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 As of June 23, 2010, the EUAs have been terminated and this information is no longer current.

Simplexa™ Influenza A H1N1 (2009)

REF MOL2500
Rev. B

A real-time RT-PCR assay intended for the *in vitro* qualitative detection and differentiation of influenza A and 2009 H1N1 influenza viral RNA.

Emergency Use Authorization
For *in vitro* Diagnostic Use

INTENDED USE

The Focus Diagnostics Simplexa™ Influenza A H1N1 (2009) assay is intended for use in CLIA High Complexity Laboratories with the ability to perform nucleic acid extraction using the Roche MagNA Pure™ LC and PCR on the 3M Integrated Cycler as part of the Microfluidic Molecular System for the in vitro qualitative detection and differentiation of influenza A and 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 Simplexa™ Influenza A H1N1 (2009) assay should not be performed unless the patient meets clinical and epidemiologic criteria for testing suspect specimens. The identification of 2009 H1N1 influenza A 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 tract 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 PCR amplification and detection system that utilizes a bi-functional fluorescent primer-probe for the detection of human influenza A virus RNA and the differential detection of 2009 influenza H1N1 virus 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) The assay is composed of two principal steps: (1) extraction of RNA from patient specimens, (2) A bi-functional fluorescent probe-primer is used together with a reverse primer to amplify a specific target (for each analyte and internal control). A well-conserved region of the matrix gene from influenza A viruses is targeted to identify both human influenza A virus and 2009 H1N1 influenza virus in the specimen. Simultaneously, a region of the hemagglutinin gene of the 2009 H1N1 influenza virus is targeted to specifically detect the presence of 2009 H1N1 influenza RNA, thereby differentiating it from seasonal human influenza A virus. An internal control is used to monitor the extraction process and to detect PCR inhibition.

MATERIALS PROVIDED

The Focus Diagnostics Simplexa™ Influenza A H1N1 (2009) kit contains sufficient reagents for 100 reactions. Upon receipt, store all kit components at -10 to -30 °C (do NOT store in 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 -30 °C. After initial use, store thawed H1N1 Primer Mix, RNA Master Mix, H1N1 Positive Control, Armored RNA Internal Control and No Template Control at 2 to 8 °C for no more than 30 days or until expiration date whichever comes first. Store the RT Mix at -10 to -30 °C until expiration date.

Table 1: Description of the kit labeling and kit components

Kit	Label
Focus Diagnostics' Simplexa™ Influenza A H1N1 (2009) (Part # MOL2500)	**ENGLISH** **Simplexa™ H1N1 Primer Mix** **Simplexa™ RNA Master Mix** **RT Mix** **Simplexa™ Armored RNA Internal Control** **Simplexa™ No Template Control** **Simplexa™ H1N1 Positive Control**

REF	EC SYMBOL	
MOL2501	REAG	A
MOL2002	REAG	B
MOL9103	REAG	C
MOL2003	CONTROL	IC
MOL2001	CONTROL	NTC
MOL2502	CONTROL	+

Components	Number of tubes per Kit	Color Code	Label			
Simplexa™ H1N1 Primer Mix (PM)	2	Brown	REF	MOL2501	Lot	Expires
Simplexa™ RNA Master Mix (RMM)	2	Green	REF	MOL2002	Lot	Expires
RT Mix (RT)	1	Yellow	REF	MOL9103	Lot	Expires
Simplexa™ Armored RNA Internal Control (AR IC)	2	Blue	REF	MOL2003	Lot	Expires
Simplexa™ No Template Control (NTC)	2	Neutral	REF	MOL2001	Lot	Expires
Simplexa™ H1N1 Positive Control (PC)	2	Red	REF	MOL2502	Lot	Expires

Table 2: Description of the kit components

Kit Component	Reactions per Kit / Vial	Volume (µL) per Vial	Component Description
Primer Mix (PM)	100/50	30	Dye-labeled fluorescent primers specific for detection of Influenza A and/or 2009 H1N1 Influenza and for the Internal Control template.
RNA Master Mix (RMM)	100/50	200	DNA polymerase, buffer and dNTP
RT Mix (RT)	100/100	50	Reverse Transcriptase Enzyme
Armored RNA Internal Control (AR IC)	100/50	250	RNA sequence encapsidated in protein.
No Template Control (NTC)	8/4	800	Nuclease-Free Water
H1N1 Positive Control (PC)	8/4	800	Inactivated 2009 H1N1 Virus

Target	Probe Fluorophore	Excitation (nm)	Emission (nm)	Targeted Gene
FLUA	FAM	495	520	*matrix*
H1N1	CFR610	590	610	*HA*
Internal Control "ARIC"	Q670	644	670	N/A

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Integrated Cycler with Integrated Cycler Studio software version 1.0 or higher
2. Universal Discs for use on the Integrated Cycler
3. Universal Disc Cover Tape
4. Roche MagNA Pure LC and associated consumables.
5. MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Cat. No 3038505001)
6. Single, multi-channel and/or repeater micropipette(s) with an accuracy range between 1-10 µL, 10-100 µL and 100-1000 µL
7. Freezer (manual defrost) at -10 to -30 °C (for kit component frozen storage)
8. Freezer (manual defrost) at -10 to -30 °C (for specimen frozen storage)
9. Laminar flow hood for extractions
10. Microcentrifuge
11. Vortex mixer
12. Sterile, RNase/DNase-free disposable aerosol-barrier micropipettor tips
13. 1.5 mL polypropylene microcentrifuge tubes and racks (RNase/DNase-free tubes are recommended but not required)
14. Disposable, powder-free gloves
15. Nuclease-Free Water
16. Cooler racks for 1.5 mL microcentrifuge tubes.

