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 As of June 23, 2010, the EUAs have been terminated and this information is no longer current.

ELITech Molecular Diagnostics 2009-H1N1 Influenza A Virus Real-Time RT-PCR

A real-time reverse-transcription polymerase chain reaction (RT-PCR) assay intended for the *in vitro* qualitative detection of 2009-H1N1 Influenza A viral RNA.

Emergency Use Authorization
For *in vitro* Diagnostic Use

Intended Use

The ELITech Molecular Diagnostics 2009-H1N1 Influenza A virus Real-Time RT-PCR assay is intended for use in ARUP Laboratories for the in vitro qualitative detection of 2009-H1N1 Influenza A viral RNA in upper respiratory tract specimens (such as nasopharyngeal swabs (NPS), nasal swabs (NS), throat swabs (TS), nasal aspirates (NA), nasal washes (NW), and dual nasopharyngeal / throat swabs (NPS/TS)), and lower respiratory tract specimens (such as broncheoalveolar lavage (BAL), bronchial aspirate (BA), bronchial wash (BW), endotracheal aspirate (EA), endotracheal wash (EW), tracheal aspirate (TA), and lung tissue) from human patients with signs and symptoms of respiratory infection in conjunction with clinical and epidemiological risk factors.

The testing format includes nucleic acid extraction using the QIAamp Virus BioRobot 9604 Kit on the Qiagen BioRobot 9604 instrument followed by real-time reverse-transcription PCR utilizing the Applied Biosystems 7900HT Real-Time PCR System. Amplification and detection are accomplished using PCR primers and Pleiades hybridization probes manufactured by Epoch BioSciences, a Division of Wescor, Inc.

Testing with the ELITech Molecular Diagnostics 2009-H1N1 Influenza A virus Real-Time RT-PCR assay should only be performed on human patients who meet the clinical and epidemiologic criteria for testing suspect specimens.

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

Principle

Influenza A viruses are members of the orthomyxoviridae family and are small enveloped particles containing a negative strand RNA genome divided into 8 segments. Classification of Influenza A is done based on the hemagglutinin and neuraminidase antigens present on the viral envelope. Current circulating strains of seasonal Influenza A include the H3N2 and H1N1 viruses. The 2009-H1N1 Influenza A virus (Novel H1N1) also referred to as Pandemic (H1N1) 2009 virus and initially as “swine-flu”, is a newly circulating influenza virus that was first identified in March 2009. This virus is a product of gene recombination from a variety of host animals which gave rise to a subtype not previously observed in human populations. The lack of immunity in the general population allowed the 2009-H1N1 virus to quickly spread and achieve worldwide pandemic status in June 2009.

Infection with the 2009-H1N1 Influenza A virus produces symptoms similar to seasonal influenza ranging from mild respiratory problems such as fever, cough, sore throat, nasal congestion, headache, chills and fatigue to more serious complications that can include death. Detection of the 2009-H1N1 Influenza A virus can be done using viral culture, DFA or RT-PCR assays, but only RT-PCR assays specific for the hemagglutinin gene in this virus can distinguish it from seasonal strains of influenza and are the recommended method of detection and confirmation.

This ELITech Molecular Diagnostics 2009-H1N1 Influenza A virus (Novel H1N1) test is a multiplex real-time reverse-transcription PCR test that amplifies and detects two regions within the genome of the 2009-H1N1 Influenza A virus in two separate reactions. The first reaction detects a region present within the hemagglutinin gene and the second reaction detects a conserved region within the Matrix Protein 1 gene. Nucleic acids are extracted from upper respiratory tract specimens (such as nasopharyngeal swabs (NPS), nasal swabs (NS), throat swabs (TS), nasal aspirates (NA), nasal washes (NW), and dual nasopharyngeal / throat swabs (NPS/TS)), and lower respiratory tract specimens (such as broncheoalveolar lavage (BAL), bronchial aspirate (BA), bronchial wash (BW), endotracheal aspirate (EA), endotracheal wash (EW), tracheal aspirate (TA), and lung tissue) in combination with a non-competitive RNA internal control, consisting of MS2 bacteriophage added during the extraction procedure. Following RNA extraction, a one-step reverse transcription and real-time PCR amplification and detection using primers and probes for the 2009-H1N1 virus and internal control are performed on the Applied Biosystems 7900HT. The 2009H1 primers and Pleiades hybridization probe were designed

to be specific for the 2009-H1N1 Influenza A virus and do not amplify or detect seasonal H3N2 or seasonal H1N1 viruses. The M1 primers and probe detect the Matrix Protein 1 gene and recognize both the 2009-H1N1 Influenza A virus and seasonal Influenza A viruses. The inclusion of an RNA internal control ensures correct nucleic acid extraction and monitors the reverse transcription and PCR processes for inhibition. .

Materials Provided

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

Table 1: Description of the Kit Labeling and Kit Components

ELITech Molecular Diagnostics 2009-H1N1 Influenza A Real-Time RT-PCR (Part # M500517)	Reactions per Vial	Minimum Volume (µL) per Vial	Number in Kit	Color Code	PN	Expires
20X 2009 H1/MS2 Probe/Primer Mix	96	240 µL	1	Clear	M500518	Three years, or end of the EUA
2009 H1 Positive Control RNA	16	200 µL	1	Green	M500514	One year, or end of the EUA
Bacteriophage MS2 Internal Control RNA	96	265 µL	4	Purple	M500516	One year, or end of the EUA
AP525 Dye Calibrator	N/A	35 µL	1	Orange	M100121	Three years, or end of the EUA
20X M1 Real Time RT-PCR Probe/Primer Mix	96	240 µL	1	Blue	800284	Three years, or end of the EUA
M1 Positive Control RNA		250 µL	1			Manufactured at ARUP Laboratories

Table 2: Description of the Kit Components

Kit Component	Description
20X 2009H1/MS2 Probe/Primer Mix	**Primers and probe targeting the 2009-H1N1 Influenza A H1N1 hemagglutinin gene. Detection is in FAM channel of instrument*.** **Primers and probe targeting the Bacteriophage MS2 A-Protein gene. Detection is in AP525 Channel of instrument.**
2009H1 Positive Control RNA	**Stabilized solution of RNA from 2009-H1N1 Influenza A virus at 2×10^6 copies/tube.**

Bacteriophage MS2 Internal Control RNA	**Stabilized solution of bacteriophage MS2 RNA (whole genome) at 2.7×10^5 PFU/tube**
AP525 Dye Calibrator	**50µM Oligo dT(10) with AP525 dye**
20X M1 Real Time RT PCR Probe/Primer Mix	**Primers and probe targeting the Matrix Protein 1 gene. Detection is in FAM channel of instrument.** **Primers and probe targeting the Bacteriophage MS2 Coat-Protein gene. Detection is in AP525 Channel of instrument.**
M1 Positive Control RNA	**Intact Influenza A (H3N2) virus at 10,000 copies/mL**

*** Applied Biosystems 7900 (ABI 7900HT). NOTE: <u>The ABI 7900HT calibration with the AP525 dye must be carried out before the first experiment</u>**

