Prodesse® Pro hMPV®+ Assay

Instructions for Use

For detection of human Metapneumovirus.

100

-70°C

CE

REF

303671

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Intended Use

The Prodesse® Pro hMPV®+ Assay is a Real-Time PCR (RT-PCR) *in vitro* diagnostic test for the qualitative detection of human Metapneumovirus (hMPV) nucleic acid isolated and purified from nasopharyngeal swab (NP) specimens obtained from individuals exhibiting signs and symptoms of acute respiratory infection. This Assay targets a highly conserved region of the Nucleocapsid gene of hMPV. The detection of hMPV nucleic acid from symptomatic patients aids in the diagnosis of human respiratory hMPV infection if used in conjunction with other clinical and laboratory findings. This test is not intended to differentiate the four genetic sub-lineages of hMPV.

Negative results do not preclude hMPV infection and should not be used as the sole basis for diagnosis, treatment or other management decisions.

Summary and Explanation

Human Metapneumovirus (hMPV) causes acute respiratory illness. Patients infected with hMPV present symptoms of acute upper and/or lower respiratory tract infections and share the same nonspecific symptoms as many other respiratory infectious agents; cough, rhinorrhea, and fever being the most common[1]. There are two distinct genetic lineages that have been established for hMPV and are designated as subtypes A and B. These lineages have further been divided into subgroups A1, A2, B1 and B2, as determined by performing phylogenetic analysis of sequence data, most often utilizing the fusion protein and G glycoprotein gene. No significant differences have been observed among patients infected with different subgroups of hMPV in terms of clinical manifestations[2]. Although information on the mode of transmission and virulence is not definitive, hMPV is likely spread by similar means as common respiratory viruses such as influenza. hMPV has been shown to co-infect with other respiratory pathogens, most commonly Respiratory Syncytial Virus at a rate of 5 to 17%, although much higher rates have been observed in hospitalized patients[1]. hMPV appears to provide only partial immunity following infection, likely due to the variety of strains and subtypes that circulate during any given season, and can re-infect individuals potentially leading to repeated episodes of illness. Infections occur mainly during late winter and early spring and the prevalence of each subtype of hMPV varies, seemingly both from year to year, and by location. Likewise, the overall incidence of hMPV can vary from year to year and its prevalence has been reported to range from 2 to 26% in patients with symptoms of respiratory infection[2]. hMPV is responsible for a significant portion of the 150,000 children hospitalized annually in the United States for bronchiolitis[3].

Principles of the Procedure

The Pro hMPV+ Assay enables detection and differentiation of hMPV and Universal Internal Control. An overview of the procedure is as follows:

1. Nasopharyngeal swab specimens are collected from patients with signs and symptoms of respiratory infection using a polyester, nylon or rayon tipped swab and placed into viral transport medium (refer to **Materials Required but not Provided**).
2. An Universal Internal Control (UIC) is added to every sample and is carried through all steps of the procedure from nucleic acid extraction through amplification and detection to monitor for inhibitors present in the specimens.
3. Extraction and purification of nucleic acids are performed using either a MagNA Pure LC System (Roche) and the MagNA Pure Total Nucleic Acid Isolation Kit (Roche) or a NucliSENS easyMAG system (bioMérieux) and the Automated Magnetic Extraction Reagents (bioMérieux).
4. The extracted and purified nucleic acids are added to Pro hMPV+ Supermix along with enzymes included in the kit. The Pro hMPV+ Supermix contains oligonucleotide primers complementary to a highly conserved region of the Nucleocapsid gene of hMPV and target-specific oligonucleotide probes dual-labeled with a reporter dye and a quencher dye.
5. After initial reverse transcription of RNA into complementary DNA (cDNA), amplification proceeds during which the probe anneals specifically to a region of the template between the forward and reverse primers. As primer extension and amplification occur, the 5' – 3' exonuclease activity of the Taq polymerase cleaves the probe separating the reporter dye from the quencher. This generates an increase in fluorescent signal upon excitation from an LED light source of appropriate wavelength. With each cycle, additional reporter dye molecules are cleaved from their respective probes, further increasing fluorescent intensity. The amount of fluorescence at any given cycle is dependent on the amount of amplification products present at that time. Fluorescent intensity is monitored during each PCR cycle by the real-time instrument.

Analyte	Gene Targeted	Probe Fluorophore	Absorbance Peak	Emission Peak	Instrument Channel
human Metapneumovirus	Nucleocapsid	FAM	495 nm	520 nm	FAM
Universal Internal Control	NA	Quasar 670	647 nm	670 nm	Cy5

Materials Provided

Pro hMPV+ Assay Kit (303671)

Reagents	Description	Quantity/ Vial	Cap Color	Cat. #	Reactions/ Vial
Pro hMPV+ Supermix	➲ Taq DNA polymerase ➲ 2 oligonucleotide primer pairs ➲ 2 oligonucleotide probes ➲ Buffer containing dNTPs (dATP, dCTP, dGTP, dTTP) ➲ $MgCl_2$ and stabilizers	1030 µL	Brown	403176	50 (2 vials provided)
M-MLV Reverse Transcriptase II	➲ 11.4 U/µL	36 µL	White	GLS32	100
RNase Inhibitor II	➲ 40 U/µL	120 µL	Green	GLS33	100
Pro hMPV+ Control (Positive Control)	➲ Non-infectious *in vitro* transcribed RNA hMPV specific viral sequences	500 µL	Yellow	403506	25
Universal Internal Control (UIC)	➲ Non-infectious *in vitro* transcribed RNA ➲ Non-infectious DNA plasmid	30 µL	Lilac	403097	100

Materials Required But Not Provided

Plasticware and Consumables

- ☐ Polyester, rayon or nylon tipped nasopharyngeal swabs
- ☐ RNase/DNase-free 1.5 mL polypropylene microcentrifuge tubes
- ☐ Sterile RNase/DNase-free filter or positive displacement micropipettor tips
- ☐ MagNA Pure LC System Disposables (Reagent Tubs, Reaction Tips, Tip Trays, Cartridges) or easyMAG System Disposables (Sample Strips and Tips)
- ☐ Biohit Pipette Tips for use with easyMAG System
- ☐ Greiner Break Four uncoated plates for use with easyMAG System
- ☐ Cepheid PCR reaction tubes, 25 µL
- ☐ Parafilm M or MagNA Pure LC Cartridge Seals

Reagents

- ☐ Roche MagNA Pure LC Total Nucleic Acid Isolation Kit *(Roche Cat. # 03038505001)* for 192 isolations or bioMérieux NucliSENS easyMAG reagents *(Buffer 1 Cat. # 280130, Buffer 2 Cat. # 280131, Buffer 3 Cat. # 280132, Magnetic Silica Cat. # 280133, and Lysis Buffer Cat. # 280134)*
- ☐ Micro Test M4 Viral Transport Medium *(Remel, Inc. Cat. # R12500)*, Micro Test M4RT Viral Transport Medium *(Remel, Inc. Cat. # R12505),* Micro Test M5 Viral Transport Medium *(Remel, Inc. Cat. # R12515)*, Micro Test M6 Viral Transport Medium *(Remel, Inc. Cat. #12530)*, BD Universal Viral Transport vial, 3mL *(Becton, Dickinson and Co. Cat. # 220220)* or Copan Universal Transport Medium *(Copan Diagnostics, Inc., Copan Cat. # 330C)*
- ☐ Molecular Grade Water *(RNase/DNase Free)*
- ☐ Extraction Control *(e.g. previously characterized positive sample or negative sample spiked with a well characterized hMPV strain)*

