

Simplexa™ Influenza A H1N1 (2009)

REF MOL2500

Rev. C



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

For *in vitro* Diagnostic Use

INTENDED USE

The Focus Diagnostics Simplexa™ Influenza A H1N1 (2009) assay is intended for use 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 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.

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

Performance characteristics for influenza A were established during the 2009-2010 influenza season when 2009 H1N1 influenza was the predominant influenza A virus in circulation. When other Influenza A viruses are emerging, performance characteristics may vary.

If infection with a novel Influenza A virus is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions for novel virulent Influenza viruses and sent to state or local health department for testing. Viral culture should not be attempted in these cases unless a BSL 3+ facility is available to receive and culture specimens.

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 viruses that contain one of three major subtypes of hemagglutinin (H1, H2, or H3) and two subtypes of neuraminidase (N1 or N2). In late March 2009, a novel influenza virus (2009 H1N1 influenza) began circulating in North America and subsequently around the world¹. 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^{2,3}.

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

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 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 identifying the subtype of 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.				
			Target	Probe Fluorophore	Excitation (nm)	Emission (nm)	Targeted Gene
			FLU A	FAM	495	520	matrix
			H1N1	CFR610	590	610	HA
Internal Control "AR IC"	Q670	644	670	N/A			
RNA Master Mix (RMM)	100/50	200	DNA polymerase, buffer and dNTPs				
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 Influenza Virus				
Simplexa™ Influenza A H1N1 (2009) Barcode Card	n/a	n/a	Assay specific parameters				

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Integrated Cycler with Integrated Cycler Studio Software version 2.1 or higher
2. Universal Discs for use on the Integrated Cycler
3. Universal Disc Cover Tape
4. ³Roche MagNA Pure LC System and associated consumables
5. ^{aT}MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Cat. No 3038505001)
6. ^{bT}QIAamp® Viral RNA Mini Kit (Qiagen Cat No. 52904 (50 extractions) or 52906 (250 extractions))
7. Single, multi-channel and/or repeater micropipette(s) with an accuracy range between 1-10 µL, 10-100 µL and 100-1000 µL
8. Freezer (manual defrost) at -10 to -30 °C (for kit component frozen storage)
9. Freezer (manual defrost) at -10 to -30 °C (for specimen frozen storage)
10. Laminar flow hood for extractions
11. Microcentrifuge
12. Vortex mixer
13. Sterile, RNase/DNase-free disposable aerosol-barrier micropipettor tips
14. 1.5 mL polypropylene microcentrifuge tubes and racks (RNase/DNase-free tubes are recommended but not required)
15. Disposable, powder-free gloves

16. Nuclease-Free Water
17. Cooler racks for 1.5 mL microcentrifuge tubes
 - ^a For use with Roche MagNA Pure LC extraction method
 - ^b For use with QIAamp Viral RNA Mini Kit extraction method
 - ^T Specific lots of the ancillary reagent of the MagNA Pure LC Total Nucleic Acid Isolation Kit and QIAamp® Viral RNA Mini Kit have been qualified for use with the Simplexa™ Influenza A H1N1 (2009) assay.

NOTE: The Simplexa™ Influenza A H1N1 (2009) assay product performance requires that only qualified manufacturer lots of the MagNA Pure LC Total Nucleic Acid Isolation Kit and QIAamp® Viral RNA Mini Kit be used with the device. Any lots not specifically qualified by Focus Diagnostics for use with the Simplexa™ Influenza A H1N1 (2009) assay are not validated for use with this assay, and may cause erroneous results.

A list of these qualified extraction reagents is available at www.focusdx.com. Please notify the reagent manufacturer of issues with the ancillary reagents and Focus Diagnostics of the impact of these issues on the performance of this Simplexa™ kit.

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. After initial use, return the RT mix to freezer (-10 to -30 °C) up to the expiration date.
6. 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 or until expiration date whichever comes first.
7. Do not refreeze Primer Mix, RNA Master Mix, Internal Control or Positive Control.
8. Do not use kits or reagents beyond their expiration dates.
9. Do not combine reagents from different kit lots.

WARNINGS AND PRECAUTIONS

1. For *In Vitro* Diagnostic Use.
2. Follow standard 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 laboratory⁴.
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 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 nasopharyngeal swabs (NPS), nasal swabs (NS), and nasopharyngeal aspirates (NPA). Specimens should be collected in accordance with collection device instructions for use and the WHO guidelines for the collection of human specimens for laboratory diagnosis of avian influenza infection⁵. Collected samples should be placed in sterile viral transport media containing protein stabilizer, antibiotics to inhibit 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.

Extraction using Roche MagNA Pure LC extraction method

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. Add 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.

Extraction using QiaGen QIAamp Viral RNA Mini Kit extraction method

The following extraction procedure is based on the Spin Protocol from the QIAamp® Viral RNA Mini Handbook (Third Edition, December, 2007). Some steps are altered for this functional assay. Follow all of the warnings and precautions listed in the QIAamp® Viral RNA Mini Handbook. Prepare all buffers according to the Handbook prior to starting the extraction procedure.

1. Prepare the Buffer AVL with carrier RNA solution by mixing the appropriate amount of Buffer AVL and carrier RNA-AVE to a tube. Volumes will depend on the number of samples to be extracted. Refer to the table below:

No. of Samples	Vol. Buffer AVL (mL)	Vol. of Carrier RNA-AVE (µL)	No. of Samples	Vol. Buffer AVL (mL)	Vol. of Carrier RNA-AVE (µL)
1	0.56	5.6	13	7.28	72.8
2	1.12	11.2	14	7.84	78.4
3	1.68	16.8	15	8.40	84.0
4	2.24	22.4	16	8.96	89.6
5	2.80	28.0	17	9.52	95.2
6	3.36	33.6	18	10.08	100.8
7	3.92	39.2	19	10.64	106.4
8	4.48	44.8	20	11.20	112.0
9	5.04	50.4	21	11.76	117.6
10	5.60	56.0	22	12.32	123.2
11	6.16	61.6	23	12.88	128.8
12	6.72	67.2	24	13.44	134.4

RNA extraction of samples using the QIAamp® Viral RNA Mini Kit:

1. Add 560 µL of prepared Buffer AVL containing carrier RNA into each tube.
2. Add 140 µL of each specimen or Positive or No Template Control into a sample extraction tube.
3. Add 5µL of Armored RNA Internal Control into each sample tube.
4. Mix by pulse-vortexing for 15 seconds and briefly centrifuge tubes.
5. Incubate at room temperature (15 – 25 °C) for 10 min.

