The information previously contained on these webpages were authorized under the 2009 H1N1 Influenza Emergency Use Authorizations (EUAs). As of June 23, 2010, the EUAs have been terminated and this information is no longer current.

Influenza A H1N1 (2009) Real Time RT-PCR

REF MOL9100
Rev. B

A real-time polymerase chain reaction (PCR) assay intended for the *in vitro* qualitative detection of 2009 H1N1 influenza virus RNA.

Emergency Use Authorization
For *in vitro* Diagnostic Use

INTENDED USE

The Focus Diagnostics Influenza A H1N1 (2009) Real Time RT-PCR is intended for use in CLIA High Complexity Laboratories with the ability to perform RT-PCR using the Roche MagNA Pure™ LC and the AB 7500 Real Time PCR System for the *in vitro* qualitative detection of 2009 H1N1 influenza viral RNA in upper respiratory tract specimens (such as nasopharyngeal swabs (NPS), nasal swabs (NS), throat swabs (TS), nasal aspirates (NA), nasal washes (NW), and dual nasopharyngeal/throat swabs (NPS/TS)), and lower respiratory tract specimens (such as broncheoalveolar lavage (BAL), bronchial aspirate (BA); bronchial wash (BW); endotracheal aspirate (EA); endotracheal wash (EW); tracheal aspirate (TA), and lung tissue) from human patients with signs and symptoms of respiratory infection in conjunction with clinical and epidemiological risk factors.

Testing with the 2009 H1N1 influenza primer and probe sets should not be performed unless the patient meets clinical and epidemiologic criteria for testing suspect specimens. The identification of 2009 H1N1 influenza should be performed along with clinical and epidemiological assessment.

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 caused by three immunologic types (A, B, and C) of RNA viruses within the Orthomyxoviridae family. Influenza A is classified further by describing two viral proteins expressed on its surface, hemagglutinin and neuraminidase. Hemagglutinin facilitates binding of the virus to respiratory epithelial cells, whereas neuraminidase functions to break those bonds with the host cell so that new virions can be released. Seasonal influenza is typically caused by three major subtypes of hemagglutinin (H1, H2, and H3) and two subtypes of neuraminidase (N1 and N2). In late March 2009, a novel influenza virus (2009 H1N1 influenza) began circulating in North America and subsequently around the world[1]. The rearranged virus has components of human influenza A virus, avian influenza A virus, and a hemagglutinin component derived from an influenza A virus known to infect swine.

Influenza classically presents with a combination of upper and lower respiratory signs and symptoms, fever, headache, myalgia, and general malaise. Illness can take on a variety of appearances, ranging from isolated respiratory findings that resemble the common cold, to severe pneumonia requiring hospitalization. Persons at higher risk for hospitalization include children aged <2, adults aged >65, and those with significant comorbidities. Flu caused by 2009 H1N1 influenza virus, like seasonal flu, may cause exacerbation of underlying medical conditions. The duration of illness is typically 2-5 days, but symptoms may last for a week or longer.

The seasonal nature of influenza, commonly referred to as “flu season”, is a widely recognized characteristic of the virus. Influenza owes its recurring nature to a process known as antigenic drift. Point mutations in the genetic makeup of the virus allow for expression of different surface proteins that permit the virus to evade immunities developed in prior seasons. A more significant change to the surface glycoproteins of the virus is known as antigenic shift. The greater the change in these antigens, the less likely that existing immunity in the population will confer protection against the new variant. It is for this reason that antigenic shift is associated with epidemics and pandemics. The changes seen in the 2009 H1N1 influenza virus are major enough to be considered antigenic shift.[2]

Testing of lower respiratory tract specimens in critically ill patients having clinical evidence suggestive of influenza may be necessary to confirm the presence of 2009 H1N1 infection. Experience (and animal studies) suggests that some patients with severe lower respiratory tract disease have high titers of virus in the lower respiratory tract, but low or absent titers in the upper respiratory tract. Therefore, accepting only upper respiratory specimens from these patients can lead to the false conclusion that the patient does not have an H1N1 infection.

PRINCIPLES OF THE PROCEDURE

The test is a real-time RT-PCR assay that utilizes fluorogenic hydrolysis probe (TaqMan®) technology for the detection of human influenza A viruses and the differential detection of 2009 H1N1 influenza virus in upper respiratory tract specimens (such as nasopharyngeal swabs (NPS), nasal swabs (NS), throat swabs (TS), nasal aspirates (NA), nasal washes (NW), and dual nasopharyngeal/throat swabs (NPS/TS)), and lower respiratory tract specimens (such as broncheoalveolar lavage (BAL), bronchial aspirate (BA); bronchial wash (BW); endotracheal aspirate (EA); endotracheal wash (EW); tracheal aspirate (TA), and lung tissue). The assay is composed of two principal steps: (1) extraction of RNA from patient specimens, (2) one-step reverse transcription and PCR amplification with human influenza A virus and 2009 H1N1 influenza virus specific primers and real-time detection with human influenza A virus and 2009 H1N1 influenza virus specific probes. The assay provides two results. A well-conserved region of the matrix gene from influenza A viruses is targeted in one set of reactions to identify both human influenza A virus and 2009 H1N1 influenza virus in the specimen. Two separate regions of the hemagglutinin gene of the 2009 H1N1 influenza virus are targeted also to differentiate the presence of seasonal human influenza A virus and the 2009 H1N1 influenza virus. An internal control is used to confirm the absence of PCR inhibition.

MATERIALS PROVIDED

The Focus Diagnostics Influenza A H1N1 (2009) Real Time RT-PCR kit contains sufficient primers and probes for 100 reactions. Upon receipt, store all kit components at -10 to -30ºC (do not use a frost-free freezer). Kit components are stable through the end of the expiration month indicated on the kit packaging when stored at -10 to -30ºC. After reagents have been prepared for use, use within one hour.

Table 1: Description of the kit labeling and kit components

Kit	Label					
Focus Diagnostics' Influenza A H1N1 (2009) Real Time RT-PCR (Part # MOL9100)	FLU Mix 1	REF	MOL9101	REAG	A	
	FLU Mix 2	REF	MOL9102	REAG	B	
	RT Mix	REF	MOL9103	REAG	C	
	Positive Control H1N1	REF	MOL9104	CONTROL	+	

Components	Number in Kit	Color Code	Label			
FLU Mix 1	4	Brown	REF	MOL9101	Lot	Expires
FLU Mix 2	4	Purple	REF	MOL9102	Lot	Expires
RT Mix	4	Yellow	REF	MOL9103	Lot	Expires
Positive Control H1N1	8	Red	REF	MOL9104	Lot	Expires

Table 2: Description of the kit components

Kit Component	Reactions per Vial	Minimum Volume (µL) per Vial	Component Description
FLU Mix 1	25	975 µL	Dye-labeled fluorescent primers and probes specific for detection of FLU A and/or H1N1 and for the Internal Control template.
FLU Mix 2	25	975 µL	Dye-labeled fluorescent primers and probes specific for detection of H1N1.
RT Mix	50	50 µL	Reverse Transcriptase Enzyme
Positive Control H1N1	1	250 µL	Inactivated 2009 H1N1 virus.