Equipment

- ☐ – 70°C Freezer
- ☐ Roche MagNA Pure LC System with software version 3.0.11 or bioMérieux NucliSENS easyMAG System with Software version 1.0.1 or 2.0
- ☐ Biohit multi-channel pipettor for use with easyMAG System
- ☐ Cepheid SmartCycler II Real Time Instrument with Dx Software version 1.7b or 3.0a/3.0b
- ☐ Micropipettors (range between 1-10 µL, 10-200 µL and 100-1000 µL)
- ☐ Mini-centrifuge with adapter for Cepheid reaction tubes
- ☐ Cepheid cooling block
- ☐ Ice/ice bucket or -20°C cold block
- ☐ Biosafety Cabinet

Warnings and Precautions

- For In Vitro Diagnostic Use only.
- Use of this product should be limited to personnel who have been trained in the techniques of Real-Time PCR.
- Performance characteristics of this Assay have only been determined with nasopharyngeal swab specimens.
- Specimens should be handled as if infectious using safe laboratory procedures such as those outlined in CDC/NIH *Biosafety in Microbiological and Biomedical Laboratories* and in the CLSI Document M29 *Protection of Laboratory Workers from Occupationally Acquired Infections*. Thoroughly clean and disinfect all surfaces with 10% bleach. Autoclave any equipment or materials that have contacted clinical specimens before discarding.
- Use pipettes with aerosol barrier or positive displacement tips for all procedures.
- Always pre-plan, organize and segregate workflow. Workflow in the laboratory should proceed in a unidirectional manner, beginning in the Pre-Amplification Area and moving to the Amplification/Detection Area.
    - Begin pre-amplification activities with reagent preparation and proceed to specimen preparation.
    - Always dedicate supplies and equipment to a specified area; no cross-movement allowed between areas.
    - Do not use equipment and supplies used for reagent preparation for specimen preparation activities or for pipeting or processing other sources of target nucleic acid.
    - Keep all amplification supplies and equipment in the Amplification/Detection Area at all times.
    - Always wear disposable gloves in each area and change them before entering a different area.
    - Do not open reaction tubes following PCR.
- Take care to preserve the purity of kit reagents. Avoid contamination from Positive Control and specimens by following good laboratory practices.
- Do not use reagent after its expiration date.
- Do not mix reagents with different lot numbers or substitute reagents from other manufacturers.
- Material Safety Data Sheets (MSDS) are available on manufacturer's website at **www.gen-probe.com**.

Reagent Storage, Handling and Stability

- Store all reagents (opened and unopened) at ≤ – 70°C until the expiration date listed on the kit.
- Always check the expiration date on the reagent tubes. For Intermediate stock of the Universal Internal Control, use the expiration date of the originating stock control vial. Do not expose Controls to more than one (1) freeze-thaw cycle.
- Pro hMPV+ Assay Kits are shipped frozen, should arrive frozen, and should be stored frozen after receipt. If the contents are not frozen, contact Customer Service for assistance.
- An internal study demonstrated that performance of Pro hMPV+ Supermix, MMLV Reverse Transcriptase II and RNase Inhibitor II are not affected for up to 5 freeze-thaw cycles.
- Visually examine reagents for adequate reagent volume before beginning any test procedures.
- Protect Pro hMPV+ Supermix from light.
- **Controls and aliquots of controls must be thawed and kept on ice at all times during preparation and use.**

Recommendation

Aliquoting of kit components to maintain less than 5 freeze/thaw cycles is recommended for labs with smaller batch sizes.

Specimen Collection, Handling and Storage

Collecting the Specimen

To obtain nasopharyngeal swab samples:

1. Insert a flexible-shaft polyester, rayon or nylon tipped swab containing a dry tip into one nostril and into the nasopharyngeal area.
2. Press the swab gently against the nasal wall to allow the swab to absorb secretions.
3. Rotate the swab two to three times and withdraw it.
4. Place the swab into a tube containing 3 mL of viral transport medium (Remel M4, M4RT, M5, or M6; Becton Dickinson UVT; or Copan UTM).
5. Break off the shaft of the swab and cap the tube.

Note

Using a smaller volume of the viral transport medium may result in inhibition.

Transporting Specimens

Ensure that when transporting human respiratory specimens, all applicable regulations for the transport of etiologic agents are met. Transport human respiratory specimens refrigerated at 2-8°C.

Storing Specimens

Store specimens refrigerated (2-8°C) for up to 72 hours before processing. Store any leftover specimens at ≤ – 70°C. If retesting a frozen specimen, thaw specimen quickly (1 to 2 minutes) in a 37°C water bath and immediately place on ice, or thaw specimen on ice.

Storing Purified Nucleic Acid

Store purified nucleic acids at ≤ – 70°C. They should be tested after no more than one (1) freeze-thaw cycle.

Note

Inadequate or inappropriate specimen collection, storage and transport are likely to yield false negative results.

Recommendation

Training in specimen collection is highly recommended because of the importance of specimen quality.

Reagent and Control Preparation

Reagents

Note

Prepare reagents from the Roche MagNA Pure LC Total Nucleic Acid Isolation Kit or the bioMérieux easyMAG Automated Magnetic Extraction Reagents following the manufacturer's instructions.

Controls

Recommendation

- *For aliquots of the Pro hMPV+ Positive Control and Intermediate stock of the Universal Internal Control, use the expiration date of the originating stock control vial.*
- *Controls and aliquots of controls must be thawed and kept on ice/cold block at all times during preparation and use. It is recommended to prepare controls in a sample prep area, such as a*

Biological Safety Cabinet.

Positive Control (PC)

Note

Include the Positive Control with each RT-PCR run.

1. Thaw Pro hMPV+ Positive Control (the yellow cap vial) on ice.
2. Make 25 aliquots of 20 µL, label and store at ≤ –70°C. Ensure that aliquots do not undergo more than one (1) freeze-thaw cycle.
3. **The Positive Control is used at the provided concentration.**

Recommendation

Do not spike Positive Control with the Universal Internal Control. Do not dilute the Positive Control. Do not take Positive Control through the nucleic acid isolation procedure.

Universal Internal Control (UIC)

1. Thaw Universal Internal Control (lilac cap vial) on ice.
2. Create Intermediate stock tubes of the Universal Internal Control using the following dilution scheme:

26 µL Universal Internal Control	+	*65 µL RNase Inhibitor*	+	*2509 µL molecular grade water*	=	*2600 µL total volume*

3. Make aliquots of 110 µL, label, and store at ≤ –70°C (this is enough volume to add to 5 samples at 20 µL per sample). Make aliquots of larger or smaller volumes based on the number of samples expected to be processed in a single run. Ensure that aliquots do not undergo more than one (1) freeze-thaw cycle.
4. Add the appropriate volume of Intermediate stock of the Universal Internal Control to each sample prior to nucleic acid isolation (see ***Step 1*** of the ***Assay Procedure***).
5. Save RNase Inhibitor II for use in ***Step 4*** of the ***Assay Procedure***.

Negative Control (NC)

1. Use Viral Transport Medium as the Negative Control.
2. Add the appropriate volume of Intermediate stock of the Universal Internal Control to the Negative Control prior to nucleic acid isolation (see ***Step 1*** of the ***Assay Procedure***).

Extraction Control (EC)

Good laboratory practice recommends including a positive extraction control (e.g. previously characterized positive sample or negative sample spiked with a well characterized hMPV strain) in each nucleic acid isolation run. The extraction control should be treated like a sample during Assay performance and analysis.

