

Nested-RT-PCR and Multiplex RT-PCR for Diagnosis of Rhinovirus Infection in Clinical Samples

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Abstract

Human rhinoviruses (HRVs) are positive-stranded RNA viruses belonging to the *Enterovirus* genus in the family of *Picornaviridae*. Identification of the specific strain in HRV disease has been difficult because the traditional serological method is insensitive, labor intensive, and cumbersome. With the fast progress in molecular biological technique, more sensitive and faster molecular methods have been developed, such as polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR, and real-time RT-PCR. To improve the technique for defining the links between illnesses and specific strains of HRV, we developed RT-PCR specific for HRV as routine base. A multiplex RT-PCR that simultaneously identifies 12 respiratory viruses including HRV is also routinely used in our lab. Here we have described the specific steps of methods for identification of HRV from clinical samples, such as sample preparation, isolation of total RNA, nested-RT-PCR for HRV, Seeplex[®] RV15 ACE Detection method, gel electrophoresis, how to use the QIAxcel[®] capillary electrophoresis system, and results interpretation.

Key words Human rhinovirus, Nucleic acid isolation, Polymerase chain reaction (PCR), Reverse transcription (RT)-PCR, Nest-PCR, Multiplex PCR

1 Introduction

Human rhinoviruses (HRVs), members within genus *Enterovirus* in the family *Picornaviridae*, have a positive-strand RNA genome of 7,200 nucleotides covalently linked at the 5' end to the viral protein 3B (VPg) and is translated cap-independently by internal ribosomal entry into a polyprotein (VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C-3D), which yields the 11 proteins through various independently functioning intermediates, upon cleavage by viral proteases [1]. HRVs were originally classified into two species, A and B, and approximately 100 serotypes [2–4]. Advances in molecular methods led to the characterization of the third HRV group, a genetically heterogeneous third species, HRV species C (HRV-C) [5, 6]. Identification of the specific strain in HRV disease has been difficult because the traditional serological method is insensitive,

labor intensive, and cumbersome [7], which requires the isolation of HRV in susceptible cell cultures and neutralization tests against all 101 serotype-specific antisera [3].

To improve the technique for defining the links between illnesses and specific strains of HRV, more sensitive and faster molecular methods have been developed. PCR, RT-PCR, and real-time RT-PCR represent the latest diagnostic methods that provide robust, reproducible results.

We present here an RT-PCR for identification of HRVs and a multiplex RT-PCR that simultaneously identifies 12 respiratory viruses; these assays are routinely used in our laboratory for diagnosis of HRV infection in clinical samples and have been optimized to enable simultaneous handling of large numbers of samples.

2 Materials

2.1 Sample Preparation

1. Sterile collection tubes with nasopharyngeal aspirates.
2. Flocked tipped swabs with nasal secretions.
3. Sterile saline (0.9 % solution of sodium chloride).
4. Microcentrifuge.
5. Eagle's Minimum Essential Medium (E-MEM) OR Virus Transport Medium (VTM) (*see Note 1*).

2.2 Isolation of Total RNA

1. Viral RNA isolation kit.
2. Ethanol (96–100 %).
3. Microcentrifuge tubes (1.5 ml).
4. Sterile, RNase-free pipette tips with aerosol barrier
5. Microcentrifuge (with rotor for 2-ml tubes).

2.3 RT-PCR

1. Ethanol (96–100 %).
2. Reverse Transcriptase Kit (e.g., SuperScript™ III, Invitrogen).
3. DNA Polymerase PCR Kit (e.g., GoTaq Green Master Mix, Promega or MangoTaq, Bioline).
4. Seeplex® RV15 ACE Detection Kit (Seegene).
5. Primers (Table 1).
6. RNase-free tips (10, 100, and 1,000 µl).
7. 96-Well plates for PCR with membranes for sealing.
8. GeneAmp PCR system 9700 (Applied Biosystems, *see Note 3*).

