510(k) SUBSTANTIAL EQUIVALENCE DETERMINATION
DECISION SUMMARY

A. 510(k) Number:
K103209

B. Purpose for Submission:
New device

C. Measurand:
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).

D. Type of Test:
Multiplex nucleic acid assay for qualitative detection and identification of Influenza A Virus, subtypes H1, H3, and 2009 H1N1, Influenza B Virus, and Respiratory Syncytial Virus A and Respiratory Syncytial Virus B target sequences in nasopharyngeal swab specimens including nucleic acid isolation, multiplex RT-PCR amplification, capture of the amplicons hybridized to gold-labeled probes on a microarray-based chip, and detection of the signal enhanced with elemental silver using the Verigene® System

E. Applicant:
Nanosphere, Inc.

F. Proprietary and Established Names:
Verigene Respiratory Virus Plus Nucleic Acid Test (RV+)

G. Regulatory Information:

1. Regulation section:
   866.3980

2. Classification:
   Class II

3. Product code:
   OCC; NSU

4. Panel:
   Microbiology (83)

H. Intended Use:

1. Intended use(s):

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.

2. Indication(s) for use:
   Same as the Intended Use

3. Special conditions for use statement(s):
   For prescription use only

4. Special instrument requirements:

Verigene® System consisting of two instruments, the Verigene Processor *SP* and the Verigene Reader

I. Device Description:

The RV+ is designed to identify virus-specific nucleic acids for Influenza A virus, Influenza A virus subtype H1, Influenza A virus subtype H3, Influenza A virus subtype 2009 H1N1, Influenza B virus, and respiratory Syncytial virus (RSV A. RSV B). The RV+ involves:

1) Sample Preparation – magnetic bead-based viral RNA extraction from nasopharyngeal swab specimens obtained from symptomatic patients;

2) Target Amplification – multiplex RT-PCR-based amplification of the eluted viral RNA targets to generate virus-specific amplicons;

3) Verigene Hybridization Test and Analysis – detection and identification of virus-specific amplicons by using gold nanoparticle probe-based technology.

The entire RV+ test is performed on the Verigene® System, which is a bench-top molecular diagnostics workstation that consists of two instruments, the Verigene Processor *SP* and the Verigene Reader. The Verigene Processor *SP* performs the assay steps on each sample by using a robotic pipettor to transfer and mix reagents within and between separate testing modules designed for nucleic acid extraction, target amplification, and the Verigene Hybridization Test. The Verigene Hybridization Test module is the same as in the original Verigene System with added modules for nucleic acid extraction and RT-PCR target amplification.

Key functions of the Verigene Processor *SP* include:

1) Reading of the barcode identification label on inserted Test Consumables to maintain positive identification of patient samples throughout processing.

2) Facilitation of nucleic acid extraction, multiplex RT-PCR target amplification, and the Verigene Hybridization Test.

3) Real-time communication of test processing status to the Reader.

The Verigene Reader is the same instrument as in the FDA-cleared RVNAT*SP*. It is a free-standing instrument with a touch screen control panel and a wand-based barcode scanner. It utilizes a graphical user interface to guide the user through the process of ordering tests and reporting results. There are no serviceable parts and no user calibration is required. Interaction with the touch screen is minimized through barcode use. This instrument also serves as the reader of the Test Cartridges using advanced optics. The key functions of the Verigene Reader include:

1) Entry and tracking of specimen identification numbers via manual keyboard input or via barcode-reader wand.

2) Test selection for each specimen.

3) Automated transfer of specimen processing instructions on Test Cartridge-specific basis to linked Processor *SP* unit(s). A single Reader unit can control up to 32 Processor units.

4) Automated imaging and analysis of Test Cartridges.

5) Results display

6) Results report generation.

RV+ consumables within each single-use disposable test kit include: (i) Tip Holder Assembly; (ii) Extraction Tray; (iii) Amplification Tray; and (iv) RV+ Test Cartridge. The kit components are inserted into the corresponding module of the Verigene Processor *SP* prior to each test, and the sample is added to the Extraction Tray. Patient information is entered into the Reader to initiate the test procedure.

1) Tip Holder Assembly – The robotic pipettor picks up pipettes from the Tip Holder Assembly. The pipettes are used for mixing and transferring reagents within the test procedure.

2) Extraction Tray – Nucleic acids are extracted from the sample by using magnetic bead-based methods within the Extraction Tray. Each Tray contains reagents for a single extraction procedure. A robotic pipette transfers reagents to designated wells within the Extraction Tray to affect the steps of lysis, capture of nucleic acids onto the magnetic beads, washing, and eluting the isolated nucleic acids from the magnetic beads.

3) Amplification Tray – The isolated nucleic acids are amplified by using multiplex RT-PCR within the Amplification Tray. Each Tray contains reagents for a single multiplex RT-PCR procedure. A robotic pipette transfers the reagents to a specific well within the Amplification Tray. A set thermal profile is then initiated to perform all of the amplification related steps including UDG-based decontamination, reverse transcription, and multiplex PCR in a single tube. Upon completion, an aliquot of the amplified sample is mixed with hybridization buffer containing the virus specific mediator probes. The sample is then transferred to the Test Cartridge.

4) RV+ Test Cartridge for Verigene Hybridization Test – The virus-specific and subtype-specific amplicons are detected and identified within a Test Cartridge by using specific nucleic acid probes in conjunction with gold nanoparticle probe-based detection technology. Each Test Cartridge is a self-contained, laboratory consumable that consists of two parts. The upper housing of each cartridge is called the "reagent pack" and contains reservoirs filled with the detection reagents. When in place with the 'substrate holder', the reagent pack creates an air-tight hybridization chamber surrounding the region of the substrate containing a target-specific capture array. As each step of the test is completed, old reagents are moved out of the hybridization chamber and new reagents are added from the reagent pack via microfluidic channels and pumps. Once the test is complete, the Test Cartridge is removed from the Verigene Processor *SP* unit and the reagent pack is snapped off and discarded. The remaining slide is now ready for imaging and analysis in the Verigene Reader.

