Prodesse ProFAST™+ Assay

Instructions for Use

For detection and discrimination of Influenza A Virus Subtypes: seasonal A/H1, seasonal A/H3, 2009 H1N1 Influenza Virus.

100

-70ºC

REF

303673

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Intended Use

The Prodesse ProFAST™+ Assay is a multiplex Real Time RT-PCR *in vitro* diagnostic test for the qualitative detection and discrimination of seasonal Influenza A/H1, seasonal Influenza A/H3 and 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.

*For users in Canada, a negative ProFAST+ Assay result is a presumptive negative result for Influenza A. These results should be confirmed by a nucleic acid-based test (NAT) detecting Influenza A licensed by Health Canada. Negative results do not preclude Influenza virus infection and should not be used as the sole basis for treatment or other patient management decisions.

Summary and Explanation

Influenza is an acute respiratory illness caused by infection with the Influenza virus, primarily types A and B.[1] Influenza A viruses are further categorized into subtypes based on the two major surface protein antigens: Hemagglutinin (HA) and Neuraminidase (N).[2] Epidemics occur yearly and although both types A and B circulate in the population, type A is usually dominant. These yearly epidemics are partly due to antigenic variation in the HA and N surface proteins of the virus. In March of 2009, a novel Influenza A virus (2009 H1N1 Influenza virus) emerged in North America and globally.[3] The 2009 H1N1 Influenza virus is considered a reassortant virus composed of two genes from Influenza viruses that normally circulate in swine in Europe and Asia in addition to bird (avian) and human genes.[4]

Transmission of Influenza is primarily via airborne droplet (coughing or sneezing). Symptoms arise on average one to two days post-exposure and include fever, chills, headache, malaise, cough, and coryza. Gastrointestinal symptoms such as nausea, vomiting, and diarrhea can occur, primarily in young children. The symptoms for the 2009 H1N1 influenza virus are similar to those of seasonal Influenza strains, however diarrhea and vomiting may be more commonly reported with the 2009 H1N1 influenza virus.[4] Complications due to Influenza include pneumonia, causing increased morbidity and mortality in pediatric, elderly, and immunocompromised populations. Young children, pregnant women, and those with underlying health conditions may be at greater risk for severe complications related to infection with the 2009 H1N1 Influenza virus. Antiviral drugs such as oseltamivir (brand name Tamiflu), zanamivir (Relenza), amantadine, and rimantadine are approved for use in the United States for treating Influenza. However, antiviral resistance varies among the different Influenza A subtypes (seasonal H1N1 Influenza A viruses are resistant to oseltamivir, but seasonal H3N2 and 2009 H1N1 Influenza viruses are not[5,6]) and the ability to differentiate the different Influenza A subtypes may impact treatment decisions.

Principles of the Procedure

The ProFAST+ Assay enables detection and discrimination of Influenza A Virus subtypes: seasonal A/H1, seasonal A/H3, and 2009 H1N1 and an Universal Internal Control.

Note

***When starting from purified nucleic acid samples that have been previously processed for testing with the ProFlu+ Assay, begin at set up of the RT-PCR reaction* (see *Step 4* of the *Assay Procedure*).**

An overview of the procedure is as follows:

1. Collect nasopharyngeal swab specimens from symptomatic patients using a polyester, rayon, or nylon tipped swab and place into viral transport medium (refer to **Materials Required but not Provided section of this Instruction for Use**).
2. Add an Universal Internal Control (UIC) 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 ProFAST+ Supermix along with enzymes included in the ProFAST+ Assay Kit. The ProFAST+ Supermix contains target-specific oligonucleotide primers and probes. The primers are complementary to conserved regions of the Hemagglutinin (HA) gene for seasonal influenza A/H1, seasonal influenza A/H3 and 2009 H1N1 Influenza Virus. 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.

Analyte	Gene Targeted	Probe Fluorophore	Absorbance Peak	Emission Peak
Seasonal H1 Influenza A	*Hemagglutinin*	FAM	495 nm	520 nm
Seasonal H3 Influenza A	*Hemagglutinin*	CAL Fluor Orange 560	540 nm	561 nm
2009 H1N1 Influenza Virus	*Hemagglutinin*	CAL Fluor Red 610	595 nm	615 nm
Universal Internal Control	*N/A*	Quasar 670	647 nm	667 nm

Materials Provided

ProFAST+ Assay Kit (Cat. # 303673)

Reagents	Description	Quantity/ Vial	Cap Color	Cat. #	Reactions/ Vial
ProFAST+ Supermix	➲ Taq DNA polymerase ➲ Oligonucleotide primers and probes ➲ Buffer containing dNTPs ➲ $MgCl_2$ and stabilizers	515 µL	Brown	HSM34	25 (4 vials provided)
M-MLV Reverse Transcriptase	➲ 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 *in vitro* transcribed RNA of specific viral sequences	300 µL	Clear	HCT34	15
Universal Internal Control (UIC)	➲ Non-infectious *in vitro* transcribed RNA ➲ Non-infectious DNA plasmid	30 µL	Lilac	403097	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 Strips 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. # 03038505001)* for 192 isolations OR bioMérieux NucliSENS easyMAG reagents *(Buffer 1 Cat. # 280130, Buffer 2 Cat. # 280131, Buffer 3 Cat. # 280132, Magnetic Silica Cat. # 280133, and Lysis Buffer Cat. #. 280134)*
- ☐ Micro Test M4 Viral Transport Medium *(Remel, Inc. Cat. # R12500),* Micro Test M5 Viral Transport Medium *(Remel, Inc. Cat. # R12515)*, Micro Test M6 Viral Transport Medium *(Remel, Inc. Cat. # R12530),* Micro Test M4RT Viral Transport Medium *(Remel, Inc. Cat. # R12505), Copan Universal Transport Medium (Copan Diagnostics, Inc. Cat. # 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)

† **NOTE: Only qualified lots of the MagNA Pure LC Total Nucleic Acid Isolation Kit can be used with the ProFAST+ Assay. Any lots not specifically qualified by Hologic for use with the ProFAST+ Assay are not verified for use with this Assay, and may cause erroneous results.**
A list of these qualified extraction reagents is available at www.hologic.com. Please notify the reagent

manufacturer of issues with this ancillary reagent and Hologic of the impact on the performance of the ProFAST+ Assay.

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
- ☐ Ice/Ice Bucket or -20°C Cold Block
- ☐ Biosafety Cabinet

Warnings and Precautions

- For *in vitro* diagnostic use only.
- Limit use of this product to personnel who are trained in the techniques of real-time PCR.
- **The ProFAST+ Assay should be performed on the Cepheid SmartCycler II instrument only.**
- **Once the RT-PCR Master mix is made, the run must be started within one hour.**
- Do not update the SmartCycler Dx Software beyond version 3.0b until Hologic communicates that the updated software version has been validated for use with the ProFAST+ Assay.
- Performance characteristics of this Assay have only been determined with nasopharyngeal swab specimens.
- A negative ProFAST+ Assay result is a presumptive negative result for Influenza A. These results should be confirmed by an FDA cleared nucleic acid test (NAT) detecting Influenza A. When all controls exhibit the expected performance and the A/H1, A/H3, and 2009 H1N1 targets are not detected, the sample should be tested with an FDA cleared Influenza A NAT assay. If the sample is positive for Influenza A, the sample has potential for containing a novel and/or newly emerging influenza A virus. The purified nucleic acid from the specimen should be re-tested with both the FDA cleared Influenza A NAT assay and the ProFAST+ Assay. If residual purified nucleic acid is not available, re-extract from residual specimen and re-test with both assays. If the re-tested sample is again positive for Influenza A, but negative for A/H1, A/H3, and 2009 H1N1, and all controls exhibit the expected performance, contact the state public health authorities or CDC Influenza Division for confirmatory testing.
- Handle all specimens as if infectious using safe laboratory procedures such as those outlined in CDC/NIH *Biosafety in Microbiological and Biomedical Laboratories* and in the CLSI Document M29 *Protection of Laboratory Workers from Occupationally Acquired Infections.* Thoroughly clean and disinfect all surfaces with 10% bleach. Autoclave any equipment or materials that have contacted clinical specimens before discarding.
- Laboratory work on clinical samples from patients who are suspected cases of 2009 H1N1 Influenza infection should be conducted in a BSL2 laboratory. All sample manipulations should be done inside a biosafety cabinet[1]. Viral isolation on clinical specimens from patients who are suspected cases of 2009 H1N1 Influenza virus infection should be performed in a BSL2 laboratory[7].
- 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.
- Real Time RT-PCR testing in general requires meticulous effort by the operator in reducing the chance of cross-contamination of samples during all steps of the procedure including: extraction, transfer of purified nucleic acids, and preparation of the RT-PCR reactions.
- Use personal protective equipment, such as (but not limited to) gloves and lab coats when handling kit

reagents while performing this Assay and handling materials including samples, reagents, pipettes, and other equipment.