2.4 Gel Electrophoresis

1. Agarose.
2. DNA gel-loading dye.
3. DNA molecular weight marker.
4. Ethidium bromide.

Table 1
Primers for HRV nested-PCR

Primer name	Primer sequence (5'–3')	Target gene	Amplicon length
First run PCR			
RV-F1	CTCCGGCCCCCTGAATRYGGCTAA		
RV-R1	TCIGGIARYTTCCASYACCAICC		
Second run PCR			
RV-F2	ACCRASACTTTGGGTRWCCGTG	5' NCR-VP4/VP2	110 bp
RV-R2	CTGTGTTGAWACYTGAGCICCCA		

Bases are shown in single-letter symbols; *A* Adenine, *C* Cytosine, *T* Thymine, *G* Guanine, *W* A/T, *S* C/G, *R* A/G, *Y* C/T, *I* Inosine

5. Electrophoretic buffers.
6. Electrophoresis apparatus.
7. UV transilluminator.
8. QIAxcel® capillary electrophoresis system.

3 Methods

3.1 Sample preparation (See Notes 4–6)

1. All samples are collected in sterile tubes in the clinic and sent to microbiology laboratory for testing within 3 h of collection. Samples should be stored at 4 °C at this time (*see Note 4*).

For respiratory virus diagnostics two kinds of samples are collected, nasopharyngeal aspirates (NPA) and nasal swabs. NPAs require specialist equipment that must be handled by trained personnel; this procedure should be performed according to local safety and clinical guidelines. Nasal swabs are collected by inserting flocked tipped swabs into nostrils and must be performed by trained personnel only and according to local safety and clinical guidelines. Swabs must be inserted into tubes with VTM immediately after collection.

2. 1.5 ml sterile saline is added into each tube of NPA; tubes are centrifuged at 14,000 rpm (20,000 × *g*) for 2 min; the supernatant is removed and pellet is resuspended in 200 µl of E-MEM, aliquoted, and kept at –70 °C.

Nasal swabs are squeezed ten times against the tube walls in VTM and removed; tubes are centrifuged at 3,000 rpm (200 × *g*) for 2 min; the supernatant is aliquoted and kept at –70 °C.

3.2 Isolation of Total RNA

The protocol presented here is based on the QIAamp viral RNA isolation kit; any kit for isolation of total RNA from cell-free body fluids may be used in its stead (*see Notes 2 and 7*).

1. Equilibrate samples and Buffer AVE to room temperature (15–25 °C).

2. Check that Buffer AW1, Buffer AW2, and Carrier RNA have been prepared according to the manufacturer's instructions. Redissolve any precipitates in Buffer AVL/Carrier RNA by heating, if necessary, and cool to room temperature before use. All centrifugation steps are carried out at room temperature.
3. Pipette 560 μ l of prepared Buffer AVL containing Carrier RNA into a 1.5-ml microcentrifuge tube. Add 140 μ l treated NPAs/nasal swab solution to the Buffer AVL/Carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s. Incubate at room temperature for 10 min.
4. Briefly centrifuge the 1.5-ml microcentrifuge tube to remove drops from the inside of the lid.
5. Add 560 μ l of ethanol (96–100 %) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5-ml microcentrifuge tube to remove drops from inside the lid.
6. Carefully apply 630 μ l of the solution from **step 5** to the QIAamp spin column (in a 2-ml collection tube) without wetting the rim. Close the cap, and centrifuge at $6,000\times g$ (8,000 rpm) for 1 min. Discard the filtrate.
7. Carefully open the QIAamp spin column, and repeat **step 6**.
8. Carefully open the QIAamp spin column, and add 500 μ l of Buffer AW1. Close the cap, and centrifuge at $6,000\times g$ (8,000 rpm) for 1 min. Place the QIAamp spin column in a clean 2-ml collection tube, and discard the tube containing the filtrate.
9. Carefully open the QIAamp spin column, and add 500 μ l of Buffer AW2. Close the cap and centrifuge at full speed (14,000 rpm; $20,000\times g$) for 3 min. Continue directly with **step 10**, or to eliminate any chance of possible Buffer AW2 carryover, perform **step 9(a)**, and then continue with **step 10**.
 - (a) (Optional): Place the QIAamp spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
10. Place the QIAamp spin column in a clean 1.5-ml microcentrifuge tube. Discard the old collection tube containing the filtrate. Carefully open the QIAamp spin column and add 60 μ l of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at $6,000\times g$ (8,000 rpm) for 1 min. Viral RNA is stable for up to 1 year when stored at $-20\text{ }^{\circ}\text{C}$ or $-70\text{ }^{\circ}\text{C}$.

