay	1.2 ml	4 x 1.2 ml
Human Sampling IC Assay	600 µl	4 x 600 µl
QN ROX™ Reference Dye	1 ml	4 x 1 ml
RNase-Free Water	2 x 1.9 ml	8 x 1.9 ml
Quick-Start Protocol	1	1

Shipping and Storage

QIAprep& Viral RNA UM Kits are shipped on dry ice. The kits should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light. When the kits are stored under these conditions and handled correctly, performance is guaranteed until the expiration date (see the quality control label inside the kit box or on the kit envelope). The QIAprep& Master Mix and the QN ROX Reference Dye can also be stored protected from light at 2 – 8°C for up to 12 months, depending on the expiry date.

If desired, ROX Reference Dye can be added to the QIAprep& Master Mix for long-term storage. For details, see “Adding ROX dye to the master mix”, page 15.

Intended Use

The QIAprep& Viral RNA UM Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAprep& Viral RNA UM Kit is tested against predetermined specifications to ensure consistent product quality.

Product Specifications

The QIAprep& Viral RNA UM Kit contains the following:

QIAprep& Viral RNA UM Prep buffer

Component	Description
Buffer	The buffer contains a proprietary list of additives making RNA molecules available and preventing degradation and PCR inhibition.

QIAprep& Viral RNA UM Master Mix

Component	Description
QuantiNova® DNA Polymerase	QuantiNova DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from <i>Thermus aquaticus</i> . QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 2 minute, 95°C incubation step.
HotStaRT-Script Reverse Transcriptase	HotStaRT-Script Reverse Transcriptase is a modified form of a recombinant 77 kDa reverse transcriptase. It is provided in an inactive state and has minimal enzymatic activity at ambient temperature. The enzyme is activated during the reverse transcription step at 50°C.
Buffer	Contains components enabling fast cycling, including Q-Bond®.
dNTP mix	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality

Other components

Component	Description
Human Sampling IC	Primer–probe mix labelled with HEX™ dye to detect Human RNA and confirms that intact RNA from the starting material is in the reaction.
RNA IC Template + Assay	Synthetic RNA and primer–probe mix labelled with fluorophore detected in the red channel on the Rotor-Gene® Q or the Cy5 channel on other cyclers for monitoring potential RT-qPCR inhibition
ROX Reference Dye	Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments from Applied Biosystems®
RNase-Free Water	Ultrapure quality, PCR-grade.

Introduction

The QIAprep& Viral RNA UM Kit is an innovative liquid-based method optimized for the preparation and detection of enveloped RNA viruses such as coronaviruses from human samples collected with a nasal, nasopharyngeal, or oropharyngeal swab stored in non-fixation transport media such as UTM, VTM, PBS, Liquid Amies medium (ESwab®), Virocult™, and 0.9% NaCl. The QIAprep& Viral RNA UM Kit combines a liquid-based sample preparation step together with one-step RT-PCR detection.

The end-to-end liquid-based workflow, consisting of only a few steps from sample to result, is easily automatable on liquid handlers and meets all throughput needs (see Figure 1). The QIAprep& Viral RNA UM Kit drastically accelerates time to results and saves time, bench space, labor, and plastics compared to other methods.

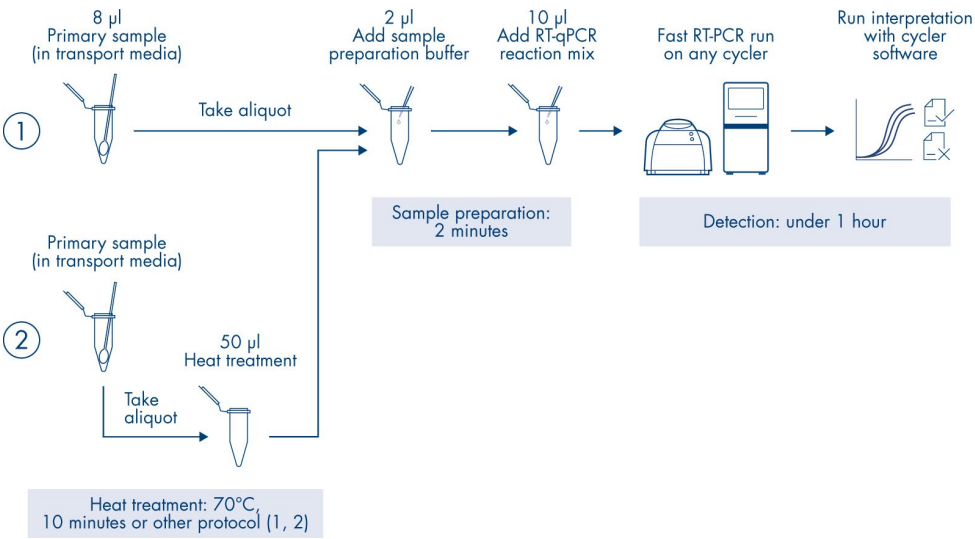


Figure 1. Overview of the QIAprep& Viral RNA UM workflow.

The kit is compatible with dual-labeled hydrolysis probes, e.g., TaqMan® probes. High specificity and sensitivity in real-time RT-PCR are achieved by a novel two-phase hot-start procedure. For high in-process safety during pathogen detection, each kit contains reagents for the simultaneous detection of user-defined targets and internal controls for confidence in results interpretation.

The QIAprep& Viral RNA UM Kit has been optimized for use with TaqMan probes in multiplex one-step RT-PCR detection of one or more targets (altogether, up to 4 assays including the internal controls).