6. Add 560 µL of ethanol (96 – 100%) to the sample, mix by pulse-vortexing for 15 seconds and briefly centrifuge the tubes.
7. Carefully apply 630 µL of the sample-ethanol mixture from step 6 to the QIAamp Mini column (in a 2 mL collection tube) without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min.
8. Place the QIAamp Mini column into a clean 2 mL collection tube and discard the tube containing the filtrate.
9. Carefully open the QIAamp Mini column and repeat steps 7 – 8.
10. Carefully open the QIAamp Mini column and add 500 µL of Buffer AW1. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min.
11. Place the QIAamp Mini column into a clean 2 mL collection tube and discard the tube containing filtrate.
12. Carefully open the QIAamp Mini column and add 500 µL of Buffer AW2. Close the cap and centrifuge at 20,000 x g (14,000 rpm) for 3 min.
13. Place the QIAamp Mini column into a new 2 mL collection tube and discard the old collection tube with filtrate. Centrifuge at 20,000 x g (14,000 rpm) for 1 min.
14. Place the QIAamp Mini column into a clean 1.5mL microcentrifuge tube. Discard the old collection tube containing filtrate.
15. Carefully open the QIAamp Mini column and add 50 µL of Buffer AVE. Close cap and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 minute.
16. Discard the QIAamp Mini column. Close caps and save the tubes containing filtrates. 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 Simplexa™ Influenza A H1N1 (2009) assay:

Note: This only needs to be performed at the first time of use or if instructed to do so.

1. Refer to Integrated Cyclor Operator Manual for details on how to manage assay definitions on the Microfluidic Molecular System.
2. Launch Integrated Cyclor Studio Software.
3. Open menu item **Tools: Scan Assay Barcode**.
4. A dialog box will display. Scan the barcode card while pressing the handheld scanner button. The scanner will beep with a successful read.
5. The IVD Assay Definition will be added to the system. If the kit has already been scanned, a dialog box will display a warning and the scan will be canceled.

Focus Diagnostics kits may contain version numbers for Assay Definitions. If the version number exists, it will be appended to the Assay Definition ie. 'Sample IVD Assay.2'

When multiple versions exist, the software automatically uses the latest version when creating runs.

Controls are pre-programmed in the Assay Definition. Focus recommends placing the Positive Control in the first well of a segment and the No Template Control in the last well of a segment. If additional control wells are programmed, the system software will use all wells designated as controls to determine run validity.

Perform the following to set up a run on the Integrated Cyclor Studio Software for the Simplexa™ 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:
 - o Select the latest version of "**Simplexa Influenza A H1N1 2009**" from the **Assay Definition** dropdown menu.
 - o Name the run under **Run Details**.
 - o Add **Lot Information** if necessary. Click **New** in the **Lot Information** section, enter the **Lot Number** and **Expiration Date**, ensure the **Predictive** button is selected and click **OK**.
 - o **Add Controls** to the **Sample List** by selecting the control and clicking the green arrow.
 - o **Add Samples** to the **Sample List**.
 - o 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

- o 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 an 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 the 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**.
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 and Include Ct Values** checkboxes 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. The Integrated Cycler Studio Software will suppress interpretation of patient results if any of the samples programmed as controls are invalid.
 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	FLU A Ct	AR IC Ct
No Template Control	0 (If ≤40 then patient results cannot be reported)	0 (If ≤40 then patient results cannot be reported)	≤40, ≠0
Positive Control	≤40, ≠0	≤40, ≠0	Not Applicable (N/A)

* See notes below for full description.

- a. If the No Template Control is:
 - i. Positive (Ct value ≤40, ≠0 for either the H1N1 or FLU A), then this indicates possible contamination of prepared samples. The control is invalid and all patient specimens must be re-extracted and re-assayed.
 - ii. Negative for H1N1 and FLU A 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 all patient specimens must be re-assayed.
 - iv. If the AR IC is detected for the No Template Control, the assay run is considered valid and acceptable.
- b. Positive Control

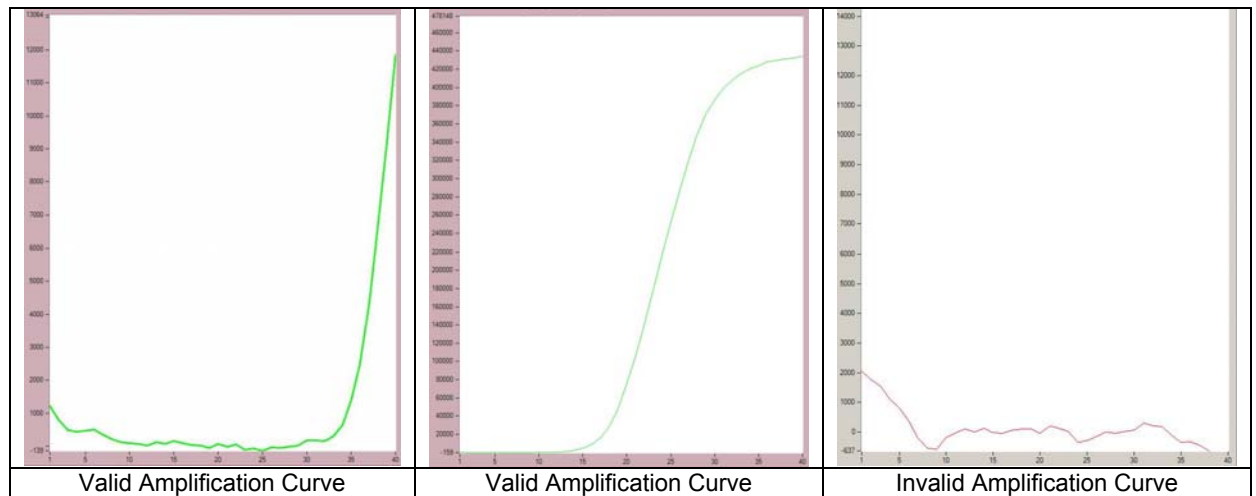
- i. If the Positive Control result is a Ct = 0 for H1N1 and / or FLU A, the assay run is considered invalid and unacceptable. All patient specimens must be re-extracted and re-assayed.
 - ii. If the Ct values for H1N1 and FLU A are, ≤ 40 , $\neq 0$ the assay run is considered valid and acceptable.
2. 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, FLU A and AR IC results must be examined for each patient specimen.

Criteria for a Valid Patient Specimen (simplified)*

Patient Specimen H1N1 Ct and FLU A Ct	Amplification Plot	AR IC Ct
Either detector or both detectors ≤ 40 , $\neq 0$	Shows exponential increase	N/A
Both detectors at 0	N/A	≤ 40 , $\neq 0$

* See notes below for full description.

- a. Amplification plots should be examined for every result with a “Data Quality” message. From the Data tab select the curve you would like to review and click **Refresh**. The software will draw the selected curves and adjust the scale of the graph. A valid amplification curve shows a smooth, exponential increase. An invalid amplification curve may be a non-exponential or linear curve or a curve with data “spikes” where the curve may cross the threshold. If the curve is valid after examination, the Ct value reported may be used to determine if FLU A or H1N1 targets are detected as indicated in section 3 below. Examples of valid and invalid curves are shown below.



