

This Procedural Bulletin is intended to provide a ready outline reference for performance of the assay. These abbreviated directions for use are not intended to replace the complete Package Insert. It is the obligation of every manufacturer of medical devices labeled FOR *IN VITRO* DIAGNOSTIC USE to provide a complete Package Insert in accordance with FDA labeling regulation (21CFR 809.10).

Quidel Corporation provides CLSI procedures for your use. The procedures are required to include the same information as listed in the Package Insert. Any modifications to this document are the sole responsibility of the Laboratory.

Lyra® RSV + hMPV

CLIA Complexity: High

INTENDED USE

The Lyra RSV + hMPV Assay is a multiplex Real-Time PCR (RT-PCR) assay for the qualitative detection and identification of respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) ribonucleic acid (RNA) extracted from nasal and nasopharyngeal swab specimens from patients with signs and symptoms of respiratory infection. This *in vitro* diagnostic test is intended to aid in the differential diagnosis of RSV and hMPV infections in humans in conjunction with clinical and epidemiological risk factors. This test is not intended to differentiate the two subtypes of RSV or the four genetic sub-lineages of hMPV.

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

Conversely, positive results do not rule-out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. The use of additional laboratory testing and clinical presentation must be considered in order to obtain the final diagnosis of respiratory viral infection.

The Lyra RSV + hMPV Assay can be performed using either the QuantStudio® Dx RT-PCR Instrument, the Applied Biosystems® 7500 Fast Dx RT-PCR Instrument or the Applied Biosystems 7500 Fast RT-PCR Instrument, or the Cepheid SmartCycler® II System.

SUMMARY AND EXPLANATION

RSV: Human respiratory syncytial virus (RSV) is a negative single-stranded RNA virus of the family *Paramyxoviridae*. RSV is the major cause of lower respiratory tract infection and hospital visits during infancy and childhood. In the United States, 60% of infants are infected during their first RSV season, and nearly all children will have been infected with the virus by 2–3 years of age.¹ Of those infected with RSV, 2% to 3% will develop bronchiolitis, necessitating hospitalization.² Natural infection with RSV induces protective immunity that wanes over time—possibly more so than other respiratory viral infections—and thus people can be infected multiple times. Sometimes an infant can become symptomatically infected more than once, even within a single RSV season. Severe RSV infections have increasingly been found among elderly patients.

RSV subtypes A and B are present either simultaneously or alternately during yearly epidemics and there are several supporting studies indicating that RSV-A induced bronchiolitis is more severe than an RSV-B induced one.³

hMPV: Human metapneumovirus (hMPV) is a negative single-stranded RNA virus of the family *Paramyxoviridae* first isolated in 2001 in the Netherlands.⁴ hMPV may be the second most common cause (after RSV) of lower respiratory infection in young children. Compared with RSV, infection with hMPV tends to occur in slightly older

children and produce disease that is less severe. Co-infection with both viruses can occur and is generally associated with more severe disease.

hMPV accounts for approximately 7.1% of respiratory tract infections.⁵ The virus appears to be distributed worldwide and have a seasonal distribution with its incidence comparable to that for the influenza viruses during winter. Serologic studies have shown that by the age of five, virtually all children have been exposed to the virus.⁶ hMPV generally causes mild respiratory tract infection. However, small children, the elderly, and immunocompromised individuals are at risk for severe disease and hospitalization.

Sequence analyses of the hMPV genome have shown that hMPV strains circulating around the world can be divided into two main genetic lineages (A and B) representing two serotypes, each comprising two sublineages (A1, A2, B1, and B2).⁷

PRINCIPLE OF THE PROCEDURE

The assay detects viral nucleic acids that have been extracted from a patient sample. A multiplex Real-time RT-PCR reaction is carried out under optimized conditions in a single tube generating amplicons for RSV, hMPV and the Process Control (PRC). Identification of RSV and hMPV and the PRC occurs by the use of target-specific primers and fluorescent-labeled probes that hybridize to conserved regions in the NS2 and polymerase gene of RSV and, polymerase gene of hMPV and the PRC.

Lyra Probe Labels	
Target	Dye
RSV	FAM
hMPV	CAL Fluor™ Red 610
PRC	Quasar® 670

The following is a summary of the procedure:

1. Sample Collection: Obtain nasal or nasopharyngeal swabs specimens using standard techniques from symptomatic patients. Transport, store, and process these specimens according to established laboratory procedures.⁸
2. Nucleic Acid Extraction: Extract nucleic acids from the specimens with the bioMérieux® NucliSENS® easyMAG® System following the manufacturer's instructions and using the appropriate reagents (See Materials Required but Not Provided). Use of other extraction systems with the Lyra RSV + hMPV Assay has not been validated. Validation of these other systems is the responsibility of the end user.

Prior to the extraction procedure, add 20 µL of the PRC to each 180 µL aliquot of specimen. The PRC serves to monitor inhibitors in the extracted specimen, assures that adequate amplification has taken place, and confirms that the nucleic acid extraction was sufficient.

3. Rehydration of Master Mix: Rehydrate the lyophilized Master Mix using the Rehydration Solution. The Master Mix contains oligonucleotide primers, fluorophore and quencher-labeled probes targeting conserved regions of RSV and hMPV, as well as the PRC sequence.
4. Nucleic Acid Amplification and Detection: Add 15 µL of the rehydrated Master Mix to each reaction tube or plate well. 5 µL of extracted nucleic acids (specimen with PRC) is then added to the reaction tube or plate well. Place the tube into the SmartCycler II instrument, or place the plate into either the Applied Biosystems 7500 Fast Dx instrument, the Applied Biosystems 7500 Fast instrument or QuantStudio Dx.

Once the reaction tube or plate is added to the instrument, initiate the assay protocol. This protocol initiates reverse transcription of the RNA targets generating complementary DNA, and the subsequent amplification of the target amplicons. The Lyra RSV + hMPV Assay is based on TaqMan® chemistry and uses an enzyme with reverse transcriptase, DNA polymerase, and 5'-3' exonuclease activities. During DNA amplification, this

enzyme cleaves the probe bound to the complementary DNA sequence, separating the quencher dye from the reporter dye. This step generates an increase in fluorescent signal upon excitation by a light source of the appropriate wavelength. With each cycle, additional dye molecules are separated from their quenchers resulting in additional signal. If sufficient fluorescence is achieved by 50 cycles on the SmartCycler II, 35 cycles on the ABI 7500 Fast and Fast Dx, or 40 cycles on the QuantStudio Dx, then the sample is reported as positive for the detected nucleic acid sequence.

MATERIALS PROVIDED

Cat. #M103

Detection Kit (96 Reactions) – Store at 2°C to 8°C

Component	Quantity
Rehydration Solution Part M5003	1 vial/kit 1.9 mL
Lyra RSV + hMPV Master Mix Part M5035 Lyophilized Contents: DNA polymerase enzyme with reverse transcriptase activity Primers and probes dNTPs Stabilizers	12 vials/kit, 8 reactions/vial
Process Control (PRC) Part M5005	1 vial/kit 2.0 mL

OPTIONAL MATERIALS

External controls for RSV and hMPV (i.e. Quidel Molecular RSV + hMPV Control Set, Cat. #M107 which serves as an external processing and extraction control)

MATERIALS REQUIRED BUT NOT PROVIDED

- Micropipettors (range between 1 to 10 µL and 100 to 1000 µL)
 - Non-aerosol pipette tips
 - QuantStudio Dx (software version 1.0 or higher) or ABI 7500Fast or Fast Dx (software version 1.4.1 or higher)
 - 96 well PCR plate
 - Optical plate films
 - Plate centrifuge
 - bioMérieux NucliSENS easyMAG software (version 2.0)
 - bioMérieux NucliSENS easyMAG Buffers 1, 2, 3
 - bioMérieux NucliSENS easyMAG Lysis Buffer
 - bioMérieux NucliSENS easyMAG Silica Magnetic Beads
 - bioMérieux NucliSENS easyMAG disposables
 - Biohit pipettor
- Or
- Micropipettors (range between 1 to 10 µL and 100 to 1000 µL)
 - Non-aerosol pipette tips
 - SmartCycler II (software version 3.0b or higher)
 - SmartCycler disposables
 - SmartCycler centrifuge
 - bioMérieux NucliSENS easyMAG software (version 2.0)

- bioMérieux NucliSENS easyMAG Buffers 1, 2, 3
- bioMérieux NucliSENS easyMAG Lysis Buffer
- bioMérieux NucliSENS easyMAG Silica Magnetic Beads
- bioMérieux NucliSENS easyMAG disposables

WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use
- The assay has been validated using
 - ▶ NucliSENS easyMAG software version 2.0,
 - ▶ SmartCycler II software version 3.0b,
 - ▶ ABI 7500 Fast and Fast Dx software version 1.4.1, and
 - ▶ QuantStudio Dx software version 1.0 or higher.
 Please contact Quidel Technical Support prior to modifying or upgrading beyond these versions of software.
- Performance characteristics of this test have been established with the specimen types listed in the **Intended Use Section** only. The performance of this assay with other specimen types or samples has not been evaluated.
- Nucleic acid extraction systems other than the NucliSENS easyMAG System have not been validated. Validation of these systems is the responsibility of the end-user.
- Using cycling conditions other than those indicated in the **Instructions for Set-up of Thermocycler Program**.
- Use of this product should be limited to personnel with sufficient training in PCR and RT-PCR techniques.
- Treat all specimen/samples as potentially infectious. Follow universal precautions when handling samples, this kit, and its contents.
- Proper sample collection, storage, and transport are essential for correct results.
- Store assay reagents as indicated on their individual labels.
- For accurate results, pipette carefully using only calibrated equipment.
- Thoroughly clean and disinfect all surfaces with a 10% bleach solution followed by molecular grade water.
- Use micropipettes with an aerosol barrier or positive displacement tips for all procedures.
- Avoid microbial and cross contamination of the kit reagents. Follow Good Laboratory Procedures.
- Do not mix reagents from kits with different lot numbers.
- Do not use reagents from other manufacturers with this kit.
- Do not use product after its expiration date.
- Proper workflow planning is essential to minimize contamination risk. Always plan laboratory workflow in a uni-directional manner, beginning with pre-amplification and moving through amplification and detection.
- Use dedicated supplies and equipment in pre-amplification and amplification areas. Do not allow cross movement of personnel or equipment between areas.
- Keep amplification supplies separate from pre-amplification supplies at all times.
- Do not open sample tubes or unseal plates post amplification.
- Do not use supplies dedicated for reagent or sample preparation for processing target nucleic acid.
- Testing should be performed in an area with adequate ventilation.
- Dispose of containers and unused contents in accordance with Federal, State and Local regulatory requirements.
- Wear suitable protective clothing, gloves, and eye/face protection when handling the contents of this kit.
- Wash hands thoroughly after handling.
- For additional information on hazard symbols, safety, handling and disposal of the components within this kit, please refer to the Safety Data Sheet (SDS) located at quidel.com.

