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Verigene® Respiratory Virus Plus Nucleic Acid Test on the Verigene® System

REF **20-005-020 (Test Kit) ● 20-012-020 (Amplification Kit)**

INTENDED USE

The Verigene® Respiratory Virus Plus Nucleic Acid Test (RV+) on the Verigene® System is a qualitative nucleic acid multiplex test intended to simultaneously detect and identify multiple respiratory virus nucleic acids in nasopharyngeal (NP) swab specimens from individuals with signs and symptoms of respiratory tract infection. The following virus types and subtypes are identified using the RV+: Influenza A, Influenza A subtype H1, Influenza A subtype H3, 2009 H1N1, Influenza B, Respiratory Syncytial Virus (RSV) subtype A, and RSV subtype B. The test is not intended to detect Influenza C virus. Detecting and identifying specific viral nucleic acids from individuals exhibiting signs and symptoms of respiratory infection aids in the diagnosis of respiratory viral infection, if used in conjunction with other clinical and laboratory findings.

Negative results for Influenza A, Influenza B, or RSV do not preclude influenza virus or RSV infection and should not be used as the sole basis for diagnosis, treatment, or 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.

Performance characteristics for Influenza A Virus were established when Influenza A/H3, A/H1, and 2009 H1N1 were the predominant Influenza A viruses circulating. These characteristics may vary when other Influenza A viruses are emerging.

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 used specifically 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.

BACKGROUND INFORMATION AND CLINICAL UTILITY

The respiratory tract is one of the most common sites for infections as it comes into contact with pathogens frequently. Influenza A and B viruses and RSV are collectively responsible for a majority of respiratory illnesses and cause significant morbidity and mortality.[1, 2, 3, 4]

Infections with Influenza A and B viruses often result in the respiratory illness commonly referred to as the 'flu.' Flu is highly contagious, and, according to the CDC, 5-20% of the population contract the flu each year. Over 200,000 people are hospitalized, and between 3,000 and 49,000 people die of complications each year, depending on the severity of the season.[5] Symptoms include fever, cough, headache, body aches, congestion, and fatigue. Flu can lead to serious complications such as pneumonia, bronchitis, sinus infections, and a general worsening of chronic conditions.[6]

In the spring of 2009 a novel quadruple-reassortant virus, now known as 2009 H1N1 Influenza, emerged in North America and quickly spread, becoming a global pandemic by the summer of 2009.[7] According to CDC estimates, the virus infected between 43 million and 89 million people between April 2009 and April 2010.[8] Importantly, this Influenza A subtype was found to be susceptible to the antiviral drug oseltamivir (brand name Tamiflu),[9] while antiviral resistance varied among other Influenza A subtypes.[10] Thus, treatment decisions may be impacted by the timely availability of Influenza A subtyping information.

Respiratory Syncytial Virus (RSV) infection is the most common cause of bronchiolitis and pneumonia in children under 1 year of age in the United States. Each year 75,000 to 125,000 children in this age group are hospitalized due to RSV infection. Symptoms of RSV infection include coughing, sneezing, runny nose, fever, and decrease in appetite. RSV is also recognized as a serious contributor to respiratory ailments in the aged and immunocompromised demographic. Flu and RSV occur as seasonal outbreaks in the United States, generally starting as early as October or November and ending as late as April or May.[5, 11]

PRINCIPLES AND PROCEDURES OF THE RV+ AND THE VERIGENE SYSTEM

The RV+ identifies virus-specific nucleic acids for Influenza A virus, Influenza B virus, and Respiratory Syncytial Virus (RSV). The RV+ targets the following genes within the viruses: matrix gene (Influenza A); hemagglutinin gene (Influenza A subtypes H1 and H3); nucleoprotein gene (Influenza A subtype 2009 H1N1); non-structural gene (Influenza B); polymerase gene (RSV A and RSV B). For each target, a set of primers amplifies a region of the gene, and a set of probes located within the amplified region detects the generated amplicons by using gold nanoparticle-based detection technology.

The entire **RV+** is performed on the Verigene® System, which is a bench-top 'sample-to-result' molecular diagnostics workstation consisting of two instruments: the Verigene® Processor *SP* and the Verigene® Reader. The Verigene Processor *SP* automates the following steps of the **RV+**: (i) Sample Preparation - Magnetic bead-based viral RNA isolation from nasopharyngeal swab specimens obtained from symptomatic patients; (ii) Target Amplification – Multiplex RT-PCR-based amplification of the eluted viral RNA to generate virus-specific amplicons; (iii) Verigene Hybridization Test – Gold nanoparticle probe-based hybridization of the virus-specific amplicons on a microarray. Gold nanoparticle probes bound specifically to target-containing spots on the microarray are silver-enhanced and light scatter from the spots is measured on the Verigene Reader and further analyzed to make decisions regarding the presence (Detected) or absence (Not Detected) of a virus/analyte. The Verigene Reader also serves as the user interface and stores and tracks sample information throughout the assay process.

The Verigene Processor *SP* utilizes single-use disposables to perform the **RV+**, including an Extraction Tray, Amplification Tray, and Verigene Test Cartridge. A separate Tip Holder Assembly contains two pipette tips that are used to transfer and mix reagents during the assay. The user tests a sample by loading the single-use disposables into the Verigene Processor *SP* and pipetting the sample into the Extraction Tray. The user initiates the test protocol on the Verigene Reader by scanning or entering the barcode ID located on the Test Cartridge along with sample information. Following assay completion, the user collects data on the Verigene Reader by scanning the barcode ID on the Test Cartridge and inserting it into the Verigene Reader for analysis.

MATERIALS PROVIDED

A. Verigene® **RV+** Test Kit; Catalog number 20-005-020
- 20 Verigene® **RV+** Test Cartridges
- 20 Verigene® **RV+** Extraction Trays (with Tip Holder Assemblies)

B. Verigene® **RV+** Amplification Kit; Catalog number 20-012-020
- 20 Verigene® **RV+** Amplification Trays

MATERIALS NEEDED BUT NOT PROVIDED

A. Instruments and Equipment
- Verigene® Reader; Catalog number 10-0000-02
- Verigene® Processor SP with Amplification; Catalog number 10-0000-07
- -70°C and -20°C freezer
- 2 – 8°C refrigerator
- Micro-pipettors & tips
- Mini-centrifuge
- Cooling block

B. Consumables and Reagents
- Nylon or Rayon tipped nasopharyngeal swabs (Copan Innovation)
- Universal Transport Medium (Catalog number 330C; Copan Innovation); Micro Test M5 Viral Transport Medium (Catalog number R12515; Remel, Inc.)

STORAGE, HANDLING, STABILITY

Component	Storage Conditions	Comments
Extraction Tray	2 – 8°C	Do not freeze.
Amplification Tray	≤-20°C	Keep frozen.
Test Cartridge	2 – 8°C	Do not freeze.
Tip Holder Assembly	2 – 30°C	Room Temperature.

PRECAUTIONS AND WARNINGS – GENERAL

- The **RV+** is for *in vitro* diagnostic use only.
- The performance of the test with viruses infecting swine and other animal hosts has not been established.
- Federal law restricts this device to sale by or on the order of a physician, or to a clinical laboratory; its use is restricted to, by, or on the order of a physician.
- Performance characteristics of the **RV+** have been determined only with nasopharyngeal swab specimens.
- 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.
- Handle supplies, reagents, and kits with powder-free gloves at all times to avoid contamination and change gloves between removal of used disposables and loading of new disposables.
- Handle samples carefully. Open one tube or sample at a time to prevent sample contamination.
- Biological samples such as tissues, body fluids, and blood of humans and other animals are potentially infectious. When handling and/or transporting human respiratory specimens, follow all applicable regulations mandated by local, state/provincial, and federal agencies for the transport of etiologic agents.
- The detection of viral nucleic acid 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.

PRECAUTIONS AND WARNINGS – INSTRUMENTS

A. General Instrument Safety
WARNING: Use this product only as specified in this document. Using this instrument in a manner not specified by Nanosphere may result in personal injury or damage to the instrument. Ensure that anyone who operates the instrument:
- Received instructions in both general safety practices for laboratories and specific safety practices for the instrument.
- Reads and understands all applicable Material Safety Data Sheets (MSDS).

B. Electrical Shock Hazard
WARNING: Severe electrical shock can result from operating the instrument without its instrument covers or back panels in place. Do not remove instrument covers or panels. High-voltage contacts are exposed when instrument covers or panels are removed from the instrument. If service is required, contact Nanosphere Technical Support at 1-888-VERIGENE (837-4436).

