

510(k) SUBSTANTIAL EQUIVALENCE DETERMINATION DECISION SUMMARY

A. 510(k) Number:

K101855

B. Purpose for Submission:

This is a new 510k application for a qualitative real-time reverse transcription-polymerase chain reaction (RT-PCR) assay used with the Cepheid SmartCycler II Real Time Instrument with Dx Software version 1.7b or 3.0a/b for the qualitative detection and discrimination of seasonal Influenza A/H1, seasonal Influenza A/H3 and 2009 H1N1 Influenza viral nucleic acids isolated and purified from nasopharyngeal (NP) swab specimens from human patients with signs and symptoms of respiratory infection. The isolation and purification of the nucleic acids is performed using either a MagNA Pure LC Instrument (Roche) and the MagNA Pure Total Nucleic Acid Isolation Kit (Roche) or a NucliSENS® easyMAG™ System (bioMérieux) and the Automated Magnetic Extraction Reagents (bioMérieux).

C. Measurand:

Target RNA sequences for the respective conserved regions of the Hemagglutinin (HA) gene for seasonal influenza A/H1, seasonal influenza A/H3 and 2009 H1N1 Influenza Virus (swine-origin), and for the transcript derived from *E. coli* Bacteriophage MS2 A-protein gene (Internal Control).

D. Type of Test:

Real-time reverse transcription-polymerase chain reaction (RT-PCR), qualitative detection and discrimination of seasonal Influenza A/H1, seasonal Influenza A/H3 and 2009 H1N1 Influenza in nasopharyngeal swabs using nucleic acid isolation (The isolation and purification of the nucleic acids is performed using either a MagNA Pure LC Instrument (Roche) and the MagNA Pure Total Nucleic Acid Isolation Kit (Roche) or a NucliSENS® easyMAG™ System (bioMérieux) and the Automated Magnetic Extraction Reagents (bioMérieux)), amplification and detection on the Cepheid SmartCycler II Real Time Instrument with Dx Software version 1.7b or 3.0a/b, which generates signals based on the acquisition of spectrofluorometric data.

E. Applicant:

Gen-Probe Prodesse Incorporated

F. Proprietary and Established Names:

Prodesse ProFAST+ Assay

G. Regulatory Information:

1. Regulation section:

866.3332

2. Classification:

Class II

3. Product code:

OQW

4. Panel:

Microbiology (83)

H. Intended Use:

1. Intended use(s):

The 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 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. This assay targets conserved regions of the Hemagglutinin (HA) gene for seasonal Influenza A/H1, seasonal Influenza A/H3 and 2009 H1N1 Influenza Virus, respectively. This assay is not intended to detect Influenza B or Influenza C Viruses.

A negative ProFAST+ Assay result is a presumptive negative result for Influenza A. These results should be confirmed by an FDA cleared nucleic acid-based test (NAT) detecting Influenza A.

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

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.

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:

To be used with the Cepheid SmartCycler II Real Time Instrument with Dx Software version 1.7b or 3.0a/b and either a MagNA Pure LC Instrument (Roche) and the MagNA Pure Total Nucleic Acid Isolation Kit (Roche) or a NucliSENS® easyMAG™ System (bioMérieux) and the Automated Magnetic Extraction Reagents (bioMérieux).

I. Device Description:

The ProFAST+ Assay is a multiplex Taqman based Real Time RT-PCR test that enables detection and differentiation of human Influenza A Virus subtypes: seasonal H1, seasonal H3, and 2009 H1N1 Influenza.

An overview of the procedure is as follows:

1. Collect NP swab specimens from symptomatic patients using a polyester, rayon or nylon tipped swab and place into viral transport medium (not provided with the kit).
2. Add an Internal Control (IC) to every sample to monitor for inhibitors present in the specimens.
3. Perform isolation and purification of nucleic acids using a MagNA Pure LC Instrument (Roche) and the MagNA Pure Total Nucleic Acid Isolation Kit (Roche), or a NucliSENS® easyMAG™ System (bioMérieux) and the Automated Magnetic Extraction Reagents (bioMérieux),
4. Add purified nucleic acids to the supermix along with enzymes included in the ProFAST+ kit. The supermix contains target-specific oligonucleotide primers and probes. The probes are dual-labeled with a reporter dye and a quencher dye (see table below).
5. Perform reverse transcription of RNA into complementary DNA (cDNA) and subsequent amplification of DNA in a Cepheid SmartCycler® II instrument. In this process, the probe anneals specifically to the template followed by primer extension and amplification. The ProFAST+ Assay is based on Taqman chemistry, which utilizes the 5' – 3' exonuclease activity of the Taq polymerase to cleave the probe thus separating the reporter dye from the quencher. This generates an increase in fluorescent signal upon excitation from a light source. With each cycle, additional reporter dye molecules are cleaved from their respective probes, further increasing fluorescent signal. The amount of fluorescence at any given cycle is dependent on the amount of amplification products present at that time. Fluorescent intensity is monitored during each PCR cycle by the real-time instrument.

ProFAST+ Assay Analyte Gene Targets and Probe Labels:

Analyte	Gene Targeted	Probe Fluorophore	Absorbance Peak	Emission Peak
Seasonal Influenza A/H1	Hemagglutinin	FAM	495 nm	520 nm
Seasonal Influenza A/H3	Hemagglutinin	Cal Orange 560	540 nm	561 nm
2009 H1N1 Influenza Virus	Hemagglutinin	Cal Red 610	595 nm	615 nm
Internal Control	<i>E.coli</i> MS2 Phage	Quasar 670	647 nm	667 nm

Materials Provided

ProFAST+ Assay Kit (Cat. # H34VK00)

Reagents	Description	Quantity/ Tube	Cap Color	Cat. #	Reactions/ Tube
ProFAST+ Supermix	- Taq DNA polymerase - Oligonucleotide primers + probes - Buffer containing dNTPs - MgCl₂ and stabilizers	515 µL	Brown	HSM34	25 (4 tubes provided)
M-MLV Reverse Transcriptase II	10 U/µL	36 µL	Red	GLS26	100
RNase Inhibitor II	40 U/µL	120 µL	Green	GLS33	100
Influenza A Subtyping RNA Control II (Positive Control)	Non-infectious <i>in vitro</i> transcribed RNA of specific viral sequences	300 µL	Clear	HCT34	15
Internal RNA Control III	Non-infectious <i>in vitro</i> transcribed RNA	30 µL	Yellow	GCT12	100

Materials Required But Not Provided

Plasticware and consumables

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

Reagents

- Roche MagNA Pure LC Total Nucleic Acid Isolation Kit (*Roche Cat. No. 03038505001*) for 192 isolations or bioMérieux NucliSENS easyMAG reagents (*Buffer 1 Cat. No. 280130, Buffer 2 Cat. No. 280131, Buffer 3 Cat. No. 280132, Magnetic Silica Cat. No. 280133, and Lysis Buffer Cat. No. 280134*)
- Micro Test[™] M4[®] Viral Transport Medium (*Remel, Inc. Cat. No. R12500*), Micro Test[™] M5[®] Viral Transport Medium (*Remel, Inc. Cat. No. R12515*), Micro Test[™]

M6[®] Viral Transport Medium (*Remel, Inc. Cat. No. R12530*), Micro Test[™] M4RT[®] Viral Transport Medium (*Remel, Inc. Cat. No. R12505*), Copan Universal Transport Medium (*Copan Cat. No. 330C*), or BD Universal Viral Transport vial, 3mL (*Becton, Dickinson and Co. Cat. # 220220*)

- Molecular Grade Water (*RNase/DNase Free*)
- Extraction Control (recommended, e.g. previously characterized positive sample or negative sample spiked with a well characterized seasonal Influenza A/H1, A/H3 or 2009 H1N1 Influenza Virus strain)

Equipment

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

Interpretation of Sample Results

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

Sample ID ¹	Assay Result	IC Result	Warning / Error Code	Seasonal A/H1 Result	Seasonal A/H3 Result	2009 H1N1 Result	Interpretation of Results
Sample ID	Negative	Pass	None	NEG	NEG	NEG	Seasonal Influenza A/ H1, Seasonal Influenza A/H3 and 2009 H1N1 Influenza RNA not detected . A negative ProFAST+ Assay result is a presumptive negative result for Influenza A. These results should be confirmed by an FDA cleared Influenza A NAT assay.
Sample ID	Positive	NA*	None	POS	NEG	NEG	Seasonal Influenza A/ H1 RNA detected
Sample ID	Positive	NA*	None	NEG	POS	NEG	Seasonal Influenza A/ H3 RNA detected
Sample ID	Positive	NA*	None	NEG	NEG	POS	2009 H1N1 Influenza RNA detected
Sample ID	Unresolved	Fail	None	NEG	NEG	NEG	Unresolved – PCR inhibition or reagent failure. Repeat testing from the purified nucleic acid,

							re-test from original sample, or collect and test a new sample.
Sample ID	Positive	NA*	None	POS	NEG	POS	Seasonal Influenza A/H1 and 2009 H1N1 Influenza RNAs detected . Multiple infections are rare. Repeat testing from the purified nucleic acid or re-test from original sample, or collect and test a new sample.
Sample ID	Positive	NA*	None	POS	POS	NEG	Seasonal Influenza A/H1 and A/H3 RNAs detected . Multiple infections are rare. Repeat testing from the purified nucleic acid, re-test from original sample, or collect and test a new sample.
Sample ID	Positive	NA*	None	NEG	POS	POS	Seasonal Influenza A/H3 and 2009 H1N1 Influenza RNAs detected . Multiple infections are rare. Repeat testing from the purified nucleic acid, re-test from original sample, or collect and test a new sample.
Sample ID	Positive	NA*	None	POS	POS	POS	Seasonal Influenza A/H1, A/H3 and 2009 H1N1 Influenza RNAs detected . Multiple infections are rare. Repeat testing from the purified nucleic acid, re-test from original sample, or collect and test a new sample.
Sample ID	Invalid		4098 ²	ND	ND	ND	Not Determined – error code 4098

¹ Columns and data not used for interpretation are not included

² An Invalid Assay run will display Error code 4098

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

J. Substantial Equivalence Information:

1. Predicate device name(s):

- xTag RVP (Respiratory Virus Panel), Luminex Molecular Diagnostics, Inc.
- FOCUS Simplexa Influenza A H1N1 (2009)
- CDC Human Influenza Virus Real Time RT-PCR Detection and Characterization Panel

2. Predicate K number(s):

(K063765, K081483, K091667), (K100148), (K080570)

3. Comparison with predicates:

Features	ProFAST+ Assay	Focus Simplexa Influenza A H1N1 (2009)	CDC Human Influenza Virus Real Time RT-PCR Detection and Characterization Panel	Luminex xTAG RVP
510(k)	K101855	K100148	K080570	K063765, K081483, K091667
Regulation	866.3332	866.3332	866.3980	866.3980
Product Code	OQW	OQW	NXD, OEP, OCC, NSU	OCC, OEM, OEP
Device Class	Class II	Class II	Class II	Class II
Intended Use	Differential qualitative detection of influenza A virus types seasonal H1, seasonal H3, and 2009 H1N1 Influenza viral nucleic acids.	For the <i>in vitro</i> qualitative detection and differentiation of influenza A and 2009 H1N1 Influenza viral RNA.	Qualitative <i>in-vitro</i> detection of influenza A/H1, A/H3, A/H5 (Asian lineage), and influenza B viral RNA.	Direct and differential qualitative detection of influenza types A and B, RSV types A and B, Parainfluenza types 1, 2 and 3, Adenovirus and Rhinovirus viral nucleic acids.
Technology/Detection	Real Time RT-PCR Detection	Real Time RT-PCR Detection	Real Time RT-PCR Detection	RT-PCR Detection: Amplified products are coupled to microspheres and detected using spectrofluorometric analysis.
Specimen Types	NP swabs	Nasopharyngeal swabs (NPS), nasal swabs (NS), and nasopharyngeal aspirates (NPA)	nasopharyngeal and/or nasal swab specimens	NP swabs
Nucleic Acid Isolation	Roche MagNA Pure LC System and bioMerieux NucliSENS easyMAG	Roche MagNA Pure LC System or Qiagen QIAamp® Viral RNA Mini Kit	Qiagen (QIAamp® Viral RNA Mini Kit or RNeasy Mini Kit) or Roche MagNA Pure LC System	NucliSENS® miniMAG extraction Kit (bioMerieux) QIAamp® MiniElute® Virus Spin Kit (Qiagen)
Instrument /Assay Platform	Cepheid SmartCycler II System	3M Integrated Cyclor as part of the Microfluidic Molecular System.	ABI 7500 Fast Dx Real-Time PCR instrument	Luminex 100 or 200
Assay Controls	Influenza positive RNA transcript control and an Internal RNA control provided	Armored RNA Internal Control (AR IC), No Template Control (NTC), and H1N1 Positive Control (PC) provided.	Human Specimen Control (HSC), Seasonal Influenza Virus Control (SIVC), and A/H5N1 Positive Control (H5VC) provided.	Bacteriophage lambda positive control and <i>E. coli</i> MS2 phage Internal Control –ancillary reagents not provided

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

- Format for Traditional and Abbreviated 510(k)s - Guidance for Industry and FDA Staff
- Nucleic Acid Based In Vitro Diagnostic Devices for Detection of Microbial Pathogens - Draft Guidance for Industry and FDA Staff Guidance for Off-the-Shelf Software Use in Medical Devices; Final

- Guidance for Off-the-Shelf Software Use in Medical Devices; Final
- Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests; Draft Guidance for Industry and FDA Reviewers
- 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
- Draft Guidance for Industry and FDA Staff: Establishing the Performance Characteristics of In Vitro Diagnostic Devices for the Detection or Detection and Differentiation of Influenza Viruses
- In Vitro Diagnostic Devices to Detect Influenza A Viruses: Labeling and Regulatory Path - Guidance for Industry and FDA Staff
- Guidance for Industry and FDA Staff - Class II Special Controls Guidance Document: Testing for Detection and Differentiation of Influenza A Virus Subtypes Using Multiplex Assays
- User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline (CLSI EP12-A 2002)
- Protocols for Determination of Limits of Detection and Limits of Quantitation (CLSI EP-17A 2004)
- Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline (CLSI MM3-A2 2006)
- Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline (CLSI MM9-A 2004)
- Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods (CLSI MM13-P 2005)
- Medical devices – Quality management systems – Requirements for regulatory purpose (ISO 13485:2003)
- Medical devices - Application of risk management to medical devices (ISO 14971:2007)
- Stability Testing of In Vitro Diagnostic Reagents (CEN 13640:2002)

L. Test Principle:

The real-time PCR process simultaneously amplifies and detects nucleic acid targets in a single closed-tube reaction. The ProFAST+ Assay enables the detection and differentiation of seasonal Influenza A/H1, seasonal Influenza A/H3, 2009 H1N1 Influenza and an Internal Control and is based on three processes: nucleic acid isolation, reverse transcription (RT) and Real Time polymerase chain reaction (PCR) amplification/detection. The Internal Control (IC) is added to each nasopharyngeal (NP) swab from symptomatic patients to monitor for inhibitors present. Nucleic acids are isolated and purified from the NP swab sample. Purified nucleic acid is added to the ProFAST+ Supermix (includes Taq polymerase) along with the appropriate enzymes. The ProFAST+ Supermix contains oligonucleotide primers complementary to highly conserved regions of the hemagglutinin genes for seasonal Influenza A/H1, seasonal Influenza A/H3, 2009 H1N1 Influenza and target-specific oligonucleotide probes dual-labeled with a reporter dye attached to the 5' end and a quencher dye attached to the 3' end. After initial reverse transcription of RNA into complementary DNA (cDNA),

amplification proceeds during which the primers and probe anneal specifically to the template (if present) followed by primer extension and amplification. The ProFAST+ Assay is based on Taqman chemistry, which utilizes the 5' – 3' exonuclease activity of the Taq polymerase to cleave the probe thus separating the reporter dye from the quencher. This generates an increase in fluorescent signal upon excitation from a light source. With each cycle, additional reporter dye molecules are cleaved from their respective probes, further increasing the fluorescent signal. The amount of fluorescence at any given cycle is dependent on the amount of amplification product present at that time. Fluorescent intensity is monitored during each PCR cycle by the real-time instrument.

M. Performance Characteristics (if/when applicable):

1. Analytical performance:

a. *Precision/Reproducibility:*

Reproducibility Study

An Inter-Laboratory Reproducibility study was conducted by extracting and testing a panel of 18 samples at three sites (two external sites and at Gen-Probe Prodesse Inc.) performed by two operators at each site for 5 days per operator (for a total of 10 runs per site). The Reproducibility Panel used in the study was prepared by spiking confirmed negative nasopharyngeal (NP) swab pools with cultured and titered stock solutions of seasonal H1 Influenza A (A/H1), seasonal H3 Influenza A (A/H3) and 2009 H1N1 Influenza Virus (A/2009 H1N1) at low positive (2 x LoD), medium positive (10 x LoD) or negative (0.001 x LoD) concentrations. Each panel included two replicates of each Influenza A subtype at each concentration. This resulted in each Reproducibility Panel consisting of 2 Medium Positives, 2 Low Positives, and 2 Negatives for each of three Influenza A strains (n = 18 total). Panel members were coded to make 18-sample test panels and to ensure sample identification was unknown to the operators. The 18-sample test panels were stored at $\leq -70^{\circ}\text{C}$ and shipped to the sites frozen.

A set of ten panels (180 total samples) was provided to each laboratory; one set was used for each of the 5 testing days per operator. Each operator thawed one panel set per day and performed nucleic acid extraction on the panel samples using either the Roche MagNA Pure LC system (Site #1) or the bioMérieux NucliSENS easyMAG (Site #2 and Site #3), and the extracted nucleic acids were tested with the ProFAST+ Assay on the Cepheid SmartCycler II. ProFAST+ Assay controls (Positive, Extraction and Negative) were also included with each panel run. Two lots of ProFAST+ Supermix were used by each of the three sites. A total of 630 data points were included in the reproducibility study data analysis (21 samples and controls/run X 1 run/day/operator X 2 operators X 5 days X 3 sites = 630). The total percent agreement for the ProFAST+ Assay was 99.7%. The ProFAST+ Assay is a qualitative assay based partially on numerical Cycle Threshold (Ct) values. The overall Ct value %CV across all sites for all samples

and controls ranged from 1.01% to 5.62% depending upon analyte type, target type, and concentration tested.