KIT REAGENTS STORAGE AND HANDLING

- Store the unopened kit at 2°C to 8°C until the expiration date listed on the outer kit box.
- The rehydrated Master Mix may be kept at room temperature for up to 24 hours or at 2°C to 8°C or ≤ -20°C for up to 5 days.

- The rehydrated Master Mix should be recapped, sealed with parafilm, and stored in an upright position. Protect the Master Mix from light during storage.

Indications of Instability or Deterioration of Reagents

Cloudiness of the Rehydration Solution may indicate deterioration of this reagent. Contact Quidel Technical Support for a replacement.

SPECIMEN COLLECTION, STORAGE AND HANDLING

Specimens used for the validation of the Lyra RSV + hMPV Assay were obtained using standard techniques from patients with upper respiratory tract infection symptoms. These specimens were collected, transported, stored, and processed according to CLSI M41-A. Briefly, samples should be transported refrigerated at 2°C to 8°C and stored refrigerated (2°C to 8°C) for up to 72 hours before processing. Any additional leftover specimen should be stored $\leq -70^{\circ}\text{C}$ for up to 56 days.

A series of studies were performed evaluating a number of routinely used viral transport medium at a volume of 2 mL: M4, M4-RT, M5, M6, and UTM. No significant difference in assay performance was seen between the five different types of viral transport media.

NUCLEIC ACID EXTRACTS STORAGE

Eluates can be stored at room temperature (20°C to 25°C) for up to 4 hours, at 2°C to 8°C for 6 hours and at $\leq -20^{\circ}\text{C}$ for 1 month. The extracted RNA is stable for up to three freeze/thaws cycles when stored at $\leq -20^{\circ}\text{C}$.

NUCLEIC ACID EXTRACTION PROGRAMMING INSTRUCTIONS

Set Up of Nucleic Acid Extraction Program – NucliSENS easyMAG System



NOTE: An RSV/hMPV positive external control (i.e. Quidel Molecular RSV + hMPV Control Set, Cat. #M107 or previously characterized positive RSV or hMPV specimen) and a negative external control (i.e., viral transport media or previously characterized RSV and hMPV negative specimen) should be included in each extraction run.


1. Turn on the instrument and wait for instrument light to appear orange. Then switch on the computer/launch NucliSENS easyMAG System software.



2. Barcode reagents after pressing the 'Instrument'  and 'Reagent Inventory'  buttons.






3. To enter samples, press the 'Daily Use'  button, which will default to the 'Define Request'  screen. Selection the following settings:

- a. Sample ID: Enter the **sample name** using the keyboard.
- b. Matrix: Select **Other** from the drop-down menu.
- c. Request: Select **Generic** from the drop-down menu.
- d. Volume (mL): Select **0.200** from the drop-down menu.
- e. Eluate (μL): Select **50** from the drop-down menu.
- f. Type: Primary
- g. Priority: Normal

4. Upon pressing the 'Save'  button, the sample will appear in the 'Unassigned Sample' window on the left side of the screen. Press the 'Enter New Extraction Request'  button, and repeat the process for


additional samples. Alternatively, multiple samples can be entered by pressing the 'Auto Create New Extraction Requests'  button.

5. Once all samples are created, go to 'Organize Runs' by clicking on the  icon near the top of the page. Create a run by pressing the 'Create Run'  button. Enter a run name, or use the default.

6. Add samples to the run by using the 'Auto Fill Run'  button (auto fills up to 24 samples from the 'Unassigned Sample list' on the left hand side of the screen). Alternatively, individual samples may be moved into and out of the run by using the left and right 'Positioning icons'   after selecting the appropriate sample. The sample order within the run may be changed using the 'Move Extraction Request Up/Down' buttons  .

7. Obtain 1 to 3 (for 8 to 24 samples, respectively) sample vessel(s), and add 20 μ L of Process Control to each sample well used.

8. Add 180 μ L of each sample to the appropriate well as designated.

9. Go to 'Load Run' by pressing the  button near the top of the screen. Insert tips and sample vessel(s) into the instrument

10. Enter the barcode(s) of the sample vessel(s).


11. Enter the barcode(s) of silica beads to be used.

12. Close the instrument lid.

13. Assign silica beads to samples as follows:


a. Click the reagents symbol below number 1 in the picture below. The lot number of the silica beads should appear below the Silica tab at number 2 in the picture below.

b. Highlight and select the samples in the run for which beads need to be assigned (in the box containing number 3 in the picture below)

c. Click the  positioning icon (below number 4 in the picture below) to assign the silica lot number to the selected samples

d. If the bead symbol to the right of number 5 in the picture below is selected, the silica bead lot number should be displayed for each sample.

14. Print work list by touching 'Load Run' icon followed by pressing the 'Print Work List' icon .

15. Press the 'Dispense Lysis'  button. The on-board lysis will take approximately 12 minutes to complete.

16. For each sample vessel, prepare magnetic particles using the Biohit pipettor and tips for up to eight reactions as follows:

a. Using 1 tip and Program 1, aspirate 550 μ L nuclease-free water and dispense into a 1.5 mL DNase / RNase free microfuge tube.

b. Vortex the magnetic silica. Using 1 tip and Program 1, aspirate 550 μ L of magnetic silica, dispense into the water and mix by vortexing.

- c. Using 1 tip and Program 2, aspirate 1050 μL of the magnetic silica mixture and dispense 25 μL back into the same tube.
- d. Dispense 125 μL magnetic silica mixture 8 times into 8 wells of an ELISA strip plate. Discard tip.
- e. After Lysis is complete (NOTE: the 'Instrument Status' at the bottom of the screen must be 'IDLE!'), using 8 tips and Program 3, aspirate 100 μL of magnetic silica mixture in strip wells, dispense 100 μL of magnetic silica mixture in strip wells, and aspirate 100 μL of magnetic silica mixture in strip wells.
- f. Insert tips into liquid within the sample vessels. Aspirate 800 μL , then dispense 900 μL of magnetic silica mixture back into vessel. Aspirate 1000 μL of magnetic silica mixture from vessel, and dispense 1000 μL of magnetic silica back into vessel. Repeat aspiration / dispensing of 1000 μL two more times.

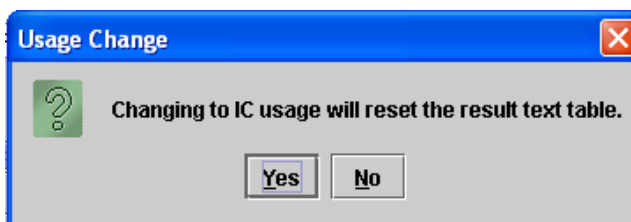


17. Close the instrument, and press the 'Start' button to begin the run.
18. Upon completion of the run, transfer purified nucleic acid to nuclease-free tubes. Eluates can be stored at room temperature (20°C to 25°C) for up to 4 hours, at 2°C to 8°C for 6 hours and at -20°C for 1 month. The extracted RNA is stable for up to three freeze/thaws cycles when stored at -20°C.

INITIAL THERMOCYCLER PROGRAMMING

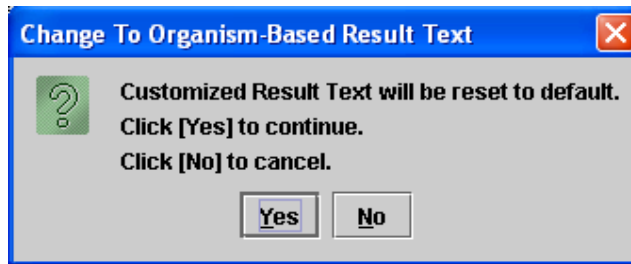
Set-up of Thermocycler Program – Cepheid SmartCycler II

1. Launch the SmartCycler II software package (version 3.0b).
2. Create the Lyra RSV + hMPV Assay
 - a. Select the **Define Assays** button from the top of the screen.
 - b. Name the assay.
 - i. Select the **New** button at the bottom left corner of the screen.
 - ii. Type in 'Lyra RSV + hMPV' and select **OK**.
 - iii. 'Lyra RSV + hMPV' will be added to the top of the **Assay Name** list located on the upper left-hand of the screen.
 - c. Set the analysis values: Under the **Assay Type: Research** section, select the **Analysis Settings** tab, and make sure the following specifications are set.
 - i. Select **FCTC25** from the **Dye Set** drop-down menu.
 - ii. The **Analysis Type** drop-down menu should be set to **Qualitative** (Default setting).
 - iii. In the **Channel Name** column, enter 'RSV' for Channel 1, 'hMPV' for Channel 3 and 'PRC' for Channel 4.
 - iv. In the **Usage** column, select **Target** from the drop-down menus for RSV and hMPV, and select Internal Control for PRC. When selecting **Internal Control**, a window below will pop up. Select the **YES** button.

