

# Simplexa™ Influenza A H1N1 (2009)

REF MOL2500  
Rev. A



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

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## Emergency Use Authorization For *in vitro* Diagnostic Use

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### 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 Cyclor as part of the Microfluidic Molecular System for the *in vitro* qualitative detection and differentiation of seasonal influenza A and 2009 H1N1 influenza viral RNA in nasopharyngeal swabs (NPS), nasal swabs (NS), and nasopharyngeal aspirates (NPA) 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<sup>1</sup>. 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.<sup>2</sup>

### 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 nasopharyngeal swabs (NPS), nasal swabs (NS), and nasopharyngeal aspirates (NPA). 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). The assay provides two results. A well-conserved region of the matrix gene from influenza A viruses is targeted to identify both seasonal 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					
	ENGLISH			REF	EC SYMBOL	
Focus Diagnostics' Simplexa™ Influenza A H1N1 (2009) (Part # MOL2500)	Simplexa™ H1N1 Primer Mix			MOL2501	REAG	A
	Simplexa™ RNA Master Mix			MOL2002	REAG	B
	RT Mix			MOL9103	REAG	C
	Simplexa™ Armored RNA Internal Control			MOL2003	CONTROL	IC
	Simplexa™ No Template Control			MOL2001	CONTROL	NTC
	Simplexa™ H1N1 Positive Control			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.				
			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			
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				

### MATERIALS REQUIRED BUT NOT SUPPLIED

- 3M Integrated Cycler with Integrated Cycler Studio software version 1.0 or higher
- Universal Discs for use on the Integrated Cycler
- Universal Disc Cover Tape
- Roche MagNA Pure LC and associated consumables.
- MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Cat. No 3038505001)
- 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<sup>3</sup>.
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 respiratory specimens (nasal swabs, nasopharyngeal swabs or nasopharyngeal aspirates) 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 Cyclor Studio software for the Influenza A H1N1 (2009) assay:

**Note: This only needs to be performed at the first time of use.**

1. Refer to Integrated Cyclor Operator Manual for details on how to define the assay on the Microfluidic Molecular System.
2. Launch Integrated Cyclor 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.

<u>Short Name</u>	<u>Long Name</u>	<u>Dye Name</u>	<u>Type</u>	<u>Threshold</u>	<u>Ct Cutoff</u>
FLUA	Influenza A	Fam	Unknown	10000	40
H1N1	H1N1 (2009)	CFR610	Unknown	15000	40
AR IC	Armored RNA IC	Q670	IC	10000	40
Joe	Joe	Joe	Spectral	0	N/A

- **Disc:** Select Universal Disc from the **Disc Type** dropdown menu.
5. In the **Define Cycles** tab.
    - **Initial Cycles**

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

- **Main Cycles**

- **Cycle Count**     **40**

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

- 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 Cyclers 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 Cyclers.
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	≤40

\* 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	≤40
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 any influenza A virus (seasonal 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  $\leq 40$  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 H1 Gene" result is reported as "Not Detected".
- f. If the H1N1 Ct value of a patient specimen is  $\leq 40$  and an amplification curve is observed, the "2009 H1N1 influenza H1 Gene" result is reported as "Detected". If the Ct value for the well is  $\leq 40$  but no amplification curve is observed in the well (nonspecific fluorescence is observed in the well), the "2009 H1N1 influenza H1 Gene" 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  $\leq 40$  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	$\leq 40$	$\leq 40$	N/A	Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Detected
2	$\leq 40$	0	N/A	Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Not Detected
3	0	$\leq 40$	N/A	Indeterminate, Repeat test
4	0	0	$\leq 40$	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
<b>Below are interpretations of specimens upon repeat testing:</b>				
6	$\leq 40$	$\leq 40$	N/A	Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Detected
7	$\leq 40$	0	N/A	<b>If H1N1 target was <u>Detected</u> in the first replicate</b> Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Detected <b>If H1N1 target was <u>Not Detected</u> in the first replicate</b> Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Not Detected
8	0	$\leq 40$	N/A	<b>If FLUA target was <u>Detected</u> in the first replicate.</b> Influenza A RNA: Detected 2009 H1N1 Influenza RNA: Detected <b>If FLUA target was <u>Not Detected</u> in the first replicate:</b> Influenza A RNA: Not Detected 2009 H1N1 Influenza RNA: Not Detected
9	0	0	$\leq 40$	Influenza A RNA: Not Detected 2009 H1N1 Influenza RNA: Not Detected

Ct = cycle threshold.

