Simplexa™ Flu A/B & RSV

REF MOL2600
Rev. E

A real-time RT-PCR assay intended for the *in vitro* qualitative detection and differentiation of influenza A, influenza B & RSV viral RNA.

For *in vitro* diagnostic use.

INTENDED USE

The Focus Diagnostics Simplexa™ Flu A/B & RSV assay is intended for use on the 3M Integrated Cycler instrument for the in vitro qualitative detection and discrimination of influenza A virus, influenza B virus, and respiratory syncytial virus (RSV) RNA in nasopharyngeal swabs (NPS) from human patients with signs and symptoms of respiratory tract infection in conjunction with clinical and epidemiological risk factors. This test is intended for use as an aid in the differential diagnosis of influenza A, influenza B, and RSV viral infections in humans and is not intended to detect influenza C.

Negative results do not preclude influenza virus or RSV infection and should not be used as the sole basis for treatment or other patient management decisions.

Performance characteristics for influenza A were established during the 2010 influenza season when 2009 H1N1 influenza was the predominant influenza A virus in circulation. When other influenza A viruses are emerging, performance characteristics may vary.

If infection with a novel Influenza A virus is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions for novel virulent Influenza viruses and sent to state or local health department for testing. Viral culture should not be attempted in these cases unless a BSL 3+ facility is available to receive and culture specimens.

SUMMARY AND EXPLANATION

Influenza is caused by three immunologic types (A, B, and C) of RNA viruses within the Orthomyxoviridae family. Influenza A is classified further by describing two viral proteins expressed on its surface, hemagglutinin and neuraminidase. Hemagglutinin facilitates binding of the virus to respiratory epithelial cells, whereas neuraminidase functions to break those bonds with the host cell so that new virions can be released. Seasonal influenza is typically caused by viruses that contain one of three major subtypes of hemagglutinin (H1, H2, or H3) and two subtypes of neuraminidase (N1 or N2). Influenza B is not classified into subtypes.

Influenza classically presents with a combination of upper and lower respiratory signs and symptoms, fever, headache, myalgia, and general malaise. Illness can take on a variety of appearances, ranging from isolated respiratory findings that resemble the common cold, to severe pneumonia requiring hospitalization. Persons at higher risk for hospitalization include children aged <2, adults aged >65, and those with significant comorbidities. Flu may cause exacerbation of underlying medical conditions. The duration of illness is typically 2-5 days, but symptoms may last for a week or longer.

Respiratory syncytial virus (RSV) infection is more prevalent in infants and toddlers but is also observed in the adult population particularly in elder care facilities or among the immunocompromised population.[1] RSV causes various respiratory tract symptoms. In infants and young children, RSV can cause cold-like symptoms, bronchitis, croup, and/or lower respiratory infections such as bronchiolitis and pneumonia. Symptomatic infection in adults is usually consistent with an upper respiratory tract infection and can include a runny nose (rhinorrhea), sore throat (pharyngitis), cough, headache, fatigue, and fever. However, some high-risk adults, such as those with certain chronic illnesses or immunosuppression, may have more severe symptoms consistent with a lower respiratory tract infection, such as pneumonia.[1]

PRINCIPLES OF THE PROCEDURE

The test is a real-time RT-PCR amplification and detection system that utilizes a bi-functional fluorescent probe-primer for the detection and differentiation of human influenza A virus RNA, human influenza B virus RNA and RSV RNA in nasopharyngeal swabs (NPS). The assay is composed of two principal steps: (1) extraction of RNA from patient specimens, (2) a bi-functional fluorescent probe-primer is used together with a reverse primer to amplify a specific target (for each analyte and the RNA internal control). The assay provides three results; conserved regions of influenza A viruses (matrix gene), influenza B viruses (matrix gene) and RSV (M gene) are targeted to identify these viruses in the specimen. An RNA internal control is used to monitor the extraction process and to detect RT-PCR inhibition.

MATERIALS PROVIDED

The Focus Diagnostics Simplexa™ Flu A/B & RSV kit contains sufficient reagents for 100 reactions. Upon receipt, store all kit components at -10 to -30 ºC (do NOT store in a frost-free freezer). Kit components are stable through the end of the expiration month indicated on the kit packaging when stored at -10 to -30 ºC. After initial use, store thawed Flu A/B & RSV Primer Mix, Master Mix, Flu A/B & RSV Positive Control, RNA Internal Control and No Template Control at 2 to 8 °C for no more than 30 days or until expiration date whichever comes first. Store the RT Mix at -10 to -30 °C until expiration date.

Description of the Kit Labeling and Kit Components

Kit	Label
Focus Diagnostics' Simplexa™ Flu A/B & RSV (Part # MOL2600)	**ENGLISH**

ENGLISH	**REF**	**EC SYMBOL**	
Simplexa™ Flu A/B & RSV Primer Mix	**MOL2601**	**REAG**	**A**
Simplexa™ TA Master Mix	**MOL2020**	**REAG**	**B**
RT Mix	**MOL9103**	**REAG**	**C**
Simplexa™ RNA Internal Control	**MOL2004**	**CONTROL**	**IC**
Simplexa™ No Template Control	**MOL2001**	**CONTROL**	**NTC**
Simplexa™ Flu A/B & RSV Positive Control	**MOL2602**	**CONTROL**	**+**

Components	Number of tubes per Kit	Color Code	Label			
Simplexa™ Flu A/B & RSV Primer Mix (PM)	2	Brown	REF	MOL2601	Lot	Expires
Simplexa™ TA Master Mix (MM)	2	Green	REF	MOL2020	Lot	Expires
RT Mix (RT)	1	Yellow	REF	MOL9103	Lot	Expires
Simplexa™ RNA Internal Control (RNA IC)	2	Blue	REF	MOL2004	Lot	Expires
Simplexa™ No Template Control (NTC)	2	Neutral	REF	MOL2001	Lot	Expires
Simplexa™ Flu A/B & RSV Positive Control (PC)	2	Red	REF	MOL2602	Lot	Expires

Description of the Kit Components

Kit Component	Reactions per Kit / Vial	Volume (µL) per Vial	Component Description
Simplexa™ Flu A/B & RSV Primer Mix (PM)	100/50	30	Dye-labeled fluorescent primers specific for detection of Influenza A, Influenza B and RSV and for the Internal Control.
Simplexa™ TA Master Mix (MM)	100/50	200	DNA polymerase, buffer and dNTPs
RT Mix (RT)	100/100	50	Reverse Transcriptase Enzyme, buffer
Simplexa™ RNA Internal Control (RNA IC)	100/50	250	Encapsulated RNA template
Simplexa™ No Template Control (NTC)	8/4	800	Nuclease-Free Water
Simplexa™ Flu A/B & RSV Positive Control (PC)	8/4	800	Inactivated Influenza A Virus, Inactivated Influenza B Virus, Inactivated RSV
Simplexa™ Flu A/B & RSV Barcode Card	n/a	n/a	Assay specific parameters