3.3 RT-PCR

3.3.1 First-Strand cDNA Synthesis

We routinely perform reverse transcription using random hexamers and SuperScriptTM III Reverse Transcriptase Kit on GeneAmp PCR system 9700 according to the manufacturer's recommendations. Any other reverse transcriptase system can be

used, keeping in mind that some optimization may be required for optimal results (*see Note 9*).

1. Briefly centrifuge each reagent provided in the kit.
2. Prepare pre-mix 1 in a sterile, nuclease-free microcentrifuge tube as below, mix by brief vortex, and centrifuge briefly:

Pre-mix 1 (for one reaction)	
5 µg Total RNA	5 µl
50 ng/µl random primer	1 µl
10 mM dNTP mix	1 µl
DNase- and RNase-free water	3 µl
Total volume	10 µl

Calculate the required total amount of each reagent based on the number of reactions (samples + controls).

3. Label a 96-well plate, as the cDNA plate. Pipette 10 µl of pre-mix into each well of the cDNA plate; cover with the sealing membrane and briefly centrifuge the plate.
4. Place the cDNA plate into GeneAmp PCR system 9700, and incubate at 65 °C for 5 min. Take the cDNA plate out and put it on the ice. Centrifuge briefly before removing the membrane seal.
5. Take another sterile, nuclease-free 1.5 ml microcentrifuge tube labeled as pre-mix 2. Add the following reagents (shown for one reaction); mix by quick vortex, and centrifuge briefly.

Pre-mix 2 (for one reaction):	
10× RT buffer	2 µl
25 mM MgCl ₂	4 µl
0.1 M DTT	2 µl
RNase OUT(40 U/µl)	1 µl
SuperScript III RT(200 U/µl)	1 µl
Total volume of pre-mix 2	10 µl

Calculate the required amount of each reagent based on the number of reactions (samples + controls).

6. Pipet 10 µl of pre-mix 2 into each well of the cDNA plate; cover it with the sealing membrane carefully and briefly centrifuge the plate.

7. Place the cDNA plate into GeneAmp PCR system 9700, run the following cycling parameter: one cycle of (25 °C, 10 min); followed by one cycle of 50 °C, 50 min; then one cycle of (85 °C, 5 min) to stop the reaction. Take the plate out and put it on the ice. Centrifuge briefly before removing the sealing membrane.
8. Pipet 1 µl of RNase H into each well of the cDNA plate; cover carefully with membrane seal and centrifuge briefly.
9. Place the cDNA plate into GeneAmp PCR system 9700, and run one cycle of (37 °C, 20 min).
10. The first-strand cDNA is synthesized and can serve as the template in the nested-PCR or the multiplex PCR or stored at -20 °C for future use.

3.3.2 Nested-PCR

This procedure consists of two PCR cycles, the first run PCR using the cDNA as template and the second run PCR using the amplimers from the first run PCR as template.

1. First Run PCR

1. Prepare pre-mix 1 in the reagent preparation room (*see Note 8*). Completely thaw and thoroughly vortex the buffer prior to use; then briefly centrifuge each reagent.
2. In a sterile, nuclease-free microcentrifuge tube combine the following components to prepare the pre-mix 1 (shown for one reaction); mix by quick vortex and centrifuge briefly.