- Use micropipettes with aerosol barrier or positive displacement tips for all procedures.
- Always pre-plan, organize and segregate workflow. Proceed with workflow in the laboratory in a unidirectional manner, beginning in the Pre-Amplification Area and moving to the Amplification/Detection Area.
    - Begin pre-amplification activities with reagent preparation and proceed to specimen preparation.
    - Always dedicate supplies and equipment to a specified area; no cross-movement allowed between areas.
    - Do not use equipment and supplies used for reagent preparation for specimen preparation activities or for pipeting or processing other sources of target nucleic acid.
    - Keep all amplification supplies and equipment in the Amplification/Detection Area at all times.
    - Always wear disposable gloves in each area and change them before entering a different area.
    - Do not open sample tubes following PCR.
- Take care to preserve the purity of kit reagents. Avoid contamination from Positive Control and specimens by following good laboratory practices.
- Do not use kit after its expiration date.
- Do not mix reagents with different lot numbers or substitute reagents from other manufacturers.
- Safety Data Sheets (SDS) are available on manufacturer's website at www.hologic.com.

Reagent Storage, Handling and Stability

- Store all reagents (opened and unopened) at ≤ -70°C until the expiration date listed on the kit.
- Always check the expiration date on the reagent tubes. For Intermediate stock of the Universal Internal Control, use the expiration date of the originating stock control vial. Do not expose controls to more than one (1) freeze-thaw cycle.
- ProFAST+ Assay Kits are shipped frozen, should arrive frozen and should be stored frozen after receipt. If the kit contents are not frozen or the tubes are compromised, contact Customer Service for assistance.
- An internal study demonstrated that performance of ProFAST+ Supermix, M-MLV Reverse Transcriptase, and RNase Inhibitor II are not affected for up to 5 freeze-thaw cycles.
- Visually examine reagents for adequate reagent volume before beginning any test procedures.
- Protect the ProFAST+ Supermix from light.
- **Controls and aliquots of controls must be thawed and kept on ice at all times during preparation and use.**

Recommendation

Aliquoting of kit components to maintain less than 5 freeze/thaw cycles is recommended for labs testing smaller numbers of samples.

Specimen Handling and Storage

Collecting the Specimen

To obtain nasopharyngeal swab samples:

1. Insert a flexible-shaft polyester, rayon or nylon tipped swab containing a dry tip into one nostril and into the nasopharyngeal area.
2. Press the swab gently against the nasopharyngeal wall to allow the swab to absorb secretions.
3. Rotate the swab two to three times and withdraw it.
4. Place the swab into a tube containing 3 mL of viral transport medium (Remel M4, M4RT, M5, M6; Copan UTM; or Becton Dickenson UVT).
5. Break off the shaft of the swab and cap the tube.


Note

Using a smaller volume of the viral transport medium may result in inhibition.

Transporting Specimens

Ensure that when transporting human respiratory specimens, all applicable regulations for the transport of etiologic agents are met. Transport human respiratory specimens refrigerated at 2-8°C.

Storing Specimens

Store specimens refrigerated (2-8°C) for up to 72 hours before processing. Specimens may also be stored frozen at ≤ -70°C before processing. Store any leftover specimens at ≤ -70°C. If retesting a frozen specimen, thaw specimen quickly (1 to 2 minutes) in a 37°C water bath and immediately place on ice or thaw specimen on ice.

Storing Purified Nucleic Acid

Store purified nucleic acids at ≤ -70°C. They should be tested after no more than one (1) freeze-thaw cycle.


Note

Inadequate or inappropriate specimen collection, storage and transport are likely to yield false negative results.


Recommendation

Training in specimen collection is highly recommended because of the importance of specimen quality.

Reagent and Control Preparation

Reagents


Note

Prepare reagents from the Roche MagNA Pure LC Total Nucleic Acid Isolation Kit or the bioMérieux easyMAG Automated Magnetic Extraction Reagents following the manufacturer's instructions.

Controls


Note

- *For aliquots of the Positive Control and Intermediate stock of the Universal Internal Control, use the expiration date of the originating stock control tube.*
- *Controls and aliquots of controls must be thawed and kept on ice/cold block at all times during preparation and use. It is recommended to prepare controls in a sample prep area, such as a Biological Safety Cabinet.*
- *It is required to include the Positive Control and the Negative Control in each RT-PCR run.*

Positive Control (PC)

1. Thaw Positive Control (the clear cap vial) on ice.
2. Make 15 aliquots of 20 µL, label and store at ≤ -70°C. **Ensure that aliquots do not undergo more than one (1) freeze-thaw cycle.**
3. **The Positive Control is used at the provided concentration.**


Note

Do not spike Positive Control with the Universal Internal Control. Do not take Positive Control through the nucleic acid isolation procedure.

Universal Internal Control (UIC)

Note

***When starting from purified nucleic acid samples previously processed for testing with the ProFlu+ Assay, begin at set up of the RT-PCR reaction* (see *Step 4* of the *Assay Procedure*).**

1. Thaw Universal Internal Control (the lilac cap vial) on ice.
2. Create Intermediate stock tubes of the Universal Internal Control using the following dilution scheme:

26 µL Universal Internal Control	+	*65 µL RNase Inhibitor*	+	*2509 µL molecular grade water*	=	*2600 µL total volume*

3. Make aliquots of 110 µL, label, and store at ≤ -70°C (this is enough volume to add to 5 samples at 20 µL per sample). Make aliquots of larger or smaller volumes based on the number of samples expected to be processed in a single run. **Ensure that aliquots do not undergo more than one (1) freeze-thaw cycle**.
4. Add the appropriate volume of Intermediate stock of the Universal Internal Control to each sample prior to nucleic acid isolation (see ***Step 1*** of the ***Assay Procedure***).
5. **Save RNase Inhibitor II for use in *Step 4* of the *Assay Procedure*. RNase Inhibitor II must be thawed and kept on ice at all times during preparation and use.**

Negative Control (NC)

1. Use viral transport medium as the Negative Control.
2. Add the appropriate volume of Intermediate stock of the Universal Internal Control to the Negative Control prior to nucleic acid isolation (see ***Step 1*** of the ***Assay Procedure***).

Extraction Control (EC)

Good laboratory practice recommends including a positive extraction control (e.g. previously characterized positive sample or negative sample spiked with a well characterized seasonal A/H1, A/H3 or A/2009 H1N1 strain) in each nucleic acid isolation run. The extraction control should be treated like a sample during Assay performance and analysis.

Assay Procedure

Assay Overview

Get Ready: Create the Assay Protocol for the Cepheid SmartCycler instrument using the Dx Software *(first time only).*

1. ***Prepare the Samples and Negative Control.***
2. ***Isolate the Nucleic Acid –*** *MagNA Pure LC System using the Total Nucleic Acid Isolation (TNAI) Kit*

   OR
3. ***Isolate the Nucleic Acid –*** *NucliSENS easyMAG System using the Automated Magnetic Extraction Reagents.*
4. ***Set up the RT-PCR Reaction.***
5. ***Run the ProFAST+ Assay.***
6. ***Print report.***

Note

- ***When starting from purified nucleic acid previously processed for testing with the ProFlu+ Assay, begin at Step 4 of the Assay Procedure.***
- *Instructions provided for the Cepheid SmartCycler II Real Time Instrument with Dx Software version 3.0a / 3.0b (Instructions for version 1.7b noted).*
- *Do NOT deviate from the protocol settings defined in this section.*

Get Ready: Create the Assay Protocol for the Cepheid SmartCycler using the Dx Software (first time only)

Note

- *The protocol is only created for first time use; it does not need to be recreated with each sample run.*
- *To **Define** and **Edit** Assay protocols, you must have administrative access rights. Otherwise, the fields are not accessible for data entry and editing (they are grayed out).*
- *Cepheid Dx Software interprets the data and reports the run as either VALID or INVALID, based on the results of the Positive and Negative Controls.*
- *You must interpret the Extraction Control result (if included) and determine if the extraction run is VALID or INVALID. You must meet all Control criteria for the RT-PCR run to be VALID (see **Interpretation of Control Results** section).*

1. Create the ProFAST+ Assay protocol:

a. Launch the Cepheid Dx software application.

b. Click the **Define Assay** box at the top of the screen.

c. Click the **New Assay** box at the bottom of the screen.

d. Enter **ProFAST+ SUBTYPING Assay** for the assay protocol in the window that opens.

e. Click **OK**.

f. Enter **Thermocycler Parameter** in the Protocol section (bottom half of ***Define Assay*** screen).