	Panel Member ID	A/H1 negative (values from IC)	A/H1 low positive	A/H1 medium positive	A/H3 negative (values from IC)	A/H3 low positive	A/H3 medium positive	A/2009 H1N1 negative (values from IC)	A/2009 H1N1 low positive	A/2009 H1N1 medium positive	Extraction Control			Influenza A Subtyping RNA Control			Negative Control	Total % Agreement
		Concentration	0.001 X LoD	2X LoD	10X LoD	0.01X LoD	2X LoD	10X LoD	0.001 X LoD	2X LoD	10X LoD	A/H1	A/H3	A/2009 H1N1	A/H1	A/H3	A/2009 H1N1	
Site 1 MP ^a	Agreement with Expected Result	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	10/10			10/10			10/10	210/210 (100%)
	Mean Ct Value	29.3	34.7	32.1	29.5	32.5	30.3	29.4	31.3	28.6	29.3	27.5	27.9	32.4	31.3	31.6	29.5	
	% CV	1.54	1.16	0.58	1.77	0.96	0.58	1.52	0.76	1.45	0.78	1.01	3.04	0.79	0.79	0.89	0.71	
Site 2 EM ^a	Agreement with Expected Result	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	10/10			10/10			10/10	210/210 (100%)
	Mean Ct Value	27.0	34.1	31.0	27.1	31.7	29.5	26.9	30.6	28.0	28.1	26.5	27.3	32.1	31.0	31.2	26.4	
	% CV	1.41	3.11	0.86	1.54	0.86	0.93	1.41	2.09	0.91	0.38	0.86	1.72	0.61	0.95	0.80	0.95	
Site 3 EM ^a	Agreement with Expected Result	20/20	19/20	20/20	20/20	19/20	20/20	20/20	20/20	20/20	10/10			10/10			10/10	208/210 (99.0%)
	Mean Ct Value	28.5	36.1	32.0	28.6	32.4	30.2	28.3	31.5	28.7	28.9	27.2	29.4	32.0	30.9	31.0	28.8	
	% CV	4.28	7.26	1.80	4.44	1.66	2.42	3.02	2.20	1.13	1.44	1.54	1.42	1.13	1.45	1.05	4.88	
	Total Agreement with Expected Result	60/60	59/60	60/60	60/60	59/60	60/60	60/60	60/60	60/60	30/30			30/30			30/30	628/630 (99.7%)
	95% CI	94.0 - 100%	91.1 - 99.7%	94.0 - 100%	94.0 - 100%	91.1 - 99.7%	94.0 - 100%	94.0 - 100%	94.0 - 100%	94.0 - 100%	88.7% - 100%			88.7% - 100%			88.7 - 100%	
	Overall Mean Ct Value	28.2	34.9	31.7	28.4	32.2	30.0	28.2	31.1	28.5	28.8	27.1	28.3	32.2	31.0	31.3	28.2	
	Overall % CV	4.40	5.08	1.97	4.49	1.59	1.94	4.11	2.12	1.66	1.99	1.95	3.86	1.01	1.19	1.21	5.62	

^aMP = Roche MagNA Pure LC extractor and EM = bioMérieux NucliSENS easyMAG extractor.

Due to the fact that the clinical trial for the ProFAST+ Assay resulted in less than 10% of the clinical samples that were positive by the ProFAST+ Assay with Ct values beyond the mean Ct values of the LoDs (approximately 39 Ct for A/H1, 34 Ct for A/H3 and 32 Ct for 2009 H1N1), but before the assay cutoff at 45 cycles, a supplemental Inter-Laboratory Reproducibility Study at three sites to evaluate inter-laboratory reproducibility of the ProFAST+ Assay with samples having a concentration below the assay's Limit of Detection (LoDs) but above the High Negatives previously tested in the reproducibility study is not necessary. However, the potential limitation of imprecision of testing extremely low levels of targets below the LoDs of the assay should still be addressed by including the following statement in the Limitation section of the ProFAST+ Assay Instructions for Use ***“As with all PCR based in vitro diagnostic tests, extremely low levels of target below the LoD of the assay may be detected, but results may not be reproducible.”***

b. Linearity/assay reportable range: Not applicable

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

Controls

The following controls are provided in the ProFAST+ Assay kit:

Positive Control (PC): The ProFAST+ Assay kit contains an Influenza A Subtyping positive RNA control that consists of one pool of three RNA transcripts (specific for each Influenza A subtype: A/H1, A/H3 and A/2009 H1N1). These transcripts are derived from plasmids containing the viral sequences of interest and are not intact virus particles. The Influenza A Subtyping PC contains equal amounts of A/H1, A/H3 and A/2009 H1N1. The approximate concentration of each transcript was 5×10^3 copies/ μ l. The PC does not go through nucleic acid isolation and purification, but is included during set-up of the RT-PCR reaction. The PC in conjunction with the IC is used to verify reagent and system performance. The PC is meant to be a control for global failure of the assay (missing reaction component, instrument failure, etc). Because the PC is assessing global failure and the customer variance components were to be determined during the clinical trial, a broad Ct range was desired. The acceptable range was set to 20.0 to 40.0 in the Clinical Trial Protocol for all Influenza A subtype targets to allow for dilution variability (e.g. pipetting) of the PC by the end users. Of a total of 75 PC tested by the ProFAST+ Assay during the clinical trial, 98.7% (74/75) of these controls gave correct results initially. The average Ct of the PCs tested was 32.35 (Min 31.50- Max 33.40), with 0.34 Standard Deviation (STDEV) and 1.05% CV; 30.54 (Min 29.80- Max 31.70), with 0.34 Standard Deviation (STDEV) and 1.12%; and 31.09 (Min 30.10- Max 32.40), with 0.35 Standard Deviation (STDEV) and 1.14 % CV for A/H1, A/H3, and A/2009H1N1 respectively. In addition, the average Ct for the PC was 32.16, 31.03, and 31.25 (n = 31) during the Reproducibility Study. The clinical study data validated the pre-determined PC Ct acceptance range of 20.0 to 40.0.

Internal Control (IC): An Internal RNA Control, a non-infectious RNA transcript, is incorporated into every sample and is carried through all steps of the procedure from nucleic acid isolation and purification through amplification to monitor for inhibitors present in the specimen or reaction tube. The IC also serves as a general process control ensuring that each step of the procedure was performed correctly, assay and instrument parameters were set correctly, and that general reagents were working. The IC is meant to assess global failure (reagent or process) and monitor for PCR inhibition. Because the IC is assessing global failure and the customer variance components were to be determined during the clinical trial, a broad Ct range was desired. The acceptable range for the Internal Control (IC) was set to 20-40 Ct in the Clinical Trial Protocol to accommodate for dilution variability (e.g., pipetting) by the user in spiking the IC, and also accommodate slight inhibition. The average Ct of all eligible prospective ProFAST+ Assay negative clinical specimens was 27.93 (Min 25.0- Max 35.20), with 1.32 Standard Deviation (STDEV) and 4.73 % CV. The average Ct of all eligible ProFAST+

Assay Negative Control was 27.36 (Min 25.50- Max 32.70), with 1.12 Standard Deviation (STDEV) and 4.09% CV. In addition, the average Ct for the IC was 28.24 (Min 25.80- Max 30.90) with 1.59 standard deviation and 5.62 % CV during the Reproducibility Study. The clinical study data validated the pre-determined IC Ct acceptance range of 20.0 to 40.0.

Final release specifications were formally established during Test Method Characterization (TMC) then validated against the appropriate analytical performance characteristics of specificity and total imprecision. Final release specifications were developed to ensure First Pass Acceptance (FPA) rates of $\geq 99\%$ at lot release and customer rejection rate of $\leq 1\%$ for a lot released at Acceptable Quality Level (AQL) (i.e. 95% probability of QC lot acceptance at lot release). For customer specifications, the maximum allowable customer lot rejection rate at the Rejectable Quality Level (RQL) Limit (i.e., 10% probability of QC acceptance at lot release) is 1%. A false valid risk of $\leq 5\%$ was used to determine the validity specifications.

QC release specification ranges were set using Operator Characteristics (OC) curve analysis. Lot, technician, instrument, run, and replicate variance components were obtained during test method characterization. Observed Ct means were adjusted for stability effects and specifications determined to meet the validity and specification requirements above.

Control	Customer Ct Specification	Kit Control Final Release Specifications
PC (A/H1)	20-40	27.18-37.24
PC (A/H3)	20-40	26.92-36.22
PC (A/2009 H1N1)	20-40	27.20-36.56
Internal Control (IC)	20-40	24.20-31.16

The following controls are not provided in the ProFAST+ Assay kit, but are required or recommended and described in the ProFAST+ Assay Instructions for Use:

Negative Control (NC): A Negative Control (NC) is not provided with the kit, but is required and described in the ProFAST+ Assay Instructions for Use. Viral transport media spiked with the IC is to be used as the negative control and processed starting from nucleic acid isolation. The negative control serves to monitor for contamination. Of a total of 78 NC tested by the ProParaflu+ Assay during the clinical trial, 97.4% (76/78) of these controls gave correct results initially. The average Ct of the IC in tested NC was 27.36 (Min 25.50- Max 32.70), with 1.12 Standard Deviation (STDEV) and 4.09% CV.

Extraction Control (EC): An Extraction Control (EC) is not provided with the kit, however, during the clinical trial, a pool of seasonal A/H1N1, seasonal A/H3N2 and 2009 H1N1 influenza viruses was included with each nucleic acid isolation

run. The EC served to monitor for lysis during nucleic acid isolation. Of a total of 77 EC tested by the ProFAST+ Assay during the clinical trial, 100% (77/77) of these controls gave correct results initially. The average Ct of the PCs tested was 28.80 (Min 27.60- Max 30.10), with 0.66 Standard Deviation (STDEV) and 2.28% CV; 26.90 (Min 25.70- Max 28.10), with 0.59 Standard Deviation (STDEV) and 2.18%; and 27.61 (Min 26.30- Max 29.20), with 0.72 Standard Deviation (STDEV) and 2.62 % CV for A/H1, A/H3, and A/2009H1N1 respectively. **The sponsor is recommending an extraction control in each nucleic acid extraction run to the end users in the package insert.**

Freeze/Thaw and Stability

Reagents and Controls stability

An Accelerated Stability study concluded that the ProFAST+ Supermix, enzymes and controls (closed and open tubes) can be stored at $\leq -70^{\circ}\text{C}$ for up to 18 months. Real time stability studies are currently ongoing at Gen-Probe Prodesse.

A Freeze/Thaw Study demonstrated that the ProFAST+ Supermix can undergo up to 5 freeze-thaws. The Controls (Positive and Internal Control) can undergo up to 2 freeze-thaws. For the Controls, the ProFAST+ Assay Package Insert specifies that controls should not undergo more than 1 freeze-thaw cycle. Although internal studies demonstrated that up to 2 freeze-thaws of controls would not adversely affect performance. To mitigate risk it is recommended they not undergo more than 1 freeze-thaw.

Specimen stability

The ProFAST+ Assay recommends that samples be stored refrigerated ($2^{\circ}\text{C} - 8^{\circ}\text{C}$) for up to 72 hours prior to processing the samples. This recommendation is supported by 1) The ProFlu+ Assay (detection of Influenza A, Influenza B and RSV nucleic acid), the Pro hMPV+ (detection of human Metapneumovirus (hMPV) nucleic acid), and the ProParaflu+ Assay (detection of human Parainfluenza virus nucleic acid) also recommend that samples be stored refrigerated ($2^{\circ}\text{C} - 8^{\circ}\text{C}$) for up to 72 hours prior to processing the samples; 2) The CLSI Guidance: Collection, Transport, Preparation and Storage of Specimens for Molecular Methods (MM13) does not specify requirements for nasopharyngeal (NP) swabs, but does recommend that bronchioalveolar lavage samples be refrigerated for up to 72 hours or frozen (at -70°C) for longer periods prior to processing for molecular methods; 3) A 72 hour timeframe is widely recommended and used (CLSI Guidance M41 Viral Culture) for viral culture. For viral culture sample stability is important as viable virus is required for growth, however, nucleic acid amplification tests (NAAT) do not require viable virus for detection.

The ProFAST+ Assay also recommends that extracted nucleic acids that are not

run immediately after extraction may be stored at $\leq -70^{\circ}\text{C}$ before running ProFAST+. Typically, purified nucleic acids may be stored for extended periods of time at $\leq -70^{\circ}\text{C}$ without marked degradation. Both CLSI guidances MM13 (Collection, Transport, Preparation and Storage of Specimens for Molecular Methods) and MM17 (Verification and Validation of Multiplex Nucleic Acid Assays) recommend storage of isolated RNA at ultralow temperature and limiting the number of freeze-thaw cycles.

Comparison between Fresh and Frozen Specimens

To determine the potential effects of freezing NP samples and purified nucleic acids may have on the ProFAST+ Assay, an analytical study was carried out. Contrived samples were generated using aliquots from a negative NP swab pool spiked with one of three Influenza A (IA) strains representing the viruses targeted by the ProFAST+ Assay which include seasonal H1 (A/H1), seasonal H3 (A/H3), and 2009 H1N1swine-origin (A/2009 H1N1). Fresh IA positive NP swab samples were not available for the study due to low prevalence of influenza during this past winter respiratory season (December 2009 – March 2010). Contrived samples were made at varying concentrations in negative NP swab matrix. For each of the three strains, twenty samples were generated (n=60) in the following manner: 2 samples each at 3 logs, 2 logs, and 1 log above LoD and 7 samples each of 3x LoD and at LoD (as determined during the Analytical Sensitivity Study). After sample generation, a portion of each sample was freshly extracted on the bioMérieux NucliSENS easyMAG. The remaining portion of the samples were frozen at $\leq -70^{\circ}\text{C}$ and then extracted again as described above. The nucleic acids were run fresh (immediately subsequent to extraction) in a single replicate. The remaining nucleic acids were frozen at $\leq -70^{\circ}\text{C}$ and later thawed on ice and subjected to RT-PCR as described above. The following tables summarize the results of the study:

Sample Stability Results — % Agreement to the Fresh NP / Fresh NA Data					
Strain	Concentration	% Agreement			
		FR NP/FR NA ^a	FR NP/FZ NA ^b	FZ NP/FR NA ^c	FZ NP/FZ NA ^d
A/H1	3 logs above LoD	2	2/2 = 100%	2/2 = 100%	2/2 = 100%
	2 logs above LoD	2	2/2 = 100%	2/2 = 100%	2/2 = 100%
	1 log above LoD	2	2/2 = 100%	2/2 = 100%	2/2 = 100%
	3x LoD	7	7/7 = 100%	7/7 = 100%	7/7 = 100%
	LoD	7	7/7 = 100%	7/7 = 100%	7/7 = 100%
	Overall	20	20/20 = 100%	20/20 = 100%	20/20 = 100%
A/H3	3 logs above LoD	2	2/2 = 100%	2/2 = 100%	2/2 = 100%
	2 logs above LoD	2	2/2 = 100%	2/2 = 100%	2/2 = 100%
	1 log above LoD	2	2/2 = 100%	2/2 = 100%	2/2 = 100%
	3x LoD	7	7/7 = 100%	7/7 = 100%	7/7 = 100%
	LoD	7	7/7 = 100%	7/7 = 100%	7/7 = 100%
	Overall	20	20/20 = 100%	20/20 = 100%	20/20 = 100%
A/2009 H1N1	3 logs above LoD	2	2/2 = 100%	2/2 = 100%	2/2 = 100%
	2 logs above LoD	2	2/2 = 100%	2/2 = 100%	2/2 = 100%
	1 log above LoD	2	2/2 = 100%	2/2 = 100%	2/2 = 100%
	3x LoD	7	7/7 = 100%	7/7 = 100%	7/7 = 100%
	LoD	7	7/7 = 100%	7/7 = 100%	7*/7 = 100%
	Overall	20	20/20 = 100%	20/20 = 100%	20/20 = 100%

^aFR NP/FR NA: Fresh contrived NP samples/Freshly extracted nucleic acids

^bFR NP/FZ NA: Fresh contrived NP samples/Frozen nucleic acids

^cFZ NP/FR NA: Frozen contrived NP samples/Fresh nucleic acids

^dFZ NP/FZ NA: Frozen contrived NP samples/Frozen nucleic acids

* Frozen (-70 °C) nucleic acids for these 7 replicates were negative for A/2009 H1N1 initially. They were thawed a second time and retested using the same lot of ProFAST+ reagent and all 7 replicates were positive for A/2009 H1N1.

Mean Ct values and Mean Differences							
Sample ID	FR NP/FR NA ^a Ct	FR NP/FZ NA ^b Ct	FZ NP/FR NA ^c Ct	FZ NP/FZ NA ^d Ct	Mean Ct Difference to initial FR NP/FR NA Data		
					FR NP/FZ NA	FZ NP/FR NA	FZ NP/FZ NA
A/2009 H1N1 at LoD	32.56	31.96	31.70	31.53*	-0.60	-0.86	-1.03*
A/2009 H1N1 3X LoD	29.39	29.53	29.21	29.61	0.14	-0.18	0.22
A/2009 H1N1 1 log above LoD	28.2	27.85	27.85	27.95	-0.35	-0.35	-0.25
A/2009 H1N1 2 logs above LoD	24.4	24.75	24.25	24.35	0.35	-0.15	-0.05
A/2009 H1N1 3 logs above LoD	21.25	21.45	21.2	21.35	0.20	-0.05	0.10
A/H1 at LoD	35.09	34.43	34.3	36.56	-0.66	-0.79	1.47
A/H1 3X LoD	32.11	31.76	31.6	31.97	-0.35	-0.51	-0.14
A/H1 1 log above LoD	30.35	30.25	30.3	30.2	-0.10	-0.05	-0.15
A/H1 2 logs above LoD	27.3	27.4	27.4	27.2	0.10	0.1	-0.10
A/H1 3 logs above LoD	24.15	24.1	24.15	24.05	-0.05	0	-0.10
A/H3 at LoD	32.2	32.04	32.11	32.14	-0.16	-0.09	-0.06
A/H3 3X LoD	30.54	30.49	30.61	30.24	-0.05	0.07	-0.30
A/H3 1 log above LoD	29.15	28.95	29.2	28.8	-0.20	0.05	-0.35
A/H3 2 logs above LoD	26.25	26	26.25	25.95	-0.25	0	-0.30
A/H3 3 logs above LoD	22.9	22.65	22.6	22.25	-0.25	-0.3	-0.65

^aFR NP/FR NA: Fresh contrived NP samples/Freshly extracted nucleic acids

^bFR NP/FZ NA: Fresh contrived NP samples/Frozen nucleic acids

^cFZ NP/FR NA: Frozen contrived NP samples/Fresh nucleic acids

^dFZ NP/FZ NA: Frozen contrived NP samples/Frozen nucleic acids

* Frozen (-70 °C) nucleic acids for these 7 replicates were negative for A/2009 H1N1 initially. They were thawed a second time and retested using the same lot of ProFAST+ reagent and all 7 replicates were positive for A/2009 H1N1.