5) End-point detection on the Verigene Reader: The test slide is inserted into the Verigene Reader wherein it is illuminated along its side. The gold-silver aggregates at the test sites scatter the light, which is in turn captured by a photosensor. The relative intensity arising from each arrayed test site is tabulated. Net signals, defined as the absolute signal intensities with background signals subtracted, are compared with thresholds determined by negative controls within the slide in order to arrive at a decision regarding the presence or absence of target. These results are linked to the test and patient information entered at the beginning of each test session to provide a comprehensive results file.

J. Substantial Equivalence Information:

1. Predicate device name(s):
   Verigene® Respiratory Virus Nucleic Acid Test
   Prodesse ProFAST+ assay

2. Predicate 510(k) number(s):
K092566, K101855

3. Comparison with predicate:

Feature	Verigene RV+ Subject Device	Verigene RVNAT SP Predicate 1	Prodesse ProFAST+ Predicate 2
510(k) #	K103209	K092566	K101855
Regulation	866.3980	866.3980	866.3332
Product Codes	OCC	OCC; NSU	OQW
Device Class	Class II	Class II	Class II
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.	The Verigene® Respiratory Virus Nucleic Acid Test ($RVNAT_{SP}$) is a qualitative multiplex *in vitro* diagnostic test for the detection and identification of Influenza A Virus, Influenza B Virus, and Respiratory Syncytial Virus (RSV) nucleic acids purified from nasopharyngeal swab specimens obtained from patients symptomatic for viral upper respiratory infection.	The Prodesse ProFAST+ Assay is a multiplex Real Time RT-PCR *in vitro* diagnostic test for the qualitative detection and discrimination of seasonal Influenza A/H1, seasonal Influenza A/H3 and Influenza A/2009 H1N1 Influenza viral nucleic acids isolated and purified from nasopharyngeal (NP) swab specimens from human patients with signs and symptoms of respiratory infection in conjunction with clinical and epidemiological risk factors.
Targets	Influenza A Influenza A/H1 Influenza A/H3 Influenza A/2009 H1N1 Influenza B RSV A RSV B	Influenza A Influenza B RSV	Influenza A/H1 Influenza A/H3 Influenza A/2009 H1N1
Specimen	Nasopharyngeal swabs in sample matrix	Nasopharyngeal swabs in sample matrix	Nasopharyngeal swabs in sample matrix
Nucleic Acid Isolation	Automated internal extraction of nucleic acids performed on the Processor SP using silica coated magnetic beads and chaotropic salts.	Automated internal extraction of nucleic acids performed on the Processor SP using silica coated magnetic beads and chaotropic salts.	External isolation of nucleic acids on the Roche MagNA Pure LC System and bioMerieux NucliSENS easyMAG.

Quality control	Internal procedural quality controls: IC1 – inhibition control; IC2 – process control; internal positive and negative controls; external positive controls	Internal procedural quality controls: PC1 (IC1) – inhibition control; PC2 (IC2) – process control; internal positive and negative controls; external positive controls	Influenza positive RNA transcript control and an internal RNA control provided
Amplification method	Internal multiplexed RT-PCR performed within the Amplification Module on the Processor SP	Internal multiplexed RT-PCR performed within the Amplification Module on the Processor SP	Real Time RT-PCR detection
	M-MLV Reverse Transcriptase	M-MLV Reverse Transcriptase	M-MLV Reverse Transcriptase
Pipetting	Pipetting module to automate fluid transfer steps	Pipetting module to automate fluid transfer steps	Mostly manual
Detection Method	Verigene Test (hybridization) is performed in the hybridization module housed in the Processor SP of the Verigene® System by using single-use Test Cartridges	Verigene Test (hybridization) is performed in the hybridization module housed in the Processor SP of the Verigene® System by using single-use Test Cartridges	In the Cepheid Smartcycler II instrument, the amount of fluorescence at a given cycle is dependent on the amount of amplification products present at the time
Decision algorithm	Target-specific signal intensities are compared to a signal threshold and ratioed against positive and negative controls for a decision.	Target-specific signal intensities are compared to a signal threshold and ratioed against positive and negative controls for a decision.	Fluorescent intensity is monitored during each PCR cycle by the real-time instrument. A specific number of cycles are defined as a threshold for presence or absence of a target.
Results	Positive or negative qualitative results	Positive or negative qualitative results	Positive or negative qualitative results
Reader	Provides the user interface, controls the Processor SP, performs image analysis, and provides results.	Provides the user interface, controls the Processor SP, performs image analysis, and provides results.	Not applicable
Software	A custom embedded software application running under the Micro-C/OS real-time operating system. Additional software programming to control the Extraction and Amplification Modules.	A custom embedded software application running under the Micro-C/OS real-time operating system. Additional software programming to control the Extraction and Amplification Modules.	Not applicable

K. Standard/Guidance Document Referenced (if applicable):

1. CLSI EP5-A2; Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline - Second Edition.
2. CLSI EP12-A; User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline - Second Edition.

3. CLSI MM3-A2; Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline – Second Edition.
4. FDA document, Guidance on Informed Consent for In Vitro Diagnostic Device Studies Using Leftover Human Specimens that are Not Individually Identifiable - Guidance for Sponsors, Institutional Review Boards, Clinical Investigators and FDA Staff
5. FDA document #337, Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices - Guidance for Industry and FDA Staff.
6. FDA document #1638, Establishing the Performance Characteristics of IVDs for the Detection and Differentiation of Influenza Viruses, February 15, 2008.
7. FDA document #1594, In Vitro Diagnostic Devices to Detect Influenza A Viruses: Labeling and Regulatory Path, May 2, 2007.
8. FDA document #1596, Class II Special Controls Guidance Document: Reagents for Detection of Specific Novel Influenza A Viruses, March 22, 2006.
9. Class II Special Controls Guidance Document: Testing for Detection and Differentiation of Influenza A Virus Subtypes Using Multiplex Nucleic Acid Assays, October 9, 2009.
10. Class II Special Controls Guidance Document: Instrumentation for Clinical Multiplex Test Systems, March 10, 2005.
11. Class II Special Controls Guidance Document: Respiratory Viral Panel Multiplex Nucleic Acid Assay, October 9, 2009.
12. Class II Special Controls Guidance Document: Respiratory Viral Panel Nucleic Acid
13. Assay: Testing for Human Metapneumovirus (hMPV) Using Nucleic Acid Assays, October 9, 2009.