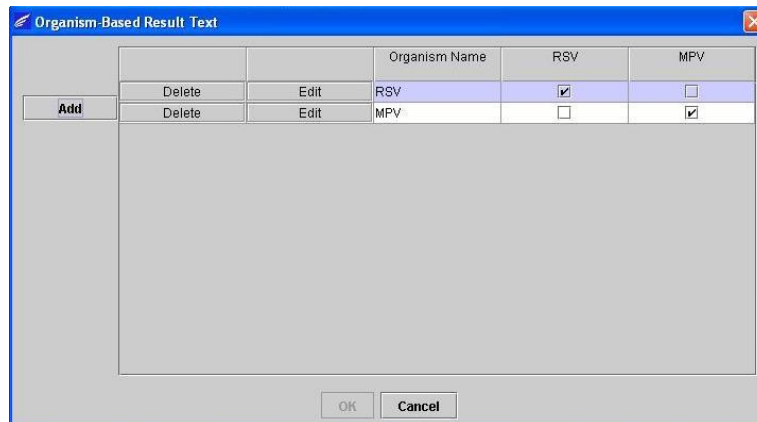


- v. In the **Curve Analysis** column, enter **Primary Curve** for each channel (RSV, hMPV, PRC) (Default setting).
- vi. In the **Thresh Setting** column, enter **Manual Threshold** for each channel (RSV, hMPV, PRC) (Default setting).
- vii. In the **Manual Thresh Fluor Units** column, enter the following thresholds:
 - a. **RSV:** 20.0
 - b. **hMPV:** 10.0
 - c. **PRC:** 10.0

- viii. In the **Valid Min Cycle** column (scroll to the right if not immediately visible), enter 5 for each channel (RSV, hMPV, PrC).
- ix. In the **Valid Max Cycle** column (scroll to the right if not immediately visible), enter 50 for each channel (RSV, hMPV, PrC).
 - x. In the **Bkgnd Sub** column, use "ON" for each channel (RSV, hMPV, PrC) (Default setting).
 - xi. In the **Bkgnd Min Cycle** column, enter 5 for each channel (RSV, hMPV, PrC).
 - xii. In the **Bkgnd Max Cycle** column, enter 35 for each channel (RSV, hMPV, PrC).
 - xiii. In the **Boxcar Avg cycles** column, keep 0 for each channel (RSV, hMPV, PrC) (Default setting).
 - xiv. In the **End Pt Threshold** column, enter **20, 10, 10** for each channel (RSV, hMPV, PrC) (Default setting).
 - xv. In the **NC IC%** column, keep "NA" for each channel (PrC) (Default setting).
 - xvi. In the **IC Delta** column, keep "NA" for each channel (RSV, hMPV) (Default setting)
- xvii. In the **Customize Result Text** section (below the table), select **Organism Based Result Text** from the drop-down menu. The warning window below will pop up. Select Yes.



- xviii. Select the **Customize** button to open the **Organism-Based Result Text** dialog window. Select the **Add** button, enter 'RSV' in the **Organism Name** column and check the **RSV** box. Select the **Add** button again, enter 'hMPV' in the **Organism Name** column and check the **hMPV** box.

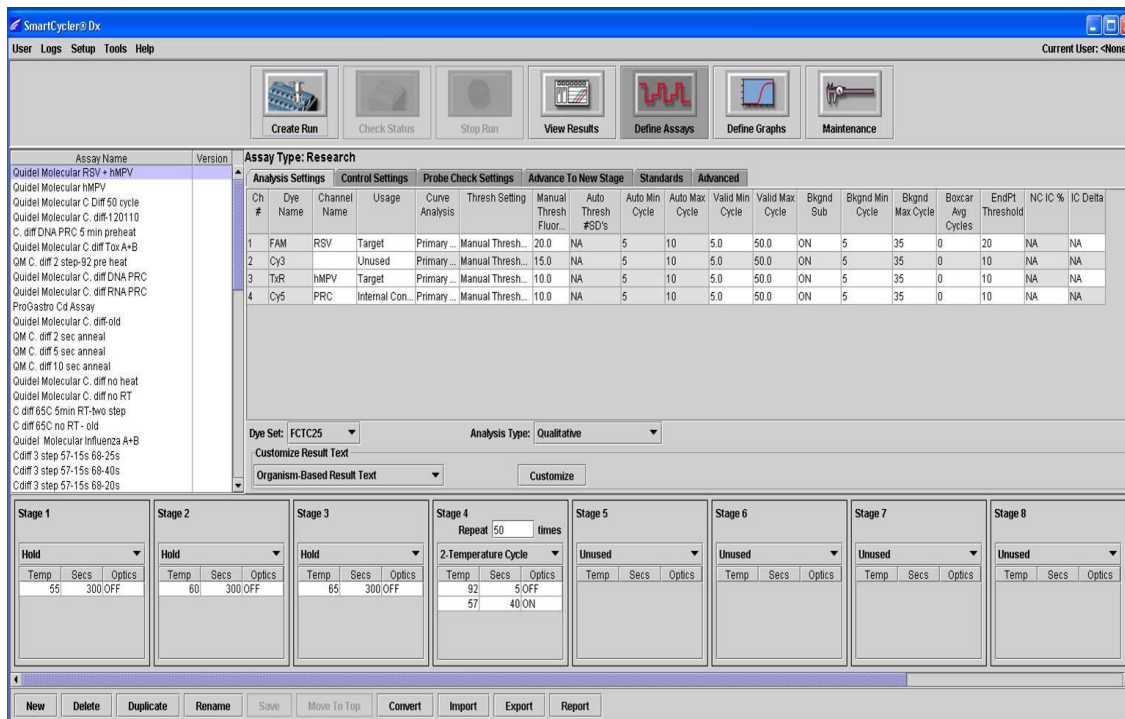


Click **OK** at the bottom of the pop-up window.

- d. Set the RT-PCR cycling times and temperatures as follows:
 - i. Stage 1
 - 1. Hold
 - 2. Temp: 55.0
 - 3. Secs: 300
 - 4. Optics: OFF
 - ii. Stage 2
 - 1. Hold
 - 2. Temp: 60.0
 - 3. Secs: 300
 - 4. Optics: OFF
 - iii. Stage 3

1. Hold
 2. Temp 65.0
 3. Secs: 300
 4. Optics: OFF
- iv. Stage 4
1. 2-Temperature Cycle
 2. Time to Repeat: 50
 3. First Temperature Row:
 - a. Temp: 92.0
 - b. Secs: 5
 - c. Optics: OFF
 4. Second Temperature Row
 - a. Temp: 57.0
 - b. Secs: 40
 - c. Optics: ON

3. Save the protocol by selecting the **Save** button at the bottom of the screen.
Figure of the completed Lyra RSV + hMPV Protocol.



Set-up of Thermocycler Program – ABI 7500 Fast and Fast Dx

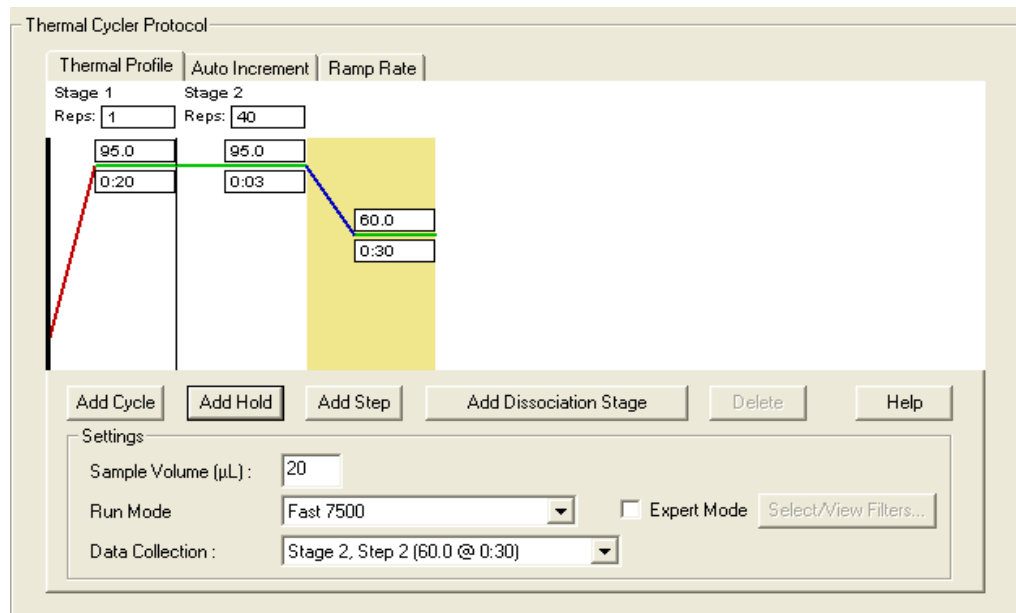
1. Launch the ABI 7500 Fast or Fast Dx software package (version 1.4.1)
2. The **Quick Startup document** dialog window will open. Select the **Create New Document** button to start the **New Document Wizard**. Follow each step to initiate the Lyra RSV + hMPV protocol.
 - a. Define Document: Most of the following should be the default setting. If not, change accordingly.
 - i. Confirm or enter the following information.