MATERIALS REQUIRED BUT NOT SUPPLIED

1 Real-Time PCR System: Applied Biosystems 7900HT Real-Time PCR System with SDS software version 2.2.2 or 2.3.
2 Qiagen BioRobot 9604 and associated consumables
3 Qiagen QuantiTect Probe RT-PCR Master mix (Qiagen Cat. No 204443)
4 QIAamp Virus BioRobot 9604 Kit (Qiagen Cat. No 965662)
5 RNase Inhibitor (Applied Biosystems Cat. No N8080119)
6 Heat-labile Uracil N-Glycosylase (Roche Cat No 11775367001)
7 MasterAmp 10X PCR Enhancer (Epicentre Cat No ME81210)
8 Single or multi-channel micropipette(s) with an accuracy range between 1-10 µL, 10-100 µL and 100-1000 µL. NOTE: Dedicated micropipettes are required for extraction, as well as for the Pre-Amplification Areas I, II and III
9 Freezer (manual defrost) at -10 to -30°C (for kit component frozen storage)
10 Freezer (manual defrost) at -10 to -30°C (for specimen frozen storage)
11 Refrigerator at 2 to 8°C
12 Laminar flow hood for extractions
13 Bench top centrifuge for low speed centrifugation of 96-well plates or other reaction vessels
14 Microcentrifuge
15 Vortex mixer
16 Sterile, RNase/DNase-free disposable aerosol-barrier micropipettor tips
17 Sterile Nuclease-free water
18 RNase/DNase-free 1.5 mL polypropylene microcentrifuge tubes and racks
19 RNase/DNase-free 96 well thermocycler plate rack or appropriate tube rack
20 96-well optical reaction plate or other reaction vessels
21 Optical adhesive cover (for use with 96-well optical reaction plate)
22 Disposable, powder-free gloves
23 Cooler racks for 1.5 mL microcentrifuge tubes and 96-well 0.2 mL PCR reaction tubes or plates

INSTRUCTIONS FOR USE

A. SPECIMEN COLLECTION

Acceptable specimen types include upper respiratory tract specimens (such as nasopharyngeal swabs (NPS), nasal swabs (NS), throat swabs (TS), nasal aspirates (NA), nasal washes (NW), and dual nasopharyngeal / throat swabs (NPS/TS)), and lower respiratory tract specimens (such as broncheoalveolar lavage (BAL), bronchial aspirate (BA), bronchial wash (BW), endotracheal aspirate (EA), endotracheal wash (EW), tracheal aspirate (TA), and lung tissue) in viral transport media tube or equivalent (UTM), or Multi Microbe Media (M4). If using swabs, use only sterile Dacron, nylon, or rayon swabs with plastic shafts. Do not use calcium alginate swabs, as they may contain substances that inhibit PCR testing.

B. SPECIMEN EXTRACTION AREA (Extraction Area)

Dedicated area for specimen extraction and preparation of the positive control. Nucleic acids are extracted from patient specimens and assay controls with the automated Qiagen BioRobot 9604 instrument and the QIAamp Virus Biorobot 9604 Kit. Refer to the manufacturer's handbook for additional information about the QIAamp Virus Biorobot 9604 Kit. Nucleic acid extraction was according to the following protocol:

REAGENT PREPARATION/CONTROLS:

1. **Qiagen Protease Stock solution**: Add 10.2 ml of protease solvent to each bottle of lyophilized Qiagen protease. Once prepared, store at 4°C.
2. **Buffer AW 1**: This buffer is supplied as a concentrate. Before using the first time, add 230 ml of 96-100% ethanol to the bottle containing AW1 concentrate. Mix by shaking before use. Store at room temperature. Once prepared, reagent is stable for one year.
3. **Buffer AW2**: This buffer is supplied as a concentrate. Before using the first time, add 640 ml of 96-100% ethanol to the bottle containing AW2 concentrate. Mix by shaking before use. Store at room temperature. Once prepared, reagent is stable for one year.
4. **Carrier RNA**: Add 800 µl buffer AVE to 1350 µg of Carrier RNA (1 vial). Once prepared, store at 4°C for 1 week.
5. **Bacteriophage MS2 Internal Control, RNA**: The MS2 RNA IC is at a concentration of 1,000 copies/µL. Prior to extraction, the IC is added to the AL lysis buffer according to Table 3.
6. **2009H1 Positive Control RNA Template:** The H1N1 2009 Positive Control RNA template is provided at a concentration of 10,000 copies/µL and is stablized in a solution of a sodium citrate guanidine thiocyanate (CEP). Make 20 µL single-use aliquots and store at -70°C. Prior to extraction, add 200 µL of molecular grade water to one single-use aliquot and extract all 220 µL. (NOTE: The positive control will not amplify without extraction due to the CEP solution and must be extracted). A Ct value in the specified range for the 2009H1 Positive Control verifies that the test materials, the extraction and the RT-PCR process are working. See interpretation of results.
7. **M1 Positive Control**: The M1 assay positive control is an Influenza A (H3N2) positive cell isolate manufactured at ARUP Laboratories. This positive isolate consists of whole intact virus that is diluted to an approximate concentration of 10,000 copies/mL prior to storage in 250 µL single-use aliquots at -70°C. For each run, 220 µL is extracted. A Ct value in the specified range for the M1 Positive Control verifies that the test materials, the extraction and the RT-PCR process are working. See interpretation of results. Both the 2009H1 and M1 Positive Controls are to be extracted and used with each run.
8. **Internal Control**: The Bacteriophage MS2 Internal Control consists of whole MS2 RNA that has been stabilized in a sodium citrate guanidine thiocyanate solution (CEP). This control is at a concentration of approximately $1x10^3$ copies/µL. This internal control (IC) is added to the lysis

buffer prior to sample extraction and is co-extracted with each sample. (NOTE: The internal control will not amplify without extraction due to the CEP solution and must be extracted). A positive IC result indicates that the test materials, the extraction and the RT-PCR process are working and that the sample does not contain inhibitors. See interpretation of results.

9. **Negative Control**: The Negative Control consists of 220 µL of molecular grade water that goes through the extraction and the RT-PCR process to verify that the test material, extraction and the RT-PCR process are free of contamination. The Bacteriophage Internal Control is added to the Negative Control prior to the nucleic acid extraction process. See interpretation of results.

EXTRACTION PROCEDURE

Brief overview of parameters for Viral RNA protocol after the sample block has been loaded with protease, water, controls, and patient samples in Lysate S-block and placed on the thermostat block:

- Flush all probes using 20 ml x 5
- Flush x 5

Dispense 240 µl lysis buffer AL

- Distribute 240 µl of lysis buffer to samples on thermostat block
- Incubate 10 minutes @ 60°C
- Flush probes x5
- Distribute 275 µl of ethanol to S-block on MP-slot 3.
- Transfer 450 µl lysate to S-block MP-slot 3.
- Time set for 5:00 minutes.