These results show that there is no negative affect due to freezing sample or nucleic acids when the sample and resultant nucleic acids have each been subjected to a freeze-thaw cycle.

d. Analytical Sensitivity (Detection limit):

LoD estimation studies using the Roche MagNA Pure LC

The analytical sensitivity (limit of detection or LoD) of the ProFAST+ Assay was estimated using quantified (TCID₅₀/mL) cultures of viral strains of A/H1, A/H3 and A/2009 H1N1 (H1N1 A/Virginia/1/06; H1N1 A/Hong Kong/2652/06; H3N2 A/Anhui/1239/05; H3N2 A/California/07/04; 2009 H1N1 Clinical Isolate #1; and 2009 H1N1 Clinical Isolate #2) serially diluted in negative nasopharyngeal (NP) pools prepared from leftover NP swab clinical samples. Each viral strain was previously cultured and titered to determine its stock concentration in TCID₅₀/mL. Each viral stock was spiked into negative NP swab pools at concentrations of 1 log above, 0.5 log above, 1 log below, 0.5 log below, and at the estimated LoD for that virus (based on preliminary studies). Each virus concentration was tested in quintuplicate for a total of 5 data points per virus concentration.

An Internal RNA Control (IC) was spiked into all dilution series samples prior to nucleic acid isolation. The IC monitors for PCR inhibition as well as reagent, procedural or instrumentation failures. A negative control, which consisted of negative NP swab matrix spiked with IC was included with each set of dilution series. Nucleic acid isolation of the negative control was performed along with the Analytical Sensitivity Panel samples. The negative control served to monitor for contamination during the testing procedure or the presence of inhibitors in the sample matrix. The Influenza A Subtyping Positive RNA Control, non-infectious *in vitro* transcribed RNA of specific A/H1, A/H3, and A/2009 H1N1 viral sequences, was included with each RT-PCR run to test for procedural errors (absence of reagent, instrument failure, etc.).

Analytical sensitivity was estimated for each virus strain. The LoD was estimated as the lowest concentration that was detected $\geq 95\%$ of the time (i.e. concentration at which at least 5 out of 5 replicates were determined to be positive). The data are presented in the following table:

Analytical Sensitivity Estimation Results					
Viral Strain	Conc TCID ₅₀ /mL	Average C _T	Standard Deviation	%CV	Replicates Detected
H1N1 A/Virginia/1/06	1x10 ¹	30.60	0.19	0.61	5/5
	5x10 ⁰	31.72	0.19	0.61	5/5
	1x10 ⁰	34.22	0.28	0.81	5/5
	5x10 ⁻¹	35.38	0.52	1.46	5/5
	1x10 ⁻¹	41.70	N/A	N/A	1/5
H1N1 A/Hong Kong/2652/06	1x10 ⁰	31.88	0.22	0.68	5/5
	5x10 ⁻¹	33.24	0.23	0.69	5/5
	1x10 ⁻¹	35.78	0.55	1.54	5/5
	5x10 ⁻²	39.76	1.25	3.15	5/5
	1x10 ⁻²	ND	N/A	N/A	0/5
H3N2 A/Anhui/1239/05	1x10 ¹	28.22	0.08	0.30	5/5
	5x10 ⁰	29.24	0.13	0.46	5/5
	1x10 ⁰	31.46	0.15	0.48	5/5
	5x10 ⁻¹	32.14	0.15	0.47	5/5
	1x10 ⁻¹	34.74	1.51	4.34	5/5
H3N2 A/California/07/04	1x10 ⁰	32.18	0.28	0.86	5/5
	5x10 ⁻¹	32.78	0.33	1.02	5/5
	1x10 ⁻¹	36.54	1.39	3.80	5/5
	5x10 ⁻²	37.18	1.84	4.95	4/5
	1x10 ⁻²	ND	N/A	N/A	0/5
2009 H1N1 Clinical Isolate #1	1x10 ³	28.18	0.11	0.39	5/5
	5x10 ²	30.10	0.07	0.23	5/5
	1x10 ²	32.44	0.25	0.77	5/5
	5x10 ¹	35.18	0.75	2.13	5/5
	1x10 ¹	ND	N/A	N/A	0/5
2009 H1N1 Clinical Isolate #5	1x10 ³	28.50	0.10	0.35	5/5
	5x10 ²	29.30	0.10	0.34	5/5
	1x10 ²	32.18	0.13	0.41	5/5
	5x10 ¹	33.38	0.29	0.88	5/5
	1x10 ¹	ND	N/A	N/A	0/5

Analytical sensitivity was estimated to be 5x10⁻¹ TCID₅₀/mL for H1N1 A/Virginia/1/06, 5x10⁻² TCID₅₀/mL for H1N1 A/Hong Kong/2652/06, 1x10⁻¹ TCID₅₀/mL for H3N2 A/Anhui/1239/05, 1x10⁻¹ TCID₅₀/mL for H3N2 A/California/07/04, 5x10¹ TCID₅₀/mL A/2009 H1N1 Clinical Isolate #1 and 5x10¹ TCID₅₀/mL A/2009 H1N1 Clinical Isolate #5.

LoD confirmation studies using the Roche MagNA Pure LC

The “estimated” LoDs were then confirmed by preparing 20 additional replicates (simulated NP swab samples) at each of the proposed LoD concentrations.

An Internal RNA Control (IC) was spiked into all dilution series samples prior to nucleic acid isolation. The IC monitors for PCR inhibition as well as reagent,

procedural or instrumentation failures. A negative control, which consisted of negative NP swab matrix spiked with IC was included with each set of dilution series. Nucleic acid isolation of the negative control was performed along with the Analytical Sensitivity Panel samples. The negative control served to monitor for contamination during the testing procedure or the presence of inhibitors in the sample matrix. The Influenza A Subtyping Positive RNA Control, non-infectious *in vitro* transcribed RNA of specific A/H1, A/H3, and A/2009 H1N1 viral sequences, was included with each RT-PCR run to test for procedural errors (absence of reagent, instrument failure, etc.).

For some of the strains, more than one concentration was included for the confirmation portion of the study, typically the two lowest concentrations that yielded 100% detection to ensure achievement of $\geq 95\%$ detection for each strain. For strain H3N2 A/Anhui/1239/05, the lowest concentration tested for the estimation portion of the study resulted in 100% detection, thus that concentration and the next two lower half-log concentrations were tested in the confirmation portion of the study. Each of the 20 replicates was subjected to the entire test system from sample preparation and extraction to RT-PCR. All samples were extracted using the MagNA Pure LC Instrument. In the event that the initial LoD estimated concentration was not confirmed (i.e. < 19 replicates were not positive), the LoD confirmation was repeated using the next half-log higher concentration. The data are presented in the following table:

Analytical Sensitivity Confirmation Results								
Viral Strain	Conc TCID ₅₀ /mL	Mean C _T	Standard Deviation	%CV	Min CT	Max Ct	Replicates Detected	% Detected
H1N1 A/Virginia/1/06	5x10 ⁻¹	35.76	0.71	1.99	34.5	37.9	20/20	100%
H1N1 A/Hong Kong/2652/06	1x10 ⁻¹	36.21	0.76	2.10	34.3	37.4	20/20	100%
	5x10 ⁻²	41.35	1.80	4.35	38.5	44.8	20/20	100%
H3N2 A/Anhui/1239/05	1x10 ⁻¹	34.78	0.83	2.39	32.1	35.7	20/20	100%
	5x10 ⁻²	36.51	0.89	2.45	35.4	38.9	18/20	90%
	1x10 ⁻³	37.5	NA	NA	37.5	37.5	1/20	5%
H3N2 A/California/07/04	5x10 ⁻¹	33.17	0.41	1.23	32.4	33.9	20/20	100%
	1x10 ⁻¹	37.16	1.60	4.30	33.4	39.8	18/20	90%
2009 H1N1 Clinical Isolate #1	1x10 ²	32.86	0.96	2.93	31.0	34.6	19/20	95%
	5x10 ¹	31.80	4.24	13.34	28.8	34.8	2/20	10%
2009 H1N1 Clinical Isolate #5	1x10 ²	31.92	0.36	1.14	31.3	32.4	20/20	100%
	5x10 ¹	33.98	0.60	1.75	33.0	35.0	18/20	90%

The sponsor confirmed LoDs are presented in the following table:

ProFAST+ Analytical Sensitivity – Limit of Detection	
Viral Strain	Limit of Detection
H1N1 A/Virginia/1/06	5×10^{-1} TCID ₅₀ /mL
H1N1 A/Hong Kong/2652/06	5×10^{-2} TCID ₅₀ /mL
H3N2 A/Anhui/1239/05	1×10^{-1} TCID ₅₀ /mL
H3N2 A/California/07/04	5×10^{-1} TCID ₅₀ /mL
2009 H1N1 Clinical Isolate #1	1×10^{-2} TCID ₅₀ /mL
2009 H1N1 Clinical Isolate #5	1×10^{-2} TCID ₅₀ /mL

Extraction Equivalency Study

To evaluate the equivalency of nucleic acid extraction of the bioMérieux NucliSENS easyMAG to the Roche MagNA Pure LC for use with the ProFAST+ Assay, an analytical study was carried out to determine “equivalency” by confirming the LoDs on the easyMAG extractor for each of the three (Seasonal A/H1, Seasonal A/H3 and A/2009 H1N1 Influenza) subtypes of Influenza A used in the Analytical Sensitivity Study (determined and confirmed using the MagNA Pure LC). Both instruments were also used throughout assay development, Clinical Trials and Reproducibility.

The Roche MagNA Pure LC is an automated nucleic acid isolation and purification system based upon binding of nucleic acids to glass particles and has the capability to process a total of 32 reactions within one run. Nucleic acid is purified in multiple plastic reaction tips and cartridges by several steps that include cell lysis and binding of nucleic acid to magnetic glass particles, wash steps, and a heated elution to unbind the nucleic acid from the glass particles.

The bioMérieux NucliSens easyMAG is an automated nucleic acid isolation and purification system that is based upon the same silica extraction technology as the MagNA Pure. The easyMAG is capable of processing a total of 24 reactions with variable sample types, sample volumes, and elution volumes within a single run. Nucleic acid is purified within a single cartridge by several steps that include lysis and binding of nucleic acid to high affinity magnetic silica beads, a series of wash steps and heated elution of purified nucleic acid from the silica beads.

One operator generated dilution series for each viral strain and spiked each diluted virus into the same negative nasopharyngeal (NP) swab pool used for the ProFAST+ Analytical Sensitivity Study at the LoD concentration confirmed in that study. Twenty independent data points from the specific (LoD) viral dilution were generated by performing 20 independent extractions on the easyMAG; each extracted sample was run in a single replicate on the Cepheid SmartCycler II. The two extraction instruments were considered equivalent if the detection rate using the easyMAG at the LoD was not statistically different (at significance level of $p < 0.05$) than the rate obtained using the MagNA Pure instrument. Twenty of twenty replicates were positive using the MagNA Pure for 5 of the 6 strains tested

and therefore, 18 replicates were required to test positive using the easyMAG ($p=0.0734$) to claim equivalency. The Prodesse 2009 H1N1 clinical isolate #1 strain only tested at 95% positive (19/20) for the LoD using the MagNA Pure and therefore, for that particular strain, 16 of 20 were required to test positive ($p=0.0757$) to claim equivalency.

The Internal RNA Control II (IC) was spiked into all samples prior to nucleic acid isolation. The IC monitors for RT-PCR inhibition as well as any reagent, procedural or instrumentation failure. The Influenza A Subtyping RNA Control II (PC) was included with each RT-PCR run to test for global errors (absence of reagent, instrument failure, etc.). The Influenza PC does not require nucleic acid isolation and was diluted just prior to set up of RT-PCR reactions. A Negative Control which consisted of Viral Transport Media (VTM) spiked with the IC was included for each extraction run. The Negative Control serves to monitor for contamination during the testing procedure or the presence of inhibitors in the sample matrix.

NucliSENS easyMAG LoD data are presented in the following table. The data from the Analytical Sensitivity Study using the MagNA Pure LC as the extraction instrument is presented in the table alongside the easyMAG data for comparison. :

LoD Summary for both MagNA Pure LC (MP)[†] and easyMAG (EM)							
Viral Strain	Extractor	Conc TCID₅₀/mL	Average C_T	Standard Deviation	%CV	Replicate s Detected	% Detected
H1N1 A/Virginia/1/06	MP	0.5	35.76	0.71	1.99	20/20	100%
	EM	0.5	36.75	1.25	3.39	20/20	100%
H1N1 A/Hong Kong/2652/06	MP	0.05	41.35	1.80	4.35	20/20	100%
	EM	0.05	40.68	2.36	5.80	20/20	100%
H3N2 A/Anhui/1239/05	MP	0.1	34.78	0.83	2.39	20/20	100%
	EM	0.1	34.49	0.77	2.24	20/20	100%
H3N2 A/California/07/04	MP	0.5	33.17	0.41	1.23	20/20	100%
	EM	0.5	32.47	0.40	1.22	20/20	100%
2009 H1N1 Clinical Isolate #1	MP	100	32.86	0.96	2.93	19/20	95%
	EM	100	32.16	1.27	3.95	16*/20	80%*
2009 H1N1 Clinical Isolate #5	MP	100	31.92	0.36	1.14	20/20	100%
	EM	100	31.42	0.61	1.96	20/20	100%

[†] The MagNA Pure LC data was previously generated during the ProFAST+ Analytical Sensitivity Study.

*Although <95% was detected, the difference is not statistically significant using the easyMAG as compared to extraction with the MagNA Pure LC.

These data demonstrated that the easyMAG extractor is not statistically different than the MagNA Pure LC instrument when testing LoD concentrations and is therefore equivalent to the MagNA Pure LC extractor. Moreover, any performance claims made using the MagNA Pure with the ProFAST+ Assay should be achievable using the easyMAG. Clinical trial and reproducibility studies also supported comparable performance of both automated extraction systems.

e. *Analytical Reactivity:*

The analytical reactivity of the ProFAST+ Assay was evaluated using multiple strains of Influenza A: 14 strains of seasonal H1, 14 strains of seasonal H3, 4 strains of 2009 H1N1. Viral strains were selected to include strains from 1933 to the present, as well as from various geographical locations, to incorporate the genetic variation that may be encountered by ProFAST+ users:

ProFAST+ Reactivity Panel List		
Strain	Target	Concentration tested (2xLoD)
A/Taiwan/42/06	Seasonal H1	2×10^0 TCID ₅₀ /mL
A/Henan/8/05	Seasonal H1	2×10^0 TCID ₅₀ /mL
A/Fujian/156/00	Seasonal H1	2×10^1 TCID ₅₀ /mL
Brazil/1137/99	Seasonal H1	2×10^5 TCID ₅₀ /mL
A/Kentucky/2/06	Seasonal H1	2×10^2 TCID ₅₀ /mL
A/Hawaii/15/01	Seasonal H1	2×10^2 TCID ₅₀ /mL
A/New Caledonia/12/99	Seasonal H1	2×10^2 TCID ₅₀ /mL
A/Brisbane/59/2007	Seasonal H1	2×10^2 TCID ₅₀ /mL
A/Solomon Islands/03/06	Seasonal H1	2×10^0 TCID ₅₀ /mL
A/Jiangxi/160/05	Seasonal H1	2×10^0 TCID ₅₀ /mL
VR 1520 A/WS/33	Seasonal H1	5.62×10^4 TCID ₅₀ /mL *†
VR 98 A1/Mal/302/54	Seasonal H1	1.78×10^6 CEID ₅₀ /mL *†
A/PR/8/34	Seasonal H1	2.0×10^6 TCID ₅₀ /mL *
VR 546 A1/Denver/1/57	Seasonal H1	1.78×10^6 CEID ₅₀ /mL *†
A/Hiroshima/52/05	Seasonal H3	2×10^0 TCID ₅₀ /mL
A/Victoria/512/05	Seasonal H3	2×10^0 TCID ₅₀ /mL
VR 822 A/Victoria/3/75	Seasonal H3	2×10^2 CEID ₅₀ /mL †
A/Brazil/02/99	Seasonal H3	2×10^3 TCID ₅₀ /mL
A/New York/55/2004	Seasonal H3	2×10^0 TCID ₅₀ /mL
A/Hong Kong/2831/05	Seasonal H3	2×10^1 TCID ₅₀ /mL
A/Port Chalmers/1/73	Seasonal H3	2×10^0 TCID ₅₀ /mL
A/Bahamas/2686/99	Seasonal H3	2×10^2 TCID ₅₀ /mL
A/Fujian/411/02	Seasonal H3	2×10^2 TCID ₅₀ /mL
A/Kentucky/03/06	Seasonal H3	2×10^2 TCID ₅₀ /mL
A/Costa Rica/07/99	Seasonal H3	2×10^2 TCID ₅₀ /mL
A/Hong Kong/218/06	Seasonal H3	2×10^1 TCID ₅₀ /mL
VR 544 A/Hong Kong/8/68	Seasonal H3	2×10^2 CEID ₅₀ /mL †
VR 547 A/Aichi/2/68	Seasonal H3	2×10^2 CEID ₅₀ /mL †
2009 H1N1 Clinical Isolate #2	Swine-Origin Influenza	2×10^3 TCID ₅₀ /mL
2009 H1N1 Clinical Isolate #3	Swine-Origin Influenza	2×10^2 TCID ₅₀ /mL
2009 H1N1 Clinical Isolate #4	Swine-Origin Influenza	2×10^1 TCID ₅₀ /mL
2009 H1N1 A/California/04/2009	Swine-Origin Influenza	2×10^2 TCID ₅₀ /mL

* Strains presumed to be non-reactive based on preliminary testing; all were tested at the highest concentration available for each strain.

† Strains not re-cultured and titered. The original culture/titer from ATCC was used in this study.

A preliminary Limit of Detection (LoD) was established for most of the strains during pre-verification studies. Four of the A/H1 strains appear non-reactive with the ProFAST+ Assay and do not have a preliminary LoD. The strains used in the study were tested at 2x LoD (as determined in pre-verification studies or at the highest concentration possible for strains that were non-reactive). One sample

was generated for each strain by spiking cultured and titered virus into aliquots of a negative nasopharyngeal (NP) swab pool. Samples were spiked with Internal Control before extraction on the MagNA Pure LC. Each sample was tested in triplicate on the Cepheid SmartCycler II.

A Negative Control, which consisted of IC spiked into VTM, was included for each set of extraction runs. The Negative Control serves to monitor for contamination during the testing procedure. The Influenza A Subtyping Positive RNA Control, non-infectious *in vitro* transcribed RNA of specific A/H1, A/H3, and A/2009 H1N1 viral sequences, was included with each RT-PCR run to test for procedural errors (absence of reagent, instrument failure, etc.).