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 the reaction mix within one hour.
5. Once thawed, store the Primer Mix, RNA Master Mix, Positive Control, Armored RNA Internal Control, and No Template Control at 2 to 8°C for no more than 30 days.
6. Do not refreeze Primer Mix, RNA Master Mix, Internal Control or Positive Control.
7. After initial use return the RT mix to freezer (-10 to -30°C) up to the expiration date.
8. Do not use kits or reagents beyond their expiration dates.
9. Do not combine reagents from different kit lots.

WARNINGS AND PRECAUTIONS

1. The performance of the test with viruses infecting swine, or specimens from humans infected with swine influenza viruses other than the 2009 H1N1 has not been established.
2. Follow universal precautions. All patient specimens and positive controls should be considered potentially infectious and handled accordingly.
3. 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 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[3].
4. 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.
5. Do not pipette by mouth.
6. Do not smoke, drink, eat, handle contact lenses or apply make-up in areas where kit reagents and/or human specimens are being used.
7. Dispose of unused kit reagents and human specimens according to local, state and federal regulations.
8. 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 to Real Time PCR amplification:
   - Begin with specimen extraction, followed by Real Time PCR instrument set-up, reagent preparation, and finally Real Time PCR amplification.
   - Do not use supplies and equipment across the dedicated areas of specimen extraction and sample preparation. No cross-movement is recommended between the different areas.
   - Supplies and equipment used for specimen preparation should not be used for reagent preparation activities or for processing amplified DNA or other sources of target nucleic acid.
   - All amplification supplies and equipment should be kept in the Real Time PCR Instrument Area at all times.
   - Personal Protective Equipment, such as laboratory coats and disposable gloves, should be area-specific.
9. Contamination of patient specimens or reagents can produce erroneous results. Use aseptic techniques.
10. Pipette and handle reagents carefully to avoid mixing of samples from adjacent wells.
11. Use proper pipetting techniques and maintain the same pipetting pattern throughout the procedure to ensure optimal and reproducible values.
12. Do not substitute or mix reagent from different kit lots or from other manufacturers.
13. Do not interchange the reagent tube caps. This may cause contamination and compromise the test results.

14. 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.
15. Assay setup should be performed at room temperature (approximate range 18 to 25°C). While mixing the reagents, keep the enzymes cold by utilizing a cooler block.
16. Do not re-use Universal Discs that have already been exposed to patient samples or reagents.
17. Dispose of used disc without detaching or removing cover tape.
18. If different Simplexa™ kits or lots are set up on the same disc, positive and No Template Controls from each kit need to be tested.
19. RNA Master Mix contains 1-10% glycerol, which may cause irritation upon inhalation or skin contact. Upon inhalation or skin contact, first aid measures should be taken.
20. Extended storage of extracted specimens at 2 – 8°C is not recommended; performance has not been established.

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 ones with a synthetic tip (e.g. Dacron, nylon, or rayon) and an aluminum or plastic shaft. Do not use calcium alginate swabs, as they may contain substances that inhibit PCR testing.

B. SPECIMEN EXTRACTION AREA

Perform in a dedicated area for specimen and control extraction.

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 Variable_elution_volume.blk" from the list. This will load the appropriate settings for the run.
3. The Sample Protocol should be "Total NA Variable_elution_volume".
4. 200 µL should be set for the 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 Protocol 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. Ensure that specimens and controls are in the correct position on the Sample Cartridge.
9. In a biosafety cabinet, pipette 200 µL of each specimen, Positive or No Template Control into the corresponding position in the sample cartridge.
10. Visually check the level of samples and controls in the Sample Cartridge to ensure sample(s) were added.
11. Pipette 5 µL of the Armored RNA Internal Control into each sample and all control wells. Change tips in between samples.
12. Transfer the sample cartridge containing the 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 removed from the MagNA Pure and sealed. Store the RNA at 2 to 8 °C prior to use. Long-term storage of extracted samples at this temperature is not recommended. Keep extracted RNA samples on a cooler block while loading disc.

C. REAL-TIME PCR INSTRUMENT SETUP

Perform the following to configure the Integrated Cycler Studio software for the Influenza A H1N1 (2009) assay:

Note: This only needs to be performed upon initial use.