Target	Probe Fluorophore	Targeted Gene	* Instrument Channel
FLU A "FLUA"	QUASAR 670	*matrix*	CY5
H1N1 (Primary) "H1N1-1"	CAL Flour Red 610	*HA*	TEXAS RED
Internal Control "QIPC"	VIC	N/A	VIC

Target	Probe Fluorophore	Targeted Gene	* Instrument Channel
H1N1 (Secondary) "H1N1-2"	FAM	*HA*	FAM

* Applied Biosystems 7500 (AB 7500)

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Real-Time PCR System: Applied Biosystems 7500 Real-Time PCR System.
2. Roche MagNA Pure LC and associated consumables.
3. MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Cat. No 3038505001)
4. Single or multi-channel micropipette(s) with an accuracy range between 1-10 µL, 10-100 µL and 100-1000 µL.
   NOTE: Dedicated micropipettes are required for extraction, as well as for the Pre-Amplification Areas I, II, and III.

5. Freezer (manual defrost) at -10 to -30°C (for kit component frozen storage)
6. Freezer (manual defrost) at -10 to -30°C (for specimen frozen storage)
7. Refrigerator at 2 to 8°C.
8. Laminar flow hood for extractions
9. Bench top centrifuge for low speed centrifugation of 96-well plates or other reaction vessels
10. Microcentrifuge
11. Vortex mixer
12. Sterile, RNase/DNase-free disposable aerosol-barrier micropipettor tips
13. Sterile Nuclease-free water
14. RNase/DNase-free 1.5 mL polypropylene microcentrifuge tubes and racks
15. RNase/DNase-free 96 well thermocycler plate rack or appropriate tube rack
16. 96-well optical reaction plate or other reaction vessels
17. Optical adhesive cover (for use with 96-well optical reaction plate)
18. Disposable, powder-free gloves
19. Cooler racks for 1.5 mL microcentrifuge tubes and 96-well 0.2mL PCR reaction tubes or plates.

SHELF LIFE AND HANDLING

1. Store reagents at -10 to -30 °C (do not use a frost-free freezer).
2. Do not use kits or reagents beyond their expiration dates.
3. Allow reagents to thaw at room temperature (approximate range 18 to 25 °C) before use.
4. After addition of RT Mix use within one hour.
5. Do not refreeze FLU Mix 1, FLU Mix 2 or Positive Control.
6. Kit reagents are stable through the end of the expiration month indicated on the kit packaging when stored at -10 to -30°C.

WARNINGS AND PRECAUTIONS

1. Follow universal precautions. All patient specimens and the positive control should be considered potentially infectious and handled accordingly.
2. Diagnostic laboratory work on clinical samples from patients who are suspected cases of 2009 H1N1 influenza virus infection should be conducted in a BSL2 laboratory. All sample and positive control manipulations should be done inside a biosafety cabinet. Viral isolation on clinical specimens from patients who are suspected cases of 2009 H1N1 influenza virus infection should be performed in a BSL2 laboratory with BSL3 practices (enhanced BSL2 conditions)[3].
3. Wear personal protective equipment, such as (but not limited to) gloves and lab coats when handling kit reagents. Wash hands thoroughly when finished performing the test.
4. Do not pipette by mouth.
5. Do not smoke, drink, eat, handle contact lenses or apply make-up in areas where kit reagents and/or human specimens are being used.
6. Dispose of unused kit reagents and human specimens according to local, state and federal regulations.
7. Workflow in the laboratory should proceed in a uni-directional manner, beginning in the Pre-Amplification areas (I, II and III) and moving to the Amplification/Detection area. Below is the sequence of events that takes place from specimen extraction (Pre-amplification Area I) to PCR set-up (Amplification/Detection area):
   - Begin Pre-amplification studies with specimen extraction (Pre-Amplification Area I) followed by reagent preparation (Pre-amplification Area II), PCR set-up (Pre-amplification Area III), and finally the instrument set-up (Amplification/Detection).
   - Do not use supplies and equipment across the dedicated areas of specimen extraction and sample preparation. No cross-movement is allowed between the different areas.
   - Supplies and equipment used for reagent preparation should not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target nucleic acid.
   - All amplification supplies and equipment should be kept in the Amplification/Detection Area at all times.
   - Personal Protective Equipment, such as laboratory coats and disposable gloves, should be area-specific.
8. Contamination of patient specimens or reagents can produce erroneous results. Use aseptic techniques.
9. Pipette and handle reagents carefully to avoid mixing of samples from adjacent wells.
10. Use proper pipetting techniques and maintain the same pipetting pattern throughout the procedure to ensure optimal and reproducible values.
11. Do not substitute or mix reagent from different kit lots or from other manufacturers.
12. Do not interchange the reagent tube caps. This may cause contamination and compromise the test results.
13. Only use the protocol described in this insert. Deviations from the protocol or the use of times or temperatures other than those specified may give erroneous results.
14. Assay setup should be performed at room temperature (approximate range 18 to 25 °C).
15. Do not re-use wells that have already been exposed to patient samples or reagents.
16. Dispose of amplified samples without opening reaction vessel.
17. FLU Mix 1 and 2 may contain 1 to 10 % glycerol which may cause irritation upon inhalation or skin contact. Upon inhalation or contact, first aid measures should be taken.

INSTRUCTIONS FOR USE

A. SPECIMEN COLLECTION

Acceptable specimen types include upper respiratory tract specimens (such as nasopharyngeal swabs (NPS), nasal swabs (NS), throat swabs (TS), nasal aspirates (NA), nasal washes (NW), and dual nasopharyngeal/throat swabs (NPS/TS)), and lower respiratory tract specimens (such as broncheoalveolar lavage (BAL), bronchial aspirate (BA); bronchial wash (BW); endotracheal aspirate (EA); endotracheal wash (EW); tracheal aspirate (TA), and lung tissue) in sterile viral transport media containing protein stabilizer, antibiotics to discourage bacterial and fungal growth, and buffer solution, (e.g. UTM, VCM, M4, M5, M6 and other media intended to transport Chlamydia, Mycoplasma and viruses). If using swabs, use only sterile Dacron, nylon, or rayon swabs with plastic shafts. Do not use calcium alginate swabs, as they may contain substances that inhibit PCR testing.