Stage 1			Stage 2			Stage 3 Repeat **5** times			Stage 4 Repeat **40** times		
Hold			**Hold**			**2- Temperature Cycle**			**2- Temperature Cycle**		
Temp	Secs	Optics	Temp	Secs	Optics	Temp	Secs	Optics	Temp	Secs	Optics
42	**1800**	**OFF**	**95**	**600**	**OFF**	**95**	**30**	**OFF**	**95**	**10**	**OFF**
						55	**60**	**ON**	**55**	**60**	**ON**

Stages 5 – 10 remain UNUSED

2. Enter information in **BOLD** in the **Analysis Settings** tab as follows:

a. Select **FTTC25** for the **Dye Set**.

b. **Analysis Type: Qualitative** (default).

c. **Customize Result Text: Target-based Result Text** (default).

Note

- *Gray boxes are default settings.*

Channel	Dye Name	Channel Name*	Usage	Curve Analysis	Thresh Setting	Manual Thresh	Auto Thresh	Auto Min. Cycle	Auto Max. Cycle	Valid Min. Cycle	Valid Max. Cycle	Bkgnd Sub	Bkgnd Min. Cycle	Bkgnd Max. Cycle	Boxcar Avg	EndPt Thresh	NC IC %	IC Delta
1	**FAM ***	**Seasonal H1**	**Target****	Primary	Manual	**60**	NA	5	10	13.0	**45.0**	ON	5	**40**	0	**60**	NA†	NA
2	**TET***	**Seasonal H3**	**Target****	Primary	Manual	**40**	NA	5	10	13.0	**45.0**	ON	5	**40**	0	**40**	NA†	NA
3	**TxR***	**2009 H1N1**	**Target****	Primary	Manual	**20**	NA	5	10	13.0	**45.0**	ON	5	**40**	0	**20**	NA†	NA
4	**Cy5***	**Universal Internal Control**	**Internal Control**	Primary	Manual	**25**	NA	5	10	13.0	**45.0**	ON	5	**40**	0	**25**	NA†	NA

** If Dx 1.7b = Target*
*** Dx 1.7b = Assay*
† Dx1.7b = 10

3. Enter information in **BOLD** in the **Control Settings** tab**.**
   - *a.* Select **NC Fails if any target criterion is positive**.
   - *b.* *Use PC1 (Pos Cntrl 1) for the Positive Control. Enter **0** replicates to inactivate Positive Controls PC 2 and PC3.*
   - *c.* *Use only one Negative Control (NC1). Enter **0** Replicates to inactivate the Negative Controls NC2 and NC3.*

Note

❖ *Gray boxes are default settings.*

Control ID	Control Name	Replicate	H1 Valid Min Cycle	H1 Valid Max Cycle	H1 EndPt Thresh	H3 Valid Min Cycle	H3 Valid Max Cycle	H3 EndPt Thresh	2009 H1N1 Valid Min Cycle	2009 H1N1 Valid Max Cycle	2009 H1N1 EndPt Thresh	UIC +/-	UIC Valid Min Cycle	UIC Valid Max Cycle	UIC EndPt Thresh
PC1	Pos Cntrl 1	**1**	**20.0**	**40.0**	**60**	**20.0**	**40.0**	**40**	**20.0**	**40.0**	**20**	**-**	**NA**	**NA**	**NA**
PC2	Pos Cntrl 2	0	13.0	45.0	10	13.0	45.0	10	13.0	45.0	10	+	13.0	45.0	10
PC3	Pos Cntrl 3	0	13.0	45.0	10	13.0	45.0	10	13.0	45.0	10	+	13.0	45.0	10
NC1	Neg Cntrl 1	**1**	**13.0**	**45.0**	**60**	**13.0**	**45.0**	**40**	**13.0**	**45.0**	**20**	**+**	**20.0**	**40.0**	**25**
NC2	Neg Cntrl 2	0	13.0	45.0	10	13.0	45.0	10	13.0	45.0	10	+	13.0	45.0	10
NC3	Neg Cntrl 3	0	13.0	45.0	10	13.0	45.0	10	13.0	45.0	10	+	13.0	45.0	10

4. Click the **Advanced** tab and select **Require Lot Number**.
5. **Probe Check Settings** tab, **Advance to New Stage** tab, and **Standards** tab are not used for the ProFAST+ Assay protocol.
6. Select **Save Assay**.

1. Prepare the Samples, Extraction Control (if included), and Negative Control
(Pre-Amplification Area)

- *a.* **Add Universal Internal Control to all samples and Extraction Control (if included).**
  - *i.* Thaw the appropriate number of aliquots of Intermediate stock of the Universal Internal Control on ice (enough volume needed for each sample, the Extraction Control, and the Negative Control).
  - *ii.* Remove 180 µL of sample from the original sample tube and pipette into a labeled 1.5 mL microcentrifuge tube. Alternately, pipet 180 µL of sample directly into sample cartridge or sample vessel.
  - *iii.* Remove 180 µL of Extraction Control (if included) from the original sample tube and pipette into a labeled 1.5 mL microcentrifuge tube. Alternately, pipet 180 µL of Extraction Control directly into sample cartridge or sample vessel.
  - *iv.* Add 20 µL of Intermediate stock of the Universal Internal Control to each sample and the Extraction Control. **Pipet up and down a minimum of 5 times to mix using a new pipet tip for each tube.**
  - *v.* Keep tubes on ice.
  - *vi.* Store any remaining sample at ≤ -70°C.

- *b.* **Add Universal Internal Control to the Negative Control.**
  - *i.* Include one (1) Negative Control in each run.
  - *ii.* Add 180 µL of viral transport medium to a labeled 1.5 mL microcentrifuge tube. Alternately, pipet 180 µL of viral transport medium directly into sample cartridge or sample vessel.
  - *iii.* Add 20 µL of Intermediate stock of the Universal Internal Control to the viral transport medium. **Pipet up and down a minimum of 5 times to mix.**
  - *iv.* Keep tube on ice.
  - *v.* Discard remaining volume of Universal Internal Control – DO NOT reuse.

Do not reuse Universal Internal Control.

2. Isolate Nucleic Acid (Pre-Amplification Area I) - MagNA Pure LC System using Total Nucleic Acid Isolation (TNAI) Kit

a. Start the instrument and software.

i. Turn power on to the MagNA Pure LC Instrument and then turn on the computer.

ii. Start the MagNA Pure LC software.

iii. From the *Main Menu* screen, select **Sample Ordering** and enter sample information in **Sample Name** column.

iv. Select the **Total NA Variable_elution_volume.blk** protocol.

v. Follow the software instructions and specify the number of samples.

vi. Type **200 µL** for the sample volume and verify that **50 µL** elution volume is selected.

vii. Select **Stage Setup** to automatically calculate the amount of each reagent required.

b. Fill the reagent tubs.

Before starting the isolation procedure, fill all reagent tubs outside the instrument with the required volume of each reagent listed on the **Start Information** screen.

Note

Use only the reagent amount needed for the number of samples entered into the software. Reagents are not stable for long-term storage in tubs. Vortex Magnetic Glass Particles (MGPs) and load the exact amount of MGPs (as listed on the **Start Information** *Screen) into the instrument just before the run starts.*

c. Load reagent tubs and disposables into the instrument.

Use the information on the **Start Information** screen to place all disposable plastics and reagent tubs necessary for the batch run on the Reagent/Sample Stage.

Recommendation

Use a colored "Positioning Frame" (provided with the TNAI kit) on the Reagent Tub Rack to help to correctly load reagents.

d. Load the samples, Extraction Control and Negative Control into the MagNA Pure Sample Cartridge.

i. Transfer all 200 µL of each sample to individual wells in the MagNA Pure Sample Cartridge.

ii. Transfer all 200 µL of the Extraction Control and the Negative Control to different wells in the MagNA Pure Sample Cartridge.

iii. Cover cartridge with Parafilm or MagNA Pure LC Cartridge Seal and keep cartridge on ice until ready to load the instrument.

e. Load the samples.