Transfer of lysate

- Flush all probes using 20 ml x5
- Transfer 700 µl from samples in MP-slot 3 to filter plate in vacuum manifold
- Vacuum pump runs for 5:00 minutes

First wash step

- Distribute 360 µl of AWI to samples on vacuum manifold top
- Vacuum pump runs for 3:00 minutes
- Flush all probes using 10 ml x5

Second wash step

- Distribute 1,000 µl of AW2 to samples on the vacuum manifold top
- Vacuum pump runs for 2:00 minutes

Third wash step

- Distribute 1,000 µl of AW2 to samples on the vacuum manifold top
- Vacuum pump runs for 2:00 minutes

Elution

- Distribute 86 µl of buffer AVE to samples on the MP slot
- Seal Plate with Airpore tape
- Time set for 00:01:00

Wash procedure

- Distribute 10,000 µl of DI water through ethanol lines to wash position
- Distribute 10,000 µl of DI water through AW1 line to wash position
- Distribute 10,000 µl of DI water through AW2 line to wash position

Step-by-step extraction parameters for the Viral RNA Protocol on the Qiagen BioRobot 9604 system:

1. Allow samples and controls to come to room temperature.
2. Mark wells on the Lysate S-block that are being used to exclude them from future use of the square well block. Cover any unused columns with clear tape.
3. Biorobot system must be turned on in the following order: Biorobot; be sure to hold probes in the up position when instrument is turned on, otherwise probe will collide with the washing station.
4. Next turn on Peristaltic pump, turn on 96 well thermostat and finally turn on computer system. AW1 and AW2 buffers should be prepared prior to starting Biorobot protocol. See instructions above under Reagent Preparation.
5. When computer is turned on, press "enter" at password prompt. Click on Qiasoft icon.
6. Select the "**Viral RNA Protocol**" from the protocol drop-down menu. The "**Viral RNA Protocol**" is located under the customized applications heading in the drop down menu.
7. Click on Run icon. The "Run Protocol: Slot Configuration" dialog box appears.
8. Click **OK**. The "Run Protocol: No. of Samples" dialog box appears.
9. Enter the number of samples to be processed (Note: samples can be extracted in multiples of 4). Be sure to enter the appropriate staring location where the samples will be extracted on the plate (e.g. Wells A4-H6). The default column will start with row A1 and calculate the number of rows automatically.

> **Info Message**
> "Do you really want to process a plate?"

10. Click **Yes**.
11. The first enter variable value appears. Enter name of report for results, using date of run and time (A, N, P) (.txt) Example: mm/dd/yyA.txt

> 12. **Enter Variable Value**
> 13. Enter name of report for results
> (.txt)

14. Click **Continue**. The second Enter Variable Value box appears.

> **Enter Variable Value**
> Operator's name:

15. Enter a name or identification code for the operator as required.

    The next message box appears.

> **Protocol Message**
> Fill system liquid container with distilled water.
> Empty waste container.
> Empty vacuum trap. Empty tip waste bag, switch on 96- well thermostat system.

16. Click **Continue**. The next protocol message appears.

> **Protocol Message**
> Place four, 2.0 mL microcentrifuge tubes w/screw caps containing 2 ml buffer AVE into positions A5-A8 of the thermoblock.
> Make sure that the vials are topped off so that the liquid level is to the very top of each vial.

17. Click **Continue**. The next protocol message appears

Protocol Message
Connect buffer bottles. Make sure that Buffer AW1 is connected to the, green adapter; Buffer AW2, to the red adapter; and ethanol to the yellow adapter, respectively.

18. Click **Continue**. A protocol message box appears.

Protocol Message
Freshly dissolve 1 tube (1,350 µg) Carrier RNA with 800 µl AVE. Transfer ___µl of reconstituted Carrier RNA to a tube containing ___µl Buffer AL.

19. Click **Continue**. A protocol message box appears.

Protocol Message
Add the appropriate amount of MS2 RNA internal control (IC) and Carrier RNA to the AL buffer based on the number of rows and AL buffer needed. See table 3 below for pre-calculated amounts.

Table 3: AL, Carrier RNA, and MS2 RNA IC Reagent Volumes

# of Samples	# of Rows	Vol. of AL	Vol. of Carrier RNA	Vol. of MS2 RNA IC[1]
8	1	4 mL	67 µL	21.5 µL
16	2	6 mL	100 µL	32.5 µL
24	3	8 mL	133 µL	43 µL
32	4	10 mL	167 µL	54 µL
40	5	12 mL	200 µL	65 µL
48	6	14 mL	233 µL	75 µL
56	7	16 mL	267 µL	86 µL
64	8	18 mL	300 µL	97 µL
72	9	20 mL	333 µL	108 µL
80	10	22 mL	367 µL	118 µL
88	11	24 mL	400 µL	129 µL
96	12	26 mL	433 µL	140 µL

1. Stock concentration of lyophilized MS2 RNA IC is 1,000 copies/µL.

20. Click **Continue**. A protocol message box appears.

Protocol Message
Gently mix AL buffer, avoiding foaming. Place 4-15 ml tubes each containing ___µl Buffer AL-carrier into reagent slots B1-B4 with lids on.

21. Click **Continue**. A protocol message box appears.

Protocol Message
Place MP-slot extension adapter (metal piece) into MP-slot-3.

22. Click **Continue**. A protocol message box appears.

Protocol Message
Place an S-block onto the MP-slot extension adapter. Any unused wells should be taped off. Make sure the wells used correspond to the same wells that will be used on the 96-well plate. Label new block "ETOH + Lysate" and the date opened. These blocks are only to be used twice (do not use same wells) and then thrown away, regardless of the number of samples processed.

23. Click **Continue**. A protocol message box appears.

Protocol Message
Place a QIAamp 96 plate into top plate of the vacuum manifold (base plate equipped with channeling block). Tape unused wells with adhesive tape and leave them sealed throughout the procedure.

24. Click **Continue.** A protocol message box appears.

Protocol Message
Place sufficient tips into the tip racks. A run of 24 samples requires 1 full rack of tips. Check the tip racks are oriented and seated correctly in the holders. (Keep container for storage of tips after run). Rack slot #1 is the front left corner of the tip racks.

25. Click **Continue**. A protocol message box appears.

Protocol Message
Get out a 2nd S-block for processing the samples. Tape off any unused /used wells. This block is to be used for 2 runs only, regardless of the number of wells processed. (Never use the same wells!) Label this block "Lysate" and with the date opened. Mark the columns that will be used for this run.

26. Click **Continue**. A protocol message box appears.

Protocol Message (manual step required)
Prepare Protease solution. Add 10.2 ml (entire vial) of Protease solvent to 1 bottle protease. Be sure to mark the "made date" on the side of the bottle. Add 40 µl protease to the wells using a Matrix pipettor.

27. Click **Continue**. A protocol message box appears.

Protocol Message (manual step required)
Using a Matrix pipettor add the following volumes of molecular grade water to each well: M1 & H1N1 – do not add water Place microtube strip caps over wells when not in use.

28. Click **Continue**. A protocol message box appears.

Protocol Message (manual step required)
Viral RNA Extraction: Use a clean filtered pipette tip, add the following volumes to each respective well:

M1 & H1N1 – 220 µl patient sample/controls Re-cover loaded wells.

29. Click **Continue**. A protocol message box appears.

Protocol Message Remove strip caps. Place the "Lysate" block onto the thermostat slot on the robot paying close attention to orientation. Open the AL buffer tubes located B1-B4.

30. Click **Continue**. A protocol message box appears.

Protocol Message Be sure to place any unused portion of protease or diluted Carrier RNA solution in 4C refrigerator for storage. Carrier RNA in solution will expire 1 week from the made date.

31. Click **Continue**. A protocol message appears:

Protocol Message Make sure that cassettes of the peristaltic pump are fitted correctly. Do not adjust the peripump control panel.

32. Click **Continue**. A protocol message appears:

Protocol Message The protocol starts now. A beeper sounds to let you know when it is time for the first user interaction.