B. SPECIMEN EXTRACTION AREA (Pre-Amplification Area I)

Dedicated area for specimen extraction and preparation of the Positive Control

NOTE: Use RNase/DNase-free tubes only

1. Nucleic acids are extracted from patient specimens and assay controls using the Roche MagNA Pure Total Nucleic Acid kit and the Roche MagNA Pure LC Automated Nucleic Acid Extractor instrument. Refer to the manufacturer's Instructions for Use for nucleic acid extraction using this kit.
2. Under the "Protocol" drop-down menu on the MagNA Pure LC System, select "Total NA", and then "Total NA External lysis.blk" from the list. This will load the appropriate settings for the run.
3. The Sample Protocol should be "Total NA External_lysis".
4. 500 µL should be set for the Lysed Sample Volume, and the elution volume should be set at 50 µL.
5. The dilution volume should be set at zero for all samples.
6. Ensure that the Post Elution Handling is set to "None".
7. Once the positive control material has thawed, vortex the vial for approximately 2 seconds, centrifuge briefly in a microcentrifuge.
8. For specimens and controls, pipette 250 µL of MagNA Pure Lysis buffer into each well of the sample cartridge that will be loaded with specimen.
9. In a biosafety cabinet, pipette 250 µL of each specimen or control into the corresponding position in the sample cartridge. Pipette up and down one time in the lysis buffer to mix.
10. Ensure that specimens and controls are in the correct position on the Sample Cartridge.
11. Visually check the level of samples and controls in the Sample Cartridge to ensure sample(s) were added.
12. Transfer the sample cartridge containing lysed samples to the MagNA Pure LC Automated Nucleic Acid extractor and begin the extraction run.
13. After nucleic acid extraction is complete, the cartridge containing the extracted controls and patient specimens can be sealed and removed from the MagNA Pure. Store the RNA for at least 15 minutes refrigerated prior to use. Extended storage at 2 – 8°C is not recommended; performance has not been established.

C. REAL-TIME PCR INSTRUMENT SETUP (Amplification/Detection Area)

Perform the following to configure the AB 7500: instrument software for the Influenza A H1N1 (2009) Real Time RT-PCR:

1. Refer to AB 7500 Instructions for Use for details on how to run the plate on the AB 7500 Real Time PCR System.
2. Launch AB 7500 Real Time PCR System software.
3. Click **Create New Document** in the **Quick Startup document** window.
4. Begin to configure the template by verifying the default settings appear as follows:
   - **Assay:** Standard Curve (Absolute Quantitation)
   - **Container:** 96-Well Clear
   - **Template:** Blank Document
   - **Run Mode:** Standard 7500
5. Click **Next** and continue to configure the template in the **Quick Startup document** window as follows:
   - Define the **Detectors** and **Reporters** as indicated below. (NOTE**:** Click **New Detector** button if necessary).

Detector Name	Reporter Dye	Quencher
H1N1-1	TEXAS RED	NONE
H1N1-2	FAM	NONE
FLUA	CY5	NONE
QIPC	VIC	TAMRA

   - Select **Passive Reference** dye to **none**.
   - Click **Next**.
   - Configure your plate as indicted in the example plate below.
     - P1: Positive Control; first sample added to the plate.
     - S: Patient Sample
     - NTC: Nuclease-Free water; last sample added to the plate.
     - Use "H1N1-1", "FLUA" and "QIPC" as the detectors for the wells containing Reaction Mix 1 and "H1N1-2" for the wells containing Reaction Mix 2.

- Click **Finish**.

Example Plate

	Wells with Reaction Mix 1						Wells with Reaction Mix 2					
	1	2	3	4	5	6	7	8	9	10	11	12
A	P1	S	S	S	S	S	P1	S	S	S	S	S
B	S	S	S	S	S	S	S	S	S	S	S	S
C	S	S	S	S	S	S	S	S	S	S	S	S
D	S	S	S	S	S	S	S	S	S	S	S	S
E	S	S	S	S	S	S	S	S	S	S	S	S
F	S	S	S	S	S	S	S	S	S	S	S	S
G	S	S	S	S	S	S	S	S	S	S	S	S
H	S	S	S	S	S	NTC	S	S	S	S	S	NTC

6. Continue template configuration in the AB 7500 main screen by selecting the **Instrument** tab and entering the following thermal cycling settings:

STAGE 1	**Cycles:** 1	**STAGE 2**	**Cycles:** 1	**STAGE 3**	**Cycles:** 50	
	Temperature: 48°C		**Temperature:** 95°C		**Temperature:** 95°C	**Temperature:** 60°C
	Time: 30:00		**Time**: 10:00		**Time**:00:15	**Time**: 1:00

7. Complete template configuration in the **Settings** section as follows:
   - Enter **Sample Volume:** 50 µL
   - Select **Run Mode:** Standard 7500
   - Select **Data Collection:** Stage 3, Step 2
8. Save file by selecting **Save As** in the **File** dropdown, name appropriately, and ensure file format is **.sds**

D. REAGENT PREPARATION AREA (Pre-Amplification Area II)

Dedicated area for preparation of Influenza A H1N1 (2009) Real Time RT-PCR Reaction Mix.

NOTE: Use RNase/DNase-free tubes only

1. Thaw the FLU Mix 1 and FLU Mix 2 at room temperature (approximate range 18 to 25°C). Each kit component vial contains sufficient volume for 25 reactions.
2. Prepare the required volume of Reaction Mix 1 and Reaction Mix 2 in separate appropriately sized polypropylene microcentrifuge tube by pipetting the volume of each component as indicated in Table 3.

Table 3: Reaction Mix volumes

Reagent	Reaction Mix 1 Volume / 1 reaction	Reaction Mix 1 Volume / 10 reactions	Reaction Mix 2 Volume / 1 reaction	Reaction Mix 2 Volume / 10 reactions)
FLU Mix 1	39.0	390 µL		--
FLU Mix 2	--	--	39.0 µL	390 µL
RT Mix	1.0 µL	10 µL	1.0 µL	10 µL
Total Volume	40.0 µL	400 µL	40.0 µL	400 µL

3. Gently mix the reaction mix by inversion or by pipetting.
4. Centrifuge for approximately five seconds to collect the contents to the bottom of the tube.
5. Use Reaction Mix 1 and Reaction Mix 2 within 1 hour of preparation.
6. Proceed to PCR Setup.

E. PCR SETUP AREA (Pre-Amplification Area III)

Dedicated area for preparation of 96-well plate for Influenza A H1N1 (2009) Real-Time RT-PCR

NOTE: Perform reaction set up on a cold rack

NOTE: Use RNase/DNase-free plates and tubes only

Refer to example plate map in section B while performing the following setup

1. Add 40.0 µL Reaction Mix 1 to one well and Reaction Mix 2 to another well for each sample.
2. Add 10.0 µL of the extracted Positive Control to the “P1” plate well.
3. Add 10.0 µL of extracted patient sample to the appropriate “S” plate well.
4. Add 10.0 µL of the Nuclease-free water to the “NTC” plate well.
5. Tightly seal the plate with the optical adhesive cover.
   Note: Make sure to handle the Optical Adhesive Cover on the edge only. Do not touch the middle part of the cover.
6. Briefly centrifuge the plate to collect the reactions at the bottom of the wells and to eliminate any air bubbles.
7. Take the plate to the AB 7500 Real Time PCR System.
8. Open the AB 7500 plate holder and carefully place the test plate in the tray inside. Position the plate so the A1 well is in the upper left corner of the plate holder. Carefully close the plate holder.
9. Click **Start** in the **Instrument** tab.