- b. If the amplification curve is valid for FLU A or H1N1, the AR IC is not required to be detected to report a positive result for FLU A or H1N1.
3. Interpretation of Results
- a. A specimen that does not contain any influenza A virus will be negative (Ct = 0) for the FLU A and H1N1 detectors. A specimen positive for influenza A virus other than 2009 H1N1 influenza will most likely have a positive result (Ct ≤ 40 , $\neq 0$) for the FLU A detector and will be negative (Ct = 0) for H1N1 detector. A specimen that is positive for 2009 H1N1 influenza will be positive for both the FLU A and the H1N1 detector.
 - b. If only the H1N1 detector is positive and not the FLU A detector, the result is indeterminate
 - c. If the FLU A 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 FLU A Ct value of a patient specimen is ≤ 40 , $\neq 0$ 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 ≤ 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 , $\neq 0$ and an amplification curve is observed and FLU A is also detected, the “2009 H1N1 Influenza RNA” result is reported as “Detected”. If the H1N1 Ct value for the well is ≤ 40 , $\neq 0$ 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 FLU A and H1N1 Ct value of a patient specimen is 0 and the AR IC Ct value is 0, the specimen must be re-assayed. If upon repeat testing, the same situation occurs, the patient result is reported as “Indeterminate due to possible 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” result is reported indeterminate.

Table 4: Interpretation of Results

Example	FLU A 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, re-assay
4	0	0	≤ 40	Influenza A RNA: Not Detected 2009 H1N1 Influenza RNA: Not Detected
5	0	0	0	Invalid, re-assay. If AR IC is still 0 on repeat, test with a new sample if clinically warranted

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

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

REPORTABLE RANGE

The 2009 H1N1 influenza positive specimens from the clinical studies have FLU A 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. For the clinical studies, the initial rate of indeterminate results was 0.16%

QUALITY CONTROL

Quality control ranges have been established as indicated in the table below. If the controls are not within these parameters, patient results should be considered invalid and the assay repeated. Each laboratory should establish its own Quality Control ranges and frequency of QC testing based on applicable local laws, regulations and standard good laboratory practice.

Table 5: Expected Control Ranges

Control Type	Simplexa™ H1N1 Positive Control FLU A 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.

LIMITATIONS

- Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
- The detection of viral nucleic acid is dependent upon proper specimen collection, handling, transportation, storage, and preparation, including extraction. Failure to observe proper procedures in any one of these steps can lead to incorrect results.
- 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.
- The prevalence of infection will affect the test's predictive values.
- As with other tests, negative results do not rule out influenza A or 2009 H1N1 influenza infections and should not be used as the sole basis for treatment or other patient management decisions.
- False negative results may occur when the infecting organism has genomic mutations, insertions, deletions, rearrangements or if the virus migrates from the upper respiratory tract to the lower respiratory tract⁶.
- False negative results may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
- False positive results may occur. Repeat testing or testing with a different device may be indicated in some settings.
- Viral nucleic acids may persist *in vivo* independent of virus viability. Detection of analyte target(s) does not imply that the corresponding viruses are infectious, or are the causative agents for clinical symptoms.
- This test is a qualitative test and does not provide the quantitative value of detected organism present.
- The performance of this test has been evaluated for use with human specimen material only.
- The performance of this test has not been evaluated for sample types other than those specified in the Intended Use.
- The performance of this test has not been evaluated for immunocompromised individuals.
- The performance of this test has not been established for patients without symptoms of influenza infection.
- The performance of this test has not been established for monitoring treatment of influenza A or 2009 H1N1 influenza infection.
- 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.
- The performance of this test has not been established with potentially interfering medications for the treatment of influenza or cold virus. The affect of interfering substances has only been evaluated for those listed in the labeling. Interference by substances other than those described below can lead to erroneous results.
- The performance of this test has not been established for individuals who have received the influenza vaccine.

19. Although limited reactivity testing has been performed with tissue cultured swine influenza viruses and inactivated avian influenza virus (H5N1), this test is not intended to differentiate avian influenza or swine influenza other than 2009 H1N1 influenza. Cross-reactivity with respiratory tract organisms other than those listed below can lead to erroneous results.
20. This test cannot rule out diseases caused by other bacterial or viral pathogens.

PERFORMANCE CHARACTERISTICS

ANALYTICAL SENSITIVITY/LIMIT OF DETECTION

The Limit of Detection (LoD) was determined for the Simplexa™ Influenza A H1N1 (2009) RT-PCR assay using quantified stocks of six Influenza A virus strains serially diluted in negative swab or aspirate matrix. Each strain was extracted with both the Roche MagNA Pure LC Total Nucleic Acid Isolation Kit and the QIAamp Viral RNA Mini Kit. The lowest concentration with ≥95% detection (at least 19 out of 20 replicates) was determined to be the limit of detection for each assay.

Table 6: Simplexa™ Influenza A H1N1 (2009) Limit of Detection – FLU A

Influenza A Strain	LoD MagNA Pure Extraction (TCID ₅₀ /mL)		LoD QIAgen Extraction (TCID ₅₀ /mL)	
	Swab	Aspirate	Swab	Aspirate
A/California/7/2009 NYMC x-179-A	1.3x10 ⁻¹	1.3x10 ⁻¹	1.3x10 ⁻¹	2.7x10 ⁻¹
A/Swine NY/02/2009 H1N1	1x10 ⁻¹	1x10 ⁻¹	1x10 ⁻¹	1x10 ⁻¹
A/Solomon Island/03/06 H1	5x10 ⁰	5x10 ⁰	1x10 ⁰	1x10 ⁰
A/Brisbane/59/07 H1	1x10 ⁰	1x10 ⁰	1x10 ⁰	1x10 ⁰
A/Brisbane/10/07 H3	1x10 ⁻¹	1x10 ⁻¹	5x10 ⁻¹	5x10 ⁻¹
A/Wisconsin/67/05 H3	1x10 ⁻¹	5x10 ⁻¹	1x10 ⁻¹	1x10 ⁻¹

Table 7: Simplexa™ Influenza A H1N1 (2009) Limit of Detection – H1N1

2009 Influenza A Strain	LoD MagNA Pure Extraction (TCID ₅₀ /mL)		LoD QIAgen Extraction (TCID ₅₀ /mL)	
	Swab	Aspirate	Swab	Aspirate
A/California/7/2009 NYMC x-179-A	1.3x10 ⁻¹	2.7x10 ⁻¹	2.7x10 ⁻¹	2.7x10 ⁻¹
A/Swine NY/02/2009 H1N1	1x10 ⁻¹	1x10 ⁻¹	1x10 ⁻¹	1x10 ⁻¹

The results show that the Simplexa™ Influenza A H1N1 (2009) assay is able to detect human influenza A virus RNA (H1N1 and H3N2) and the 2009 H1N1 influenza virus RNA using both extraction methods.