Assay Procedure

Assay Overview

Get Ready: Create the Assay protocol for the Cepheid SmartCycler Instrument using the Dx Software *(first time only).*

1. ***Prepare the Samples and Negative Control.***
2. ***Isolate the Nucleic Acid –*** *MagNA Pure LC System using the Total Nucleic Acid Isolation (TNAI) Kit*

   OR
3. ***Isolate the Nucleic Acid –*** *NucliSENS easyMAG System using the Automated Magnetic Extraction Reagents.*
4. ***Set up the RT-PCR Reaction.***
5. ***Run the Pro hMPV+ Assay protocol.***
6. ***Print report.***

Note

- *Instructions provided for the Cepheid SmartCyclerII Instrument with Dx Software version 3.0a/3.0b.(Instructions for version 1.7b noted).*
- *Do NOT deviate from the protocol settings defined in this section.*

Get Ready: Create the Assay protocol for the Cepheid SmartCycler Instrument using the Dx Software (first time only)

Note

- *The protocol is only created for first time use; it does not need to be recreated with each sample run.*
- *Refer to SmartCycler Dx Software Operator Manual for assistance in defining assay protocols.*
- *To **Define** and **Edit** Assay protocols, the user must have administrative access rights. Otherwise, the fields will not be accessible for data entry and editing (they will be grayed out).*
- *Cepheid Dx Software interprets the data and reports the run as either VALID or INVALID, based on the results of the Positive and Negative Control. Enter the Extraction control as if it was a sample.*
- *Interpret the control results and determine if the run is VALID or INVALID. All Control criteria must be met in order for the run to be VALID (see **Interpretation of Results** section).*

1. Create the Pro hMPV+ Assay protocol**:**

- ***a.*** Launch the Cepheid Dx software application.
- ***b.*** Click on the **Define Assay** box at the top of the screen.
- ***c.*** Click on the **New Assay** box at the bottom of the screen.
- ***d.*** Enter **Pro hMPV+ Assay** for the assay protocol in the window that opens.
- ***e.*** Click **OK**.
- ***f.*** Enter **Thermocycler Parameter** in the Protocol section (bottom half of ***Define Assay*** screen).

Stage 1			**Stage 2**			**Stage 3** Repeat **5** times			**Stage 4** Repeat **30** times		
Hold			**Hold**			**2- Temperature Cycle**			**2- Temperature Cycle**		
Temp	Secs	Optics	Temp	Secs	Optics	Temp	Secs	Optics	Temp	Secs	Optics
42	**1800**	**OFF**	**95**	**600**	**OFF**	**95**	**30**	**OFF**	**95**	**10**	**OFF**
						55	**60**	**ON**	**55**	**60**	**ON**

Stages 5 – 10 remain UNUSED

2. Enter information in **BOLD** in the **Analysis Settings** tab as follows:

- ***a.*** Select **FTTC25** for the **Dye Set**.
- ***b.*** **Analysis Type: Qualitative** (default).
- ***c.*** **Customize Result Text: Target-based Result Text** (default).

Note

❖ *Gray boxes are default settings.*

Channel	Dye Name	Channel Name*	Usage	Curve Analysis	Thresh Setting	Manual Thresh	Auto Thresh	Auto Min. Cycle	Auto Max. Cycle	Valid Min. Cycle	Valid Max. Cycle	Bkgnd Sub	Bkgnd Min. Cycle	Bknd Max. Cycle	Boxcar Avg	EndPt Thresh	NC IC %	IC Delta
1	FAM*	hMPV	Target**	Primary Curve	Manual Threshold	30	NA	5	10	13	35	On	5	35	0	30	NA†	NA
2	TET	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	TxR	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	Cy5*	Universal Internal Control	Internal Control	Primary Curve	Manual Threshold	22	NA	5	10	17	35	On	5	35	0	22	NA†	NA

* *If Dx 1.7b = Target*
** *Dx 1.7b = Assay*
† *Dx 1.7b = 10*

3. Enter information in **BOLD** in the **Control Settings** tab**.**
   a. Select **NC Fails if any target criterion is positive**.
   b. *Use PC1 (Pos Cntrl 1) for Pro hMPV+ Positive Control. Enter **0** replicates to inactivate Positive Controls PC 2 and PC3.*
   c. *Use only one Negative Control (NC1). Enter* **0** *Replicates to inactivate the Negative Controls NC2 and NC3.*

Note

❖ *Gray boxes are default settings.*

Control ID	Control Name	Replicate	hMPV Valid Min Cycle	hMPV Valid Max Cycle	hMPV EndPt Thresh	UIC +/-	UIC Valid Min Cycle	UIC Valid Max Cycle	UIC EndPt Thresh
PC1	Pos Cntrl 1	**1**	**13.0**	**35.0**	**30**	**-**	**17.0**	**35.0**	**22**
PC2	Pos Cntrl 2	0	13.0	45.0	10	+	13.0	45.0	10
PC3	Pos Cntrl 3	0	13.0	45.0	10	+	13.0	45.0	10
NC1	Neg Cntrl 1	**1**	**13.0**	**35.0**	**30**	**+**	**17.0**	**35.0**	**22**
NC2	Neg Cntrl 2	0	13.0	45.0	10	+	13.0	45.0	10
NC3	Neg Cntrl 3	0	13.0	45.0	10	+	13.0	45.0	10

4. Click on the **Advanced** tab and select **Require Lot Number**.
5. **Probe Check Settings** tab, **Advance to New Stage** tab, and **Standards** tab are not used for the Pro hMPV+ Assay protocol.
6. Select **Save Assay**.

1. Prepare the Samples, Extraction Control, and Negative Control (Pre-Amplification Area)

a. **Add Universal Internal Control to all samples.**

i. Thaw the appropriate number of aliquots of Intermediate stock of the Universal Internal Control (enough volume needed for each sample, Extraction Control, and the Negative Control) on ice.

ii. Remove 180 µL of sample from the original sample tube and pipet into a labeled 1.5 mL microcentrifuge tube. Alternately, pipet 180 µL of sample directly into sample cartridge or sample vessel.

iii. Remove 180 µL of Extraction Control from the original sample tube and pipet into a labeled 1.5 mL microcentrifuge tube. Alternately, pipet 180 µL of sample directly into sample cartridge or sample vessel.

iv. Add 20 µL of Intermediate stock of the Universal Internal Control to each sample**. Pipet up and down a minimum of 5 times to mix using a new pipet tip for each sample.**

v. Keep tubes on ice.

vi. Store any remaining sample at ≤ – 70°C.

b. **Add Universal Internal Control to the Negative Control.**

i. Include one (1) Negative Control in each run.

ii. Add 180 µL of Viral Transport Medium to a labeled 1.5 mL microcentrifuge tube. Alternately, pipet 180 µL of Viral Transport Medium directly into sample cartridge or sample vessel.

iii. Add 20 µL of Intermediate Stock of Universal Internal Control to the Viral Transport Medium. **Pipet up and down a minimum of 5 times to mix.**

iv. Keep tube on ice.

v. Discard remaining volume of Universal Internal Control – DO NOT reuse.

Do not reuse Universal Internal Control.

2. Isolate the Nucleic Acid (Pre-Amplification Area I) – MagNA Pure LC System using the Total Nucleic Acid Isolation (TNAI) Kit

a. **Start the instrument and software.**

i. Turn power on to the MagNA Pure LC instrument and then turn on the computer.

ii. Start the MagNA Pure LC software.

iii. From the *Main Menu* Screen, select **Sample Ordering** and enter sample information in **Sample Name** column.

iv. Select the **Total NA Variable_elution_volume.blk** protocol.

v. Follow the software instructions and specify the number of samples.

vi. Type in **200 µL** for the sample volume and verify that **50 µL** elution volume is selected.

vii. Select **Stage Setup** and the software will automatically calculate the amount of each reagent that is required.

b. **Fill the reagent tubs.**

Before starting the isolation procedure, fill all reagent tubs outside the instrument with the required volume of each reagent listed on the **Start Information** screen.