The kit has been optimized for use with any real-time cyclers. The QN ROX Reference Dye is provided in a separate tube and can be added if using a cycler that requires ROX as a passive reference dye (see Table 1 below).

Table 1. Assays and detection channels

Assay	Targets	Dye/color channel	Supply
Inhibition control	Synthetic transcript	Detected in the red channel on RGQ or Cy5 in other cyclers/Red	Included in the kit, optional
Sampling control	Human B2M and RNase P genes	HEX™/Yellow (the two targets are detected in the same channel)	Included in the kit, optional
Passive reference dye	-	ROX/Orange	Included in the kit, optional
Viral RNA assay	User defined	(Recommended: FAM™; Alexa Fluor® 488 and/or other dyes in the green, blue, or NIR channel; maximum 4-plex)	Provided by user

Principle and procedure

Viral RNA sample preparation

The QIAprep& Viral RNA UM Prep Buffer contains an innovative liquid-based RNA extraction method from nasal, oropharyngeal, and nasopharyngeal swabs in transport media such as UTM, VTM, PBS, Liquid Amies medium (ESwabs), Virocult, and 0.9% NaCl. This sample preparation step prepares the viral RNA genome for RT-qPCR detection, protects the RNA molecule from degradation, and prevents inhibition. The incubation step is only 2 minutes and can be done either on ice or at room temperature.

PCR and one-step RT-PCR

The QIAprep& Viral RNA UM Kit contains a highly concentrated 4x Master Mix, which allows use of larger volumes of template in order to increase assay sensitivity. The use of the QIAprep& Viral RNA UM Master Mix allows both reverse transcription and PCR to take place in a single tube. All reagents required for both reactions are contained in the Master Mix, so there is no need to open the tube once the reverse transcription reaction has been started. The assay setup can be done at room temperature and should be processed immediately after sample addition. If heat treatment was performed, storage up to 1 hour at room temperature or for a longer period, frozen at -30 to -15°C , is possible.

Viral RNA UM Master Mix

The components of the Viral RNA UM Master Mix include HotStarT-Script Reverse Transcriptase, QuantiNova DNA Polymerase, buffer, and dNTPs. The optimized master mix ensures fast RT-PCR amplification with high specificity and sensitivity.

HotStaRT-Script Reverse Transcriptase

The QIAprep& Viral RNA UM Master Mix contains HotStaRT-Script Reverse Transcriptase for heat-mediated activation of the reverse transcription step. The HotStaRT-Script Reverse Transcriptase is associated with an RT-blocker, rendering the enzyme almost inactive at ambient temperature. This allows the RT-PCR reaction setting up at room temperature without the risk of primer-dimer formation by the reverse transcriptase. Upon starting the RT-PCR protocol with the RT step at 50°C, the RT-blocker is released from the reverse transcriptase and cDNA synthesis is initiated (Figure 2).

Novel, antibody-mediated hot-start mechanism

The QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient or higher temperatures. The enzyme remains completely inactive during the reverse transcription reaction and does not interfere with it. The antibody-mediated hot-start mechanism prevents the formation and extension of nonspecific RT-PCR products and primer-dimers during reaction setup, reverse transcription, and the first denaturation step. Therefore, this mechanism allows higher PCR specificity and accurate quantification. At low temperatures, the QuantiNova DNA Polymerase is kept in an inactive state by the QuantiNova Antibody and Guard, which stabilizes the complex and improves the stringency of the hot-start. After reverse transcription and within 2 minutes of raising the temperature to 95°C, the QuantiNova Antibody and Guard are denatured and the QuantiNova DNA Polymerase is activated, enabling PCR amplification (Figure 2). This two-phase hot-start enables rapid and convenient room-temperature setup and allows both steps to be performed sequentially in a single tube.

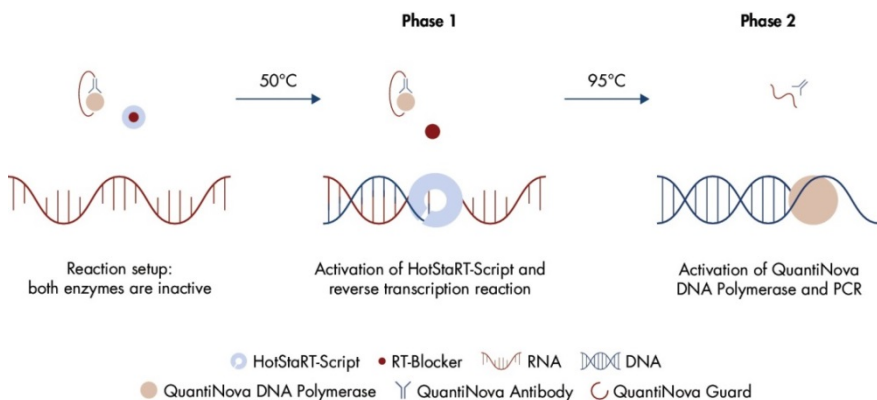


Figure 2. Principle of the novel QuantiNova two-phase hot-start mechanism. At ambient temperature, the HotStaRT-Script is inhibited by the RT-Blocker and the QuantiNova DNA Polymerase is kept inactive by QuantiNova Antibody and QuantiNova Guard. At 50°C, the RT is activated while the QuantiNova DNA polymerase remains inactive. At 95°C, the RT enzyme is denatured and the DNA polymerase is activated.