REPRODUCIBILITY

Three investigative sites assessed the device's inter-laboratory reproducibility and inter/intra-assay reproducibility. Each of the three laboratories tested eighteen samples, the Positive Control and the No Template Control, in triplicate on five different days. Each site had two operators who each ran the assay once per day, for a total of two runs per day. Two sites performed the extraction using the MagNA Pure LC Total Nucleic Acid Isolation Kit; one site performed the extraction step using the QIAamp Viral RNA Mini Kit. Combined results for all sites are presented in the tables below. For the purposes of calculating averages and variance components, samples that offered a negative result (Ct=0) were assigned to a value of 40.0, as a value of 40.0 is more representative of negative samples which have Ct values at the upper limit of the range. Invalid sample results, including pipetting errors reported by sites, were excluded from the analysis.

Table 8: Reproducibility – FLU A

Sample	Site 1			Site 2			Site 3			Total Agreement with Expected Results	95% CI
	Agreement with Expected Results	Avg. CT	% CV	Agreement with Expected Results	Avg. CT	% CV	Agreement with Expected Results	Avg. CT	% CV		
No Template Control	30/30	40.0	0.0	29/30 ¹	40.0	0.3	30/30	40.0	0.0	89/90 (98.9%)	94% - 99.8%
Positive Control	29/29 ²	27.9	0.3	30/30	27.3	0.5	30/30	28.9	0.9	89/89 (100%)	95.9% - 100%
Swabs											
2009 H1N1 Flu high negative	25/29 ³	39.8	0.6	20/30	39.5	2.0	19/30	39.6	2.0	64/89 (71.9%)	61.8% - 80.2%
2009 H1N1 Flu low positive	26/28 ³	33.7	1.9	28/29 ³	33.5	4.2	29/29 ³	34.0	1.1	83/86 (96.5%)	90.2% - 98.8%
2009 H1N1 Flu medium positive	27/27 ³	29.9	1.7	30/30	30.1	0.9	30/30	30.3	1.8	87/87 (100%)	95.8% - 100%

Sample	Site 1			Site 2			Site 3			Total Agreement with Expected Results	95% CI
	Agreement with Expected Results	Avg. CT	% CV	Agreement with Expected Results	Avg. CT	% CV	Agreement with Expected Results	Avg. CT	% CV		
Influenza A (H1N1) high negative	26/30	39.8	0.7	27/30	39.9	0.8	27/30	39.9	1.1	80/90 88.9%	80.7% - 93.9%
Influenza A (H1N1) low positive	25/29 ³	33.8	2.6	27/30	33.2	7.7	29/30	33.9	3.6	81/89 (91.0%)	83.3% - 95.4%
Influenza A (H1N1) medium positive	29/30	29.7	2.1	30/30	28.9	0.5	30/30	30.2	0.6	89/90 (98.9%)	94% - 99.8%
Influenza A (H3N2) high negative	25/28 ³	39.8	0.7	29/30	40.0	0.0	28/30	39.9	1.0	82/88 (93.2%)	85.9% - 96.8%
Influenza A (H3N2) low positive	18/24 ³	35.0	2.0	29/30	33.8	3.8	30/30	34.1	1.1	77/84 (91.7%)	83.8% - 95.9%
Influenza A (H3N2) medium positive	30/30	29.8	0.3	27/27 ³	29.6	1.6	30/30	30.7	1.0	87/87 (100%)	95.8% - 100%
Aspirate											
2009 H1N1 Flu high negative	25/25 ³	40.0	0.0	29/29 ⁴	40.0	0.0	29/29 ³	40.0	0.0	83/83 (100%)	95.6% - 100%
2009 H1N1 Flu low positive	24/30	34.7	2.7	29/30	32.8	4.2	28/28 ³	34.3	1.3	81/88 (92.0%)	84.5% - 96.1%
2009 H1N1 Flu medium positive	30/30	29.4	0.4	29/30	29.4	6.8	30/30	30.6	0.8	89/90 (98.9%)	94% - 99.8%
Influenza A (H1N1) high negative	25/28 ³	39.8	0.6	28/30	40.0	0.4	28/30	39.9	1.3	81/88 (92.0%)	84.5% - 96.1%
Influenza A (H1N1) low positive	26/30	34.7	2.0	29/30	33.5	3.9	29/29 ³	34.7	1.4	84/89 (94.4%)	87.5% - 97.6%
Influenza A (H1N1) medium positive	29/29 ³	30.2	0.3	27/30	30.8	9.7	28/28 ³	31.2	0.6	84/87 (96.6%)	90.3% - 98.8%
Influenza A (H3N2) high negative	30/30	40.0	0.0	26/27 ⁴	40.0	0.3	28/29 ³	39.8	3.2	84/86 (97.7%)	91.9% - 99.4%
Influenza A (H3N2) low positive	30/30	36.4	1.8	30/30	35.2	2.3	30/30	38.0	4.0	90/90 (100%)	95.9% - 100%
Influenza A (H3N2) medium positive	30/30	30.2	0.8	30/30	29.5	0.5	30/30	30.8	1.0	90/90 (100%)	95.9% - 100%
Total Agreement All	539/576 (93.6%)			563/592 (95.1%)			572/592 (96.6%)			1674/1760 (95.1%)	94% - 96%

- 1) The 1st replicate of the NTC for Site 2, Day 4, Run 1, was detected in the Flu A channel with a Ct value of 39.5. This well appeared to have a valid amplification curve. The other two replicates were negative, and the data from this run are included in the analysis.
- 2) The 2nd replicate of the Positive Control for Site 1, Day 4, Run 1 was invalid. The other two replicates of this run were positive, and the data from this run are included in the analysis.
- 3) Well(s) had an invalid result, and is (are) not used in the estimation of Ct variability.
- 4) Sample results have been excluded from the analysis due to pipetting errors reported by the laboratory sites.