The majority of strains analyzed in this study tested positive by the ProFAST+ Assay as expected. The strains that tested negative during pre-verification studies and were not expected to be reactive towards the assay remained non-reactive. Mean Cts and standard deviations for reactive strains were calculated:

ProFAST+ Analytical Reactivity Panel Results					
Strain	Target	Concentration Tested	A/H1 Detection Mean Ct±Standard Deviation	A/H3 Detection Mean Ct±Standard Deviation	A/2009 H1N1 Detection Mean Ct±Standard Deviation
A/Taiwan/42/06	A/H1	2x10 ⁰ TCID ₅₀ /mL	37.63 ± 0.91	-	-
A/Henan/8/05	A/H1	2x10 ⁰ TCID ₅₀ /mL	36.37 ± 0.61	-	-
A/Fujian/156/00	A/H1	2x10 ¹ TCID ₅₀ /mL	38.67 ± 0.21	-	-
Brazil/1137/99	A/H1	2x10 ⁵ TCID ₅₀ /mL	34.10 ± 0.44	-	-
A/Kentucky/2/06	A/H1	2x10 ² TCID ₅₀ /mL	32.10 ± 0.10	-	-
A/Hawaii/15/01	A/H1	2x10 ² TCID ₅₀ /mL	35.70 ± 0.85	-	-
A/New Caledonia/12/99	A/H1	2x10 ² TCID ₅₀ /mL	38.40 ± 1.42	-	-
A/Brisbane/59/2007	A/H1	2x10 ² TCID ₅₀ /mL	33.97 ± 0.45	-	-
A/Solomon Islands/03/06	A/H1	2x10 ⁰ TCID ₅₀ /mL	33.47 ± 0.25	-	-
A/Jiangxi/160/05	A/H1	2x10 ⁰ TCID ₅₀ /mL	37.97 ± 1.42	-	-
VR 1520 A/WS/33	A/H1	5.62x10 ⁴ TCID ₅₀ /mL	-	-	-
VR 98 A1/Mal/302/54	A/H1	1.78x10 ⁶ CEID ₅₀ /mL	-	-	-
A/PR/8/34	A/H1	2.0x10 ⁶ TCID ₅₀ /mL	-	-	-
VR 546 A1/Denver/1/57	A/H1	1.78x10 ⁶ CEID ₅₀ /mL	-	-	-
A/Hiroshima/52/05	A/H3	2x10 ⁰ TCID ₅₀ /mL	-	31.30 ± 0.00	-
A/Victoria/512/05	A/H3	2x10 ⁰ TCID ₅₀ /mL	-	32.70 ± 0.10	-
VR 822 A/Victoria/3/75	A/H3	2x10 ² CEID ₅₀ /mL	-	33.57 ± 0.29	-
A/Brazil/02/99	A/H3	2x10 ³ TCID ₅₀ /mL	-	31.30 ± 0.10	-
A/New York/55/2004	A/H3	2x10 ⁰ TCID ₅₀ /mL	-	32.27 ± 0.12	-
A/Hong Kong/2831/05	A/H3	2x10 ¹ TCID ₅₀ /mL	-	34.47 ± 0.72	-
A/Port Chalmers/1/73	A/H3	2x10 ⁰ TCID ₅₀ /mL	-	38.67 ± 1.11	-
A/Bahamas/2686/99	A/H3	2x10 ² TCID ₅₀ /mL	-	34.23 ± 0.15	-
A/Fujian/411/02	A/H3	2x10 ² TCID ₅₀ /mL	-	31.10 ± 0.20	-
A/Kentucky/03/06	A/H3	2x10 ² TCID ₅₀ /mL	-	30.43 ± 0.15	-
A/Costa Rica/07/99	A/H3	2x10 ² TCID ₅₀ /mL	-	33.30 ± 0.10	-
A/Hong Kong/218/06	A/H3	2x10 ¹ TCID ₅₀ /mL	-	35.80 ± 0.70	-
VR 544 A/Hong Kong/8/68	A/H3	2x10 ² CEID ₅₀ /mL	-	32.30 ± 0.35	-
VR 547 A/Aichi/2/68	A/H3	2x10 ² CEID ₅₀ /mL	-	32.93 ± 0.06	-

ProFAST+ Analytical Reactivity Panel Results					
Strain	Target	Concentration Tested	A/H1 Detection Mean Ct±Standard Deviation	A/H3 Detection Mean Ct±Standard Deviation	A/2009 H1N1 Detection Mean Ct±Standard Deviation
2009 H1N1 Clinical Isolate #2	S-OIV	2x10 ³ TCID ₅₀ /mL	-	-	27.17 ± 0.12
2009 H1N1 Clinical Isolate #3	S-OIV	2x10 ² TCID ₅₀ /mL	-	-	31.90 ± 0.10
2009 H1N1 Clinical Isolate #4	S-OIV	2x10 ¹ TCID ₅₀ /mL	-	-	33.67 ± 0.55
2009 H1N1 A/California/04/2009	S-OIV	2x10 ² TCID ₅₀ /mL	-	-	28.47 ± 0.25

There were four strains that were not reactive with the ProFAST+ Assay. These results were expected, as the strains were not reactive with the ProFAST+ Assay during pre-verification work. All four strains are reactive with the FDA cleared Prodesse ProFlu+ Assay (targeting the matrix gene) validating that the non-reactivity with the ProFAST+ Assay is not due to nucleic acid degradation. It was not surprising that the older A/H1 strains did not react with ProFAST+ as the design emphasized more contemporary strains. It is difficult to find regions of the hemagglutinin gene that are conserved over time considering the mutability of the virus. The only sequence available for these older strains was for VR 546 A1/Denver/1/57 which showed several mismatches to the primers. However, the issue of the ProFAST+ assay may not react with non-contemporary influenza A strains should be addressed in the product package insert as the following: *“The ProFAST+ Assay may not react with non-contemporary influenza A strains”*

f. Analytical specificity:

The analytical specificity of the ProFAST+ Assay was evaluated by testing a panel of 38 viruses (including the three targeted Influenza A viruses, four influenza viruses of swine origin, and two H5N1 strains), 25 bacteria, and 1 yeast strain representing common respiratory pathogens or flora commonly present in the nasopharynx. Bacteria and yeast were tested at concentrations of 10⁶ to 10⁷ CFU/mL. Viruses were tested at concentrations of 10³ to 10⁶ TCID₅₀/mL. Samples were extracted and tested in triplicate.

Aliquots of a negative nasopharyngeal (NP) swab pool matrix were spiked with the analytical specificity organisms at concentrations of at 1.0x10³ – 10⁶ TCID₅₀/ml (except Epstein Barr Virus, which was tested at 10⁸ copies/mL) and the bacterial and yeast strains at 10⁶ – 10⁷ CFU/ml, all of which are clinically relevant concentrations. Cultured and titered strains of Influenza A H1N1 (A/H1), Influenza A H3N2 (A/H3), and 2009 H1N1 Influenza A (A/2009 H1N1) were also included in the panel and tested at 2 logs above their respective Limit of Detection, and served as Extraction Controls for each target channel:

Analytical Specificity Panel Samples			
Organism	Concentration Tested	Cultured By	Original Source
Virus Strains			
A/H1 A/Henan/8/05	10 ² TCID ₅₀ /mL	TriCore	CDC, Atlanta, GA
A/H3 A/California/07/04	10 ¹ TCID ₅₀ /mL	Tricore	University of Wisconsin Madison, Madison, WI
A/2009 H1N1 Prodesse Novel H1N1 clinical isolate #2	10 ⁵ TCID ₅₀ /mL	Tricore	WMH
Adenovirus 1/Adenoid 71	10 ⁶ TCID ₅₀ /mL	Tricore	ATCC
Adenovirus 7	10 ⁶ TCID ₅₀ /mL	Tricore	ATCC
Coronavirus 229E	10 ⁶ TCID ₅₀ /mL	Tricore	ATCC
Coxsackie B4	10 ⁴ TCID ₅₀ /mL	Tricore	ATCC
Coxsackie B5/10/2006	10 ⁵ TCID ₅₀ /mL	Tricore	ATCC
Cytomegalovirus	10 ⁴ TCID ₅₀ /mL	Tricore	ATCC
Echovirus 2	10 ⁶ TCID ₅₀ /mL	Tricore	ATCC
Echovirus 3	10 ⁵ TCID ₅₀ /mL	Tricore	ATCC
Echovirus 6	10 ⁵ TCID ₅₀ /mL	Tricore	ATCC
Echovirus 11	10 ⁶ TCID ₅₀ /mL	Tricore	ATCC
Enterovirus 68	10 ³ TCID ₅₀ /mL	Tricore	ATCC
Enterovirus 70	10 ³ TCID ₅₀ /mL	Tricore	ATCC
Ebstein Barr Virus	10 ⁸ copies/mL	Zeptomatrix	Zeptomatrix
HSV Type 1 MacIntyre Strain	10 ⁵ TCID ₅₀ /mL	Tricore	ATCC
HSV Type 2 G strain	10 ³ TCID ₅₀ /mL	Tricore	ATCC
Human Metapneumovirus A2	10 ⁴ TCID ₅₀ /mL	Zeptomatrix	University of Iowa, Iowa City, IA
Human Rhinovirus 1a	10 ³ TCID ₅₀ /mL	Tricore	ATCC
Human Rhinovirus	10 ³ TCID ₅₀ /mL	Tricore	ATCC
Influenza B/Wisconsin	10 ⁴ TCID ₅₀ /mL	Tricore	CDC, Atlanta, GA
Measles/7/2000	10 ⁴ TCID ₅₀ /mL	Tricore	ATCC
Mumps Virus	10 ⁴ TCID ₅₀ /mL	Tricore	ATCC
Parainfluenza Type 1	10 ⁴ TCID ₅₀ /mL	Tricore	ATCC
Parainfluenza Type 2	10 ⁶ TCID ₅₀ /mL	Tricore	ATCC
Parainfluenza Type 3	10 ⁶ TCID ₅₀ /mL	Tricore	ATCC
Parainfluenza Type 4	10 ⁴ TCID ₅₀ /mL	Tricore	ATCC
Poliovirus 1	10 ⁶ TCID ₅₀ /mL	Tricore	ATCC
RSV A Strain Long	10 ⁴ TCID ₅₀ /mL	Tricore	ATCC
RSV B Strain Wash	10 ⁴ TCID ₅₀ /mL	Tricore	ATCC
Varicella Zoster Virus	10 ⁴ TCID ₅₀ /mL	Tricore	ATCC
Bacteria Strains			
<i>Bordetella pertussis</i>	10 ⁷ cfu/mL	Microbiologics	ATCC
<i>Bordetella bronchoiseptica</i>	10 ⁷ cfu/mL	Microbiologics	ATCC
<i>Chlamydia pneumonia</i>	10 ³ TCID ₅₀ /mL	Tricore	ATCC
<i>Legionella micdadei</i>	10 ⁶ cfu/mL	Tricore	ATCC
<i>Legionella pneumophila</i>	10 ⁶ cfu/mL	Microbiologics	ATCC
<i>Mycobacterium intracellulare</i>	10 ⁷ cfu/mL	Microbiologics	ATCC
<i>Mycobacterium tuberculosis</i>	10 ⁶ cfu/mL	Gen-Probe, San	ATCC

Analytical Specificity Panel Samples			
Organism	Concentration Tested	Cultured By	Original Source
		Diego, CA	
<i>Mycoplasma pneumonia</i>	10 ⁶ cfu/mL	Gen-Probe, San Diego, CA	ATCC
<i>Haemophilus influenza</i>	10 ⁷ cfu/mL	RMC	ATCC
<i>Pseudomonas aeruginosa</i>	10 ⁷ cfu/mL	WMH	ATCC
<i>Proteus vulgaris</i>	10 ⁷ cfu/mL	WMH	ATCC
<i>Proteus mirabilis</i>	10 ⁷ cfu/mL	RMC	ATCC
<i>Neisseria gonorrhoeae</i>	10 ⁷ cfu/mL	RMC	ATCC
<i>Neisseria meningitidis</i>	10 ⁷ cfu/mL	RMC	ATCC
<i>Neisseria mucosa</i>	10 ⁷ cfu/mL	Microbiologics	ATCC
<i>Klebsiella pneumonia</i>	10 ⁷ cfu/mL	WMH	ATCC
<i>Escherichia coli</i>	10 ⁷ cfu/mL	WMH	ATCC
<i>Moraxella catarrhalis</i>	10 ⁷ cfu/mL	Microbiologics	ATCC
<i>Corynebacterium diphtheriae</i>	10 ⁶ cfu/mL	Microbiologics	ATCC
<i>Lactobacillus plantarum</i>	10 ⁶ cfu/mL	Microbiologics	ATCC
<i>Streptococcus pneumoniae</i>	10 ⁷ cfu/mL	RMC	ATCC
<i>Streptococcus pyogenes</i>	10 ⁷ cfu/mL	RMC	ATCC
<i>Streptococcus salivarius</i>	10 ⁶ cfu/mL	Microbiologics	ATCC
<i>Staphylococcus epidermidis</i>	10 ⁷ cfu/mL	WMH	ATCC
<i>Staphylococcus aureus</i>	10 ⁷ cfu/mL	WMH	ATCC
Yeast Strain			
<i>Candida albicans</i>	10 ⁷ cfu/mL	WMH	ATCC
Influenza Virus of Swine Origin			
VR 897 A/New Jersey/8/76	2x10 ⁴ TCID ₅₀ /mL	ND*	ATCC
A/South Dakota/03/2008	2x10 ³ TCID ₅₀ /mL	Tricore	CDC
A/Wisconsin/10/1998	2x10 ³ TCID ₅₀ /mL	Tricore	CDC
A/Iowa/2006	2x10 ³ TCID ₅₀ /mL	Tricore	CDC
Influenza A Virus of Avian Origin			
H5N1/VN/1203	2.7ng/μL RNA	NA	University of Wisconsin, Madison, WI
H5N1/HK/486	1.4ng/μL RNA	NA	University of Wisconsin, Madison, WI

* This strain was not recultured and titered. The ATCC provided titer was used and the strain was tested at as high a concentration as possible. Tricore = TriCore Reference Laboratories, Albuquerque, NM

Zeptomatrix – Zeptomatrix, Inc., Buffalo, NY

Microbiologics = MicroBioLogics, Inc., St. Cloud, MN

RMC = Resurrection Medical Center, Chicago, IL

WMH = Waukesha Memorial Hospital, Waukesha, WI

ATCC = American Tissue Culture Collection, Manassas, VA

ND = Not Done

A Negative Control, which consisted of the M4 viral transport media spiked with the IC, was included with Analytical Specificity Panel samples. The Influenza A Subtyping Positive Control and was included with each detection run to test for global failure (absence of reagents, instrument failure, etc.). The Analytical

Specificity Panel samples (except for the four influenza viruses of swine origin and the two H5N1 strains, which were extracted on the MagNA Pure LC) and the Negative Control were extracted on the bioMérieux NucliSENS easyMAG and tested in triplicate on a Cepheid Smartcycler II using one lot of ProFAST+ reagents.

The ProFAST+ Assay did not cross-react with any of the Analytical Specificity Panel samples tested, except for two of the four influenza viruses of swine origin. Detailed Analytical Specificity results are presented in the following table:

Analytical Specificity Results				
Organism	Concentration Tested	A/H1 Detection Mean Ct/Standard Deviation	A/H3 Detection Mean Ct/Standard Deviation	A/2009 H1N1 Detection Mean Ct/Standard Deviation
A/H1	10 ² TCID ₅₀ /mL	28.60/0.17	-	-
A/H3	10 ¹ TCID ₅₀ /mL	-	28.67/0.12	-
A/2009 H1N1	10 ⁵ TCID ₅₀ /mL	-	-	20.57/0.15
Adenovirus 1/Adenoid 71	10 ⁶ TCID ₅₀ /mL	-	-	-
Adenovirus 7	10 ⁶ TCID ₅₀ /mL	-	-	-
Coronavirus 229E	10 ⁶ TCID ₅₀ /mL	-	-	-
Coxsackie B4	10 ⁴ TCID ₅₀ /mL	-	-	-
Coxsackie B5/10/2006	10 ⁵ TCID ₅₀ /mL	-	-	-
Cytomegalovirus	10 ⁴ TCID ₅₀ /mL	-	-	-
Echovirus 2	10 ⁶ TCID ₅₀ /mL	-	-	-
Echovirus 3	10 ⁵ TCID ₅₀ /mL	-	-	-
Echovirus 6	10 ⁵ TCID ₅₀ /mL	-	-	-
Echovirus 11	10 ⁶ TCID ₅₀ /mL	-	-	-
Enterovirus 68	10 ³ TCID ₅₀ /mL	-	-	-
Enterovirus 70	10 ³ TCID ₅₀ /mL	-	-	-
Epstein Barr Virus	10 ⁸ copies/mL	-	-	-
HSV Type 1 MacIntyre Strain	10 ⁵ TCID ₅₀ /mL	-	-	-
HSV Type 2 G strain	10 ⁵ TCID ₅₀ /mL	-	-	-
Human Metapneumovirus A2	10 ⁴ TCID ₅₀ /mL	-	-	-
Human Rhinovirus 1a	10 ³ TCID ₅₀ /mL	-	-	-
Human Rhinovirus	10 ³ TCID ₅₀ /mL	-	-	-
Influenza B/Wisconsin	10 ⁴ TCID ₅₀ /mL	-	-	-
Measles/7/2000	10 ⁴ TCID ₅₀ /mL	-	-	-
Mumps Virus	10 ⁴ TCID ₅₀ /mL	-	-	-
Parainfluenza Type 1	10 ⁴ TCID ₅₀ /mL	-	-	-
Parainfluenza Type 2	10 ⁶ TCID ₅₀ /mL	-	-	-
Parainfluenza Type 3	10 ⁶ TCID ₅₀ /mL	-	-	-
Parainfluenza Type 4	10 ⁴ TCID ₅₀ /mL	-	-	-
Poliovirus 1	10 ⁶ TCID ₅₀ /mL	-	-	-
RSV A Strain Long	10 ⁴ TCID ₅₀ /mL	-	-	-
RSV B Strain Wash	10 ⁴ TCID ₅₀ /mL	-	-	-
Varicella Zoster Virus	10 ⁴ TCID ₅₀ /mL	-	-	-
<i>Bordetella pertussis</i>	10 ⁷ cfu/mL	-	-	-

Analytical Specificity Results				
Organism	Concentration Tested	A/H1 Detection Mean Ct/Standard Deviation	A/H3 Detection Mean Ct/Standard Deviation	A/2009 H1N1 Detection Mean Ct/Standard Deviation
<i>Bordetella bronchoiseptica</i>	10 ⁷ cfu/mL	-	-	-
<i>Chlamydia pneumonia</i>	10 ³ TCID ₅₀ /mL	-	-	-
<i>Legionella micdadei</i>	10 ⁶ cfu/mL	-	-	-
<i>Legionella pneumophila</i>	10 ⁶ cfu/mL	-	-	-
<i>Mycobacterium intracellulare</i>	10 ⁷ cfu/mL	-	-	-
<i>Mycobacterium tuberculosis</i>	10 ⁶ cfu/mL	-	-	-
<i>Mycoplasma pneumonia</i>	10 ⁶ cfu/mL	-	-	-
<i>Haemophilus influenza</i>	10 ⁷ cfu/mL	-	-	-
<i>Pseudomonas aeruginosa</i>	10 ⁷ cfu/mL	-	-	-
<i>Proteus vulgaris</i>	10 ⁷ cfu/mL	-	-	-
<i>Proteus mirabilis</i>	10 ⁷ cfu/mL	-	-	-
<i>Neisseria gonorrhoeae</i>	10 ⁷ cfu/mL	-	-	-
<i>Neisseria meningitidis</i>	10 ⁷ cfu/mL	-	-	-
<i>Neisseria mucosa</i>	10 ⁷ cfu/mL	-	-	-
<i>Klebsiella pneumonia</i>	10 ⁷ cfu/mL	-	-	-
<i>Escherichia coli</i>	10 ⁷ cfu/mL	-	-	-
<i>Moraxella catarrhalis</i>	10 ⁷ cfu/mL	-	-	-
<i>Corynebacterium diphtheriae</i>	10 ⁶ cfu/mL	-	-	-
<i>Lactobacillus plantarum</i>	10 ⁶ cfu/mL	-	-	-
<i>Streptococcus pneumoniae</i>	10 ⁷ cfu/mL	-	-	-
<i>Streptococcus pyogenes</i>	10 ⁷ cfu/mL	-	-	-
<i>Streptococcus salivarius</i>	10 ⁶ cfu/mL	-	-	-
<i>Staphylococcus epidermidis</i>	10 ⁷ cfu/mL	-	-	-
<i>Staphylococcus aureus</i>	10 ⁷ cfu/mL	-	-	-
<i>Candida albicans</i>	10 ⁷ cfu/mL	-	-	-
VR 897 A/New Jersey/8/76	2x10 ⁴ TCID ₅₀ /mL*	-	-	31.57/0.12
A/South Dakota/03/2008	2x10 ³ TCID ₅₀ /mL	-	-	-
A/Wisconsin/10/1998	2x10 ³ TCID ₅₀ /mL	-	-	29.43/0.06
A/Iowa/2006	2x10 ³ TCID ₅₀ /mL	-	-	-
H5N1/VN/1203	2.7ng/μL RNA	-	-	-
H5N1/HK/486	1.4ng/μL RNA	-	-	-

* This strain was not recultured and titered. The ATCC provided titer was used and the strain was tested at as high a concentration as possible.