Target	Probe Fluorophore	Excitation (nm)	Emission (nm)	Targeted Gene
Flu A	FAM	495	520	matrix
Flu B	JOE	520	548	matrix
RSV	CFR610	590	610	M gene
Internal Control "RNA IC"	Q670	644	670	N/A

MATERIALS REQUIRED BUT NOT SUPPLIED

1. 3M Integrated Cycler with Integrated Cycler Studio Software version 3.0 or higher
2. Universal Discs for use on the Integrated Cycler
3. Universal Disc Sealer
4. [a] Roche MagNA Pure LC System and associated consumables.
5. [aT] Roche MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Cat. No 3038505001)
6. [b] bioMérieux NucliSENS® easyMAG™ instrument and associated consumables and reagents
7. [b] Biohit/ bioMérieux multi-channel pipette
8. [b] ELISA strip plate
9. Single, multi-channel and/or repeater micropipette(s) with an accuracy range between 1-10 µL, 10-100 µL and 100-1000 µL

10. Freezer (manual defrost) at -10 to -30 °C (for kit component frozen storage)
11. Freezer (manual defrost) at -10 to -30 °C (for specimen frozen storage)
12. Refrigerator at 2 to 8 °C (for thawed kit components)
13. Biosafety cabinet (laminar flow hood) for extractions
14. Microcentrifuge
15. Vortex mixer
16. Sterile, RNase/DNase-free disposable aerosol-barrier micropipettor tips
17. 1.5 mL polypropylene microcentrifuge tubes and racks (RNase/DNase-free tubes are recommended but not required)
18. Disposable, powder-free gloves
19. Nuclease-Free Water
20. Cooler racks for 1.5 mL microcentrifuge tubes

[a] For use with Roche MagNA Pure LC extraction method

[b] For use with bioMérieux easyMAG extraction method

[T] **NOTE (U.S. Customers): If you choose to use the MagNA Pure extraction method, the Simplexa™ Flu A/B & RSV assay product performance requires that only qualified manufacturer lots of the MagNA Pure LC Total Nucleic Acid Isolation Kit be used with the device. Any lots not specifically qualified by Focus Diagnostics for use with the Simplexa™ Flu A/B & RSV assay are not validated for use with this assay, and may cause erroneous results.**

A list of these qualified extraction reagents is available at www.focusdx.com. Please notify the reagent manufacturer of issues with the ancillary reagents and Focus Diagnostics of the impact of these issues on the performance of this Simplexa™ kit.

SHELF LIFE AND HANDLING

1. Store reagents at -10 to -30 °C (do not use a frost-free freezer).
2. Do not use kits or reagents beyond their expiration dates.
3. Allow Primer Mix, Master Mix, Positive Control, RNA Internal Control, and No Template Control to thaw at room temperature (approximate range 18 to 25 °C) before use.
4. After addition of RT Mix, use the reaction mix within one hour.
5. If PCR Setup will not be performed immediately after the Reaction Mix is prepared, store Reaction Mix at 2 to 8 °C until ready to proceed with PCR Setup (within one hour).
6. After each use, return the RT mix to freezer (-10 to -30 °C) up to the expiration date.
7. Once thawed, store the Primer Mix, Master Mix, Positive Control, RNA Internal Control, and No Template Control at 2 to 8 °C for no more than 30 days.
8. Do not refreeze Primer Mix, Master Mix, RNA Internal Control or Positive Control.
9. Do not combine reagents from different kit lots.

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic use.
2. Follow standard precautions. All patient specimens and positive controls should be considered potentially infectious and handled accordingly.
3. Wear personal protective equipment, such as (but not limited to) gloves and lab coats when handling kit reagents. Wash hands thoroughly when finished performing the test.
4. Do not pipette by mouth.
5. Do not smoke, drink, eat, handle contact lenses or apply make-up in areas where kit reagents and/or human specimens are being used.
6. Dispose of unused kit reagents and human specimens according to local, state and federal regulations.
7. Workflow in the laboratory should proceed in a uni-directional manner, beginning in the Pre-Amplification areas and moving to the Amplification/Detection area. Below is the sequence of events that takes place from specimen extraction to Real-Time PCR amplification:
   - Begin with specimen extraction, followed by Real-Time PCR instrument set-up, reagent preparation, and finally Real-Time PCR amplification.
   - Do not use supplies and equipment across the dedicated areas of specimen extraction and sample preparation. No cross-movement is recommended between the different areas.
   - Supplies and equipment used for specimen preparation should not be used for reagent preparation activities or for processing amplified DNA or other sources of target nucleic acid.
   - All amplification supplies and equipment should be kept in the Real-Time PCR Instrument Area at all times.
   - Personal Protective Equipment, such as laboratory coats and disposable gloves, should be area-specific.
8. Contamination of patient specimens or reagents can produce erroneous results. Use aseptic techniques.
9. Pipette and handle reagents carefully to avoid mixing of samples from adjacent wells.
10. Use proper pipetting techniques and maintain the same pipetting pattern throughout the procedure to ensure optimal and reproducible values.
11. Do not substitute or mix reagent from different kit lots or from other manufacturers.
12. Do not interchange the reagent tube caps. This may cause contamination and compromise the test results.
13. Use only the protocol described in this insert. Deviations from the protocol or the use of times or temperatures other than those specified may give erroneous results.

14. Assay setup should be performed at room temperature (approximate range 18 to 25 °C). While mixing the reagents, keep the enzymes cold by utilizing a cooler block.
15. Do not re-use Universal Discs that have already been exposed to patient samples or reagents.
16. Dispose of used disc without detaching or removing cover tape.
17. If different Simplexa™ kits or lots are set up on the same disc, Positive and No Template Controls from each kit need to be tested.
18. Master Mix and RT Mix contain > 1% glycerol which may cause irritation upon inhalation or skin contact. Upon inhalation or skin contact, first aid measures should be taken.
19. Extended storage of extracted specimens at 2 to 8 °C is not recommended; performance has not been established.