Table 9: Reproducibility – H1N1

Sample	Site 1			Site 2			Site 3			Total Agreement with Expected Results	95% CI
	Agreement with Expected Results	Avg. CT	% CV	Agreement with Expected Results	Avg. CT	% CV	Agreement with Expected Results	Avg. CT	% CV		
No Template Control	30/30	40.0	0.0	30/30 ¹	40.0	0.0	30/30	40.0	0.0	90/90 (100%)	95.9% - 100%
Positive Control	29/29 ²	29.2	2.6	30/30	28.5	0.9	30/30	29.0	0.7	89/89 (100%)	95.9% - 100%
Swabs											
2009 H1N1 Flu high negative	29/29 ³	40.0	0.0	28/30	39.9	0.7	25/30	39.6	2.2	82/89 (92.1%)	84.6% - 96.1%
2009 H1N1 Flu low positive	25/28 ³	34.8	6.1	28/29 ³	34.0	4.1	29/29 ³	33.9	1.6	82/86 (95.3%)	88.6% - 98.2%
2009 H1N1 Flu medium positive	27/27 ³	30.6	5.1	30/30	30.6	1.0	30/30	30.1	2.4	87/87 (100%)	95.8% - 100%
Influenza A (H1N1) high negative	30/30	40.0	0.0	29/30	39.9	1.1	30/30	40.0	0.0	89/90 (98.9%)	94% - 99.8%
Influenza A (H1N1) low positive	29/29 ³	40.0	0.0	30/30	40.0	0.0	30/30	40.0	0.0	89/89 (100%)	95.9% - 100%
Influenza A (H1N1) medium positive	30/30	40.0	0.0	30/30	40.0	0.0	30/30	40.0	0.0	90/90 (100%)	95.9% - 100%
Influenza A (H3N2) high negative	27/28 ³	40.0	0.7	29/30	39.9	0.8	30/30	40.0	0.0	86/88 (97.7%)	92.1% - 99.4%
Influenza A (H3N2) low positive	23/24 ³	40.0	0.2	29/30	40.0	0.2	29/30	40.0	0.1	81/84 (96.4%)	90% - 98.8%
Influenza A (H3N2) medium positive	30/30	40.0	0.0	27/27 ³	40.0	0.0	30/30	40.0	0.0	87/87 (100%)	95.8% - 100%
Aspirate											
2009 H1N1 Flu high negative	25/25 ³	40.0	0.0	29/29	40.0	0.0	29/29 ³	40.0	0.0	83/83 (100%)	95.6% - 100%
2009 H1N1 Flu low positive	24/30	35.2	6.4	29/30	33.7	3.7	28/28 ³	34.3	3.2	81/88 (92.0%)	84.5% - 96.1%
2009 H1N1 Flu medium positive	30/30	30.2	1.1	29/30	30.0	3.2	30/30	30.4	1.6	89/90 (98.9%)	94% - 99.8%
Influenza A (H1N1) high negative	28/28 ³	40.0	0.0	30/30	40.0	0.0	30/30	40.0	0.0	88/88 (100%)	95.8% - 100%
Influenza A (H1N1) low positive	30/30	40.0	0.0	30/30	40.0	0.0	29/29 ³	40.0	0.0	89/89 (100%)	95.9% - 100%
Influenza A (H1N1) medium positive	29/29 ³	40.0	0.0	30/30	40.0	0.0	28/28 ³	40.0	0.0	87/87 (100%)	95.8% - 100%
Influenza A (H3N2) high negative	30/30	40.0	0.0	27/27 ⁴	40.0	0.0	29/29 ³	40.0	0.0	86/86 (100%)	95.7% - 100%
Influenza A (H3N2) low positive	30/30	40.0	0.0	30/30	40.0	0.0	30/30	40.0	0.0	90/90 (100%)	95.9% - 100%
Influenza A (H3N2) medium positive	30/30	40.0	0.0	30/30	40.0	0.0	30/30	40.0	0.0	90/90 (100%)	95.9% - 100%
Total Agreement All	565/576 (98.1%)			584/592 (98.6%)			586/592 (99%)			1735/1760 (98.6%)	97.9% - 99%

1) The 1st replicate of the NTC for Site 2, Day 4, Run 1, was detected in the Flu A channel with a Ct value of 39.5. This well appeared to have a valid amplification curve. The other two replicates were negative, and the data from this run are included in the analysis.

2) The 2nd replicate of the Positive Control for Site 1, Day 4, Run 1 was invalid. The other two replicates of this run were positive, and the data from this run are included in the analysis.

3) Well(s) had an invalid result, and is (are) not used in the estimation of Ct variability.

4) Sample results have been excluded from the analysis due to pipetting errors reported by the laboratory sites.

ANALYTICAL REACTIVITY / CROSS REACTIVITY

Analytical Reactivity

In addition to the six strains tested for LoD, various dilutions (in swab matrix) of five additional influenza A strains were tested for reactivity with the Simplexa™ Influenza A H1N1 (2009) RT-PCR assay. Quantified virus was serially diluted and extracted three times using the MagNA Pure LC Total Nucleic Acid Isolation Kit. The lowest concentration at which all three replicates were positive was treated as the tentative LoD. Results of screening with the additional strains are presented in the table below.

Table 10: Analytical Reactivity with Additional Influenza A Strains

Influenza A Strain	Lowest Concentration Detected (TCID ₅₀ /mL)	Result
A/PR/8/34 H1N1	1x10 ⁰	Positive for FLU A
A/New Caledonia/20/99 H1N1	1x10 ⁰	Positive for FLU A
A/Taiwan/42/06 H1N1	1x10 ¹	Positive for FLU A
A/WS/33 H1N1	1x10 ⁰	Positive for FLU A
A/Hong Kong/8/68 H3N2	1x10 ⁰	Positive for FLU A
Tissue Culture Adapted Influenza A/Swine/Iowa/15/30	1x10 ¹	Positive for FLU A
Tissue Culture Adapted Influenza A/1976/31	1x10 ¹	Positive for FLU A
Influenza A/H5N1 (Asian Lineage) Positive Control	Unknown	Positive for FLU A

In addition to laboratory testing, bioinformatics resources and computer simulations were used to predict reactivity of additional influenza A strains. Comparison of the sequence of the bi-functional fluorescent probe-primer and the reverse primer for FLU A and sequences of the matrix gene of the influenza A strains identified in the table below shows that the bi-functional fluorescent probe-primer and reverse primers have a near perfect match to all subtypes, and the forward primer has a maximum of 3 mismatches, which would theoretically enable the assay to detect all subtypes listed.

Table 11: Simulated Analytical Reactivity (sequence matches) with Additional Influenza A Strains

Influenza A Strain	GenBank	Simulated Reactivity
A/California/VRDL72/2009 (H1N1)	CY055480.1	Positive for FLU A
A/swine/Italy/306907/2003 (H1N1)	FJ975097.1	Positive for FLU A
A/mallard/Korea/GH171/2007 (H7N7)	FJ959087.1	Positive for FLU A
A/swine/Sweden/1021/2009 (H1N2)	GQ495135.1	Positive for FLU A
A/Baden-Wuerttemberg/20/03 (H1N2)	EU249175.1	Positive for FLU A
A/Thailand/CU-B1697/2009 (H3N2)	GU271985.1	Positive for FLU A
A/ruddy turnstone/New Jersey/563/2006 (H7N2)	GQ257382	Positive for FLU A
A/swine/Hong Kong/NS857/2001 (H1N2)	GQ229350.1	Positive for FLU A

Cross Reactivity

The Simplexa™ assay's analytical specificity was evaluated by testing the ability to exclusively identify influenza A virus and/or H1N1 influenza virus with no cross-reactivity to organisms that are closely related, or cause similar clinical symptoms, or present as normal flora in the specimen types of interest.