The cross-reactivity observed with two of the four influenza viruses of swine origin is expected, since the target for the 2009 H1N1 detection is the *HA* gene. It is anticipated that targeting the *HA* gene may also detect other viruses from the North American Swine lineage. However, swine-origin influenza viruses have

historically rarely surfaced in humans. Based on the literature, from 1958 until the current 2009 H1N1 Influenza outbreak, there have been 64 cases of S-OIV in humans. Fifty-seven of these cases were H1N1, six were characterized as belonging to the H3N2 lineage, and one was a triple-reassortant H1N2. Thirteen of these cases occurred in military personnel during a contained and short-lived (22 days) outbreak at Fort Dix in 1976. Excluding the Fort Dix outbreak, S-OIV cases in humans have averaged 1 to 2 per year and nearly all have been linked to exposure to swine. However, the issue of potential reactivity with other swine origin Influenza A strains should be addressed in the product package insert as the following: *“The ProFAST+ 2009H1N1 reagents may also react with other swine origin Influenza A strains. However, literature review indicated that Influenza Virus of Swine Origin have historically rarely surfaced in humans.”*

Ideally, seasonal Influenza Viruses (A/H1 and A/H3) and 2009 H1N1 Influenza strains, such as those tested in the analytical reactivity study, should also be tested at high virus titer (e.g., 10^6 TCID₅₀/mL) in the analytical specificity study. However, the prospective and retrospective clinical studies included a total of 258 Influenza A positive NPS samples, and no cross-reactivity between seasonal Influenza Viruses (A/H1 and A/H3) and 2009 H1N1 Influenza markers was observed. A substantial number of these Influenza A positive samples represented clinically high viral levels based on Ct values generated from the FDA cleared ProFlu+ Assay. Therefore, an additional analytical specificity study testing high titers of seasonal Influenza Viruses (A/H1 and A/H3) and 2009 H1N1 Influenza strains is not necessary.

In addition to laboratory testing, bioinformatics resources were used to predict cross reactivity of additional influenza A strains with the seasonal A/H1, seasonal A/H3 and A/2009 H1N1 targets. A significant number of mismatches are observed for each primer and probe when compared to the sequences of other Influenza A subtypes listed below. Optimal primer/probe annealing temp is 2°C - 5°C below the melt temperature (T_m) of the primer/probe. Due to the number of base pair mismatches for each Influenza A strain, the T_m for each analyte primer/probe set is estimated to be >20°C below the actual primer/probe T_m. This T_m difference would prevent amplification and/or detection of the subtypes listed below with the ProFAST+ A/H1, A/H3, or A/2009 H1N1 primer/probe sets. The ProFAST+ Assay primer and probe sequences for each Influenza A subtype, A/H1, A/H3 and A/2009 H1N1, are unique and are not expected to cross-react with the other Influenza A subtypes listed in the table below:

Simulated Analytical Specificity (sequence matches) with Additional Influenza A Strains		
Influenza A Strain	GenBank	Simulated Cross Reactivity
A/Cambodia/R0405050/2007 (H5N1)	FJ225472	Negative for A/H1, A/H3 and 2009 H1N1
A/Japanese white-eye/Hong Kong/1038/2006 (H5N1)	DQ992842	Negative for A/H1, A/H3 and 2009 H1N1
A/chicken/India/NIV33487/06 (H5N1)	EF362418	Negative for A/H1, A/H3 and 2009 H1N1
A/chicken/Vietnam/NCVD-016/2008 (H5N1)	FJ842476	Negative for A/H1, A/H3 and 2009 H1N1
A/chicken/Yunnan/1251/2003 (H5N1)	CY028979	Negative for A/H1, A/H3 and 2009 H1N1

Simulated Analytical Specificity (sequence matches) with Additional Influenza A Strains		
Influenza A Strain	GenBank	Simulated Cross Reactivity
A/common magpie/Hong Kong/645/2006 (H5N1)	DQ992839	Negative for A/H1, A/H3 and 2009 H1N1
A/duck/Hunan/795/2002 (H5N1)	CY028963	Negative for A/H1, A/H3 and 2009 H1N1
A/chicken/Pennsylvania/Sg-00426/2004 (H2N2)	CY036576	Negative for A/H1, A/H3 and 2009 H1N1
A/duck/PA/486/1969 (H6N1)	EU743286	Negative for A/H1, A/H3 and 2009 H1N1
A/mallard/Korea/GH171/2007 (H7N7)	FJ750872	Negative for A/H1, A/H3 and 2009 H1N1
A/turkey/Wisconsin/1/1966 (H9N2)	AB295601	Negative for A/H1, A/H3 and 2009 H1N1
A/swine/Hong Kong/NS857/2001 (H1N2)	GQ229348	Negative for A/H1, A/H3 and 2009 H1N1
A/swine/Sweden/1021/2009 (H1N2)	GQ495132	Negative for A/H1, A/H3 and 2009 H1N1
A/swine/Italy/306907/2003 (H1N1)	GQ175971	Negative for A/H1, A/H3 and 2009 H1N1
A/Swine/Wisconsin/125/97 (H1N1)	AF222026	Negative for A/H1, A/H3 and 2009 H1N1

g. Assay cut-off:

The “cutoff value” represents the fluorescent intensity signal level (reported in Relative Fluorescent Units) at which a “positive” reaction reaches a relative fluorescent intensity above the background or baseline of a “negative” reaction. If a sample exceeds the threshold in a detection channel during PCR, the sample is considered positive for that channel. If the sample does not exceed the threshold for a detection channel by the last PCR cycle, the sample is considered negative for that channel.

Cutoff Determination and Confirmation studies were conducted with two operators using two different lots of ProFAST+ Supermix.

Cutoff values (RFUs) of the ProFAST+ Assay were determined upon completion of the Cutoff Determination Study which included a training set of 58 negative and simulated positive samples prepared from individual NP swab clinical samples. Specifically, individual samples (n = 6) were spiked with each type of Influenza A virus (seasonal H1, seasonal H3 and 2009 H1N1) at 3 logs above the preliminary Limit of Detection (LoD) representing high positive NP swab samples. Individual samples (n = 18) were spiked with each of the three Influenza A strains at 2 times the preliminary LoD representing low positive NP swab samples. 34 samples were left unspiked. The high and low positive samples, as well as 30 negative samples were spiked with the Internal Control. Four (4) negative samples were not spiked with the Internal Control. The samples were evenly split into two aliquots to be run by two operators.

Each operator evenly divided their Cutoff Determination Study Panel samples into two series. Samples were extracted using the Roche MagNA Pure LC or the bioMérieux NucliSENS easyMAG. A negative viral transport media control spiked with IC was included with each extraction run. The extracted nucleic acids and the Influenza A Subtyping Positive RNA Control and the IC diluted in water were run using two different lots of ProFAST+ Supermix on the Cepheid

SmartCycler II.

The cutoff values were then verified in the Cutoff Confirmation Study against a set of retrospective clinical samples and controls (32 A/H1 positive, 32 A/H3 positive, 32 A/2009 H1N1 positive, 36 Influenza A negative NP swab samples, 18 Influenza A Subtyping Positive RNA Controls, 16 ICs in TM and 4 Negative Controls). The determined cutoff was confirmed if $\geq 90\%$ sensitivity and specificity was attained for each channel with the above mentioned set of retrospective clinical samples and controls. The Cutoff Confirmation Study Panel was split between two Operators.

Each Operator divided their Cutoff Confirmation Study Panel samples into two series. Internal Control (IC) was spiked into the appropriate samples prior to extraction. Samples were split evenly and extracted on the Roche MagNA Pure LC and the bioMérieux easyMAG. The negative controls (viral transport medium spiked with IC and unspiked) were included with each extraction run. The Influenza A Subtyping Positive RNA Control was included in each RT-PCR run.

In the FAM, TET, TxR and Cy5 channels, the final RFUs for all samples of the positives and negatives demonstrated minimal overlap. For a given detection, a range of possible cutoffs was generated by determining robust lower and upper boundaries of the threshold, based on final RFU that would minimize false positives and false negatives. Population distribution, Quantiles, and ROC curves were used in selecting cutoff thresholds for A/H1, A/H3, A/2009 H1N1 and Internal Control threshold settings.

Acceptable ranges for the potential cutoff were generated in the following manners: 1) ROC curves were generated and analyzed for all channels used with the ProFAST+ Assay. The threshold ranges that yielded the maximum accuracy were identified. 2) The distributions of the negatives and positives were first assessed for normality. a) If normal, the averages and standard deviations of the final RFU were calculated. For the lower limit of the acceptable range of cutoff, negative samples were analyzed. The lower threshold was defined as the average final RFU plus three standard deviations of the negatives, that is, the point at which greater than 99.7% of true negatives are expected to fall below assuming normality. Similarly, the upper limit of the acceptable range of cutoff threshold was determined using positives and was defined as the average final RFU minus three standard deviations. This is the point at which at least 99.7% of true positives are expected to be greater than, assuming normality. b) If the distribution was not normal, a Quantile approach was taken. The lower limit of the cutoff threshold range was defined minimally as the 99.5% quantile of the negative samples. The upper limit of the cutoff threshold range was defined maximally as the 0.5% quantile of the positive samples. 3) After settings were finalized the retrospective clinical samples were evaluated for sensitivity and specificity.

The cutoffs used in the Cutoff Determination and Confirmation Studies are

presented in the following table:

Cutoffs used in Cutoff Determination and Confirmation Studies

Channel	Target	EndPt Threshold (RFU)	
		Preliminary Cutoff used in Cutoff Determination Study*	Cutoff used in Cutoff Confirmation Study
FAM	A/H1	50	60
TET	A/H3	40	40
TxR	A/2009 H1N1	15	20
Cy5	Internal Control	25	25

* Established during product development

Analysis settings and threshold settings were determined for FAM (A/H1) = 60, TET (A/H3) = 40, TxRed (A/2009 H1N1) = 20, Cy5 (IC) = 25. These settings produced 100% positive agreement and 100% negative agreement in the set of retrospective clinical samples used during Cutoff Confirmation and produced 100% positive and negative agreement for A/H1, A/H3, and IC; and 96.9% positive agreement and 100% negative agreement for A/2009 H1N1, and met all acceptance criteria for the Cutoff Confirmation study. Therefore, cutoff values were confirmed as acceptable for use during the clinical trials and all subsequent analytical studies.

h. General Assay Analysis Settings:

Cepheid SmartCycler II				
1.7b, Dx Software				
Channel:	1	2	3	4
Dye Name:	FAM	TET	TxR	Cy5
Target:	Seasonal A/H1	Seasonal A/H3	A/2009 H1N1	Internal Control
Usage:	Assay**	Assay**	Assay**	Internal Control
Curve Analysis:	Primary Curve	Primary Curve	Primary Curve	Primary Curve
Thresh Setting:	Manual	Manual	Manual	Manual
Manual Thresh:	60	40	20	25
Auto Thresh:	N/A	N/A	N/A	N/A
Auto Min Cycle:	5	5	5	5
Auto Max Cycle:	10	10	10	10
Valid Min Cycle:	13	13	13	13
Valid Max Cycle:	45	45	45	45
Backgrd Subtract:	On	On	On	On
Bkgnd Min Cycle	5	5	5	5
Bkgnd Max Cycle	40	40	40	40
Boxcar Avg	0	0	0	0
EndPt Thresh	60	40	20	25
NC IC %	10*	10*	10*	10*
IC Delta	N/A	N/A	N/A	N/A

** Dx 3.0a/3.0b = Target

* Dx 3.0a/3.0b = NA

i. *Interfering Substances:*

An interfering substances study was carried out to examine whether a panel of endogenous and exogenous potential RT-PCR inhibitors affected the performance of the ProFAST+ Assay. Blood, mucin, or medications (prescription and over-the-counter) for relief of congestion, sore throat, allergy and asthma symptoms were spiked into simulated Influenza A positive NP swab matrix. A single strain of seasonal H1 influenza A, H1N1 A/Virginia/1/06, was used and spiked into negative NP pools at 2X Limit of Detection (LoD). High clinically relevant amounts of the potential inhibiting substances were added to spiked samples. An Internal Control (IC) was added to samples. Nucleic acid from the samples was extracted with the bioMérieux easyMAG. RT-PCR was performed in triplicate reactions for each sample on the Cepheid SmartCycler II. Any substance in which detection of A/H1 or the Internal RNA Control (IC) was not observed was considered an inhibitor of PCR. A Negative Control, which consisted of M4 viral transport media spiked with an IC, was included with the Interfering Substances Panel samples. The Influenza A Subtyping RNA Positive Control was included with each detection run to test for global errors.

The following table shows the interfering substances used for this study. As described in the justification column, the concentrations spiked directly into samples ranged from approximately 5% to 10% of recommended dose for some substances, while others were tested at concentrations reported in scientific literature or in references from other IVD package inserts. The substances consisted of nasal sprays (liquid and powder), ingestible pills and lozenges, injectables, and endogenous substances (blood and mucin).

Interfering Substances Test Concentrations			
Substance Name	Active Ingredient	Concentration Tested	Justification
Mucin (Bovine Submaxillary gland, type I-S)	Purified mucin protein	5%, 1%, and 0.1% w/v**	The amount possibly present on an NP swab and then diluted in ~3.0mL of VTM*
Blood (human), EDTA anticoagulated	N/A	2% (volume/volume)	Other Respiratory IVD's Package Insert*
Neo-Synephrine®	Phenylephrine HCl	15% (volume/volume)	10% of total recommended dose (45µl)*
Walgreens Original Anefrin Nasal Spray	Oxymetazoline Hydrochloride	15% (volume/volume)	10% of total recommended dose (45µl)*
Zicam Homeopathic Non-Drowsy Allergy Relief No Drip Liquid Nasal gel	Luffa Operculata, Galphimia Glauca, Histaminum Hydrochloricum, Sulphur	5% (volume/volume)	10% of total recommended dose (15µl)*
Walgreens Saline Nasal Spray	Sodium chloride with preservatives	15% (volume/volume) of dose	10% of total recommended dose (45µl)*
Beconase AQ®	Beclomethasone dipropionate	5% volume/volume	10% of total recommended dose*
Walgreens Sore Throat Lozenges, Cherry	Oral anesthetic/analgesic	0.62mg/ml; 1/20 drop, crushed; active ingredients:	5% of total dose*

Interfering Substances Test Concentrations			
Substance Name	Active Ingredient	Concentration Tested	Justification
		1.0mg/ml benzocaine, 1.7mg/ml menthol	
Relenza®	Zanamivir	5mg/ml	10% of total spray dose*
Tobramycin	Tobramycin	4.0µg/ml	10% of total recommended dose*
Bactroban Nasal ointment	Mupirocin	10mg/ml	10% of total recommended dose in Mupirocin ointment*
TamiFlu	Oseltamivir	7.5mg/ml	10% of total recommended dose*

* Concentration tested is 10% of suggested dose in a 0.3ml sample, or otherwise stated in table above.

**Mucin was tested at 3 different concentrations

The Interfering Substances Study results demonstrated that with the exception of mucin at 5% and 1% w/v, the interfering substances used in this study do not inhibit the detection of A/H1 nucleic acid using the ProFAST+ Assay. Inhibition of A/H1 at 2X LoD was observed for mucin at 5% and 1% w/v concentrations. A mucin concentration of 0.1% w/v did not inhibit the assay. Interfering Substances Study results are presented in the following table:

Interfering Substances Test Results				
Interfering Substance	Mean A/H1 Ct	Std Dev A/H1	Mean IC Ct	Std Dev IC
Mucin 5%*	ND	NA	ND	NA
Mucin 1%*	ND	NA	33.80	0.1
Mucin 0.1%	33.57	0.4	27.23	0.4
Blood	37.60	2	29.47	0.4
Neo-Syneprine	35.20	0.4	28.07	0.2
Anefrin Nasal Spray	34.73	0.6	27.83	0.1
Zicam	35.63	0.3	28.17	0.06
Saline Nasal Spray	35.07	0.6	27.90	0.1
Beconase AQ	35.63	1	28.23	0.1
Chloraseptic ® Sore Throat Lozenges	34.53	0.8	27.80	0.3
Relenza	35.07	0.6	27.87	0.3
Tobromycin	35.57	0.5	28.20	0.1
Mupirocin	34.87	0.7	27.57	0.3
TamiFlu	33.60	0.5	28.33	0.1
No Interfering Substance	34.87	0.5	28.10	0.2
Negative NP	0	0	28.33	0.2

*These samples inhibited the detection of A/H1 (both concentrations of mucin) and IC (in the 5% samples).