L. Test Principle:

RV+ is a molecular test and relies on the specific detection of target nucleic acids. For each of the viruses/analytes detected in RV+, at least 4 sets of oligonucleotides are required: (i) and (ii) Specific forward and reverse primers for the generation of virus-/analyte-specific amplicons; (iii) Capture oligonucleotides; and (iv) Mediator oligonucleotides. The Capture oligonucleotides (or captures) and Mediator oligonucleotides (or mediators) are needed for the endpoint gold nanoparticle probe-based detection process. The Capture oligonucleotides (or captures) are printed on the Test Substrate (or the microarray) and are designed to specifically bind to one part of the target amplicon. The Mediator oligonucleotides (or mediators) bind to another part of the same target amplicon and allow the binding of a gold nanoparticle probe to a portion complementary to a gold nanoparticle probe. Notably, in a multiplexed detection system, numerous unique target-specific mediators can co-exist and form unique 'sandwich' hybridizations at the different captures on the microarray. Since all the mediators have a target specific region and a gold nanoparticle probe region, a single 'universal' gold nanoparticle probe is sufficient. Specific silver enhancement of the bound gold nanoparticle probes at the capture sites results in gold-silver aggregates that scatter light with high efficiency. Light scatter from the capture spots is imaged by the Verigene Reader and intensities from the microarray spots are used to make decisions regarding the presence (Detected) or absence (Not Detected) of a virus/analyte.

M. Performance Characteristics (if/when applicable):

1. <u>Analytical performance:</u>

a. Precision/Reproducibility:

The reproducibility panel was developed by using 6 unique virus strains to represent all of the analytes in the RV+. The 6 virus strains were combined to generate 12 unique combinations. The combinations were designed such that each virus strain was represented at 3 levels: High Negative, Low Positive, and Moderate. 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). In order to prepare the samples, the strains were diluted at the desired concentrations in the above-mentioned combinations into Universal Transport Medium, aliquoted into individual test aliquots, and stored frozen at ≤ -70°C until the time of use. Panel A and Panel B composition is outlined in the table below.

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

Reproducibility testing was carried out at three sites. One site was Nanosphere, Inc, while the other two were external testing sites. 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 duplicate resulting in 24 tests per day.

The internal site running the 12-day precision study 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 each day for. The precision study was run for a total testing period of 12 non-consecutive days. The following table summarizes the cumulative data for RV+ across the three test sites. The Confidence Interval (CI) was calculated using the exact binomial method in SAS 9.1.3.

Clinical Reproducibility Summary

Site		Site 1	CV, %		Site 2	CV, %		Site 3	CV, %		All 3 Sites		
Specific Panel Member	Level	Agreement	Analyte	Value	Agreement	Analyte	Value	Agreement	Analyte	Value	Total Agreement	% Agreement	95% CI
INFA/H1-MT	MP	12/12	INFA	0.074%	12/12	INFA	0.073%	46/48[1,2]	INFA	0.132%	70/72	97.2%	90.4% - 99.2%
			H1	0.530%		H1	0.627%		H1	0.381%			
	LP	12/12	INFA	0.097%	12/12	INFA	0.120%	48/48	INFA	0.030%	72/72	100%	94.9% - 100%
			H1	0.542%		H1	1.292%		H1	0.271%			
	HN	11/12[3]	INFA	N/A	12/12	INFA	N/A	48/48	INFA	N/A	71/72	98.6%	92.5% - 99.8%
			H1	N/A		H1	N/A		H1	N/A			
INFA/H3	MP	12/12	INFA	0.151%	12/12	INFA	0.033%	48/48	INFA	0.067%	72/72	100%	94.9% - 100%
			H3	0.343%		H3	0.346%		H3	0.067%			
	LP	12/12	INFA	0.337%	12/12	INFA	0.965%	47/48[4]	INFA	0.005%	71/72	98.6%	92.5% - 99.8%
			H3	0.572%		H3	0.621%		H3	0.172%			
	HN	12/12	INFA	N/A	12/12	INFA	N/A	48/48	INFA	N/A	72/72	100%	94.9% - 100%
			H3	N/A		H3	N/A		H3	N/A			
INFA/2009H1N1-MT	MP	12/12	INFA	0.081%	12/12	INFA	0.035%	48/48	INFA	0.001%	72/72	100%	94.9% - 100%
			2009H1N1	0.547%		2009H1N1	0.050%		2009H1N1	0.001%			
	LP	12/12	INFA	0.136%	12/12	INFA	0.067%	48/48	INFA	0.013%	72/72	100%	94.9% - 100%
			2009H1N1	0.274%		2009H1N1	0.273%		2009H1N1	0.181%			
	HN	12/12	INFA	N/A	12/12	INFA	N/A	47/48[5]	INFA	N/A	71/72	98.6%	92.5% - 99.8%
			2009H1N1	N/A		2009H1N1	N/A		2009H1N1	N/A			
INFB	MP	12/12	INFB	0.149%	12/12	INFB	0.086%	48/48	INFB	0.002%	72/72	100%	94.9% - 100%
	LP	12/12		0.704%	12/12		0.066%	48/48		0.025%	72/72	100%	94.9% - 100%
	HN	12/12		N/A	11/12[a]		N/A	48/48		N/A	71/72	98.6%	92.5% - 99.8%
RSVA	MP	12/12	RSVA	0.422%	12/12	RSVA	0.245%	48/48	RSVA	0.192%	72/72	100%	94.9% - 100%
	LP	12/12		0.682%	12/12		0.500%	46/48[b,4]		0.176%	70/72	97.2%	90.4% - 99.2%
	HN	12/12		N/A	12/12		N/A	48/48		N/A	72/72	100%	94.9% - 100%
RSVB	MP	12/12	RSVB	0.127%	12/12	RSVB	0.087%	48/48	RSVB	0.002%	72/72	100%	94.9% - 100%
	LP	11/12[c]		0.163%	12/12		0.302%	47/48[d]		0.060%	70/72	97.2%	90.4% - 99.2%
	HN	11/12[e]		N/A	12/12		N/A	48/48		N/A	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 2009H1N1 which was not expected. [5]One INFA/2009H1N1-MT HN sample did not detect Influenza A and 2009H1N1 which was expected as this is a high negative sample. However, Influenza B and RSV B were also detected which was not expected. [6]One INFA/H3/RSVA LP sample detected Influenza A, H3 RSV A which was expected as this is a low positive. However, 2009H1N1 was 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.