C. Maintenance of the Verigene Reader and Verigene Processor *SP*
For general maintenance and cleaning instructions, please refer to the Verigene System User's Manual.

PRECAUTIONS AND WARNINGS – REAGENTS AND TEST CARTRIDGES

A. Toxicity of Reagents
- Exposure to chemicals sealed inside the Test Cartridge is hazardous in case of skin contact and of ingestion. Protective disposable gloves, laboratory coats, and eye protection should be worn when handling specimens, Extraction Trays, Amplification Trays, and Verigene Test Cartridges.
- See Material Safety Data Sheets (MSDS) for toxicity information. Material Safety Data Sheets (MSDS) are available upon request from Nanosphere, Inc.

B. Waste Disposal
- Dispose unused reagents and waste in accordance with federal, state, and local regulations.

METHODS

A. Specimen Collection & Storage
- Use Nylon or Rayon tipped nasopharyngeal swabs for specimen collection.
- Place swab into Viral Transport Medium. Break swab shaft and cap the tube.
- Inadequate or inappropriate specimen collection, storage, or transport may yield false negative results.
- Training in specimen collection and handling is highly recommended because of the importance of specimen quality.
- Store specimens refrigerated at 2 – 8ºC for up to 72 hours before processing. Store leftover specimens at ≤ -70ºC.
- Transport human respiratory specimens refrigerated at 2 – 8ºC. When transporting human respiratory specimens, ensure that all applicable regulations for the transport of etiologic agents are met.

B. **RV+** Test Procedure

This section has step-wise instructions for testing samples using the **RV+** assay on the Verigene System. Please refer to the Verigene System User's Manual for additional details on performing tests on the Verigene System including daily maintenance.

1. Create a Verigene Session
   a. Login to the system as a 'user'.
   b. From the Menu Bar, Session tab, select Start New Session. The Session Setup window will appear.
   c. Touch the Session ID button and enter information by using the data entry keyboard. The Session ID can be any unique identifier in a format defined by the lab. The operator ID is automatically entered as the currently logged in operator.
   d. Touch the Processing option on the Navigation Bar at the bottom of the screen.
2. Performing a **RV+** test
   a. For each sample to be tested, retrieve an **RV+** Test Kit and an **RV+** Amplification Kit from storage and set aside at room temperature. Each **RV+** Test requires an **RV+** Test Cartridge, **RV+** Extraction Tray (and Tip Holder Assembly), and an **RV+** Amplification Tray.
   b. Open the Drawer Assembly of the Verigene Processor *SP* by pressing the open/close button on the front of the instrument.
   c. Lift the Drawer Clamp so that the trays and Tip Holder Assembly can be inserted into the Verigene Processor *SP*.
   d. Shake the Extraction Tray to mix the reagents and tap to settle the reagents. Place the Extraction Tray onto the Extraction Module.
   e. Remove the Tip Holder Assembly from the plastic pouch and place the assembly onto the Tip Module.
   f. Thaw the Amplification Tray, gently vortex, and tap to settle the reagents. Place the Amplification Tray onto the Amplification Module.
   g. Lower the Drawer Clamp over the trays and latch it to secure the trays in their positions. NOTE: If the Drawer Clamp is not latched appropriately, the drawer will not close.
   h. Set the **RV+** Test Cartridge on a level surface and remove the cover that seals the Test Cartridge by holding the Substrate Holder handle with the left hand, pressing the palm of the right hand along one edge, and using fingers to lift up the opposite edge.

i. Before loading the Test Cartridge into the Verigene Processor *SP*, scan the **RV+** Test Cartridge identification number into the Verigene Reader using the attached barcode scanner. Upon being prompted, enter sample information into the Verigene Reader.
j. The Verigene Reader will display the viral targets for which results will be reported upon successful completion of the assay. De-select any undesired targets from the list. NOTE: Once the test process is initiated the results for any de-selected targets cannot be retrieved
k. Select viruses and/or virus subtypes to be tested.
l. Tap the Test Cartridge and insert into Verigene Processor *SP*. NOTE: If the Test Cartridge is not seated properly, the drawer will not close.
m. Pipette 200 μL of sample into the Sample Well of the Extraction Tray.
n. Close the drawer of the Verigene Processor *SP* by pressing the open/close button on the front of the instrument. The Verigene System will perform a series of checks and automatically initiate the test process.
o. Once the test process is complete, remove the **RV+** Test Cartridge from the Verigene Processor *SP*.
p. Remove the Reagent Pack from the Substrate Holder.
NOTE: Handle only the Substrate Holder. Do not touch the substrate's surface.
q. Re-scan the **RV+** Test Cartridge identification number into the Verigene Reader using the attached barcode scanner.
r. Immediately before analysis, remove the protective tape from the back of the Substrate Holder and insert the Substrate Holder into the Verigene Reader for analysis. Refer to the Verigene System User's Manual for specific instructions on starting the analysis and obtaining results.
s. Open the Drawer Clamp and remove the used trays and Tip Holder Assembly from the Verigene Processor *SP*.
t. Dispose of each consumable in the appropriate waste receptacle.

QUALITY CONTROL

Quality control, as a component of an overall quality assurance program, consists of tests and procedures for monitoring and evaluating the analytical performance of a measurement system to ensure the reliability of patient test results.

A. Quality Control - Verigene System
The Verigene System uses a series of automated on-line quality measurements to monitor instrument functionality, software performance, fluidics, test conditions, reagent integrity, and procedural steps each time a test is performed. A series of automated on-line procedural checks guide the user through the testing process each time a test is performed. **RV+** test barcode and sample information are linked upon entry into the Verigene Reader to help prevent misreporting of results.

B. Quality Control – **RV+**
The **RV+** is a 'sample-to-result' detection system wherein viral RNA is isolated from nasopharyngeal swabs and amplified prior to specific detection on a microarray housed within the Test Cartridge. All reagents are prepackaged in single use disposable reagent trays and cartridges to prevent reagent dispensing errors. Several layers of controls built into the **RV+** ensure that failures at any step within the **RV+** are identified during the procedure or in the end-point image analysis of the Test Cartridge.

An MS2 bacteriophage sample processing control (IC2) is added to each sample automatically prior to extraction to monitor failure in the Sample Extraction and Target Amplification steps (see Table below). An Inhibition Control (IC1) is included in the Primer Mix reagent and added automatically to monitor PCR inhibition. A valid 'Call' is made only after both inhibition control (IC1) and process control (IC2) are verified during analysis of each test, signifying that the extraction and target amplification processes performed correctly. If IC1 or IC2 are 'Not Detected' a 'No Call – INT CTL 1' or a 'No Call – INT CTL 2' is provided respectively. If both IC1 and IC2 are 'Not Detected', a 'NO CALL – INT CTL' result is provided. The following exception exists: IC2 detection alone is sufficient for a valid call if any of the viral targets are also detected; there is no requirement for IC1 to also be 'Detected'. The recourse for the 'No Call – INT CTL decision is to repeat the **RV+** test. In addition, separate internal positive and negative controls are present within each Test Cartridge that inform regarding the proper functioning of the Test Cartridge.

C. External Controls
Regardless of the choice of quality control materials, all external quality control requirements and testing should be performed in conformance with local, state, and federal regulations or accreditation organizations as applicable and should

follow the user's laboratory's standard quality control procedures. External Controls can be prepared by using Influenza A, Influenza B, and RSV viral particles diluted in negative clinical matrix or Viral Transport Media. It is recommended that the user refer to CLSI document C24-A2, Statistical Quality Control for Quantitative Measurements: Principles and Definitions: [Approved Guideline-Second Edition] or other published guidelines for general quality control recommendations. For further guidance on appropriate quality control practices, refer to 42CFR 493.1202(c).

Control	Description	Function
Inhibition Control (IC1)	Double-stranded DNA target present in the primer mix. Amplified with every RT-PCR reaction.	Controls for PCR inhibition due to sample- or process-related inhibitors or due to reagent failures.
Process Control (IC2)	MS2 bacteriophage with an intact viral RNA genome. Added automatically to each test sample including external positive and negative controls.	Controls for sample isolation step (or nucleic acid extraction step) and the RT-PCR step.
External Positive Control	Any of the three viral particles (Influenza A, Influenza B, or RSV).	Serve as external controls for the extraction, target amplification, and detection steps. Used to verify reagent performance: (a) during installation, system validation, and when troubleshooting dictates (see Verigene System User's Manual); (b) to verify the performance of a new lot/batch of reagents; (c) when the integrity of storage conditions is in question.
External Negative Control	Viral Transport Media	Controls for reagent and/or environmental contamination.