Assay:	Standard Curve (Absolute Quantitation)
Container:	96-Well Clear
Template:	Blank Document
Run Mode:	Fast 7500
Operator:	<i>your operator name</i>
Comments:	SDS v1.4.1

Plate Name:	'Lyra RSV + hMPV'
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- ii. Select the **Next** button.
 - iii. Select Detectors: **New detectors** for hMPV, RSV and the PRC, must be added. For each target, select the **New Detector** button to open the **New Detector** pop-up window. Alternatively, use the **Create Another** button from within the New Detector pop-up window for the last two detectors. Enter the following information for each detector:

Name	Reporter Dye	Quencher Dye	Color
RSV	FAM	(none)	(Select)
hMPV	Texas Red	(none)	(Select)
PRC	Cy5	(none)	(Select)
 - iv. Select a unique color to represent each detector.
 - v. Highlight the new detectors and added to the **Detectors in Document** column using the **Add** button.
 - vi. Select (none) from the **Passive Reference** drop-down menu.
 - vii. Select the **Next** button.
 - viii. Select the **Finish** button without setting any wells.
- b. The wizard will close, and the software will open, starting with the **Setup** tab. This will show the sample plate that was set up during the quick start. For the initial set up, nothing needs to be changed here.
- c. Define the Thermocycler Protocol:
- i. Select the **Instrument** tab to set up the Lyra RSV + hMPV RT-PCR Assay cycling times and temperatures.
 - ii. Under **Thermal Profile** there should be a default 2-stage protocol (see figure below). Each stage will have 3 user-editable text boxes. The top box value represents the number of reps or cycles for that stage. The middle box value represents the temperature (°C), and the lowest box value represents the time (minutes: seconds).

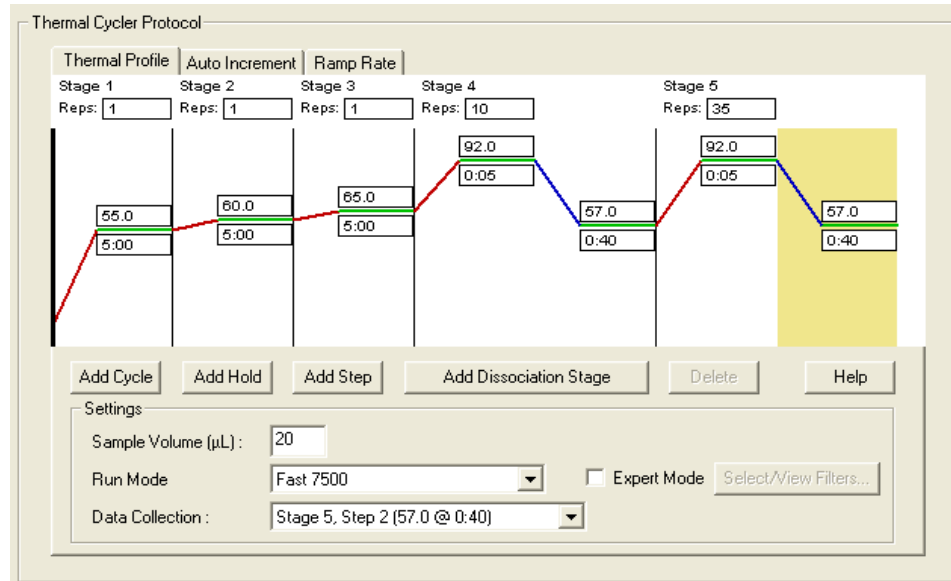


- iii. Make the following changes to the default **Thermal Cycler Protocol**:
 1. Stage 1
 - a. Reps: 1
 - b. Temp: 55
 - c. Time: 5:00

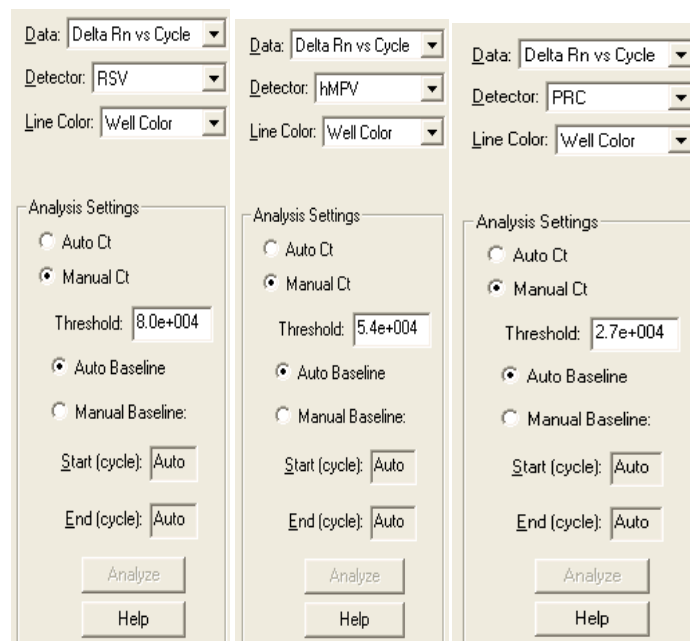
2. Select the bar between Stage 1 and Stage 2. Select the **Add Hold** button to add another stage.
 3. Stage 2
 - a. Reps: 1
 - b. Temp: 60
 - c. Time: 5:00
 4. Select the bar between Stage 2 and Stage 3. Select the **Add Hold** button to add another stage.
 5. Stage 3
 - a. Reps: 1
 - b. Temp: 65
 - c. Time: 5:00
 6. Stage 4 (2-Step Amplification Stage)
 - a. Reps: 10
 - b. Step 1
 - i. Temp: 92
 - ii. Time: 0:05
 - c. Step 2
 - i. Temp: 57
 - ii. Time: 0:40
 7. Select the bar to the right of Stage 4. Select the **Add Cycle** button to add another stage.
 8. Stage 5 (2-Step Amplification Stage)
 - a. Reps: 35
 - b. Step 1
 - i. Temp: 92
 - ii. Time: 0:05
 - c. Step 2
 - i. Temp: 57
 - ii. Time: 0:40
 9. If a wrong stage is added, the stage may be removed by pressing the **Delete** button after highlighting the stage between the vertical lines.
- iv. Under Settings, enter the following:

Sample Volume (μ L):	20 (default)
Run Mode:	7500 Fast (default)
Data Collection:	Stage 5, Step 2 (57.0 @ 0:40)
NOTE: Do not check the box next to 'Expert Mode.'	

- v. A figure of the final protocol is shown below.



- d. Set threshold for each analyte as follows (see figure below):
 - i. Select the **Results** tab.
 - ii. Select the **Amplification Plot** tab.
 - iii. Select RSV from the **Detector** tab in the top right corner.
 - iv. In the Analysis Settings block, set the **Threshold** to **8.0e4**.
 - v. Select the **Auto Baseline** radio button.
 - vi. Repeat steps iii-v for hMPV setting the **Threshold** to **5.4e4**.
 - vii. Repeat steps iii-v for PRC setting the **Threshold** to **2.7e4**.



- e. Save the new protocol as a template for future uses.
 - i. At the top of the screen select **File** and then **Save As**.
 - ii. **Save In:** D:\Applied Biosystems\7500 Fast System\Templates\.
 - iii. **File name:** 'Lyra RSV + hMPV'.
 - iv. **Save as type:** 'SDS Templates (*.sdt).'
- f. Exit the software.

Set-up of Thermocycler Program – QuantStudio Dx

Lyra provides a pre-defined template for the Lyra RSV + hMPV RT-PCR Assay on a CD that must be uploaded to the QuantStudio Dx. Please contact a Quidel Representative at 800.874.1517 (in the U.S.) or 858.552.1100 (outside the U.S.), Monday through Friday, from 8:00 a.m. to 5:00 p.m. (Eastern Time) to obtain this CD. These templates contain the run parameters such that no instrument programming is needed to get started. To install a test definition document:

1. From the QuantStudio Dx Software **Home** tab, click **Manage Test** in the **Tools** panel.
2. From the **Test Menu**, click **Install**.
3. Navigate to your test definition document (.tdd) file, select the file, and click **Open**. The QuantStudio Dx Software automatically adds the selected test to the **Test Menu**.
4. Click **Close** to close the **Test Menu** and save your changes.

ASSAY PROCEDURE

Run the following procedures at the controlled room temperature of 20°C to 25°C.

Nucleic Acid Extraction Procedure

Refer to the section titled: Set-Up of Nucleic Acid Extraction Program –NucliSENS easyMAG System (above).

1. Add 20 µL of the PRC to the sample extraction well.
2. Add 180 µL of patient sample or external control to a sample extraction well.
3. Follow extraction procedure as per manufacturer’s instructions.
4. Eluates can be stored at room temperature (20°C to 25°C) for up to 4 hours, at 2°C to 8°C for 6 hours and at –20°C for 1 month. The extracted RNA is stable for up to three freeze/thaws cycles when stored at –20°C.

Master Mix Rehydration Procedure

1. Determine the number of specimens to be tested, and obtain the correct number of eight-test lyophilized Master Mix vials for testing.
2. Return unused reagents to the appropriate storage conditions.
3. Open Master Mix carefully to avoid disruption of the pellet.
4. Add 135 µL of Rehydration Solution to the Master Mix.
5. Place vial at room temperature for 1-2 minutes to allow rehydration of pellet.
6. Gently pipette up and down 2-3 times (avoiding the formation of bubbles) prior to dispensing into the first PCR tube or plate well.