INSTRUCTIONS FOR USE

A. SPECIMEN COLLECTION

Acceptable specimen types include nasopharyngeal swabs (NPS) in sterile viral transport media containing protein stabilizer, antibiotics to inhibit bacterial and fungal growth, and buffer solution, (e.g. UTM, VCM, M4, M5, M6 and other media intended to transport Chlamydia, Mycoplasma and viruses). Use only swabs with a synthetic tip (e.g. Dacron, nylon, or rayon) and an aluminum or plastic shaft. Do not use calcium alginate swabs, as they may contain substances that inhibit PCR testing.

B. SPECIMEN EXTRACTION AREA

Perform in a dedicated area for specimen and control extraction. Sample preparation for extraction should be performed in a biosafety cabinet.

Extraction using Roche MagNA Pure LC extraction method

1. Nucleic acids are extracted from patient specimens and assay controls using the Roche MagNA Pure Total Nucleic Acid kit and the Roche MagNA Pure LC Automated Nucleic Acid Extractor instrument. Refer to the manufacturer's Instructions for Use for nucleic acid extraction using this kit.
2. Under the "Protocol" drop-down menu on the MagNA Pure LC System, select "Total NA", and then "Total NA Variable_elution_volume.blk" from the list. This will load the appropriate settings for the run.
3. The Sample Protocol should be "Total NA Variable_elution_volume".
4. 200 µL should be set for the Sample Volume, and the elution volume should be set at 50 µL.
5. The dilution volume should be set at zero for all samples.
6. Ensure that the Post Elution Protocol is set to "None".
7. Ensure that specimens and controls are in the correct position on the Sample Cartridge.
8. Vortex each sample and the Positive Control for 2 to 4 seconds and briefly centrifuge to pull contents down to bottom of tube.
9. Pipette 200 µL of each specimen, Positive or No-Template Control into the corresponding position in the sample cartridge.
10. Visually check the level of samples and controls in the Sample Cartridge to ensure sample(s) were added.
11. Pulse vortex RNA Internal control 2 times and briefly centrifuge to pull contents down to bottom of tube.
12. Pipette 5 µL of the RNA Internal Control into each sample and all control wells. Change tips in between samples.
13. Transfer the sample cartridge containing the samples to the MagNA Pure LC Automated Nucleic Acid extractor and begin the extraction run.
14. After nucleic acid extraction is complete, the cartridge containing the extracted controls and patient specimens can be removed from the MagNA Pure and sealed. Store the extracted RNA at 2 to 8 °C prior to use. Long-term storage of extracted samples at this temperature is not recommended. Keep extracted RNA samples on a cooler block while loading disc.

Extraction using bioMérieux NucliSENS® easyMAG™extraction method

1. Refer to the NucliSENS® easyMAG™ User Manual for operation of the instrument and software.
2. Choose the Generic template on the NucliSENS® easyMAG™ software with the following settings;
   Default Request: Generic 2.0.1 (or equivalent)
   Run Name Prefix: (as appropriate)
   Sample ID prefix: (as appropriate)
   Sample Type: Primary
   Workflow Defaults: On-board lysis Incubation
   On-board Silica Incubation
   Sample Addition Guidance Off
   Reagent Tracking: Lysis, Silica, Internal Control reagent tracking disabled
3. Enter individual sample information into Extraction Request screen as below.
   Sample ID: (Enter sample name)
   Request: Generic 2.0.1 (or equivalent)
   Volume (mL): 0.200
   Eluate(µL): 50
   Type: Primary
   Priority: Normal
   Matrix: Other

4. Create Extraction Run in NucliSENS® easyMAG™ software per User Manual.
5. Vortex each sample and the Positive Control for 2 to 4 seconds and briefly centrifuge to pull contents down to bottom of tube.
6. Pipette 200µL of sample, Positive, or No Template Control to sample vessel(s).
7. Pulse vortex RNA Internal Control 2 times and briefly centrifuge to pull contents down to bottom of tube.
8. Pipette 5µL of RNA Internal Control to each sample and all control wells. Change tips in between samples.
9. Load sample vessel(s), new aspirator disposables, and reagents onto the easyMAG™ instrument per User Manual.
10. Initiate the on-board lysis and incubate the lysed samples for 10 minutes before addition of magnetic silica mixture.
11. During lysis incubation period, prepare magnetic silica mixture. Mix silica and dilute in nuclease-free water by adding 1 part magnetic silica to 3 parts nuclease-free water (e.g., 270µL of magnetic silica + 810µL nuclease-free water). Prepare minimally 135µL of magnetic silica mixture per sample.
12. To transfer magnetic silica mixture into ELISA strip wells, mix magnetic silica mixture and use 1 tip and operating mode P2 of the Biohit pipette. Press **Start** to aspirate 1050µL of the magnetic silica mixture and press **Start** again to dispense the first shot back into magnetic silica mixture tube. Press **Start** to dispense 125µL of the magnetic silica mixture into 8 individual wells of the ELISA strip. Repeat as necessary for additional ELISA strips.
13. After the 10 minute lysis incubation, use 8 tips (per ELISA strip) and operating mode P3 of the Biohit pipette to transfer 100µL of magnetic silica mixture to each sample in the sample vessel. Place tips into the ELISA strip wells and press **Start** to mix and aspirate magnetic silica mixture.
14. Transfer magnetic silica mixture to appropriate sample vessel and place pipette tip(s) into samples below the liquid level. Press **Start** to aspirate, dispense and mix (x3) the magnetic silica and samples. Ensure pipette tips remain below the liquid level to ensure proper mixing.
15. Repeat steps 11 and 12 for additional sample vessels using new tip(s).
16. After addition of magnetic silica mixture to all sample vessels, start the extraction run.
17. Upon completion of run, remove sample vessel(s) from the instrument. If samples are not going to be used immediately, transfer into individual tubes to minimize chance of magnetic silica falling back into sample. Store the extracted RNA at 2 to 8ºC prior to use. Long-term storage of extracted samples at this temperature is not recommended. Keep extracted RNA on a cooler block while loading disc.

C. REAL-TIME PCR INSTRUMENT SETUP

1. Refer to the Integrated Cycler Operator Manual for details on how to configure the Integrated Cycler Studio Software to add an assay definition, set up and analyze runs on the Integrated Cycler.

D. REAGENT PREPARATION AREA

Dedicated area for preparation of Simplexa™ Flu A/B & RSV assay reaction mix.

1. Thaw the Primer Mix and the Master Mix at room temperature (approximate range 18 to 25 °C). Each kit component vial contains sufficient reagents for 50 reactions. Prior to each use, gently mix the Primer Mix and Master Mix kit components by inverting 6 to 8 times and briefly centrifuge to pull contents down to bottom of tube.
2. Prepare the required volume of the Reaction Mix in an appropriately sized polypropylene microcentrifuge tube by pipetting the volume of each component as indicated in the table below.