TROUBLESHOOTING

Refer to the Troubleshooting section of the Verigene System User's Manual.

LIMITATIONS

- Performance characteristics of this product were determined with nasopharyngeal swab specimens only.
- A trained health care professional should interpret assay results together with the patient's medical history, clinical signs and symptoms, and the results of other diagnostic tests.
- Viral nucleic acid may persist in vivo, independent of virus viability. Detection of analyte target(s) does not imply that the corresponding virus(es) are infectious, or are the causative agents for clinical symptoms.
- The detection of viral nucleic acid is dependent on proper specimen collection, handling, transport, storage, and preparation, including extraction. Failure to observe proper procedures in any of these steps could lead to incorrect results.
- There is a risk of false negative results due to sequence variants in the viral targets of the assay, procedural errors, amplification inhibitors in the specimen, or inadequate viral concentration for amplification.
- A specimen yielding a negative result may contain respiratory viruses other than Influenza A, Influenza B, or RSV.
- There is a risk of false positive results due to cross-contamination by target viruses, their nucleic acids or amplified product, or from non-specific signals in the assay.

- Performance characteristics of the **RV+** in a paired comparison study of fresh and 3x 'freeze thaw' cycles demonstrated positive percent agreement of 100% for Influenza A, Influenza A subtype H1, Influenza A subtype H3, Influenza A subtype 2009 H1N1, Influenza B, RSV A and RSV B. The negative percent agreement was 100% Influenza A, Influenza A subtype H1, Influenza A subtype H3, Influenza A subtype 2009 H1N1, Influenza B, RSV A and RSV B. However, freeze-thaw cycles may compromise specimen stability, especially at very low virus titers.
- The **RV+** is a qualitative test and does not provide the quantitative value of the detected organism.
- The **RV+** has not been evaluated for patients without signs and symptoms of upper respiratory infection.
- The **RV+** has not been evaluated for monitoring treatment of Influenza or RSV infection.
- The **RV+** has not been evaluated for screening of blood or blood product for the presence of influenza.
- The affect of interfering substances has only been evaluated for those listed within. Interference by substances other than those described can lead to erroneous results.
- The assay performance has not been established in individuals who received nasally administered or injectable influenza vaccine. FluMist Influenza Vaccine Live, Intranasal (MedImmune) contains live attenuated reassortants of each of the three strains: A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008. FluMist is administered intranasally. FluLaval (GlaxoSmithKline/ID Biomedical) is administered as an intramuscular shot and contains inactivated strains of each of the three strains: A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) (an A/Perth/16/2009-like virus), and B/Brisbane/60/2008. In the dilution series for FluMist, Influenza A and the H1 and H3 subtypes were not detected at or below an approximate $1{:}5x10^5$ dilution while Influenza B was not detected at or below an approximate $1{:}5x10^6$ dilution. In the dilution series for FluLaval, the H1 subtype was not detected at or below the $1{:}1x10^5$ dilution, Influenza A and the H3 subtype were not detected at or below an approximate $1{:}1x10^6$ dilution, and Influenza B was not detected at or below an approximate $1{:}1x10^7$ dilution.
- The assay performance has not been established in immunocompromised individuals.

INTERPRETATION OF RESULTS

RV+ provides a qualitative result for the presence (Detected) or absence (Not Detected) of the following viruses/analytes: Influenza A, Influenza B, RSV A, RSV B. For Influenza A positive samples, **RV+** provides subtyping information as H1, H3, or 2009 H1N1.

In order to obtain results, the intensities from target-specific oligonucleotide spots on the microarray are imaged and analyzed on the Verigene Reader. Additionally, two Internal Controls, IC1 – inhibition control and IC2 – process controls guide decisions regarding the validity of the test process and their presence is verified before a valid result is provided. Together, they ensure that the isolation and amplification steps performed satisfactorily. If the internal controls fail verification, a No Call – INT CTL 1 (for IC1 failure) or a No Call – INT CTL 2 (for IC2 failure) or a No Call – INT CTL (for failure of both IC1 and IC2) are provided. If the internal controls are verified, the presence or absence of individual viruses is reported based on the cut-off criteria. For each valid test, a 'Detected' or 'Not Detected' result is provided for each of the viruses/analytes in the **RV+**.

If the specimen contains a novel Influenza A virus that is unsubtypeable for H1, H3, or 2009 H1N1, RV+ provides a Detected result for Influenza A and a Not Detected result for H1, H3, and 2009 H1N1; the unsubtypeable result for Influenza A is an inferred result or 'inferred unsubtypeable'. A fresh specimen should be tested for confirmation of the 'inferred unsubtypeable' result.

For assays that differentiate between Influenza A subtypes, a positive test result for Influenza A in the presence of a negative test result for an Influenza subtype (i.e., H1 or H3 or 2009 H1N1) necessitates immediate notification of appropriate local, state or federal public health authorities to determine necessary measures for verification of results in accordance with the MMWR notice (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5613a4.htm and http://www.cste.org/ps/2007pdfs/novelfluanndssjan10final23.pdf), to determine whether the questionable Flu A specimen represents a novel strain of Influenza A.

Calls for Valid Tests

Virus	Internal Controls Status	Calls	Notes
INFA/H1	Verified	Influenza A; H1	--
INFA/H3	Verified	Influenza A; H3	--
INFA/2009H1N1	Verified	Influenza A; 2009 H1N1	--
INFA UNSUBTYPEABLE	Verified	Influenza A	'Inferred unsubtypeable' Influenza A strain - rare event. Repeat recommended (see INTERPRETATION OF RESULTS section)
INFB	Verified	Influenza B	--
RSVA	Verified	RSV A	--
RSVB	Verified	RSV B	--
No Target	Verified	Not Detected for all viruses/analytes	--

Error Calls and Recourse

Error Call	Reason	Recourse	Notes
No Call – INT CTL 1	IC1 Not Detected.	Repeat **RV+**	Inhibition during Target Amplification
No Call – INT CTL 2	IC 2 Not Detected	Repeat **RV+**	Processing and/or Target Amplification Issues
No Call – INT CTL	IC1 and IC2 Not Detected	Repeat **RV+**	Processing and/or Target Amplification Issues
No Call – NO GRID	Reader unable to image Test Substrate	Ensure protective silver tape has been removed from the back of the Test Substrate. Adjust Test Substrate, repeat image analysis. If the call persists, repeat **RV+**	--
No Call – VARIATION	An inability to obtain the test result because of high variability in the target-specific signals.	Repeat **RV+**	--
No Call – BKGD			
No Call – NEG CTL			
Processing Error	Pre-analytical error	Power cycle Processor*SP*. Repeat **RV+**.	Internal checks within the Processor *SP* detected an unexpected event.

EXPECTED VALUES

While the timing and duration of influenza and RSV seasons can vary, the fall and winter months are the peak times in the US. RSV infections in the US typically occur during annual community outbreaks in the late fall, winter and early spring, and there may be variation in the timing of outbreaks between regions and between communities in the same region.

During the 2008-2009 and 2009-2010 flu seasons, 21% of 519,543 samples and 20% of 456,302 samples tested for influenza were positive for either Influenza A or Influenza B [based on data from laboratories in the US which collaborated with World Health Organization (WHO) and National Respiratory and Enteric Virus Surveillance System (NREVSS)].[12] During the 2006-2007 and 2007-2008 flu seasons, the prevalence was 13%[13] of 179,268 samples tested and 18%[14] of 225,329 samples tested, respectively.

According to data reported to the NREVSS, the prevalence of RSV was 15%[15] in the 404,798 samples tested for RSV during the 2008-2009 season and 16%[16] in the 369,944 samples tested during the 2007-2008 season.

In the **RV+** multi-site methods comparison study, which analyzed 1022 samples collected during the 2008-2009 and 2009-2010 flu season, the prevalence of Influenza A was 30.0%, for Influenza B was 4.3%, and of RSV was 10.2%. No dual infections were detected by culture and DFA, but 0.2% (or 2 specimens) of the total infections were found to contain dual infections by the **RV+** (and confirmed subsequently by bi-directional sequencing); one specimen was positive for RSV A and RSV B and a second specimen was positive for Influenza A and RSV A. It is recommended that the samples undergo repeat testing if nucleic acids from all three analytes, Influenza A, Influenza B, and RSV are detected in a single sample.