The issue of inhibitory effect of mucin at concentrations of 5% and 1% w/v should be addressed in the product package insert as the following: “*Highly viscous sample may have an inhibitory effect on detection of influenza A subtypes and the Internal RNA Control (IC) near the LoDs using the ProFAST+ Assay*”.

j. *Internal Control Interference:*

Competitive inhibition of the ProFAST+ Assay due to the presence of the IC was assessed. Simulated samples were tested with and without IC to determine if the presence of the IC inhibited the reaction. Three cultured and titrated (TCID₅₀/mL) strains of Influenza A (H1N1 A/Virginia/1/06, H3N2 A/California/07/04, and 2009 H1N1 Clinical Isolate) were used and were spiked into aliquots of a negative nasopharyngeal (NP) swab pool at one log above LoD, at LoD and at one log below the LoD for each strain as determined in the Analytical Sensitivity Study. Each concentration for each strain was prepared two different ways: spiked with the IC and without any Internal Control (IC) added. All dilution series were extracted in duplicate on the bioMérieux NucliSENS easyMAG. A Negative Control, Viral Transport Media (VTM) spiked with IC, was included for each extraction run. The Negative Control serves to monitor for contamination during the testing procedure. The Influenza A Subtyping RNA Control II (Positive Control) was included with each RT-PCR run to test for global errors (absence of reagent, instrument failure, etc.). The Positive Control does not require nucleic acid isolation.

The extracted nucleic acids were tested by RT-PCR in quintuplicate using one lot of ProFAST+ Real Time Assay reagents on the Cepheid SmartCycler II. This resulted in a total of 10 data points for each viral strain at each concentration spiked with IC and 10 data points for each viral strain at each concentration without the IC. The data were analyzed and the effects of competitive inhibition due to the presence of the IC were assessed. Competitive inhibition was to be considered if the difference in the LoD of the samples with and without IC was greater than one log and the mean Ct for concentrations with and without IC were greater than 3.3 CTs for all concentrations tested.

IC Interference Study results are presented in the following table:

Strains	Conc. (TCID ₅₀ /mL)	Results	RNA IC			No IC			ΔCT*
			Avg. CT	SD	% CV	Avg. CT	SD	% CV	
H1N1 A/Virginia/1/06 1x10 ⁴ TCID ₅₀ /mL	5x10 ⁰	10/10	31.25	0.03	0.66	30.96	0.2	0.57	0.29
	5x10 ⁻¹	10/10	39.02	2	4.90	37.97	0.7	1.92	1.05
	5x10 ⁻²	0/10	ND	NA	NA	ND	NA	NA	NA
H3N2 A/California/07/04 1x10 ^{4.5} TCID ₅₀ /mL	5x10 ⁰	10/10	29.23	0.1	0.33	29.34	0.1	0.37	-0.11
	5x10 ⁻¹	10/10	32.24	0.2	0.69	32.37	0.2	0.70	-0.13
	5x10 ⁻²	0/10	ND	NA	NA	ND	NA	NA	NA
2009 H1N1 Clinical Isolate #5 1x10 ^{6.5} TCID ₅₀ /mL	1x10 ³	10/10	27.49	0.5	1.65	27.69	0.3	1.04	-0.20
	1x10 ²	10/10	32.85	0.6	1.70	32.28	0.7	2.24	0.57
	1x10 ¹	0/10	ND	NA	NA	ND	NA	NA	NA

* The ΔCT column is the average CT data column for the IC samples minus the average CT data column for the No IC samples.

There was no evidence of competitive inhibition at the assay LoDs for detection of A/H1, A/H3 or A/2009 H1N1 in the presence IC using the ProFAST+ Assay. The assay demonstrated equivalent detection of LoD concentrations for three stains of influenza A with mean Ct value differences for each strain and

concentration ranging from -0.2 to 1.05.

k. *Competitive Inhibition Study*

Effects of competitive inhibition on the ProFAST+ Assay when two of the Assay's target organisms are present in a single sample were not evaluated in a Competitive Inhibition Study. However, multiple infections with different subtypes of influenza A are rare. The prospective and retrospective clinical studies conducted at four geographically diverse U.S. clinical laboratories for this 510k submission included a total of 1002 NPS samples, of which 258 were Influenza A positive samples. All of the 258 Influenza A positive samples represented samples positive for only one influenza A subtype, and no true co-infections of seasonal A/H1, A/H3 and A/2009 H1N1 was observed in the clinical studies. Therefore, an analytical Competitive Inhibition Study to assess the potential competition between Influenza A subtypes is not necessary.

l. *Carry-Over Contamination:*

To evaluate the degree of carry-over/cross-contamination that occurs with the use of the ProFAST+ Assay in association with nucleic acid extraction on the Roche MagNA Pure LC and the bioMérieux NucliSens easyMAG instruments and PCR on the Cepheid SmartCycler II thermocycler, an internal Carry-Over study was carried out by testing simulated human Influenza A (2009 H1N1 Influenza A) high positive samples run in series with A/2009 H1N1 high negative samples over 5 runs. The High Positive samples consisted of negative nasopharyngeal (NP) swab matrix spiked with a concentration 3 logs above the Limit of Detection (LoD) of a cultured and titered strain: Prodesse Influenza A 2009 H1N1 (Swine) Clinical Isolate #3. A maximum Ct value of 23 was targeted for the High Positive samples as a Ct of 23 or lower represented the lower end of the "clinical range" of the ProFAST+ Assay as seen in development. High Negative samples consisted of negative NP swab matrix spiked with a concentration of A/2009 H1N1 below the analytical LoD concentration (3 logs below LoD) such that > 95% of samples should be negative. The samples were processed and extracted in a High Positive/High Negative alternating fashion (i.e. checkerboard pattern) on the extraction instruments and likewise processed and run on the Cepheid SmartCycler II instrument in an alternating fashion. Eleven (11) High Positive (HP) and 11 High Negative (HN) samples and a Negative Control were extracted per run on the easyMAG and on the MagNA Pure LC for a total of 55 High Positive samples and 55 High Negative samples over 5 runs for each extraction instrument.

Carry-Over Contamination Study results are presented in the following table:

	easyMAG					MagNA Pure LC				
		A/2009 H1N1	IC				A/2009 H1N1	IC		
Sample ID	Total Number Correct	Average Ct Value	Std. Dev.	Average Ct Value	Std. Dev.	Total Number Correct	Average Ct Value	Std. Dev.	Average Ct Value	Std. Dev.
High Positive	55/55 (100%)	22.09	0.2	NA	NA	55/55 (100%)	22.97	0.2	NA	NA
High Negative	55/55 (100%)	NA	NA	28.89	0.6	55/55 (100%)	NA	NA	28.83	0.5

The results of this study demonstrated no evidence of carryover/cross-contamination over a 5 day course of processing High Positive samples alongside High Negative samples using the ProFAST+ Assay. 100% of the High Positive and High Negative samples were accurately detected and the mean Ct value for High Positive samples was ≤ 23 Ct (22.5).

m. Comparator/Reference Assays Analytical Validation Studies

The ProFAST+ Comparator/Reference Assays used as the comparison methods to the ProFAST+ Assay for the ProFAST+ Clinical Study include three independent 2-Step RT-PCR followed by b-directional sequencing assays which target conserved regions of the hemagglutinin (HA) gene of Seasonal A/H1, Seasonal A/H3 and A/2009 H1N1 Influenza A (Swine-Origin) subtypes. Although the gene target is the same as for the ProFAST+ Assay by necessity, the primers for the comparator/reference assays target different regions of the HA gene. The seasonal A/H1 target for the Reference Assay encompasses the ProFAST+ target in the HA gene. Targeting a different region within the HA gene was not possible due to the lack of conservation throughout the gene. The primers for the A/H1 Reference Assay are located outside the region detected by ProFAST+ resulting in detection of a total of 218 unique (additional) bases by the Reference Assay. A total of 19 Seasonal A/H1, 14 Seasonal A/H3 and 8 A/2009 H1N1 Swine-Origin primer sets were initially designed to target conserved regions of the *Hemagglutinin (HA)* gene. A single primer set for each target was chosen based on conservation of primers to the Influenza A subtype specific alignments generated from available sequences at NCBI, BLAST analysis, amplicon length, melting temperature, hairpin/self dimerization of the selected primers, and analytical performance as compared to the ProFAST+ Assay. Each Influenza A subtype PCR Supermix underwent optimization of primer concentration, enzyme concentration and cycling parameters. The RT mix which uses random hexamers and MuLV Reverse Transcriptase had been previously optimized for reverse transcription for the Pro hMPV+ Reference Assays, therefore, the same reagents and reactions conditions were used. PCR product generated using the ProFAST+ Reference Assays were analyzed on the Agilent 2100 Bioanalyzer for the presence of the correct size band for each Influenza A subtype.

The following analytical studies were independently performed for each of the three comparators: Analytical Sensitivity, Reactivity and Analytical Specificity.

Analytical Sensitivity

The analytical sensitivity (limit of detection - LoD) of the ProFAST+ Comparator/Reference Assays was determined using two strains of each of the three Influenza A subtypes (Seasonal H1, Seasonal H3 and 2009 H1N1 Influenza A(Swine-Origin)). The viral strains used in this study were cultured and titered by an independent virology lab. Each virus was serially diluted and spiked at concentrations of 2 logs above, 1 log above, at, and 1 log below the expected LoD (as predetermined during assay development) into negative NP swab matrix. Each viral dilution was tested in quintuplicate using the appropriate Reference Assay (i.e. A/H1 virus dilution series was tested with the A/H1 Reference Assay only) starting from sample extraction through RT-PCR. Extraction was performed using the Roche MagNA Pure LC. Complementary DNA (cDNA) was reverse transcribed from the extracted nucleic acid followed by RT-PCR using the appropriate individual master mix for each subtype of Influenza A. PCR products generated with the ProFAST+ Reference Assays were analyzed using the Qiagen QIAxcel (capillary electrophoresis). Samples demonstrating the appropriate size PCR product were confirmed by bi-directional genetic sequencing and sequences verified using NCBI BLAST Analysis (www.ncbi.nlm.nih.gov/blast/Blast.cgi). A total of five data points per virus concentration were generated. Analytical Sensitivity was determined to be the lowest concentration of virus detected $\geq 95\%$ of the time (5/5 replicates). This concentration was then confirmed by preparing 20 additional replicates at the proposed LoD concentration using the same negative NP swab matrix pool as the Determination portion of the study. In the event that the initial LoD concentration could not be confirmed (i.e. <19 replicates were not positive), the LoD confirmation was repeated using the next log higher concentration. At least 95% of the 20 replicates were required to test positive by Qiagen QIAxcel and a representative sample confirmed as positive by sequencing and BLAST Analysis to confirm the LoD for each viral strain.

Detailed analytical sensitivity data for the reference assays are presented in the following tables:

Analytical Sensitivity Determination Results					
Viral Strain	Concentration TCID ₅₀ /mL	# Replicates detected by QIAxcel	% detected by QIAxcel	# Replicates by Sequencing	% detected by Sequencing
H1N1 A/Virginia/1/06	1×10^1	5/5	100%	5/5	100%
	1×10^0	4/5	80%	4/5	80%
	1×10^{-1}	4/5	80%	4/5	80%
	1×10^{-2}	0/5	0%	N/T	N/T
H1N1 A/Hong Kong/2652/06	1×10^1	5/5	100%	N/T	N/T
	1×10^0	5/5	100%	N/T	N/T

Analytical Sensitivity Determination Results					
Viral Strain	Concentration TCID ₅₀ /mL	# Replicates detected by QIAxcel	% detected by QIAxcel	# Replicates by Sequencing	% detected by Sequencing
	1x10 ⁻¹	5/5	100%	5/5	100%
	1x10 ⁻²	1/5	20%	2/5	40%
H3N2 A/Anhui/1239/05	5x10 ¹	5/5	100%	N/T	N/T
	5x10 ⁰	5/5	100%	5/5	100%
	5x10 ⁻¹	3/5	60%	2/5	40%
	5x10 ⁻²	0/5	0%	N/T	N/T
H3N2 A/California/07/04	1x10 ²	5/5	100%	N/T	N/T
	1x10 ¹	5/5	100%	N/T	N/T
	1x10 ⁰	5/5	100%	5/5	100%
	1x10 ⁻¹	0/5	0%	0/5	0%
2009 H1N1 Clinical Isolate #1	1x10 ²	5/5	100%	5/5	100%
	1x10 ¹	2/5	40%	3/5	60%
	1x10 ⁰	0/5	0%	N/T	N/T
	1x10 ⁻¹	0/5	0%	N/T	N/T
2009 H1N1 Clinical Isolate #5	1x10 ²	5/5	100%	5/5	100%
	1x10 ¹	4/5	80%	4/5	80%
	1x10 ⁰	1/5	20%	N/T	N/T
	1x10 ⁻¹	1/5	20%	N/T	N/T

Analytical Sensitivity Confirmation Results			
Viral Strain	Conc TCID ₅₀ /mL	Replicates Detected by QIAxcel	% Detected by QIAxcel
H1N1 A/Virginia/1/06	1x10 ¹	20/20	100%
	1x10 ⁰	17/20	85%
H1N1 A/Hong Kong/2652/06	1x10 ⁻¹	20/20	100%
H3N2 A/Anhui/1239/05	5x10 ⁰	20/20	100%
H3N2 A/California/07/04	1x10 ⁰	20/20	100%
2009 H1N1 Clinical Isolate #1	1x10 ²	19/20	95%
2009 H1N1 Clinical Isolate #5	1x10 ²	20/20	100%

The following table shows the LoD comparison between the ProFAST+ Assay and the Comparator/Reference Assays. The LoD of the seasonal A/H3 and A/2009 H1N1 strains tested was the same for both the ProFAST+ Assay and their respective Comparator/Reference Assay. The LoD for the seasonal A/H1 was 1.5 log and 0.5 log less sensitive with the A/H1 Comparator/Reference Assay as compared to the ProFAST+ Assay.

ProFAST+ Reference Analytical Sensitivity – Limit of Detection in comparison to the ProFAST+ Assay		
Viral Strain	ProFAST+ Reference Assay Limit of Detection	ProFAST+ Real Time Assay Limit of Detection
H1N1 A/Virginia/1/06	1×10^1 TCID ₅₀ /mL	5×10^{-1} TCID ₅₀ /mL
H1N1 A/Hong Kong/2652/06	1×10^{-1} TCID ₅₀ /mL	5×10^{-2} TCID ₅₀ /mL
H3N2 A/Anhui/1239/05	5×10^0 TCID ₅₀ /mL	5×10^0 TCID ₅₀ /mL
H3N2 A/California/07/04	1×10^0 TCID ₅₀ /mL	1×10^0 TCID ₅₀ /mL
2009 H1N1 Clinical Isolate #1	1×10^2 TCID ₅₀ /mL	1×10^2 TCID ₅₀ /mL
2009 H1N1 Clinical Isolate #5	1×10^2 TCID ₅₀ /mL	1×10^2 TCID ₅₀ /mL

Analytical Reactivity

The reactivity or inclusivity of the ProFAST+ Comparator/Reference Assays was evaluated using multiple strains of Influenza A: 14 strains of seasonal H1, 14 strains of seasonal H3 and 8 strains of swine-origin (four 2009 H1N1 isolates and 4 Swine-Origin). Viral strains were selected to include strains from 1933 to the present, as well as from various geographical locations, to incorporate the genetic variation that may be encountered during the ProFAST+ Clinical Study. The panel included the same strains tested in the ProFAST+ Reactivity Study to confirm detection of similar subtypes and strains. Remaining nucleic acid from the ProFAST+ Assay Reactivity study was used or new dilutions and extractions were made at a higher concentration and retested if necessary. The Reactivity Panel consisted of cultured and titrated strains of Influenza A, spiked with Internal RNA Control and diluted in negative nasopharyngeal (NP) swab pool to a concentration of 2x LoD of the ProFAST+ Assay and extracted on the Roche MagNA Pure LC. Each sample was tested in duplicate reactions using the series of Reference Assays. Complementary DNA (cDNA) was reverse transcribed from the extracted nucleic acid using random hexamers and MuLV Reverse Transcriptase. PCR followed using an individual master mix for each subtype of Influenza A. The master mix contained the required components for PCR (buffer, MgCl₂, dNTP's, and Taq DNA Polymerase and subtype specific primer set). The PCR product generated with the Reference Assay Supermixes was analyzed for the appropriate sized product using the QIAGEN QIAxcel. A single replicate at the lowest concentration as determined by the QIAxcel was confirmed as positive for the appropriate subtype by bi-directional genetic sequencing and NCBI BLAST analysis.

The majority of strains analyzed in this study tested positive by the Comparator/Reference Assay as expected. The strains that tested negative during pre-verification studies and were not expected to be reactive towards the assay remained non-reactive. The following table summarizes the results:

ProFAST+ Reference Reactivity Panel Results					
Strain	Target	Concentration Detected	A/H1 Detection	A/H3 Detection	A/2009 H1N1 Detection
A/Taiwan/42/06	A/H1	2x10 ⁰ TCID ₅₀ /mL	+	-	-
A/Henan/8/05	A/H1	2x10 ⁰ TCID ₅₀ /mL	+	-	-
A/Fujian/156/00	A/H1	2x10 ¹ TCID ₅₀ /mL	+	-	-
Brazil/1137/99	A/H1	2x10 ⁵ TCID ₅₀ /mL	+	-	-
A/Kentucky/2/06	A/H1	2x10 ² TCID ₅₀ /mL	+	-	-
A/Hawaii/15/01	A/H1	2x10 ² TCID ₅₀ /mL	+	-	-
A/New Caledonia/12/99	A/H1	2x10 ² TCID ₅₀ /mL	+	-	-
A/Brisbane/59/2007	A/H1	2x10 ² TCID ₅₀ /mL	+	-	-
A/Solomon Islands/03/06	A/H1	2x10 ⁰ TCID ₅₀ /mL	+	-	-
A/Jiangxi/160/05	A/H1	2x10 ⁰ TCID ₅₀ /mL	+	-	-
VR 1520 A/WS/33	A/H1	5.62x10 ⁴ TCID ₅₀ /mL	-	-	-
VR 98 A1/Mal/302/54	A/H1	1.78x10 ⁶ CEID ₅₀ /mL	-	-	-
A/PR/8/34	A/H1	2.0x10 ⁶ TCID ₅₀ /mL	+	-	-
VR 546 A1/Denver/1/57	A/H1	1.78x10 ⁶ CEID ₅₀ /mL	-	-	-
A/Hiroshima/52/05	A/H3	2x10 ⁰ TCID ₅₀ /mL	-	+	-
A/Victoria/512/05	A/H3	2x10 ⁰ TCID ₅₀ /mL	-	+	-
VR 822 A/Victoria/3/75	A/H3	2x10 ² CEID ₅₀ /mL	-	+	-
A/Brazil/02/99	A/H3	2x10 ³ TCID ₅₀ /mL	-	+	-
A/New York/55/2004	A/H3	2x10 ⁰ TCID ₅₀ /mL	-	+	-
A/Hong Kong/2831/05	A/H3	2x10 ¹ TCID ₅₀ /mL	-	+	-
A/Port Chalmers/1/73	A/H3	2x10 ⁰ TCID ₅₀ /mL	-	+	-
A/Bahamas/2686/99	A/H3	2x10 ² TCID ₅₀ /mL	-	+	-
A/Fujian/411/02	A/H3	2x10 ² TCID ₅₀ /mL	-	+	-
A/Kentucky/03/06	A/H3	2x10 ² TCID ₅₀ /mL	-	+	-
A/Costa Rica/07/99	A/H3	2x10 ² TCID ₅₀ /mL	-	+	-
A/Hong Kong/218/06	A/H3	2x10 ¹ TCID ₅₀ /mL	-	+	-
VR 544 A/Hong Kong/8/68	A/H3	2x10 ² CEID ₅₀ /mL	-	+	-
VR 547 A/Aichi/2/68	A/H3	2x10 ² CEID ₅₀ /mL	-	+	-
2009 H1N1 Clinical Isolate #2	S-OIV	2x10 ³ TCID ₅₀ /mL	-	-	+
2009 H1N1 Clinical Isolate #3	S-OIV	2x10 ² TCID ₅₀ /mL	-	-	+
2009 H1N1 Clinical Isolate #4	S-OIV	2x10 ¹ TCID ₅₀ /mL	-	-	+
2009 H1N1 A/California/04/2009	S-OIV	2x10 ² TCID ₅₀ /mL	-	-	+
VR 897 A/New Jersey/8/76	S-OIV	2x10 ⁴ TCID ₅₀ /mL	-	-	-
A/South Dakota/03/2008	S-OIV	2x10 ³ TCID ₅₀ /mL	-	-	+
A/Wisconsin/10/1998	S-OIV	2x10 ³ TCID ₅₀ /mL	-	-	+
A/Iowa/2006	S-OIV	2x10 ³ TCID ₅₀ /mL	-	-	+

There were four strains that were not reactive with the ProFAST+ Comparator/Reference Assays. The first 3 listed are also not reactive with the ProFAST+ Assay, several base pair mismatches occur between the A/New Jersey/8/76 strain and the primers for A/2009 H1N1 which is why that strain is not detected.