Note

Use only the reagent amount needed for the number of samples entered into the software. Reagents are not stable for long-term storage in tubs. Vortex Magnetic Glass Particles (MGPs) and load the exact amount of MGPs (as listed on the **Start Information** *Screen) into the instrument just before the run starts.*

c. **Load reagent tubs and disposables into the instrument.**

Use the information on the **Start Information** screen to place all disposable plastics and reagent tubs necessary for the batch run on the Reagent/Sample Stage.

Recommendation

Use a colored "Positioning Frame" (provided with the TNAI kit) on the Reagent Tub Rack to help to correctly load reagents.

d. **Load the samples, Extraction Control, and Negative Control into the MagNA Pure Sample Cartridge.**

i. Transfer all 200 µL of each sample to the proper individual wells in the MagNA Pure Sample Cartridge.

ii. Transfer all 200 µL of the Extraction Control and Negative Control to different wells in the MagNA Pure Sample Cartridge.

iii. Cover cartridge with Parafilm or MagNA Pure LC Cartridge Seal and keep cartridge on ice until ready to load the instrument.

e. **Load the samples.**

Transfer the Sample Cartridge containing the samples, Extraction Control, and Negative Control into the MagNA Pure LC instrument.

f. **Start the run.**

i. Start the Batch Run by confirming the correct placement of all disposable plastics and reagents by mouse-clicking the respective text boxes on the **Start Information** screen.

ii. Select the **OK** button to start the automated isolation procedure. The instrument will automatically dispense all reagents and process the samples.

g. **Store the eluted total nucleic acid.**

After completing the run, place the Storage Cartridge containing the eluted nucleic acids immediately on ice or transfer eluted nucleic acid to 1.5 mL tubes and store for longer durations at ≤ – 70°C.

Note

Do not store purified nucleic acids in the Storage Cartridge on the Cooling Unit 1.

3. Isolate the Nucleic Acid (Pre-Amplification Area I) – NucliSENS easyMAG System using the Automated Magnetic Extraction Reagents

a. Start Instrument and Software.

Turn power on to the easyMAG instrument and once the LED on the instrument turns green, turn on the computer and log into the software.

b. Prepare the software for a run.

To prepare for a run, touch the "**Settings**"  icon in the main toolbar which defaults to the "Application Settings" icon and choose the following run settings:

Default Protocol: Generic 1.0.6 or 2.0.1 (for software version 1.0.1 or 2.0, respectively)
Run Name Prefix: N/A (leave as default)
Sample ID prefix: N/A (leave as default)
Sample Type: Primary (on-board lysis)
Default On-board Lysis Dispensing: Yes
Default On-board Lysis Incubation: Yes
Sample Addition Guidance: Off
Reagent Tracking: Off

c. Input buffer information.

Touch the "**Instrument**"  icon to default to the "**Reagent Inventory**"  icon and input the buffer barcodes by first scanning the instrument position (A, B, C, or D) and then its corresponding buffer. For example, scan position A and then scan the bottle of Lysis buffer in that position and then move on to position B and its corresponding bottle.

d. Create a worklist.

i. Touch the "**Daily Use**"  icon which will default to the "**Define Extraction Request"**  icon and select the following settings:

Sample ID: Manually enter the sample name.
Matrix: Other
Protocol: Generic 1.0.6 or 2.0.1 (for software version 1.0.1 or 2.0, respectively)
Volume (mL): 0.200 (input volume of sample)
Eluate (µL): 55
Type: Primary
Priority: Normal

ii. Press **Enter** on the keyboard or touch the "**New Extraction Request**"  icon after each manual sample addition. The settings above will remain as the default settings for each subsequent entry as long as you do not navigate to other pages.

e. Create a run and add samples from the worklist.

Touch the "**Organize Runs**"  icon and then the "**Create Run**"  icon which will bring up the *New Run Window.* In this screen, name the run appropriately and verify that the **auto-number** box is left unchecked (NOT SELECTED) and that **Yes** is selected for both the On-Board Lysis Dispensing and On-Board Lysis Incubation options. Touch **OK** and the *New Run Window* will close and you will be in the "**Organize Runs**" screen. Assign samples to run with the positioning (arrow)  icons. Touch the "**Load Run"**  icon and select the run. Print the worklist with the "**Print worklist"**  icon. This worklist will aid in keeping track of the order of the samples to be loaded into the sample vessel wells.

f. Load the samples and tips and barcode the sample strip(s).

Add all of the 200 µL from each sample into the proper vessels of the sample strip(s) as noted in the worklist. Insert tips into sample vessel(s) in the correct order as noted in the worklist and scan the sample strip(s) position on the instrument and then the sample strip itself. For example, scan position A and then the sample strip in that position, then B and then C, if necessary. After scanning the sample strip(s), the indicator will change from red to green on the screen.

g. **On-Board Lysis Dispensing.**

Once the samples and tips are loaded and the strip(s) scanned, close the lid and touch the **"Dispense Lysis"**  icon. The instrument will dispense 2 mL of Lysis Buffer and incubate for 10 minutes.

h. **Prepare the magnetic silica to add to the sample vessel.**

During the 10-minute lysis incubation, use the Biohit multi-channel pipettor to prepare the magnetic silica. This procedure will need to be performed for each sample vessel used in the run (1, 2, or 3 times).

i. Set the pipettor to **Program 1** and place a Biohit pipette tip on position 1. Program 1 provides the means to aspirate and dispense 550 µL of liquid. The magnetic silica is prepared in a 1:1 ratio of Molecular Biology Grade Water to Magnetic Silica.

ii. Using Program 1 of the pipettor, press the **start** button to aspirate and then again to dispense 550 µL of water into a microcentrifuge tube. Vortex the tube of magnetic silica briefly to mix and use Program 1 of the pipettor to aspirate and then dispense 550 µL of magnetic silica into the same microcentrifuge tube as the water. Eject the tip, cap the tube and vortex to mix.

iii. Set the pipettor to **Program 2** and place a Biohit pipette tip on position 1. Program 2 will transfer 8 volumes of the previous mix to the 8 vessels of a strip on an ELISA plate (1 strip/sample vessel). Press the **start** button to aspirate the mix. Press the **start** button again to dispense the remaining mixture back into the tube containing the mix to reset the pipette.

iv. Press the **start** button 8 separate times to dispense the remaining mix in each of 8 vessels of an ELISA plate strip and eject the tip.

v. After the 10 minute lysis incubation is done, set the pipettor to **Program 3** and place 8 Biohit pipette tips on the multichannel pipettor (or however many samples are present in the specific sample strip). Make sure that the filter tips are very well connected with the multichannel pipettor to prevent leakage errors. Program 3 first mixes the magnetic silica mixture in the ELISA plate and then aspirates it to be delivered to the vessels of the sample strip where it will be mixed. Press the **start** button once and the pipette will mix the silica in the ELISA plate and then aspirate it for addition to the sample vessel. Verify that each tip has the same volume of silica mix before placing in the sample vessel. Place the pipettor over the sample vessel strip so the tips are below the liquid level of each sample and press the **start** button again, which will then aspirate 800 µL out of each sample vessel and perform 3 mix cycles with 1000 µL. At this point be sure to hold the pipette steady below the liquid/air interface as it is mixing so as not to introduce bubbles to the sample.

vi. Repeat for each sample strip in the run.

i. **Start the run.**

Touch the "**Start**"  icon to begin the run. The instrument will perform 5 washes and heat and elute. The purified nucleic acids need to be transferred to appropriate storage tubes (1.5 mL microcentrifuge tubes) on ice within 30 minutes of extraction completion to avoid contamination by the magnetic silica stuck to the front wall of the sample vessel(s). Use immediately or store at ≤ –70˚C.