Reaction Mix Volumes

Reagent	Reaction Mix Volume / 1 reaction	Reaction Mix Volume / 10 reactions
Simplexa™ TA Master Mix	4.0 µL	40 µL
Simplexa™ Flu A/B & RSV Primer Mix	0.5 µL	5 µL
RT Mix	0.5 µL	5 µL
Total Volume	5.0 µL	50 µL

3. Gently mix the Reaction Mix by inversion or by pipetting 8 to 10 times.
4. Briefly centrifuge to pull contents down to bottom of tube.
5. Proceed to PCR Setup.
6. Use the Reaction Mix within one hour of preparation. If PCR Setup will not be performed immediately after the Reaction Mix is prepared, store Reaction Mix at 2 to 8 °C until ready to proceed with PCR Setup (within one hour).

E. REAL TIME PCR AMPLIFICATION AREA

Perform in a dedicated area for preparation of the 96-well Universal Disc for Simplexa™ Flu A/B & RSV assay.

1. Add 5.0 µL of the reaction mix to each well.
2. Add 5.0 µL of the extracted Positive Control to the “PC” disc well.
3. Add 5.0 µL of extracted patient sample to the appropriate “S” disc well.
4. Add 5.0 µL of extracted No-Template Control to the “NTC” disc well.
5. Cover the disc with the Universal Disc Cover Tape.
6. Load the sealed Universal Disc in the Integrated Cycler and start the run.

F. DATA ANALYSIS

1. When the run finishes, click **Analyze**.
2. Review Channels one at a time or **All Channels** at once.
3. Press the **Print Preview** button (bottom right) then check the **Include Graphs and Include Ct Values** checkboxes to review a summary of the Ct values and the amplification plots. Scroll from page to page using the arrow buttons in the top left corner of the Print preview window.
4. Print or Save the Report as needed.
5. Export the Ct values if needed.

REPORTING RESULTS

Reporting results is a three step process.

1. Examination of controls to determine if the run is valid. The Integrated Cycler Studio Software will suppress interpretation of patient results if any of the samples programmed as controls are invalid.
2. Examination of validity of patient specimen results.
3. Interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.

4. Determine if the run is valid by examining the Flu A/B and RSV Positive Control, No Template Control, and RNA Internal Control

Criteria for a Valid Control (simplified)*

Control	Flu A Ct	Flu B Ct	RSV Ct	RNA IC Ct
No Template Control	0	0	0	≤40, ≠0
Positive Control	≤40, ≠0	≤40, ≠0	≤40, ≠0	Not Applicable (N/A)

* See notes below for full description.

a. No-Template Control:
   i. If the No Template Control is positive (Ct value ≤40, ≠0 for Flu A, Flu B or RSV), then this indicates possible contamination of prepared samples. The control is invalid and all patient specimens must be re-assayed.
   ii. If the RNA IC is not detected in the No Template Control, the assay run is invalid and all patient specimens must be re-assayed.
   iii. If the No Template Control is negative for Flu A, Flu B or RSV detector (Ct = 0) AND if the RNA IC is detected for the No Template Control, then this control is valid and acceptable.

b. Positive Control
   i. If the Positive Control result is a Ct = 0 for Flu A, Flu B or RSV, the assay run is considered invalid and unacceptable. All patient specimens must be re-assayed.
   ii. If the Ct values for Flu A, Flu B and RSV A are ≤40, ≠0 the assay run is considered valid and acceptable.

c. RNA Internal Control:
   i. Detection of the Simplexa™ RNA Internal Control is required to report a negative result.
   ii. Detection of the Simplexa™ RNA Internal Control is not required to report a positive result.

2. Examination of Patient Specimen Results
   Examination of clinical specimen results should be performed after the Positive and No-Template Controls have been examined and determined to be valid and acceptable. Flu A, Flu B, RSV and RNA IC results must be examined for each patient specimen.

   a. Amplification plots should be examined for every result with a "Data Quality" message. From the **Data** tab select the curve you would like to review and click **Refresh.** The software will draw the selected curves and adjust the scale of the graph. A valid amplification curve shows a smooth, exponential increase. An invalid amplification curve may be a non-exponential or linear curve or a curve with data "spikes" where the curve may cross the threshold. If the curve is valid after examination, the Ct value reported may be used to determine if Flu A, Flu B, or RSV targets are detected as indicated in section 3 below\
   b. If the amplification curve is valid for Flu A, Flu B, or RSV, the RNA IC is not required to be detected to report a positive result.

3. Interpretation of Results

Interpretation of Results

Example	Flu A Ct value	Flu B Ct value	RSV Ct value	RNA IC Ct value*	Interpretation
1	0	0	0	≤40, ≠0	Flu A, Flu B and RSV Not Detected
2	≤40, ≠0	0	0	N/A	Flu A Detected
3	0	≤40, ≠0	0	N/A	Flu B Detected
4	0	0	≤40, ≠0	N/A	RSV Detected

Example	Flu A Ct value	Flu B Ct value	RSV Ct value	RNA IC Ct value*	Interpretation
5	≤40, ≠0	≤40, ≠0	0	N/A	Flu A, Flu B Detected
6	0	≤40, ≠0	≤40, ≠0	N/A	Flu B, RSV Detected
7	≤40, ≠0	0	≤40, ≠0	N/A	Flu A, RSV Detected
8	≤40, ≠0	≤40, ≠0	≤40, ≠0	N/A	Flu A, Flu B and RSV Detected**
9	0	0	0	0	Invalid, re-extract and repeat.

Ct = cycle threshold. Detected is a Ct ≤40, ≠0. Not Detected is a Ct = 0, * Detection of the Simplexa™ RNA Internal Control is not required to report a positive result. ** Because the incidence of a triple infection of influenza A, influenza B, and RSV is low, it is recommended that repeat testing be carried out on samples from which nucleic acids from all three analytes are detected.

REPORTABLE RANGE

The positive specimens from the clinical studies have Flu A Ct values in the range of 14.9 - 31.2, and Flu B Ct values in the range of 15.9 - 33.5 and RSV Ct values in the range of 14.4 - 35.3. The majority of specimens have Ct values <35 for each target.

QUALITY CONTROL

Quality control ranges have been established as indicated in the table below. If the controls are not within these parameters, patient results should be considered invalid and the assay repeated. Each laboratory should establish its own Quality Control ranges and frequency of QC testing based on applicable local laws, regulations and standard good laboratory practice.