Transfer the Sample Cartridge containing the samples, Extraction Control and Negative Control into the MagNA Pure LC instrument.

f. Start the run.

i. Start the Batch Run by confirming the correct placement of all disposable plastics and reagents by mouse-clicking the respective text boxes on the **Start Information** screen.

ii. Select the **OK** button to start the automated isolation procedure. The instrument automatically dispenses all reagents and processes the samples.

g. Store the eluted total nucleic acid.

After completing the run, place the Storage Cartridge containing the eluted nucleic acids immediately on ice or transfer eluted nucleic acid to 1.5 mL tubes and store for longer durations at ≤ -70°C.

Note

- *Do not store purified nucleic acids in the Storage Cartridge on the Cooling Unit 1.*
- *Do not use nucleic acids for RT-PCR if the extraction instrument/run failed.*

3. Isolate Nucleic Acid (Pre-Amplification Area I) - NucliSENS easyMAG System using Automated Magnetic Extraction Reagents

a. Start Instrument and Software.

Turn power on to the easyMAG instrument and once the LED on the instrument turns green, turn on the computer and log into the software.

b. Prepare the software for a run.

To prepare for a run, touch "**Settings**"  in the main toolbar to default to the "Application Settings" icon and choose the following run settings:

Default Protocol: Generic 1.0.6 or 2.0.1 (for software version 1.0.1 or 2.0, respectively)
Run Name Prefix: N/A (leave as default)
Sample ID prefix: N/A (leave as default)
Sample Type: Primary (on-board lysis)
Default On-board Lysis Dispensing: Yes
Default On-board Lysis Incubation: Yes
Sample Addition Guidance: Off
Reagent Tracking: Off

c. Input buffer information.

Touch "**Instrument**"  to default to the "**Reagent Inventory**"  icon and input the buffer barcodes by first scanning the instrument position (A, B, C, or D) and then its corresponding buffer. For example, scan position A and then scan the bottle of Lysis buffer in that position and then move on to position B and its corresponding bottle.

d. Create a worklist.

a. Touch "**Daily Use**"  to default to the "**Define Extraction Request"**  icon and select the following settings:

Sample ID: Manually enter the sample name.
Matrix: Other
Protocol: Generic 1.0.6 or 2.0.1 (for software version 1.0.1 or 2.0, respectively)
Volume (mL): 0.200 (input volume of sample)
Eluate (µL): 55
Type: Primary
Priority: Normal

b. Press **Enter** on the keyboard or touch "**New Extraction Request**"  after each manual sample addition. The settings above remain as the default settings for each subsequent entry as long as you do not navigate to other pages.

e. Create a run and add samples from the worklist.

Touch "**Organize Runs**"  and then "**Create Run**"  to display the *New Run Window.* In this screen, name the run appropriately and verify that the **auto-number** box is left unchecked (not selected) and that **Yes** is selected for both the On-Board Lysis Dispensing and On-Board Lysis Incubation options. Touch **OK**; the *New Run Window* closes and the "**Organize Runs**" screen is displayed. Assign samples to run using the positioning arrows.

Touch "**Load Run"**  and select the run. Touch "**Print worklist"**  to print the list. Use this worklist to keep track of the order of the samples to load into the sample vessel wells.

f. **Load the samples and tips and barcode the sample strip(s).**

Add all of the 200 µL from each sample into the proper vessels of the sample strip(s) as noted in the worklist. Insert tips into sample vessel(s) in the correct order as noted in the worklist and scan the sample strip(s) position on the instrument and then the sample strip itself. For example, scan position A and then the sample strip in that position, then B and then C, if necessary. After scanning the sample strip(s), the indicator changes from red to green on the screen.

g. **On-Board Lysis Dispensing.**

Once the samples and tips are loaded and the strip(s) scanned, close the lid and touch **"Dispense Lysis"**  . The instrument dispenses 2 mL of Lysis Buffer and incubates for 10 minutes.

h. **Prepare the magnetic silica to add to the sample vessel.**

During the 10-minute lysis incubation, use the Biohit multi-channel pipettor to prepare the magnetic silica. Perform this procedure for each sample vessel used in the run (1, 2, or 3 times).

i. Set the pipettor to **Program 1** and place a Biohit pipette tip on position 1. Program 1 provides the means to aspirate and dispense 550 µL of liquid. The magnetic silica is prepared in a 1:1 ratio of Molecular Biology Grade Water to Magnetic Silica.

ii. Using Program 1 of the pipettor, press the **start** button to aspirate and then again to dispense 550 µL of water into a microcentrifuge tube. Vortex the tube of magnetic silica briefly to mix and use Program 1 of the pipettor to aspirate and then dispense 550 µL of magnetic silica into the same microcentrifuge tube as the water. Eject the tip, cap the tube and vortex to mix.

iii. Set the pipettor to **Program 2** and place a Biohit pipette tip on position 1. Program 2 transfers 8 volumes of the previous mix to the 8 vessels of a strip on an ELISA plate (1 strip/sample vessel). Press **start** to aspirate the mix. Press **start** again to dispense the remaining mixture back into the tube containing the mix to reset the pipette.

iv. Press **start** 8 separate times to dispense the remaining mix in each of 8 vessels of an ELISA plate strip and eject the tip.

v. After the 10 minute lysis incubation is done, set the pipettor to **Program 3** and place 8 Biohit pipette tips on the multichannel pipettor (or however many samples are present in the specific sample strip). Make sure the filter tips are very well connected with the multichannel pipettor to prevent leakage errors. Program 3 first mixes the magnetic silica mixture in the ELISA plate and then aspirates it for delivery to the vessels of the sample strip where it is mixed.

Press **start** once and the pipette mixes the silica in the ELISA plate and then aspirates it for addition to the sample vessel. Verify that each tip has the same volume of silica mix before placing in the sample vessel. Place the pipettor over the sample vessel strip so the tips are below the liquid level of each sample and press **start** again to aspirate 800 µL out of each sample vessel and perform 3 mix cycles with 1000 µL. As it is mixing, hold the pipette steady below the liquid/air interface to avoid introducing bubbles to the sample.

vi. Repeat for each sample strip in the run.

i. **Start the run.**

Touch "**Start**"  to begin the run. The instrument performs 5 washes and heat and elute. Transfer the purified nucleic acids to appropriate storage tubes (1.5 mL microcentrifuge tubes) on ice within 30 minutes of extraction to avoid contamination by the magnetic silica stuck to the front wall of the sample vessel(s). Use immediately or store at ≤ -70°C.


Note

Do not use nucleic acids for RT-PCR if the extraction instrument/run failed.

4. Set up the RT-PCR Reaction (Pre-Amplification Area II)


Note

Start the SmartCycler ProFAST+ Assay run within 1 hour of making the RT-PCR Master mix.

a. Thaw the Positive Control.

i. Thaw one (1) aliquot of the Positive Control on ice.

ii. Keep tube on ice.

b. Prepare the RT-PCR Master mix.


Note

The RT-PCR Master mix must be prepared FRESH for each RT-PCR run.

i. Calculate the amount of each reagent needed based on the number of reactions (samples + controls):

19.45 µL	ProFAST+ Supermix
+0.30 µL	M-MLV Reverse Transcriptase
+0.25 µL	RNase Inhibitor
20.00 µL	per reaction

ii. Thaw the ProFAST+ Supermix on ice and **mix by pipetting up and down a minimum of 5 times before use.**

iii. Remove M-MLV Reverse Transcriptase and RNase Inhibitor enzymes from the freezer and keep on ice during use. M-MLV Reverse Transcriptase should be spun down and pipetted from the top.

iv. Prepare the RT-PCR Master mix by combining the reagents listed above in a 1.5 mL microcentrifuge tube. **Pipet up and down a minimum of 5 times to mix**.

v. Keep the RT-PCR Master mix on ice and protected from light before adding to SmartCycler tubes.