The panel of thirty four (34) cross-reactants were individually spiked into a swab matrix at clinically relevant concentrations. The unspiked matrix was also tested to serve as a baseline. Samples were tested in triplicate to screen for cross reactivity. If signal was detected in either the FLU A channel or H1N1 (2009) channel in any of the three replicates, an additional 5 replicates were tested for confirmation. One extraction was used to make the original three replicates and the confirmatory five replicates.

No cross reactivity was detected for either Influenza A or H1N1 (2009), after confirmatory testing.

Table 12: Cross Reactivity

Cross-Reactant	Testing Concentration	Units	Flu A	H1N1
Adenovirus 1	1.02 x10 ⁵	TCID ₅₀ /mL	– ¹	–
Adenovirus 7A	4.57 x10 ⁵	TCID ₅₀ /mL	–	–
<i>Bordetella pertussis</i>	5.80 x10 ⁶	cfu/mL	–	–
<i>Chlamydia pneumoniae</i>	1.00 x10 ⁵	TCID ₅₀ /mL	–	–
Coronavirus 229E	1.00 x10 ⁵	TCID ₅₀ /mL	–	–
<i>Corynebacterium diphtheriae</i>	2.87 x10 ⁶	cfu/mL	–	–
Cytomegalovirus	1.04 x10 ⁵	TCID ₅₀ /mL	–	–
Enterovirus 71	1.00 x10 ⁵	TCID ₅₀ /mL	–	–
Epstein Barr Virus	6.04 x10 ⁵	copies/mL ²	–	–

Cross-Reactant	Testing Concentration	Units	Flu A	H1N1
<i>Escherichia coli</i> , O157H7	2.34 x10 ⁶	cfu/mL	–	–
<i>Haemophilus influenzae</i>	1.04 x10 ⁶	cfu/mL	–	–
Influenza B (B/Florida/04/2006)	1.26 x10 ⁵	TCID ₅₀ /mL	–	–
Influenza B (B/Malaysia/2506/04)	1.26 x10 ⁵	TCID ₅₀ /mL	–	–
<i>Lactobacillus plantarum</i> , 17-5	1.75 x10 ⁶	cfu/mL	–	–
<i>Legionella longbeachae</i>	7.10 x10 ⁶	cfu/mL	–	–
Measles	1.26 x10 ⁵	TCID ₅₀ /mL	–	–
Metapneumovirus	1.04 x10 ⁵	TCID ₅₀ /mL	–	–
<i>Moraxella catarrhalis</i> , Ne 11	6.83 x10 ⁶	cfu/mL	–	–
Mumps	1.51 x10 ⁵	TCID ₅₀ /mL	–	–
<i>Mycobacterium tuberculosis</i>	2.20 x10 ⁶	cfu/mL	–	–
<i>Mycoplasma pneumoniae</i> , Strain M129	1.13 x10 ⁵	TCID ₅₀ /mL	–	–
<i>Neisseria elongata</i>	1.99 x10 ⁶	cfu/mL	–	–
<i>Neisseria meningitidis</i>	1.63 x10 ⁶	cfu/mL	–	–
Parainfluenza 1	1.32 x10 ⁵	TCID ₅₀ /mL	–	–
Parainfluenza 2	1.18 x10 ⁶	TCID ₅₀ /mL	–	–
Parainfluenza 3	1.32 x10 ⁵	TCID ₅₀ /mL	–	–
<i>Pseudomonas aeruginosa</i>	1.05 x10 ⁶	cfu/mL	–	–
RSV-B	1.51 x10 ⁵	TCID ₅₀ /mL	–	–
Rhinovirus 16	1.00 x10 ⁵	TCID ₅₀ /mL	–	–
<i>Staphylococcus aureus</i> , COL	1.68 x10 ⁶	cfu/mL	–	–
<i>Staphylococcus epidermidis</i>	3.80 x10 ⁶	cfu/mL	–	–
<i>Streptococcus pneumoniae</i>	5.54 x10 ⁶	cfu/mL	–	–
<i>Streptococcus pyogenes</i>	1.55 x10 ⁶	cfu/mL	–	–
<i>Streptococcus salivarius</i>	1.14 x10 ⁶	cfu/mL	–	–

1) One of the original triplicates for adenovirus was detected in the Influenza A channel. The baseline matrix that this sample was spiked into also gave a positive signal in the same run, indicating possible contamination. Five additional replicates from the same extraction were tested, offering 2 detected, one indeterminate, and 2 not detected results. In order to evaluate the possibility of contamination of the sample during extraction versus true reactivity of adenovirus 1, the cross reactant was spiked into fresh matrix and a new extraction performed. Both adenovirus 1 and the baseline matrix were not detected in all replicates.

2) The EBV virus is grown in a transformed cell line (marmoset leukocytes). Transformed cells are not an appropriate cell line for quantitation using TCID₅₀/mL, instead, copies/mL is calculated using a quantitative PCR method.

In addition to laboratory testing, bioinformatics resources and computer simulations were used to predict cross reactivity of additional influenza A strains with the 2009 H1N1 target. Comparison of the sequence of the bi-functional fluorescent probe-primer and the reverse primer for H1N1 shows that bi-functional fluorescent probe-primer and reverse primers designed to detect the 2009 H1N1 influenza strain have a significant number of mismatches when compared to the sequences of all other listed subtypes. The 2009 H1N1 influenza strain is unique, and primers directed to the H1N1 sequence would not detect the other subtypes listed in the table below.

Table 13: Simulated Analytical Cross Reactivity (sequence matches) with Additional Influenza A Strains

Influenza A Strain	GenBank	Simulated Cross Reactivity
A/California/VRDL72/2009 (H1N1)	CY055479.1	Positive for H1N1 (2009)
A/New York/417/2002(H1N2)	CY003769.1	Negative for 2009 H1N1 influenza
A/swine/Italy/30073/2006 H1N2	FJ770267.1	Negative for 2009 H1N1 influenza
A/chicken/New A/chicken/New York H7N2	CY035946.1	Negative for 2009 H1N1 influenza
A/mallard/Geumgang/1/2007 H7N7	FJ767719.1	Negative for 2009 H1N1 influenza

The performance of this test has not been established with potentially interfering medications for the treatment of influenza or cold virus. The medications listed below were reported by the study subjects at the time of specimen collection. There was no evidence that the medications listed below interfered with the Simplexa assay.