4. Set up the RT-PCR Reaction (Pre-Amplification Area II)


Note

Start the SmartCycler Pro hMPV+ Assay run within 1 hour of making the RT-PCR master mix.

a. Thaw the Pro hMPV+ Positive Control.

i. Include the Positive Control with each run. Thaw one (1) aliquot of the Positive Control on ice.

ii. Keep tube on ice.

b. Prepare the RT-PCR master mix.

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

19.45 µL	Pro hMPV+ Supermix
+0.30 µL	M-MLV Reverse Transcriptase II
+0.25 µL	RNase Inhibitor II
20.00 µL	per reaction

ii. Thaw the Pro hMPV+ Supermix on ice and **mix by pipetting up and down a minimum of 5 times before use**.

iii. Remove M-MLV Reverse Transcriptase II and RNase Inhibitor II enzymes from the freezer and keep on ice during use. M-MLV Reverse Transcriptase should be spun down and pipetted from the top.

iv. Prepare the RT-PCR master mix by combining the reagents listed above in a 1.5 mL microcentrifuge tube. **Pipet up and down a minimum of 5 times to mix.**

v. Keep the RT-PCR master mix on ice and protected from light before adding to SmartCycler tubes.

c. Add 20 µL of RT-PCR master mix to each SmartCycler tube.

i. Load the required number of tubes into the Cepheid Cooling Block.

ii. Pipet the RT-PCR master mix into the upper part of the SmartCycler tubes. Discard any unused RT-PCR master mix.

d. Add 5 µL of each sample's nucleic acid to individual SmartCycler tubes containing RT-PCR master mix.

i. After adding the sample's nucleic acid to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube. Use a new pipette tip for each sample.

e. Add 5 µL of the Extraction Control nucleic acid to a separate SmartCycler tube containing RT-PCR master mix.

i. After adding the extraction control nucleic acid to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube.

f. Add 5 µL of Pro hMPV+ Positive Control to a separate SmartCycler tube containing RT-PCR master mix.

i. After adding the Positive Control to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube** using a new pipet tip for each control tube.

ii. Close the tube.

iii. Discard remaining volume of Positive Control – DO NOT reuse.

Do not reuse Positive Control aliquot.

g. **Add 5 µL of the Negative Control nucleic acid to the last SmartCycler tube containing RT-PCR master mix.**

i. After adding the Negative Control nucleic acid to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube.

h. **Centrifuge all tubes.**

i. Appropriately label the SmartCycler tubes on the caps.

ii. Centrifuge all tubes for 5 to 10 seconds using the Cepheid microcentrifuge specially adapted to fit the SmartCycler tubes.

iii. Return tubes to the cooling block.

i. **Keep the tubes on the Cepheid cooling block before loading them into the SmartCycler instrument.**

5. Run the Pro hMPV+ Assay (Amplification/Detection Area)

a. Create a new run by clicking on the **Create Run** icon at the top of the screen. This will open the *Create Run* screen.

b. Under **Run Name** in the left panel of the *Create Run* screen, enter a unique run identifier.

c. Click on the **Assay** arrow in the left panel of the *Create Run* screen and select the **Pro hMPV+ Assay** protocol from the drop-down menu.

d. Under **Assay Information** in the left panel of the *Create Run* screen, enter the **Lot Number** and **Expiration Date** (YYYY/MM/DD) of the Pro hMPV+ Assay kit.

e. In the left panel of the *Create Run* screen, enter the number of specimens (excluding the Positive and Negative Control) and click on **Apply**. This will display the **Site Table** and the SmartCycler Dx Software will automatically select the **I-Core** sites.

f. In the **Site Table** under the **Sample ID** column, enter the Sample Identifier for the appropriate I-Core sites.

g. Insert each reaction tube into an I-Core site of the SmartCycler by pressing down firmly on all tubes and close each lid. Verify that the Positive Control (PC1) and Negative Control (NC1) are loaded into the correct I-Core site.

h. Select the **Start Run** button located at the bottom left corner of the screen. Verify that the LED is on for the appropriate I-Core sites.

6. Print Report

a. Click **Report** at bottom of screen to open the **Report Preview** screen.

b. Click **Print** at the top of the screen.

Interpretation of Control Results

Validation of Run


Note

The user must interpret the Extraction Control (if included) results to determine whether the extraction run is VALID; the SmartCycler Dx software will automatically interpret the Positive and Negative Control results.

For a VALID Extraction run, the following conditions must be met:

Sample ID[1]	Assay Result	UIC Result	Warning / Error Code	Sample Type	UIC Ct	hMPV Result	hMPV Ct
Extraction Control	Positive	NA		SPEC	NA	Positive	13-35
Neg Control	Valid[2]	Pass		NC1	17-35	Valid	0

[1] *Columns and data not used for interpretation are not included.*

[2] *(Typical) An Invalid Assay will display Error Code 4098.*

For a VALID RT-PCR run, the conditions in the table below must be met. If the run is valid, specimens should be interpreted using the next section *Interpretation of Specimen Results*.

Sample ID[1]	Assay Result	UIC Result	Warning / Error Code	Sample Type	UIC Ct	hMPV Result	hMPV Ct
Pos Control	Valid	NA		PC1	NA	POS	13-35
Neg Control	Valid[2]	Pass		NC1	17-35	Valid	0

[1] *Columns and data not used for interpretation are not included.*

[2] *(Typical) An Invalid Assay will display Error Code 4098.*

Invalid Extraction Run

If the conditions for a Valid Extraction run are not met (i.e. the Extraction Control is not positive or the Negative Control is invalid), repeat the entire extraction run. Start from original sample(s) using a new Extraction Control and a new Negative Control (starting at ***Step 1*** of the ***Assay Procedure***).

Invalid RT-PCR Run

If the Positive Control is invalid but the Negative Control is valid, prepare all new reactions using remaining purified nucleic acids and a new Positive Control (starting with PCR at ***Step 4*** of the ***Assay Procedure*****)**.

If the Negative Control is invalid (i.e. positive in the hMPV channel), prepare new reactions using remaining purified nucleic acids from the Negative Control and sample(s) as appropriate.

Interpretation of Specimen Results

The SmartCycler Dx software automatically determines the specimen results. The interpretation of the Assay specimen results is as follows:

Sample ID[1]	Assay Result	UIC Result	Warning / Error Code	hMPV Result	Interpretation of Results
Sample ID	Negative	Pass	NONE	**NEG**	hMPV nucleic acid **not detected**
Sample ID	Positive	NA*	NONE	**POS**	hMPV nucleic acid **detected**
Sample ID	Unresolved	Fail	NONE	**NEG**	Unresolved – PCR inhibition or reagent failure. Repeat testing from the purified nucleic acid or collect and test a new sample.
Sample ID	Invalid		4098[2]	ND	Not Determined – error code 4098

[1] *Columns and data not used for interpretation are not included*

[2] *An Invalid Assay run will display Error Code 4098*

* *Detection of the Universal Internal Control in the Cy5 detection channel is not required for positive result. High viral load can lead to reduced or absent Universal Internal Control signal.*

Not Determined Samples

If an Assay result of **ND** (Not Determined) is reported with an instrument failure other than Warning/Error Code 3079, repeat testing from the purified nucleic acids (starting with PCR, see ***Step 4 (a)*** of the ***Assay Procedure***). Refer to the Cepheid Dx Software Operator Manual for interpretation of Warning Codes.