Recommendation

If setup is prolonged, keep the cold blocks on ice while adding reagents to the SmartCycler tubes.

c. Add 20 µL of RT-PCR Master mix to the SmartCycler tubes.

i. Load the required number of tubes into the Cepheid Cooling Block.

ii. Pipet the RT-PCR Master mix into the upper part of the SmartCycler tubes. Discard any unused RT-PCR Master mix.

d. Add 5 µL of each sample's nucleic acid to individual SmartCycler tubes containing RT-PCR Master mix.

i. After adding the sample's nucleic acid to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube. Use a new pipette tip for each sample.

e. Add 5 µL of the Positive Control to a separate SmartCycler tube containing RT-PCR Master mix.

i. After adding the Positive Control to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube.

iii. Discard remaining volume of Positive Control – DO NOT reuse.

Do not reuse Positive Control aliquot.

f. **Add 5 µL of the Extraction Control (if included) nucleic acid to a separate SmartCycler tube containing RT-PCR Master mix.**

i. After adding the Extraction Control nucleic acid to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube.

g. **Add 5 µL of the Negative Control nucleic acid to the last SmartCycler tube containing RT-PCR Master mix.**

i. After adding the Negative Control nucleic acid to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube.

h. **Centrifuge all tubes.**

i. Appropriately label the SmartCycler tubes on the caps.

ii. Centrifuge all tubes for 5 to 10 seconds using the Cepheid microcentrifuge specially adapted to fit the SmartCycler tubes.

iii. Return tubes to the cooling block.

i. **Keep the tubes on the Cooling Block before loading them into the SmartCycler instrument.**

5. Run the ProFAST+ Assay (Amplification/Detection Area)

a. Create a new run by clicking **Create Run** at the top of the screen. The *Create Run* screen is displayed.

b. Under **Run Name** in the left panel, enter a unique run identifier.

c. Click the **Assay** arrow in the left panel and select **ProFAST+ SUBTYPING Assay** from the drop-down menu.

d. Under **Assay Information** in the left panel, enter the **Lot Number** and **Expiration Date** of the ProFAST+ Assay Kit.

e. In the left panel, enter the number of specimens (including the Extraction Control - if included, but excluding the Positive and Negative Controls) and click **Apply**. The **Site Table** is displayed and the SmartCycler Dx automatically selects the **I-Core** sites.

f. In the **Site Table** under the **Sample ID** column, enter the Sample Identifier or Extraction Control Identifier for the appropriate I-Core sites.

g. Insert each reaction tube into an I-Core site of the SmartCycler by pressing down firmly on all tubes; close each lid. Verify that the Positive Control (PC1) and Negative Control (NC1) are correctly loaded into the specified I-Core sites.

h. Select **Start Run** located at the bottom left corner of the screen. Verify that the LED is on for the appropriate I-Core sites.

6. Print Report

a. Click **Report** at the bottom of screen to open the **Report Preview** screen.

b. Click **Print** at the top of the screen.

Interpretation of Control Results

Validation of Run

Note

You must interpret the Extraction Control (if included) results to determine whether the extraction run is VALID; the SmartCycler Dx software automatically interprets the Positive Control (PC1) and Negative Control (NC1) results to determine if the RT-PCR run is VALID.

For a VALID Extraction run, the following conditions must be met:

Sample ID[1]	Assay Result	UIC Result	Warning / Error Code	Sample Type	UIC Ct	H1 or H3 or 2009 H1N1 Result[3]	H1 or H3 or 2009 H1N1 Ct[3]
Extraction Control	Valid	NA		SPEC	NA	POS	13-45
Neg Control	Valid[2]	Pass		NC1	20-40	Valid	0

[1] *Columns and data not used for interpretation are not included.*

[2] *(Typical) An Invalid Assay will display Error Code 4098.*

[3] *The expected result depends on the organism or sample used as the Extraction Control.*

For a VALID RT-PCR run, the conditions in the table below must be met:

Sample ID[1]	Assay Result	UIC Result	Warning / Error Code	Sample Type	UIC Ct	H1 Result	H1 Ct	H3 Result	H3 Ct	2009 H1N1 Result	2009 H1N1 Ct
Pos Control	Valid	NA		PC1	0	POS	20-40	POS	20-40	POS	20-40
Neg Control	Valid[2]	Pass		NC1	20-40	Valid	0	Valid	0	Valid	0

[1] *Columns and data not used for interpretation are not included.*

[2] *(Typical) An Invalid Assay will display Error Code 4098.*

Invalid Extraction Run

If the conditions for a Valid Extraction run are not met (i.e., the Extraction Control is not positive or the Negative Control is Invalid), repeat the entire extraction run. Start from original sample(s) using a new Extraction Control and a new Negative Control (starting at ***Step 1*** of the ***Assay Procedure***).

Invalid RT-PCR Run

If the Positive Control is Invalid but the Negative Control is Valid, prepare all new reactions using remaining purified nucleic acids and a new Positive Control (starting with PCR at ***Step 4*** of the ***Assay Procedure***).

If the Negative Control is invalid, prepare all new extractions starting from original sample(s) using a new Extraction Control and a new Negative Control (starting at ***Step 1*** of the ***Assay Procedure***).

Interpretation of Individual Specimen Results

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

Sample ID[1]	Assay Result	UIC Result	Warning / Error Code	H1 Result	H3 Result	2009 H1N1 Result	Interpretation of Results
Sample ID	Negative	Pass		**NEG**	**NEG**	**NEG**	Seasonal Influenza A/ H1, Seasonal Influenza A/H3 and 2009 H1N1 Influenza viral 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*		**POS**	NEG	NEG	Seasonal Influenza A/ H1 RNA **detected**
Sample ID	Positive	NA*		NEG	**POS**	NEG	Seasonal Influenza A/ H3 RNA **detected**
Sample ID	Positive	NA*		NEG	NEG	**POS**	2009 H1N1 Influenza RNA **detected**
Sample ID	Unresolved	Fail		**NEG**	**NEG**	**NEG**	Unresolved – PCR inhibition or reagent failure. Repeat testing from the purified nucleic acid or collect and test a new sample.
Sample ID	Positive	NA*		**POS**	NEG	**POS**	Seasonal Influenza A/H1 and 2009 H1N1 Influenza RNAs **detected.** Multiple infections are rare. Repeat testing from the purified nucleic acid, from original sample, or collect and test a new sample.
Sample ID	Positive	NA*		**POS**	**POS**	NEG	Seasonal Influenza A/H1 and A/H3 RNAs **detected.** Multiple infections are rare. Repeat testing from the purified nucleic acid, from original sample, or collect and test a new sample.
Sample ID	Positive	NA*		NEG	**POS**	**POS**	Seasonal Influenza A/H3 and 2009 H1N1 Influenza RNAs **detected.** Multiple infections are rare. Repeat testing from the purified nucleic acid, from original sample, or collect and test a new sample.
Sample ID	Positive	NA*		**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, 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 Universal Internal Control in the Cy5 detection channel is not required for positive result. High viral load can lead to reduced or absent Universal Internal Control signal.*

Influenza A Positive (as determined by an FDA cleared Influenza A NAT assay), but Seasonal Influenza A/H1, Seasonal Influenza A/H3, and 2009 H1N1 Influenza Negative Samples

When all controls exhibit the expected performance and the A/H1, A/H3, and 2009 H1N1 targets are not detected, the sample should be tested with an FDA cleared Influenza A NAT assay. If the sample is positive for Influenza A, the sample has potential for containing a novel and/or newly emerging influenza A virus. The purified nucleic acid from the specimen should be re-tested with both the FDA cleared Influenza A NAT assay and the ProFAST+ Assay. If residual purified nucleic acid is not available, re-extract from residual specimen and re-test with both assays. If the re-tested sample is again positive for Influenza A, but negative for A/H1, A/H3, and 2009 H1N1, and all controls exhibit the expected performance, contact the state public health authorities or CDC Influenza Division for confirmatory testing.

Invalid Samples

If an Assay result of **ND** (Not Determined) is reported with an instrument Warning/Error Code 4098, repeat testing from the purified nucleic acids (starting with PCR, see ***Step 3 (a)*** of the ***Assay Procedure***). Refer to the Cepheid Dx Software Operator Manual for interpretation of Warning Codes.

Multiple Positive Samples

Multiple positive results can occur when multiple Influenza A subtypes are co-circulating. Repeat testing of multiple positives is required. Repeated multiple positives should be further confirmed by other FDA cleared influenza subtyping tests and 2009 H1N1 influenza tests.