QIAprep& Viral RNA UM PCR Buffer

The PCR buffer from the QIAprep& Viral RNA UM Kit is specifically designed to facilitate both efficient reverse transcription and fast real-time PCR using sequence-specific probes. The buffer additive, Q-Bond, allows for short cycling times. Q-Bond increases the affinity of the DNA Polymerase for short single-stranded DNA, reducing the time required for primer–probe annealing to a few seconds. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times.

The QIAprep& Viral RNA Master Mix is also based on the unique QIAGEN PCR buffer system. The buffer composition is extremely robust to various salt concentrations brought to the reaction from the transport media used in the primary sample tube, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. When

using this master mix, primer annealing is only marginally influenced by the MgCl_2 concentration, so optimization by titration of Mg^{2+} is not required.

The master mix also contains Factor MP, which facilitates multiplex PCR. This synthetic molecule increases the local concentration of primers and probes at the DNA template and stabilizes specifically bound primers and probes, allowing efficient annealing and extension. The combination of these various components of the QIAprep& Viral RNA UM PCR Buffer prevents multiple amplification reactions from affecting each other.

The composition of the novel RT stabilizing buffer allows room-temperature RT-PCR reaction setup without the need for cooling. The assay setup can be done at room temperature and should be processed immediately after sample addition. If heat treatment was performed, storage up to 1 hour at room temperature or for a longer period, frozen at -30 to -15°C , is possible.

RNA Internal Control (RNA IC) and Assay

For increased in-process safety in pathogen-detection assays, an internal positive control is detected in the same tube as the pathogen RNA targets during multiplex RT-PCR. The RNA IC is a synthetic RNA with a unique and artificial sequence that can be used to monitor successful amplification. The RNA IC is intended to report instrument or chemistry failures, errors in assay setup, and the presence of inhibitors. Inhibitors may be brought to the reaction from the transport media used in the primary sample tube or from patient samples.

The primer and probe sequences for the detection of the RNA Internal Control have been bioinformatically validated for non-homology against hundreds of eukaryotic and prokaryotic organisms. Additionally, they have been experimentally tested against a multitude of human, mouse, and rat RNA samples from multiple tissues and cell lines.

The RNA IC RNA is detected as a 200 bp amplicon. The RNA Internal Control Assay contained in the kit employs a reporter dye with an excitation/emission maxima of 647/664 nm and can be detected in the red channel on the Rotor-Gene Q or the Cy5 channel on other cyclers.

The RNA IC Template + Assay can be used optionally, but is highly recommended. It should be added during reaction setup.

Please note that adding the RNA IC Template + assay to the reaction mix will result in positive signals in the no-template-control (NTC) tubes in the red channel. These signals serve as a reference to assure that the internal control has been successfully amplified. If the internal control signal is detected in the NTCs, but not in sample reactions, this may indicate the presence of inhibitors or other issues with the sample reaction.

Human Sampling IC (Internal Control) Assay

For increased confidence in interpretation of negative samples (no target amplification), the Human Sampling Internal Control targets two human transcripts of the B2M and RNase P genes, detected in the same channel (primer–probe mix labelled with HEX dye). The Human Sampling Internal Control is intended to report that the primary sample tube contains human material and that RNA materials have not been degraded.

The Human Sampling IC Probe Assay contained in the kit employs HEX as a reporter dye. With excitation/emission maxima of 535/556 nm, the HEX dye has a spectral profile allowing detection in the yellow channel on the Rotor-Gene Q or the HEX, JOE™, or VIC® channel on other cyclers and therefore can be used with most real-time cyclers.

The Human Sampling IC Assay can be used optionally, but is highly recommended. It should be added during reaction setup.

If the human sampling internal control is not detected in an unknown sample but the RNA IC is detected in the same sample, this may indicate that RNA is degraded in the primary tube or the primary sample does not contain human material in the first place.

Passive reference dye

For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR-related variations in fluorescence detection. Fluorescence from ROX dye does not change during the course of real-time PCR but provides a stable baseline to which PCR-related fluorescent signals are normalized. Thus, ROX dye compensates for differences in fluorescence detection due to slight variations in well reaction volume or to differences in well position. ROX dye does not interfere with real-time PCR since it is not involved in the reaction and has an emission spectrum different from fluorescent dyes commonly used for probes.

The use of ROX dye is necessary for instruments from Applied Biosystems. The QIAprep&Viral RNA UM Kit is provided with a separate tube of ROX Reference Dye. ROX dye should be used as a 20x concentrated solution 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.

Refer to Table 2 for details on real-time cyclers that require high or low ROX concentrations. If desired, ROX Reference Dye can be added to the QIAprep&Viral RNA Master Mix for long-term storage (Table 3). For details, see “Adding ROX dye to the master mix”, page 15.

Table 2. Real-time cyclers requiring high/low concentrations of ROX dye

High ROX dye concentration (1:20 dilution of ROX Reference Dye in 1x reaction)	Low ROX dye concentration (1:200 dilution of ROX Reference Dye in 1x reaction)
ABI PRISM® 7000	Applied Biosystems 7500
Applied Biosystems 7300	Applied Biosystems ViiA™ 7
Applied Biosystems 7900	Applied Biosystems QuantStudio® Systems
Applied Biosystems StepOne™	
Applied Biosystems StepOne Plus	

Adding ROX dye to the master mix

If only using cyclers from Applied Biosystems with the QIAprep& Viral RNA UM Kit, ROX Reference Dye can be added to QIAprep& Viral RNA Master Mix for long-term storage, if desired. For information on the concentration of ROX required for Applied Biosystems instruments, refer to Table 2. For reaction setups with master mix that already contains a high concentration of added ROX Reference Dye, refer to “Appendix A: Reaction Setup Using Master Mix Containing a High Concentration of ROX”, page 29.

