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 As of June 23, 2010, the EUAs have been terminated and this information is no longer current.

CDC swH1N1 (swine) Influenza Virus

Real-time RT-PCR Detection Panel

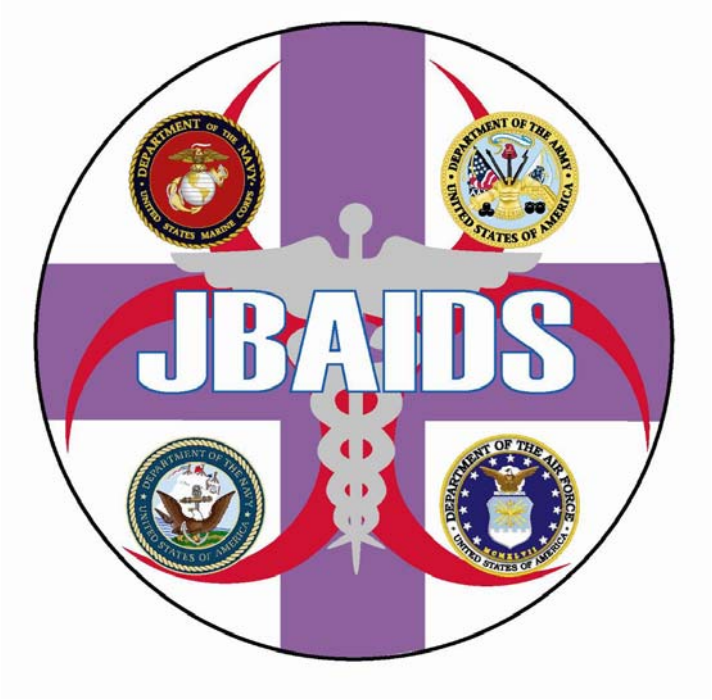
(rRT-PCR Swine Flu Panel)

ON

JBAIDS

(Joint Biological Agent Identification & Diagnostic System)

Instruction Booklet



December 18, 2009

Manufactured by the Centers for Disease Control & Prevention (CDC)
for
Department of Defense (DoD) / Joint Program Executive Office for Chemical Biological Defense (JPEO-CBD) / Chemical Biological Medical Systems (CBMS) / Medical Identification & Treatment Systems (MITS) / Joint Biological Agent Identification and Diagnostic System (JBAIDS)

ATTACHMENT 3

Table of Contents

1. INTENDED USE STATEMENT

The CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel (rRT-PCR Swine Flu Panel) on the JBAIDS instrument is intended for use in real-time RT-PCR assays on a JBAIDS instrument in conjunction with clinical and epidemiological information:

- For the qualitative detection of influenza virus type A viral RNA in upper respiratory tract specimens (URTS), 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 (LRTS), such as broncheoalveolar lavage (BAL), bronchial aspirate (BA), bronchial wash (BW), endotracheal aspirate (EA), endotracheal wash (EW), tracheal aspirate (TA), and lung tissue, from patients with signs and symptoms of respiratory infection or in viral culture.
- For the identification of the 2009 H1N1 Influenza virus viral RNA in upper respiratory tract specimens (URTS), 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 (LRTS), such as broncheoalveolar lavage (BAL), bronchial aspirate (BA), bronchial wash (BW), endotracheal aspirate (EA), endotracheal wash (EW), tracheal aspirate (TA), and lung tissue, from patients with signs and symptoms of respiratory infection or in viral culture. from patients with signs and symptoms of respiratory infection or in viral culture, in conjunction with clinical and epidemiological risk factors.

Testing with the swine influenza swInfA and swH1 primer and probe sets should not be performed unless the patient meets clinical and epidemiologic criteria for testing suspect specimens. The identification of 2009 H1N1 influenza virus should be performed along with clinical and epidemiological assessment.

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

All users, analysts, and any person reporting diagnostic results from use of this device should be trained to perform and interpret the results from this procedure by JBAIDS instructors or designees prior to use. Use of this device is limited to designated Department of Defense (DoD) laboratories equipped with the JBAIDS instruments.

2. INTRODUCTION

The rRT-PCR Swine Flu Panel has been qualified on the JBAIDS in order to augment existing testing capacity by providing swine influenza H1N1 test capability to DoD sites that currently have PCR capability on the JBAIDS.