Expected Control Ranges

Control Type	Simplexa™ Flu A/B & RSV Positive Control Flu A Ct value	Simplexa™ Flu A/B & RSV Positive Control Flu B Ct value	Simplexa™ Flu A/B & RSV Positive Control RSV Ct value	Simplexa™ RNA Internal Control (RNA IC)
No Template Control	Ct = 0	Ct = 0	Ct = 0	Ct < 40
Positive Control	Ct ≤40, ≠0	Ct ≤40, ≠0	Ct ≤40, ≠0	Not applicable*

* Detection of the Simplexa™ RNA Internal Control (RNA IC) is not required for a valid result.

LIMITATIONS

1. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
2. The detection of viral nucleic acid is dependant upon proper sample 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.
3. All results from this and other tests must be correlated with the clinical history, epidemiological data and other data available to the clinician evaluating the patient.
4. The prevalence of infection will affect the test's predictive value.
5. As with other tests, negative results do not rule out influenza A, influenza B or RSV infections and should not be used as the sole basis for treatment of other patient management decisions.
6. False-negative results may occur when the infecting organism has genomic mutations, insertions, deletions, or rearrangements or when performed very early in the course of illness.
7. False-negative results may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
8. False-positive results may occur. Repeat testing or testing with a different device may be indicated in some settings.
9. Viral nucleic acids may persist *in vivo* independent of virus viability. Detection of target analyte(s) does not imply that the corresponding viruses are infectious or are the causative agents for clinical symptoms.
10. This test is a qualitative test and does not provide the quantitative value of detected organism present.
11. The performance of this test has been evaluated for use with human specimen material only.
12. The performance of this test has not been evaluated for sample types other than nasopharyngeal swabs.
13. The performance of this test has not been established for immunocompromised individuals.
14. The performance of this test has not been established for patients without symptoms of viral respiratory tract infection.
15. The performance of this test has not been established for monitoring treatment of influenza A, influenza B or RSV infection.
16. The performance of this test has not been established for screening of blood or blood product for the presence of influenza A, Influenza B or RSV.
17. The performance of this test has not been established with potentially interfering medications for the treatment of influenza or cold virus.
18. The performance of this test has not been established for individuals who have received the influenza vaccine.
19. This test cannot rule out diseases caused by other bacterial or viral pathogens.

PERFORMANCE CHARACTERISTICS

METHOD COMPARISON

Three external testing sites participated in the Clinical Agreement Study. Reference results for influenza A and influenza B viruses were generated using a high performance FDA cleared nucleic acid test (NAT). Reference results for RSV were

generated using culture/DFA. Culture/DFA results were carried forward from the results obtained at the time of sample collection or banking. A total of 735 nasopharyngeal swabs specimens were obtained from a combination of prospectively collected specimens (n = 558) from patients with signs and symptoms of viral respiratory tract infection and retrospectively banked specimens from patients with signs and symptoms of viral respiratory tract infection. Prospective samples were collected in Southern United States from 26-February-2010 to 24-March-2010 and in Australia from 04-July-2010 to 17-August-2010. Three (3) samples were excluded from the prospective analysis due to "Unresolved" status on the reference nucleic acid assay and two (2) samples were excluded due to "invalid" status on the Simplexa assay. The samples remained unresolved or invalid upon repeat testing. One (1) sample was excluded from the prospective analysis due to lack of culture/DFA result for RSV. Due to the low prevalence rate of influenza during the collection period and the rareness of RSV in the adult population, retrospectively collected specimens from the 2008 – 2009 and early 2010 influenza and RSV seasons in Eastern and Mid-Western United States from patients with signs and symptoms of viral respiratory tract infection were also evaluated (n = 177); of the 177 samples collected, 47 were not analyzed by culture/DFA for influenza A or influenza B.

Influenza A Clinical Agreement Summary – Prospective Samples

*High Perf. NAT[1]		Simplexa		
	n	Detected	Not Detected	% Agreement
Detected	25	25	0	100%(25/25) 95% CI:86.7-100%
Not Detected	528	1	527	99.8%(527/528) 95% CI:98.9-100%

Culture/DFA		Simplexa		
	n	Detected	Not Detected	Sensitivity / Specificity
Detected	22	22	0	100%(22/22) 95% CI:85.1-100%
Not Detected	534	4	530	99.3%(530/534) 95% CI:98.1-99.7%

Influenza B Clinical Agreement Summary – Prospective Samples

*High Perf. NAT		Simplexa		
	n	Detected	Not Detected	% Agreement
Detected	2	1	1	50%(1/2) 95% CI:9.5-90.5%
Not Detected	551	1	550	99.8%(550/551) 95% CI:99-100%

Culture/DFA		Simplexa		
	n	Detected	Not Detected	Sensitivity / Specificity
Detected	1	1	0	100%(1/1) 95% CI:20.7-100%
Not Detected	555	1	554	99.8%(554/555) 95% CI:99-100%

RSV Clinical Agreement Summary – Prospective Samples

NAT		Simplexa		
	n	Detected	Not Detected	% Agreement
Detected	111	110	1	99.1%(110/111) 95% CI:95.1-99.8%
Not Detected	442	2	440	99.5%(440/442) 95% CI:98.4-99.9%

*Culture/DFA		Simplexa		
	n	Detected	Not Detected	Sensitivity / Specificity
Detected	100	98	2	98%(98/100) 95% CI:93-99.4%
Not Detected	455	14	441	96.9%(441/455) 95% CI:94.9-98.2%

Influenza A Clinical Agreement Summary – Retrospective Samples

*High Perf. NAT		Simplexa		
	n	Detected	Not Detected	% Agreement
Detected	79	79	0	100%(79/79) 95% CI:95.4-100%
Not Detected	98	1	97	99%(97/98) 95% CI:94.4-99.8%

Culture/DFA		Simplexa		
	n	Detected	Not Detected	Sensitivity / Specificity
Detected	80	80	0	100%(80/80) 95% CI:95.4-100%
Not Detected	50	0	50	100%(50/50) 95% CI:92.9-100%

Influenza B Clinical Agreement Summary – Retrospective Samples

*High Perf. NAT		Simplexa		
	n	Detected	Not Detected	% Agreement
Detected	50	50	0	100%(50/50) 95% CI:92.9-100%
Not Detected	127	0	127	100%(127/127) 95% CI:97.1-100%

Culture/DFA		Simplexa		
	n	Detected	Not Detected	Sensitivity / Specificity
Detected	50	50	0	100%(50/50) 95% CI:92.9-100%
Not Detected	93	0	93	100%(93/93) 95% CI:96-100%

RSV Clinical Agreement Summary – Retrospective Samples

NAT		Simplexa		
	n	Detected	Not Detected	% Agreement
Detected	22	22	0	100%(22/22) 95% CI:85.1-100%
Not Detected	155	1	154	99.4%(154/155) 95% CI:96.4-99.9%

*Culture/DFA		Simplexa		
	n	Detected	Not Detected	Sensitivity / Specificity
Detected	22	22	0	100%(22/22) 95% CI:85.1-100%
Not Detected	25	1	24	96%(24/25) 95% CI:80.5-99.3%

[1]High Perf. NAT = High Performance FDA Cleared Nucleic Acid Test
*Reference method for clinical performance evaluation for 510(k) clearance.