b. Linearity/assay reportable range:
Not Applicable/ Qualitative Assay

c. *Traceability, Stability, Expected values (controls, calibrators, or methods):*

Blanks or no template control

Not required as the RV+ is performed in a single-test use disposables. Also, the RV+ employs a thermolabile UDG enzyme that eliminates the potential of amplicon cross-over and potential contamination between tests. Moreover, the RV+ is an end-point detection test and high background within the Test Cartridge owing to the amplification step (or related steps) results in a 'No Call – BKGD'.

Negative Sample Control

External Negative Control – In the RV+ is Universal Transport Media. The External Negative Control is the equivalent of the proposed Negative Sample Control in the Guidance document. Since the RV+ is an endpoint detection test, non-specific priming during the target amplification step, if any, does not interfere with the test since detection requires binding of specific captures and mediators. The expected calls after test analysis are 'Not Detected' for all the viruses/analytes. The frequency of running this test is dictated by the test site. It is recommended that the RV+ kit be tested upon receipt by running the external negative control to ensure proper performance of the kit.

Note that in the processing of the External Negative Control, RNA from IC2 gets extracted and is amplified along with IC1, which is present in the amplification mix. The inability to detect either IC1 or IC2 indicates issues with isolation or the target amplification within the particular test alone. The recourse is to isolate and amplify a fresh external negative control sample, just the same as if it were a sample. NC or Negative Control is a set of non-target oligonucleotide spots that serve as negative controls and are used to normalize signal intensities at target spots (Ratio-to-NC)

Positive control for complete assay

External positive controls – In the RV+, 'positive control for the complete assay' are

suggested to be inactivated intact viral particles with altered surface proteins making them unable to infect a host cell. Obtained from Zeptometrix Corporation, these viral particles, referred to as NATtrol, possess viral integrity and an intact viral genome. Thus, in the RV+, the NATtrol serves as a control for the extraction, the target amplification, and detection steps.

Positive control for amplification/detection

IC1 serves as the internal inhibition control or 'positive control for amplification/detection'. It is a 99-mer duplex DNA synthetic construct present in the primer mix and is amplified along with every test sample (along with potential targets and IC2). It independently controls for inhibitors to the PCR step in each test. The presence of IC1 in the analysis indicates that the reaction components performed as expected. PC or Imaging control is a set of oligonucleotide spots on the substrate that act as a quality check for proper fluid control and movement between the Test Cartridge and the instrument. If the imaging controls are absent, then a "No Call – No Grid" is an output. The imaging control signal is due to signal probe hybridization to an oligonucleotide on the substrate. Signal will only be present if the probe reagent and the signal enhancement reagents are pumped into and held in the hybridization chamber for the specified time period. The PC also serves to normalize signal intensities at target spots (Ratio-to-PC).

Internal Control

IC2 (Escherichia coli MS2 bacteriophage) serves as an internal process control in the RV+. The IC2 has an intact viral genome in a viral capsid and therefore controls for the sample isolation or the nucleic acid extraction step and the target amplification or the RT-PCR step. In the context of the RV+, IC2 is added to every test sample and is co-extracted with the sample. The extracted RNA undergoes RT-PCR and controls for inhibitors within the test sample.

NOTE: 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.

The following table summarizes the controls found in the RV+ with a description of the composition of the control as well as the function.

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.

d. Detection limit:

The analytical sensitivity of RV+ was assessed and confirmed by using virus strains with established titers to represent all analytes in the test. The strains were previously cultured and titered (see the table below for stock titers). In order to determine the Limits of Detections (LODs), the strains were serially diluted into negative pools of nasopharyngeal (NP) swab samples. Each concentration level in the dilution series was tested in the RV+ in triplicate to establish an estimated LOD.

For confirmation, 20 replicate samples were prepared at the estimated LOD level chosen from the dilution series experiment and tested in the RV+. Acceptance criteria for LOD confirmation required that at least 19 of the 20 samples tested gave a 'Detected' call for the analyte. The confirmed LOD levels results for the different analytes and the associated virus strains are presented in the table below.