Quality Control

- Quality control requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard quality control procedures. It is recommended that the user refer to CLSI document C24-A3, *Statistical Quality Control for Quantitative Measurement Procedures: Principles and Definitions:* [Approved Guideline – Third Edition] or other published guidelines for general quality control recommendations. For further guidance on appropriate quality control practices, refer to 42 CFR 493.1205.
- Quality control procedures are intended to monitor reagent and Assay performance.

Control Type	Used to Monitor
Positive	Substantial reagent failure including primer and probe integrity
Negative	Reagent, environmental, or carry-over contamination
Extraction	Failure in lysis and extraction procedure
Internal	PCR inhibition in individual samples, reagent failure or process error

- Dilute the Universal Internal Control and test both the Positive Control and Universal Internal Control prior to running samples with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice recommends including a positive Extraction Control and a Negative Control in each nucleic acid isolation run. The Extraction Control should be treated as a sample.
- Never run the Positive Control through nucleic acid isolation.
- Always include one Negative Control (containing Universal Internal Control) and one Positive Control in each amplification/detection run performed.
- Failure of Controls (Positive, Negative and/or Extraction) invalidates the run and results should not be reported.
- If the Positive Control is not positive within the specified Ct range but the Negative Control is valid, repeat testing starting from the purified nucleic acid using a new aliquot of the Positive Control. If repeat results are still invalid, do not report results; repeat testing from the original sample or collect and test a new sample.
- If the Extraction Control is not positive within the specified Ct range or the Negative Control is invalid, repeat testing starting from the original sample using a new Extraction Control and a new Negative Control. If repeat results are still invalid, do not report results; collect and test a new sample.

Limitations

- Results from this test must be correlated with the clinical history, epidemiological data and other data available to the clinician evaluating the patient.
- The ProFAST+ Assay targets the hemagglutinin gene only; it does not detect or differentiate the neuraminidase gene.
- The detection of viral nucleic acid is dependent upon proper specimen collection, handling, transportation, storage, and preparation (including extraction). Failure to observe proper procedures in any one of these steps can lead to incorrect results. There is a risk of false negative values resulting from improperly collected, transported, or handled specimens.
- There is a risk of false negative values due to the presence of sequence variants in the viral targets of the Assay, procedural errors, amplification inhibitors in specimens, or inadequate numbers of organisms for amplification.
- Negative results do not rule out seasonal influenza A/H1, A/H3 or 2009 H1N1 infections and should not be used as the sole basis for treatment or other patient management decisions.
- Positive and negative predictive values are highly dependent on prevalence. False negative test results are more likely during peak activity when prevalence of disease is high. False positive test results are more likely during periods of low influenza activity when prevalence is moderate to low.
- The performance of this test has not been established for individuals who have received the influenza vaccine.
- The Universal Internal Control is included in the ProFAST+ Assay to aid in the identification of specimens that contain inhibitors to PCR amplification. The Universal Internal Control does not indicate whether or not nucleic acid has been lost due to inadequate collection, transport or storage of specimens.
- A specimen yielding a negative result may contain respiratory viruses other than Influenza A or bacterial pathogens.
- There is a risk of false positive values resulting from cross-contamination by target organisms, their nucleic acids or amplified product, or from non-specific signals in the Assay.
- Highly viscous sample may have an inhibitory effect on detection of influenza A subtypes (seasonal influenza A/H1, A/H3 or 2009 H1N1) and the Universal Internal Control (UIC) near the LoDs using the ProFAST+ Assay.
- Viral nucleic acids may persist *in vivo* independent of virus viability. Detection of analyte target(s) do not imply that the corresponding viruses are infectious, or are the causative agents for clinical symptoms.
- This test is a qualitative test and does not provide the quantitative value of detected organism present.
- The performance of the test has been evaluated for use with human specimen material only.
- The performance of this test has not been evaluated for sample types other than nasopharyngeal (NP) swab specimens.
- The performance of this test has not been evaluated for immunocompromised individuals.
- The performance of this test has not been established for patients without symptoms of influenza infection.
- The performance of this test has not been established for monitoring treatment of seasonal influenza A/H1, A/H3 or 2009 H1N1 infections.
- The performance of this test has not been established for screening of blood or blood product for the presence of seasonal influenza A/H1, A/H3 or 2009 H1N1 influenza.
- The performance of this test has not been established with potentially interfering medications for the treatment of influenza or cold viruses. The affect of interfering substances has only been evaluated for those listed in the labeling. Interference by substances other than those described in the “Interference” section below can lead to erroneous results.
- Cross-reactivity with respiratory tract organisms other than those listed in the “Analytical Specificity” section below may lead to erroneous results.
- The ProFAST+ Assay may not react with non-contemporary influenza A strains.
- The ProFAST+ 2009 H1N1 primers and probe may also react with other swine-origin Influenza A strains. However, literature review indicated that Influenza Virus of Swine Origin have historically rarely infected humans.

Expected Values

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 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.

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	Number Positive By the ProFAST+ Assay			Observed Prevalence		
		A/H1	A/H3	2009 H1N1	A/H1	A/H3	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	6	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%**

Performance Characteristics

Clinical Performance

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 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. All specimens used in the study meeting the inclusion 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.

Demographic details for the patient population included in the prospective study are summarized in the following table.

Gender and Age Demographic Detail for ProFAST+ Assay Prospective Study

Sex	Number of Subjects
Female	439 (52.1%)
Male	403 (47.9%)
Age	
≤ 5 years	439 (52.1%)
6 - 21 years	184 (21.9%)
22 – 59 years	168 (20.0%)
≥ 60 years	51 (6.0%)

Performance of the ProFAST+ Assay was assessed and compared to the composite comparator/reference method of the ProFlu+ Assay 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 2009 H1N1). “True” seasonal A/H1, A/H3 or 2009 H1N1 RNA positives were considered as any sample that tested positive for Influenza A by the ProFlu+ Assay, 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 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 2009 H1N1 RNA negatives were considered as any sample that tested negative for Influenza A by the ProFlu+ Assay, or any sample that tested positive for Influenza A by the ProFlu+ Assay, but tested negative by the respective Influenza A subtype specific RT-PCR assay followed by bi-directional sequencing. Nucleic acid extractions on the clinical samples were carried out using either the Roche MagNA Pure LC system or the bioMérieux NucliSENS easyMAG during the clinical study.

A total of 874 prospective NP swab specimens were initially included in the prospective clinical trial. Thirty two (32) samples 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, resulting in a total 842 eligible prospective specimens to be included in the prospective clinical study data analysis.

Of the prospective specimens run using the ProFAST+ Assay, 98.9% (864/874) of these specimens were successful on the first attempt. The remaining 10 (10/874 = 1.1%) gave “Unresolved” results on the first attempt. 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 nucleic acid for retest, only 50.0% (5/10) gave a valid result on the second attempt. The remaining 5 were “Unresolved” on the second attempt.

Seasonal Influenza A/H1 Comparison Results

		ProFlu+/Sequencing			
		Positive	**Negative**	**Total**	
ProFAST+ Assay	**Positive**	53	8[a]	**61**	Positive Percent Agreement=100.0% (93.2% - 100.0%) 95% CI
	Negative	0	781	**781**	Negative Percent Agreement=99.0% (98.0% - 99.5%) 95% CI
	Total	**53**	**789**	**842**	

[a] 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-subtyptable, 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 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 Comparison Results

		ProFlu+/Sequencing			
		Positive	**Negative**	**Total**	
ProFAST+ Assay	**Positive**	25	3[a]	**28**	Positive Percent Agreement=100.0% (86.7% - 100.0%) 95% CI
	Negative	0	814	**814**	Negative Percent Agreement=99.6% (98.9% - 99.9%) 95% CI
	Total	**25**	**817**	**842**	

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

2009 H1N1 Influenza Comparison Results

		ProFlu+/Sequencing			
		Positive	**Negative**	**Total**	
ProFAST+ Assay	**Positive**	62	0	**62**	Positive Percent Agreement=95.4% (87.3% - 98.4%) 95% CI
	Negative	3	777	**780**	Negative Percent Agreement=100.0% (99.5% - 100.0%) 95% CI
	Total	**65**	**777**	**842**	

Retrospective Study

In addition to the prospective clinical study, two clinical sites 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 study.

Demographic details for this patient population are summarized in the table below.

Gender and Age Demographic Detail for ProFAST+ Assay Retrospective Study

Sex	Number of Subjects
Female	74 (46.3%)*
Male	84 (52.5%)*
Age	
≤ 5 years	25 (15.6%)
6 - 21 years	24 (15.0%)
22 – 59 years	91 (56.9%)
≥ 60 years	20(12.5%)

*For two subjects, the gender was unknown.