Detected is a Ct  $\leq 40$ . 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. As with other tests, 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. As with other tests, 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. The performance of this test has not been established for patients without symptoms of influenza infection.
10. The performance of this test has not been established for monitoring treatment of influenza A or 2009 H1N1 influenza infection.
11. The performance of this test has not been established for screening of blood or blood product for the presence of influenza A or 2009 H1N1 influenza.
12. The performance of this test has not been established with potentially interfering medications for the treatment of influenza or cold virus.
13. The performance of this test has not been established for individuals who have received the influenza vaccine.
14. This test cannot rule out diseases caused by other bacterial or viral pathogens.

## ANALYTICAL PERFORMANCE CHARACTERISTICS

### ANALYTICAL SENSITIVITY/LIMIT OF DETECTION

The Limit of Detection (LoD) 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 ( $3 \times 10^7$  TCID<sub>50</sub>/mL)] and seasonal influenza A/PR/8/34 H1N1 ( $10^{9.5}$  TCID<sub>50</sub>/mL) and Influenza A/Hong Kong/8/68 H3N2 ( $10^{7.5}$  TCID<sub>50</sub>/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.

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

TCID <sub>50</sub> /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<sub>50</sub>/mL

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

TCID <sub>50</sub> /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<sub>50</sub>/mL

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

TCID <sub>50</sub> /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



TCID <sub>50</sub> /mL	Focus Diagnostics Assay		CDC Assay	
	Initial Screening	Confirmation of LoD	Initial Screening	Confirmation of LoD
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<sub>50</sub>/mL

**Table 8: LoD Screening Seasonal Influenza A (H2N2)**

Influenza A/Japan/305/57 (H2N2)

TCID <sub>50</sub> /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 <sub>50</sub> /mL	FLUA Ct value	H1N1 Ct value	AR IC Ct value
5.62x10 <sup>5</sup>	18.0	0	0
5.62x10 <sup>4</sup>	21.5	0	36.0
5.62x10 <sup>4</sup>	21.2	0	36.1
5.62x10 <sup>3</sup>	24.9	0	33.8
5.62x10 <sup>3</sup>	24.8	0	33.7
5.62x10 <sup>2</sup>	28.3	0	33.1
5.62x10 <sup>2</sup>	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.

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).

## 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<sup>7</sup> TCID<sub>50</sub>/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<sup>6</sup> 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  $C_t$  values.

**Table 10: Intra-Assay Precision of 2009 H1N1 influenza in Transport Medium**

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

\*One replicate was omitted due to operator error.

**Table 11: 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
<b>N</b>	10	10	10	10	10	10
<b>Average</b>	0	0	0	25.9	29.4	33.0
<b>Std Dev</b>	0	0	0	0.22	0.16	0.29
<b>% CV</b>	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  $C_t$  values.