REPRODUCIBILITY

Three investigative sites assessed the device's inter-laboratory reproducibility and inter/intra-assay reproducibility. Each of the three laboratories tested nine samples plus the Positive Control and the No Template Control, each in triplicate on five different days. Each site had two operators who each ran the assay once per day, for a total of two runs per day. One site performed the extraction using the MagNA Pure LC Total Nucleic Acid Isolation Kit; two sites performed the extraction step using the bioMérieux NucliSENS® easyMAG™. Combined results for all sites are presented in the tables below.

Reproducibility – Flu A

Sample	Site 1			Site 2			Site 3				
	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Total Agreement with expected results	95% CI
No Template Control	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Positive Control	30/30	30.2	1.6	30/30	29.6	1.5	30/30	29.8	1.3	90/90 (100.0%)	95.9% - 100.0%
Flu A High Negative	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu A Low Positive	30/30	33.0	0.9	30/30	33.3	1.3	30/30	33.5	2.1	90/90 (100.0%)	95.9% - 100.0%
Flu A Medium Positive	30/30	29.6	0.9	30/30	29.4	0.6	30/30	29.5	0.8	90/90 (100.0%)	95.9% - 100.0%
Flu B High Negative	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%

Sample	Site 1			Site 2			Site 3				
	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Total Agreement with expected results	95% CI
Flu B Low Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu B Medium Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
RSV High Negative	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
RSV Low Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
RSV Medium Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Total Agreement	330/330 (100.0%)			330/330 (100.0%)			330/330 (100.0%)			990/990 (100.0%)	99.6% – 100.0%

Reproducibility – Flu B

Sample	Site 1			Site 2			Site 3				
	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Total Agreement with expected results	95% CI
No Template Control	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Positive Control	30/30	28.8	1.2	30/30	28.4	1.0	30/30	28.1	1.5	90/90 (100.0%)	95.9% - 100.0%
Flu A High Negative	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu A Low Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu A Medium Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu B High Negative	30/30	NA	NA	29/30	39.2	NA	30/30	NA	NA	89/90 (98.9%)	94.0% - 100.0%
Flu B Low Positive	30/30	33.8	1.2	26/30	34.6	2.4	25/30	34.0	2.2	81/90/90.0%)	82.1% - 100.0%
Flu B Medium Positive	30/30	30.4	0.7	30/30	30.1	0.5	30/30	29.9	1.2	90/90 (100.0%)	95.9% - 100.0%
RSV High Negative	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
RSV Low Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
RSV Medium Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Total Agreement	330/330 (100.0%)			325/330 (98.5%)			325/330 (98.5%)			980/990 (99.0%)	98.2% - 99.5%

Reproducibility – RSV

Sample	Site 1			Site 2			Site 3				
	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Total Agreement with expected results	95% CI
No Template Control	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Positive Control	30/30	31.1	2.9	30/30	29.4	3.2	30/30	28.4	2.7	90/90 (100.0%)	95.9% - 100.0%
Flu A High Negative	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu A Low Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu A Medium Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu B High Negative	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu B Low Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
Flu B Medium Positive	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%
RSV High Negative	30/30	NA	NA	30/30	NA	NA	30/30	NA	NA	90/90 (100.0%)	95.9% - 100.0%

Sample	Site 1			Site 2			Site 3				
	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Agreement with expected results	Avg. Ct	Total %CV	Total Agreement with expected results	95% CI
RSV Low Positive	30/30	33.8	4.5	30/30	33.3	3.6	30/30	32.3	2.0	90/90 (100.0%)	95.9% - 100.0%
RSV Medium Positive	30/30	30.4	4.3	30/30	28.6	4.4	30/30	28.5	4.1	90/90 (100.0%)	95.9% - 100.0%
Total Agreement	330/330 (100.0%)			330/330 (100.0%)			330/330 (100.0%)			990/990 (100.0%)	99.6% – 100.0%

ANALYTICAL SENSITIVITY/LIMIT OF DETECTION

The Limit of Detection (LoD) was determined for the Simplexa™ Flu A/B & RSV assay using quantified stocks of influenza A, influenza B and RSV virus strains serially diluted in negative swab matrix. Each strain was extracted with both the Roche MagNA Pure LC Total Nucleic Acid Isolation Kit and the bioMérieux NucliSENS® easyMAG™. The lowest concentration with ≥95% detection (at least 19 out of 20 replicates) was determined to be the limit of detection for each assay.

Simplexa™ Flu A/B & RSV Limit of Detection

Viral Strain	LoD MagNA Pure extraction ($TCID_{50}$/mL)	LoD NucliSENS easyMAG extraction ($TCID_{50}$/mL)
Influenza A/PR/8/34 (H1N1)	1.0×10^{-2}	1.0×10^{-2}
Influenza A/Hong Kong/8/68 (H3N2)	1.0×10^{1}	1.0×10^{1}
Influenza B/Great Lakes/1739/54	1.0×10^{0}	5.0×10^{0}
Influenza B/Malaysia/2506/2004	1.0×10^{1}	1.0×10^{1}
RSV A2	1.0×10^{0}	1.0×10^{0}
RSV B1	1.0×10^{0}	5.0×10^{0}

ANALYTICAL REACTIVITY / CROSS REACTIVITY

Analytical Reactivity

Different strains of influenza A including H1 and H3 subtypes, Influenza B and RSV including A and B subtypes were evaluated. The most recent strains and geographically diverse strains were chosen. Quantified viral material was spiked into negative swab matrix at a single dilution near the LoD with a concentration of approximately 1.0×10^{2} $TCID_{50}$/mL and assayed in triplicate.