Pre-mix 1 (for one reaction)	
Nuclease-free water	8.25 µl
5× colorless GoTaq flexi buffer	5 µl
Mgcl ₂ solution, 25 mM	2 µl
PCR nucleotide mix, 10 mM each	0.5 µl
Primers	1 µl
Go Taq DNA polymerase (5 u/µl)	0.25 µl
Total volume of pre-mix 1	17 µl

Calculate the necessary amount of each reagent based on the number of reactions (samples + controls).

3. Label a 96-well plate as the first run PCR plate. Pipet 17 µl of pre-mix 1 into each well of the first run PCR plate; Transfer the first run PCR plate from the reagent preparation room to the template preparation room. Open the cDNA plate and pipet 3 µl of cDNA template from the cDNA plate into each corresponding well of the first run PCR plate.

4. Cover the 96-well plates with the membrane for PCR plate carefully; briefly centrifuge the plate.
5. Place the first run PCR plate into the GeneAmp PCR system 9700 in the nucleic acid amplification room, and run the following cycling parameter:

94 °C 30 s	
55 °C 1 min	45 cycles
72 °C 1 min	
72 °C 10 min	

2. Second run PCR.

1. Prepare pre-mix 2 in the reagent preparation room. Completely thaw and thoroughly vortex the buffer prior to use; then briefly centrifuge each reagent.
2. In a sterile, nuclease-free microcentrifuge tube combine the following components to prepare the pre-mix 2 (shown for one reaction); mix by quick vortex and centrifuge briefly.

Pre-mix 2 (for one reaction)	
Nuclease-free water	10.25 µl
5× colorless GoTaq flexi buffer	5 µl
MgCl ₂ solution, 25 mM	2 µl
PCR nucleotide mix, 10 mM each	0.5 µl
Primers	1 µl
Go Taq DNA polymerase(5u/µl)	0.25 µl
Total volume of pre-mix 2	19 µl

Calculate the necessary amount of each reagent based on the number of reactions (samples + controls).

3. Label another 96-well plate as the second run PCR plate. Pipet 19 µl of pre-mix 2 into each well of the second run PCR plate.
4. Transfer the second run PCR plate from the reagent preparation room to the template preparation room. Open the first run PCR plate and pipet 1 µl of DNA template from the first run PCR plate into each corresponding well of the second run PCR plate.
5. Cover the second run PCR plate with the membrane for PCR plate carefully; briefly centrifuge the plate.

- Place the second run PCR plate into the GeneAmp PCR system 9700 in the nucleic acid amplification room, and run the following cycling parameter:

94 °C 30 s	
55 °C 1 min	35 cycles
72 °C 1 min	
72 °C 10 min	

- After analysis of the PCR products by electrophoresis, irradiate the PCR products with UV light (365 nm) for 20 min to prevent carryover contamination.

3.3.3 Multiplex PCR for 15 Respiratory Viruses

We usually use the Seeplex RV15 ACE Detection Kit to simultaneously detect 15 common respiratory viruses. The Seeplex kit is very widely used in clinical microbiology laboratories around the world. The viruses included influenza (Flu) A/B, respiratory syncytial viruses (RSV) A/B, human metapneumovirus (hMPV), human parainfluenza viruses (PIV) 1/2/3/4, human adenovirus (AdV), human rhinovirus (HRV), human coronavirus 229E/NL63 and OC43/HKU1, human bocavirus (HBoV) and human enterovirus.

- Prepare master mix in the reagent preparation room. Completely thaw and thoroughly vortex the buffer prior to use, and then briefly centrifuge each reagent.
- Add the following reagents (shown for one reaction) to a sterile, nuclease-free microcentrifuge tube, mix by quick vortex, and centrifuge briefly:

5× RV 15 ACE PM	4 µl
2× Multiple master mix	2 µl
8-MOP solution	3 µl
Water	8 µl
Total volume of master mix	17 µl

Calculate the necessary amount of each reagent based on the number of reactions (samples + controls).