3. EQUIPMENT AND MATERIALS

3.1 Equipment

Freezer, -20°C
Refrigerator, 4°C
Pipettes (ranges from 0.5 µl-1000 µl)
Vortexer (VWR 58810-163 or equivalent)
Microcentrifuge (Eppendorf 5415C or equivalent)
Capillary chiller (Roche)
JBAIDS (Model JB01)
JBAIDS Software (Version 2.5 or later)
Class II Biosafety cabinet
Capillary capping tool or forceps

3.2 Materials

3.2.1 The rRT-PCR Swine Flu Panel – Catalog Number: FLUSW03. The kit reagents consist of the following:

Components	***Part Number***	***Quantity per vial (nmol)***
InfA-F (forward primer)	MR-023	20.0
InfA-R (reverse primer)	MR-024	20.0
InfA-P (probe)	MR-025	5.0
swInfA-F (forward primer)	MR-029	20.0
swInfA-R (reverse primer)	MR-030	20.0
swInfA-P (probe)	MR-031	5.0
swH1-F (forward primer)	MR-026	20.0
swH1-R (reverse primer)	MR-027	20.0
swH1-P (probe)	MR-028	5.0
RP-F (forward primer)	MR-032	20.0
RP-R (reverse primer)	MR-033	20.0
RP-P (probe)	MR-034	5.0
CDC Swine Influenza Positive Control (SIPC)—store at -20°C		
CDC Human Specimen Control (HSC)—store at -20°C		

- Probe contains 6-FAM reporter and BHQ1 quencher.
- Approximate number of test (samples and control tests) per kit: 1000.
- CDC reagents are shipped in a lyophilized state; store at 2-8°C before reconstitution; Store at -20°C after rehydration
- Store CDC Swine Influenza Positive Control (SIPC) and CDC Human Specimen Control (HSC) at -20°C upon receipt. After first thaw, aliquot into single-use volumes and store at -20°C. Minimize repeated freeze-thaw cycles.

3.2.2 Other Materials Not Provided by CDC

Powder-free gloves
Tube racks; microcentrifuge tube racks
1.5 mL microcentrifuge tubes
Assorted aerosol barrier filter, nuclease-free pipette tips
Bovine Serum Albumin, 20 mg/mL solution (Sigma)
Molecular grade water (nuclease-free water)
Absolute Ethanol (96-100%)
SuperScript III Platinum One-Step RT-PCR kit (Invitrogen Cat. No. 12574-026) – store at -20°C
Qiagen QIAamp Viral RNA Mini sample purification kits (Qiagen Cat. No. 52906)
LightCycler glass capillaries (Roche)

3.3 Points of Contact

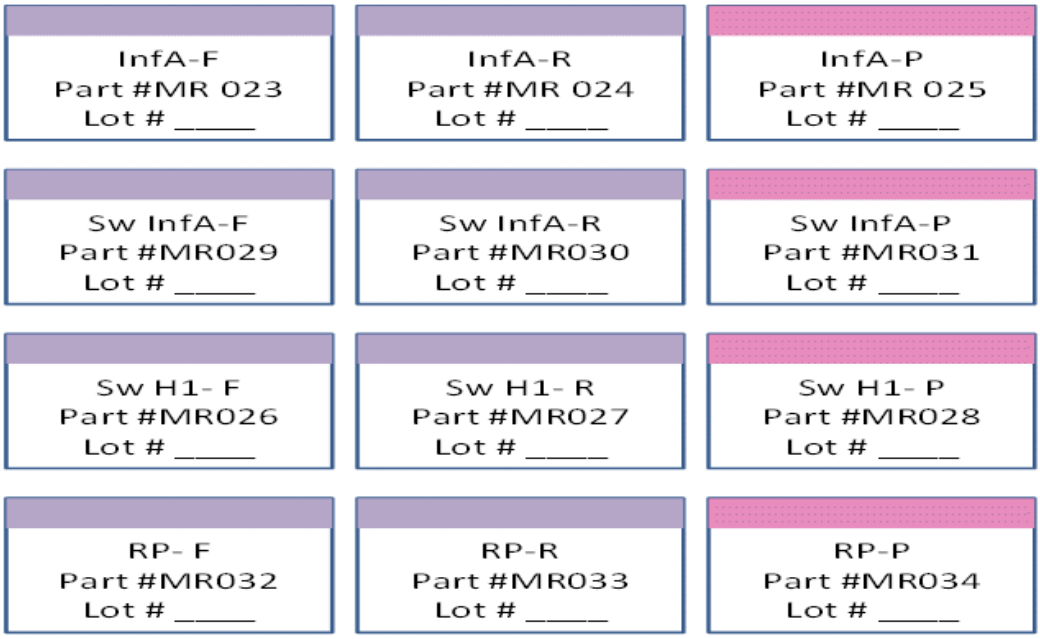
3.3.1 For questions regarding components of the CDC swH1N1 (swine) Influenza Virus Real-Time RT-PCR Detection Panel configured for use on the JBAIDS Instrument and ordering of supplies send an email to JBAIDSswFlu@amedd.army.mil.