33. The protocol will proceed with the following with no user interaction for Viral RNA protocol.

-Add 240 µl AL buffer to the wells

-Incubate @ 60 °C for 10 minutes

-Add 96% ETOH to S-block on MP-slot 3

-Transfer lysate from thermostat slot to S-block on MP-slot 3 containing ETOH

-Mix lysate and ETOH

-Transfer lysate/ETOH mixture to 96-well plate on vacuum manifold

-Vacuum 5 min.

-Click **"**Continue."

-Add 360 µl of AW1 Buffer

-Vacuum for 3min.

-Add 1,000 µl AW2 Buffer

-Vacuum for 2min.

-Add 1,000 µl AW2 Buffer

-Vacuum for 2min.

34. Click **Continue**. A protocol message appears:

Protocol Message
Load the QIAamp 96 plate on top of an S-block and place them together in the centrifuge. Do not place any tape on the plate. Centrifuge at 6000 rpm for 10 min. Seal used portions of both S-blocks (Lysate, Lysate + ETOH blocks) w/adhesive tape. Mark the used portions of block.

35. Following centrifugation**,** click **Continue**. A protocol message appears:

Protocol Message
Remove MP-slot extension adapter.
Place a new collection microtube rack into MP-slot 3 in the correct orientation. In the following dialog box enter or scan in the identification barcode (ID) of the rack.

36. Click **Continue**. An enter variable value box appears:

Enter variable value
Identification barcode of the collection microtube rack= (ID), enter the date here and time (A, N, P).

37. Click **Continue**. A protocol message appears:

Protocol Message
Identification barcode of the collection microtube rack = {ID}

38. Click **Continue**. A protocol message appears:

Protocol Message
Place the QIAamp 96 plate in the correct orientation onto the collection microtube rack located on the MP-slot 3.

39. Click **Continue**. A protocol message appears:

Protocol Message
Open the 2ml microcentrifuge tubes containing Buffer AVE.

40. Click **Continue**.
41. The instrument will now distribute 86 µl of AVE to each of the identified wells and incubate at room temperature for one minute. At the end of the incubation the instrument will beep.
42. Click **Continue**. A protocol message appears:

Protocol Message
Seal the QIAamp 96 plate with AirPore tape, and then transfer the QIAamp 96 plate with the collection microtube rack into the centrifuge. Centrifuge at 6000 rpm for 3 min.

43. Click **Continue**.

44. Remove the assembly from the centrifuge bucket. Discard the QIAamp 96 plate if it has been fully used. If the plate is going to be used again, remove AirPore tape and seal with Qiagen tape from “Tape Pad”. Mark used portion of the block.

45. Visually inspect elution plate to ensure all wells have eluted properly. If wells appear to have not eluted properly, re-centrifuge. It re-centrifugation fails, add a small volume of AVE (~40 µl) and re-centrifuge. Continue until proper elution occurs.

46. Seal the collection microtubes with the caps provided. Store the collection microtube rack containing the purified RNA at -20°C until required for use. DNA is stored at 2-8°C for one week, and at -20°C for one month.

47. Click **Continue**. The Report File appears, in which comments can be inserted.

48. Click **Continue**. The BioRobot 9604 protocol continues. A beeper sounds to let you know the main protocol has finished.

WASH PROCEDURE

1. Click "Continue". A protocol message appears:

> **Protocol Message**
> Attach the buffer bottle connectors to the adapters on the wash bottle. The wash bottle should be filled with distilled water.
> Click "Continue" to start the wash procedure. Place any unused portion of tips back into original container for storage.

2. The robot will now wash with water from the system liquid container through both the peripump and the dilutor syringes. 50 ml of water will pass through the peripump and the dilutors will be flushed 5 times.
3. Click **Continue**. After the wash procedure is finished, a protocol message appears:

> **Protocol Message**
> Rinse balance block with bleach, distilled water, and ETOH
> Remove all 3 pieces of the vacuum manifold. Rinse ***thoroughly*** w/ distilled water. Dry thoroughly with paper towel. Do not use bleach or ethanol on the manifold.

4. After the wash procedure is finished, a protocol message appears:

> **Protocol Message**
> Process finished!
> Close all buffer bottles.
> Release cassettes of the peristaltic pump.
> Switch off 96-well thermostat system.

5. Click **Continue**. A protocol message appears:

> **Protocol Message (This is an optional step)**
> Turn off robot, while holding up the probes so that they do not crash into wash station.
> Gently lower probes down into an unobstructed area.
> Shut down the computer in the appropriate manner.

C. **REAL-TIME PCR INSTRUMENT CALIBRATION WITH AP525 DYE**

Perform the following to calibrate the Applied Biosystems 7900HT Real-Time PCR System with the AP525 dye calibrator used in the ELITech Molecular Diagnostics 2009-H1N1 Influenza A virus Real-

Time RT-PCR. Refer to Applied Biosystems 7900HT instructions manual for detailed instructions on how to calibrate the instrument.

1. The first time the H1N1 2009 assay is run on the Applied Biosystems 7900HT, the instrument requires calibration with the AP525 dye calibrator.

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

Perform the following to configure the Applied Biosystems 7900HT Real-Time PCR System for the ELITech Molecular Diagnostics 2009-H1N1 Influenza A virus Real-Time RT-PCR. Refer to Applied Biosystems 7900HT instructions manual for detailed instructions on how to operate/run a plate on the instrument.

1. The first time the H1N1 2009 assay is run on the Applied Biosystems 7900HT, the instrument requires calibration with the AP525 dye calibrator.
2. Launch Applied Biosystems 7900HT Real-Time PCR System SDS software.
3. From the **File** menu, click **New Document**.
4. Configure the template by selecting the settings as follows:
    - **Assay**: Absolute Quantification (Standard Curve)
    - **Container**: 96 Wells Clear Plate
    - **Template**: Blank Document
5. Click **OK**
6. From the Tools menu, select **Detector Manager** and select the Detectors as indicated below. (NOTE: Click **New Detector** button if necessary to create these detectors – see instruction manual for further details if necessary).

Detector Name	Reporter Dye	Quencher
2009H1	FAM	Non-Fluorescent
Internal Control	AP525	Non-Fluorescent
M1	FAM	Non-Fluorescent

7. Select **Copy To Plate Document** and then **Done**.
8. Apply the detectors to the wells containing samples and controls as indicated in the example below. (NOTE: Each well should contain either both the H1N1 and Internal Control detectors or M1 and Internal Control detectors).

	Wells with 2009H1 Reaction Mix						**Wells with M1 Reaction Mix**					
	1	2	3	4	5	6	7	8	9	10	11	12
A	S	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	NTC	S	S	S	S	S	NTC
H	S	S	S	S	S	Pos	S	S	S	S	S	POS

9. Ensure that ROX is selected as the Passive Reference dye.
10. Select the Instrument tab and configure the thermal cycler protocol as follows:
    - Mode – Standard
    - Sample Volume (µL) – 50
    - Under the Thermal Profile tab enter the following setting:

Stage 1	Stage 2	Stage 3	Stage 4 - 50 repeats			Stage 5		
20°C	50°C	95°C	95°C	56°C	76°C	95°C	45°C	95°C
10:00	30:00	15:00	0:15	0:30	0:30	0:15	0:15	0:15

- Under the Ramp Rate tab enter:

Stage 1	Stage 2	Stage 3	Stage 4 - 50 cycles			Stage 5		
20°C	50°C	95°C	95°C	56°C	76°C	95°C	45°C	95°C
100%	100%	100%	100%	100%	100%	100%	100%	5%

- Under the Data Collection tab ensure data collection is occurring at Stage 4 Step 2 and during the ramp between Stage 5 Step 2 and Step 3.