1. Refer to Integrated Cycler Operator Manual for details on how to define the assay on the Microfluidic Molecular System.
2. Launch Integrated Cycler Studio software, and log in as an Administrator or Lab Manager.
3. Click **Create Assay Definition** in the **Quick Pick Menu**.
4. In the **Overview** tab:
   - **Basics:** Define assay **Name** as **Simplexa Influenza A H1N1 2009**. .
     Check **Share Assay** box.
   - **Add Targets:** Fill in the boxes with the information below, and then click on the green plus sign (+) after entering the information for an entire row.

Short Name	Long Name	Dye Name	Type	Threshold	Ct Cutoff
FLUA	Influenza A	FAM	Unknown	10000	40
H1N1	H1N1 (2009)	CFR610	Unknown	15000	40

AR IC	Armored RNA IC	Q670	IC	10000	40

- **Disc:** Select Universal Disc from the **Disc Type** dropdown menu.

5. In the **Define Cycles** tab.
   - **Initial Cycles**

	Time (s)	Temperature (°C)	Ramp Rate (C/s)	Name
Initial 1 √	900	47	10	Hold
Initial 2 √	600	97	10	Activate

   - **Main Cycles**
     - **Cycle Count** 40

	Time (s)	Temperature (°C)	Ramp Rate (C/s)	
Denature	15	97	10	
Anneal/Extend	30	60	10	Capture

     - Ensure **Capture** radial button is checked.
     - Ensure **Extend 1** and **Extend 2** boxes are unchecked.
   - Ensure **Final Cycles** are unchecked.
6. Click **Save** and then **Close**.

Perform the following to set up a run on the Integrated Cycler Studio software for the Influenza A H1N1 (2009) assay:

1. Set up the run by selecting **Setup Run** under Tasks.
2. Complete the configuration in the **Setup Run** section as follows:
   - Select "**Simplexa Influenza A H1N1 2009**" from the **Assay Definition** dropdown menu.
   - Name the run under **Run Details**
   - **Add Samples** to the **Sample List.**
   - Click **Move to Disc.**

Example Disc Layout

	Spoke 1	Spoke 2	Spoke 3	Spoke 4	Spoke 5	Spoke 6	Spoke 7	Spoke 8	Spoke 9	Spoke 10	Spoke 11	Spoke 12
A	PC	S	S	S	S	S	S	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	S	S	S	S	S	S	NTC

   - Click **Save.**

D. REAGENT PREPARATION AREA

Dedicated area for preparation of Simplexa™ Influenza A H1N1 (2009) assay reaction mix.

1. Thaw the Primer Mix and the RNA Master Mix at room temperature (approximate range 18 to 25°C). Each kit component vial contains sufficient reagents for 50 reactions.
2. Prepare the required volume of the Reaction Mix in 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 Volume / 1 reaction	Reaction Mix Volume / 10 reactions
Simplexa™ RNA Master Mix	4.0 µL	40 µL
Simplexa™ H1N1 Primer Mix	0.5 µL	5 µL
RT Mix	0.5 µL	5 µL
Total Volume	5.0 µL	50 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 the Reaction Mix within one hour of preparation.
6. Proceed to PCR Setup.

E. REAL TIME PCR AMPLIFICATION AREA

Perform in a dedicated area for preparation of 96-well Universal Disc for Simplexa™ Influenza A H1N1 (2009) assay.
Refer to example disc layout in section C while performing the following setup:

1. Add 5.0 µL of the reaction mix to each well.
2. Add 5.0 µL of the extracted Positive Control to the "PC" disc well.

3. Add 5.0 µL of extracted patient sample to the appropriate "S" disc well.
4. Add 5.0 µL of extracted No Template Control to the "NTC" disc well.
5. Cover the disc with the Universal Disc Cover Tape.
6. Open the lid of the Integrated Cycler.
7. Place the sealed Universal Disc onto the platen.
8. Close the lid gently.
9. Click **Run**.
10. Click **Start**.

F. DATA ANALYSIS

1. When the run finishes, click **Analyze**, or to review a prior run, select it from **Analyze Completed Runs** or **Tasks/Browse Runs.**
2. Review Channels one at a time or **All Channels** at once.
3. Press the Print Preview button (bottom right) then check the include Graphs checkbox to review a summary of the Ct values and the amplification plots. Scroll from page to page using the arrow buttons in the top left corner of the Print preview window.
4. Print or Save the Report as needed.
5. Export the Ct values if needed.

REPORTING RESULTS

Reporting results is a three step process.

1. Examination of controls to determine if the run is valid,
2. Examination of validity of patient specimen results
3. Interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.