VIRUS	ANALYTE	VIRAL STRAINS – ANALYTICAL STUDIES	LOD ($TCID_{50}$/mL)	20 REPS
INFA	INFA	Influenza A/Virginia/01/2006 (H1N1)	1	19/20
		Influenza A/2009H1N1 Clinical Isolate (H1N1)	10	20/20
		Influenza A/Brisbane/59/2007 Clinical Isolate (H1N1)	5	20/20
		Influenza A/California/04/2009 (H1N1)	1	20/20
		Influenza A/Wisconsin/67/05 (H3N2)	0.1	20/20
	H1	Influenza A/Virginia/01/2006 (H1N1)	5	20/20
		Influenza A/Brisbane/59/2007 Clinical Isolate (H1N1)	5	19/20
	2009H1N1	Influenza A/2009H1N1 Clinical Isolate (H1N1)	10	19/20

		Influenza A/California/04/2009 (H1N1)	1	20/20
	H3	Influenza A/Wisconsin/67/05 (H3N2)	0.1	20/20
INFB	INFB	Influenza B/Wisconsin/2/2006	0.1	20/20
		Influenza B/Florida/02/06	1	20/20
RSVA	RSVA	RSV A Strain A2	10	20/20
RSVB	RSVB	RSV B Strain Wash/18537/62	1	20/20

e. Analytical specificity:

Analytical specificity studies were performed to assess potential cross-reactivity of the RV+ with common respiratory pathogens and other microorganisms commonly present in the respiratory tract. In the cross-reactivity studies, 23 bacterial strains were tested at concentrations between 10^5 to10^7 cfu/mL while 29 virus strains were tested at concentrations between 10^4 to10^6 $TCID_{50}$/mL. The bacterial strains were obtained from ATCC, re-grown and their titers confirmed. The virus strains were obtained from several sources including ATCC, Zeptometrix, Intelligent MDx, and Tricore. All virus strains were re-grown and their titers were confirmed. The microorganisms were diluted into a sample matrix, Universal Transport Medium (Copan), at the concentrations indicated and tested subsequently in the RV+.

In the RV+, IC1 and IC2 are verified before providing a valid call. IC1 is an inhibition control for the Target Amplification step and IC2 is a process control (bacteriophage, MS2) that is added to the sample automatically prior to the extraction step. The IC2 goes through all steps of RV+: Sample Extraction, Target Amplification, and Verigene Hybridization Test. 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+.

RV+ did not cross-react with any of the microorganisms chosen to represent pathogens residing in the respiratory tract. The table below details the test results from the study.

Viruses	Strain	pfu/mL	INF A	H1	H3	2009 H1N1	INF B	RSV A	RSV B
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	$2.2x10^9$ copies/m	-	-	-	-	-	-	-
Herpes Simplex virus Type 1	MacIntyre	$2.4x10^5$	-	-	-	-	-	-	-
Measles	Edmonst-on	$1.1x10^6$	-	-	-	-	-	-	-
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**	**INF A**	**H1**	**H3**	**2009 H1N1**	**INF B**	**RSV A**	**RSV B**
Acinetobacter Baumannii		$3.0x10^5$	-	-	-	-	-	-	-
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. Analytical Reactivity:

The analytical reactivity of the RV+ was evaluated using multiple strains of Influenza A (8 seasonal H1 strains, 7 seasonal H3 strains, 4 2009 H1N1 strains, 2 swine origin H1N1 strains, 1 H5N1 strain, and 1 H7N7 strain collectively representing temporal and geographical diversity), 9 Influenza B strains, 2 RSV A strains and 3 RSV B strains.

In order to demonstrate 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 Media (Copan), to a nominal concentration of 100 $TCID_{50}$/mL (or $CEID_{50}$/mL). Each strain dilution was tested in triplicate in the RV+. Most strains were reactive at 100 TCID50/mL; however, concentration levels for a few strains were increased in order for all three replicate tests to yield 'Detected'. All the Influenza A/H3, Influenza A/H1, and Influenza A/2009 H1N1 strains yielded expected subtyping decisions.

The two swine H1N1 strains of non-human origin gave a 'Detected' result for Influenza A and for H1 at 100 CEID50/mL and gave a 'Not Detected' result for 2009 H1N1. Higher concentrations of these strains ($1x10^3$ $CEID_{50}$/mL and $1x10^4$ $CEID_{50}$/mL) were also tested to assess cross-reactivity at 2009 H1N1 but none was found as evidenced by a 'Not Detected' call for 2009 H1N1.

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 untypeable in the RV+.

In summary, the RV+ demonstrated good reactivity against a range of panel viruses. Against, Influenza viruses H5 and H7, RV+ gave a 'Detected' call for Influenza A but these viruses did not cross-react with any of the subtyping systems even at very high concentrations demonstrating high assay specificity. Similarly, the two swine-origin H1N1 strains gave 'Detected' calls for Influenza A and HI only; no cross-reactivity was observed even at very high titers. Finally, no cross-reactivity was observed within the remaining panel viruses.

Virus Strains	**Titer ($TCID_{50}$/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)	$1X10^3$	+	+	-	-	-	-	-
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)	1X102	+	+	-	-	-	-	-
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) **	$2.3X10^2$	+	-	-	-	-	-	-
Influenza A/Netherlands/219/2003 (H7N7) **	$1.1X10^4$	+	-	-	-	-	-	-

Influenza B	Titer ($TCID_{50}$/mL)	INFA	H1	H3	2009 H1N1	INFB	RSVA	RSVB
Influenza B/ Wisconsin/2/2006	1X102	-	-	-	-	+	-	-
Influenza B/ Florida/02/2006	1X102	-	-	-	-	+	-	-
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	$1X10^2$	-	-	-	-	+	-	-
Influenza B/Florida/02/2006	$1X10^2$	-	-	-	-	+	-	-
RSV A	**Titer ($TCID_{50}$/mL)**	**INFA**	**H1**	**H3**	**2009 H1N1**	**INFB**	**RSVA**	**RSVB**
RSV A Strain A2	$1X10^2$	-	-	-	-	-	+	-
RSV A Strain Long	$1X10^2$	-	-	-	-	-	+	-
RSV B	**Titer ($TCID_{50}$/mL)**	**INFA**	**H1**	**H3**	**2009 H1N1**	**INFB**	**RSVA**	**RSVB**
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 $1x10^4$ $TCID_{50}$/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.

g. Assay cut-off:

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.

Image analysis of the Test Substrate provides image intensities from target-specific capture spots. Two Internal Controls guide decisions regarding the validity of the test. The Internal Controls, IC1 – inhibition control and IC2 – the process control are also represented by unique captures and are treated as targets and their presence is verified before a valid result is provided.