Seasonal Influenza A/H1 Comparison Results

		ProFlu+/Sequencing			
		Positive	**Negative**	**Total**	
ProFAST+ Assay	**Positive**	17	1[a]	**18**	Positive Percent Agreement=94.4% (74.3% - 99.0%) 95% CI
	Negative	1	141	**142**	Negative Percent Agreement=99.3% (96.1% - 99.9%) 95% CI
	Total	**18**	**142**	**160**	

[a] 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 Comparison Results

		ProFlu+/Sequencing			
		Positive	**Negative**	**Total**	
ProFAST+ Assay	**Positive**	72	0	**72**	Positive Percent Agreement=100.0% (94.9% - 100.0%) 95% CI
	Negative	0	88	**88**	Negative Percent Agreement=100.0% (95.8% - 100.0%) 95% CI
	Total	**72**	**88**	**160**	

2009 H1N1 Influenza A Comparison Results

		ProFlu+/Sequencing			
		Positive	**Negative**	**Total**	
ProFAST+ Assay	**Positive**	25	0	**25**	Positive Percent Agreement=100.0% (86.7% - 100.0%) 95% CI
	Negative	0	135	**135**	Negative Percent Agreement=100.0% (97.2% - 100.0%) 95% CI
	Total	**25**	**135**	**160**	

Reproducibility

The reproducibility of the ProFAST+ Assay was evaluated at 3 laboratory sites. Reproducibility was assessed using a panel of 18 simulated samples that included medium positive, low positive (near the Assay limit of detection, ≥ 95% positive), and negative samples for each of the three Influenza A subtypes detected by the Assay. Panels and controls were tested at each site by 2 operators for 5 days (21 samples and controls/run X 1 run/day/operator X 2 operators X 5 days X 3 sites = 630 data points). Nucleic acid extraction was carried out using either the Roche MagNA Pure LC System (Site 1) or the bioMérieux NucliSENS easyMAG System (Sites 2 and 3). The overall percent agreement with the expected result for the ProFAST+ Assay was 99.7%.

	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	2009 H1N1 negative (values from IC)	2009 H1N1 low positive	2009 H1N1 medium positive	Extraction Control			Influenza A Subtyping RNA Control			Negative Control	Total % Agreement
											A/H1	A/H3	2009 H1N1	A/H1	A/H3	2009 H1N1		
	Concentration	0.001 X LoD	2 X LoD	10X LoD	0.001 X LoD	2 X LoD	10X LoD	0.001 X LoD	2 X LoD	10X LoD	N/A			N/A			N/A	
Site 1	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	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	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%	98.8% - 99.9%
	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	

Analytical Sensitivity

The analytical sensitivity (limit of detection or LoD) of the ProFAST+ Assay was determined using quantified ($TCID_{50}$/mL) cultures of seasonal Influenza A/H1, seasonal Influenza A/H3, and 2009 H1N1 Influenza A (2 strains per subtype) serially diluted in nasopharyngeal clinical matrix. Each viral strain was extracted using the Roche MagNA Pure LC System and tested in replicates of 20 per concentration of virus.

Analytical sensitivity (LoD), defined as the lowest concentration at which ≥ 95% of all replicates tested positive, ranged from $5x10^{-2}$ – $1x10^{2}$ $TCID_{50}$/mL.

Viral Strain	Limit of Detection
H1N1 A/Virginia/1/06	$5x10^{-1}$ $TCID_{50}$/mL
H1N1 A/Hong Kong/2652/06	$5x10^{-2}$ $TCID_{50}$/mL
H3N2 A/Anhui/1239/05	$1x10^{-1}$ $TCID_{50}$/mL
H3N2 A/California/07/04	$5x10^{-1}$ $TCID_{50}$/mL
2009 H1N1 Clinical Isolate #1	$1x10^{2}$ $TCID_{50}$/mL
2009 H1N1 Clinical Isolate #5	$1x10^{2}$ $TCID_{50}$/mL

Reactivity

The reactivity of the ProFAST+ Assay was evaluated against multiple strains and subtypes of Influenza A. The panel consisted of 14 Influenza A subtype H1N1, 15 Influenza A subtype H3N2, and 4 2009 H1N1.
Each viral strain was extracted using the Roche MagNA Pure LC System and then tested in triplicate.

Viral Strain	Concentration	A/H1	A/H3	2009 H1N1
A/Taiwan/42/06 (H1N1)	$2x10^{0}$ $TCID_{50}$/mL	+	-	-
A/Henan/8/05 (H1N1)	$2x10^{0}$ $TCID_{50}$/mL	+	-	-
A/Fujian/156/00 (H1N1)	$2x10^{1}$ $TCID_{50}$/mL	+	-	-
Brazil/1137/99 (H1N1)	$2x10^{5}$ $TCID_{50}$/mL	+	-	-
A/Kentucky/2/06 (H1N1)	$2x10^{2}$ $TCID_{50}$/mL	+	-	-
A/Hawaii/15/01 (H1N1)	$2x10^{2}$ $TCID_{50}$/mL	+	-	-
A/New Caledonia/12/99 (H1N1)	$2x10^{2}$ $TCID_{50}$/mL	+	-	-
A/Brisbane/59/2007 (H1N1)	$2x10^{2}$ $TCID_{50}$/mL	+	-	-
A/Solomon Islands/03/06 (H1N1)	$2x10^{0}$ $TCID_{50}$/mL	+	-	-
A/Jiangxi/160/05 (H1N1)	$2x10^{0}$ $TCID_{50}$/mL	+	-	-
VR 1520 A/WS/33 (H1N1)	$5.62x10^{4}$ $TCID_{50}$/mL *†	-	-	-
VR 98 A1/Mal/302/54 (H1N1)	$1.78x10^{6}$ $CEID_{50}$/mL *†	-	-	-
A/PR/8/34 (H1N1)	$2.0x10^{6}$ $TCID_{50}$/mL *	-	-	-
VR 546 A1/Denver/1/57 (H1N1)	$1.78x10^{6}$ $CEID_{50}$/mL *†	-	-	-
A/Hiroshima/52/05 (H3N2)	$2x10^{0}$ $TCID_{50}$/mL	-	+	-
A/Victoria/512/05 (H3N2)	$2x10^{0}$ $TCID_{50}$/mL	-	+	-
VR 822 A/Victoria/3/75 (H3N2)	$2x10^{2}$ $CEID_{50}$/mL†	-	+	-
A/Brazil/02/99 (H3N2)	$2x10^{3}$ $TCID_{50}$/mL	-	+	-
A/New York/55/2004 (H3N2)	$2x10^{0}$ $TCID_{50}$/mL	-	+	-
A/Hong Kong/2831/05 (H3N2)	$2x10^{1}$ $TCID_{50}$/mL	-	+	-
A/Port Chalmers/1/73 (H3N2)	$2x10^{0}$ $TCID_{50}$/mL	-	+	-
A/Bahamas/2686/99 (H3N2)	$2x10^{2}$ $TCID_{50}$/mL	-	+	-
A/Fuijan/411/02 (H3N2)	$2x10^{2}$ $TCID_{50}$/mL	-	+	-
A/Kentucky/03/06 (H3N2)	$2x10^{2}$ $TCID_{50}$/mL	-	+	-
A/Costa Rica/07/99 (H3N2)	$2x10^{2}$ $TCID_{50}$/mL	-	+	-
A/Hong Kong/218/06 (H3N2)	$2x10^{1}$ $TCID_{50}$/mL	-	+	-
A/Indiana/10/2011 (H3N2v)**	10^{3} $TCID_{50}$/mL	-	+	-

Viral Strain	Concentration	A/H1	A/H3	2009 H1N1
VR 544 A/Hong Kong/8/68 (H3N2)	$2x10^2$ $CEID_{50}$/mL†	-	+	-
VR 547 A/Aichi/2/68 (H3N2)	$2x10^2$ $CEID_{50}$/mL†	-	+	-
2009 H1N1 Clinical Isolate #2 (2009 H1N1 S-OIV)	$2x10^3$ $TCID_{50}$/mL	-	-	+
2009 H1N1 Clinical Isolate #3 (2009 H1N1 S-OIV)	$2x10^2$ $TCID_{50}$/mL	-	-	+
2009 H1N1 Clinical Isolate #4 (2009 H1N1 S-OIV)	$2x10^1$ $TCID_{50}$/mL	-	-	+
2009 H1N1 A/California/04/2009 (2009 H1N1 S-OIV)	$2x10^2$ $TCID_{50}$/mL	-	-	+

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

** Although this test has been shown to detect Influenza A/ Indiana/10/2011 (H3N2v) virus cultured from positive human respiratory specimens, the performance characteristics of this device with clinical specimens that are positive for H3N2v Influenza virus have not been established.