3.3.2 For questions regarding overall procedures using JBAIDS – JBAIDS Training Staff/SME at 210-536-3248/3254 (DSN 240).

4. KIT LABELS & STORAGE OF CONTENTS

4.1 Examples of Labels

4.1.1 Labels Found on the CDC Reagent Vials



4.1.2 Labels on the Invitrogen Vials (Not included in the CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel)

4.2 Storage Instructions

4.2.1 Prior to rehydration, store CDC kits at 2-8°C, store Superscript III and 2X Reaction Mix at -20°C.

4.2.2 Do not use any product past the labeled expiration date.

4.3 Instructions for Rehydrating and Aliquoting CDC Primers and Probes

4.3.1 Rehydrate each tube with 500 µl of molecular grade water (nuclease-free).

4.3.2 Dispense into aliquots

- For example, if 25 µl of reagent is dispensed into a cryotube, then the user should have approximately 20 aliquots.
- Place proper labels on each of the tubes clearly indicating a name and date.
- Store aliquots of primers and probes at -20°C or below for up to 12 months.
- Thawed aliquots of primers and probes may be stored **in the dark** for up to 6 months at 2-8°C.
- Do not store in frost-free freezers. Store in a non-defrosting freezer

4.4 Specimens will be Extracted using the Qiagen QIAamp Viral RNA Mini Kit

Each extracted specimen should be tested with each of the primer/probe sets in the rRT-PCR Swine Flu Panel.

4.5 Reagents

- **InfA** detects universal influenza A in upper respiratory specimens from patients with signs and symptoms of respiratory infection and/or positive viral culture.
- **swInfA** specifically detects swine influenza A strains (NP gene) in upper respiratory specimens from patients with signs and symptoms of respiratory infection and/or positive virus culture.
- **swH1** is specific for swine influenza A, subtype H1 (HA gene) in upper respiratory specimens from patients with signs and symptoms of respiratory infection and/or positive viral culture.

The rRT-PCR Swine Flu Panel also includes the following control materials:

- **RNase P (RP)** detects human RNase P and is used as a positive control with human clinical specimens to indicate that adequate isolation of nucleic acid resulted from the extraction of the clinical specimen.
- **Swine Influenza Panel Real Time RT-PCR Positive Control (SIPC)** is a Positive Template Control (PTC) designed to react with all the primer and probe sets including RNase P. This material consists of irradiated virus material and must be extracted according to the Qiagen QIAamp Viral RNA Mini kit protocol prior to use.

The extracted material should be diluted 1:10 (v/v) in nuclease-free water before adding to the PCR reaction mixture.

- **Human specimen control (HSC)** consists of inactivated cultured human cells that are provided by the CDC that monitors the extraction step. The HSC should only be positive for the RNase P assay.

Other controls that should be included in the run:

- **Negative Processing control (Pr. Ctl)** is a blank water sample and will serve as an external negative specimen processing control.
- **Negative template control (NTC)** will be prepared in the reagent area and will serve as a check for contamination in that designated area.

5. PROCEDURE

Note: Current CDC guidelines state that procedures involving live swH1N1 virus and/or patient samples suspected of containing live swH1N1 virus should be performed at BSL-2. Prior to extraction, all samples should be handled in a Class II biological safety cabinet. See current guidance at: http://www.cdc.gov/h1n1flu/guidelines_labworkers.htm

5.1 Obtaining Samples

5.1.1 Appropriate upper respiratory tract and lower respiratory tract samples are collected in viral transport medium, previously tested for influenza virus using the FDA cleared CDC Human Influenza Virus Real-time RT-PCR Detection and Characterization Panel.