In order to provide a 'Detected' and 'Not Detected' result in RV+, three conditions or filters are examined. These conditions serve as a single-set of clinical cut-off criteria.

- Filter 1: Signal > Noise Threshold
- Filter 2: Normalized 'Ratio to Negative Control' > 0.85
- Filter 3: Normalized 'Ratio to Positive Control' ≥ 0.4
- A True Positive (or a False Positive) requires that all three filter conditions are met.
- A True Negative (or a False Negative) is provided if any or all the filter conditions are not met.

For a result, the decision tree verifies the presence of IC1 and IC2 in conjunction with each of the viruses. 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.

Cut-off verification

A set of retrospective clinical samples (71) positive by DFA/Culture for either Influenza A, Influenza B, or RSV was tested in the RV+ to verify the cut offs.

The plots of the logistic fit of Expected Results by Ratio-to-PC and Ratio-to-NC for these retrospective samples was provided for this data set along with the predictive value of the algorithm. Decisions were made based on the three-filter method by using the cut-off limits for Ratio-to-PC and Ratio-to-NC determined in the above study. The results from the RV+ showed perfect agreement with the decisions obtained by culture/DFA for Influenza A, Influenza B, and RSV. Moreover, the Influenza A subtype decisions (H1, H3, and 2009 H1N1) and RSV A and RSV B decisions made by RV+ were all justified by sequencing. Overall, the three-filter method provided excellent predictive power. The cut-offs determined in these studies were used throughout the analytical studies, the reproducibility and precision studies, and in the methods comparison studies.

h. Fresh vs. Frozen equivalency:

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 RVNAT+ 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 RVNAT+. 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 RVNAT+ (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 RVNAT+ (see Table 13 & 14)). The negative NP swab samples were not pooled; each virus was represented by 10 unique NP swab samples.

Each unique sample had sufficient volume to allow ~4 tests. Thus, each sample was tested four (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-containing tube was frozen by placing it at or below -70 °C. For the first freeze thaw, the sample-containing tube was thawed and an aliquot was removed and tested in the RV+. This activity constituted the first 'freeze-thaw' cycle, and the sample-containing tube was placed again at or below -70 °C. The 'freeze-thaw' process was repeated for the second and third 'freeze-thaw' cycles.

The performance of each virus/analyte after each freeze-thaw cycle was compared to the corresponding results obtained when the sample was 'fresh' or unfrozen. Of the 240 tests, 236 tests gave valid results (98.3%). The results for all the viruses/analytes within this set of valid results were in perfect agreement between the paired 'fresh' and 'frozen' samples at each of the three 'freeze-thaw' cycles. Overall, the results showed that the RV+ detected the viruses/analytes correctly even after the sample was subjected to three sets of 'freeze-thaw' cycles. There was no trend of decreased performance with the number of 'freeze-thaw' cycles.

i. Competitive Inhibition Studies:

Competitive inhibition or interference in the RV+ was assessed in clinically relevant co-infections. By definition, competitive inhibition or interference in a co-infection setting refers to the potential ability of the virus present at a high concentration to inhibit efficient target amplification.

Considering the RV+ is a multiplexed test designed to detect multiple viruses simultaneously, combinations of Influenza A viruses and RSV A/B viruses were identified as 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. 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	$TCID_{50}$/mL	Virus	$TCID_{50}$/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

The samples were created by diluting stocks for each virus strain with verified titers into a sample matrix (Universal Transport Media, Copan). Each binary combination was tested in triplicate in the RV+. The expected calls were compared to the Verigene Calls to assess inhibition. All the calls for the low titer sample and the high titer sample would have to be 'Detected' in a sample for there to be no competitive inhibition.

In the RV+, IC1 and IC2 are verified before providing a valid call. IC1 is an inhibition control for the Target Amplification step and IC2 is a Process Control (bacteriophage, MS2) that is added to the sample. The IC2 goes through all steps of RV+: Sample Extraction, Target Amplification, and the Verigene Hybridization Test. Both IC1 and IC2 undergo Target Amplification along with the targets in the sample and may be prone to competitive inhibition effects similar to the low titer sample. In all the samples tested, a valid test result was provided indicating that the presence of the high titer target did not inhibit the internal controls during the different steps of the RV+. Moreover, all the replicates within the binary combinations gave expected calls for both viruses in each sample. The presence of the higher titer viruses did not inhibit detection of the lower titer virus at the levels tested. Thus, in the binary combinations tested in these studies there was no evidence of competitive inhibition.

j. Carry-Over / Cross-Contamination Study

A 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 Processor *SP* units were used in the study and each Processor *SP* was subjected to a comprehensive set of tests.

Viruses and virus subtypes, as well as specific virus strains used in the study are presented in the table below. The concentrations associated with the High Positive (HP) and High Negative (HN) for the virus strains are also listed. A total of six (6) unique strains, representing all the analytes in the RV+, were tested at High Positive and High Negative concentrations.

Viruses used for Carry-over/Cross-Contamination Study

Virus Type	Virus Strain	Level	$TCID_{50}$/mL
INFA/H3	Influenza A/Wisconsin/67/05	High Positive	200
		High Negative	0.001
INFA/H1	Influenza A /Virginia/01/2006	High Positive	2000
		High Negative	0.01
INFA/2009 H1N1	Influenza A/California/04/2009	High Positive	2000
		High Negative	0.01
INFB	Influenza B/Wisconsin/02/2006	High Positive	500
		High Negative	0.001
RSVA	RSV A/Strain A2	High Positive	5000

		High Negative	0.01
RSVB	RSV B/Washington/18537/62	High Positive	1000
		High Negative	0.001

The samples were divided into two sets as described. Each set comprised a total of six (6) unique alternating High Positive and High Negative samples. The samples within each set were tested sequentially in the order described. Each set was tested in triplicate on separate Processor *SP*s (2 Sets x 3 Processor *SP*s = 6 Processor *SP*s. Thus, a total of 36 samples [6 strains x 2 (HP+HN) x 3 replicates] were tested across 6 Processor *SP*s over 6 sequential test runs. The 6 test runs were conducted over a 3 day period.