**Table 12: Inter-Assay Precision of 2009 H1N1 influenza in Transport Medium**

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

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

	H1N1 Ct Value			FLUA Ct Value		
	High	Medium	Low	High	Medium	Low
<b>N</b>	47	47	47	47	47	47
<b>Average</b>	0	0	0	25.9	29.4	32.8
<b>SD</b>	0	0	0	0.24	0.21	0.40
<b>% CV</b>	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 ( $\geq 10^5$  TCID<sub>50</sub>/mL or  $\geq 10^6$  CFU/mL for cultured organisms or pathogen  $C_t \leq 30$  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 14 and 15. 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 14: 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 15: Cross Reactivity**

Pathogen	Source	H1N1 Ct Value	FLUA Ct value
Adenovirus 2	ATCC	0	0
Adenovirus 7	ATCC	0	38.3
		0	0
<i>Bordetella parapertussis</i>	ATCC	0	0
<i>Bordetella pertussis</i>	ATCC	0	0
<i>Chlamydomphila pneumoniae</i>	ATCC	0	0
Coronavirus 229E	ATCC	0	0
Coronavirus OC43	ATCC	0	0
<i>Corynebacterium diphtheriae</i>	ATCC	0	0
<i>Corynebacterium xerosis</i>	ATCC	0	0
<i>Coxiella burnetii</i>	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
<i>Escherichia coli</i>	ATCC	0	0
<i>Haemophilus influenzae</i>	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
<i>Lactobacillus acidophilus</i>	Focus Diagnostics	0	0
<i>Legionella micdadei</i>	ATCC	0	0
<i>Legionella pneumophila</i>	Focus Diagnostics	0	0
Measles	ATCC	0	0
<i>Moraxella catarrhalis</i>	ATCC	0	0
Methicillin-resistant <i>S. aureus</i>	ATCC	0	0
Methicillin-sensitive <i>S. aureus</i>	ATCC	0	0
Mumps	ATCC	0	0
<i>Mycobacterium tuberculosis</i>	Focus Diagnostics (3 specimens)	0*	0*
		0	0
<i>Mycoplasma pneumoniae</i>	ATCC	0*	0*
		0	0
<i>Mycoplasma pneumoniae</i>	ATCC (genomic DNA)	0	0

Pathogen	Source	H1N1 Ct Value	FLUA Ct value
<i>Mycoplasma orale</i>	ATCC	0	0
<i>Mycoplasma salivarium</i>	ATCC	0	0
<i>Mycoplasma fermentans</i>	ATCC	0	0
<i>Mycoplasma genitalium</i>	ATCC	0	0
<i>Mycoplasma hominis</i>	ATCC	0	0
<i>Neisseria meningitides</i>	ATCC	0	0
Parainfluenza -1	ATCC	0	0
Parainfluenza -2	ATCC	0	0
Parainfluenza -3	ATCC	0	0
<i>Pseudomonas aeruginosa</i>	ATCC	0	0
Rhinovirus -16	ATCC	0*	0*
		0	0
RSV A	ATCC	0	0
RSV B	ATCC	0	0
<i>Staphylococcus aureus</i>	ATCC	0	0
<i>Staphylococcus epidermidis</i>	ATCC	0	0
<i>Streptococcus pneumoniae</i>	ATCC	0	0
<i>Streptococcus pyogenes</i>	ATCC	0	38.1
		0	0
<i>Streptococcus salivarius</i>	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 16 and 17 below.

**Table 16: 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	
	<b>Total</b>	60	120	180	% Negative Agreement 98.3% (119/120) 95% CI: 95.4-99.9

\*One sample was detected by CDC assay with Ct  $\geq$ 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  $\geq$ 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 17: Concordance for Seasonal Influenza A**

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

\*One sample was detected by Simplexa™ assay with Ct >37.0 for FLUA A 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 18. 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 18: 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.

**REFERENCES**

- Update: Novel Influenza A (H1N1) Virus Infections – Worldwide, May 6, 2009. *MMWR Morb Mortal Wkly Rep.* 2009; **58**:400. <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5817a1.htm>
- Gallaher, WR. Towards a sane and rational approach to management of Influenza H1N1 2009. *Virology Journal.* 2009 May 7; 6:51.
- H1N1 Influenza Virus Biosafety Guidelines for Laboratory Workers. [http://www.cdc.gov/h1n1flu/guidelines\\_labworkers.htm](http://www.cdc.gov/h1n1flu/guidelines_labworkers.htm).

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