The age distribution of the patient population in the **RV+** multi-site methods comparison study is presented in the Table below:

Age Categorization (yrs)	Number of Subjects	Proportion (%)
0-2	269	26.3%
3-5	118	11.5%
6-11	147	14.4%
12-18	124	12.1%
19-64	297	29.1%
≥ 65	67	6.6%
All Ages	1022	100%

PERFORMANCE CHARACTERISTICS OF RV+

A. Method Comparison Studies for **RV+**

A total of 1022 nasopharyngeal swab specimens were collected prospectively during the 2008-2009 and 2009-2010 respiratory seasons for routine influenza or RSV testing by DFA/culture methods. The residual specimens were frozen and later tested at three clinical sites (between Site 1 – 314; Site 2 – 385; Site 3 – 323), using the **RV+**. The results for each target, including the Sensitivity and Specificity, for the comparison study, are presented in the following Tables. The **RV+** performance was compared to a culture-based reference method followed by direct fluorescent antibody (DFA) identification of all culture positive specimens. A small number of specimens (see footnotes in the 2x2 tables) were tested by using an

FDA-cleared Nucleic Acid Amplification Test (NAAT). Subtyping results for Influenza A and RSV specimens and discordant results between the **RV+** and the reference method were confirmed and/or analyzed respectively by using bi-directional sequencing at an independent reference laboratory (described in Table footnotes).

Samples with an initial 'No Call' result were re-tested successfully by following the recommendations in the 'Interpretation of Results' section. A total of 34 samples (3.3%) generated a "No Call" result; all but two of the samples (0.2%) resolved upon retest. A total of 25 samples (2.4%) resulted in a pre-analysis error ('pre-ae'). A 'pre-ae' may occur due to a number of causes including a user- initiated procedure termination (for example, procedure termination upon realizing that an incorrect sample was loaded) or an instrument-initiated procedure termination (for example, because internal checks detect an unexpected event), or non-availability of the Test Substrate for analysis (due to test consumable breakage, etc). All the specimens with an initial 'pre-ae' result resolved upon retest.

Influenza A

A: Influenza A Results: **RV+** vs. Culture/DFA

INFA		Culture/DFA			
		Positive	**Negative**	**Total**	**Total**
RV+	**Positive**	311 [a, c]	48 [b]	359	Sensitivity = 98.7% (96.8%-99.5%) 95% CI
	Negative	4 [d, e, f]	659	663	Specificity = 93.2% (91.1%-94.8%) 95% CI
	Total	315	707	1022 [g]	

B: Influenza A Subtype H3 Results: **RV+** vs. Culture/DFA/Sequencing

INFA/H3		Culture/DFA/Sequencing			
		Positive	**Negative**	**Total**	**Total**
RV+	**Positive**	108	0	108	Sensitivity = 100% (96.6%-100%) 95% CI
	Negative	0	909	909	Specificity = 100% (99.6%-100%) 95% CI
	Total	108	909	1017	

C: Influenza A Subtype H1 Results: **RV+** vs. Culture/DFA/Sequencing

INFA/H1		Culture/DFA/Sequencing			
		Positive	**Negative**	**Total**	**Total**
RV+	**Positive**	39	1[c]	40	Sensitivity = 100% (91.0%-100%) 95% CI
	Negative	0	977	977	Specificity = 99.9% (99.4%-100%) 95% CI
	Total	39	978	1017	

D: Influenza A Subtype 2009 H1N1 Results: **RV+** vs. Culture/DFA/Sequencing

INFA/2009 H1N1		Culture/DFA/Sequencing			
		Positive	**Negative**	**Total**	**Total**
RV+	**Positive**	206	0	206	Sensitivity = 99.5% (97.3%-99.9%) 95% CI
	Negative	1[c]	810	811	Specificity = 100% (99.5%-100%) 95% CI
	Total	207	810	1017	

E: Influenza A and Subtype Discordant Summary

[a] 1 specimen was positive for Influenza A by both culture and **RV+**. No sub-typing was observed on the **RV+** result. Also, the specimen failed subtype sequencing for H1, H3 and 2009 H1N1. [b] 4 specimens were Influenza A positive by **RV+**. No sub-typing was observed on the **RV+**. All 4 specimens were culture negative and failed sequencing. [c] 1 specimen was Influenza A/H1 by **RV+** and positive for Influenza A by culture. Sequencing resulted in a positive result for Influenza A/2009H1N1. [d] 1 specimen was negative by **RV+** for Flu A and positive for Flu B. Initial culture/DFA of the specimen was Flu A positive. By sequencing, the specimen was positive for Flu B and negative for Flu A. By NAAT and upon repeat culture/DFA, the specimen was positive for Flu B and negative for Flu A and RSV. [e] 1 specimen was negative for Flu A and positive for RSV B by **RV+**. By initial culture/DFA, the specimen was Flu A positive. By sequencing the specimen was positive for RSV B and negative for Flu A. By NAAT and upon repeat culture/DFA the specimen was positive for RSV and negative for Flu A and Flu B. [f] 2 specimens were negative by **RV+** but Flu A positive by culture. By sequencing both specimens were negative for Flu A. Both specimens were negative for Flu A (and negative for Flu B and RSV B) by NAAT. [g] 5 specimens failed sequencing or were not subtyped by the **RV+** assay and are not included in the Subtyping Tables.

Influenza B

A: Influenza B Results: **RV+** vs. Culture/DFA results

INFB		Culture/DFA			
		Positive	**Negative**	**Total**	**Total**
RV+	**Positive**	43	3 [a, b]	46	Sensitivity = 100% (91.8%-100%) 95% CI
	Negative	0	976	976	Specificity = 99.7% (99.1%-99.9%) 95% CI
	Total	43	979	1022	

B: Influenza B Discordant Summary

[a] 1 specimen was positive for Influenza B by **RV+** and negative for Influenza B by culture/DFA. This specimen was tested by NAAT assay and found to be positive for Influenza B. Specimen was positive for Influenza B by sequencing and upon repeat culture/DFA. [b] 2 specimens were positive for Influenza B by **RV+** and negative for Influenza B by culture. Both specimens were positive for Influenza B by sequencing.

RSV

A: RSV Results: **RV+** vs. Culture/DFA results

RSV		Culture/DFA			
		Positive	**Negative**	**Total**	**Total**
RV+	**Positive**	104	5 [a, d]	109	Sensitivity = 97.2% (92.1%-99.0%) 95% CI
	Negative	3 [b, c]	910	913	Specificity = 99.5% (98.7%-99.8%) 95% CI
	Total	107	915	1022 [e]	

B: RSV A Subtype Results: **RV+** vs. Culture/DFA/Sequencing

RSV A		Culture/DFA/Sequencing			
		Positive	**Negative**	**Total**	**Total**
RV+	**Positive**	57 [f]	0	57	Sensitivity = 100% (93.7%-100%) 95% CI
	Negative	0	962	962	Specificity = 100% (99.6%-100%) 95% CI
	Total	57	962	1019	

C: RSV B Subtype Results: **RV+** vs. Culture/DFA/Sequencing

RSV B		Culture/DFA/Sequencing			
		Positive	**Negative**	**Total**	**Total**
RV+	**Positive**	53 [f]	0	53	Sensitivity = 100% (93.2%-100%) 95% CI
	Negative	0	966	966	Specificity = 99.9% (99.6%-100%) 95% CI
	Total	53	966	1019	

D: RSV Discordant Summary

[a] 1 specimen was positive for RSV B by **RV+** and by sequencing but negative for RSV by culture/DFA. Specimen was positive for RSV by NAAT and upon repeat culture/DFA. [b] 1 specimen was negative for RSV by **RV+** and by sequencing but positive for RSV by culture and NAAT assay. [c] 2 specimens were negative for RSV by **RV+** and positive for RSV by culture. The specimens were negative for RSV by NAAT assay and failed sequencing. [d] 4 specimens were positive for RSV A or RSV B by **RV+** but negative by culture: 1 was positive for RSV A and 3 were positive for RSV B by **RV+** and by sequencing. [e] 3 specimens failed subtype sequencing and are not included in the Subtyping Tables. [f] 1 specimen was positive for both RSV A and RSV B (dual infection) by **RV+** and was culture positive for RSV. By sequencing specimen was positive for both RSV A and RSV B.