F. DATA ANALYSIS

1. When the run finishes, click **OK** from the window.
2. Click on **Analysis** menu and choose **Analysis settings**.
3. Detectors should be set at “**All Detectors**”.

4. **Manual Ct** should be selected
5. **Manual Baseline** should be selected (Start at 3, Stop at 15)
6. Set the **threshold** for all detectors at **40,000.**
7. Click the **Analyze** icon (►) from the **toolbar**
   Note: Wait for a few minutes for the analysis process to be complete.
8. Click the **Results** Tab.
9. Click the **Amplification Plot** tab.
10. Choose **H1N1-1, FLUA, QIPC** and **H1N1-2** from the **Detector** window.
11. Click one well containing a specimen at a time and look at the Amplification plot and Component plot to check for the accuracy of the result.
12. Choose Save option from the File menu after analyzing all the wells.
13. Export the Results to the LIS, if desired.
14. Print the results for records, if desired.

REPORTING RESULTS

1. All assay controls must be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.
2. Examine the 2009 H1N1 Flu / Influenza A RT-PCR Positive and Negative Controls
   a. If the Negative Control is:
      i. Positive (C_T value less than 50 for either the H1N1-1, H1N1-2 or FLUA detector probe), then this control is invalid. This indicates possible contamination of prepared samples. Positive patient results cannot be reported. Positive specimens on this run must be repeated. Negative specimens may be reported given that all other assay run criteria are met.
      ii. Negative (C_T value of 50 or listed as "Undetermined"), then this control is valid and acceptable.
   b. Positive Controls
      i. If the Positive Control is above the upper value of your laboratory's predetermined acceptance criteria for FLUA, H1N1-1 or H1N1-2, the assay run is considered invalid and unacceptable. All patient specimens must be re-assayed.
      ii. If the C_T values for FLUA, H1N1-1 AND H1N1-2 are within or below their respective acceptable criteria, the assay run is considered valid and acceptable.
3. Examination of Patient Specimen Results
   a. Examination of clinical specimen results should be performed after the Positive and Negative Controls have been examined and determined to be valid and acceptable. H1N1-1, H1N1-2, FLUA and QIPC results must be examined for each patient specimen.
   b. Amplification plots should be examined for every positive sample (those with C_T value below 50). If the amplification plot shows an exponential increase, the amplification curve is valid.
4. Interpretation of Results
   a. A specimen that does not contain influenza A virus (pan A or 2009 H1N1 influenza virus) will be negative for the FLUA, H1N1-1 and H1N1-2 detectors. A specimen positive for seasonal influenza A virus will have a positive result for the FLUA detector and will be negative for both H1N1-1 and H1N1-2 detectors. In the majority of specimens, a specimen positive for 2009 H1N1 will be positive for the FLUA detector, the H1N1-1 detector and the H1N1-2 detector. (see Table 4 below; examples 1-3).
   b. Validation studies have shown that specimens containing influenza A viruses (seasonal or 2009 H1N1 influenza) will most likely have C_T values below 38 for the FLUA, H1N1-1 and/or H1N1-2 detectors. Specimens with C_T values in the range of 38.00 – 49.99 for any of these detectors most likely represent specimens with very low influenza A virus concentrations. Repeat testing on these specimens must be performed to confirm positive results.
   c. Validation studies have shown that specimens containing 2009 H1N1 influenza virus will be positive for both the H1N1-1 AND H1N1-2 detectors. If only one of these 2009 H1N1 influenza virus RT-PCR reactions is positive, the specimens must be retested to confirm the result. If upon repeat testing the same result is obtained, the specimen can be reported as positive for 2009 H1N1 influenza virus.
   d. If the FLUA C_T value of a patient sample is ≥ 50 or is listed as "Undetermined" and the QIPC C_T value falls within or below the acceptable range, the "Influenza A RNA" result is reported as "Not Detected".
   e. If the FLUA C_T value of a patient specimen is less than 38 and an amplification curve is observed for the well, the "Influenza A RNA" result is reported as "Detected". If the C_T value for the well is less than 50 but no amplification curve is observed (nonspecific fluorescence is observed in the well), the "Influenza A RNA" result is reported as "Not Detected"
   f. If the H1N1-1 C_T value and the H1N1-2 C_T value of a patient sample are both ≥ 50 or are listed as "Undetermined" and the QIPC C_T value falls within or below the acceptable range, the "2009 H1N1 Influenza H1 Gene" result is reported as "Not Detected".
   g. If the H1N1-1 C_T value and the H1N1-2 C_T values of a patient specimen are less than 38 and an amplification curve is observed for each well, the "2009 H1N1 Influenza H1 Gene" result is reported as "Detected". If the C_T value for each well is less than 50 but no amplification curve is observed in either well (nonspecific fluorescence is observed in the well), the "2009 H1N1 Influenza H1 Gene" result is reported as "Not Detected".

h. If only one of the H1N1-1 or H1N1-2 C_T values of a patient specimen is less than 38, the specimen must be retested to verify the result (see Table 4 below).
i. If the FLUA, H1N1-1 or H1N1-2 C_T value of a patient specimen is in the range of 38–49.99 and an amplification plot is observed for the well, the specimen must be retested to verify the positive result for the appropriate RNA target.
j. If upon retesting, the FLUA C_T value is < 50 for the FLUA and an amplification plot is observed for the well, the "Influenza A RNA" result is reported out as "Detected".
k. If upon retesting, if the H1N1-1 and the 2009 H1N1 2 C_T values are < 50 and an amplification plot is observed for each well, the "2009 H1N1 Influenza H1 Gene" result is reported out as "Detected".
l. If upon retesting the FLUA, H1N1-1 or H1N1-2 C_T value of a patient specimen is ≥ 50 or is listed as "Undetermined" and the QIPC C_T value falls within the acceptable range, the respective result is interpreted as "Not Detected".
m. If upon repeat testing, the interpretation of the result for FLUA, H1N1-1 or H1N1-2 changes, a third replicate should be tested, and the consensus of the three replicates should be reported.
n. If the FLUA, H1N1-1 or H1N1-2 C_T value of a patient specimen is ≥ 50 or is listed as "Undetermined" but the QIPC C_T value falls above the upper value of the acceptable range, the specimen must be retested. If upon repeat testing the same situation occurs the patient result is reported as "Indeterminate due to inhibition" with the additional comment: "After repeat analysis, non-amplification of the internal control suggests the presence of PCR inhibitors in the patient sample. An additional sample should be submitted for testing if clinically warranted."