Analytical Reactivity with Additional Viral Strains

Viral Strain	Lowest Concentration Detected ($TCID_{50}$/mL)	Result
Influenza A/Wisconsin/67/05 H3	1.0×10^{2}	Flu A Detected
Influenza A/New Caledonia/20/99	1.0×10^{2}	Flu A Detected
Influenza A/Brisbane/10/07 H3	1.0×10^{2}	Flu A Detected
Influenza A/Solomon Island/03/06 H1	1.0×10^{2}	Flu A Detected
Influenza A/Taiwan/42/06 H1N1	1.0×10^{2}	Flu A Detected
Influenza A/Brisbane/59/07 H1	1.0×10^{2}	Flu A Detected
Influenza A/Swine NY/02/2009 H1	1.0×10^{2}	Flu A Detected
Influenza A/WS/33 H1N1	1.0×10^{2}	Flu A Detected
Influenza A/Port Chalmers/1/73 H3N2	1.0×10^{2}	Flu A Detected
Influenza A/California/7/2009 NYMC X-179A	1.0×10^{2}	Flu A Detected
Influenza B/Florida/02/06	1.0×10^{2}	Flu B Detected
Influenza B/Florida/04/06	2.0×10^{2}	Flu B Detected
Influenza B/Florida/07/04	1.0×10^{2}	Flu B Detected
Influenza B/Lee/40	1.0×10^{2}	Flu B Detected
Influenza B/Maryland/1/59	1.0×10^{2}	Flu B Detected
Influenza B/Hong Kong/5/72	1.0×10^{2}	Flu B Detected
Influenza B/Allen/45	1.0×10^{2}	Flu B Detected
Influenza B/Taiwan/2/62	2.0×10^{2}	Flu B Detected
Influenza B/Panama/45/90	2.0×10^{2}	Flu B Detected
RSV A-Long	1.0×10^{2}	RSV Detected
RSV B-Wash/18537/62	1.0×10^{2}	RSV Detected

Viral Strain	Lowest Concentration Detected ($TCID_{50}$/mL)	Result
RSV B-WV/14617/85	1.0×10^2	RSV Detected
RSV B-9320	1.0×10^2	RSV Detected

Cross Reactivity (Analytical Specificity)

The Simplexa™ assay's analytical specificity was evaluated by testing the ability to exclusively identify influenza A virus and/or influenza B virus and/or RSV with no cross reactivity to organisms that are closely related, or cause similar clinical symptoms, or present as normal flora in the specimen types of interest.

The panel of thirty-two (32) potential cross reactants were individually spiked into a swab matrix at clinically relevant concentrations. The unspiked matrix was also tested to serve as a baseline. Samples were tested in triplicate to screen for cross reactivity. If signal was detected in any detection channel (Flu A, Flu B, RSV) in any of the three replicates, an additional 5 replicates were tested for confirmation.

No cross reactivity was detected for Flu A, Flu B or RSV.

Simplexa Flu A/B & RSV – Cross Reactivity

Cross Reactant	Testing Concentration	Units	Flu A	Flu B	RSV
Adenovirus 1	1.02×10^5	$TCID_{50}$/mL	–	–	–
Adenovirus 7A	4.57×10^5	$TCID_{50}$/mL	–	–	–
Bordetella pertussis	5.80×10^5	cfu/mL	–	–	–
Chlamydia pneumoniae	1.52×10^5	IFU/mL	–	–	–
Coronavirus 229E	2.45×10^5	$TCID_{50}$/mL	–	–	–
Coronavirus OC43	1.70×10^5	$TCID_{50}$/mL	–	–	
Corynebacterium diphtheriae	2.87×10^6	cfu/mL	–	–	–
Cytomegalovirus	3.55×10^5	$TCID_{50}$/mL	–	–	–
Enterovirus 71	1.41×10^5	$TCID_{50}$/mL	–	–	–
Epstein Barr Virus	6.04×10^5	copies/mL[1]	–	–	–
Escherichia coli, O157H7	2.34×10^6	cfu/mL	–	–	–
Haemophilus influenzae	2.60×10^6	cfu/mL	–	–	–
Lactobacillus plantarum, 17-5	1.75×10^6	cfu/mL	–	–	–
Legionella longbeachae	7.10×10^6	cfu/mL	–	–	–
Measles	1.26×10^5	$TCID_{50}$/mL	–	–	–
Metapneumovirus	5.01×10^5	$TCID_{50}$/mL	–	–	–
Moraxella catarrhalis, Ne 11	6.83×10^6	cfu/mL	–	–	–
Mumps	8.51×10^5	$TCID_{50}$/mL	–	–	–
Mycobacterium tuberculosis	2.20×10^6	cfu/mL	–	–	–
Mycoplasma pneumoniae, Strain M129	5.63×10^6	$TCID_{50}$/mL	–	–	–
Neisseria elongata	1.99×10^6	cfu/mL	–	–	–
Neisseria meningitides	1.63×10^6	cfu/mL	–	–	–
Parainfluenza 1	6.61×10^5	$TCID_{50}$/mL	–	–	–
Parainfluenza 2	5.89×10^5	$TCID_{50}$/mL	–	–	–
Parainfluenza 3	6.61×10^5	$TCID_{50}$/mL	–	–	–
Pseudomonas aeruginosa	1.05×10^6	cfu/mL	–	–	–
Rhinovirus 1A	3.16×10^5	$TCID_{50}$/mL	–	–	–
Staphylcoccus aureus, COL	8.40×10^6	cfu/mL	–	–	–
Staphylococcus epidermidis	3.80×10^6	cfu/mL	–	–	–
Streptococcus pneumoniae	5.54×10^6	cfu/mL	–	–	–
Streptococcus pyogenes	1.55×10^6	cfu/mL	–	–	–
Streptococcus salivarius	1.14×10^6	cfu/mL	–	–	–

1) The EBV virus is grown in a transformed cell line (marmoset leukocytes). Transformed cells are not an appropriate cell line for quantitation using $TCID_{50}$/mL, instead, copies/mL is calculated using a quantitative PCR method.

INTERFERENCE

The performance of this assay was evaluated with potentially interfering substances that may be present in nasopharyngeal swabs at the concentrations indicated in the table below. The potentially interfering substances were evaluated in influenza A (influenza A/Hong Kong/8/68), and influenza B (influenza B/Malaysia/2506/2004) at a concentration of 50 $TCID_{50}$/mL and RSV A2 at a concentration of 5 $TCID_{50}$/mL. There was no evidence of interference caused by the substances tested.