Table 14: Medications Reported by Study Subjects.

Afrin (nasal spray)	Augmentin	Codral	Ketoprofen	Nystatin	Penicillin	Stemetil
Albuterol	Azithromycin	Codril	Keflex	Omnicef	Pepto Bismol	Triaminic
Amoxicillin	Bactrim	Cortef	Neoral	Oroxine	Prevacid	Vicodin
Amoxil	Bisacodyl	Demazin	Nexium	Panadol	Robitussin	Voltaren
Antihistamine	Buitroban	Diastat	Nuprin	Paracetamol	Rondec	
Atuss Hs	Carba - XP	Ipratropium	NyQuil	Acetaminophen	Sigmacort	

METHOD COMPARISON

Specimens were prospectively collected at three sites from patients with signs and symptoms of influenza like illness (Austin, TX (September 2009) and the New South Wales region of Australia (July – September 2009). Specimens were blinded and randomly distributed to 3 U.S. clinical laboratories for testing. Specimens were determined to be positive for 2009 H1N1 influenza by a composite reference method for the Flu A target including the Luminex xTAG RVP Flu A target, a validated PCR assay using primer and probe sequences published by the CDC and a well characterized PCR followed by sequencing. The sequencing data were used to determine the 2009 H1N1 subtype. Two results were generated for each specimen, an influenza A result and a 2009 H1N1 influenza sub-typing result. Both results must be positive to determine that a specimen is 2009 H1N1 influenza positive.

299 prospectively collected nasal/nasopharyngeal swabs and 112 nasopharyngeal aspirates were analyzed using the Simplexa™ Influenza A H1N1 (2009) assay. The data presented below are stratified by both result and specimen type. Fifteen (15) specimens (10 swabs and 5 aspirates) were excluded from the analysis because there was no consensus among the reference assays for the influenza A result. Twelve (12) specimens (9 swabs and 3 aspirates) were excluded from the 2009 H1N1 Influenza Clinical Agreement Summary tables as sequencing data used to determine subtype was not available. These 12 specimens are included in the Influenza A Clinical Agreement Summary tables.

Table 14: 2009 H1N1 Influenza Clinical Agreement Summary
Simplexa™ Influenza A H1N1 (2009) vs. Composite Reference Result – Prospectively Collected Swabs¹

		H1N1 Result - Simplexa™ Influenza A H1N1 (2009)			
Composite Reference Result		n	H1N1 Detected	H1N1 Not Detected	% Agreement
	H1N1 Detected	101	101	0	% Positive Agreement 100%(101/101) 95% CI:96.3-100%
	H1N1 Not Detected	179	8	171	% Negative Agreement 95.5%(171/179) 95% CI:91.4-97.7%

1) Ten (10) samples were excluded from the analysis because there was no consensus among the influenza A reference assays. Nine (9) samples were excluded because sequencing results to determine sub-types were not available.

Table 15: 2009 H1N1 Influenza Clinical Agreement Summary
Simplexa™ Influenza A H1N1 (2009) vs. Composite Reference Result – Prospectively Collected Aspirates¹

		H1N1 Result - Simplexa™ Influenza A H1N1 (2009)			
Composite Reference Result		n	H1N1 Detected	H1N1 Not Detected	% Agreement
	H1N1 Detected	24	24	0	% Positive Agreement 100%(24/24) 95% CI:86.2-100%
	H1N1 Not Detected	80	6	74	% Negative Agreement 92.5%(74/80) 95% CI:84.6-96.5%

1) Five (5) samples were excluded from the analysis because there was no consensus among the influenza A reference assays. Three (3) samples were excluded because sequencing results to determine sub-types were not available.

Table 16: Influenza A Clinical Agreement Summary
Simplexa™ Influenza A H1N1 (2009) vs. Composite Reference Result – Prospectively Collected Swabs¹

Influenza A Result - Simplexa™ Influenza A H1N1 (2009)					
Composite Reference Result		n	Influenza A Detected	Influenza A Not Detected	% Agreement
	Influenza A Detected	116	116	0	% Positive Agreement 100%(116/116) 95% CI:96.8-100%
	Influenza A Not Detected	173	13	160	% Negative Agreement 92.5%(160/173) 95% CI:87.6-95.6%

1) Due to the low prevalence of other strains of influenza A during the testing period, all FLU A responses from prospectively collected swabs were combined to demonstrate the performance of the FLU A bi-functional fluorescent primer-probe. Of the 116 specimens determined to be positive for FLU A: 101 were 2009 H1N1 influenza positive, zero (0) were H1N1, four (4) were H3N2, two (2) were not detected by the alternate PCR and could not be sequenced, and nine (9) were not sub-typed. Ten (10) samples were excluded from the analysis because there was no consensus among the influenza A reference assays.

Table 17: Influenza A Clinical Agreement Summary
Simplexa™ Influenza A H1N1 (2009) vs. Composite Reference Result – Prospectively Collected Aspirates¹

Influenza A Result - Simplexa™ Influenza A H1N1 (2009)					
Composite Reference Result		n	Influenza A Detected	Influenza A Not Detected	% Agreement
	Influenza A Detected	31	31	0	% Positive Agreement 100%(31/31) 95% CI:89-100%
	Influenza A Not Detected	76	3	73	% Negative Agreement 96.1%(73/76) 95% CI:89-98.6%

1) Due to the low prevalence of other strains of influenza A during the testing period; all FLU A responses from prospectively collected aspirates were combined to demonstrate the performance of the FLU A bi-functional fluorescent primer-probe. Of the 31 specimens determined to be positive for FLU A, 24 were 2009 H1N1 influenza positive, one (1) was sequenced but the sub-type could not be determined, three (3) were not detected by the alternate PCR and could not be sequenced, three (3) did not have sufficient volume to sequence to determine sub-type. Five (5) samples were excluded from the analysis because there was no consensus of the influenza A reference assays.

An additional 214 retrospectively collected nasal/nasopharyngeal swabs and 2 nasal washes from the Focus Sample Bank were also tested at 3 sites. 3 swab specimens were excluded from the analysis because there was no consensus among the reference assay results. 1 swab specimen was excluded from the 2009 H1N1 Influenza Clinical Agreement Summary tables as sequencing data used to determine subtype was not available. This specimen is included in the Influenza A Clinical Agreement Summary tables.