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

Twenty Eight (28) of the 32 strains used for this study were reactive with the ProFAST+ Assay. There were 4 strains that were not reactive with the ProFAST+ Assay as expected based on design and/or sequence homology to more contemporary influenza A strains. The following strains are not reactive with the ProFAST+ Assay, and they all represent older, non-contemporary (1933 to 1957) influenza A/H1 strains that are not in circulation:

- VR 1520 A/WS/33 (A/H1)
- VR 98 A1/Mal/302/54 (A/H1)
- A/PR/8/34 (A/H1)
- VR 546 A1/Denver/1/57 (A/H1)

Analytical Specificity

The analytical specificity of the ProFAST+ Assay was evaluated by testing a panel of 38 viruses, 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 10^6 to10^7 CFU/mL. Viruses were tested at concentrations of 10^3 to10^6 $TCID_{50}$/mL. Influenza A viruses (A/H1, A/H3, and 2009 H1N1) were tested at 2 logs above LoD. Samples were extracted and tested in triplicate. 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.

Strains	Concentration Tested	A/H1	A/H3	2009 H1N1
Influenza A H1N1 Strain A/Henan/8/05	10^2 $TCID_{50}$/mL	+	-	-
Influenza A H3N2 Strain A/California/07/04	10^1 $TCID_{50}$/mL	-	+	-
Influenza A/2009 H1N1 Clinical Isolate #2	10^5 $TCID_{50}$/mL	-		+
VR 897 A/New Jersey/8/76 H1N1	$2x10^4$ $TCID_{50}$/mL	-	-	+
A/South Dakota/03/2008 H1N1	$2x10^3$ $TCID_{50}$/mL	-	-	-
A/Wisconsin/10/1998 H1N1	$2x10^3$ $TCID_{50}$/mL	-	-	+
A/Iowa/2006 H1N1	$2x10^3$ $TCID_{50}$/mL	-	-	-
A/VN/1203 H5N1 RNA	2.7ng/µL	-	-	-
A/HK/486 H5N1 RNA	1.4ng/µL	-	-	-
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	-	-	-

Strains	Concentration Tested	A/H1	A/H3	2009 H1N1
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	-	-	-
Bordetella pertussis	10^7 cfu/mL	-	-	-
Bordetella bronchoiseptica	10^7 cfu/mL	-	-	-
Chlamydia pneumonia	10^3 $TCID_{50}$/mL	-	-	-
Legionella micdadei	10^6 cfu/mL	-	-	-
Legionella pneumophila	10^6 cfu/mL	-	-	-
Mycobacterium intracellulare	10^7 cfu/mL	-	-	-
Mycobacterium tuberculosis	10^6 cfu/mL	-	-	-
Mycoplasma pneumonia	10^6 cfu/mL	-	-	-
Haemophilus influenza	10^7 cfu/mL	-	-	-
Pseudomonas aeruginosa	10^7 cfu/mL	-	-	-
Proteus vulgaris	10^7 cfu/mL	-	-	-
Proteus mirabilis	10^7 cfu/mL	-	-	-
Neisseria gonorrhoeae	10^7 cfu/mL	-	-	-
Neisseria meningitides	10^7 cfu/mL	-	-	-
Neisseria mucosa	10^7 cfu/mL	-	-	-
Klebsiella pneumonia	10^7 cfu/mL	-	-	-
Escherichia coli	10^7 cfu/mL	-	-	-
Moraxella catarrhalis	10^7 cfu/mL	-	-	-
Corynebacterium diptheriae	10^6 cfu/mL	-	-	-
Lactobacillus plantarum	10^6 cfu/mL	-	-	-
Streptococcus pneumoniae	10^7 cfu/mL	-	-	-
Streptococcus pyogenes	10^7 cfu/mL	-	-	-
Streptococcus salivarius	10^6 cfu/mL	-	-	-
Staphylococcus epidermidis	10^7 cfu/mL	-	-	-
Staphylococcus aureus	10^7 cfu/mL	-	-	-
Candida albicans	10^7 cfu/mL	-	-	-

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 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. The ProFAST+ Assay primer and probe sequences for each Influenza A subtype, A/H1, A/H3 and 2009 H1N1, are unique and are not expected to cross-react with the other Influenza A subtypes listed in the table below.

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
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

Interference

Mucin, whole blood and a number of other potentially interfering exogenous substances (medications and over the counter (OTC) products) that may be present in the nasopharynx were evaluated with the ProFAST+ Assay. Seasonal Influenza A/H1 was spiked into negative NP pools at 2X LoD. Clinically relevant amounts of the potential interfering substances were added to spiked samples. An Internal Control (IC) was also added to each sample. The ProFAST+ Assay was performed in triplicate reactions for each sample. The following table shows the potential interfering substances used for this study. The substances consisted of nasal sprays (liquid and powder), ingestible pills and lozenges, injectables, and endogenous substances:

Substance Name	Active Ingredient	Concentration Tested
Mucin	Purified mucin protein	5%, 1%, and 0.1% w/v
Blood (human)	N/A	2% (volume/volume)
Neo-Synephrine	Phenylephrine HCl	15% (volume/volume)
Anefrin Nasal Spray	Oxymetazoline Hydrochloride	15% (volume/volume)
Zicam Nasal gel	Luffa Operculata, Galphimia Glauca, Histaminum Hydrochloricum, Sulfur	5% (volume/volume)
Saline Nasal Spray	Sodium chloride with preservatives	15% (volume/volume) of dose
Beconase AQ®	Beclomethasone dipropionate	5% volume/volume
Walgreens Sore Throat Lozenges	Oral anesthetic/analgesic	0.62mg/ml, active ingredients: 1.0mg/ml benzocaine, 1.7mg/ml menthol
Relenza	Zanamivir	5mg/ml
Tobramycin	Tobramycin	4.0µg/ml
Bactroban Nasal ointment	Mupirocin	10mg/ml
TamiFlu	Oseltamivir	7.5mg/ml

The ProFAST+ Assay was not affected by the presence of a panel of endogenous and exogenous potential RT-PCR inhibitors with the exception of mucin. Results indicate that mucin at a concentration of 5% and 1% w/v added to NP swab matrix, resulting in a highly viscous sample, had an inhibitory effect on detection of influenza A subtypes and the Internal RNA Control (IC) near the LoD using the ProFAST+ Assay.

Extractor Equivalency

Equivalency of the bioMérieux NucliSENS easyMAG and the Roche MagNA Pure LC instruments for nucleic acid extraction was evaluated by performing a limit of detection study using the easyMAG and comparing those results to the results obtained during the Analytical Sensitivity Study which was performed using the MagNA Pure LC. Six strains of Influenza A (2 each subtype: A/H1, A/H3, and 2009 H1N1) were used for this study. Twenty independent samples of each strain at the LoD determined during the Analytical Sensitivity Study were spiked with the IC, extracted, and tested using the ProFAST+ Assay.

The ProFAST+ Assay demonstrated comparable performance (statistically not different, $p>0.05$) with respect to the limit of detection on the bioMérieux NucliSens easyMAG and Roche MagNA Pure LC instruments.

Carry-over/Cross-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 RT-PCR on the Cepheid SmartCycler II thermocycler, a Carry-Over study was performed. Simulated Influenza A/2009 H1N1 high positive samples were run in series alternating with Influenza A/2009 H1N1 high negative samples. The results of this study demonstrate no evidence of carryover/cross-contamination over a 5 day course of processing High Positive samples alongside High Negative samples using the ProFAST+ Assay.

Disposal

Dispose of hazardous or biologically contaminated materials according to the practices of your institution.

References

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The following symbols may appear on the packaging and labeling:

Symbol	Definition	Symbol	Definition
REF	Reference Number or Catalog number	Σ	Contains sufficient for < n > tests
LOT	Batch code or Lot Number		Do not reuse
	Use By Date or Expiration Date		Manufacturer
	Upper Storage Temperature Limitation	EC REP	Authorized Representative
CONT	Contents		

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