Table 4. Interpretation of RT-PCR Results

Example	FLUA C_T value	H1N1-1 C_T value	H1N1-2 C_T value	Interpretation
1	50*	50	50	Influenza A RNA: Not detected 2009 Influenza H1 Gene: Not detected Specimen negative for 2009 H1N1 influenza viral RNA
2	<38	50	50	Influenza A RNA: Detected 2009 Influenza H1 Gene: Not detected Specimen negative for 2009 H1N1 influenza viral RNA
3	<38	<38	<38	Influenza A RNA: Detected 2009 Influenza H1 Gene: Detected Specimen positive for 2009 H1N1 influenza viral RNA
4	<38	50	<38	Repeat testing to confirm
5	<38	<38	50	Repeat testing to confirm
6	50	<38	<38	Repeat testing to confirm
7	If FLUA, H1N1-1 or H1N1-2 C_T value is 38-49.99, repeat testing to confirm			
Below are interpretations of specimens upon repeat testing:				
8	50	50	50	Influenza A RNA: Not detected 2009 Influenza H1 Gene: Not detected
9	<50	50	50	Influenza A RNA: Detected 2009 Influenza H1 Gene: Not detected
10	<50	<50	<50	Influenza A RNA: Detected 2009 Influenza H1 Gene: Detected
11	<50	<50	50	Influenza A RNA: Detected 2009 Influenza H1 Gene: Detected
12	<50	50	<50	Influenza A RNA: Detected 2009 Influenza H1 Gene: Detected
13	50	<50	<50	Influenza A RNA: Not detected 2009 Influenza H1 Gene: Not detected
14	50	<50	50	Influenza A RNA: Not detected 2009 Influenza H1 Gene: Not detected
15	50	50	<50	Influenza A RNA: Not detected 2009 Influenza H1 Gene: Not detected
16	If FLUA, H1N1-1 or H1N1-2 interpretation changes upon repeat testing, a third replicate should be tested, and the consensus of the three replicates should be reported.			

* the AB 7500 printout will read "undetermined"

LIMITATIONS

1. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
2. All results from this and other tests must be correlated with the clinical history, epidemiological data and other data available to the clinician evaluating the patient.
3. The prevalence of infection will affect the test's predictive value.
4. Negative results do not rule out Influenza A or 2009 H1N1 influenza infections.
5. False negative results may occur when the infecting organism has genomic mutations, insertions, deletions, or rearrangements or when performed very early in the course of illness.
6. False negative results may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
7. False positive results may occur. Repeat testing or testing with a different device may be indicated in some settings.
8. This test is a qualitative test and does not provide the quantitative value of detected organism present.
9. This test is intended for patients with signs and symptoms of respiratory infection.
10. This test is not intended for monitoring treatment of influenza A or 2009 H1N1 influenza infection.
11. This test is not intended for screening of blood or blood product for the presence of influenza A or 2009 H1N1 influenza.
12. This test has not been evaluated with potentially interfering medications for the treatment of influenza or cold virus.
13. This test has not been evaluated for individuals who have received the influenza vaccine.
14. This test cannot rule out diseases caused by other bacterial or viral pathogens.
15. The use of sputum has not been evaluated with this assay.

ANALYTICAL PERFORMANCE CHARACTERISTICS

ANALYTICAL SENSITIVITY/LIMIT OF DETECTION

The Limit of Detection for upper respiratory tract specimens was determined for each primer and probe set in the Influenza A H1N1 (2009) RT-PCR assay by limiting dilution studies using viral stocks of 2009 H1N1 influenza (~ $9x10^7$ gene copies/mL) and seasonal influenza A H1N1 (~$9.1x10^7$ gene copies/mL) and H3N2 (~$3.6x10^8$ gene copies/mL). The results were compared to the analytical sensitivity results of the CDC rRT-PCR Swine Flu Panel. The lowest concentration in which 95% detection was reached was determined to be the limit of detection for each assay. The result summary is presented in Tables 5 to 7.

Table 5. Summary of Comparative LoD data: Focus Assay vs. CDC Assay for 2009 H1N1 Influenza Virus Detection

Estimated Gene Copies/mL	Focus Diagnostics Assay		CDC Assay	
	Initial Screening	Confirmation of LoD	Initial Screening	Confirmation of LoD
$4.5x10^5$	3 of 3	N/A	3 of 3	N/A
$9.0x10^4$	3 of 3	N/A	3 of 3	N/A
$4.5x10^4$	3 of 3	20 of 20	3 of 3	20 of 20
$2.3x10^4$	N/A	19 of 20	N/A	20 of 20
$9x10^3$	1 of 3	N/A	1 of 3	N/A

Table 6. Summary of Comparative LoD data: Focus Assay vs. CDC Assay for Seasonal Influenza A (H1N1) Virus Detection

Estimated Gene Copies/mL	Focus Diagnostics Assay		CDC Assay	
	Initial Screening	Confirmation of LoD	Initial Screening	Confirmation of LoD
$9.1x10^4$	3 of 3	N/A	3 of 3	N/A
$4.6x10^4$	3 of 3	20 of 20	3 of 3	N/A
$2.3x10^4$	N/A	7 of 20	N/A	N/A
$9.1x10^3$	2 of 3	N/A	3 of 3	20 of 20
$4.6x10^3$	1 of 3	N/A	2 of 3	20 of 20
$9.1x10^2$	0 of 3	N/A	0 of 3	N/A

Table 7. Summary of Comparative LoD data: Focus Assay vs. CDC Assay for Seasonal Influenza A (H3N2) Virus Detection

Estimated Gene Copies/mL	Focus Diagnostics Assay		CDC Assay	
	Initial Screening	Confirmation of LoD	Initial Screening	Confirmation of LoD
$3.6x10^5$	3 of 3	N/A	3 of 3	N/A
$1.8x10^5$	3 of 3	N/A	3 of 3	N/A

Estimated Gene Copies/mL	Focus Diagnostics Assay		CDC Assay	
	Initial Screening	Confirmation of LoD	Initial Screening	Confirmation of LoD
$3.6x10^4$	3 of 3	20 of 20	3 of 3	N/A
$1.8x10^4$	2 of 3	20 of 20	3 of 3	19 of 20
$9.0x10^3$	N/A	N/A	N/A	18 of 20
$3.6x10^3$	0 of 3	N/A	0 of 3	N/A

The Limit of Detection results for upper respiratory tract specimens in the Focus assay were equivalent to the CDC results for 2009 H1N1 influenza and seasonal Influenza A H3N2 and within a 10 fold difference for Seasonal H1N1.

Limit of Detection screening studies in lower respiratory tract specimens were performed by spiking virus into negative bronchial alveolar lavage and negative endotracheal aspirate matrices. Results of the LoD screening for lower respiratory tract specimens are presented in Table 8 and 9.