Table 18: 2009 H1N1 Influenza Clinical Agreement Summary
Simplexa™ Influenza A H1N1 (2009) vs. Composite Reference Result – Retrospectively Collected Swabs¹

H1N1 Result - Simplexa™ Influenza A H1N1 (2009)						
Composite Reference Result		n	H1N1 Detected	H1N1 Not Detected	Indeterminate	% Agreement
	H1N1 Detected	57	57	0	0	% Positive Agreement 100%(57/57) 95% CI:93.7-100%
	H1N1 Not Detected	153	13	139	1	% Negative Agreement 90.8%(139/153) 95% CI:85.2-94.5%

1) Three (3) samples were excluded from the analysis because there was no consensus of the influenza A reference assays. One (1) sample was excluded from the analysis because sequencing results there was insufficient sample to perform sequencing.

Two retrospectively collected washes were found to be positive for 2009 H1N1 influenza by the composite reference method and by the Simplexa assay.

Table 19: Influenza A Clinical Agreement Summary
Simplexa™ Influenza A H1N1 (2009) vs. Composite Reference Result – Retrospectively Collected Swabs¹

		Influenza A Result - Simplexa™ Influenza A H1N1 (2009)				
Composite Reference Result		n	Influenza A Detected	Influenza A Not Detected	Indeterminate	% Agreement
	Influenza A Detected	132	131	0	1	% Positive Agreement 99.2%(131/132) 95% CI:95.8-99.9%
	Influenza A Not Detected	79	13	66	0	% Negative Agreement 83.5%(66/79) 95% CI:73.9-90.1%

1) Due to the low prevalence of other strains of influenza A during the testing period; all FLU A responses from retrospectively collected samples were combined to demonstrate the performance of the FLU A bi-functional fluorescent primer-probe. Of the 132 specimens determined to be positive for FLU A, 57 were 2009 H1N1 influenza positive, two (2) were H1N1, 59 were H3N2, one (1) was sequenced but the sub-type could not be determined, one (1) was indeterminate by Simplexa, 11 were not detected by the alternate PCR and could not be sequenced, and one (1) did not have sufficient volume to sequence to determine sub-type. Three (3) samples were excluded from the analysis because there was no consensus of the influenza A reference assays.

Two retrospectively collected washes were found to be positive for influenza A by the composite reference method and by the Simplexa assay.

EXTRACTION EQUIVALENCY

Extraction equivalency of the Roche MagNA Pure LC and Qiagen QIAamp® Viral RNA Mini Kit was evaluated by performing both a limit of detection study and a method comparison study. Limit of detection study results are presented in the Analytical Sensitivity/Limit of Detection section. For the method comparison study, 139 clinical samples were extracted by the Roche MagNA Pure LC and the Qiagen QIAamp® Viral RNA Mini Kit and assayed using the Simplexa™ Influenza A H1N1 (2009) assay. Results from each extraction method were compared and are presented in the tables below.

Table 20: Extraction Method Concordance for 2009 H1N1 influenza using Simplexa™ Influenza A H1N1 (2009) Assay.

		Roche MagNA Pure LC Extraction Method			
Qiagen QIAamp®Viral RNA Mini Kit Extraction Method		2009 H1N1 Positive	2009 H1N1 Negative	Total	% Positive Agreement 100.0% (62/62) 95% CI: 94.2- 100.0
	2009 H1N1 Positive	62	1*	63	
	2009 H1N1 Negative	0	76	76	% Negative Agreement 98.7% (76/77) 95% CI: 93.0- 99.8
	Total	62	77	139	

*All three targets were detected for one specimen with QIAamp extraction, whereas only FLU A and IC targets, but not H1N1 target, were detected with MagNA Pure extraction. Upon re-extraction of frozen clinical specimen, Simplexa™ Influenza A H1N1 (2009) assay detected the specimen as positive for H1N1 with both extraction methods.

Table 21: Extraction Method Concordance for influenza A using Simplexa™ Influenza A H1N1 (2009) Assay.

		Roche MagNA Pure LC Extraction Method			
Qiagen QIAamp®Viral RNA Mini Kit Extraction Method		Influenza A Positive	Influenza A Negative	Total	% Positive Agreement 100% (96/96) 95% CI: 96.2-100
	Influenza A Positive	96	2**	98	
	Influenza A Negative	0	41	41	% Negative Agreement 95.3% (41/43) 95% CI: 84.5-98.7
	Total	96	43	139	

**FLU A and AR IC targets were detected for two specimens with QIAamp extraction, whereas only AR IC was detected for both specimens with MagNA Pure extraction. Upon re-extraction of frozen clinical specimens, Simplexa™ assay did not detect the FLU A target with either extraction method.

CARRY-OVER CONTAMINATION

The amplification carry-over study is designed to test for the presence of contamination in high negative samples caused by high positive samples in the run. The study was designed by alternately placing high negative and high positive (7.5×10^5 TCID₅₀/mL) samples on each disc. A total of 180 high negative samples were tested across five runs.

The carryover effect was evaluated by comparing the observed negative rate for the high negative sample with the expected rate under normal reproducibility conditions (i.e. 95%, the % negative rate outside of contamination). It is assumed that a certain

amount of high negative samples results will be positive; hence the purpose of analysis is to determine if the positive rate is elevated beyond what is probable. A one sample, one sided test for a proportion (binomial) was used to test for significance for both the FLU A and H1N1 channels.

The high negative sample was created immediately prior to this study; therefore, a baseline run characterized the sample's reproducibility. The sample was negative approximately 95% of the time for both FLU A and H1N1 channels at baseline (6.7% positive for FLU A, 4.4% positive for H1N1, across 90 replicates). No significant carry-over contamination effect was seen in either channel. Results are summarized in the table below.

Table 22: Carry Over Contamination

Results Summary	FLU A	H1N1 ¹
Positive Count	12	13
Negative Count	168	167
Total observations ²	180	180
Percent positive	6.7%	7.2%
One-sided binomial test (p-value)	0.19	0.12

1) Only two (2) of the H1N1 results were actual H1N1 positives per the PI, as the FLU A channel was negative for 11 of detected H1N1 samples. These results were still included in the statistical testing for the purpose of detecting contamination of that channel.

2) One (1) of the 180 replicates of high negative came up as invalid due to a "Not Detected" ARIC result. With this replicate excluded, the one-sided binomial test still offered a non-significant result for both FLU A and H1N1 (p-value of 0.19 and 0.12, respectively).

EXPECTED VALUES

The prevalence of influenza varies each year with flu-season occurring during the fall and winter months in the US. Variables that affect the rate of positivity observed in respiratory testing include: the efficiency and timing of specimen collection, handling and transport of the specimen, the time of year, age of the patient, and local disease prevalence. Prospective specimens used in our clinical study were obtained from the Texas region of the United States and the New South Wales region of Australia. The prevalence of influenza A in Texas (Region VI) ranged from 25.6 – 29.4% during the September 2009 collection period; 99% of those cases were 2009 H1N1 influenza⁷. In New South Wales, the prevalence during the July to September collection period ranged from 20-42%, with 83-92% of these cases representing 2009 H1N1 influenza⁸.

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