11. From the **File** menu, select **Save**, name the file ensuring the file format is .sds and click **Save**.

E. **REAGENT PREPARATION (Pre-Amplification Area)**

Keep reagents on cold block (or ice) while preparing master mix. The master mixes must be made in dedicated area with dedicated pipettes using aerosol resistant pipette tips and RNase/DNase-free tubes.

1. Prepare the required volume of 2009H1 Master Mix by pipetting the volume of each component into a microcentrifuge tube as indicated in the following table:

Table 4: 2009H1 Master Mix Reagent Volumes

Reagent	Per Rxn.	Per 10 Rxns.
20X 2009H1/MS2 Probe/Primer Mix	2.50 µL	25.0 µL
RNase Inhibitor	0.25 µL	2.5 µL
HL-UNG (1U/µl stock)	1.00 µL	10.0 µL
Water (Molecular Biological Grade)	10.75 µL	107.5 µL
2X QuantiTect Probe RT-PCR Master Mix	25.00 µL	250.0 µL
QuantiTect RT Mix	0.50 µL	5.0 µL
Total	40 µL	400 µL

2. Mix by inversion and shaking, and spin to remove solution off the sides of the tube.
3. Pipette 40 µL of this reaction mixture into the appropriate wells of an Applied Biosystems 96-well reaction plate that has been placed in a cold block.
4. Prepare the required volume of the M1 Master Mix by pipetting the volume of each component into a microcentrifuge tube as indicated in the following table:

Table 5: M1 Master Mix Reagent Volumes

Reagent	Per Rxn.	Per 10 Rxns.
20X M1 Detection Reagent LDT	2.50 µL	25.0 µL
RNase Inhibitor	0.25 µL	2.50 µL
HL-UNG (1U/µl stock)	1.00 µL	10.0 µL
PCR Enhancer	5.00 µL	50.0 µL
Water (Molecular Biological Grade)	5.75 µL	325 µL
2X QuantiTect Probe RT-PCR Master Mix	25.0 µL	250 µL
QuantiTect RT Mix	0.50 µL	5.00 µL
Total	40 µL	400 µL

5. Mix by inversion and shaking, and spin to remove solution off the sides of the tube.

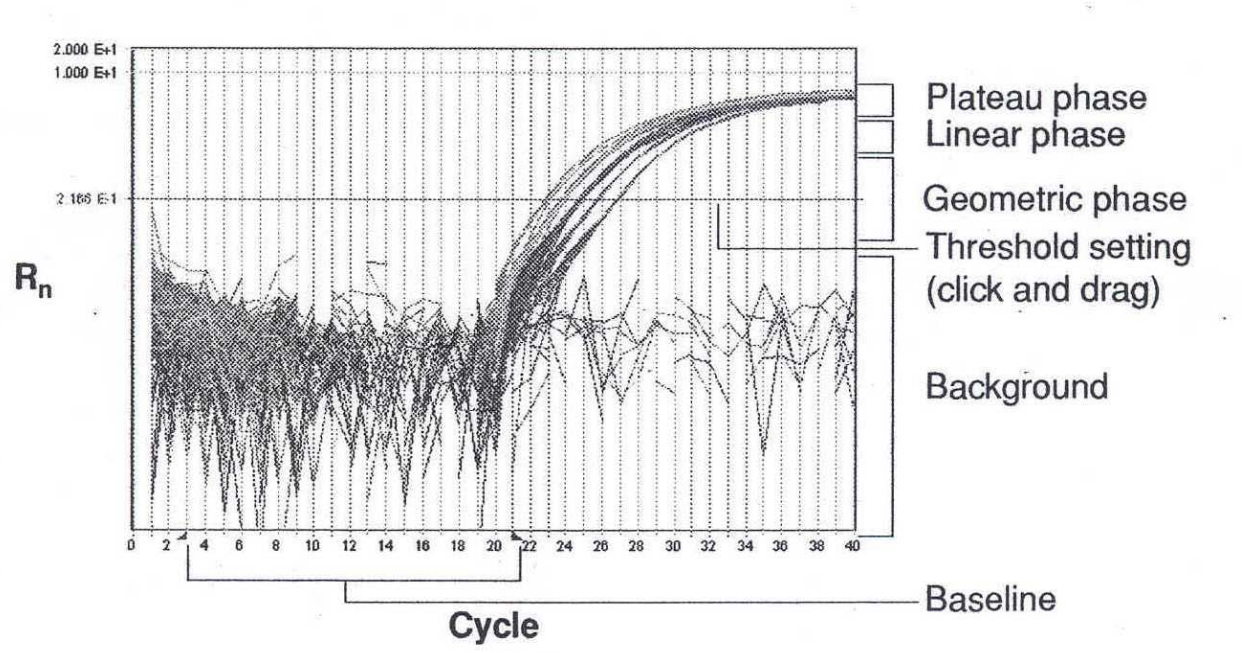
6. Pipette 40 μL of this reaction mixture into the appropriate wells of an Applied Biosystems 96-well reaction plate that has been placed in a cold block.

F. **PCR SETUP (Specimen Inoculation Area)**

1. Load 10 μL of each control and patient sample RNA into the appropriate well of an Applied Biosystems 96-well reaction plate using a pipette with the corresponding aerosol resistant tips.
2. Seal plate with Applied Biosystems Optical Clear Adhesive Cover.
3. Briefly centrifuge the plate using a disposable base to collect the reactions at the bottom of the wells and to eliminate any air bubbles that may be present.
4. Take the sealed plate to the Applied Biosystems 7900HT and place a compression pad over the optical tape (NOTE: the gray side facing the plate, and brown side up).
5. Open instrument tray and place the plate into the instrument tray. (NOTE: the A1 position is located in the top-left side of the instrument).
6. Click **Start**.

F. DATA ANALYSIS

1. When the run finishes, click **OK**.
2. Select all 2009H1 wells in the plate grid.
3. From the toolbar, click the Analyze icon (▸).
4. Select the Results tab.
5. Set the baseline:
    - The lower limit of the baseline should be set at cycle 3.
    - Set the upper limit of the baseline to 4 cycles earlier than the highest samples (earliest crossing threshold)
6. Identify the components of the amplification curve and set the threshold so that it is:
    - Above the background as defined by the negative controls or water (for IC).
    - Below the plateau and linear regions.
    - Within the geometric phase of the amplification curve (SEE GRAPH BELOW).
7. From the Detector pop-up menu, select the internal control detector and set the baseline and threshold by repeating steps 4-6.
8. From the Detector pop-up menu, select the M1 detector and set the baseline and threshold by repeating steps 4-6.
9. Visually check that each sample, which has been assigned a crossing threshold (C_T) by the SDS software, exhibits actual amplification.
10. Click on the Dissociation Curve tab and the software displays results of the run in a melt curve plot. (NOTE: The dissociation curve is the first derivative of reporter fluorescence versus temperature obtained during the melting analysis – Stage 5).
11. Visually examine the dissociation curve for each 2009H1 result by selecting each well in the plate grid. A true negative sample should display no corresponding melting peak.
12. From the Detector pop-up menu, select the M1 detector and repeat steps 10-11.
13. Save the analyzed run.
14. Print the run report if desired.