Quality Control

- Quality control requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard quality control procedures. It is recommended that the user refer to CLSI document C24-A3, *Statistical Quality Control for Quantitative Measurements: Principles and Definitions:* [Approved Guideline – Third Edition] or other published guidelines for general quality control recommendations. For further guidance on appropriate quality control practices, refer to 42 CFR 493.1205.
- Quality control procedures are intended to monitor reagent and Assay performance.

Control Type	Used to Monitor
Positive	Substantial reagent failure including primer and probe integrity
Negative	Reagent, environmental, or carry-over contamination
Extraction	Failure in lysis and extraction procedure
Internal	PCR inhibition in individual samples and Reagent failure or process error

- Dilute the Universal Internal Control and test both the Positive Control and the Universal Internal Control prior to running samples with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice recommends including a positive Extraction Control and a Negative Control in each nucleic acid isolation run. The Extraction Control should be treated as a sample.
- Never run the Positive Control through nucleic acid isolation.
- Always include a Negative Control *(containing Universal Internal Control)* and a Positive Control in each amplification/detection run performed.
- Failure of Controls (Positive, Negative and/or Extraction) invalidates the run and results should not be reported.
- If the Positive Control is invalid but the Negative Control is valid, repeat testing should be done starting from the purified nucleic acid and using a new aliquot of the Positive Control. If repeat results are still invalid, results should not be reported and testing should be repeated from the original sample or a new sample should be collected and tested.
- If the Extraction Control is not positive within the specified Ct range or the Negative Control is invalid, repeat testing should be done starting from the original sample and using a new Extraction Control and a new Negative Control. If repeat results are still invalid, results should not be reported and a new sample should be collected and tested.

Limitations

- This test does not differentiate hMPV subtypes (i.e., A1, A2, B1, and B2); additional testing is required to identify and differentiate hMPV subtypes.
- Negative results do not preclude hMPV infection and should not be used as the sole basis for diagnosis, treatment or other management decisions.
- False negative results may occur due to loss of nucleic acid. The Universal Internal Control has been added to the test to aid in the identification of specimens that contain inhibitors to PCR amplification. The Universal Internal Control does not indicate whether or not nucleic acid has been lost due to inadequate collection, transport or storage of specimens.
- A trained health care professional should interpret Assay results in conjunction with the patient's medical history, clinical signs and symptoms, and the results of other diagnostic tests.
- Analyte target (hMPV RNA sequence) may be present persistently in vivo, independent of virus viability. Detection of hMPV RNA does not imply that the hMPV is infectious, nor is the causative agent for clinical symptoms.
- The detection of hMPV RNA sequences is dependent upon proper specimen collection, handling, transportation, storage, and preparation (including extraction). Failure to observe proper procedures in any one of these steps can lead to incorrect results.
- **Once the RT-PCR master mix has been made, the run must be started within one hour.**
- Optimal Assay performance requires strict adherence to the Assay procedure described in this insert.
- Reliable results are dependent on adequate specimen collection, transport, storage and processing procedures.
- There is a risk of false negative values resulting from improperly collected, transported, or handled specimens.
- There is a risk of false positive values resulting from cross-contamination by target organism or its nucleic acids.
- There is a risk of false negative values due to the presence of sequence variants in the viral target of the Assay, procedural errors, amplification inhibitors in specimens, or inadequate numbers of organisms for amplification.
- The performance of the Pro hMPV+ Assay has not been established in immunocompromised patients.
- Positive and negative predictive values are highly dependent on prevalence. The Assay performance was established during the 2008 respiratory virus season (January – March). The performance may vary depending on the prevalence and population tested.

Expected Values

hMPV appears to have a similar seasonality as RSV in the US occurring during the winter/early spring months of the year[4]. Incidences of lower respiratory and upper respiratory infections in children have been reported between 5% - 15%[5]. In the Pro hMPV+ Assay multicenter prospective study, hMPV prevalence varied from 1% to 9% by site and averaged 5% overall. The number and percentage of hMPV RNA positive cases determined by the Pro hMPV+ Assay during this study, stratified by patient age group, are presented in the following table:

Age Group	Total (N)	Total # hMPV Positive By the Pro hMPV+ Assay	Observed Prevalence
< 1 year	296	25	8.4%
1-5 years	300	30	10.0%
6-10 years	115	7	6.1%
11-15 years	57	3	5.3%
16-21 years	82	1	1.2%
> 21 years	425	6	1.4%
Total	1275	72	5.6%

The performance of the modified Pro hMPV+ Assay has been demonstrated using a panel of selected retrospective hMPV positive and negative nasopharyngeal (NP) swab samples collected during 2011 - 2012. These were selected to include a total of 45 hMPV positive and 138 negative samples.

Performance Characteristics

Clinical Performance

Performance characteristics of the Pro hMPV+ Assay were established during a prospective study at 4 U.S. clinical laboratories during the 2008 respiratory virus season (January - March). Specimens used in the study represented excess nasopharyngeal (NP) swab specimens that were prospectively collected from symptomatic individuals suspected of respiratory infection, and were submitted for routine care or analysis by each site. Demographic details for this patient population are summarized in the following table:

Gender	Number of Subjects (Percentage of Total)
Female	617 (48.4%)
Male	654 (51.3%)
Not Determined	4 (0.3%)
Age	
≤ 5 years	596 (46.7%)
6 - 21 years	254 (19.9%)
22 – 59 years	219 (17.2%)
≥ 60 years	206 (16.2%)

Performance of the Pro hMPV+ Assay was assessed and compared to a predetermined algorithm that used composite reference methods. The composite reference methods consisted of two independent molecular (RT-PCR) tests for two separate gene targets of hMPV followed by bi-directional genetic sequencing. The two comparator methods targeted the Nucleocapsid gene (different region of the gene than targeted by the Pro hMPV+ Assay) and the Fusion gene. True hMPV RNA positives were considered as any sample that had bi-directional sequencing data meeting pre-defined quality acceptance criteria for one or both gene targets that matched hMPV sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov). True hMPV RNA negatives were considered as any sample that was tested negative by both of the comparator methods. Nucleic acid extractions on the clinical samples were carried out using either the Roche MagNA Pure LC System or the bioMérieux NucliSENS easyMAG System during the clinical study.

A total of 1275 eligible NP swab samples were tested with the Pro hMPV+ Assay at the four clinical sites and by the composite reference methods at Prodesse. Of the Pro hMPV+ Assay run on all eligible specimens, 98.1% (1273/1298) of these specimens were successful on the first attempt. The remaining 25 specimens gave "Unresolved" results on the first attempt. Unresolved results occur when the sample is negative for both hMPV and the Internal Control, indicating potentially PCR-inhibiting samples. Of the 25 "Unresolved" specimens on the first attempt with sufficient sample for retest, 8.0% (2/25) gave a valid "negative" result on the second attempt. The remaining 23 samples were "Unresolved" on the second attempt, therefore, were not included in the analysis below. All 23 samples were tested negative by the composite reference methods.

		Composite Reference Methods			
		Positive	**Negative**	**Total**	**Comments**
Pro hMPV+ Assay	**Positive**	64	8	**72**	Percent Positive Agreement 95.5% (87.6% - 98.5%) 95% CI
	Negative	3	1200	**1203**	Percent Negative Agreement 99.3% (98.7% - 99.7%) 95% CI
	Total	**67**	**1208**	**1275**	

Clinical Comparison Results

The Pro hMPV+ Assay's supermix was reformulated and performance characteristics were established by comparing the reformulated Assay to the original Pro hMPV+ Assay. One hundred eighty-three retrospective nasopharyngeal swab samples collected during 2011 – 2012 at two sites (Milwaukee, WI and Chicago, IL) were used for this study. hMPV positive and negative NP swab samples were selected for inclusion based on previous site specific molecular test results. One sample was not used in the final analysis as it was Unresolved upon initial and repeat testing with both the original and reformulated Pro hMPV+ Assays.