- Label a 96-well plate as the RV15 PCR plate. Aliquot 17 µl of master mix into each well the RV15 PCR plate.
- Transfer the RV15 PCR plate from the Reagent preparation room to the template preparation room. Pipet 3 µl of cDNA template from the cDNA plate into each corresponding well of the RV 15 PCR plate. Use 3 µl of ACE NC for the negative control PCR. Use 3 µl of ACE PC for the positive control PCR.



Fig. 1 Gel image of rhinovirus nested-RT-PCR products. The nasopharyngeal aspirate (NPA) samples (S) 1, 3, 5, 9, 10, 21, and 32 were collected from seven children with lower respiratory tract infection. Respiratory epithelial cells were collected from NPAs and processed for RNA isolation followed by nested-RT-PCR as described in Subheadings 2 and 3. PCR products were separated on 2 % agarose gel electrophoresis and visualized by ethidium bromide staining; the relevant DNA marker is indicated

5. Cover the RV 15 PCR plate with sealing membrane and briefly centrifuge the plate.
6. Place the RV 15 PCR plate into the preheated 94 °C GeneAmp PCR system 9700 in the nucleic acid amplification room, and immediately run the PCR reaction using the following program:

94 °C 0.5 min	
60 °C 1.5 min	40 cycles
72 °C 1.5 min	
72 °C 10 min	

3.3.4 Analysis of PCR Products

PCR products may be analyzed by agarose gel electrophoresis or by capillary electrophoresis. Figure 1 depicts a typical pattern obtained by gel electrophoresis, while Fig. 2 depicts a typical pattern obtained by capillary electrophoresis.

1. Separate the PCR products from the second run PCR by 2 % agarose gel electrophoresis and visualize by UV transillumination (*see* Chapter 3 for detailed method).
 - (a) For reactions containing the 5× Green GoTaq™ Reaction Buffer, load samples onto the gel directly after amplification.
 - (b) For reactions not containing any indicator dyes, add gel-loading dye (e.g., bromophenol blue) before loading on the gel.
 - (c) Please *see* Fig. 1 for a picture of a sample gel of nested-PCR products from nasal swabs collected from children with suspected lower respiratory tract infection.
 - (d) There are single bright bands at the 110 bp size location of the positive control (PC), lanes labeled S5, S10, S21, and S32, but no bands around the same location in the negative control (NC), S1, S3, and S9 lanes.