RESULT REPORTING

Positive and negative controls must be run on each run with the patient samples. If the controls are not valid, patient results cannot be interpreted.

1. Positive Control Review – FAM Channel (2009H1 and M1)
    - If the positive control exhibits amplification at a crossing threshold within the established range (±2 S.D.) for a given lot/preparation of positive control material then the run is valid.
    - If the positive control exhibits amplification at a crossing threshold later than or earlier than the established range for that given lot/preparation the run is considered invalid and all patient samples must be repeated.
2. Negative Control Review – AP525 Channel (MS2 IC)
    - If the negative control exhibits a crossing threshold in the FAM channel (2009H1 or M1) then this control is considered invalid and indicates possible contamination. All positive samples must be repeated. Negative sample results may be reported if all other run criteria are met.
    - If the negative control does not exhibit a crossing threshold in the FAM channel (2009H1 or M1) and exhibits amplification in the AP525 channel (MS2 IC) at a crossing threshold within the established range (±2 S.D.) for a given lot/preparation of internal control material then this control is valid.
3. Patient Sample Review
    - If the assay controls are valid, patient results should be carefully examined in both the FAM and AP525 channels. Always ensure that when a sample is assigned a crossing threshold, it also has a curve displayed on the amplification plot.
    - All samples that are negative in the FAM channel (2009 H1 and M1) should be positive in the AP525 channel (MS2 IC), indicating that the Internal Control has amplified. The internal control should exhibit amplification within the established range (±2 S.D.) for a given lot/preparation of internal control material.

Interpretation of Results

1. If both 2009H1 and M1 detectors have Ct ≤ 36, the sample is scored ***Detected*** for 2009-H1N1 Influenza A RNA, irrespective of the Ct value for IC.

2. If both 2009H1 and M1 detectors have Ct > 46 and the Ct for the IC is within or below the specified range, then the sample is ***Not Detected*** for 2009-H1N1 Influenza A RNA.

3. If the 2009H1 detector is negative (Ct > 46) and the M1 detector is positive (Ct ≤ 36), the sample is scored ***Not Detected*** for 2009-H1N1 Influenza A viral RNA.

4. Clinical testing of patient samples containing 2009-H1N1 Influenza A virus has shown that the Ct values for 2009H1 and M1 detectors will usually be below 36. If the Ct value for 2009H1 or M1 detector is in the range 36 to 46, then repeat testing with the specimen and detector must be carried out, irrespective of the Ct value for IC.

    - If the Ct value on repeat testing is ≤ 46, then the result is scored ***Detected*** for the specimen with that detector irrespective of the Ct value for IC.
    - If the Ct value on repeat testing is > 46 and the Ct value for IC is within or below the specified range, then the result is scored ***Not Detected*** for the specimen with that detector.
    - If the Ct value on repeat testing is > 46 and the Ct value for IC is above the specified range, then the result is scored ***Inhibited*** for the specimen with that detector. If more sample is available, a third replicate should be tested from the original sample and the consensus of the replicates should be reported.

5. Both 2009H1 and M1 detectors must give positive results for the sample to be scored positive for 2009-H1N1 Influenza A RNA. If the 2009H1 detector is scored positive and the M1 detector is scored negative, then repeat testing must be carried out.

    - If repeat testing is again positive for 2009H1 detector and negative for M1 detector, the sample is scored ***Indeterminate*** for 2009-H1N1 Influenza RNA.
    - If repeat testing provides positive results for both detectors, the sample is scored ***Detected*** for 2009-H1N1 Influenza RNA.

6. If either 2009H1 and M1 detectors have Ct > 46 and the Ct for the IC is above the specified range, then the sample is scored ***IC Failure*** and repeat extraction and amplification of sample must be carried out.

    - If repeat testing again gives Ct > 46 for either 2009H1 and M1 detectors and the Ct for IC is still above the specified range, the result is reported as ***Inhibited***.
    - If repeat testing gives Ct for IC within or below the specified range, then interpretation of detector results is carried out as described above.

Table 6: Summary of Interpretation of Results

Initial Result	2009H1 Ct	M1 Ct	IC Ct	Interpretation
1	≤ 36	≤ 36	N/A	2009-H1N1 Influenza A RNA ***Detected***
2	> 46	> 46	In range	2009-H1N1 Influenza A RNA ***Not Detected***
3	> 46	≤ 36	In range	2009-H1N1 Influenza A RNA ***Not Detected***
4*	≤ 36	> 36 but ≤ 46	N/A	Repeat Extraction & PCR
4*	> 36 but ≤ 46	≤ 36	N/A	Repeat Extraction & PCR
5*	≤ 36	> 46	In range	Repeat Extraction & PCR
6*	> 46 OR > 46		Above range	IC Failure. Repeat Extraction & PCR*

* For guidance on repeating the test refer to the corresponding number in Interpretation of Results Section on page 17 of Product Insert.

LIMITATIONS

1 This test has not been evaluated for detection of swine influenza viruses other than the 2009-H1N1 virus in specimens from humans.
2 This test has not been evaluated for detection of viruses infecting swine.
3 Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
4 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.
5 The prevalence of infection will affect the test's predictive value.
6 As with other tests, negative results do not rule out 2009-H1N1 influenza A infections.
7 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.
8 False negative results may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
9 As with other tests, false positive results may occur. Repeat testing or testing with a different device may be indicated in some settings.
10 This test is a qualitative test and does not provide the quantitative value of detected organism present.
11 This test is not intended for patients without signs and symptoms of respiratory infection
12 This test is not intended for monitoring treatment of 2009-H1N1 influenza infection.
13 This test is not intended for screening of blood or blood product for the presence of 2009-H1N1 influenza.
14 This test cannot rule out diseases caused by other bacterial or viral pathogens.
15 The use of sputum has not been evaluated with this assay.

ASSAY PERFORMANCE

LIMIT OF DETECTION (UPPER RESPIRATORY SPECIMENS)

A positive sample, confirmed as 2009-H1N1 Influenza A virus positive by the CDC rRT-PCR assay, was grown to a high titer by viral culture to serve as a positive control material. This material was quantified by standard $TCID_{50}$ protocols as well as using 2009-H1N1 Influenza A RNA transcripts. Two-fold dilution series to end-point of this control material was made in swab samples. These samples were extracted and amplified in triplicate. The lowest dilution detected in triplicate was established as the initial limit of detection. The limit of detection was confirmed by extraction and amplification of 20 replicates. The limit of detection was determined to be the dilution at which 95% of the replicates were positive. This dilution corresponds to 1 $TCID_{50}$/mL or 2.9×10^4 copies/mL. A summary of this data can be found in the following tables.

Table 7: Preliminary limit of detection for 2009H1 detector.

$TCID_{50}$/mL	Copies/mL	2009H1 C_T	Avg. C_T (n=3)
15	$4.6x10^5$	34.37	34.50
		34.54	
		34.60	
7	$2.3x10^5$	37.80	37.96
		37.67	
		38.42	
4	$1.1x10^5$	39.20	38.79
		38.04	
		39.14	
2	$5.8x10^4$	38.62	39.14
		38.64	
		40.17	
1	$2.9x10^4$	40.34	40.12
		39.16	
		40.87	
0.5	$1.4x10^4$	39.81	NA
		50.00	
		41.13	

Table 8: Summary of limit of detection for 2009H1 detector.