Note: The rehydrated Master Mix is sufficient for eight reactions.

Note: The rehydrated Master Mix may be kept at room temperature for up to 24 hours or at 2°C to 8°C or ≤–20°C for up to 5 days. (See “Storage and Handling of Kit Reagents” section for additional storage options).

RT-PCR Set-up Procedure

1. Add 15 µL of the rehydrated Master Mix to each reaction tube or plate well.
2. Add 5 µL of extracted nucleic acid (specimen with the PRC) into the reaction tubes or plate wells. Mixing of reagents is not required.
Note: Use a micropipettor with a new non-aerosol tip with each extracted specimen.
3. Close the reaction tubes or seal the plate.
Note: Quidel suggests each thermocycler run should include a reaction tube or well with RSV and hMPV External Positive Control and Negative Control. Run controls in keeping with your lab practices and policies.
4. Centrifuge the reaction tubes or plate for a minimum of 15 seconds. Ensure that all liquid is at the bottom of the tube.
5. Insert tubes or plate into the thermocycler.

Amplification Protocol – SmartCycler II

1. Switch on SmartCycler II block(s).
2. Launch the SmartCycler Dx software package (version 3.0b).
3. Select the **Create Run** button from the top of the screen to set up the run.
4. Under **Run Name**, enter a name for the current run (e.g. YYMMDD-Lyra RSV + hMPV).
5. Under **Notes**, enter any notes about the run for future reference.
6. Under **Assay**, select the 'Lyra RSV + hMPV' assay from the drop-down menu.
7. Under **Assay Information**, enter lot number and expiration date of the kit.
8. To select the wells that will be used, do one of the following:
 - a. To automatically assign wells do the following:
 - i. Under **Number of specimens**, enter the number of samples in the provided text box.
 - ii. Select the **Apply** button. The entered number of rows will appear in the Site Table.
 - b. To manually choose wells on the SmartCycler II block(s), do the following:
 - i. Select the **Add/Remove Sites** button towards the bottom of the screen.
 - ii. This will open the **Select Sites** pop-up window with two columns. The column on the left (Sites) lists all available sites, and the column on the right (**Selections**) holds all selected sites.
 - iii. To select all sites, click the **Select All Sites** button.
 - iv. To select specific sites, highlight one or more sites, and select the right arrow to add the site(s) to the **Selections** column.
 - v. Select the **OK** button to close the window. The selected sites will appear in the **Site Table**.
9. Enter the sample identifiers under the **Sample ID** column within the **Site Table** (this can also be done after the run is started).
10. Enter any notes under the **Notes** column, and leave the **Sample Type** column entries as 'SPEC.'
11. Select the **Start Run** button at the bottom of the screen.
12. Select the **View Results** button to view the progress of the run.
13. Save the run after it is finished and prior to exiting the software.

Amplification Protocol – ABI 7500 Fast and Fast Dx

1. Switch on ABI 7500 Fast or Fast Dx.
2. Launch the ABI 7500 Fast or Fast Dx software package (version 1.4.1).
3. The Quick Startup document dialog window will open.
4. Click on Create a new document.
5. Most of the following should be the default setting. If not, change accordingly.

Assay:	Standard Curve (Absolute Quantitation)
Container:	96-Well Clear
Template:	Lyra RSV + hMPV
Run Mode:	Fast 7500
Operator:	<i>your operator name</i>
Comments:	SDS v1.4.1 (<i>add more if needed</i>)
Plate Name:	YYMMDD-Lyra RSV + hMPV

6. The Set Up Sample Plate:
 - a. Under the **Setup** and **Plate** tabs the plate setup will appear.
 - b. Select all wells that will contain sample, right-click and select the **Well Inspector** from the drop-down menu. When the **Well Inspector** pop-up window opens, select the detectors for RSV, hMPV and PRC.
 - c. Use the **Well Inspector** to enter the sample names. Patient IDs may be entered in the **Well Inspector** window; however, it is recommended that this is done prior to resuspending the lyophilized master mix,

- post run, or using the import function to minimize the time the PCR reactions will sit at room temperature prior to starting the run.
- d. Save the run as **YYMMDD-Lyra RSV + hMPV.sds**.
 - e. A window will open asking for the “Reason for change of entry.” Enter “**Setup**” and any other comments relevant to the run.
7. Starting the PCR:
 - a. Select the **Instrument** tab.
 - b. Insert the 96 well PCR plate into the machine.
 - c. Under **Instrument Control**, select the **Start** button to initiate the run.
 8. Post PCR:
 - a. **IMPORTANT:** When the run is finished, press OK. **Analyze** the data by pressing the “Analyze” button in the top menu, and save the file.
 - b. Save the file by pressing **Save Document** in the task bar. A window will open asking for the “Reason for change of entry.” Enter “**Data analysis post run**” and any other comments relevant to the run.

Amplification Protocol – QuantStudio Dx

1. Switch on QuantStudio Dx.
2. Choose IVD mode on the instrument.
3. Launch the QuantStudio Dx IVD software package (version 1.0).
4. Enter the system **Username** included every extraction and **Password** when prompted.
5. The **Home screen** window will open.
6. In the **Setup** box, highlight the previously loaded test name “**Lyra RSV + hMPV.**”
7. Click the **Setup** button to begin a RT-PCR run.
8. The **Setup, Test Properties** screen will be displayed. Enter run information accordingly.
 - a. Enter the **Experiment Name** (default setting launches the run with a date and time stamp).
 - b. Enter the **Plate Barcode** information.
 - c. Record material lot numbers under **Reagent Information**.
 - d. Save the run with a unique identifier as an “.sds” file (e.g. **YYMMDD-runID#-LyraRSV + hMPV.sds**).
 - e. A window will open asking for the “Reason for change of entry.” Enter “**Setup**” and any other comments relevant to the run.
9. In the left menu bar, select **Define**.
10. Edit sample information.
 - a. Enter specific sample information for each well by deleting the default identifier (Patient 1, Patient 2, etc.) and entering new information, OR
 - b. Select **Import from File** across the top of the display to upload a predefined plate map from a Text (tab delimited) file.
11. In the left menu bar, select **Assign** to verify proper plate setup.
12. Loading the sample plate.
 - a. Eject the instrument tray.
 - b. Insert the 96-well PCR plate into the machine with the A1 well positioned in the top, left corner.
 - c. Retract the instrument tray.
13. Starting the run.
 - a. In the left menu bar, select **Run**.
 - b. Click the green **Start Run** button at the top of the screen.
 - i. If prompted, select the serial number specific to the instrument being used.
14. When the run is complete, select **Analysis** in the left menu bar.
 - a. Save the file by pressing **Save** in the task bar. A window will open asking for the “Reason for change of entry.” Enter “**Data analysis post run**” and any other comments relevant to the run.
 - b. The **Amplification Plot** will show by default. To view other plot types, select them from the left menu bar.
 - c. To view run information with Ct values, select the **Well Table** tab in the right side of the screen.

15. Printing a report.
 - a. In the top menu bar, select **Print Report**. Customize the report contents by selecting or deselecting boxes from the report window.
 - b. Select the “**Print Report**” button at the bottom of the dialogue box.
16. Exporting data files.
 - a. In the left menu bar, select **Export**.
 - b. Enter the **Export File Location** OR click **Browse** to locate the desired path.
 - c. The **Export File Name** will default to that of the saved run.
 - d. Select Excel as the file type.
 - e. Customize the exported data report by toggling across the provided tabs and selecting or deselecting options.
 - f. Select Start Export along the bottom of the screen.

INTERPRETATION OF RESULTS

Interpretation of Results – SmartCycler II

1. Select **View Results** tab after the run is finished.
2. Select **Sample Results** tab.
3. The SmartCycler II software will automatically report whether RSV and/or hMPV viral RNA has been detected in the samples or whether the run was invalid (unresolved).
4. More detailed information may be found under the respective tabs of each analyte in the same window. If there is a positive call for either RSV or hMPV (or both), the PRC result is not applicable. The PRC is only required for negative calls.

Interpretation of the Lyra RSV + hMPV Assay Results using the SmartCycler II				
Assay Result	Detector: RSV	Detector: hMPV	Detector: PRC	Interpretation of Results
Negative	NEG	NEG	Pass	RSV and hMPV nucleic acid not detected; PRC Detected
RSV Positive	POS	NEG	NA*	RSV nucleic acid detected
hMPV Positive	NEG	POS	NA*	hMPV nucleic acid detected
RSV and hMPV Positive	POS	POS	NA*	RSV and hMPV nucleic acid detected**
Invalid	NEG	NEG	Fail	Unresolved – PCR inhibition or reagent failure. Retest the same purified sample. If this test is also invalid, re-extract and retest another aliquot of the same sample or obtain a new sample and retest.

*No value is required for the PRC to make a positive call.

**Dual infections are rare. Repeat testing using the same purified sample. If the retest confirms this result, collect and test new specimen. Contact Quidel if multiple samples provide this result.

Error Code 3079: Warning/Error Code 3079 may be observed with RSV and/or hMPV positive samples. Warning/Error Code 3079 occurs when the fluorescence (RFU) signal is too high. In this case, all results for that sample are reported by the Dx software as ND (Not Determined). If a Ct value ≥ 5 is reported for either virus, the sample results may be recorded as Positive for the corresponding virus. If the Ct value is < 5 the corresponding virus is reported as Negative.