Considering each High Positive test was followed by a High Negative test of a different viral type, 'Observed Results' that differed from 'Expected Results' for the High Negative test would be considered as carry over/cross-contamination. The only exception to this would be if a High Negative sample gave a positive result for that particular High Negative viral strain as by definition, a High Negative sample should be detected < 5%. Regardless, this would be distinguished easily from the previous viral type because of the order of testing.

Decisions regarding the presence or absence of a virus were based on the 'Calls' ('Detected' or 'Not Detected') displayed by the Verigene® Reader. Carry over/cross-contamination would be a probable cause if the Verigene® System detected a virus other than or in addition to the one that was intended in the sample.

All High Positive samples yielded the expected 'Detected' results for the intended virus and 'Not Detected' results for the other viruses or analytes. All but one of the High Negative samples yielded a 'Not Detected' for the intended virus as well as all other viruses. One Influenza B high negative sample gave a 'Detected' call for Influenza B. High Negative samples are expected to be positive < 5% of the time; this result was expected. Moreover, the High Positive preceding this sample was Influenza A 2009H1N1 confirming that the Influenza B result was not due to sample carry over. Overall, the studies confirmed that there was no evidence of carry over/cross-contamination from the High Positive samples, or any other internal or external sources, in any of the three steps of the RV+: Sample Extraction, Target Amplification, and Verigene Test.

k. Interfering Substances Studies

The potential inhibitory effects of interfering substances or interferents that may be encountered in nasopharyngeal specimens on the RV+ was assessed. A set of 22 interferents and two types of influenza vaccines were identified for a total of 24 potential interferents.

All the interferents (except the vaccines) were dissolved into a sample matrix, Universal Transport Medium (Copan), to make intermediate stocks. The concentration of the interferent was based on the maximum dosage and the interferent's solubility in the sample matrix. Next, each interferent was spiked into samples containing different combinations of viral strains. A set of four unique virus combinations were chosen to represent all the analytes in the RV+. The different target combinations and interferents amounted to 88 unique samples (22 interferents x 4 unique samples). The titers chosen

for each virus strain were near their limits of detection. Samples containing viruses and the interferents were then processed through the RV+. The vaccines were tested as a dilution series.

In the RV+, Internal Control (IC1) and Internal Control 2 (IC2) are verified before providing a valid call. IC1 is an inhibition control for the Target Amplification step and IC2 is a process control (bacteriophage, MS2) that is added to the sample automatically prior to the extraction step. The IC2 goes through all steps of RV+: Sample Extraction, Target Amplification, and the Verigene Hybridization Test. In all the samples tested, a valid test result was provided indicating that the interferents did not pose 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 containing the interferents.

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
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 ~5x106 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 ~1x107 dilution.*

2. Comparison studies:

   a. Method comparison with predicate device:
   Not applicable, performance of the assay was evaluated in comparison to the gold standard/reference method, viral culture followed by DFA and/or viral culture followed by sequencing

   b. Matrix comparison: Not Applicable.

3. Clinical studies:

   a. Clinical Sensitivity and Specificity:
   The clinical performance of the RV+ assays was established through a study conducted at three external clinical sites: TriCore Reference Laboratory, Albuquerque, NM (TC); Midwest Research Institute, Palm Bay, FL (MRI); and Medical College of Wisconsin, Milwaukee, WI (MCW). Across the three sites, there were one Verigene Reader and 12 Verigene Processor SPs utilized per site for testing during the study. The testing for this study was performed using multiple lots (at least 4 lots at each site) of Test Cartridges, Extraction Trays and Amplification Trays at each site. Verigene Processor SPs and Verigene Readers used in these studies were standard production instruments. The software revisions were validated for intended use according to documented test protocols and developed according to Standard Operating Procedures.

   Clinical nasopharyngeal specimens for the clinical study were collected prospectively at three collection laboratory sites during the 2008-2009 and 2009-2010 respiratory seasons. The three collection sites were TriCore Reference Laboratories (TC), Esoterix/LabCorp (ES) and University of North Carolina (UNC). Two of the sites, Esoterix and UNC collected specimens in universal transport medium (UTM) and one site, TriCore, collected specimens in M5 medium.

   Culture-based/DFA confirmation was performed on these prospectively-collected specimens while still fresh and in an unfrozen state as part of normal laboratory practice. Frozen specimens were only used when confirmation was performed by using nucleic acid amplification tests (NAAT). Each site then saved the residual from these specimens, which were de-identified on-site, frozen at -70°C, and shipped on dry ice to Nanosphere. At Nanosphere, the specimens were stored at or below -70 °C.

   Prior to distribution to the test sites, the specimens were randomized by using the random generator function in Microsoft Excel (2007). Each specimen was blinded and labeled with a Nanosphere-generated unique identifier while removing the collection site identifier. All specimen culture/DFA were kept blinded to all study personnel until RV+ testing was completed. The specimens were shipped to the testing sites overnight on dry ice. The sites stored the specimens at -70°C before and after testing.

A total of 1022 specimens were tested at the three study sites (TC—314; MRI—385; MCW— 323). All tests were performed by laboratory personnel at the three sites using the RV+ on the Verigene System. Multiple lots of Amplification Trays, Extraction Trays and Test Cartridges were used at each site. The Influenza A, Influenza B and RSV results in the methods comparison study were compared to results obtained for the specimens using culture-based methods confirmed with a FDA-cleared DFA test Influenza A subtyping and RSV subtyping results were compared to results obtained for the specimens utilizing bi-directional sequencing. Discordant results were resolved utilizing bi-directional sequencing or an acceptable NAAT–based comparator. Bi-directional sequencing was performed at an independent laboratory (ACGT; Wheeling, IL) and footnoted in the performance comparison tables.