Table 8: LoD Screening Bronchial Alveolar Lavage Matrix – 2009 H1N1 Influenza Virus RNA

Influenza A/California/7/2009 (H1N1) reassortant virus

$TCID_{50}$/mL	FLUA C_T value	SWINE1 C_T value	SWINE2 C_T value	QIPC C_T value
5600	27.30	25.50	27.04	27.75
5600	27.47	25.84	28.06	27.85
5600	27.34	25.54	27.17	27.71
560	30.62	29.12	30.43	27.87
560	30.85	29.10	30.54	28.05
560	30.98	29.19	30.01	28.09
56	33.84	31.73	33.88	28.07
56	34.54	32.73	34.05	28.11
56	34.21	32.86	35.05	28.21
5.6	38.39	37.64	Undetermined	28.22
5.6	37.01	36.04	Undetermined	28.25
5.6	37.88	35.22	Undetermined	28.11

Table 9: LoD Screening Endotracheal Aspirate Matrix – 2009 H1N1 Influenza Virus RNA

Influenza A/California/7/2009 (H1N1) reassortant virus

$TCID_{50}$/mL	FLUA C_T value	SWINE1 C_T value	SWINE2 C_T value	QIPC C_T value
56	36.38	35.26	37.57	28.15
56	34.23	32.75	35.66	27.56
56	35.21	34.08	33.87	28.06
5.6	38.35	38.31	Invalid*	27.64
5.6	37.28	35.61	36.38	27.70
5.6	Undetermined	36.62	Undetermined	28.07

* Irregular response curve, insufficient sample for repeat.

The results show that the Influenza A H1N1 (2009) RT-PCR assay is able to detect 2009 H1N1 influenza virus RNA in lower respiratory specimens. .

REPRODUCIBILITY

Within Run Precision

Three pools of 2009 H1N1 influenza virus were prepared by making dilutions of a patient specimen identified as positive for 2009 H1N1 influenza by the CDC protocol. The initial **C_T** values for a 1 to 5 dilution of the patient sample were as follows: H1N1-1 = 22.13, H1N1-2 = 26.08 and FLUA = 22.37. The original patient specimen was diluted 200 fold to create the high pool, the medium pool was created from a 10 fold dilution of the high pool, and the low pool was created from a ten fold dilution of the medium pool. Similarly, pools with high, medium and low concentrations of seasonal influenza A virus were prepared by making dilutions of influenza A virus (ATCC #VR-1520) stock in viral transport media. The high pool was created by making a 2500 fold dilution of the stock, the medium pool was created from a 10 fold dilution of the high pool, and the low pool was created from a ten fold dilution of the medium pool. Three aliquots of each sample were assayed in a single run to determine intra-assay precision. Standard deviation and %CV values were calculated based on the C_T values obtained from the amplification reactions.

Table 10. Intra-Assay Precision of 2009 H1N1 Influenza in Transport Medium

Replicate #	H1N1-1 Target C_T Value				H1N1-2 Target C_T Value				Pan-Influenza A C_T Value		
	High	Medium	Low		High	Medium	Low		High	Medium	Low
1	28.68	33.65	38.56		34.09	40.63	42.68		28.93	33.67	37.39
2	29.07	32.84	35.43		35.28	38.78	41.36		29.12	33.27	36.71
3	30.17	33.40	36.92		35.47	39.42	44.95		30.04	33.37	36.49
Average	29.31	33.30	36.97		34.95	39.61	43.00		29.36	33.44	36.86
SD	0.77	0.41	1.57		0.75	0.94	1.82		0.59	0.21	0.47
% CV	2.64	1.25	4.23		2.14	2.37	4.22		2.02	0.62	1.27

Table 11. Intra-Assay Precision of Seasonal Influenza A Virus in Transport Medium

Replicate #	H1N1-1 Target C_T Value				H1N1-2 Target C_T Value				Pan-Influenza A C_T Value		
	High	Medium	Low		High	Medium	Low		High	Medium	Low
1	50	50	50		50	50	50		28.87	32.51	35.84
2	50	50	50		50	50	50		29.21	32.78	36.56
3	50	50	50		50	50	50		29.48	32.08	36.06
Average	N/A	N/A	N/A		N/A	N/A	N/A		29.19	32.46	36.15
SD	N/A	N/A	N/A		N/A	N/A	N/A		0.31	0.35	0.37
% CV	N/A	N/A	N/A		N/A	N/A	N/A		1.05	1.09	1.02

Between Run Precision

Three pools with high, medium and low concentrations of 2009 H1N1 influenza virus were prepared by making dilutions of a patient specimen identified as positive for 2009 H1N1 influenza by the CDC method. Similarly, samples with high, medium and low concentrations of seasonal influenza A virus were prepared by making dilutions of influenza A virus (ATCC® #VR-1520™) stock in transport media. Three aliquots of each sample were assayed in three separate assay runs to determine intra-assay precision. Standard deviation and %CV values were calculated based on the C_T values obtained from the amplification reactions. Standard deviation and %CV values were calculated based on the C_T values obtained for each amplification.

Table 12. Inter-Assay Precision of 2009 H1N1 Influenza in Transport Medium

Run	Replicate #	H1N1-1 Target C_T Value				H1N1-2 Target C_T Value				Pan-Influenza A C_T Value		
		High	Medium	Low		High	Medium	Low		High	Medium	Low
1	1	28.68	33.65	38.56		34.09	40.63	42.68		28.93	33.67	37.39
	2	29.07	32.84	35.43		35.28	38.78	41.36		29.12	33.27	36.71
	3	30.17	33.4	36.92		35.47	39.42	44.95		30.04	33.37	36.49
2	1	29.63	32.74	39.11		34.18	37.43	43.84		29.60	33.16	38.14
	2	30.68	32.69	36.01		35.26	38.80	42.10		30.37	33.21	36.50
	3	29.72	36.67	37.10		34.03	40.04	44.16		30.03	34.39	37.66
3	1	28.14	34.13	37.24		34.54	40.01	42.90		28.93	33.76	37.09
	2	29.32	32.34	35.81		35.51	38.46	41.05		29.38	33.17	36.70
	3	29.30	32.53	37.00		34.65	38.93	44.25		29.45	32.75	36.98
	Average	29.41	33.44	37.02		34.78	39.17	43.03		29.54	33.42	37.07
	SD	0.76	1.34	1.22		0.61	0.97	1.36		0.52	0.47	0.56
	% CV	2.58	4.01	3.28		1.75	2.48	3.16		1.75	1.41	1.52

Table 13. Inter-Assay Precision of Seasonal Influenza A Virus in Transport Medium

Run	Replicate #	H1N1-1 Target C_T Value				H1N1-2 Target C_T Value				Influenza A C_T Value		
		High	Medium	Low		High	Medium	Low		High	Medium	Low
1	1	50	50	50		50	50	50		28.87	32.51	35.84
	2	50	50	50		50	50	50		29.21	32.78	36.56
	3	50	50	50		50	50	50		29.48	32.08	36.06