1. Determine if the run is valid by examining the H1N1 Positive Control, No Template Control, and Armored RNA Internal Control

Criteria for a Valid Control (simplified)*

Control	H1N1 Ct	FLUA Ct	AR IC Ct
No Template Control	0 (If ≤40 then positive patient results cannot be reported)	0 (If ≤40 then positive patient results cannot be reported)	≤40
Positive Control	≤40	≤40	Not Applicable (N/A)

* See notes below for full description.

a. If the No Template Control is:
   i. Positive (Ct value ≤40 for either the H1N1 or FLUA), 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 maybe reported given that all other assay run criteria are met.
   ii. Negative for H1N1 and FLUA detector (Ct = 0), then this control is valid and acceptable.
   iii. If the AR IC is not detected in the No Template Control, the assay run is invalid and needs to be repeated.
   iv. If the AR IC is detected for the No Template Control, the assay run is considered valid and acceptable.

b. Positive Control
   v. If the Positive Control result is a Ct = 0 for H1N1 and FLUA, the assay run is considered invalid and unacceptable. All patient specimens must be re-assayed.
   vi. If the Ct values for H1N1 and FLUA are within or below their respective acceptable criteria, the assay run is considered valid and acceptable.

2. Examination of Patient Specimen Results
Examination of clinical specimen results should be performed after the Positive and No Template Controls have been examined and determined to be valid and acceptable. H1N1, FLUA and AR IC results must be examined for each patient specimen.

Criteria for a Valid Patient Specimen (simplified)*

Patient Specimen H1N1 Ct and FLUA Ct	Amplification Plot	AR IC Ct
Either detector or both detectors ≤40	Shows exponential increase	N/A
Both detectors at 0	NA	≤40

* See notes below for full description.

a. Amplification plots should be examined for every positive sample. If the amplification plot shows an exponential increase, the amplification curve is valid.
b. If the amplification curve is valid for FLUA or H1N1, the AR IC is not required to be detected to report a positive result for FLUA or H1N1.

3. Interpretation of Results
   a. A specimen that does not contain influenza A virus (pan A or 2009 H1N1 influenza virus) will be negative (Ct = 0) for the FLUA and H1N1 detectors. A specimen positive for seasonal influenza A virus will have a positive result (Ct ≤40) for the FLUA detector and will be negative (Ct = 0) for H1N1 detectors. A specimen positive for 2009 influenza H1N1 will be positive for both the FLUA and the H1N1 detector.
   b. Validation studies have shown that specimens containing 2009 influenza A H1N1 virus will be positive for the H1N1 and the FLUA detector. If only the H1N1 detector is positive and not the FLUA detector, the specimens must be retested to confirm the result.
   c. If the FLUA Ct value of a patient sample is not detected and the AR IC Ct value falls within or below the acceptable range, the "Influenza A RNA" result is reported as "Not Detected".
   d. If the FLUA Ct value of a patient specimen is ≤40 and an amplification curve is observed for the well, the "Influenza A RNA" result is reported as "Detected". If the Ct value for the well is less than 40 but no amplification curve is observed (nonspecific fluorescence is observed in the well), the "Influenza A RNA" result is reported as "Not Detected"
   e. If the H1N1 Ct value of a patient sample is listed as "0" and the AR IC Ct value falls within or below the acceptable range, the "2009 H1N1 Influenza RNA" result is reported as "Not Detected".
   f. If the H1N1 Ct value of a patient specimen is ≤40 and an amplification curve is observed, the "2009 H1N1 Influenza RNA" result is reported as "Detected". If the Ct value for the well is ≤40 but no amplification curve is observed in the well (nonspecific fluorescence is observed in the well), the "2009 H1N1 Influenza RNA" result is reported as "Not Detected".
   g. If the FLUA or H1N1 Ct value of a patient specimen is 0 and the AR IC Ct value is 0, 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."
   h. If upon repeat testing the result is still indeterminate for H1N1 then the 2009 H1N1 Influenza RNA results is reported as "Not Detected."
   I If upon repeat testing a Ct ≤ 40 has been obtained for both FLUA and H1N1 in one of the two replicates then the result is reported as "Detected" for both Influenza A and 2009 H1N1

Table 4: Interpretation of Results

Example	FLUA Ct value	H1N1 Ct value	AR IC Ct value	Interpretation
1	≤ 40	≤ 40	N/A	Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Detected
2	≤ 40	0	N/A	Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Not Detected
3	0	≤ 40	N/A	Indeterminate, Repeat test
4	0	0	≤ 40	Influenza A RNA: Not Detected 2009 H1N1 Influenza RNA: Not Detected
5	0	0	0	Invalid, Repeat Test. If AR IC is still 0 on repeat, test with a new sample if clinically warranted
Below are interpretations of specimens upon repeat testing:				
6	≤ 40	≤ 40	N/A	Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Detected
7	≤ 40	0	N/A	**If H1N1 target was Detected in the first replicate** Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Detected **If H1N1 target was Not Detected in the first replicate** Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Not Detected
8	0	≤ 40	N/A	**If FLUA target was Detected in the first replicate.** Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Detected **If FLUA target was Not Detected in the first replicate:** Influenza A RNA: Not Detected 2009 H1N1 Influenza RNA: Not Detected
9	0	0	≤ 40	Influenza A RNA: Not Detected 2009 H1N1 Influenza RNA: Not Detected

Ct = cycle threshold.
Detected is a Ct ≤40. Not Detected is a Ct = 0.