Table 3. Addition of QN ROX Reference Dye to master mix

Volume of Viral RNA Master Mix (without ROX dye)	Volume of ROX dye for high ROX concentration/low ROX concentration
1.5 ml	300/30 µl

Equipment and Reagents to Be Supplied by User

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

- A programmable real-time PCR thermocycler with at least three detection channels, compatible with the QIAprep& Viral RNA UM Kit (Table 1)
- Plastic PCR consumables compatible with the abovementioned thermocyclers
- Calibrated micropipettes for volumes ranging from 2 µl to 50 µl and tips or an automated liquid handler
- A probe-based assay for the detection of one or more targets from RNA viruses, compatible with the QIAprep& Viral RNA UM Kit (Table 1). This is referred to as primer–probe mix in the reaction setup table (Table 4).

We suggest to use FAM or another equivalent dye that can be detected simultaneously with the Internal Controls Assays contained in this kit in a multiplex PCR.

To guarantee optimal performance of multiplex amplification of the viral target and the Internal Controls, we recommend designing the viral target assay to follow certain specifications. These specifications are in accordance with general recommendations for the design of optimal real-time PCR and RT-PCR primers and probes, and are therefore likely to already apply for the majority of proven and established (e.g., literature-derived) real-time RT-PCR assays. For more details on target assay design, see “Appendix B: Assay Design and Optimization”, page 30.

Important Notes

- The QIAprep& Viral RNA UM Kit is an innovative liquid-based method optimized for the preparation and detection of viral RNA targets from samples such as nasal, nasopharyngeal, or oropharyngeal swabs that are stored in non-fixation transport media such as UTM, VTM, PBS, Liquid Amies medium (ESwabs), Virocult, and 0.9% NaCl.
- Samples can be kept at room temperature during preparation steps and reaction setup. Sample preparation can conveniently be performed directly in the PCR vessel prior to the addition of the PCR reaction. The assay setup can be done at room temperature and should be processed immediately after sample addition. If heat treatment was performed, storage up to 1 hour at room temperature or for a longer period, frozen at –30 to –15°C, is possible.
- The Viral RNA UM Prep Buffer prepares the samples for the detection step but is not a virus inactivation solution.
- The protocol in this handbook includes a recommended heat treatment step before the sample preparation step. This workflow step is intended to inactivate viral particles in an aliquot of the primary sample in transport media (1, 2). QIAGEN cannot guarantee that this heat treatment step will inactivate 100% of viral particles. The inactivation of virus needs to be verified and validated by users. This heat treatment can be substituted by other heat treatments.
- The RT-qPCR protocol uses TaqMan probes in a multiplex reaction that works with any real-time cyclers. For fluorescence normalization, ROX dye might be required at the following concentrations:
 - **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
 - **No requirement for ROX dye:** Rotor-Gene Q, QIAquant, Bio-Rad® CFX, Roche® LightCycler® 480, and Agilent® Technologies Mx instruments. The QN 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.

For the highest efficiency in real-time RT-PCR using TaqMan probes, amplicons should ideally be 60–150 bp in length.

Before performing multiplex analyses, choose suitable combinations of reporter dyes and quenchers that are compatible with the detection optics of your real-time cyclers.

Important: Always start with the cycling conditions and primer concentrations specified in this protocol.

- The PCR section of the RT-qPCR protocol must start with an initial incubation step of 2 minutes at 95°C to activate the DNA Polymerase.
- For viral targets, it is recommended to prepare a 20x primer–probe mix containing target-specific primers and probes. Viral sequences can be detected in the green channel on the Rotor-Gene Q or in the FAM dye channel on other real-time PCR instruments. We recommend to use a final concentration of 0.8 μM primers (forward/reverse) and a 0.25 μM probe in the reaction. For further information, or to download the handbook or supplementary protocols, please visit the product page (www.qiagen.com/qiaprepamp-viral-rna-um-kit).
- The RNA IC Template + Assay is an inhibition control using a synthetic RNA template. It is a 200 bp IC template detected in the red channel on the Rotor-Gene Q or in the Cy5 channel on other real-time PCR instruments.
- The Human Sampling IC Assay (Sampling Control) is intended to report that the primary sample tube contains intact human genetic material. For this purpose, two different human RNA targets are both detected in the yellow channel on the Rotor-Gene Q or in the VIC/HEX dye channel on other real-time PCR instruments.
- The use of the supplied Internal Controls is optional but recommended for confident interpretation of results.
- Before use, thaw the Viral RNA UM Prep Buffer, Viral RNA Master Mix, RNA IC Template + Assay, Human Sampling IC Assay, ROX Reference Dye (if required), and RNase-Free Water. Mix the individual solutions.

For both 96-well and 384-well block cyclers, we recommend a final reaction volume of 20 μl in order to maximize input volumes of template from the primary sample and increase assay sensitivity.