$TCID_{50}$/mL	Copies/mL	Initial Screen	Confirmation
15	$4.6x10^5$	3/3	NA
7	$2.3x10^5$	3/3	NA
4	$1.1x10^5$	3/3	NA
2	$5.8x10^4$	3/3	20/20
1	$2.9x10^4$	3/3	20/20
0.5	$1.4x10^4$	2/3	18/20

The sensitivity of the M1 detection system was compared to two test kits approved by the FDA for detection of seasonal influenza A viral RNA. The comparison was made using log dilutions of an H3N2 positive isolate. The crossing thresholds for the M1 detector and the comparator kits are shown in the table below.

Table 9: Limit of detection for M1 detector

$TCID_{50}$/mL	M1 C_T	Comparator 1	Comparator 2
$1x10^4$	29.3	Pos	NA
$1x10^3$	32.6	Pos	NA
$1x10^2$	36.7	Pos	33.5
$1x10^1$	40.4	No Call	37
1	43.5	Neg	38.4
$1x10^{-1}$	Neg	Neg	Neg

The M1 detection system is equivalent in sensitivity to the comparator kits and detected the H3N2 isolate at a level of 1 $TCID_{50}$/mL

ANALYTICAL STUDIES (LOWER RESPIRATORY SPECIMENS)

Analytical studies were carried out by spiking 2009-H1N1 Influenza A virus into individual clinical samples of BAL and sputum at High (297 $TCID_{50}$/mL), Medium (29.7 $TCID_{50}$/mL), and Low (2.97 $TCID_{50}$/mL) levels of virus and then testing with the ELITech Molecular Diagnostics 2009-H1N1 Influenza A Virus Real-Time RT-PCR test. The Low level of virus is close to the LoD estimated from the Limit of Detection study (Tables 7 and 8) described above. The results of this study are summarized in Tables 10 and 11.

Table 10: Analytical Performance Evaluation with Spiked BAL

	EXPECTED RESULT				
ELITech Molecular Diagnostics 2009-H1N1 Influenza A Virus Real-Time RT-PCR test		2009-H1N1 Positive*	2009-H1N1 Negative	Total	Positive Agreement 100% (9/9) 95% CI: 79.6 – 100
	2009-H1N1 Detected	15	0	15	
	2009-H1N1 Not Detected	0	11	11	Negative Agreement 100% (11/11) 95% CI: 74.1 - 100
	Indeterminate*	0	0	0	
	Total	15	11	N = 26	

*Samples = 4 at High Level, 4 at Medium Level, 7 at Low Level

Table 11: Analytical Performance Evaluation with Spiked Sputum					
	EXPECTED RESULT				
ELITech Molecular Diagnostics 2009-H1N1 Influenza A Virus Real-Time RT-PCR test		2009-H1N1 Positive*	2009-H1N1 Negative	Total	Positive Agreement 93.8.0% (30/32) 95% CI: 79.8 – 98.3
	2009-H1N1 Detected	30	0	30	
	2009-H1N1 Not Detected	0	10	10	Negative Agreement 100% (10/10) 95% CI: 72.2 - 100
	Indeterminate**	2	0	2	
	Total	32	10	N = 42	

* Samples = 5 at High Level, 7 at Medium Level, 20 at Low Level

** The two Indeterminate results were from samples spiked at Low Level (2.97 $TCID_{50}$/mL)

The results from the study described above indicate that the ELITech Molecular Diagnostics 2009-H1N1 Influenza A Virus Real-Time RT-PCR test has comparable analytical performance with upper and lower respiratory samples. Additional LoD studies with BAL and sputum indicate that the LoD for 2009-H1N1 Influenza A virus in BAL and sputum are 1 $TCID_{50}$/mL and 2 $TCID_{50}$/mL, respectively. Limited clinical testing with BAL clinical specimens also indicate that the ELITech Molecular Diagnostics 2009-H1N1 Influenza A Virus Real-Time RT-PCR test should detect 2009 H1N1 viral RNA in patients with levels of 2009 H1N1 virus close to LoD in lower respiratory samples.

ANALYTICAL SPECIFICITY (CROSS-REACTIVITY)

High titer positive control material for the following viral and bacterial specimens was tested to determine if there is any cross-reactivity of the 2009H1 and M1 detectors with nucleic acid of other micro-organisms. The pathogen tested, source of the material, and crossing thresholds for the 2009H1 and M1 probes are summarized in Table 12. No cross-reactivity was observed for the other respiratory pathogens tested and the appropriate reactivities for 2009-Influenza A and Influenza A (H3N2) were observed.

Table 12: Analytical Specificity

Pathogen	Source	titer (copies/rxn)	2009H1 C_T		titer (copies/rxn)	M1 C_T
2009-H1N1 Influenza A	ARUP Laboratories	$2x10^4$	35.21		$2x10^4$	36.73
Influenza A (H3N2)	ARUP Laboratories	$1x10^4$	50		$2x10^5$	29.30
B. pertussis	ARUP Laboratories	$1x10^4$	50		$1x10^3$	50
C. pneumoniae	ARUP Laboratories	$1x10^7$	50		$1x10^6$	50
hMPV	ARUP Laboratories	$1x10^6$	50		$1x10^5$	50
L. pneumophila	ARUP Laboratories	$1x10^4$	50		$1x10^3$	50
M. pneumoniae	ARUP Laboratories	$1x10^7$	50		$1x10^6$	50

Influenza B	ARUP Laboratories	$1x10^5$	50		$6x10^5$	50
RSV	ARUP Laboratories	$1x10^7$	50		$5x10^4$	50
Parainfluenza 1	ARUP Laboratories	$1x10^7$	50		$1x10^6$	50
Parainfluenza 2	ARUP Laboratories	$1x10^5$	50		$1x10^4$	50
Parainfluenza 3	ARUP Laboratories	$1x10^7$	50		$1x10^6$	50
Parainfluenza 4	ATCC (VR-1377)	$1x10^5$	50		$1x10^4$	50
N. lactamica	ATCC (23970)	$2x10^6$	50		$2x10^6$	50
S. epidermidis	ATCC (12288)	$2x10^6$	50		$2x10^6$	50
E. coli	ATCC (25922)	$2x10^6$	50		$2x10^6$	50
N. meningitides	ATCC (13090)	$2x10^6$	50		$2x10^6$	50
P. aeruginosa	ATCC (27853)	$2x10^6$	50		$2x10^6$	50
M. catarrhalis	ATCC (25238)	$2x10^6$	50		$2x10^6$	50
S. aureus	ATCC (29213)	$2x10^6$	50		$2x10^6$	50
H. influenzae	ATCC (49247)	$2x10^6$	50		$2x10^6$	50
Strep. pneumoniae	ATCC (49619)	$2x10^6$	50		$2x10^6$	50
Strep. pyogenes	ATCC (19615)	$2x10^6$	50		$2x10^6$	50
C. striatum	ARUP Laboratories	$2x10^6$	50		$2x10^6$	50

A total of 54 seasonal influenza A positive samples (H1N1 [N=29] and H3N2 [= 25]) were also tested to verify that the 2009H1 detector does not react with a variety of strains of seasonal influenza A and to verify reactivity of the M1 detector with these same strains. The samples were typed using either WHO antisera, fluorescent monoclonal antibodies or in 6 cases using the CDC rRT-PCR assay. Crossing thresholds for the 2009H1 probe and the M1 probe together with the approximate year of isolation are shown in Table 13. The 2009H1 detector did not react with any of the strains while the M1 detector was reactive in all cases.