B. Reproducibility / Precision Studies

The Reproducibility/Precision study was conducted at three sites (two external and one internal) to investigate the inter-laboratory reproducibility of the **RV+**. The reproducibility panels (see Table below) were developed by using 6 unique virus strains that together represented all of the analytes in the **RV+**. The 6 virus strains were combined to generate combinations such that each virus strain was represented at 3 levels: HN- High Negative, LP - Low Positive, and MP - Moderate Positive. For two of the unique samples, virus strains were combined as in the case of INFA/H3 and RSVA and Influenza B and RSV B. For the Reproducibility/Precision study, the Test Panel comprised the 12 unique samples in duplicate for a total of 24 samples. The Test Panel samples were then divided equally into Panel A (12 samples) and Panel B (12 samples).

Viral Panel	Virus Strain	Level	Samples
A	INFB + RSVB	High Negative	1
			2
		Low Positive	3
			4
		Moderate Positive	5
			6
	INFA/2009H1N1-MT	High Negative	7
			8
		Low Positive	9
			10
		Moderate Positive	11
			12
B	INFA/H1-MT	High Negative	1
			2
		Low Positive	3
			4
		Moderate Positive	5
			6
	INFA/H3 + RSVA	High Negative	7
			8
		Low Positive	9
			10
		Moderate Positive	11
			12

At the two external study sites, Test Panel A and Test Panel B were tested on separate days. Testing each day involved 2 operators testing the same Test Panel in two replicate runs. Both Test Panels A and B were tested for a total testing period of six non-consecutive days.

The internal site ran a 12-day precision study and utilized the same four unique samples at the three levels. The complete Test Panel (Test Panel A and B) was tested separately by two operators. The precision study was run for a total testing period of 12 non-consecutive days. The cumulative results from the Reproducibility/Precision Studies are summarized. Data is presented for each individual site and then combined to provide collective results. The Table contains the agreement between the expected results and the obtained results for each virus strain in the Test Panel. These are grouped further based on their concentration levels (MP, LP, and HN). NOTE: Though some samples in the Test Panels contained combinations of viruses, results are stratified by individual virus strains.

Site		Site 1	Site 2	Site 3	All 3 Sites		
Specific Panel Member	Level	Agreement	Agreement	Agreement	Total Agreement	% Agreement	95% CI
INFA/H1-MT	MP	12/12	12/12	46/48[1,2]	70/72	97.2%	90.4% - 99.2%
	LP	12/12	12/12	48/48	72/72	100%	94.9% - 100%
	HN	11/12[3]	12/12	48/48	71/72	98.6%	92.5% - 99.8%
INFA/H3	MP	12/12	12/12	48/48	72/72	100%	94.9% - 100%
	LP	12/12	12/12	47/48[4]	71/72	98.6%	92.5% - 99.8%
	HN	12/12	12/12	48/48	72/72	100%	94.9% - 100%
INFA/2009H1N1 -MT	MP	12/12	12/12	48/48	72/72	100%	94.9% - 100%
	LP	12/12	12/12	48/48	72/72	100%	94.9% - 100%
	HN	12/12	12/12	47/48[5]	71/72	98.6%	92.5% - 99.8%
INFB	MP	12/12	12/12	48/48	72/72	100%	94.9% - 100%
	LP	12/12	12/12	48/48	72/72	100%	94.9% - 100%
	HN	12/12	11/12[a]	48/48	71/72	98.6%	92.5% - 99.8%
RSVA	MP	12/12	12/12	48/48	72/72	100%	94.9% - 100%
	LP	12/12	12/12	46/48[b,4]	70/72	97.2%	90.4% - 99.2%
	HN	12/12	12/12	48/48	72/72	100%	94.9% - 100%
RSVB	MP	12/12	12/12	48/48	72/72	100%	94.9% - 100%
	LP	11/12[c]	12/12	47/48[d]	70/72	97.2%	90.4% - 99.4%
	HN	11/12[e]	12/12	48/48	71/72	98.6%	92.5% - 99.8%

Expected Calls: [a]One (1) INFB + RSVB HN detected Influenza B. [b]One (1) INFA/H3 + RSVA LP sample detected Influenza A and H3 but did not detect RSV A. [c]One (1) INFB + RSVB LP sample detected Influenza B but did not detect RSV B. [d]One INFB + RSVB LP sample detected Influenza B but did not detect RSV B. [e]One INFB + RSVB HN sample detected RSV B but did not detect Influenza B. Additional Positive Calls: [1]One (1) INFA/H1-MT MP sample detected both Influenza A and H1 as expected, but also detected H3 which was unexpected. [2]One (1) INFA/H1-MT MP sample detected both Influenza A and H1 as expected, but also detected H3, Flu B and RSV A which was unexpected. [3]One (1) INFA/H1-MT HN sample detected Influenza A, but did not detect H1 which was expected as this is a high negative sample. However, RSV A was also detected which was unexpected. [4]One INFA/H3 + RSVA LP sample detected both

Influenza A, H3 and RSV A as expected for a low positive sample, but also detected 2009 H1N1 which was not expected. [5]One INFA/2009H1N1-MT HN sample did not detect Influenza A and 2009 H1N1 which was expected as this is a high negative sample. However, Influenza B and RSV B were also detected which was not expected. NOTE: All the samples with additional positive calls gave the expected results for the intended virus; there were no mis-calls. The source of the additional positives is likely cross-contamination artifacts during the Test Panel preparation.

There were 14 "No Calls" and 2 "pre-analytical errors" in the study. These 16 samples were repeat tested successfully. In this study the "No Call" rate was 1.6% (14/864) and the "pre-analysis error" failure rate was 0.2% (2/864). Out of the 864 samples tested, the percent agreement for all panel members for the combined sites ranged from 97.2%-100% (95% CI range from 90.3% - 99.7% to 95.0% - 100.0%, respectively).

C. Analytical Sensitivity – **RV+**

The analytical sensitivity of **RV+** was assessed and confirmed by using virus strains with established titers to represent all analytes in the test. In order to determine the Limits of Detections (LODs), the strains were serially diluted into negative pools of nasopharyngeal (NP) swab samples around the estimated LOD and replicates were tested in the **RV+**. The lowest concentration level where all the replicates were detected was chosen as the LOD. For confirmation, 20 replicate samples were prepared at the LOD level chosen. Confirmation of LOD required that at least 19 of the 20 samples tested gave a 'Detected' call for the analyte. The confirmed LOD levels for the different analytes and the associated virus strains are presented.

Virus	Analyte	Strain	LOD (TCID50/ML)
INFA	INFA	Influenza A/Virginia/01/2006 (H1N1)	1
		Influenza A/2009H1N1 Clinical Isolate (H1N1)	10
		Influenza A/Brisbane/59/2007 Clinical Isolate (H1N1)	5
		Influenza A/California/04/2009 (H1N1)	1
		Influenza A/Wisconsin/67/05 (H3N2)	0.1
	H1	Influenza A/Virginia/01/2006 (H1N1)	5
		Influenza A/Brisbane/59/2007 Clinical Isolate (H1N1)	5
	2009H1N1	Influenza A/2009H1N1 Clinical Isolate (H1N1)	10
		Influenza A/California/04/2009 (H1N1)	1
	H3	Influenza A/Wisconsin/67/05 (H3N2)	0.1
INFB	INFB	Influenza B/Wisconsin/2/2006	0.1
		Influenza B/Florida/02/06	1
RSVA	RSVA	RSV A Strain A2	10
RSVB	RSVB	RSV B Strain Wash/18537/62	1

D. Analytical Reactivity

The analytical reactivity of the **RV+** was evaluated using multiple strains of Influenza A (7 seasonal H1 strains, 7 seasonal H3 strains, 4 2009 H1N1 strains, 2 swine-origin H1N1 strains, 3 H5N1 strain, and 2 H7N7 strain), 8 Influenza B strains, 2 RSV A strains and 3 RSV B strains. Viral strains for Influenza A were selected to include strains representing temporal and geographical diversity. In order to assess analytical reactivity, cultured and titered strains of Influenza A/H3, Influenza A/H1, Influenza A/2009H1N1, Influenza B, RSV A, and RSV B were diluted in sample matrix (Universal Transport Medium, Copan) at the concentrations listed in the adjoining Table and tested in triplicate in the **RV+**. All the Influenza A/H3, Influenza A/H1, and Influenza A/2009 H1N1 strains yielded expected subtyping results.