Protocol: Multiplex Real-Time RT-PCR Using Both Internal Controls as Sampling and Inhibition Controls

Important points before starting

- The QIAprep& Viral RNA UM procedure consists of two main workflow steps, the liquid-based sample preparation step and the RT-qPCR reaction step. All workflow steps are liquid-based and can be carried out manually or automated on a liquid handler such as the QIAgility instrument.
- We recommend to include the optional heat treatment (step 4) for safety reasons and to improve robustness against inhibitory effects in subsequent PCR reaction. This can avoid repetition of negative results when RNA IC signal is absent.
- The RNA IC Template + Assay is added to the reaction mix. The addition of this internal control as an inhibition control will result in positive signals in NTCs. These signals serve as a reference to assure that the internal control has been successfully amplified. If the internal control signal is detected in the NTCs, but not in sample reactions, this may indicate the presence of inhibitors or other issues with the sample reaction. The pre-mixed formulation (10x) contains the synthetic RNA template, the forward and reverse primers, and TaqMan probe.
- The Human Sampling IC is added to the reaction mix. The addition of this internal control serves as a positive amplification control to report that the primary sample tube contains human material and that RNA materials have not been degraded. If the internal control signal is not detected in the unknown samples, but is detected in relevant controls, this may indicate RNA degradation in the primary tube or that the primary sample did not contain human materials in the first place. The pre-mixed formulation (20x) contains forward and reverse primers and TaqMan probes.

Procedure

1. Before use, thaw the Viral RNA UM Prep Buffer, Viral RNA Master Mix, RNA IC Template + Assay, Human Sampling IC Assay, ROX Reference Dye (if required), and RNase-Free Water. Mix the individual solutions.
2. Prepare a reaction mix according to Table 4. Because of the two-phase hot-start of both the RT and the PCR reactions, it is not necessary to keep samples on ice during reaction setup.

Table 4. Reaction setup

Component	96/384-well block	Final concentration
Viral RNA Master Mix, 4x	5 µl	1x
20x primer–probe mix†	1 µl	1x
RNA IC Template + Assay, 10x‡	2 µl	1x
Human Sampling IC Assay, 20x	1 µl	1x
ROX Reference Dye (ABI instruments only)	1 µl/0.1 µl*	1x
RNase-Free Water	Fill up to 10 µl	–
Prepared sample (combined at step 7)	10 µl	–
Total reaction volume	20 µl	–

* To be used as a 20x concentrate for high-ROX dye cyclers (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900, and StepOne Real-Time PCR Systems) and as a 200x concentrate for low ROX-dye cyclers (i.e., Applied Biosystems 7500, ViiA 7, and QuantStudio Real-Time PCR Systems).

† 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. 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.

‡ detects RNA Internal Control.

3. Vortex vigorously the primary sample tube containing the swab in transport media.
4. **Optional sample heat treatment (recommended):**
 - 4a. Either the entire primary sample or an aliquot of 50 µl can be submitted for heat treatment. Ensure the complete sample volume is appropriately heated.
 - 4b. Incubate at 70°C for 10 min.
 - 4c. Centrifuge the plate/tube briefly.
5. Dispense 2 µl of Viral RNA UM Prep Buffer into each PCR tube or wells of a PCR plate.

6. Transfer 8 µl of the sample to the same PCR tube or wells containing the Viral RNA UM Prep Buffer. Mix by pipetting up and down at least twice. Incubate at room temperature for 2 min.

Note: Incubation time starts after adding the last sample to the Viral RNA UM Prep Buffer.

Do not increase incubation time for more than 6 h.

7. Add 10 µl of the reaction master mix prepared in step 2 (Table 4) to the same PCR tubes or wells.

8. Important considerations:

- 8a. Seal the plate/tube thoroughly to prevent cross-contamination. In case an adhesive film is used, make sure to apply pressure uniformly across the entire plate, to obtain a tight seal across individual wells.
 - 8b. Mix gently by vortexing for 10–30 s at medium speed. Place the plate in different positions while vortexing, to ensure an equal contact with the vortex platform.
 - 8c. Centrifuge the plate/tube briefly to collect liquid at the bottom of the plate/tube.
 - 8d. Immediately proceed to step 9. The complete reaction can be stored only after heat treatment up to 1 h at room temperature or for a longer period, frozen at –30 to –15°C.
9. Place the tubes or plates in the real-time cycler and perform cycling according to below conditions (Table 5).

Program the real-time cycler before reaction setup according to Table 5.

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

Table 5. Cycling conditions

Step	Time	Temperature	Ramp rate
RT-step	10 min	50°C	Maximal/fast mode
PCR initial heat activation	2 min	95°C	Maximal/fast mode
2-step cycling (40 cycles)			
Denaturation	5 s	95°C	Maximal/fast mode
Combined annealing/extension	30 s	58°C*	Maximal/fast mode

* Annealing temperatures can be adapted between 55–62°C depending on the primer/probe set used. For further details on cycling conditions, primer/probe concentrations, and annealing temperature, visit the product page (www.qiagen.com/qiaprepamp-viral-rna-um-kit).

10. For results interpretation, refer to Table 6.

Analysis and Interpretation of the Internal Controls

After amplification, perform data analysis as recommended for your real-time PCR instrument.

RNA IC Template + Assay

The C_T value for the RNA IC Template + Assay in the QIAprep& Viral RNA UM Kit depends on the real-time PCR instrument used and can usually be expected within a C_T range of 30 ± 4 .

If the internal control signal is detected in the NTCs, but not in sample reactions, this may indicate inhibition or PCR setup failure. In such case, we recommend the following:

- a. Check equipment for accurate performance and repeat sample/experiment to rule out pipetting or handling errors.
- b. Repeat the experiment with lower template input volume. This may dilute potential inhibitors to a concentration they do not significantly inhibit the PCR.

Human Sampling Internal Control

In samples containing template, the Human Sampling Internal Control is expected to have C_T values in a wide range from 20 to 35 depending on the initial quantity of human materials in the primary sample tube. Higher C_T values indicate degradation or sampling failure. In such case, we recommend the following:

- a. C_T values >38 may indicate that RNA materials from the primary sample tube have been partially or entirely degraded during transport or storage, or that sampling failed and no human material was present at all. Consider repeating the test or new sampling.
- b. Check equipment for accurate performance and repeat sample/experiment to rule out pipetting or handling errors.