5.1.2 Samples should be of sufficient volume to support testing for all targets in singlet (one capillary per test), with some reserve for retesting or re-extraction if necessary. To support the extraction protocol as described below (70 µl input volume) an initial minimum sample volume of 150 µl is recommended.

5.2 RNA Extraction using the QIAamp® Viral RNA Mini Kit

Note: Specimens can be extracted in sets of 4, plus a water blank serving as an external sample processing control.

Note: Qiagen Buffer AVL with Carrier RNA is stable at room temperature for 3-4 hours; however, store reconstituted Buffer AVL/Carrier RNA at 2-8°C for longer periods. A precipitate will form and must be redissolved by warming at 80°C before use. DO NOT warm solution more than 6 times and DO NOT incubate for more than 5 minutes. (See manufacturer guidelines for more information.)

NOTE: Do not forget to reconstitute buffers AW1 and AW2 with ethanol. (See manufacturer guidelines for more information.)

5.2.1 Pipette **280 µl of prepared Buffer AVL containing carrier RNA** into a 1.5 mL labeled microcentrifuge tube.

5.2.2 Add **70 µl of sample (i.e. viral transport medium) or control** to be extracted to a microcentrifuge tube and mix by pulse-vortexing for 15 seconds.

5.2.2.1 Controls may include the HSC, Pr. Ctl, and the PTC. The PTC is included in the CDC kit as the Swine Influenza Positive Control (SIPC), but it must be extracted using the QIAamp Viral RNA Mini kit prior to use. Once extracted, the PTC can be aliquoted and placed in the freezer for further use.

5.2.3 Incubate sample and/or controls at room temperature (15–25°C) for 10 minutes.

5.2.4 Briefly centrifuge the tube to remove drops from the inside of the lid.

5.2.5 Add **280 µl of ethanol** (96–100%) to the sample, and mix by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

5.2.6 For each sample/control, place a QIAamp spin column into a 2 mL collection tube (from the QIAamp Viral RNA Mini Kit).

5.2.7 Carefully transfer the mixture from step 5 (4.2.5), including any precipitate, to the QIAamp spin column WITHOUT moistening the rim of the column.

5.2.8 Spin 1-2 minutes at 6000 x g. If the sample has not cleared the filter after first spin, repeat until sample has cleared the filter.

5.2.9 For each sample/control, place the QIAamp spin column into a second, clean 2 mL collection tube (from the QIAamp Mini Kit) and add **500 µl of Buffer AW1**. Discard the tube containing the filtrate from the previous step.

5.2.10 Spin 1-2 minutes at 6000 x g. If the buffer has not cleared the filter after 1-2 minutes then repeat until buffer has cleared the filter.

5.2.11 Place each QIAamp spin column into a third clean 2 mL collection tube (from the QIAamp Mini Kit). Carefully open the QIAamp spin column and add **500 µl of Buffer AW2.**

5.2.12 Centrifuge at full speed (approx. 14,000 x g) for 3 minutes. Discard the tube containing the filtrate from the previous step.

5.2.13 To eliminate any possible Buffer AW2 carryover, place the column into a new collection tube, discard the old collection tube, and spin at full speed for 1 minute.

5.2.14 Place the QIAamp Mini column in a clean 1.5 mL microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate.

5.2.15 Carefully open the QIAamp Mini column and **add 70 µl of Buffer AVE** equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 minute.

5.2.16 Centrifuge at 6000 x g (8000 rpm) for 1 minute. RNA is now present in the eluate and ready to test. Store samples and controls at 2-8°C until master mixes are prepared.

5.2.17 Extracted samples should be tested within 6 hours of completing the extraction process. Residual unextracted samples should be stored at 2-8°C while testing is in progress. Long-term storage (>6 hours) should be at -20°C (preferably -80°C). Freeze-thaw cycles should be minimized and should not exceed three cycles.