Two swine-origin H1N1 strains (of non-human origin), Influenza A/Swine/Iowa/15/30 and Influenza A/Swine/1976/31 gave a 'Detected' result for Influenza A and for H1 but gave a 'Not Detected' result for 2009 H1N1. Higher concentrations (1- and 2-logs) did not cross-react with 2009 H1N1 demonstrating good assay specificity. The H5 and H7 Influenza strains in this study were grown in culture, titered, and inactivated before they were tested in the **RV+**. The H5 and H7 strains gave a 'Detected' result for Influenza A and a 'Not Detected' result for H1, H3, and 2009 H1N1. As expected, these strains were unsubtypeable in the **RV+**. Due to the inactivation process used for the H5 and H7 strains, a loss in the original titer was noted. While lower dilutions than those reported in the Table below were not tested, higher dilutions (1- and 2-logs higher concentrations) were examined. No cross-reactivity with H1, H3, or 2009 H1N1 was observed at any of these higher dilutions, again demonstrating good assay specificity.

Virus Strains	Titer (TCID50/mL)	INFA	H1	H3	2009 H1N1	INFB	RSVA	RSVB
Influenza A								
Influenza A/New Caledonia/20/99 (H1N1)	$1X10^2$	+	+	-	-	-	-	-
Influenza A/PR/8/34 (H1N1)	$1X10^2$	+	+	-	-	-	-	-
Influenza A1/FM/1/47 (H1N1)	$5X10^2$	+	+	-	-	-	-	-
Influenza A/NWS/33 (H1N1)	$1X10^2$	+	+	-	-	-	-	-
Influenza A1/Denver/1/57 (H1N1)	$1X10^3$	+	+	-	-	-	-	-
Influenza A/Hawaii/15/01 (H1N1)	$1X10^2$	+	+	-	-	-	-	-
Influenza A/Brisbane/59/2007 Clinical Isolate (H1N1)	$1X10^2$	+	+	-	-	-	-	-
Influenza A/ Virginia/01/2006 (H1N1)	$1X10^2$	+	+	-	-	-	-	-
Influenza A/Port Chalmers/1/73 (H3N2)	$1X10^2$	+	-	+	-	-	-	-
Influenza A/Hong Kong/8/68 (H3N2)	$1X10^2$	+	-	+	-	-	-	-
Influenza A/Aichi2/68 (H3N2)	$1X10^2$	+	-	+	-	-	-	-
Influenza A/Victoria/3/75 (H3N2)	$1X10^2$	+	-	+	-	-	-	-

Influenza A/Wisconsin/67/05 (H3N2)	$1X10^2$	+	-	+	-	-	-	-
Influenza A/Hiroshima/52/05 (H3N2)	$1X10^2$	+	-	+	-	-	-	-
Influenza A/NY/55/04 (H3N2)	$1X10^2$	+	-	+	-	-	-	-
Influenza A/California/04/2009 (H1N1)	$1X10^2$	+	-	-	+	-	-	-
Influenza A/2009H1N1/PSF1 Clinical Isolate 09 (H1N1)	$1X10^2$	+	-	-	+	-	-	-
Influenza A/Wisconsin/629-D01606/ 2009 (H1N1)	$1X10^2$	+	-	-	+	-	-	-
Influenza A/2009H1N1 Clinical Isolate (H1N1)	$1X10^2$	+	-	-	+	-	-	-
Influenza A/Swine/Iowa/15/30*	$1X10^2$	+	+	-	-	-	-	-
Influenza A/Swine/1976/31*	$1X10^2$	+	+	-	-	-	-	-
Influenza A/Duck/Hunan/795/02 (H5N1)**	$9X10^2$	+	-	-	-	-	-	-
Influenza A/Chicken/Korea/IS/2006 (H5N1)**	$3X10^3$	+	-	-	-	-	-	-
Influenza A/Scaly Breasted Munia/Hong Kong/2006 (H5N1)**	$4X10^1$	+	-	-	-	-	-	-
Influenza A/Netherlands/219/2003 (H7N7)**	$3X10^4$	+	-	-	-	-	-	-
Influenza A/New York/107/2003 (H7N2)**	$5X10^0$	+	-	-	-	-	-	-
Influenza B								
Influenza B/ Wisconsin/2/2006	$1X10^2$	-	-	-	-	+	-	-
Influenza B/ Florida/02/2006	$1X10^2$	-	-	-	-	+	-	-
Influenza B/Malaysia/2506/2004	$1X10^2$	-	-	-	-	+	-	-
Influenza B/Ohio/1/2005	$1X10^2$	-	-	-	-	+	-	-
Influenza B/ Lee/40	$1X10^2$	-	-	-	-	+	-	-
Influenza B/GL/1739/54	$1X10^2$	-	-	-	-	+	-	-
Influenza B/Taiwan/2/62	$1X10^2$	-	-	-	-	+	-	-
Influenza B/Hong Kong/5/72	$1X10^2$	-	-	-	-	+	-	-
Influenza B/Maryland/1/59	$1X10^2$	-	-	-	-	+	-	-

RSV A								
RSV A Strain A2	$1X10^2$	-	-	-	-	-	+	-
RSV A Strain Long	$1X10^2$	-	-	-	-	-	+	-
RSV B								
RSV B Strain Wash/18537/62	$1X10^2$	-	-	-	-	-	-	+
RSV B Strain B-1 Wild Type	$1X10^2$	-	-	-	-	-	-	+
RSV B Strain 9320	$1X10^2$	-	-	-	-	-	-	+

* In addition to the above testing, both swine-origin H1N1 strains were tested at 1- and 2-logs higher concentrations ($1x10^3$ and $1x10^4$ TCID50/mL). No cross-reactivity to 2009 H1N1 was observed. ** In addition to the above testing, the H5 and H7 strains were tested at 1- and 2-logs higher concentration. No cross-reactivity to any sub-types was observed.

E. Analytical Specificity/Cross-Reactivity

Analytical specificity studies were performed to assess potential cross-reactivity of the **RV+** with respiratory pathogens and other microorganisms commonly present in the respiratory tract. A total of 53 organisms of interest were identified as respiratory pathogens with which the majority of the population may be infected. These organisms included 24 bacterial strains that were tested between 10^5 to10^7 cfu/mL and 29 virus strains that were tested between 10^4 to10^6 TCID50/mL. The microorganisms were diluted into a sample matrix (Universal Transport Medium, Copan) at the concentration levels described in the Table below and tested subsequently in the **RV+**.

In all the samples tested, a valid test result was provided indicating that the microorganisms did not negatively effect the **RV+** processing. Significantly, all the microorganisms tested gave a 'Not Detected' call for each analyte on the **RV+**; the microorganisms tested did not demonstrate cross reactivity with any of the analytes in the **RV+**.

Viruses	Strain	pfu/mL	INFA	H1	H3	2009 H1N1	INFB	RSVA	RSVB
Human Adenovirus Type 1	Adenoid 71	$6.2x10^5$	-	-	-	-	-	-	-
Human Adenovirus Type 2	Adenoid 6	$1.0x10^5$	-	-	-	-	-	-	-
Human Adenovirus Type 3	G.B.	$1.0x10^6$	-	-	-	-	-	-	-
Human Adenovirus Type 4	RI-67	$1.0x10^6$	-	-	-	-	-	-	-
Human Adenovirus Type 5	Adenoid 75	$5.5x10^6$	-	-	-	-	-	-	-
Human Adenovirus Type 7	Gomen	$6.2x10^5$	-	-	-	-	-	-	-
Human Adenovirus Type 11	Slobitski	$1.9x10^5$	-	-	-	-	-	-	-
Human Adenovirus Type 14	de Wit	$5.5x10^6$	-	-	-	-	-	-	-
Human Adenovirus Type 31	1315	$3.5x10^5$	-	-	-	-	-	-	-
Human Adenovirus Type 35	holden	$1.1x10^5$	-	-	-	-	-	-	-
Human Coronavirus (OC43)	OC43	$1.1x10^6$	-	-	-	-	-	-	-
Human coronavirus (229E)	229E	$1.1x10^5$	-	-	-	-	-	-	-
Human coronavirus (NL63)	NL63	$9.9x10^4$	-	-	-	-	-	-	-
Cytomegalovirus	68-1	$2.0x10^5$	-	-	-	-	-	-	-