Potential Interferents	Active Ingredient	Interferent Concentration
Afrin Nasal Spray	Oxymetazoline	15% (v/v)
Anti-viral drug-Relenza	Zanamivir	3.3 mg/ml
Anti-viral drug-Tamiflu	Oseltamivir	25 mg/ml
Antibacterial, systemic	Tobramycin	4.0 ug/ml
Antibiotic, nasal ointment	Mupirocin	6.6 mg/ml
Blood	N/A	2% (v/v)
Mucin Bovine Submaxillary Glad Type I-S	Purified Mucin Protein	60 ug/ml
Nasal Corticosteroid-Beconase AQ	Beclomethasone	5% (v/v)
Nasal Corticosteroid-Fluticasone	Fluticasone	5% (v/v)
Zicam Nasal Gel	Luffa Opperculata, Galphimia glauca, histaminum hydrochloricum	5% (v/v)

COMPETITIVE INTERFERENCES

The Competitive Interference study evaluated the effects of clinically relevant co-infections with each of the analytes probed by the assay. The study assessed whether a high concentration of one virus in the specimen could potentially affect the Simplexa assay performance for another target present at low levels in the multiplex assay. A low sample was contrived at approximately 4 to 6 times the LoD for each target (influenza A, influenza B and RSV), and a baseline Ct was determined for each sample. A high level of each potential concomitant infecting virus was spiked into the low level specimen according to the table below.

Baseline Strain (Concentration)	Competitive Strain (Concentration)
Influenza A/PR/8/34 (8.80×10^1 $TCID_{50}$/mL)	RSV A2 (1.60×10^4 $TCID_{50}$/mL)
Influenza B/Malaysia/2506/2004 (1.29×10^2 $TCID_{50}$/mL)	RSV A2 (1.60×10^4 $TCID_{50}$/mL)
RSV A2 (<10 $TCID_{50}$/mL)	Influenza A/PR/8/34 (2.50×10^5 $TCID_{50}$/mL)*
	Influenza B/Malaysia/2506/2004 (2.58×10^5 $TCID_{50}$/mL)

*In the rare instance when very high levels of influenza A are present with low levels of RSV, the signal from the RSV reaction may not be adequate to be detected, due to competitive interference. Please note that when all three viruses are present at moderate levels (as is the case in our positive control) there is no evidence of competitive interference.

INHIBITION BY OTHER MICROORGANISMS

The Simplexa™ assay was evaluated by testing the ability to identify influenza A virus, influenza B virus, and RSV when potentially inhibitory organisms are present.

The panel of thirty two (32) potentially inhibitory organisms were individually spiked into a pool with a low concentration (approximately 2 times LoD) of influenza A (Influenza A/PR/8/34), influenza B (Influenza B/Malaysia/2506/2004) and RSV (A2). Samples were tested in triplicate to screen for inhibition. If signal was not detected in any detection channel (Flu A, Flu B, RSV) in any of the three replicates, an additional 5 replicates were tested for confirmation.

No inhibitory effects were confirmed for influenza A, influenza B, or RSV.

Microorganism	Microorganism Concentration	Concentration Units	Flu A	Flu B	RSV
Adenovirus 1	1.1×10^5	$TCID_{50}$/mL	+	+	+
Adenovirus 7A	1.1×10^5	$TCID_{50}$/mL	+	+	+
Bordetella pertussis	1.1×10^6	cfu/mL	+	+	+

Microorganism	Microorganism Concentration	Concentration Units	Flu A	Flu B	RSV
Chlamydia pneumoniae	1.1 x10^6	copies/mL	+	+	+
CMV	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Coronavirus 229E	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Coronavirus OC43[1]	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Corynebacterium diphtheriae	1.1 x10^6	cfu/mL	+	+	+
E. coli O157	1.1 x10^6	cfu/mL	+	+	+
EBV	1.1 x10^5	copies/mL	+	+	+
Enterovirus 71	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Haemophilus influenzae	1.1 x10^6	cfu/mL	+	+	+
Lactobacillus plantarum, 17-5	1.1 x10^6	cfu/mL	+	+	+
Legionella longbeachae	1.1 x10^6	cfu/mL	+	+	+
Measles	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Metapneumovirus[1]	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Moraxella catarrhalis Ne11	1.1 x10^6	cfu/mL	+	+	+
Mumps	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Mycobacterium tuberculosis	1.1 x10^6	cfu/mL	+	+	+
Mycoplasma pneumoniae M129	1.1 x10^6	$TCID_{50}$/mL	+	+	+
Neisseria elongata	1.1 x10^6	cfu/mL	+	+	+
Neisseria meningitides	1.1 x10^6	cfu/mL	+	+	+
Parainfluenza 1	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Parainfluenza 2	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Parainfluenza 3	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Pseudomonas aeruginosa	1.1 x10^6	cfu/mL	+	+	+
Rhinovirus 1A	1.1 x10^5	$TCID_{50}$/mL	+	+	+
Staphylococcus aureus, COL	1.1 x10^6	cfu/mL	+	+	+
Staphylococcus epidermidis	1.1 x10^6	cfu/mL	+	+	+
Streptococcus pneumoniae	1.1 x10^6	cfu/mL	+	+	+
Streptococcus pyogenes	1.1 x10^6	cfu/mL	+	+	+
Streptococcus salivarius	1.1 x10^6	cfu/mL	+	+	+

1) Initial testing appeared to show possible inhibition, upon repeat testing there was no evidence of inhibition.

CARRY-OVER CONTAMINATION

The amplification carry-over study searched for the presence of contamination in high negative samples. The study was designed by alternately placing a high positive and a high negative sample on each disc. The carryover effect was evaluated by comparing the observed negative rate for the high negative sample with the expected rate under normal reproducibility conditions. No carry-over contamination effect was seen in the Flu A, Flu B or RSV channels.

EXPECTED VALUES

The prevalence of influenza varies each year with flu-season occurring during the fall and winter months in the US. Variables that affect the rate of positivity observed in respiratory testing include: the efficiency and timing of specimen collection, handling and transport of the specimen, the time of year, age of the patient, and local disease prevalence. Prospective specimens used in our clinical study were obtained from the United States and Australia. The prevalence of all influenza viruses in the US[2] during the February to March 2010 collection period ranged from 3.5 to 6.4%. Among influenza positives, 97 to 99.6% were positive for Influenza A and 0 to 1.5% for Influenza B. Outbreaks of RSV occur each year, usually lasting 3–4 months during the fall, winter, and/or spring months. The exact timing of the RSV season can vary by region[3]. The prevalence of RSV in the United States during the February to March 2010 collection period ranged from 5 to 12%[4].

Prevalence observed during our clinical study is indicated in the table below.

Region	Flu A	Flu B	RSV
Texas	10.2%	0%	18.0%
Ohio	0%	1.0%	49.0%
Australia	1.6%	0.4%	5.6%

REFERENCES

1. http://www.cdc.gov/rsv/clinical/description.html
2. http://www.cdc.gov/flu/weekly/fluactivity.htm
3. http://www.cdc.gov/Features/dsRSV/
4. http://www.cdc.gov/surveillance/nrevss/rsv/state.html

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