"True" hMPV positives were considered as any sample that tested positive for hMPV by the original Pro hMPV+ Assay. "True" hMPV negatives were considered as any sample that tested negative for hMPV by the original Pro hMPV+ Assay. Discrepant analysis for samples where the reformulated Pro hMPV+ Assay and the original Pro hMPV+ Assay results were in disagreement was performed using RT-PCR with hMPV specific primers targeting the hMPV phosphoprotein gene followed by bi-directional genetic sequencing.

hMPV Comparison Results

		Current Pro hMPV+ Assay			
		Positive	**Negative**	**Total**	**Comments**
Reformulated Pro hMPV+ Assay	**Positive**	43	2*	**45**	Percent Positive Agreement 100% (91.80%-100%) 95% CI
	Negative	0	137	**137**	Percent Negative Agreement 98.6% (94.91%-99.61%) 95% CI
	Total	**43**	**139**	**182**	

* Two samples tested positive for hMPV by bi-directional sequencing.

Reproducibility

The reproducibility of the Pro hMPV+ Assay was evaluated at 3 laboratory sites. Reproducibility was assessed using a panel of 9 simulated samples that included medium positive, low positive (near the Assay limit of detection) and "high negative" hMPV samples. Panels and controls were tested at each site by 2 operators for 5 days (9 samples and 3 controls X 2 operators X 5 days X 3 sites = 360). Nucleic acid extraction on the test panel samples were carried out using either the Roche MagNA Pure LC System (Clinical Trial Site #4) or the bioMérieux NucliSENS easyMAG System (Site #1 and Site #2). The overall percent agreement with the expected result for the Pro hMPV+ Assay was 99.2%.

	Panel Member ID	hMPV A2 High Negative [a]	hMPV A2 Low Positive	hMPV A2 Moderate Positive	hMPV B2 High Negative [a]	hMPV B2 Low Positive	hMPV B2 Moderate Positive	hMPV RNA Control	Negative Control[a]	Extraction Control hMPV A2	Total % Agreement
	Concentration	0.01 X LoD	2 X LoD	10 X LoD	0.01 X LoD	2 X LoD	10 X LoD	NA	NA	NA	
		1×10^{0} $TCID_{50}$/mL	2×10^{2} $TCID_{50}$/mL	1×10^{3} $TCID_{50}$/mL	1×10^{-1} $TCID_{50}$/mL	2×10^{1} $TCID_{50}$/mL	1×10^{2} $TCID_{50}$/mL				
Site 1	Agreement with Expected result	15/15 (100%)	15/15 (100%)	15/15 (100%)	15/15 (100%)	15/15 (100%)	15/15 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	**120/120 (100%)**
	Average Ct Value	26.6	29.2	27.1	27.5	29.3	26.6	32.5	26.2	33.1	
	% CV	1.53	2.84	1.21	1.97	2.20	1.68	0.81	0.80	2.73	
Site 2	Agreement with Expected result	15 /15 (100%)	14/15 (93.3%)	15/15 (100%)	15/15 (100%)	13/15 (86.7%)	15/15 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	**117/120 (97.5%)**
	Average Ct Value	25.8	30.7	26.9	26.9	30.7	26.3	32.8	25.6	32.9	
	% CV	0.54	3.95	2.88	1.44	4.14	1.25	1.37	0.98	4.86	
Site 4	Agreement with Expected result	15/15 (100%)	15/15 (100%)	15/15 (100%)	15/15 (100%)	15/15 (100%)	15/15 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	**120/120 (100%)**
	Average Ct Value	27.4	30.5	27.8	28.5	29.4	27.0	33.6	27.6	28.8	
	% CV	1.45	2.15	2.13	3.00	3.80	2.50	1.09	1.87	3.08	
	Total Agreement with Expected result	45/45 (100%)	44/45 (97.8%)	45/45 (100%)	45/45 (100%)	43/45 (95.6%)	45/45 (100%)	30/30 (100%)	30/30 (100%)	30/30 (100%)	**357/360 (99.2%)**
	95% CI	92.1% - 100%	88.4% - 99.6%	92.1% - 100%	92.1% - 100%	85.2% - 98.8%	92.1% - 100%	88.6% - 100%	88.6% - 100%	88.6% - 100%	**97.6% - 99.7%**
	Overall Average Ct Value	**26.6**	**30.1**	**27.63**	**27.6**	**29.7**	**26.6**	**33.0**	**26.5**	**31.6**	
	Overall %CV	**2.85**	**3.73**	**2.57**	**3.29**	**3.97**	**2.16**	**1.72**	**3.49**	**7.36**	

[a]Average Ct value calculated for the Internal Control (IC)

Analytical Sensitivity

The analytical sensitivity (limit of detection or LoD) of the Pro hMPV+ Assay was determined using quantified ($TCID_{50}$/mL) cultures of 2 hMPV (subtype A2 and subtype B2) strains serially diluted in nasopharyngeal clinical matrix. Each viral strain was extracted using the Roche MagNA Pure LC instrument and tested in replicates of 20 per concentration of virus. Analytical sensitivity (LoD) as defined as the lowest concentration at which ≥ 95% of all replicates tested positive, ranged from 10^2 – 10^1 $TCID_{50}$/mL. The LoD's for the reformulated Pro hMPV+ Assay were determined using the bioMérieux NucliSENS EasyMAG system for extraction and the LoD was identical to the original Pro hMPV+ Assay for hMPV A2 and 0.5 log lower for hMPV B2.

Viral Strain	LoD Concentration	LoD Concentration (Reformulated Pro hMPV+ Assay)
hMPV subtype A2	10^2 $TCID_{50}$/mL	10^2 $TCID_{50}$/mL
hMPV subtype B2	10^1 $TCID_{50}$/mL	$10^{0.5}$ $TCID_{50}$/mL

Reactivity

The reactivity of the Pro hMPV+ Assay was evaluated against four strains of hMPV (subtypes A1, A2, B1 and B2). Each viral strain was extracted using the Roche MagNA Pure LC instrument and tested in triplicate. All viral cultures of the panel were detected by the Pro hMPV+ Assay.

Viral Strain	Concentration	hMPV (FAM)
hMPV subtype A1	10^1 $TCID_{50}$/mL	+
hMPV subtype A2	10^1 $TCID_{50}$/mL	+
hMPV subtype B1	10^1 $TCID_{50}$/mL	+
hMPV subtype B2	$10^1$$TCID_{50}$/mL	+

Analytical Specificity

The analytical specificity of the Pro hMPV+ Assay was evaluated by testing a panel of 52 cultures consisting of 28 viruses, 23 bacteria, and 1 yeast strain representing common respiratory pathogens or flora commonly present in the nasopharynx. Bacteria and yeast were tested at concentrations of 10^6 to $7.4x10^7$ CFU/mL. Viruses were tested at concentrations of 10^2 to 10^6 $TCID_{50}$/mL. Samples were extracted using the Roche MagNA Pure LC instrument and tested in triplicate. Analytical specificity of the Pro hMPV+ Assay was 100%.