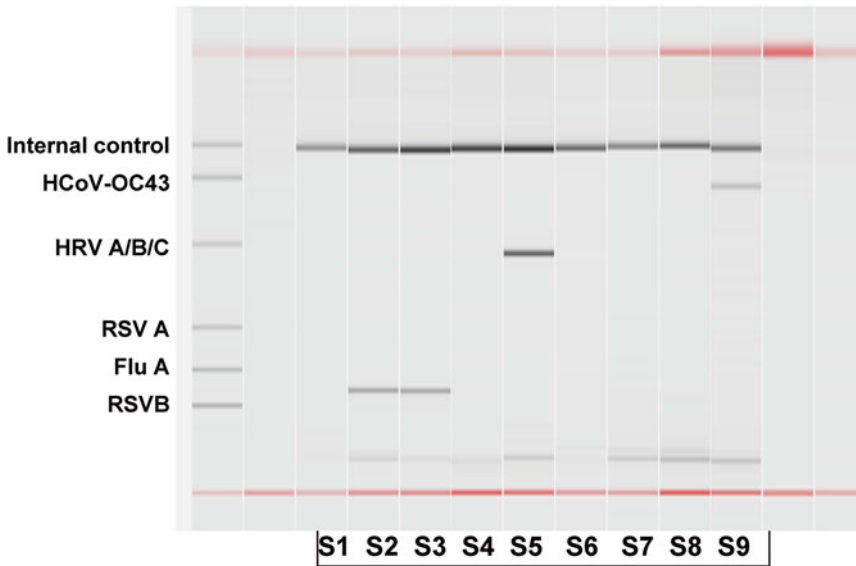


Fig. 2 Image of Seeplex RV 15 Multiplex products using QIAxcel capillary electrophoresis. NPA samples (S) 1–9 were collected from nine children with lower respiratory tract infection. NPA samples were processed for cell isolation, RNA extraction, and reverse transcription as in Fig. 1. Seeplex RV 15 Multiplex PCR was performed as described in the text and PCR products separated by the QIAxcel® Novel 12-channel capillary electrophoresis system. The reference table for RV15 ACE Detection (B set) marker is indicated

2. Capillary electrophoresis using QIAxcel system (*see Note 10*). The 96-well plates can be run directly in the QIAxcel® capillary electrophoresis system. Separation is performed in a capillary of a precast gel cartridge. Each sample is automatically loaded (according to voltage and time parameters) into an individual capillary and voltage is applied. As the molecules migrate through the capillary, they pass a detector that measures the fluorescent signal. A photomultiplier detector converts the emission signal into electronic data, which are then transferred to the computer for further processing using BioCalculator software. After processing, the data are displayed as an electropherogram and a gel image.
 - (a) The 0.2-ml 12-tube strips containing QX Alignment Marker and QX Intensity Calibration Marker (if required) should fit loosely in the MARKER1 and MARKER2 position.
 - (b) QX Alignment Markers should be replaced every 15–20 runs or as needed.
 - (c) When not in use, the 12-tube strip containing QX Alignment Marker should be stored at -20°C . It should be equilibrated to operating temperature ($20\text{--}25^{\circ}\text{C}$) and centrifuged briefly before use.

- (d) If the QIAxcel gel cartridge is being used for the first time, intensity calibration should be performed (*see* **Note 11**).
- (e) Preparation of the gel cartridge:
Add 10 ml QX Wash Buffer to both reservoirs of the QX Cartridge Stand (provided with the QIAxcel instrument) and cover with 3 ml mineral oil (supplied).
Remove the QIAxcel gel cartridge from its packaging and carefully wipe off any soft gel debris from the capillary tips using a soft tissue.
Remove the purge cap seal from the back of the QIAxcel gel cartridge and place it in the QX Cartridge Stand.
Incubate new cartridges in the QX Cartridge Stand for 20 min prior to use.
- (f) Preparing the buffer tray:
Allow all reagents to equilibrate to room temperature (15–25 °C) before use.
Wash the buffer tray with hot water and rinse thoroughly with deionized water.
Fill the WP and WI positions of the buffer tray with 8 ml QX Wash Buffer.
Fill the BUF position of the buffer tray with 18 ml QX Separation Buffer.
Carefully add mineral oil to all three positions to prevent evaporation: add 2 ml mineral oil to positions WP and WI and 4 ml mineral oil to position BUF. All three positions should be covered with mineral oil.
Insert the buffer tray into the buffer tray holder so that the slots for the 12-tube strips face the front of the instrument.
- (g) Preparing QX Alignment Markers:
Load 15 µl QX Alignment Marker into each well of a QX 0.2 ml 12-tube strip.
Add one drop of mineral oil to each well, and insert the strip into the MARKER1 position of the buffer tray.
Important: The 12-tube strip should fit loosely in the MARKER1 position on the buffer tray.
- (h) Installing a QIAxcel gel cartridge and smart key:
Remove the QIAxcel gel cartridge from the QX Cartridge Stand; open the cartridge door and insert the QIAxcel gel cartridge into the QIAxcel system. The cartridge description label should face the front and the purge hole should face the back of the system.
Insert the smart key into the smart key socket. The smart key can be inserted in either direction.
Close the cartridge door. The cartridge ID, number of runs remaining, and cartridge type will be displayed

automatically in the “Instrument Control” window once the smart key is latched. The system will not recognize the cartridge and will not operate if the smart key is not inserted.