Interpretation of Results

Possible outcomes are summarized in Table 6 below.

Table 6. Possible outcome

Viral RNA assay	Internal control	Sampling control	Status	Result
+	+	+	VALID	Positive
+	+	–	VALID	Positive
+	–	–	VALID	Positive
+	–	+	VALID	Positive
–	+	+	VALID	Negative, virus not detected
–	+	–	Inconclusive	Repeat test using a new sample
–	–	+	PCR inhibited	Repeat test using a lower-sample volume (down to 2 µl)
–	–	–	PCR inhibited	Repeat test using a lower-sample volume (down to 2 µl)

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, the scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit www.qiagen.com).

Comments and suggestions

No signal or one or more signals detected late in PCR

- | | |
|---|---|
| a) Incorrect cycling conditions | Always start with the optimized cycling conditions specified in the protocols. Be sure that the PCR step of your cycling conditions include the initial step for activation of the QuantiNova DNA Polymerase (95°C for 2 minutes) and the specified times for denaturation and annealing/extension. |
| b) QuantiNova DNA Polymerase not activated | Ensure that the cycling program includes the QuantiNova DNA Polymerase activation step (2 minutes at 95°C) as described in the protocols. |
| c) Pipetting error or missing reagent | Check the concentrations and storage conditions of the reagents, including primers, probes, and template nucleic acid. See "Appendix B: Assay Design and Optimization", page 30, for details on evaluating the concentration of primers and probes. Repeat the PCR. |
| d) Wrong or no detection step | Ensure that fluorescence detection takes place during the combined annealing/extension step when using hybridization probes. |
| e) Primer or probe concentration is not optimal | Use optimal primer concentrations. For TaqMan assays, use each primer at 0.8 µM. In most cases, a probe concentration of 0.25 µM provides satisfactory results. Check the concentrations of primers and probes by spectrophotometry (see "Appendix B: Assay Design and Optimization", page 30). |
| f) Problems with starting template | Check the concentration, storage conditions, and quality of the starting template (see "Appendix B: Assay Design and Optimization", page 30). |
| g) Insufficient amount of starting template | Increase the amount of template, if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample. If necessary, concentrate or make new serial dilutions of template nucleic acid from the stock solutions. Repeat the PCR. |
| h) Insufficient number of cycles | Increase the number of cycles. We recommend 40 cycles. |
| i) Reaction volume is too high | For both 96-well and 384-well block cyclers, we recommend a final reaction volume of 20 µl. |
| j) RT-PCR product is too long | Increase the annealing/extension time. |

Comments and suggestions

k) Primer design is not optimal	For optimal results, RT-PCR products should be between 60 and 150 bp. RT-PCR products should not exceed 300 bp.
l) Probe design is not optimal	If the amplification reaction was successful, there may be a problem with the probe. Review the probe design guidelines (see "Appendix B: Assay Design and Optimization", page 30).
m) Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter setting is chosen for the reporter dye.
n) No detection activated	Check that fluorescence detection was activated in the cycling program.
o) Probe synthesis is not optimal	Check the quality of dual-labeled probes by incubation with DNase I. A correctly synthesized probe, containing both fluorophore and quencher, will show a significant increase in fluorescence after DNase I incubation.
p) Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
q) Incorrect temperature for RT reaction	We recommend performing the RT reaction at 50°C. However, if this temperature does not yield satisfactory results, the temperature can be adjusted between 42°C and 50°C.
r) High concentration of PCR inhibitors	We recommend repeating the experiment with lower template input volume. This may dilute potential inhibitors to a concentration they do not significantly inhibit the PCR.

Increased fluorescence or C_T value for "No Template" control

a) Contamination of reagents	Discard all the components of the assay (e.g., master mix, primers, and probes). Repeat the assay using new components.
b) Contamination during reaction setup	Take appropriate precautions during reaction setup, such as using aerosol-barrier pipette tips.
c) Minimal probe degradation, leading to sliding increase in fluorescence	Check the amplification plots, and adjust the threshold settings.

Varying fluorescence intensity

a) Contamination of real-time cyclers	Decontaminate the real-time cycler according to the manufacturer's instructions.
b) Real-time cycler is no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.

Comments and suggestions

All cyclers systems:

- | | |
|---|--|
| a) Wavy curve at high template amounts for highly expressed targets | In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template. |
| b) Carry-over contamination | If the negative control (without template RNA) shows an RT-PCR product or a smear, exchange all reagents. Use disposable pipette tips containing hydrophobic filters to minimize cross-contamination. Set up all reaction mixtures in an area separate from that used for RNA preparation or PCR product analysis. |

Applied Biosystems instruments only:

- | | |
|--|---|
| ΔR_n values are unexpectedly too high or too low | The concentration of the QN ROX Reference Dye is incorrect. To choose the right ROX concentration for your cycler, refer to Table 3, page 15. |
|--|---|

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Appendix A: Reaction Setup Using Master Mix Containing a High Concentration of ROX

This appendix is only relevant for a reaction setup using a master mix containing a high concentration of ROX that has been added according to Table 3, page 15. When using a master mix containing low concentration of ROX, the volume of ROX added is negligible and the standard reaction setup as described in Table 4 (page 20) should be used; however, for high concentration of ROX, the standard reaction setup as described in Table 7 should be used.