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 (LoD) in upper respiratory tract specimens was determined for the Simplexa™ Influenza A H1N1 (2009) RT-PCR assay by limiting dilution studies using viral stocks of 2009 Pandemic H1N1 reassortant [Influenza A/California/7/2009 NYMC X-179-A ($3x10^7$ $TCID_{50}$/mL)] and seasonal influenza A/PR/8/34 H1N1 ($10^{9.5}$ $TCID_{50}$/mL) and Influenza A/Hong Kong/8/68 H3N2 ($10^{7.5}$ $TCID_{50}$/mL). The results were compared to the analytical sensitivity results of the CDC rRT-PCR Swine Flu Assay (EUA). The lowest concentration with ≥95% detection was determined to be the limit of detection for each assay. The result summary is presented in Tables 5 to 7. In addition to the three strains tested for LoD, various dilutions of two additional influenza A strains were tested for reactivity with the Simplexa™ Influenza A H1N1 (2009) RT-PCR assay. Results of the LoD screening with the additional strains are presented in Tables 8 to 9.

Limit of Detection screening studies in lower respiratory tract specimens were performed by spiking virus into negative endotracheal aspirate matrix and negative bronchial alveolar lavage matrix. Results of the LoD screening for lower respiratory tract specimens are presented in Tables 10 to 13.

Table 5: Summary of Comparative LoD data – 2009 H1N1 Influenza Virus RNA

$TCID_{50}$/mL	Focus Diagnostics Assay		CDC Assay	
	Initial Screening	Confirmation of LoD	Initial Screening	Confirmation of LoD
30	2 of 2	20 of 20	N/A	N/A
7.5	4 of 4	20 of 20	N/A	18 of 20
3	1 of 6	14 of 20	N/A	3 of 20

LoD = 7.5 $TCID_{50}$/mL

Table 6: Summary of Comparative LoD data – Seasonal Influenza A (H1N1) Virus RNA

$TCID_{50}$/mL	Focus Diagnostics Assay		CDC Assay	
	Initial Screening	Confirmation of LoD	Initial Screening	Confirmation of LoD
31.6	2 of 2	N/A	N/A	N/A
15.8	4 of 4	N/A	N/A	N/A
3.16	6 of 6	20 of 20	N/A	18 of 20
1.58	8 of 8	18 of 20	N/A	17 of 20

LoD = 3.16 $TCID_{50}$/mL

Table 7: Summary of Comparative LoD data – Seasonal Influenza A (H3N2) Virus RNA

$TCID_{50}$/mL	Focus Diagnostics Assay		CDC Assay	
	Initial Screening	Confirmation of LoD	Initial Screening	Confirmation of LoD
31.6	2 of 2	N/A	N/A	N/A
3.16	2 of 2	N/A	N/A	N/A
1.58	2 of 2	20 of 20	N/A	20 of 20
0.316	2 of 2	20 of 20	N/A	18 of 20
0.158	2 of 2	10 of 20	N/A	11 of 20

LoD = 0.316 $TCID_{50}$/mL
The Limit of Detection (LoD) results for the Simplexa™ Influenza A H1N1 2009 assay were equivalent to the CDC assay results for 2009 H1N1 influenza and seasonal influenza A (H1N1 and H3N2 strains).

Table 8: LoD Screening Seasonal Influenza A (H2N2)

Influenza A/Japan/305/57 (H2N2)

$TCID_{50}$/mL	FLUA Ct value	H1N1 Ct value	AR IC Ct value
5623	27.1	0	32.7
5623	26.9	0	32.8
562	30.9	0	32.9
562	30.8	0	32.4
281	32.1	0	32.7
281	32.0	0	32.4
56.2	33.8	0	32.3
56.2	34.5	0	33.5
28.1	38.3	0	32.9
28.1	36.7	0	32.4
5.62	37.0	0	32.7
5.62	0	0	33.1
2.81	0	0	32.7
2.81	38.2	0	32.4

Table 9: LoD Screening Seasonal Influenza A (H1N1)

Influenza A/WS/33 (H1N1)

$TCID_{50}$/mL	FLUA Ct value	H1N1 Ct value	AR IC Ct value
$5.62x10^5$	18.0	0	0
$5.62x10^4$	21.5	0	36.0
$5.62x10^4$	21.2	0	36.1
$5.62x10^3$	24.9	0	33.8
$5.62x10^3$	24.8	0	33.7
$5.62x10^2$	28.3	0	33.1
$5.62x10^2$	28.0	0	32.7
56.2	31.9	0	33.2
56.2	32.1	0	33.1
5.62	35.0	0	33.3
5.62	36.5	0	33.1

The results show that the Simplexa™ Influenza A H1N1 (2009) assay is able to detect seasonal human influenza A virus RNA (H1N1 and H3N2) and the 2009 H1N1 influenza virus RNA in upper respiratory tract specimens.