- VR 1520 A/WS/33 (A/H1)
- VR 98 A1/Mal/302/54 (A/H1)
- VR 546 A1/Denver/1/57 (A/H1)
- VR 897 A/New Jersey/8/76(A/S-OIV)

Based on the analytical validations (i.e., sensitivity and reactivity), these Comparator/Reference Assays performed equivalently to the ProFAST+ Assay for Analytical Sensitivity and Reactivity. They are therefore acceptable methods to be used as a part of composite reference method in determining “clinical diagnostic truth” for the ProFAST+ Clinical Trial.

2. Comparison studies:

a. *Method comparison with predicate device:*

Not applicable.

b. *Matrix Description and Comparison:*

Analytical performance of the ProFAST+ Assay using six different viral transport media (VTM) was evaluated. Analytical sensitivity was determined using cultured and titered strains of human Influenza A viruses (A/H1, A/H3, and A/2009 H1N1) spiked into each of the six different VTMs.

The intended use of Remel M4, M5, and M6, Copan Universal Transport Medium (UTM), and Becton Dickinson Universal Viral Transport (UVT) is for the transport of clinical specimens containing viruses, chlamydiae, mycoplasma, and ureaplasma from the collection site to the laboratory for microbiological procedures. Remel M4RT VTM is for the transport of clinical specimens containing viruses or chlamydiae, but is not recommended for the transport of mycoplasmas and ureaplasmas. The VTMs have essentially the same components as shown in the table below:

Composition of Viral Transport Media						
	Remel M4	Remel M5	Remel M6	Remel M4 RT	Copan UTM	BD UTV
Components	Hank's Balanced Salts Bovine Serum Albumin Gelatin	Hank's Balanced Salts Bovine Serum Albumin Protein Stabilizers	Hank's Balanced Salts Bovine Serum Albumin Gelatin	Hank's Balanced Salts Bovine Serum Albumin Gelatin	Hank's Balanced salts Bovine Serum Albumin Gelatin	Hank's Balanced Salts Bovine Serum Albumin Gelatin
	Sucrose L-Glutamic acid HEPES Buffer Phenol Red Vancomycin Amphotericin B Collistin	Sucrose L-Glutamic acid HEPES Buffer Phenol Red Vancomycin Amphotericin B Collistin	Sucrose L-Glutamic acid HEPES Buffer Phenol Red Vancomycin Amphotericin B Collistin	Sucrose L-Glutamic acid HEPES Buffer Phenol Red Gentamicin Amphotericin B	Sucrose L-Glutamic Acid HEPES Buffer Phenol Red Vancomycin Amphotericin B Colistin	Sucrose L-Glutamic acid HEPES Buffer Phenol Red Vancomycin Amphotericin B Collistin

					L- Cysteine	L-Cysteine
pH	7.3 +/- 0.2 @ 25°C	7.3 +/- 0.2 @ 25°C	7.3 +/- 0.2 @ 25°C	7.3 +/- 0.2 @ 25°C	7.3 +/- 0.2 @ 25°C	7.3 +/- 0.2 @ 25°C
Storage Temperature	Frozen at -25°C or 2-8°C until use	Frozen at -25°C or 2-8°C until use	2-30°C until use	2-30°C until use	2-25°C until use	2-25°C until use
Volume per Vial	3mL	3mL	1.5 ml	3.0 ml	3mL	3mL

Two cultured and titered Influenza A viral isolates (A/H1 and A/2009 H1N1) were spiked at LoD, and one, and two logs below the LoD and the A/H3 viral isolate was spiked at one log above, at, and one log below LoD (as determined in the ProFAST+ Analytical Sensitivity Study) into each VTM type. The testing was performed at lower concentrations for A/H1 and A/2009 H1N1 because initial testing at 1 log below the LoD (as determined in the ProFAST+ Analytical Sensitivity Study) resulted in 100% detection. Differences in Analytical Sensitivity between NP swab matrix (as used in the Analytical Sensitivity Study) and VTM alone (as used in the present study) are not unusual and VTM alone typically results in a lower LoD.

All VTMs were compared to Remel M4 to determine equivalence because that is the VTM used in all other analytical verification studies, including the Analytical Sensitivity study, as well as by the majority of the Clinical Trial sites. For a VTM to be considered equivalent to M4:

1. All three replicates for at least one concentration were required to be positive and within one log of the lowest concentration in which all 3 replicates of M4 are positive.
2. The average CT values at all concentrations in which 3 of 3 replicates are positive shall not differ by more than 3.3 CTs from the average CT of that concentration diluted in M4. If the sample differs by more than 3.3 CTs the media will not be considered equivalent.

Each of the VTM performed “equivalently” to Remel M4 as they all met the required criteria described above. Detailed data are presented in the following table:

VTM Study Results Summary Comparison														
		Conc TCID ₅₀ /mL	Remel M4		Remel M5		Remel M6		Remel M4RT		Copan UTM		BD UVT	
			Avg Ct	Ct SD	Avg Ct	Ct SD	Avg Ct	Ct SD	Avg Ct	Ct SD	Avg Ct	Ct SD	Avg Ct	Ct SD
Influenza A Strain	A/H1	5x10 ⁻¹	35.37	0.2	35.17	0.2	35.40	0.4	35.93	0.8	34.77	0.4	35.20	0.2
		5x10 ⁻²	38.73	0.4	38.90	0.6	39.20	1	38.33	0.5	38.17	0.9	38.87	0.7
		5x10 ⁻³	0.00	NA	40.80*	NA	0.00	NA	40.40*	NA	39.90**	1	40.10*	NA
	A/H3	5x10 ⁰	30.67	0.1	30.70	0.1	30.73	0.1	30.70	0.3	30.57	0.2	30.60	0.1
		5x10 ⁻¹	34.03	0.3	33.93	0.3	34.30	0	34.13	0.3	33.67	0.7	33.87	0.2
		5x10 ⁻²	36.47	0.4	37.17	0.5	37.40	1	36.73	0.2	37.53	0.5	36.40**	0.3
		5x10 ⁻³	0.00	NA	38.65**	0.07	0.00	NA	0.00	NA	0.00	NA	0.00	NA
		5x10 ⁻⁴	0.00	NA	0.00	NA	0.00	NA	0.00	NA	0.00	NA	0.00	NA
	A/2009 H1N1	1x10 ²	32.73	0.3	32.80	0.4	33.50	0.5	33.07	0.7	33.40	0.8	32.33	0.2
		1x10 ¹	36.07	0.4	36.10	0.1	36.97	0.7	36.90	2	35.80	0.5	35.73	0.4
		1x10 ⁰	38.95**	0.07	37.30*	NA	38.60*	NA	38.55**	0.4	40.50*	NA	38.30*	NA

*One of three replicated detected.

**Two of three replicates detected.

All six VTMs evaluated are compatible with the ProFAST+ Assay.

3. Clinical studies:

a. *Prospective Clinical studies*

The clinical performance of the ProFAST+ Assay was established during prospective studies at 4 U.S. clinical laboratories. NP swab samples were collected and tested at three U.S. clinical laboratories (Sites 1, 2, and 4) during December 2009 thru May 2010. Due to the absence of seasonal (H1N1 or H3N2) and 2009 H1N1 Influenza A during the typical 2009-2010 winter season, prospectively collected archived samples were also included in the prospective studies. These samples were collected from January – March, 2008, February – March, 2009 and October – November, 2009, and tested at two U.S. clinical laboratories (Sites 1 and 3). All specimens used in the study meeting the inclusion and exclusion criteria represented excess, remnants of nasopharyngeal (NP) swab specimens that were prospectively collected from symptomatic individuals suspected of respiratory infection, and were submitted for routine care or analysis by each site, and that otherwise would have been discarded. Individual specimens were delinked from all patient identifiers and given a study sample code. All clinical sites were granted waivers of informed consent by their IRBs for this study. Inclusion criteria included, but were not limited to: the specimen was from a symptomatic patient (for respiratory infection) that was submitted for routine respiratory pathogen testing; the specimen was a NP swab in appropriate viral transport media (i.e., Remel M4 or M5 viral transport medium); the specimen contained adequate volume for comparator/reference methods and the ProFAST+ Assay; age and gender information were available; specimen was stored properly (refrigerated at 2°C – 8°C until nucleic acid extraction was performed within 72 hours of the sample collection time. Or, samples were stored frozen at ≤ -70°C within 72 hours of collection until

processing through ProFAST+ and comparator/reference assays); and initiation of the ProFAST+ Assay (nucleic acid extraction) took place within 72 hours of sample collection, or samples were frozen at $\leq -70^{\circ}\text{C}$ within 72 hours.

The specimens and controls (underwent nucleic acid extraction using the bioMérieux NucliSENS easyMAG Instrument using bioMérieux NucliSENS easyMAG reagents (Sites 1 and 3) or using the Roche MagNA Pure LC Instrument using the Total Nucleic Acid Isolation kit (Sites 2 and 4). Purified nucleic acids were tested with the ProFAST+ Assay and the ProFlu+ Assay on the Cepheid SmartCycler II. (Note: Purified nucleic acids were further processed immediately following extraction or were stored frozen ($\leq -70^{\circ}\text{C}$) and thawed prior to ProFAST+ Assay and ProFlu+ Assay set-up). Remaining nucleic acids and original samples were sent back to Gen-Probe Prodesse for sequencing analysis.

Reverse transcription was performed on remaining nucleic acids that had been stored at $\leq -70^{\circ}\text{C}$. PCR was performed on the resulting cDNA using all three Comparator/Reference Assays and the PCR products were analyzed by capillary electrophoresis (QIAxcel, QIAGEN). PCR products with the appropriate base pair band size were sent for further analysis by genetic sequencing. Sequencing results that met acceptance criteria (>100 contiguous bases, QV score (i.e., PHRED) > 20 , E-value scores $<1\text{e-}20$) for both the forward and reverse primer were recorded as positive for the specific Influenza A subtype.

Performance of the ProFAST+ Assay was compared to the composite comparator/reference method of the FDA cleared ProFlu+ Assay IVD (K081030) and individual well characterized Influenza A subtype specific RT-PCR assays followed by bi-directional sequencing. The sequencing assays targeted different regions of the hemagglutinin gene than the ProFAST+ Assay and were specific for each of the Influenza A subtypes (A/H1, A/H3, and A/2009 H1N1). “True” seasonal A/H1, A/H3 or A/2009 H1N1 RNA positives, were considered as any sample that was tested positive for Influenza A by the ProFlu+ Assay IVD, and had bi-directional sequencing data meeting pre-defined quality acceptance criteria, for both the forward and the reverse sequences that matched seasonal A/H1, A/H3, and A/2009 H1N1 sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov), respectively, with acceptable E-values. “True” seasonal A/H1, A/H3 or A/2009 H1N1 RNA negatives were considered as any sample that was tested negative for Influenza A by the ProFlu+ Assay IVD, or any sample that was tested positive for Influenza A by the ProFlu+ Assay IVD, but was tested negative by the respective Influenza A subtype specific RT-PCR assay. The E-values generated from the clinical trial range from a low of $<1\text{e-}180$ to a high of $2\text{e-}35$. The E-Value from NCBI BLAST Alignment indicates the statistical significance of a given pair-wise alignment and reflects the size of the database and the scoring system used. The lower the E-Value, the more significant the hit. A sequence alignment that has an E-Value of $1\text{e-}3$ means that this similarity has a 1 in 1000 chance of occurring by chance alone. (<http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=handbook.section.614>).

Therefore an E-Value ranging from $<2e-35$ to $1e-180$ has a very low probability of occurring purely by chance.

The overall assay failure rate of the ProFAST+ Assay during the clinical trials (both the prospective and the retrospective trials) due to failure of Positive, Negative or Extraction Controls not related to procedural errors was 4.0% (total of 3 of 75 runs across all 4 sites). Two of the run failures were due to the Negative Control contamination (one run at Site 3 and one run at Site 4). The other invalid run was due to the PC failure (none of the analytes were detected) and it is hypothesized that the PC was mistakenly not added to the ProFAST+ master mix. All samples, the Negative Control and the Extraction Control were re-run using the ProFAST+ Assay starting from the purified nucleic acid and repeat test results were “Valid”.

A total of 874 prospective NP swab specimens were initially included in the prospective clinical trial (400 samples from Site 1, 129 samples from Site 2, 300 samples from Site 3, and 45 samples from Site 4). Thirty two (32) samples (18 samples from Site 1, 10 samples from Site 2, 2 samples from Site 3, and 2 samples from Site 4) were excluded from the prospective clinical study data analysis because they remained “Unresolved” after repeat testing for either the ProFlu+ Assay (comparator assay), or the ProFAST+ Assay, or both assays, resulted in a total 842 eligible prospective specimens to be included in the prospective clinical study data analysis.

Of the ProFAST+ Assay run on all prospective specimens, 98.9% (864/874) of these specimens were successful on the first attempt (Site 1: $392/400 = 98.0\%$; Site 2: $128/129 = 99.2\%$; Site 3: $299/300 = 99.7\%$; Site 4: $45/45 = 100\%$). The remaining 10 ($10/874 = 1.1\%$) gave “Unresolved” results on the first attempt (8 from Site 1, 1 from Site 2, 1 from Site 3, and none from Site 4). Unresolved results occur when the sample is negative for all three Influenza A subtype markers and the Internal Control, indicating potentially PCR-inhibiting samples. Of the 10 “Unresolved” specimens on the first attempt with sufficient sample for retest, only 50.0% (5/10) gave a valid result on the second attempt (3 from Site 1, 1 from site 2, and 1 from Site 3). The remaining 5 were “Unresolved” on the second attempt.

The following tables represent the prospective clinical data by site and by influenza A subtypes:

Seasonal Influenza A/H1

ProFAST+ A/H1 Results Site 1	Composite Reference		
	Positive	Negative	Total
Positive	14	1*	15
Negative	0	367	367
Total	14	368	382
Positive Percent Agreement: 14/14 100.0% (95%CI: 76.8%-100.0%)			
Negative Percent Agreement: 367/368 99.7% (95%CI: 98.5%-100.0%)			

* One (1) sample was negative for Influenza A by the ProFlu+ Assay, but positive for seasonal A/H1 by bi-directional sequence analysis.

ProFAST+ A/H1 Results Site 2	Composite Reference		
	Positive	Negative	Total
Positive	0	0	0
Negative	0	119	119
Total	0	119	119
Positive Percent Agreement: 0/0			N/A
Negative Percent Agreement: 119/119			100.0% (95%CI: 96.9%-100.0%)

ProFAST+ A/H1 Results Site 3	Composite Reference		
	Positive	Negative	Total
Positive	39	7*	46
Negative	0	252	252
Total	39	259	298
Positive Percent Agreement: 39/39			100.0% (95%CI: 91.0%-100.0%)
Negative Percent Agreement: 252/259			97.3% (95%CI: 94.5%-98.9%)

* One (1) sample was negative for Influenza A by the ProFlu+ Assay, but positive for seasonal A/H1 by bi-directional sequence analysis. One (1) sample was negative for Influenza A by the ProFlu+ Assay, and negative for seasonal A/H1 by bi-directional sequence analysis, but positive for Influenza A, un-subtypable, by the FDA cleared CDC rRT-PCR Influenza Panel. Five (5) samples were positive for Influenza A by the ProFlu+ Assay, negative for A/H1, A/H3 and A/2009 H1N1 by bi-directional sequence analysis, but positive for A/H1 by the FDA cleared CDC rRT-PCR Influenza Panel.

ProFAST+ A/H1 Results Site 4	Composite Reference		
	Positive	Negative	Total
Positive	0	0	0
Negative	0	43	43
Total	0	43	43
Positive Percent Agreement: 0/0			N/A
Negative Percent Agreement: 43/43			100.0% (95%CI: 91.8%-100.0%)

Seasonal Influenza A/H3

ProFAST+ A/H3 Results Site 1	Composite Reference		
	Positive	Negative	Total
Positive	16	0	16
Negative	0	366	366
Total	16	366	382
Positive Percent Agreement: 16/16			100.0% (95%CI: 79.4%-100.0%)
Negative Percent Agreement: 366/366			100.0% (95%CI: 99.0%-100.0%)

ProFAST+ A/H3 Results Site 2	Composite Reference		
	Positive	Negative	Total
Positive	0	0	0
Negative	0	119	119
Total	0	119	119
Positive Percent Agreement: 0/0			N/A
Negative Percent Agreement: 119/119			100.0% (95%CI: 96.9%-100.0%)

ProFAST+ A/H3 Results Site 3	Composite Reference		
	Positive	Negative	Total
Positive	9	2*	11
Negative	0	287	287
Total	9	289	298
Positive Percent Agreement: 9/9 100.0% (95%CI: 66.4%-100.0%)			
Negative Percent Agreement: 287/289 99.3% (95%CI: 97.5%-99.9%)			

*One (1) sample was positive for Influenza A by the ProFlu+ Assay, negative for A/H1, A/H3 and A/2009 H1N1 by bi-directional sequence analysis, but positive for A/H3 by the FDA cleared CDC rRT-PCR Influenza Panel. One (1) sample was positive for Influenza A by the ProFlu+ Assay, positive for A/H1 and negative for A/H3 and A/2009 H1N1 by bi-directional sequence analysis.