Interpretation of Results – ABI 7500 Fast and Fast Dx

Interpretation of the Lyra RSV + hMPV Assay Results using the 7500 Fast and Fast Dx			
Detector: RSV	Detector: hMPV	Detector: PRC	Interpretation of Results
Ct < 5.0 or Ct > 35.0	Ct < 5.0 or Ct > 35.0	$5.0 \leq \text{Ct} \leq 35.0$	Negative – No RSV or hMPV viral RNA detected; PRC detected
$5.0 \leq \text{Ct} \leq 35.0$	Ct < 5.0 or Ct > 35.0	NA*	RSV Positive – RSV viral RNA detected

Interpretation of the Lyra RSV + hMPV Assay Results using the 7500 Fast and Fast Dx			
Detector: RSV	Detector: hMPV	Detector: PRC	Interpretation of Results
Ct <5.0 or Ct >35.0	5.0 ≤ Ct ≤ 35.0	NA*	hMPV Positive – hMPV viral RNA detected
5.0 ≤ Ct ≤ 35.0	5.0 ≤ Ct ≤ 35.0	NA*	RSV and hMPV Positive - RSV and hMPV viral RNA detected**
Ct <5.0 or Ct >35.0	Ct <5.0 or Ct >35.0	Ct <5.0 or Ct >35.0	Invalid – No RSV or hMPV viral RNA and no PRC detected; invalid test. Retest the same purified sample. If this test is also invalid, re-extract and retest another aliquot of the same sample or obtain a new sample and retest.
Undetermined	Undetermined	Undetermined	Invalid – Not determined. Retest the same purified sample. If the test is also invalid, re-extract and retest another aliquot of the same sample or obtain a new sample and retest.

*No Ct value is required for the PRC to make a positive call.

**Dual infections are rare. Repeat testing using the same purified sample. If the retest confirms this result, collect and test a new specimen. Contact Quidel if multiple samples provide this result.

Interpretation of Results – QuantStudio Dx

Interpretation of the Lyra RSV + hMPV Assay Results using the QuantStudio Dx			
Detector: RSV	Detector: hMPV	Detector: PRC	Interpretation of Results
Ct <15 or Ct >40	Ct <15 or Ct >40	15 ≤ Ct ≤ 40	Negative – No RSV or hMPV viral RNA detected; PRC detected
15 ≤ Ct ≤ 40	Ct <15 or Ct >40	NA*	RSV Positive – RSV viral RNA detected
Ct <15 or Ct >40	15 ≤ Ct ≤ 40	NA*	hMPV Positive – hMPV viral RNA detected
15 ≤ Ct ≤ 40	15 ≤ Ct ≤ 40	NA*	RSV and hMPV Positive – RSV and hMPV viral RNA detected**
Ct <15 or Ct >40	Ct <15 or Ct >40	Ct <15 or Ct >40	Invalid – No RSV or hMPV viral RNA and no PRC detected; invalid test. Retest the same purified sample. If this test is also invalid, re-extract and retest another aliquot of the same sample or obtain a new sample and retest.
Undetermined	Undetermined	Undetermined	Invalid – No RSV or hMPV viral RNA and no PRC detected; invalid test. Retest the same purified sample. If this test is also invalid, re-extract and retest another aliquot of the same sample or obtain a new sample and retest.

**No Ct value is required for the PRC to make a positive call.

**Dual infections are rare. Repeat testing using the same purified sample. If the retest confirms this result, collect and test a new specimen. Contact Quidel if multiple samples provide this result.

QUALITY CONTROL

The Lyra RSV+hMPV Assay incorporates several controls to monitor assay performance.

1. The Process Control (PRC) should be used during extraction and amplification in the assay. This control should be added to each sample aliquot prior to extraction. Please refer to the section titled **Principle of Procedure** for further information.
2. Commercially available external positive RSV/hMPV controls may be treated as a patient specimen and should be used in accordance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard quality control procedures. Previously characterized positive RSV or hMPV specimens may be used in lieu of a commercial RSV/hMPV control.
3. Viral transport media (UTM, M4, M4-RT, M5, or M6) or a previously characterized negative specimen may be used as an external negative control. This must be treated as a patient specimen and should be performed

in accordance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard quality control procedures.

4. Always include one negative control (containing a PRC) and at least one positive control in each amplification/detection run performed.
5. Failure of controls (positive, negative, and/or PRC) invalidates the run and results should not be reported.
6. If the positive control is not positive within the specified Ct range 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.
7. If the PRC is not positive or the negative control is invalid, repeat testing should be done starting from the original sample using new aliquots of the PRC and 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 is not intended to differentiate RSV or hMPV subtypes. Additional testing is required if subtype differentiation is required.
- Negative results do not preclude infection with RSV or hMPV and should not be the sole basis of a treatment decision.
- As with other assays of this type, there is a risk of false negative results due to the presence of sequence variants in the viral target.
- Improper collection, storage, or transport may lead to false negative results.
- Inhibitors present in the sample and/or errors in following the assay procedure may lead to false negative results.
- 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 targets (viral sequences) may persist *in vivo*, independent of virus viability. Detection of analyte target(s) does not imply that the corresponding virus(es) are infectious, nor are the causative agents for clinical symptoms.
- The detection of viral 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. 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 organisms, their nucleic acids or amplified product, or from non-specific signals in the assay.
- There is a risk of false negative values due to the presence of sequence variants in the viral targets of the assay.
- It is recommended that specimens found to be negative be confirmed by an alternate method (e.g. cell culture).
- The performance of the assay has not been established in individuals who received nasally administered corticosteroids.
- The performance of the assay has not been established in individuals who received nasally administered Influenza vaccine.
- hMPV inhibition at 2x LOD: Results from the analytical studies show that, at 2x LOD, detection of the A2 subtype of hMPV was inhibited by RSV-A at a level 10,000x above LOD on the ABI 7500 Fast Dx and the SmartCycler II System. At 2x LOD, the B2 subtype of hMPV was inhibited by RSV-A at a level of 10,000x above LOD on the QuantStudio Dx. RSV inhibition at 2x LOD: Results from the analytical studies show that, at 2x LOD, detection of RSV-A may be inhibited by the A2 subtype of hMPV at a level of 1,000x above the LOD on the QuantStudio Dx. The incidence of co-infection with RSV and hMPV seen in the two clinical studies for the Lyra RSV + hMPV Assay was 0.1% (1/949; ABI 7500 Fast Dx and SmartCycler II in winter 2012) and 0.1% (1/711; QuantStudio Dx in winter 2013).
- The assay performance was not established in immunocompromised patients.

- Positive and negative predictive values are highly dependent on prevalence and may vary depending on the population tested. Performance of the assay using the SmartCycler II System and the ABI 7500 Fast Dx was established during the 2012 winter season. Performance of the assay using the QuantStudio Dx was established during the 2013 winter season.

EXPECTED VALUES

Clinical studies were performed with the Lyra RSV + hMPV Assay using the SmartCycler II System and the ABI 7500 Fast Dx. Testing was performed with prospective specimens received from throughout the United States in the winter of 2012 (January 2012 to March 2012). The table below provides the expected value for each virus on the two instruments.

Expected Values for SmartCycler II (Winter 2012)						
Age Group (Years)	Total (n)	Total # RSV Positive	RSV Detection Rate	Total (n)	Total # hMPV Positive	hMPV Detection Rate
	<1					
1 to 5	352	67	19.0%	341	73	21.4%
6 to 10	134	6	4.5%	130	16	12.3%
11 to 15	62	3	4.8%	60	6	10.0%
16 to 21	33	3	9.1%	31	1	3.2%
>21	175	8	4.6%	153	17	11.1%
Total	1014	158	15.6%	960	148	15.4%

Expected Values for ABI 7500 Fast Dx (Winter 2012)						
Age Group (Years)	Total (n)	Total # RSV Positive	RSV Detection Rate	Total (n)	Total # hMPV Positive	hMPV Detection Rate
	<1					
1 to 5	354	72	19.9%	341	76	22.3%
6 to 10	134	6	4.5%	130	17	13.1%
11 to 15	62	3	4.8%	60	7	11.7%
16 to 21	33	3	9.1%	31	1	3.2%
>21	175	10	5.7%	153	18	11.8%
Total	1012*	166	16.4%	960	157	16.4%

*Two specimens were invalid.

A clinical study was performed with the Lyra RSV + hMPV Assay using QuantStudio Dx. Testing was performed with prospective specimens received from throughout the United States in the winter of 2013 (January 2013 to February 2013). The table below provides the expected value for each virus on the instrument.

Expected Values for QuantStudio Dx (Winter 2013)						
Age Group (Years)	Total (n)	Total # RSV Positive	RSV Detection Rate	Total (n)	Total # hMPV Positive	hMPV Detection Rate
	<1					
1 to 5	200	58	29.0%	200	26	13.0%
6 to 10	91	4	4.4%	91	3	3.3%
11 to 15	74	5	6.8%	74	3	4.1%
16 to 21	33	0	0%	33	0	0%
>21	227	17	7.5%	227	13	5.7%
Total	711*	116	16.3%	711*	59	8.3%

*Two specimens were invalid.

CLINICAL PERFORMANCE

Clinical Performance – SmartCycler II and the ABI 7500 Fast Dx

Performance characteristics of the Lyra RSV + hMPV Assay using the SmartCycler II System and the ABI 7500 Fast Dx were established during a prospective study during the 2012 respiratory virus season (January to March

2012). Samples used for this study were fresh (414) and frozen (600) nasal and nasopharyngeal swab specimens that were collected for routine respiratory virus testing four sites across the United States. A single specimen was collected per patient and tested for RSV immediately after collection using direct specimen DFA and culture with DFA. The residual samples were extracted with the NucliSENS easyMAG System and tested with Lyra RSV + hMPV Assay using both the ABI 7500 Fast Dx and the SmartCycler II. Aliquots of each specimen were sent to a central location for testing with an FDA-cleared hMPV molecular test.