Add the DNA size marker (supplied with the kit) into the black well (*see Note 12*).

Load the 96-well plate containing the samples. The minimum sample volume required for analysis is 10 μl . Less than 0.1 μl of the sample will be loaded onto the QIAxcel gel cartridge for analysis. The remaining DNA can be kept for reanalysis or downstream processing.

Results for nine nasal swab samples assayed by the Seeplex RV15 multiplex PCR products are shown in Fig. 2. According to the reference table provided by the manufacturer, samples 2 and 3 are probably positive for influenza A (a second specific PCR and/or sequencing of the PCR product needs to be performed to confirm), sample 5 is positive for HRV, and sample 9 is positive for CoV-OC43. The size of samples 2 and 3 is equal to influenza A, the size of sample 5 is equal to human rhinovirus A/B/C, and the size of sample 9 is equal to human coronavirus OC43. Samples 4, 6, 7, and 8 are negative for all the respiratory viruses detected by Seeplex assay.

4 Notes

1. E-MEM or VTM can be used to collect samples for virus testing. If desired, commercial VTM preparations can be used.
2. The protocol described here has been optimized for the Roche MagnaPure LC 2.0 system; however, any comparable system for isolation of total RNA from clinical samples may be used, but may require modification of the protocol to optimize results.
3. Any PCR equipment that is able to handle multiple samples in a 96-well format will be suitable.
4. The clinical samples collected from patients with lower respiratory tract infection should be kept in 4 °C refrigerator and sent to microbiological laboratory within 8 h post-collection; If samples cannot be sent to laboratory within 8 h post-collection, they should be frozen at -70 °C.
5. Appropriate personal protective equipment (laboratory coat, mask, gloves) should be worn at all times when handling human specimens.
6. All clinical samples collected from patients with lower respiratory tract infection should be handled in a biological safety cabinet.

7. When the number of samples to be analyzed is less than 10, it is quicker and more convenient to isolate the total RNA from samples manually by using the QIAamp® Viral RNA Mini Kit. When the number of samples is more than 10, it is laborious and time consuming to isolate the total RNA from samples manually and an automated system, e.g., the Roche MagnaPure LC 2.0, may be used.
8. Prepare the PCR pre-mix only in the pre-mix preparation room. Assay the first-strand cDNA synthesis and perform template DNA/RNA or cDNA pipetting in the template room. Do not apply the first-strand cDNA synthesis in the pre-mix preparation room to avoid contamination of the following PCR test. Ensure that the plates are covered and sealed with a membrane before they are transferred to PCR machine in the PCR amplification room.
9. The 8-channel micropipette is preferred when using 96-well plates. It is less laborious and more accurate to aliquot reagent and pipet template DNA/RNA or cDNA into each well. Using multichannel micropipette also helps to reduce the contamination during PCR assay process.
10. The QIAxcel system offers a number of advantages over traditional slab-gel electrophoresis, including higher detection sensitivity, less sample wastage (minimal sample input volumes), fast analysis of up to 96 samples, and automated loading and analysis, making it the preferred method for analysis of DNA products when large numbers of samples are processed.
11. This step is not necessary if the QIAxcel gel cartridge has already been calibrated, unless it is being used on a different QIAxcel instrument or a different computer is used to operate the instrument. If a different computer is used, the calibration log file must be transferred from the computer used to operate the instrument to the new computer so that it is not necessary to run the calibration again.
12. Once created, a single DNA reference marker table can be used for the entire life of the cartridge. However, variations in separation temperature of the cartridge and buffer can introduce variations in DNA size determination. For optimal results, we recommend creating a new DNA reference marker table every 8 runs or after each 96-well plate.

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