Table 10: LoD Screening Endotracheal Aspirate Matrix – 2009 H1N1 Influenza Virus RNA

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

$TCID_{50}$/mL	FLUA Ct value	H1N1 Ct value	AR IC Ct value
150	32.0	32.7	33.7
150	32.3	33.3	33.2
150	31.7	32.7	33.4
75	33.7	35.4	34.1
75	32.7	33.7	32.4
75	33.1	34.3	32.6
30	34.9	35.7	32.7
30	35.1	35.2	32.5
30	34.1	36.0	33.2
7.5	35.1	37.0	32.6
7.5	37.3	38.6	32.4
7.5	38.7	39.3	33.0

Table 11: LoD Screening Endotracheal Aspirate Matrix – Seasonal Influenza Virus RNA

Influenza A/PR/8/34 (H1N1)

$TCID_{50}$/mL	FLUA Ct value	H1N1 Ct value	AR IC Ct value
31.6	31.4	0	32.6
31.6	31.3	0	32.3
31.6	31.7	0	33.1
15.8	33.9	0	33.1
15.8	34.5	0	32.9
15.8	34.4	0	32.6
3.16	39.6	0	31.5
3.16	35.9	0	32.5
3.16	35.1	0	33.1
1.58	38.3	0	32.7
1.58	36.1	0	32.5
1.58	37.3	0	33.4

Table 12: LoD Screening Bronchial Alveolar Lavage Matrix – 2009 H1N1 Influenza Virus RNA

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

$TCID_{50}$/mL	FLUA Ct value	H1N1 Ct value	AR IC Ct value
150	31.0	31.6	33.6
150	31.5	32.3	33.2
150	31.5	32.3	33.7
75	33.0	33.0	33.6
75	32.2	33.4	33.9
75	32.5	33.2	33.1
30	33.6	34.4	33.7
30	34.3	34.4	33.7
30	34.0	33.7	34.3
7.5	35.5	36.0	34.3
7.5	36.1	36.4	34.0
7.5	36.5	36.0	34.7

Table 13: LoD Screening Bronchial Alveolar Lavage Matrix – Seasonal Influenza Virus RNA

Influenza A/PR/8/34 (H1N1)

$TCID_{50}$/mL	FLUA Ct value	H1N1 Ct value	AR IC Ct value
31.6	31.4	0	34.1
31.6	31.1	0	33.5
31.6	31.3	0	34.1
15.8	32.1	0	34.4
15.8	32.6	0	33.7
15.8	32.9	0	33.4
3.16	34.7	0	33.8
3.16	35.4	0	33.4
3.16	37.4	0	33.2
1.58	35.6	0	33.1
1.58	36.7	0	33.1
1.58	34.8	0	33.3

The results show that the Simplexa™ Influenza A H1N1 (2009) assay is able to detect seasonal human influenza A virus RNA and the 2009 H1N1 influenza virus RNA in lower respiratory tract specimens.

REPRODUCIBILITY

Within Run Precision

Three pools of 2009 influenza A H1N1 virus were prepared by making dilutions of a cultured virus stock of 2009 H1N1 influenza A/California/7/2009 NYMC X-179-A ($3x10^7$ $TCID_{50}$/mL). The virus stock was diluted 10,000 fold with Universal Transport Medium (Copan Diagnostics) to create the high pool. The medium pool was created from a ten-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 (Influenza A/PR/8/34 H1N1) stock in Universal Transport Medium. The high pool was created by preparing a $2x10^6$ fold dilution of the stock. The medium pool was created from a ten-fold dilution of the high pool, and the low pool was created from a ten-fold dilution of the medium pool. Five aliquots of each sample were extracted and assayed in duplicate 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.

Intra-assay variation was <3% CV based on Ct values.

Table 14: Intra-Assay Precision of 2009 H1N1 influenza in Transport Medium

	H1N1 target Ct Value			FLUA target Ct Value		
	High	**Medium**	**Low**	**High**	**Medium**	**Low**
N	9*	10	10	10	10	10
Average	27.78	31.13	34.98	27.30	30.71	34.27
Std Dev	0.15	0.70	0.68	0.18	0.52	0.32
% CV	0.53	2.24	1.94	0.66	1.71	0.92

*One replicate was omitted due to operator error.

Table 15: Intra-Assay Precision of Seasonal Influenza A Virus in Transport Medium

	H1N1 target Ct Value			FLUA target Ct Value		
	High	**Medium**	**Low**	**High**	**Medium**	**Low**
N	10	10	10	10	10	10
Average	0	0	0	25.9	29.4	33.0
Std Dev	0	0	0	0.22	0.16	0.29
% CV	N/A	N/A	N/A	0.84	0.53	0.88

Between Run Precision

Three pools with high, medium and low concentrations of 2009 H1N1 influenza virus were prepared by making dilutions of a cultured virus stock as described above. Similarly, samples with high, medium and low concentrations of seasonal influenza A virus were prepared by making dilutions of influenza A virus stock in Universal Transport Medium. A minimum of four aliquots of

each sample were assayed in two separate assay runs per day, for five days to determine inter-assay precision. Standard deviation and %CV values were calculated based on the C_t values obtained from the amplification reactions.