Run	Replicate #	H1N1-1 Target C_T Value				H1N1-2 Target C_T Value				Influenza A C_T Value		
		High	Medium	Low		High	Medium	Low		High	Medium	Low
2	1	50	50	50		50	50	50		29.23	32.21	35.37
	2	50	50	50		50	50	50		28.98	32.66	35.6
	3	50	50	50		50	50	50		28.67	32.13	35.18
3	1	50	50	50		50	50	50		28.65	32.28	35.5
	2	50	50	50		50	50	50		28.66	32.29	35.49
	3	50	50	50		50	50	50		28.88	32.39	36.06
	Average	N/A	N/A	N/A		N/A	N/A	N/A		28.96	32.37	35.74
	SD	N/A	N/A	N/A		N/A	N/A	N/A		0.29	0.24	0.43
	% CV	N/A	N/A	N/A		N/A	N/A	N/A		1.01	0.74	1.20

ANALYTICAL SPECIFICITY / CROSS REACTIVITY

Genomic DNA or RNA of a variety of organisms (≥5 x 10^4 copies/PCR reaction for cultured organisms or pathogen C_T ≤ 30 for clinical specimens) was assayed to show no cross-reactivity of the Influenza A primers/probe or 2009 H1N1 influenza primers / probe with nucleic acids of other organisms. The results are shown in Table 14. Given that the RT-PCR assay uses 50 cycles of amplification and detection, no cross-reactivity was seen to any of the respiratory pathogens tested, with the exception of two deviations in an original experiment. The influenza A virus RT-PCR reaction gave a weak signal with the parainfluenza virus subtype 2 stock tested. The 2009 H1N1 influenza primary RT-PCR reaction gave a weak signal with the *Mycoplasma pneumoniae* stock tested. Given that a weak signal was seen in only one of the three-target RT-PCR reactions used in the assay, these weak signals were considered to be deviations. The assay was repeated on the parainfluenza-2 genomic RNA and the *M. pneumoniae* genomic DNA. Upon repeat testing, no amplification was detected for the Influenza A target or either of the 2009 H1N1 influenza targets.

Table 14. Analytical Specificity

Pathogen	Source	H1N1-1 Target C_T Value	H1N1-2 Target C_T Value	Influenza A C_T value
Influenza B	A26347376	50	50	50
Influenza B	A26349245	50	50	50
Influenza B	A26352671	50	50	50
RSV A	ATCC	50	50	50
RSV B	ATCC	50	50	50
Parainfluenza-1	ATCC	50	50	50
Parainfluenza-2	ATCC	50 50	50 50	41.37 50
Parainfluenza-3	ATCC	50	50	50
hMPV	Focus Diagnostics	50	50	50
Coronavirus OC43	ATCC	50	50	50
Coronavirus 229E	ATCC	50	50	50
Rhinovirus-16	ATCC	50	50	50
B. pertussis	Focus Diagnostics	50	50	50
B. parapertussis	Focus Diagnostics	50	50	50
M. pneumoniae	ATCC	42.42 50	50 50	50 50
C. pneumoniae	ATCC	50	50	50
L. pneumophila	Focus Diagnostics	50	50	50
MTB	Focus Diagnostics	50	50	50
MRSA	ATCC	50	50	50
MSSA	ATCC	50	50	50
Enterovirus 71	ATCC	50	50	50
C. burnetii	Virion/Serion	50	50	50
Echovirus-7	ATCC	50	50	50
Adenovirus-7	ATCC	50	50	50
Mumps virus	ATCC	50	50	50

CLINICAL PERFORMANCE CHARACTERISTICS

METHOD COMPARISON

Sixteen samples were included in this Method Comparison, thirteen (13) specimens previously determined to be Influenza A positive and three (3) specimens previously determined to be Influenza B positive. Each specimen was submitted to Focus Diagnostics as a respiratory swab in viral transport media. The test requisitions did not state the exact anatomical location of the material present on each swab. The Focus assay was compared to the CDC panels for seasonal Flu A/B and the CDC rRT-PCR for 2009 H1N1 influenza. The results show that the Influenza A H1N1 (2009) Real-Time RT-PCR Assay is able to detect both seasonal human influenza A viruses and the new 2009 H1N1 influenza virus, and that the assay can differentiate between seasonal H1/H3 influenza A viruses and the newly discovered 2009 H1N1 influenza virus.

Experience (and animal studies) suggests that some patients with severe lower respiratory tract disease have high titers of virus in the lower respiratory tract, but low or absent titers in the upper respiratory tract. Nine lower respiratory tract specimens from critically ill patients with influenza symptoms were tested using the Focus assay. Two of the specimens were negative and two of the samples were positive for H1N1 (2009) in both lower and upper respiratory tract specimens. Four of the specimens were positive for H1N1 (2009) in lower respiratory tract specimens and negative in paired upper respiratory tract specimens. The ninth sample was positive for H1N1 (2009) in a lower respiratory tract specimen; a corresponding upper respiratory tract specimen was not available for testing.

Table 15. METHOD COMPARISION (Accuracy/Bias)

Sample ID	Focus Diagnostics Reference Lab Influenza A/B RNA Real-Time RT-PCR		FLU Subtype per California DoPH VRD Lab CDC panel	CDC rRT-PCR for 2009 H1N1 Influenza Result	Focus Influenza A H1N1 (2009)Real-Time RT-PCR H1N1 Differentiation	
	Influenza A	Influenza B			Influenza A	2009 H1N1
1	Detected	Not Detected	Human H1	N/A	Detected	Not Detected
2	Detected	Not Detected	Human H1	N/A	Detected	Not Detected
3	Detected	Not Detected	Human H1	N/A	Detected	Not Detected
4	Detected	Not Detected	Human H3	N/A	Detected	Not Detected
5	Detected	Not Detected	Human H3	N/A	Detected	Not Detected
6	Detected	Not Detected	Human H3	N/A	Detected	Not Detected
7	Detected	Not Detected	Human H3	N/A	Detected	Not Detected
8	Detected	Not Detected	Human H3	N/A	Detected	Not Detected
9	Detected	Not Detected	Unsubtypable	2009 H1N1	Detected	Detected
10	Detected	Not Detected	Unsubtypable	2009 H1N1	Detected	Detected
11	Detected	Not Detected	Unsubtypable	2009 H1N1	Detected	Detected
12	Detected	Not Detected	Unsubtypable	2009 H1N1	Detected	Detected
13	Detected	Not Detected	Unsubtypable	2009 H1N1	Detected	Detected
14	Not Detected	Detected	N/A	N/A	Not Detected	Not Detected
15	Not Detected	Detected	N/A	N/A	Not Detected	Not Detected
16	Not Detected	Detected	N/A	N/A	Not Detected	Not Detected

Method Comparison, Influenza A: 100% positive agreement with California VRDL + CDC result (13/13), 95% CI: 77.2, 100
Method Comparison, Novel H1N1: 100% positive agreement with CDC result (5/5), 95% CI: 56.6, 100

CONFIRMATION OF POSITIVES

After completion of assay validation testing, an additional fifty nine (59) specimens reported by Focus as “Detected” for the “Influenza A RNA” result field and also reported out as “Detected” for the “2009 H1N1 Influenza H1 Gene” result field using the Focus Diagnostics Influenza A H1N1 (2009) Real Time RT-PCR were sent to the public health laboratory in the state where the patient resides. Each of the specimens was confirmed to be positive for the novel 2009 H1N1 influenza virus by the state public health laboratories using the CDC protocol. The assay demonstrated 100% agreement (59/59), 95% CI: 93.9, 100.