Gender*	Number of Subjects (Percentage of Total)
Female	482 (47.5%)
Male	528 (52.1%)

*4 specimens did not list a gender (0.4%)

Age	Number of Subjects (Percentage of Total)
≤5	610 (60.2%)
6-21	229 (22.6%)
22-59	60 (5.9%)
≥60	115 (11.3%)

Prospective Clinical Study – SmartCycler II

One thousand and fourteen (1014) specimens were tested by both the subject and comparator method for RSV. One (1) specimen was contaminated and three (3) were toxic in cell culture (0.4%). These four (4) specimens have been excluded from analysis. Results for the remaining one thousand and ten (1010) specimens are detailed in the table below.

Combined Site – Respiratory syncytial virus								
DSFA & Cell Culture w/DFA							95% CI	
Lyra RSV + hMPV Assay		POS	NEG	Total	Sensitivity	97.9%	93.9%	99.3%
	POS	137	21*	158	Specificity	97.6%	96.3%	98.4%
	NEG	3	849	852				
	Total	140	870	1010				

*All originally discordant specimens were positive for RSV by an FDA-cleared RT-PCR assay.

Nine hundred and sixty (960) specimens were tested by both the subject and comparator device for hMPV (the comparator device was unavailable to complete comparison testing for fifty-four (54) specimens). Nine (9) specimens were invalid in the comparator device (0.9%). These nine (9) specimens have been excluded from analysis. Results for the remaining nine hundred and fifty-one (951) specimens are detailed in the table below.

Combined Site – Human metapneumovirus								
FDA-cleared hMPV Molecular Test							95% CI	
Lyra RSV + hMPV Assay		POS	NEG	Total	Positive percent agreement	96.7%	92.4%	98.6%
	POS	145	3	148	Negative percent agreement	99.6%	98.9%	99.9%
	NEG	5	798	803				
	Total	150	801	951				

Prospective Clinical Study – ABI 7500 Fast Dx

One thousand and fourteen (1014) specimens were tested by both the subject and comparator methods for RSV. One (1) specimen was contaminated and three (3) were toxic in cell culture (0.4%). Two (2) specimens were invalid in the subject methods (0.2%). These six (6) specimens have been excluded from analysis. Results for the remaining one thousand and eight (1008) specimens are detailed in the table below.

Combined Site - Respiratory syncytial virus								
DSFA & Cell Culture w/DFA							95% CI	
Lyra RSV + hMPV Assay		POS	NEG	Total	Sensitivity	98.6%	94.9%	99.6%
	POS	138	28*	166	Specificity	96.8%	95.4%	97.8%
	NEG	2	840	842				
	Total	140	868	1008				

*25 of 28 originally discordant specimens were positive for RSV by a FDA-cleared RT-PCR assay.

Nine hundred and sixty (960) specimens were tested by both the subject and comparator devices for hMPV (the comparator device was unavailable to complete comparison testing for fifty-four (54) specimens). Nine (9) specimens were invalid in the comparator device (0.9%). Two (2) specimens were invalid in the subject methods (0.2%). These eleven (11) specimens have been excluded from analysis. Results for the remaining nine hundred and forty-nine (949) specimens are detailed in the table below.

Combined Site - Human metapneumovirus								
FDA-cleared hMPV Molecular Test							95% CI	
Lyra RSV + hMPV		POS	NEG	Total	Positive percent agreement	98.0%	94.3%	99.3%
	POS	147	9	156	Negative percent agreement	98.9%	97.9%	99.4%
	NEG	3	790	793				
	Total	150	799	949				

Clinical Performance – QuantStudio Dx

Performance characteristics of the Lyra RSV + hMPV Assay using the QuantStudio were established during a prospective study during the 2013 respiratory virus season (January to March 2013). Samples used for this study were (713) nasal or nasopharyngeal swab specimens that were collected and extracted fresh for routine respiratory virus testing at three sites across the United States. Sample extracts were stored at -70°C until the time of testing. A single specimen was collected per patient. The specimens were extracted with the NucliSENS easyMAG System and tested with the Lyra RSV + hMPV Assay using the QuantStudio Dx and the comparator devices (FDA-cleared molecular tests for hMPV and RSV). Sites 1 and 2 extracted each specimen and tested them with the subject and comparator devices. Aliquots of each specimen extracted at Site 3 were sent to Site 1 for testing with the comparator devices.

Gender	Number of Subjects (Percentage of Total)
Female	375 (52.6%)
Male	338 (47.4%)

Age	Number of Subjects (Percentage of Total)
≤ 5	286 (40.1%)
6-21	200 (28.1%)
22-59	116 (16.3%)
≥ 60	111 (15.6%)

Prospective Clinical Study – QuantStudio Dx

Seven hundred and thirteen (713) swab specimens were tested by both the subject and comparator device for RSV viral RNA. A total of thirteen (13) invalid specimens have been removed from the analysis. Two (2) of these specimens were invalid on initial and repeat testing with the Lyra RSV + hMPV Assay (0.3%). Eleven (11) specimens were invalid on initial and repeat testing (as per the device's PI) on the comparator device (1.5%). The table below details the results for the remaining seven hundred (700) specimens.

Respiratory syncytial virus								
FDA-cleared RSV Molecular Test							95% CI	
Lyra RSV + hMPV Assay		POS	NEG	Total	Positive percent agreement	93.8%	87.7%	96.9%
	POS	105	11	116	Negative percent agreement	98.1%	96.7%	99.0%
	NEG	7	577	584				
	Total	112	588	700				

Seven hundred and thirteen (713) swab specimens were tested by both the subject and comparator device for hMPV viral RNA. A total of six (6) invalid specimens have been removed the analysis. Two (2) of these specimens were invalid on initial and repeat testing with the Lyra RSV + hMPV Assay (0.3%). Four (4) specimens were invalid on initial and repeat testing (as per the device's PI) on the comparator device (0.6%). The table below details the results for the remaining seven hundred and seven (707) specimens.

Human metapneumovirus								
FDA-cleared hMPV Molecular Test							95% CI	
Lyra RSV + hMPV Assay		POS	NEG	Total	Positive percent agreement	98.2%	90.6%	99.7%
	POS	55	4	59	Negative percent agreement	99.4%	98.4%	99.8%
	NEG	1	647	648				
	Total	56	651	707				

ANALYTICAL PERFORMANCE

Level of Detection

The limit of detection (LOD) of the Lyra RSV + hMPV Assay was determined using quantified (TCID₅₀/mL) cultures of two (2) RSV strains and four (4) hMPV strains serially diluted in negative nasopharyngeal matrix. Each dilution was extracted in replicates of 20 per concentration of virus using the NucliSENS easyMAG System and tested on the ABI 7500 Fast Dx, the SmartCycler II, and the QuantStudio Dx. LOD is defined as the lowest concentration at which 95% of all replicates tested positive.

LOD Value Summary			
Virus	LOD TCID ₅₀ /mL ABI 7500 Fast Dx	LOD TCID ₅₀ /mL SmartCycler II	LOD TCID ₅₀ /mL QuantStudio Dx
RSV A	6.29E-01	1.89E+00	6.29E-01
RSV B	7.5E-01	2.25E+00	2.25E-01
hMPV-A1	1.7E+01	2.65E+01	8.73E+00
hMPV-A2	4.17E+00	4.17E+00	2.91E+00
hMPV-B1	1.05E+00	7.88E+00	2.25E+00
hMPV-B2	3.21E+00	9.63E+00	2.25E+00

Analytical Reactivity (Inclusivity)

The reactivity of the Lyra RSV + hMPV Assay was evaluated with a panel of five (5) RSV strains and 11 hMPV strains. Each panel member was extracted using the NucliSENS easyMAG System and tested in triplicate on both the ABI 7500 Fast Dx and the SmartCycler II.

RSV Inclusivity Panel			
Subtype	Strain	TCID ₅₀ /mL	Lyra RSV + hMPV assay
A (NIBSC)	A-2	N/A	Positive
A	A-2	5.67E+00	Positive
B	Washington	2.25E+00	Positive
B	9320	2.25E+00	Positive
B	CH9318(1B)	2.25E+00	Positive

hMPV Inclusivity Panel			
Subtype	Strain	TCID ₅₀ /mL	Lyra RSV + hMPV assay
A1	IA3-2002 G Gene	7.95E+01	Positive
A1	IA10-2003	7.95E+01	Positive
A2	IA14-2003 G Gene	1.25E+1	Positive
A2	Clinical Isolate	1.25E+1	Positive
B1	Peru2-2002 G Gene	2.36E+01	Positive

hMPV Inclusivity Panel			
Subtype	Strain	TCID ₅₀ /mL	Lyra RSV + hMPV assay
B1	Peru3-2003 G Gene	2.36E+01	Positive
B2	Peru1-2002 G Gene	9.63E+00	Positive
B2	Peru6-2003	9.63E+00	Positive
B2	IA18-2003 G Gene	9.63E+00	Positive
B2	IA18-2003 G Gene	9.63E+00	Positive
hMPV (NIBSC)	N/A	N/A	Positive

The Lyra RSV + hMPV Assay detected 100% of the RSV and hMPV strains tested.