Enterovirus, H. coxsackievirus B4	J.V.B.	$1.9x10^6$	-	-	-	-	-	-	-
Enterovirus, echovirus 11		$1.4x10^5$	-	-	-	-	-	-	-
Epstein Barr Virus	B95-8	$2.2x10^9$ cp/ml	-	-	-	-	-	-	-
Herpes Simplex virus Type 1	MacIntyre	$2.4x10^5$	-	-	-	-	-	-	-
Measles	Edmonston	$1.1x10^6$	-	-	-	-	-	-	-
Metapneumovirus 21	A1	$1.1x10^4$	-	-	-	-	-	-	-
Metapneumovirus	A2	$1.1x10^4$	-	-	-	-	-	-	-
Metapneumovirus	B2	$3.5x10^5$	-	-	-	-	-	-	-
Mumps virus	Enders	$1.1x10^4$	-	-	-	-	-	-	-
Human Parainfluenza Type 1	C35	$1.2x10^5$	-	-	-	-	-	-	-
Human Parainfluenza Type 2	Greer	$2.2x10^5$	-	-	-	-	-	-	-
Human Parainfluenza Type 3	C243	$1.1x10^5$	-	-	-	-	-	-	-
Human Parainfluenza Type 4	M-25	$7.0x10^5$	-	-	-	-	-	-	-
Human Rhinovirus Type 1A	2060	$1.1x10^5$	-	-	-	-	-	-	-
Varicella-Zoster virus		$8.0x10^5$	-	-	-	-	-	-	-
Bacteria	**Strain**	**cfu/mL**	**INFA**	**H1**	**H3**	**2009 H1N1**	**INFB**	**RSVA**	**RSVB**
Acinetobacter Baumannii		$3.0x10^6$	-	-	-	-	-	-	-
Bordetella bronchiseptica		$3.0x10^5$	-	-	-	-	-	-	-
Bordetella pertussis		$3.7x10^7$	-	-	-	-	-	-	-
Chlamydia pneumoniae	CM-1	$1.1x10^6$	-	-	-	-	-	-	-
Corynebacterium pseudodiphtheriticum		$8.2x10^7$	-	-	-	-	-	-	-
Escherichia coli		$1.5x10^7$	-	-	-	-	-	-	-
Haemophilus influenzae		$1.0x10^7$	-	-	-	-	-	-	-
Klebsiella pneumoniae		$5.0x10^5$	-	-	-	-	-	-	-
Lactobacillus acidophilus		$1.1x10^6$	-	-	-	-	-	-	-
Legionella pneumophila		$2.1x10^6$	-	-	-	-	-	-	-
Listeria innocua		$1.2x10^6$	-	-	-	-	-	-	-
Moraxella catarrhalis		$6.5x10^7$	-	-	-	-	-	-	-
Mycoplasma pneumoniae		$3.0x10^7$	-	-	-	-	-	-	-
Mycobacterium tuberculosis, attenuated		$2.6x10^6$	-	-	-	-	-	-	-
Neisseria gonorrhoeae		$5.0x10^6$	-	-	-	-	-	-	-
Neisseria meningitidis		$1.0x10^5$	-	-	-	-	-	-	-
Proteus vulgaris		$2.0x10^6$	-	-	-	-	-	-	-
Pseudomonas aeruginosa	Boston 41501	$3.5x10^6$	-	-	-	-	-	-	-
Staphylococcus aureus		$6.1x10^7$	-	-	-	-	-	-	-

Staphylococcus epidermidis		$3.7x10^6$	-	-	-	-	-	-	-
Streptococcus pneumoniae		$9.0x10^6$	-	-	-	-	-	-	-
Streptococcus agalactiae	O90R	$2.0x10^6$	-	-	-	-	-	-	-
Streptococcus pyogenes		$1.3x10^6$	-	-	-	-	-	-	-
Streptococcus salivarius		$5.5x10^7$	-	-	-	-	-	-	-

F. Determination of Clinical Cut-Off and Call Algorithm

The **RV+** uses a microarray-based platform in which the viruses, the inhibition control (IC1) and process control (IC2) are represented by recognition elements on the Test Substrate. The recognition elements or spots are virus-specific oligonucleotide sequences that bind to the amplified viral targets, which in turn bind to gold nanoparticle probes via additional recognition elements. A gold nanoparticle probe-specific signal enhancement reaction deposits silver at the virus-specific spots. The scatter from the spots is detected by the Verigene Reader and registered as signal intensity. In addition to the above recognition elements, the Test Substrate has spots specific to positive control (PC) and negative control (NC). Three conditions were identified that together served as a single set of clinical cutoff criteria.

Condition 1: Noise Threshold

Condition 2: Normalized 'Ratio to Negative Control' (Ratio-to-NC) – intensity at the virus-specific recognition element normalized against the intensity values at the negative control elements.

Condition 3: Normalized 'Ratio to Positive Control' (Ratio-to-PC) – intensity at the virus-specific recognition element normalized against the intensity values at the positive control elements.

The Noise Threshold was determined empirically. The cut-offs for the normalized ratios, 'Ratio-to-NC' and the 'Ratio-to-PC', were determined by using ROC curves. For a positive 'Detected' decision the following criteria apply:

Condition 1: Signal intensity is above the noise threshold

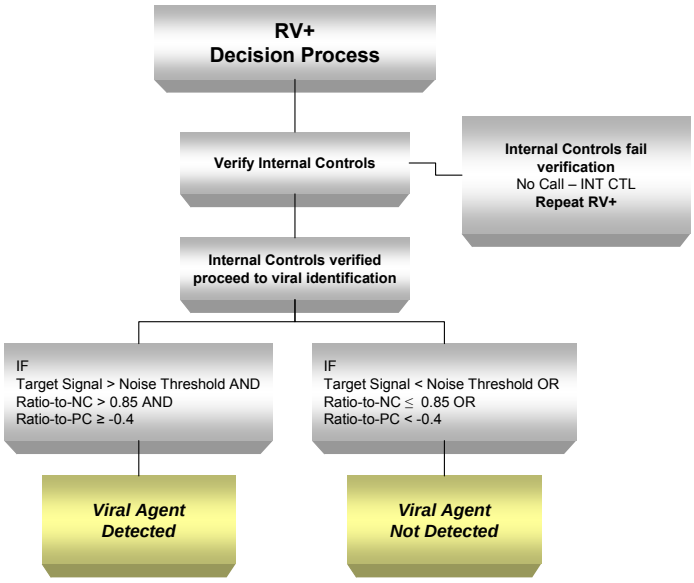
Condition 2: Ratio-to-NC > 0.85

Condition 3: Ratio-to-PC ≥ -0.4

If any one of these criteria is not met, a negative 'Not Detected' decision is provided. Criteria set for each of the three conditions are required to be met for a 'Detected' call.

For a result, the decision tree verifies the presence of IC1 and IC2 in conjunction with each of the viruses (see Schematic). Both IC1 and IC2 signal intensities have to meet the detection criteria for a valid call. A valid 'Call' is made only after both inhibition control (IC1) and process control (IC2) are verified during analysis of each test, signifying that the extraction and target amplification processes performed correctly. If IC1 or IC2 are 'Not Detected' a 'No Call – INT CTL 1' or a 'No Call – INT CTL 2' is provided respectively. If both IC1 and IC2 are 'Not Detected', a 'NO CALL – INT CTL' result is provided. The following exception exists: IC2 detection alone is sufficient for a valid call if any of the viral targets are also detected; there is no requirement for IC1 to also be 'Detected'. The recourse for the 'No Call – INT CTL' decision is to repeat the **RV+** test. In addition, separate internal positive and negative controls are present within each Test Cartridge that inform regarding the proper functioning of the Test Cartridge.

RV+ Decision Process



G. Competitive Inhibition – **RV+**

Competitive inhibition or interference in the **RV+** was assessed in clinically relevant co-infections. A set of 12 unique 'high titer-low titer' combination samples, each containing a virus at a high titer and another virus at a low titer, were generated. The samples represented combinations of Influenza A subtypes (H1, H3, and 2009 H1N1) and RSV A/B viruses (see the Table below). The levels for individual strains were chosen based on the limits of detection determined for the individual strains. The high titers for the strains were at least 3 log orders higher than their limits of detection and the low titers were held close to the limits of detection.