CONFIRMATION OF NEGATIVES

After completion of assay validation testing, an additional fifty (50) specimens reported by Focus as “Undetected” for the “2009 H1N1 Influenza H1 Gene” result field using the Focus Diagnostics Influenza A H1N1 (2009) Real Time RT-PCR were sent to the California Department of Public Health Viral & Rickettsial Disease Laboratory (California VRDL). Each of the specimens was confirmed to be negative for the 2009 H1N1 influenza virus by the California VRDL using the CDC protocol. The assay demonstrated 100% agreement (50/50), 95% CI: 92.9, 100.

SUMMARY OF CLINICAL PERFORMANCE EVALUATION

Data from the Method Comparison study, and the Confirmation of Positive and Negative studies were pooled to demonstrate concordance of the Focus assay with the CDC methods for Seasonal Flu A and 2009 H1N1 influenza. Summary data are presented in Tables 16 and 17 below.

Table 16: Concordance of H1N1 results

	CDC REALTIME RT-PCR FOR 2009 H1N1 INFLUENZA				
		2009 H1N1 Positive	**2009 H1N1* Negative**	**Total**	% Positive Agreement
Focus Influenza A H1N1 (2009) Real-Time RT-PCR	**2009 H1N1 Positive**	64	0	64	100% (64/64) 95% CI: 94.3, 100
	2009 H1N1 Negative	0	58	58	% Negative Agreement
	Total	64	58	122	100% (58/58) 95% CI: 93.8, 100

*** Samples reported as Inconclusive H1N1 by either assay are indicated as negative.**

Table 17: Concordance of Flu A Results

	CDC HUMAN INFLUENZA VIRUS REAL-TIME RT- PCR DETECTION AND CHARACTERIZATION PANEL				
		Flu A Positive	**Flu A Negative**	**Total**	% Positive Agreement
Focus Influenza A H1N1 (2009) Real-Time RT-PCR	**Flu A Positive**	87	0	87	97.8% (87/89) 95% CI: 92.2, 99.4
	Flu A Negative	2**	33	35	% Negative Agreement
	Total	89	33	122	100% (33/33) 95% CI: 89.6, 100

**** 1 sample was positive by the CDC panel for Seasonal Flu A, but a flu A subtype could not be determined. The second sample was determined to be Flu A positive but inconclusive for H1N1**

REFERENCE INTERVAL

The Reference Interval was determined by analyzing 2009 H1N1 influenza negative specimens. Influenza A or B results were determined by a separate real-time RT-PCR assay. All 2009 H1N1 influenza negative specimens should have a C_T value of 50 for the primary and secondary 2009 H1N1 influenza RT-PCR targets. The results are shown in Table 18.

Table 18. Reference Range

Sample	Focus Diagnostics Reference Lab Influenza A/B RNA Real-Time RT-PCR		CA Department of Health Result	H1N1-1 Target C_T Value	H1N1-2 Target C_T Value	Influenza A C_T Value
	Influenza A	Influenza B				
1	Detected	Not Detected	Human H1	50	50	33.40
2	Detected	Not Detected	Human H1	50	50	24.03
3	Detected	Not Detected	Human H1	50	50	36.76
4	Detected	Not Detected	Human H3	50	50	29.23
5	Detected	Not Detected	Human H3	50	50	22.24
6	Detected	Not Detected	Human H3	50	50	31.96
7	Detected	Not Detected	Human H3	50	50	28.33
8	Detected	Not Detected	Human H3	50	50	23.40
9	Not Detected	Positive	N/A	50	50	50
10	Not Detected	Positive	N/A	50	50	50
11	Not Detected	Positive	N/A	50	50	50

REPORTABLE RANGE

Twenty-one 2009 H1N1 influenza-positive clinical specimens were analyzed using the Influenza A H1N1 (2009) Real-Time RT-PCR. These specimens were confirmed to be positive for the 2009 H1N1 influenza by the state health laboratories in California, Texas or Illinois using CDC RT-PCR methods available in their laboratories. The C_T values observed for the Influenza A, H1N1-1 and H1N1-2 RT-PCR targets in these specimens should be indicative of positive results for this assay. The results are shown in Table 19. The confirmed 2009 H1N1 influenza positive specimens have Influenza A C_T values in the range of 17.59-32.59, H1N1-1 C_T values in the range of 17.81-32.01 and H1N1-2 C_T values in the range of 19.87-35.63. The majority of specimens have CT values <30 for all three targets.

Table 19. Reportable Range

Specimen #	Specimen Type	Influenza A Target C_T Value	H1N1-1 Target C_T Value	H1N1-2 Target C_T Value
1	Swab	24.71	24.38	27.09
2	Swab	32.33	32.70	37.89
3	Swab	21.61	20.95	24.25
4	Swab	22.22	21.75	24.52
5	Swab	24.50	21.75	27.31
6	Swab	18.21	17.66	19.65
7	Swab	24.20	24.20	26.37
8	Nasal	19.54	19.01	21.36
9	Swab	17.59	17.81	19.87
10	Swab	32.59	32.01	35.63
11	Nasal swab	23.74	22.20	25.23
12	Swab	19.35	18.08	20.32
13	Swab	21.89	21.18	22.46
14	Swab	21.70	20.57	23.24
15	Swab	21.75	20.52	22.84
16	Swab	24.45	23.11	25.56
17	Throat swab	21.19	20.78	23.05
18	Throat swab	21.28	20.90	23.11
19	Swab	19.56	19.69	21.34
20	Throat swab	23.06	22.54	25.00
21	Throat swab	20.70	20.15	22.32

QUALITY CONTROL RANGES:

An unassayed positive control is provided with the kit, sterile nuclease-free water may be used as a negative control. Each laboratory should establish their own Quality Control ranges and frequency of QC testing based on applicable local laws, regulations and standard good laboratory practice.

REFERENCES

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3. H1N1 Influenza Virus Biosafety Guidelines for Laboratory Workers. http://www.cdc.gov/h1n1flu/guidelines_labworkers.htm.

TECHNICAL ASSISTANCE

If questions arise concerning the kit or its reagents, please contact Focus Diagnostics Technical Services personnel.

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