Table 7. Reaction setup

Component	96/384-well block	Final concentration
Viral RNA Master Mix, 4x after ROX addition*	6 µl	1x
20x primer–probe mix†	1 µl	1x
Human Sampling IC Assay (optional)	1 µl	1x
RNA IC Template + Assay (optional)	2 µl	1 x
RNase-Free Water	Variable	–
Template DNA or RNA	Variable	Variable
Total reaction volume	20 µl	1x

* Contains a 1:20 dilution for high ROX instruments (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900, and StepOne Real-Time PCR Systems).

† 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.

Appendix B: Assay Design and Optimization

Important factors for successful quantitative, single-plex and duplex real-time RT-PCR include the design of optimal primer pairs and probes, the use of appropriate primer and probe concentrations, and the correct storage of primers and probes.

Assay design

Guidelines for the optimal design of primers and probes are given below. It is particularly important to minimize nonspecific annealing of primers and probes. This can be achieved through careful assay design.

T_m of primers for TaqMan assays

- Use specialized design software (e.g., Primer Express® Software) to design primers and probes.
- T_m of all primers should be 55–62°C and within 2°C of each other.
- T_m of probes should be 8–10°C higher than the T_m of the primers.
- Avoid a guanidine at the 5' end of probes, next to the reporter, since this causes quenching.
- Avoid runs of 4 or more of the same nucleotide, especially of guanidine.
- Choose the binding strand so that the probe has more C than G bases.
- All assays should be designed using the same settings to ensure that they will work optimally under the same cycling conditions (60°C annealing/extension).

Primer sequence

- Length: 18–30 nucleotides.
- GC content: 30–70%.

- Always check the specificity of primers by performing a BLAST® search (www.ncbi.nlm.nih.gov/blast). Ensure that primer sequences are unique for your template sequence.
- Check that primers and probes are not complementary to each other.
- Try to avoid highly repetitive sequences.
- Avoid complementarity of two or three bases at the 3' ends of primer pairs to minimize primer-dimer formation.
- Avoid mismatches between the 3' end of primers and the template sequence.
- Avoid runs of three or more Gs and/or Cs at the 3' end. Avoid complementary sequences within a primer sequence and between the primer pair.

Product size

Ensure that the length of RT-PCR products is 60–150 bp. Some longer amplicons may amplify efficiently in duplex RT-PCR, with minimal optimization.

Handling and storing primers and probes

Guidelines for handling and storing primers and probes are given in **Error! Reference source not found.** For optimal results, we recommend only combining primers of comparable quality.

Table 8. Guidelines for handling and storing primers and probes

Description	
Storage buffer	Lyophilized primers should be dissolved in a small volume of low-salt buffer to give a concentrated stock solution (e.g., 100 µM). We recommend using TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) for standard primers and probes labeled with most fluorescent dyes. However, probes labeled with fluorescent dyes such as Cy3, Cy3.5, Cy5, and Cy5.5 should be stored in TE buffer, pH 7.0, since they tend to degrade at higher pH.
Storage	<p>Primers should be stored in sterile, nuclease-free TE buffer in small aliquots at –30 to –15°C. Standard primers are stable under these conditions for at least 1 year. Fluorescently labeled probes are usually stable under these conditions for at least 6–9 months. Repeated freeze–thaw cycles should be avoided, since they may lead to degradation.</p> <p>For easy and reproducible handling of primer–probe sets used in duplex assays, we recommend preparing 20x primer–probe mixes, each containing two primers and one probe for a particular target at the suggested concentrations (see protocols).</p>
Dissolving primers and probes	<p>Before opening a tube containing lyophilized primer or probe, centrifuge the tube briefly to collect all material at the bottom of the tube. To dissolve the primer or the probe, add the required volume of sterile, nuclease-free TE buffer, mix and leave for 20 minutes to allow the primer or probe to completely dissolve. Mix again and determine the concentration by spectrophotometry as described below.</p> <p>We do not recommend dissolving primers and probes in water. They are less stable in water than in TE buffer and some may not dissolve easily in water.</p>
Concentration	<p>Spectrophotometric conversion for primers and probes: 1 A_{260} unit = 20–30 µg/ml</p> <p>To check primer concentration, the molar extinction coefficient can be used: $A_{260} = \epsilon_{260} \times \text{molar concentration of primer or probe}$</p> <p>If the ϵ_{260} value is not given on the data sheet supplied with the primers or probes, it can be calculated from the primer sequence using the following formula: $\epsilon_{260} = 0.89 \times [(A \times 15,480) + (C \times 7340) + (G \times 11,760) + (T \times 8850)]$</p> <p>Example</p> <p>Concentration of diluted primer: 1 µM = 1×10^{-6} M</p> <p>Primer length: 24 nucleotides with 6 each of A, C, G, and T bases</p> <p>Calculation of expected A_{260}: $0.89 \times [(6 \times 15,480) + (6 \times 7340) + (6 \times 11,760) + (6 \times 8850)] \times (1 \times 10^{-6}) = 0.232$</p> <p>The measured A_{260} should be within +/– 30% of the theoretical value. If the measured A_{260} is very different to the theoretical value, we recommend recalculating the concentration of the primers or probes, or having the primers or probes resynthesized.</p> <p>For probes, the fluorescent dye does not significantly affect the A_{260} value.</p>
Primer quality	The quality of 18–30 mers can be checked on a 15% denaturing polyacrylamide gel; a single band should be seen. Please contact QIAGEN Technical Services for a protocol.
Probe quality	The quality of the fluorescent label and the purity of TaqMan probes can be determined by comparing fluorescence before and after DNase digestion. Incubate probes with or without 5 units DNase at 37°C for 1 hour. A significant difference in fluorescence following DNase treatment should be detectable.