Inter-assay variation was <3% CV based on Ct values.

Table 16: Inter-Assay Precision of 2009 H1N1 influenza in Transport Medium

	H1N1 Ct Value			FLUA Ct Value		
	High	Medium	Low	High	Medium	Low
N	47	47	47	47	47	47
Average	27.8	31.3	34.6	27.2	30.7	34.1
SD	0.34	0.50	0.68	0.21	0.37	0.42
% CV	1.24	1.60	1.98	0.78	1.21	1.23

Table 17: Inter-Assay Precision of Seasonal Influenza A Virus in Transport Medium

	H1N1 Ct Value			FLUA Ct Value		
	High	Medium	Low	High	Medium	Low
N	47	47	47	47	47	47
Average	0	0	0	25.9	29.4	32.8
SD	0	0	0	0.24	0.21	0.40
% CV	N/A	N/A	N/A	0.92	0.70	1.21

ANALYTICAL REACTIVITY / CROSS REACTIVITY

Genomic DNA or RNA of a variety of organisms (≥10^5 $TCID_{50}$/mL or ≥10^6 CFU/mL for cultured organisms or pathogen Ct ≤ 30 for clinical specimens) was assayed to verify lack of cross-reactivity of the Simplexa assay with nucleic acids of other organisms. The results are shown in Tables 18 and 19. Within 40 cycles of amplification and detection, no cross-reactivity was seen with respiratory pathogens tested, with the exception of two deviations (Adenovirus type 7 and *Streptococcus pyogenes*) in an original experiment. Both organisms gave weak signal with FLUA probe of Simplexa™ assay. The assay was repeated on Adenovirus type 7 and the *S. pyogenes* genomic DNA. Upon repeat testing, no amplification was detected for the FLUA target or for the 2009 H1N1 target with either organism.

Table 18: Analytical Reactivity

Pathogen	Source	Strain	H1N1 Result	FLUA Result
Influenza A/A/WS/33	ATCC	H1N1	Not Detected	Detected
Influenza A/PR/8/34	Advanced Biotechnologies, Inc	H1N1	Not Detected	Detected
Influenza A/Japan/305/57	Advanced Biotechnologies, Inc	H2N2	Not Detected	Detected
Influenza A/Hong Kong/8/68	Advanced Biotechnologies, Inc	H3N2	Not Detected	Detected
Influenza Virus/A/California/ 7/2009 NYMC X-179-A	Virapur	H1N1 (2009)	Detected	Detected

Table 19: Cross Reactivity

Pathogen	Source	H1N1 Ct Value	FLUA Ct value
Adenovirus 2	ATCC	0	0
Adenovirus 7	ATCC	0	38.3
		0	0
Bordetella parapertussis	ATCC	0	0
Bordetella pertussis	ATCC	0	0
Chlamydophila pneumoniae	ATCC	0	0
Coronavirus 229E	ATCC	0	0
Coronavirus OC43	ATCC	0	0
Corynebacterium diptheriae	ATCC	0	0

Pathogen	Source	H1N1 Ct Value	FLUA Ct value
Corynebacterium xerosis	ATCC	0	0
Coxiella burnetii	Virion/Serion	0	0
Cytomegalovirus	Advanced Biotechnologies	0	0
Echovirus 7	ATCC	0	0
Enterovirus 71	ATCC	0	0
Epstein Barr Virus	Advanced Biotechnologies	0	0
Escherichia coli	ATCC	0	0
Haemophilus influenzae	ATCC	0	0
Human metapneumovirus	Focus Diagnostics	0	0
Influenza B (B/Lee/40)	ATCC	0	0
Influenza B (B/Brisbane/60/2008)	Clinical Trials Vaccine Stock	0	0
Influenza B (B/Malaysia/2506/2004)	Clinical Trials Vaccine Stock	0	0
Lactobacillus acidophilus	Focus Diagnostics	0	0
Legionella micdadei	ATCC	0	0
Legionella pneumophila	Focus Diagnostics	0	0
Measles	ATCC	0	0
Moraxella catarrhalis	ATCC	0	0
Methicillin-resistant *S. aureus*	ATCC	0	0
Methicillin-sensitive *S. aureus*	ATCC	0	0
Mumps	ATCC	0	0
Mycobacterium tuberculosis	Focus Diagnostics (3 specimens)	0*	0*
		0	0
Mycoplasma pneumoniae	ATCC	0*	0*
		0	0
Mycoplasma pneumoniae	ATCC (genomic DNA)	0	0
Mycoplasma orale	ATCC	0	0
Mycoplasma salivarium	ATCC	0	0
Mycoplasma fermentans	ATCC	0	0
Mycoplasma genitalium	ATCC	0	0
Mycoplasma hominis	ATCC	0	0
Neisseria meningitides	ATCC	0	0
Parainfluenza -1	ATCC	0	0
Parainfluenza -2	ATCC	0	0
Parainfluenza -3	ATCC	0	0
Pseudomonas aeruginosa	ATCC	0	0
Rhinovirus -16	ATCC	0*	0*
		0	0
RSV A	ATCC	0	0
RSV B	ATCC	0	0
Staphylococcus aureus	ATCC	0	0
Staphylococcus epidermidis	ATCC	0	0
Streptococcus pneumoniae	ATCC	0	0
Streptococcus pyogenes	ATCC	0	38.1
		0	0
Streptococcus salivarius	ATCC	0	0

*AR IC was not detected on the first run, but repeat extraction and testing showed that AR IC was detected.