Sample	High Titer		Low Titer	
	Virus	TCID50/mL	Virus	TCID50/mL
1	RSVA	$2X10^4$	INFA/H3	5
2	RSVA	$2X10^4$	INFA/H1	25
3	RSVA	$2X10^4$	INFA/2009H1N1	10
4	RSVB	$5X10^3$	INFA/H3	5
5	RSVB	$5X10^3$	INFA/H1	25
6	RSVB	$5X10^3$	INFA/2009H1N1	10
7	INFA/H3	$2.5X10^3$	RSVA	100
8	INFA/H3	$2.5X10^3$	RSVB	5
9	INFA/H1	$2X10^4$	RSVA	100
10	INFA/H1	$2X10^4$	RSVB	5
11	INFA/2009H1N1	$1X10^4$	RSVA	100
12	INFA/2009H1N1	$1X10^4$	RSVB	5

Viral Strains**:** INFA/H3: Influenza A/Wisconsin/67/05; INFA/H1: Influenza A/Virgina/01/2006; INFA/2009H1N1: Influenza A/California/04/2009; RSV A: RSV A Strain A2; RSVB: RSV B/Wash/18537/62

H. Fresh vs. Frozen

Freezing virus-containing samples can potentially compromise viral integrity, which may translate to a lower effective concentration in frozen samples. In order to determine the impact of freezing samples on **RV+** performance, unfrozen negative nasopharyngeal swab samples were obtained and spiked with Influenza A, Influenza B, and RSV viruses to represent all the analytes in the **RV+**. Each virus was tested at multiple concentrations. Paired comparisons were conducted for each sample by testing it before and after freezing. The impact of 2 additional freeze-thaw cycles on the **RV+** (for a total of 3 freeze-thaw cycles) was also assessed.

The 'Fresh-vs-Frozen' comparison study involved testing a total of 60 negative nasopharyngeal (NP) swab samples across 6 unique virus types representing all the targets and analytes in the **RV+**. The negative NP swab samples were not pooled; each virus was represented by 10 unique NP swab samples (10 x 6 = 60 unique samples). Each virus type was tested at 2 or 3 different concentrations to represent levels ~1 log above the limits of detection, ~2 logs above the limit of detection, and greater than 2 logs above the limit of detection for all but 2 viruses.

Each unique sample was tested 4 times: once while fresh or unfrozen and once after each of the three (3) freeze-thaw cycles. Logistically, after a sample was spiked with the appropriate virus, an aliquot of this sample was tested in the **RV+** while the sample was 'fresh.' The residual sample was frozen by placing the sample at or below -70 °C. For the first freeze thaw, the residual sample was thawed and an aliquot was tested in the **RV+**. This constituted the first 'freeze-thaw' cycle. This process was repeated for the second and third 'freeze-thaw' cycles. Thus, the study involved testing 60 samples x 4 separate tests (1 fresh + 3 frozen) = 240 samples. The performance of each virus/analyte after each freeze-thaw cycle was compared to the corresponding result obtained when the sample was 'fresh' or unfrozen. The results from this paired comparison showed 100% agreement between the results obtained while the sample was 'fresh' and those obtained after each freeze-thaw cycle at all the concentration levels tested.

Overall, the results showed that the **RV+** detected the viruses/analytes correctly even after the sample was subjected to 3 sets of 'freeze-thaw' cycles. The viral analytes yielded a positive percentage agreement of 100% after each freeze/thaw cycle when compared to the results from the corresponding 'fresh' samples.

I. Carry-Over / Cross-Contamination Study

The carry-over study was performed to assess the carry over/cross-contamination of the **RV+** by alternately running 'High Positive' samples followed by 'High Negative' samples. Multiple Verigene Processors *SP* s used in the study and each Processor *SP* was subjected to a comprehensive set of tests.

Based on the collective data, there was no evidence of cross-contamination. Moreover, there was no evidence of any cross over within the Verigene Processor *SP* modules when high titer samples were alternated with low titer samples.

J. Interferences

The potential inhibitory effect of interfering substances or interferents that may be encountered in nasopharyngeal specimens on the **RV+** was assessed. The viral strains and the titers used in the studies are listed along with the interferents and the amount employed in the tests in the following two Tables.

Unique Sample	Viral Strain	Expected 'Detected' Calls	Concentration (TCID50/mL)
INFA/H3 + RSV A	Influenza A/Wisconsin/67/05	Influenza A; H3	2
	RSV A/ Strain A2	RSV A	100
INFB + RSV B	Influenza B/Wisconsin/2/06	Influenza B	5
	RSV B/Wash/18537/62	RSV B	10
INFA/H1	Influenza A/Virginia/01/2006	Influenza A; H1	20
INFA/2009 H1N1	A/California/04/2009	Influenza A; 2009 H1N1	20

Active Interferent	Source	Amount
No Interferent	Universal Transport Media	Not Applicable
Human Blood	Human Blood	5% v/v
Phenylephrine	WalFour Nasal Spray	10% v/v
Oxymetazoline	Anefrin Nasal Spray	10% v/v
NaCl	Saline Nasal Spray	10% v/v
Luffa opperculata	Similasan Sinus Relief	1.0% v/v
Benzocoaine	Anbesol	0.5% v/v of sample
Beclomethasone dipropionate	Beclomethasone dipropionate	16 µg/mL
Dexamethasone	Dexamethasone	50 µg/mL
Flunisolide	Flunisolide	58 µg/mL
Triamcinolone acetonide	Triamcinolone acetonide	5.5 µg/mL
Budesonide	Budesonide	25 µg/mL
Mometasone Furoate	Mometasone Furoate	2.5 µg/mL
Fluticasone propionate	Fluticasone propionate	5 µg/mL
Sulphur	Boiron	4.5 mg/mL
Menthol	Menthol	0.5 mg/mL
Mupirocin	Mupirocin	5 µg/mL
Tobramycin	Tobramycin	0.150 mg/mL
Mucin	Mucin	0.1 mg/mL
Oseltamamivir Phosphate	Tamiflu	33 µg/mL
Zanamivir*	Relenza	10 mg/mL
Fluticasone furoate	Veramyst	10% v/v
Galphimia Glauca Homeopathic Remedy	Boiron	115 µg/mL
Histaminum Hydrochloricum	Boiron	115 µg/mL
FluMist Influenza Vaccine Live, Intranasal	Medimmune	▪Influenza A and subtypes H1 and H3: No detection at or below ~$5x10^5$ dilution.* ▪Influenza B: No detection at or below ~$5x10^6$ dilution.*
FluLaval	GlaxoSmithKline/ID Biomedical	▪Influenza A and subtypes H1 and H3: No detection at or below ~$1x10^6$ dilution.* ▪Influenza B: No detection at or below ~$1x10^7$ dilution.*
** See below for additional information.*		

In addition to the interferents, the impact of two sets of Influenza vaccines in the **RV+** was also assessed. FluMist Influenza Vaccine Live, Intranasal (MedImmune) contains live attenuated reassortants of each of the three strains: A/California/07/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008. FluMist is administered intranasally. FluLaval (GlaxoSmithKline/ID Biomedical) is administered as an intramuscular shot and contains inactivated strains of each

of the three strains: A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) (an A/Perth/16/2009-like virus), and B/Brisbane/60/2008. Both vaccine samples were diluted into a sample matrix, Universal Transport Medium (Copan), and log dilution series were generated for each vaccine.

Both Influenza vaccines gave 'Detected' signals in the **RV+** for Influenza A, H3, H1, and Influenza B. As the viruses in these vaccines were reassortants, neither vaccines gave a 'Detected' call for 2009 H1N1. In the dilution series for FluMist, Influenza A and the H1 and H3 subtypes were not detected at or below an approximate $1{:}5x10^5$ dilution while Influenza B was not detected at or below an approximate $1{:}5x10^6$ dilution (see Table). In the dilution series for FluLaval, the H1 subtype was not detected at or below the $1{:}1x10^5$ dilution, Influenza A and the H3 subtype were not detected at or below an approximate $1{:}1x10^6$ dilution, and Influenza B was not detected at or below an approximate $1{:}1x10^7$ dilution.

In all the other samples tested, a valid test result was provided indicating that the interferents did not present a problem during the different steps of the **RV+**. Moreover, in each of these samples the expected calls were obtained for each of the analytes tested; none of the interferents at the tested concentration prevented the **RV+** from making the correct 'Detected' calls for the virus strains and analytes present in samples.

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TEST KIT LABELING

The contents of a Test Kit may use symbols defined below on labels.

REF	Catalog number
	Use by YYYY-MM-DD
LOT	Batch code
SN	Serial number
IVD	*In vitro* diagnostic medical device

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