Assay and Multiplex Assay Optimization

The QIAprep& Viral RNA UM Kit works well with most existing probe systems that have been designed using standard design methods. However, for optimal performance of a probe system in real-time RT-PCR, some considerations need to be made, including the quality of the primers and probes, the concentration of primers and probes, and the annealing temperature and the choice of a compatible combination of reporter dyes (i.e., the fluorophores on the probes). Please read the following guidelines before starting.

- Some real-time cyclers require you to perform a calibration procedure for each reporter dye. Check whether the reporter dyes you selected for your multiplex assay are part of the standard set of dyes already calibrated on your cycler. If they are not, perform a calibration procedure for each dye before using them for the first time (for details, refer to the manufacturer's instructions for your real-time cycler).
- Check the real-time cycler user manual for the correct setup of the cycler. Be sure to activate the detector for each reporter dye used.
- Check the functionality of each set of primers and probe. Check the functionality of each set of primers and probe in individual assays before combining the different sets in a multiplex assay. For multiplex analysis, the use of non-fluorescent quenchers (e.g., Black Hole Quencher® [BHQ] on TaqMan probes) is preferred over fluorescent quenchers (e.g., TAMRA™ fluorescent dye). For details about fluorescent dyes, see “Appendix C: Suitable Combinations of Reporter Dyes on the Rotor-Gene Q Instrument”, page 35.
- Perform appropriate controls for evaluating the performance of your multiplex assays (e.g., amplifying each target individually and comparing the results with those for the multiplex assay).

Always start with the concentrations for primers and probes specified in the protocol. In some cases, it may be preferable to test the optimal concentration for primers and probes. We recommend testing combinations of different primers and probe concentrations using Table 9.

Table 9. Primers and probe concentration

Final concentration				
Forward primer	0.3 μ M	0.3 μ M	0.8 μ M	0.8 μ M
Reverse primer	0.3 μ M	0.8 μ M	0.3 μ M	0.8 μ M
Probe concentration	0.25 μ M			
Forward primer	0.3 μ M	0.3 μ M	0.8 μ M	0.8 μ M
Reverse primer	0.3 μ M	0.8 μ M	0.3 μ M	0.8 μ M
Probe concentration	0.1 μ M			

- Always start with the cycling conditions specified in the protocol. Depending on the melting temperature of primers and probes, the annealing/extension temperature can be between 55°C and 62°C.
- When performing multiplex experiments with a high number of targets, the annealing/extension time may be prolonged to 45 seconds.
- Optimal analysis settings (i.e., baseline settings and threshold values) for each reporter dye are a prerequisite for accurate quantification data. For details, refer to the manufacturer's instructions for your real-time cycler.

Appendix C: Suitable Combinations of Reporter Dyes on the Rotor-Gene Q Instrument

Multiplex real-time PCR requires the simultaneous detection of up to five different fluorescent reporter dyes. For accurate detection, the fluorescence spectra of the dyes should be well separated or exhibit only minimal overlap (Table 10). For up to 4-plex analysis, we recommend using the core channels: Green, Yellow, Orange, and Red. If a higher multiplex degree (5-plex, 6-plex) is performed, extend the spectral range to the blue channel and/or the crimson channel. These channels require less frequently used fluorophores, which will not be detected on all commonly used real-time PCR instruments.

Note: To find out which reporter dyes can be used in multiplex analyses if using other real-time PCR instruments, please refer to the user manual or the manufacturer's instructions for your real-time cycler.

Table 10. Dyes commonly used in multiplex real-time PCR on Rotor-Gene Q

Channel	Excitation (nm)	Detection (nm)*	Examples of fluorophores detected
Blue	365 ± 20	460 ± 20	Marina Blue®, Edans, Bothell Blue, Alexa Fluor 350, AMCA-X, ATTO 390
Green	470 ± 10	510 ± 5	FAM, Alexa Fluor 488
Yellow	530 ± 5	557 ± 5	JOE, VIC, HEX, TET™, CAL Fluor® Gold 540, Yakima Yellow®
Orange	585 ± 5	610 ± 5	ROX, CAL Fluor Red 610, Cy3.5, Texas Red®, Alexa Fluor 568
Red	625 ± 10	660 ± 10	Cy5, Quasar® 670, LightCycler Red 640, Alexa Fluor 633
Crimson	680 ± 5	712 high pass	Quasar 705, LightCycler Red 705, Alexa Fluor 680

* Emission spectra may vary depending on the buffer conditions.

Ordering Information

Product	Contents	Cat. no.
QIAprep& Viral RNA UM Kit (600)	For 600 x 20 µl reactions: 1.2 ml Viral RNA UM Prep Buffer; 2 x 1.5 ml Viral RNA Master Mix, 4x; 1.2 ml RNA IC Template + Assay; 0.6 ml Human Sampling IC Assay; 1 ml QN ROX; 2 x 1.9 ml RNase-Free Water	221415
QIAprep& Viral RNA UM Kit (2400)	For 2400 x 20 µl reactions: 4 x 1.2 ml Viral RNA UM Prep Buffer; 8 x 1.5 ml Viral RNA Master Mix, 4x; 4 x 1.2 ml RNA IC Template + Assay; 4 x 0.6 ml Human Sampling IC Assay, 4 x 1 ml QN ROX; 8 x 1.9 ml RNase-Free Water	221417

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Document Revision History

Date	Changes
11/2020	Initial release

Notes

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