Table 13: Additional specificity using seasonal Influenza A positives

Seasonal Influenza A		H1N1 C_T	M1 C_T	Seasonal Influenza A		H1N1 C_T	M1 C_T
Type	Year			Type	Year		
H1N1	2009	50	28.00	H1N1	2006-2007	50	26.04
H1N1	2009	50	22.80	H1N1	1986	50	33.78
H3N2	2009	50	28.00	H3N2	1995	50	34.49
H1N1	2009	50	21.50	H3N2	1995	50	32.69
H3N2	2009	50	27.30	H3N2	1994	50	39.00
H1N1	2009	50	20.80	H3N2	1995	50	32.67
H1N1	2006-2007	50	28.97	H3N2	1989	50	30.88
H1N1	2006-2007	50	21.85	H3N2	1989	50	36.37
H1N1	2006-2007	50	23.24	H3N2	1989	50	24.60
H1N1	2006-2007	50	28.00	H3N2	1991	50	31.84
H1N1	2006-2007	50	28.29	H1N1	1991	50	43.45
H1N1	2006-2007	50	28.37	H3N2	1989	50	23.17
H1N1	2006-2007	50	34.06	H3N2	1991	50	20.04
H1N1	2006-2007	50	29.46	H3N2	1994	50	24.62
H1N1	2006-2007	50	32.11	H3N2	1990	50	26.07
H1N1	2006-2007	50	24.94	H1N1	1991	50	31.32
H1N1	2006-2007	50	27.50	H3N2	1990	50	29.80
H1N1	2006-2007	50	32.00	H3N2	1989	50	39.49
H1N1	2006-2007	50	25.73	H3N2	1992	50	33.09
H1N1	2006-2007	50	27.74	H3N2	1991	50	32.00
H1N1	2006-2007	50	28.93	H3N2	1991	50	33.83
H1N1	2006-2007	50	24.80	H3N2	1990	50	22.02
H1N1	2006-2007	50	28.34	H3N2	1990	50	37.82
H1N1	2006-2007	50	29.65	H3N2	1994	50	25.99
H1N1	2006-2007	50	25.93	H3N2	1995	50	27.75
H1N1	2006-2007	50	26.74	H3N2	1994	50	30.53
H1N1	2006-2007	50	22.48	H3N2	1994	50	28.70

CLINICAL PERFORMANCE – Comparison with the CDC rRT-PCR assay.

Clinical performance was assessed by testing a total of one hundred upper respiratory specimens that had previously been tested by the Utah Public Health Laboratory with the CDC rRT-PCR assay for 2009-H1N1 Influenza. The patient samples included fifty positive and fifty negative samples. Prior to testing in the ELITech Molecular Diagnostics 2009-H1N1 Influenza A Virus Real-Time RT-PCR assay all samples were de-identified and blinded.

A summary of the comparative results between the CDC rRT-PCR and the ELITech Molecular Diagnostics 2009-H1N1 Influenza A Virus Real-Time RT-PCR assay is shown in Table 14.

Table 14: Clinical Performance Evaluation

	CDC Real Time RT-PCR H1N1-09 Test				
ELITech Molecular Diagnostics 2009-H1N1 Influenza A Virus Real-Time RT-PCR test		2009-H1N1 Positive	2009-H1N1 Negative	Total	Positive Agreement 96.0% (48/50) 95% CI: 86.5 – 98.9
	2009-H1N1 Detected	48	0	48	
	2009-H1N1 Not Detected	0	50	50	Negative Agreement 100% (47/47) 95% CI: 92.9 - 100
	Indeterminate*	2	0	2	
	Total	50	50	N = 100	

* The two samples that were scored as ***Indeterminate*** gave positive results for 2009H1 detector and negative for M1 detector.

REPRODUCIBILITY (PRECISION) – Inter-run and Intra-run

2009 H1 Intra-run Precision
Four samples positive for 2009-H1N1 Influenza A by the CDC rRT-PCR assay were extracted and amplified in triplicate. Crossing thresholds, means, standard deviations, and %C.V. are shown in the table below.

Table 15: 2009H1 Intra-Run Precision

Sample #	Rep 1	Rep 2	Rep 3	Mean	StDev	%C.V.
1	27.93	28.03	28.05	28.00	0.06	0.23
2	28.81	29.94	30.03	29.59	0.68	2.29
3	29.62	29.81	30.11	29.85	0.25	0.83
4	32.77	33.23	32.80	32.93	0.26	0.78
					0.31	1.03

2009 H1 Inter-Run Precision
Four samples positive for 2009-H1N1 Influenza A by the CDC rRT-PCR assay were amplified on three different runs. Crossing thresholds, means, standard deviations, and %C.V. are shown in the table below.

Table 16: 2009 H1 Inter-Run Precision

Sample #	Run 1	Run 2	Run 3	Mean	StDev	%C.V.
1	26.63	28.00	28.53	27.72	0.98	3.55
2	28.59	29.59	29.15	29.11	0.50	1.72
3	28.05	29.85	28.87	28.92	0.90	3.12
4	32.07	32.93	32.51	32.50	0.43	1.33
					0.70	2.43

Aliquots of the 2009H1 Positive Control material were extracted and run on every run. Reproducibility was based on crossing threshold. Means, standard deviations, and %C.V. for each positive are shown below.

N = 16	2009H1 Pos
Mean	34.44
StDev	0.85
%C.V.	2.47

Negative controls (extracted) were assayed on each validation run. Reproducibility of the internal control was based on crossing thresholds. The mean, standard deviation, and %C.V. for the negative control are shown below.

N = 24	IC
Mean	36.34
StDev	0.57
%C.V.	1.5

M1 assay Intra-run Precision
Ten seasonal Influenza A positive samples were extracted and amplified in triplicate. Crossing thresholds, means, standard deviations, and %C.V. are shown in the table below.

Table 17: M1 Intra-Run Precision

Sample Name	Sample Type	Virus	Rep 1	Rep 2	Rep 3	Mean	StDev	%C.V.
wh-rsp032206-02	Swab	Flu A	36.65	36.39	36.58	36.54	0.13	0.37
wh-rsp032206-09	Swab	Flu A	27.89	26.93	27.45	27.42	0.48	1.74
wh-rsp032206-13	Swab	Flu A	31.89	31.24	31.95	31.69	0.40	1.25

M1 assay Inter-run Precision
Ten positive samples were extracted and amplified on two different runs. Crossing thresholds, means, standard deviations, and %C.V. are shown in the table below.

Table 18: M1 Inter-Run Precision

Sample Name	Sample Type	Virus	Rep 1	Rep 2	Mean	StDev	%C.V.
wh-rsp032206-02	Swab	Flu A	36.81	36.65	36.73	0.11	0.30
wh-rsp032206-09	Swab	Flu A	26.72	27.89	27.30	0.83	3.03
wh-rsp032206-13	Swab	Flu A	31.90	31.89	31.89	0.01	0.03

Points of contact
For questions regarding the components of this kit, for additional instructions for use or to report any adverse events contact:

Dr. Walt Mahoney
Epoch BioSciences
Bothell, WA
(425) 482 5173
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