Per protocol, if a test resulted in a "No Call" or a "pre-analysis error" ("pre-ae"), the specimen could be repeat tested. There were 34 "No Calls" and 25 "pre-aes" in the study. Of these, 57 of the 59 specimens were repeat tested successfully. The other two "No Call" specimens repeated as "No Calls". Thus, in this study the total "No-Call" rate was 3.3% (34/1022) and the "pre-ae" failure rate was 2.4% (25/1022). The "pre-ae", by definition, is any test that did not go to completion regardless of the cause. A "pre-ae" may occur due to a number of causes including a user- initiated procedure termination (for example, upon realizing that an incorrect specimen was loaded) or an instrument-initiated procedure termination (for example, because internal checks detect an unexpected event), or due to an instrument failure, or non-availability of the Test Substrate for analysis (due to test consumable breakage, etc).

Combined results for the methods comparison study across the three sites are presented and reflect the results after all "no call" results were repeated per the protocol and the proposed package insert guidance. Results from the RV+ were initially compared to results from culture-based DFA methods. An FDA cleared NAAT was utilized to resolve seven discrepant results between the RV+ and culture/DFA which had failed sequencing. Bi-directional sequencing was used to subtype Influenza A, differentiate RSV A and RSV B and for discordant testing. The tables below show results for Influenza A, Influenza B, and RSV compared to culture/DFA with discordant resolution footnoted. For subtyping results for Influenza A and RSV specimens, RV+ results are compared to a composite comparator result obtained from culture/DFA followed by bi-directional sequencing. Percent positive and negative agreement for each table are provided as well as the lower and upper two-sided 95% confidence limits that were calculated using the exact binomial methods in SAS 9.1.3 from: http://172.16.20.9/stat.html

The Influenza A combined results demonstrate strong results for the RV+ assay with a Sensitivity of 98.7% (96.8-99.5%) and Specificity of 93.2% (91.1-94.8%). The lower value for Percent Negative Agreement is due to a higher number of "false positives (FPs)" that are apparent when the RV+ result is compared to culture/DFA/NAAT. Almost all of these (FPs) are considered TPs (true positives) when compared to bi-directional sequencing.

All 1022 patient nasopharyngeal swab specimens are accounted for in the combined tables for Influenza A, Influenza B, and RSV. However, a small number of Influenza A specimens that failed sequencing or did not produce a subtype result could not be placed in the subtyping tables. For the Influenza A subtypes, 5 out of 1022 results (0.49%) were

either not subtyped by the RV+ or failed sequencing (i.e., results for only 1017 specimens in the Influenza A subtype tables). Influenza B did not require sub-typing and, therefore, the table contains all 1022 specimen results. Three specimens failed RSV subtype sequencing and could not be included in the RSV subtyping tables (i.e., results for only 1019 specimens in the RSV subtype tables).

Influenza A Combined Results

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]	

[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. By culture/DFA the specimen was Flu A positive. By sequencing the specimen was positive for Flu B and negative for Flu A. By NAAT 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 culture/DFA, the specimen was Flu A positive. By sequencing the specimen was positive for RSV B and negative for Flu A. By NAAT 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 and are not included in the Subtyping Tables.

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	

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	

[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]	

[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. [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.

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	

[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 0300 was positive for both RSV A and RSV B.

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	

[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 0300 was positive for both RSV A and RSV B.

4. Clinical cut-off**:**

   Not Applicable

5. Expected values/Reference range:

While the timing and duration of flu 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)]. During the 2006 2007 and 2007- 2008 flu seasons, the prevalence was 13% of 179,268 samples tested and 18% of 225,329 samples tested, respectively.

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

In the Verigene Respiratory Virus Plus Nucleic Acid Test 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, however, one specimen was detected by the Verigene Respiratory Virus Plus Nucleic Acid Test to have both RSV A and RSV B. It is recommended that the samples undergo repeat testing if nucleic acids from all three analytes are detected in a single sample.

N. Instrument Name:

Verigene System

O. System Descriptions:

1. Modes of Operation:

   The RV+ on the Verigene platform functions as a sample-to-answer system in which all sample processing steps as well as detections is carried out on a single use, disposable test cartridge.

2. Software:

   FDA has reviewed applicant's Hazard Analysis and software development processes for this line of product types:

   Yes __X__ or No _____

3. Specimen Identification:
   Each test cartridge handles one sample and has a unique barcode identifier that is scanned into the Verigene system. Upon scanning the user is prompted to enter sample information via barcode-reader or touch-screen interface thus linking patient information to test cartridge. The cartridge and consumables are loaded onto the Verigene system followed by the patient sample into the designated sample well and the test is initiated.

4. Specimen Sampling and Handling:
   The steps automated within the Processor SP use test reagents that are self contained in the test cartridge and include:
   1. Sample Preparation/Extraction – Magnetic bead-based viral RNA isolation from nasopharyngeal swab specimens
   2. Target Amplification – Multiplex RT-PCR-based amplification of the eluted viral RNA to generate virus-specific amplicons
   3. Verigene Hybridization Test – Gold nanoparticle probe-based detection of the virus-specific amplicons on a microarray

   Upon completion of Specimen processing the cartridge is removed form the Verigene processor and placed into the Verigene Reader for analysis

5. Calibration:

Not required. Test cartridges are single use and part of a closed system.

6. Quality Control:
   A series of internal controls used in conjunction with procedural checks monitors instrument functionality, performance, fluidics, reagent integrity, and result determination based on a pre-defined decision algorithm.

P. Other Supportive Instrument Performance Characteristics Data Not Covered In the "Performance Characteristics" Section above:

None

Q. Proposed Labeling:

The labeling is sufficient and it satisfies the requirements of 21 CFR Part 809.10.

R. Conclusion:

The submitted information in this premarket notification is complete and supports a substantial equivalence decision.