Strains	Concentration	hMPV (FAM)
hMPV A2	10^3 $TCID_{50}$/mL	+
hMPV B2	10^2 $TCID_{50}$/mL	+
Adenovirus 1/Adenoid 71	10^6 $TCID_{50}$/mL	-
Coronavirus 229E	10^6 $TCID_{50}$/mL	-
Coxsackie B4	10^4 $TCID_{50}$/mL	-
Coxsackie B5/10/2006	10^5 $TCID_{50}$/mL	-
Cytomegalovirus	10^4 $TCID_{50}$/mL	-
Echovirus 2	10^6 $TCID_{50}$/mL	-
Echovirus 3	10^5 $TCID_{50}$/mL	-
Echovirus 6	10^5 $TCID_{50}$/mL	-
Echovirus 11	10^5 $TCID_{50}$/mL	-
Enterovirus 68	10^3 $TCID_{50}$/mL	-
Enterovirus 70	10^3 $TCID_{50}$/mL	-
HSV Type 1 MacIntyre Strain	10^5 $TCID_{50}$/mL	-
HSV Type 2 G strain	10^5 $TCID_{50}$/mL	-
Human Rhinovirus 39	10^3 $TCID_{50}$/mL	-
Human Rhinovirus	10^4 $TCID_{50}$/mL	-
Influenza A/Port Chalmers	10^4 $TCID_{50}$/mL	-
Influenza B/Wisconsin	10^4 $TCID_{50}$/mL	-
Measles/7/2000	10^4 $TCID_{50}$/mL	-
Mumps Virus	10^4 $TCID_{50}$/mL	-
Parainfluenza Type 1	10^4 $TCID_{50}$/mL	-
Parainfluenza Type 2	10^5 $TCID_{50}$/mL	-
Parainfluenza Type 3	10^5 $TCID_{50}$/mL	-
Parainfluenza Type 4	10^4 $TCID_{50}$/mL	-
RSV A Strain Long	10^4 $TCID_{50}$/mL	-
RSV B Strain Wash	10^4 $TCID_{50}$/mL	-
Varicella Zoster Virus	10^4 $TCID_{50}$/mL	-
Bordetella pertussis	10^6 CFU/mL	-
Chlamydia pneumoniae	10^4 $TCID_{50}$/mL	-
Chlamydia trachomatis	10^4 $TCID_{50}$/mL	-
Legionella pneumophila	10^6 CFU/mL	-
Mycobacterium intracellulare	10^7 CFU/mL	-
Mycobacterium tuberculosis	10^7 CFU/mL	-
Haemophilus influenza	10^6 CFU/mL	-
Pseudomonas aeruginosa	10^6 CFU/mL	-
Proteus vulgaris	10^6 CFU/mL	-
Proteus mirabilis	10^6 CFU/mL	-
Neisseria gonorrhoeae	10^6 CFU/mL	-
Neisseria meningitidis	10^6 CFU/mL	-
Neisseria mucosa	$7.4x10^7$ CFU/mL	-
Klebsiella pneumoniae	10^6 CFU/mL	-
Escherichia coli	10^6 CFU/mL	-
Moraxella catarrhalis	10^7 CFU/mL	-
Corynebacterium diptheriae	$3x10^7$ CFU/mL	-
Lactobacillus plantarum	10^6 CFU/mL	-
Streptococcus pneumoniae	10^6 CFU/mL	-
Streptococcus pyogenes	10^6 CFU/mL	-
Streptococcus salivarius	$2x10^6$ CFU/mL	-
Staphylococcus epidermidis	10^6 CFU/mL	-
Staphylococcus aureus	10^6 CFU/mL	-
Candida albicans	10^6 CFU/mL	-

Note: Fresh cultured and titered stocks of organisms were used for the analytical specificity study, except for *C. pneumoniae* and *C. trachomatis*. ATCC frozen cultures and ATCC supplied titers were used for both due to technical difficulties in re-growing and re-titering these organisms.

Interference

Whole blood and a number of other potentially interfering substances (medications and over the counter (OTC) products) that may be present naturally or artificially introduced in the nasopharynx were evaluated in the Pro hMPV+ Assay. Two subtypes of hMPV (A2 and B2) were used and spiked into NP pools at either 2X LoD or 10X LoD. The hMPV strains were re-titered prior to interference testing. Clinically relevant amounts of the potential interfering substances were added to spiked samples. The Internal RNA Control III (IC) was also added to each sample. Nucleic acid from the samples was extracted with the Roche MagNA Pure LC instrument. The Pro hMPV+ Assay was performed in triplicate reactions for each sample on the Cepheid SmartCycler II. The following table shows the potential interfering substances used for this study. The substances consisted of nasal sprays (liquid and powder), ingestible pills and lozenges, injectables, and endogenous substances:

Substance Name	Active Ingredient	Concentration Tested
Mucin	Purified mucin protein	60µg/mL
Blood (human)	N/A	2% (volume/volume)
Neo-Synephrine	Phenylephrine HCl	15% (volume/volume)
Anefrin Nasal Spray	Oxymetazoline Hydrochloride	15% (volume/volume)
Zicam Nasal gel	Luffa Operculata, Galphimia Glauca, Histaminum Hydrochloricum,	5% (volume/volume)
Saline Nasal Spray	Sodium chloride with preservatives	15% (volume/volume) of dose
Chloraseptic Throat Lozenges	Oral anesthetic/analgesic	0.63mg/mL; active ingredients: 1.0mg/mL benzocaine, 1.7mg/mL menthol
Relenza	Zanamivir	3.3mg/mL
Tobramycin	Tobramycin	4.0µg/mL
Mupirocin	Mupirocin	6.6mg/mL
Rebitol	Ribavirin	20mg/mL
TamiFlu	Oseltamivir	25mg/mL
Beconase AQ	Beclomethasone dipropionate	5% (volume/volume)

All of the exogenous or endogenous potentially interfering substances tested were found to have no effect on Pro hMPV+ Assay results when present in simulated respiratory samples at the concentrations indicated.

Extraction Equivalency

Extraction equivalency of the bioMérieux NucliSENS easyMAG and Roche MagNA Pure LC instruments was evaluated by performing a limit of detection study. Single cultured and titered strains of hMPV A2 and hMPV B2 were spiked (along with an IC) into individual aliquots of negative NP matrix pools at concentrations of 1 log above, at, and 1 log below the previously determined LoD. Each viral strain dilution was extracted in replicates of 10 on each automated extractor and tested using the Pro hMPV+ Assay.

The bioMérieux NucliSens easyMAG instrument and the Roche MagNA Pure LC instrument performed equivalently with respect to limit of detection.

Carry-over/Contamination

To evaluate the degree of carry-over/cross-contamination that occurs with the use of the Pro hMPV+ Assay in association with nucleic acid extraction on the Roche MagNA Pure LC and the bioMérieux NucliSens easyMAG instruments and RT-PCR on the Cepheid SmartCycler II thermocycler, an internal Carry-Over study was carried out by testing simulated human Metapneumovirus (hMPV) high positive samples run in series alternating with hMPV high negative samples. The hMPV high positive samples in this study represented the lower Cycle Threshold (Ct) range (higher sample titer range) obtained in the Pro hMPV+ Assay clinical trials (lowest Ct = 17.9). The high-negative samples include a low amount of hMPV that is detectable no more than 5% of the time. One out of twenty-two high-negative samples tested showed potential hMPV contamination when extracted using the MagNA Pure extraction system in the study. Potential contamination could have occurred during creation of the sample, during sample preparation for extraction, during extraction or when transferring the purified nucleic acid samples from the sample cartridge to microfuge tubes. The possibility that this sample falls in the 5% category could not be ruled out.

Disposal

Dispose of hazardous or biologically contaminated materials according to the practices of your institution.

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Understanding the Symbols

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Symbol	Definition	Symbol	Definition
REF	Reference Number or Catalog number	Σ	Contains sufficient for < n > tests
LOT	Batch code or Lot Number		Do not reuse
	Use By Date or Expiration Date		Manufacturer
	Upper Storage Temperature Limitation	EC REP	Authorized Representative
CONT	Contents		

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