CLINICAL PERFORMANCE CHARACTERISTICS

METHOD COMPARISON

One hundred eighty (180) clinical samples were compared using the Focus assay and the CDC rRT-PCR for 2009 H1N1 influenza. The study included 60 specimens previously determined to be seasonal influenza A positive and 60 specimens previously determined to be 2009 H1N1 influenza positive by Focus Diagnostics real-time PCR assay (EUA) as well as 60 negative specimens. Specimens were submitted to Focus Diagnostics as a respiratory swab in viral transport medium. All previously reported results were blinded to the operators. The results show that the Simplexa™ Influenza A H1N1 (2009) assay is able to detect and differentiate seasonal human influenza A virus RNA and the 2009 H1N1 influenza virus RNA. Summary data are presented in Tables 20 and 21 below.

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. Five lower respiratory tract specimens from critically ill patients with influenza symptoms were tested using the Simplexa 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. The fifth sample was positive for H1N1 (2009) in a lower respiratory tract specimen; a corresponding upper respiratory tract specimen was not available for testing. All lower respiratory testing results matched results from the Focus Diagnostics Influenza A H1N1 (2009) Real-Time RT-PCR (REF: MOL9100). Four additional samples were tested with MOL9100 and were positive for H1N1 (2009) in lower respiratory tract specimens and negative in paired upper respiratory tract specimens.

Table 20: Concordance for 2009 H1N1 influenza

		CDC REALTIME RT-PCR FOR 2009 H1N1 INFLUENZA			
		2009 H1N1 Positive	**2009 H1N1 Negative**	**Total**	
Simplexa™ Influenza A H1N1 (2009)	**2009 H1N1 Positive**	59	1**	60	% Positive Agreement 98.3% (59/60) 95% CI: 91.1-99.7
	2009 H1N1 Negative	1*	119	120	% Negative Agreement 99.2% (119/120) 95% CI: 95.4-99.9
	Total	60	120	180	

*One sample was detected by CDC assay with Ct ≥38.0 for all three target detectors, whereas Simplexa™ assay detected Ct 39.0 for FLUA target and no Ct for H1N1 target. Upon retesting of frozen clinical specimen, both assays detected the sample as positive for influenza A.
**One sample was detected by Simplexa™ assay with Ct ≥36.0 for both target detectors, whereas CDC assay did not detect Ct value for any target detectors. Upon retesting of frozen clinical specimens, both assays did not detect influenza A or 2009 H1N1.

Table 21: Concordance for Influenza A

		CDC REALTIME RT-PCR FOR 2009 H1N1 INFLUENZA			
		Flu A Positive	**Flu A Negative**	**Total**	
Simplexa™ Influenza A H1N1 (2009)	**Flu A Positive**	118	2*	120	% Positive Agreement 100% (118/118) 95% CI: 96.8 -100
	Flu A Negative	0	60	60	% Negative Agreement 96.8% (60/62) 95% CI: 89.0 -99.1
	Total	118	62	180	

*One sample was detected by Simplexa™ assay with Ct >37.0 for FLUA detector, whereas CDC assay did not detect Ct value for any target detector. Upon retesting of frozen clinical specimens, Simplexa™ assay did not detect Ct value for any target detector, whereas CDC assay detected the influenza A target with Ct ≥36.0. One sample was detected by Simplexa™ assay with Ct ≥36.0 for both target detectors, whereas CDC assay did not detect Ct value for any target detectors. Upon retesting of frozen clinical specimens, both assays did not detect influenza A or 2009 H1N1.

REPORTABLE RANGE

The Ct values observed for the influenza A and 2009 H1N1 influenza H1 targets in these specimens should be indicative of positive results for this assay. The 2009 H1N1 influenza positive specimens have FLUA Ct values in the range of 17.6 -39.0, and H1N1 Ct values in the range of 18.4 -39.2. The majority of specimens have Ct values <35 for both targets.

QUALITY CONTROL

Quality control ranges have been established as indicated in Table 22. If the controls are not within these parameters, patient results should be considered invalid and the assay repeated. 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.

Table 22: Expected Control Ranges

Control Type	Simplexa™ H1N1 Positive Control FLUA Ct value	Simplexa™ H1N1 Positive Control H1N1 Ct value	Simplexa™ Armored RNA Internal Control (AR IC)
No Template Control	Ct = 0	Ct = 0	Ct < 40
Positive Control	Ct < 40	Ct < 40	Not applicable*

* Detection of the Simplexa™ Armored RNA Internal Control (AR IC) is not required for a valid result.

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