Reproducibility Study

The reproducibility of the Lyra RSV + hMPV Assay was evaluated at 3 laboratory sites (two external, one in-house). Reproducibility was assessed using a panel of 4 simulated samples that include medium positive and low positive, high negative and negative samples. Separate panels were constructed for RSV and hMPV, using the RSV-A and hMPV-A2 strains, respectively. The panels and controls were extracted using the NucliSENS easyMAG System and tested on the SmartCycler II, the ABI 7500 Fast Dx, and the QuantStudio Dx. Panels and controls were tested at each site by 2 operators for 5 days (triplicate testing x 2 operators x 5 days x 3 sites = 90 results per level for each virus). The LOD values were based on the values obtained in the LOD study.

Reproducibility Results – SmartCycler II										
Panel Member ID	Site 1			Site 2			Site 3			Total Positive Results
	Positive Results	AVE Ct*	%CV	Positive Results	AVE Ct*	%CV	Positive Results	AVE Ct*	%CV	
RSV High Negative 0.05x LOD	8/30	44.3	9.3	6/30	47.4	5.4	1/30	48.7	N/A	15/90
RSV Low Positive 2x LOD	30/30	31.2	7.3	30/30	31.4	4.1	30/30	30.9	1.5	90/90
RSV Med Positive 5x LOD	30/30	29.6	5.7	30/30	29.7	3.1	30/30	29.5	2.0	90/90
RSV Negative	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
RSV Negative Control	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
RSV Positive Control	30/30	30.0	1.8	30/30	31.0	3.3	30/30	30.6	1.2	90/90
hMPV High Negative 0.15x LOD	12/30	34.3	8.3	6/30	41.8	6.3	8/30	34.9	6.4	26/90
hMPV Low Positive 2x LOD	30/30	31.1	6.7	30/30	32.2	6.2	30/30	31.1	3.3	90/90
hMPV Med Positive 5x LOD	30/30	29.3	2.0	30/30	29.9	3.2	30/30	29.8	3.4	90/90
hMPV Negative	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
hMPV Negative Control	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
hMPV Positive Control	30/30	30.3	1.4	30/30	30.9	1.9	30/30	30.8	2.5	90/90

*Average Ct values based on positive results. Ct values of 0 were left out of this calculation.

Reproducibility Results – ABI 7500 Fast Dx										
Panel Member ID	Site 1			Site 2			Site 3			Total Positive Results
	Positive Results	AVE Ct*	%CV	Positive Results	AVE Ct*	%CV	Positive Results	AVE Ct*	%CV	
RSV High Negative 0.05x LOD	14/30	30.7	8.3	3/30	33.8	2.4	9/30	32.3	4.0	26/90
RSV Low Positive 2x LOD	30/30	23.7	7.4	30/30	23.8	3.1	30/30	23.4	4.5	90/90

Reproducibility Results – ABI 7500 Fast Dx										
Panel Member ID	Site 1			Site 2			Site 3			Total Positive Results
	Positive Results	AVE Ct*	%CV	Positive Results	AVE Ct*	%CV	Positive Results	AVE Ct*	%CV	
RSV Med Positive 5x LOD	30/30	20.5	4.0	30/30	21.8	2.2	29/29	21.1	2.2	89/89
RSV Negative	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
RSV Negative Control	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
RSV Positive Control	30/30	19.7	6.5	30/30	20.0	8.5	30/30	19.6	1.9	90/90
hMPV High Negative 0.15x LOD	14/30	24.9	19.5	6/30	29.1	10.2	10/30	25.1	16.9	30/90
hMPV Low Positive 2x LOD	30/30	21.0	8.7	30/30	21.5	4.1	30/30	21.6	5.8	90/90
hMPV Med Positive 5x LOD	30/30	18.9	3.3	30/30	19.6	3.0	29/29	19.2	2.6	89/89
hMPV Negative	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
hMPV Negative Control	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
hMPV Positive Control	30/30	19.7	3.9	30/30	20.0	1.7	30/30	19.9	1.4	90/90

*Average Ct values based on positive results. Ct values of 0 were left out of this calculation.

Reproducibility Results – QuantStudio Dx										
Panel Member ID	Site 1			Site 2			Site 3			Total Positive Results
	Positive Results	AVE Ct*	%CV	Positive Results	AVE Ct*	%CV	Positive Results	AVE Ct*	%CV	
RSV High Negative 0.3x LOD	15/30	37.6	3.7	1/30	37.7	N/A	23/30	36.7	3.7	39/90
RSV Low Positive 2x LOD	30/30	32.3	5.3	29/30	34.9	5.0	30/30	32.1	2.7	89/90
RSV Med Positive 5x LOD	30/30	30.3	1.9	30/30	31.5	5.5	30/30	29.9	1.6	90/90
RSV Negative	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
RSV Negative Control	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
RSV Positive Control	30/30	30.9	1.7	30/30	33.0	5.1	30/30	31.9	10.2	90/90
hMPV High Negative 0.15x LOD	20/30	35.9	4.0	11/30	35.2	5.9	21/30	36.6	4.0	52/90
hMPV Low Positive 2x LOD	30/30	30.3	5.0	30/30	30.2	2.5	30/30	30.4	2.1	90/90
hMPV Med Positive 5x LOD	30/30	28.9	2.0	30/30	28.4	1.2	30/30	28.3	3.7	90/90
hMPV Negative	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
hMPV Negative Control	0/30	N/A	N/A	0/30	N/A	N/A	0/30	N/A	N/A	0/90
hMPV Positive Control	30/30	28.7	0.6	30/30	28.1	2.3	30/30	28.3	4.4	90/90

*Average Ct values based on positive results. Ct values of 0 were left out of this calculation.

Analytical Specificity – Cross-reactivity

The analytical specificity of the Lyra RSV + hMPV Assay was evaluated by testing a panel consisting of 34 viruses, 26 bacteria and 1 yeast strain representing common respiratory pathogens or flora commonly present in the nasopharynx. Samples were extracted using the NucliSENS easyMAG System and tested in triplicate on both the ABI 7500 Fast Dx and the SmartCycler II. Analytical specificity of the Lyra RSV + hMPV Assay was 100%.

Organisms used for the study were the following:

Viruses

Influenza A/Mexico/4108/2009, Influenza B/Florida/04/2006, Adenovirus 1/Adenoid 71, Adenovirus 2, Adenovirus 3, Adenovirus 4, Adenovirus 5, Adenovirus 7, Adenovirus 11, Adenovirus 14, Adenovirus 31, Coronavirus NL63, Coronavirus 229E, Coronavirus OC43, Coxsackievirus B4, Coxsackievirus B5/10/2006, Cytomegalovirus, Echovirus 7, Echovirus 9, Echovirus 6, Echovirus 11, Enterovirus 71, Enterovirus 70, Epstein Barr Virus, HSV Type 1 MacIntyre strain, Human Rhinovirus, HSV Type 2 G strain, Measles virus, Mumps virus, Parainfluenza Type 1, Parainfluenza Type 2, Parainfluenza Type 3, Parainfluenza Type 4, Varicella Zoster Virus

Bacteria

Bordetella pertussis, *Bordetella bronchiseptica*, *Chlamydomphila pneumonia*, *Chlamydia trachomatis*, *Legionella pneumophila*, *Mycobacterium intracellulare*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycoplasma pneumoniae*, *Haemophilus influenza*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Proteus mirabilis*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Neisseria mucosa*, *Klebsiella pneumonia*, *Escherichia coli*, *Moraxella catarrhalis*, *Corynebacterium diphtheriae*, *Lactobacillus plantarum*, *Streptococcus pneumonia*, *Streptococcus pyogenes*, *Streptococcus salivarius*, *Staphylococcus epidermidis*, *Staphylococcus aureus*.

Yeast

Candida albicans

Analytical Specificity – Interfering Substances

The performance of Lyra RSV + hMPV Assay was evaluated using both the ABI 7500 Fast Dx and the SmartCycler II for potentially interfering substances that may be present in nasopharyngeal specimens. The potentially interfering substances were evaluated using the RSV B and hMPV B1 strains at a concentration of 3x and 10x LOD. There was no evidence of interference caused by the substances tested at 3x or 10x LOD.

Substance Name	Concentration Tested
Mucin (Bovine Submaxillary Gland, type I-S)	60 µg/mL
Blood (human), EDTA anticoagulated	2% (vol/vol)
Neo-Synephrine	15% (vol/vol)
Afrin Nasal Spray	15% (vol/vol)
Zicam Homeopathic Non-Drowsy Allergy Relief No Drip Liquid Nasal Gel	5% (vol/vol)
Saline Nasal Spray	15% (vol/vol) of dose
Throat Lozenges	0.68 g/mL; 1/18 drop, crushed; active ingredients: 1.7 mg/mL menthol
Zanamivir	3.3-5 mg/mL
Tobramycin	4.0 µg/mL
Mupirocin	6.6-10 mg/mL
Oseltamivir phosphate	7.5-25 mg/mL

Carryover and Cross-contamination Studies

In an internal study there was no evidence of carry-over/cross contamination on either the ABI 7500 Fast Dx, the SmartCycler II, when the Lyra RSV + hMPV Assay was used to detect the presence of high concentrations of RSV-A and hMPV-A2 (1,000x LOD and 10,000x LOD, respectively) extracted with the NucliSENS easyMAG System.

In an internal study there was no evidence of carry-over/cross contamination on the QuantStudio Dx thermocycler platform when the Lyra RSV + hMPV Assay was used to detect the presence of high concentrations of RSV-B and hMPV-A1 (either 2.57E+06 or 3.16E+07, TCID₅₀/mL respectively) extracted with the NucliSENS easyMAG System.

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