ProFAST+ A/H3 Results Site 4	Composite Reference		
	Positive	Negative	Total
Positive	0	1*	1
Negative	0	42	42
Total	0	43	43
Positive Percent Agreement: 0/0 N/A			
Negative Percent Agreement: 42/43 97.7% (95%CI: 87.7%-99.9%)			

* One (1) sample was negative for Influenza A by the ProFlu+ Assay, also negative for seasonal A/H1 and A/H3, and A/2009 H1N1 by bi-directional sequence analysis.

2009 H1N1 Influenza Virus

ProFAST+ A/2009 H1N1 Results Site 1	Composite Reference		
	Positive	Negative	Total
Positive	31	0	31
Negative	0	351	351
Total	31	351	382
Positive Percent Agreement: 31/31 100.0% (95%CI: 88.8%-100.0%)			
Negative Percent Agreement: 351/351 100.0% (95%CI: 99.0%-100.0%)			

ProFAST+ A/2009 H1N1 Results Site 2	Composite Reference		
	Positive	Negative	Total
Positive	0	0	0
Negative	0	119	119
Total	0	119	119
Positive Percent Agreement: 0/0 N/A			
Negative Percent Agreement: 119/119 100.0% (95%CI: 96.9%-100.0%)			

ProFAST+ A/2009 H1N1 Results Site 3	Composite Reference		
	Positive	Negative	Total
Positive	31	0	31
Negative	3	264	267
Total	34	264	298
Positive Percent Agreement: 31/34 91.2% (95%CI: 76.3%-98.1%)			
Negative Percent Agreement: 264/264 100.0% (95%CI: 98.6%-100.0%)			

ProFAST+ A/2009 H1N1 Results Site 4	Composite Reference		
	Positive	Negative	Total
Positive	0	0	0
Negative	0	43	43
Total	0	43	43
Positive Percent Agreement:	0/0	N/A	
Negative Percent Agreement:	43/43	100.0%	(95%CI: 91.8%-100.0%)

The prospective performance data from all study sites combined are presented in the following tables by influenza A subtype:

Seasonal Influenza A/H1

ProFAST+ A/H1 Results Combined	Composite Reference		
	Positive	Negative	Total
Positive	53	8*	61
Negative	0	781	781
Total	53	789	842
Positive Percent Agreement:	53/53	100.0%	(95%CI: 93.2%-100.0%)
Negative Percent Agreement:	781/789	99.0%	(95%CI: 98.0%-99.5%)

* Two (2) samples were negative for Influenza A by the ProFlu+ Assay, but positive for seasonal A/H1 by bi-directional sequence analysis. One (1) sample was negative for Influenza A by the ProFlu+ Assay, and negative for seasonal A/H1 by bi-directional sequence analysis, but positive for Influenza A, un-subtypable, by the FDA cleared CDC rRT-PCR Influenza Panel. Five (5) samples were positive for Influenza A by the ProFlu+ Assay, negative for A/H1, A/H3 and A/2009 H1N1 by bi-directional sequence analysis, but positive for A/H1 by the FDA cleared CDC rRT-PCR Influenza Panel.

Seasonal Influenza A/H3

ProFAST+ A/H3 Results Combined	Composite Reference		
	Positive	Negative	Total
Positive	25	3*	28
Negative	0	814	814
Total	25	817	842
Positive Percent Agreement:	25/25	100.0%	(95%CI: 86.7%-100.0%)
Negative Percent Agreement:	814/817	99.6%	(95%CI: 98.9%-99.9%)

* One (1) sample was negative for Influenza A by the ProFlu+ Assay, also negative for seasonal A/H1 and A/H3, and A/2009 H1N1 by bi-directional sequence analysis. *One (1) sample was positive for Influenza A by the ProFlu+ Assay, negative for A/H1, A/H3 and A/2009 H1N1 by bi-directional sequence analysis, but positive for A/H3 by the FDA cleared CDC rRT-PCR Influenza Panel. One (1) sample was positive for Influenza A by the ProFlu+ Assay, positive for A/H1 and negative for A/H3 and A/2009 H1N1 by bi-directional sequence analysis.

2009 H1N1 Influenza Virus

ProFAST+ A/2009 H1N1 Results Combined	Composite Reference		
	Positive	Negative	Total
Positive	62	0	62
Negative	3	777	780
Total	65	777	842
Positive Percent Agreement:	62/65	95.4%	(95%CI: 87.3%-98.4%)
Negative Percent Agreement:	777/777	100.0%	(95%CI: 99.5%-100.0%)

The general demographic data for all eligible prospective specimens (N=842) are presented in the following table:

Sex	Number of Subjects
Male	439/842 (52.1%)
Female	403/842 (47.9%)
Age (yrs)	
≤ 5 years	439/842 (52.1%)
6 - 18 years	184/842 (21.9%)
19 – 64 years	168/842 (20.0%)
≥ 65 years	51/842 (6.0%)

b. Retrospective Clinical studies

In addition to the prospective clinical studies, two clinical sites (Site 2 and site 4) also performed testing using retrospective samples that were collected from January – March, 2008, January – November 2009, and March 2010. The ProFAST+ Assay was compared to the same composite comparator/reference method that was employed for the prospective study to determine clinical Percent Positive Agreement and Percent Negative Agreement. A total of 160 retrospective nasopharyngeal (NP) swab samples were included in the retrospective studies (60 samples at Site 2 and 100 samples at site 4). The E-values generated from the retrospective clinical trial range from a low of <1e-180 to a high of 7e-45. The E-Value from NCBI BLAST Alignment indicates the statistical significance of a given pair-wise alignment and reflects the size of the database and the scoring system used. The lower the E-Value, the more significant the hit. A sequence alignment that has an E-Value of 1e-3 means that this similarity has a 1 in 1000 chance of occurring by chance alone.

(<http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=handbook.section.614>).

Therefore an E-Value ranging from 7e-45 to <1e-180 has a very low probability of occurring purely by chance.

These data were analyzed separately from the prospective samples and are summarized in the following tables by site and by influenza A subtype:

Seasonal Influenza A/H1

ProFAST+ A/H1 Result Site 2	Composite Reference		
	Positive	Negative	Total
Positive	17	0	17
Negative	1	42	43
Total	18	42	60
Positive Percent Agreement:	17/18	94.4%	(95%CI: 72.7%-99.9%)
Negative Percent Agreement:	42/42	100.0%	(95%CI: 91.6%-100.0%)

ProFAST+ A/H1 Results Site 4	Composite Reference		
	Positive	Negative	Total
Positive	0	1*	1
Negative	0	99	99
Total	0	100	100
Positive Percent Agreement:	0/0	N/A	
Negative Percent Agreement:	99/100	99.0%	(95%CI: 94.5%-100.0%)

* One (1) sample was negative for Influenza A by the ProFlu+ Assay, but positive for seasonal A/H1 by bi-directional sequence analysis.

Seasonal Influenza A/H3

ProFAST+ A/H3 Results Site 2	Composite Reference		
	Positive	Negative	Total
Positive	34	0	34
Negative	0	26	26
Total	34	26	60
Positive Percent Agreement:	34/34	100.0%	(95%CI: 89.7%-100.0%)
Negative Percent Agreement:	26/26	100.0%	(95%CI: 86.8%-100.0%)

ProFAST+ A/H3 Results Site 4	Composite Reference		
	Positive	Negative	Total
Positive	38	0	38
Negative	0	62	62
Total	38	62	100
Positive Percent Agreement:	38/38	100.0%	(95%CI: 90.7%-100.0%)
Negative Percent Agreement:	62/62	100.0%	(95%CI: 94.2%-100.0%)

2009 H1N1 Influenza Virus

ProFAST+ A/2009 H1N1 Results Site 2	Composite Reference		
	Positive	Negative	Total
Positive	5	0	5
Negative	0	55	55
Total	5	55	60
Positive Percent Agreement:	5/5	100.0%	(95%CI: 47.8%-100.0%)
Negative Percent Agreement:	55/55	100.0%	(95%CI: 93.5%-100.0%)

ProFAST+ A/2009 H1N1 Results Site 4	Composite Reference		
	Positive	Negative	Total
Positive	20	0	20
Negative	0	80	80
Total	20	80	100
Positive Percent Agreement:	20/20	100.0%	(95%CI: 83.2%-100.0%)
Negative Percent Agreement:	80/80	100.0%	(95%CI: 95.5%-100.0%)

The retrospective performance data from all study sites combined are presented in the following tables by influenza A subtypes:

Seasonal Influenza A/H1

ProFAST+ A/H1 Results Combined	Composite Reference		
	Positive	Negative	Total
Positive	17	1*	18
Negative	1	141	142
Total	18	142	160
Positive Percent Agreement:	17/18	94.4%	(95%CI: 74.3%-99.0%)
Negative Percent Agreement:	141/142	99.3%	(95%CI: 96.1%-99.9%)

* One (1) sample was negative for Influenza A by the ProFlu+ Assay, but positive for seasonal A/H1 by bi-directional sequence analysis.

Seasonal Influenza A/H3

ProFAST+ A/H3 Results Combined	Composite Reference		
	Positive	Negative	Total
Positive	72	0	72
Negative	0	88	88
Total	72	88	160
Positive Percent Agreement:	72/72	100.0%	(95%CI: 94.9%-100.0%)
Negative Percent Agreement:	88/88	100.0%	(95%CI: 95.8%-100.0%)

2009 H1N1 Influenza Virus

ProFAST+ A/2009 H1N1 Results Combined	Composite Reference		
	Positive	Negative	Total
Positive	25	0	25
Negative	0	135	135
Total	25	135	160
Positive Percent Agreement:	25/25	100.0%	(95%CI: 86.7%-100.0%)
Negative Percent Agreement:	135/135	100.0%	(95%CI: 97.2%-100.0%)

The general demographic data for all eligible retrospective specimens (N=160) are presented in the following table:

Sex*	Number of Subjects
Male	74/160 (46.3%)
Female	84/160 (52.5%)
Age (yrs)	
≤ 5 years	25/160 (15.6%)
6 - 18 years	24/160 (15.0%)
19 – 64 years	91/160 (56.9%)
≥ 65 years	20/160 (12.5%)

*For two of the subjects the gender was unknown

c. *Other clinical supportive data (when a. and b. are not applicable):*

Not applicable.

4. Clinical cut-off:

Not applicable.

5. Expected values/Reference range:

In the prospective ProFAST+ Assay clinical study, a total of 842 eligible prospective nasopharyngeal (NP) swab specimens were tested from four U.S. clinical laboratories across the United States. Of the total of 842 eligible NP swab samples, 257 were collected and tested at three U.S. clinical laboratories (Sites 1, 2, and 4) during December 2009 thru May 2010. Due to the absence of seasonal (H1N1 or H3N2) and 2009 H1N1 Influenza A during the typical 2009-2010 winter season, a total of 585 prospectively collected archived NP swab samples were also included in the prospective studies. Of the total of 585 prospectively collected archived NP swab samples, 195 samples were collected from January – March, 2008; 196 samples were collected from February – March, 2009; and 194 samples were collected from October – November, 2009. The prospectively collected archived NP swab samples were tested at two U.S. clinical laboratories (Sites 1 and 3).

The number and percentage of seasonal A/H1, seasonal A/H3 and A/2009 H1N1 RNA positive cases as determined by the ProFAST+ Assay, calculated by age group and prospective sample collection time period, are presented in the following table:

Age Group	Total N	A/H1 Positive By the ProFAST+ Assay	A/H3 Positive By the ProFAST+ Assay	A/2009 H1N1 Positive By the ProFAST+ Assay			
		Number Positive	Number Positive	Number Positive	Observed Prevalence A/H1	Observed Prevalence A/H3	Observed Prevalence A/2009 H1N1
December 2009 thru May 2010							
< 2 years	102	0	0	0	0%	0%	0%
2-5 years	30	0	0	0	0%	0%	0%
6-11 years	23	0	0	1	0%	0%	4.3%
12-18 years	12	0	0	0	0%	0%	0%
19-64 years	68	0	1	0	0%	1.5%	0%
> 65 years	22	0	0	0	0%	0%	0%
Total	257	0	1	1	0%	0.4%	0.4%

January 2008 thru March 2008							
< 2 years	94	8	11	0	8.5%	11.7%	0%
2-5 years	24	2	3	0	8.3%	12.5%	0%
6-11 years	24	4	3	0	16.7%	12.5%	0%
12-18 years	19	2	3	0	10.5%	15.8%	0%
19-64 years	25	3	4	0	12.0%	16.0%	0%
> 65 years	9	2	0	0	22.0%	0%	0%
Total	195	21	24	0	10.8%	12.3%	0%
February 2009 thru March 2009							
< 2 years	88	9	1	0	10.2%	1.1%	0%
2-5 years	25	9	1	0	36.0%	4.0%	0%
6-11 years	31	9	0	0	29.0%	0%	0%
12-18 years	16	4	0	0	25.0%	0%	0%
19-64 years	30	8	1	0	26.7%	3.3%	0%
> 65 years	6	1	0	0	16.7%	0%	0%
Total	196	40	3	0	20.4%	1.5%	0%
October 2009 thru November 2009							
< 2 years	40	0	0	9	0%	0%	22.5%
2-5 years	36	0	0	12	0%	0%	33.3%
6-11 years	38	0	0	19	0%	0%	50.0%
12-18 years	21	0	0	7	0%	0%	33.3%
19-64 years	45	0	0	13	0%	0%	28.9%
> 65 years	14	0	0	1	0%	0%	7.1%
Total	194	0	0	61	0%	0%	31.4%
Combined							
< 2 years	324	17	12	9	5.2%	3.7%	2.8%

2-5 years	115	11	4	12	9.6%	3.5%	10.4%
6-11 years	116	13	3	20	11.2%	2.6%	17.2%
12-18 years	68	6	3	7	8.8%	4.4%	10.3%
19-64 years	168	11	5	13	6.5%	3.6%	7.7%
> 65 years	51	3	0	1	5.9%	0%	2.0%
Total	842	61	28	62	7.2%	3.3%	7.4%

N. Instrument Name:

Cepheid SmartCycler II Real Time Instrument with Dx Software version 1.7b or 3.0a or 3.0b

MagNA Pure LC Instrument (Roche)

NucliSENS® easyMAG™ System (bioMérieux)

O. System Descriptions:

1. Modes of Operation:

The Cepheid SmartCycler II Real Time instrument with Dx software version 1.7b or 3.0a or 3.0b is used to perform real time reverse transcription, PCR amplification and detection of nucleic acid. Seven other nucleic acid amplification tests that use the SmartCycler II instrument have received 510(k) clearance: Prodesse's ProParaflu+ Assay (K091053), ProGastro Cd Assay (K090239), ProhMPV+ Assay (K082688) and ProFlu+ Assay (K081030), IDI-MRSA test (K033415), the IDI-Strep B Assay (K022504), and the Influenza A/H5 (Asian lineage) Virus Real-Time RT-PCR Primer and Probe Set (K060159). The Cepheid SmartCycler instrument is an integrated nucleic acid amplification and detection instrument system based on Cepheid's proprietary microprocessor-controlled I-CORE module. For purified RNA samples, the SmartCycler instrument enables reverse-transcriptase (RT) to transcribe target viral RNA into cDNA, polymerase chain reaction (PCR) for the amplification of cDNA, and hybridization of fluorogenic target-specific probes for the detection of the amplified cDNA.

The Roche MagNA Pure LC is an automated nucleic acid isolation and purification system based upon binding of nucleic acids to glass particles and has the capability to process a total of 32 reactions within one run. Nucleic acid is purified in multiple plastic reaction tips and cartridges by several steps that include cell lysis and binding of nucleic acid to magnetic glass particles, wash steps, and a heated elution to unbind the nucleic acid from the glass particles. Six other tests that use the Roche MagNA

Pure LC system have received 510(k) clearance: Prodesse's ProParaflu+ Assay (K091053), ProGastro Cd Assay (K090239), ProhMPV+ Assay (K082688) and ProFlu+ Assay (K081030), Roche Factor V Leiden Kit (K033607) and the Roche Factor II (Prothrombin) G20210A Kit (K033612).

The bioMérieux NucliSens easyMAG is an automated nucleic acid isolation and purification system that is based upon the same silica extraction technology as the MagNA Pure. The easyMAG is capable of processing a total of 24 reactions with variable sample types, sample volumes, and elution volumes within a single run. Nucleic acid is purified within a single cartridge by several steps that include lysis and binding of nucleic acid to high affinity magnetic silica beads, a series of wash steps and heated elution of purified nucleic acid from the silica beads. Prodesse's ProFlu+ Assay (K081030) that received Special 510(k) clearance and Prodesse's ProhMPV+ Assay (K082688), ProGastro Cd Assay (K090239) and ProParaflu+ (K091053) also use the easyMAG system for automated nucleic acid extraction.

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:

User enters Patient ID/Sample ID by typing it in.

4. Specimen Sampling and Handling:

Not applicable

5. Calibration:

Not applicable

6. Quality Control:

Positive Control (PC): The ProFAST+ Assay kit contains an Influenza A Subtyping positive RNA control that consists of one pool of three RNA transcripts (specific for each Influenza A subtype: A/H1, A/H3 and A/2009 H1N1). These transcripts are derived from plasmids containing the viral sequences of interest and are not intact virus particles. The Influenza A Subtyping PC contains equal amounts of A/H1, A/H3 and A/2009 H1N1. The approximate concentration of each transcript was 5×10^3 copies/ μ l. The PC does not go through nucleic acid isolation and purification, but is included during set-up of the RT-PCR reaction. The PC in conjunction with the IC is used to verify reagent and system performance. The PC is meant to be a control

for global failure of the assay (missing reaction component, instrument failure, etc).

Internal Control (IC): An Internal RNA Control, a non-infectious RNA transcript, is incorporated into every sample and is carried through all steps of the procedure from nucleic acid isolation and purification through amplification to monitor for inhibitors present in the specimen or reaction tube. The IC also serves as a general process control ensuring that each step of the procedure was performed correctly, assay and instrument parameters were set correctly, and that general reagents were working. The IC is meant to assess global failure (reagent or process) and monitor for PCR inhibition.

Negative Control (NC): A Negative Control (NC) is not provided with the kit, but is required and described in the ProFAST+ Assay Instructions for Use. Viral transport media spiked with the IC is to be used as the negative control and processed starting from nucleic acid isolation. The negative control serves to monitor for contamination.

Extraction Control (EC): An Extraction Control (EC) is not provided with the kit, however, during the clinical trial, a pool of seasonal A/H1N1, seasonal A/H3N2 and 2009 H1N1 influenza viruses was included with each nucleic acid isolation run. The EC served to monitor for lysis during nucleic acid isolation. **The sponsor is also recommending an extraction control in each nucleic acid extraction run to the end users in the package insert.**

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

Not applicable

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.