5.3 Testing Samples on JBAIDS

5.3.1 Reaction Setup

5.3.1.1 Reaction assay mixtures are made as a cocktail and dispensed into the individual 1.5 mL reaction tubes.

5.3.1.2 Water and extracted nucleic acid or positive template controls are then added to the appropriate test reactions and controls.

5.3.1.3 Label one 1.5 mL microcentrifuge tube for each primer/probe set (4 tubes total – InfA, swInfA, swH1, & RNase P).

5.3.1.4 Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction cocktail to allow for the NTC, PTC, HSC reactions, and pipetting error. See below:

- If number of samples (n) including controls = 1 to 14, then N = n + 1
- If number of samples (n) including controls > 15, then N = n + 2

5.3.1.5 Master Mix: calculate the amount of each reagent to be added for each primer/probe set reaction master mix. The calculations are as follows:

Reagent	Volume of Reagent Added per Reaction
2X PCR Master Mix	N x 10.0 µl
Molecular Grade (nuclease-free) Water	N x 3.15 µl
20 mg/mL Bovine Serum Albumin in Nuclease-free water	N x 0.25 µl
Forward Primer	N x 0.4 µl
Reverse Primer	N x 0.4 µl
Probe	N x 0.4 µl
SuperScript™ III RT/Platinum® Taq Mix	N x 0.4 µl
Total Volume	N x 15.0 µl

- In order to minimize contamination, it is suggested to add contents in the order provided in the table.
- Due to the viscosity of the Taq Mix, pipette more slowly and mix by pipetting up and down.

5.3.1.6 After addition of the components, mix reaction mixtures by pipetting up and down. Do not vortex.

5.3.1.7 Pulse centrifuge at full speed for 5 seconds to collect contents at bottom of the tube, and then place the tube in cold rack (2-8°C).

5.3.1.8 Set up capillaries or PCR reaction tubes as appropriate in a cooler rack.

5.3.1.9 Dispense 15 µl of each master mix into each capillary as indicated in the diagrams below. Dispense one master mix into all of the required positions before moving on to the next master mix. For example, dispense 15 µl InfA master mix into the appropriate capillaries before moving on to the swInfA master mix.

5.3.2 Run Setup

5.3.2.1 Test Setup table is shown for a 32-position Carousel-based system. This test assumes singlet testing. Once all master mixes are prepared, it is pertinent that the user place the appropriate master mix in the correct positions. The user will add all of the master mix combinations from the first table into the appropriate positions. Once this is complete, samples and controls will then be added according to the Rotor 1/Rotor 2 illustrations below.

Note that there are two setups illustrated:

(A) 5 to 10 specimens in two rotors
(B) 1 to 4 specimens in one rotor

(A) 6 to 10 specimens (2 rotors):

Table 1. Shows the positions for all four of the master mixes: For example, the Influenza A (InfA) master mix should be dispensed into positions 1, 5, 9, 13, 17, 21, 25, & 29.

Position		Position		Position		Position	
1	InfA	9	InfA	17	InfA	25	InfA
2	swInfA	10	swInfA	18	swInfA	26	swInfA
3	swH1	11	swH1	19	swH1	27	swH1
4	RP	12	RP	20	RP	28	RP
5	InfA	13	InfA	21	InfA	29	InfA
6	swInfA	14	swInfA	22	swInfA	30	swInfA
7	swH1	15	swH1	23	swH1	31	swH1
8	RP	16	RP	24	RP	32	RP

Assuming singlet testing, up to 10 specimens can be extracted and batched on 2 rotors. In this example, a maximum of 5 samples can be tested on rotor 1 and another 5 samples on rotor 2.

Rotor 1 (contains 5 specimens plus NTC, PTC, & HSC)

Position		Position		Position		Position	
1	NTC	9	S2	17	S4	25	PTC
2	NTC	10	S2	18	S4	26	PTC
3	NTC	11	S2	19	S4	27	PTC
4	NTC	12	S2	20	S4	28	PTC
5	S1	13	S3	21	S5	29	HSC
6	S1	14	S3	22	S5	30	HSC
7	S1	15	S3	23	S5	31	HSC
8	S1	16	S3	24	S5	32	HSC

Rotor 2 (contains 5 specimens plus NTC, PTC, & external negative processing control (Pr Ctl)). *[If fewer than 5 specimens, PTC & Pr.Ctl. must be moved to lower numbered positions so that no gaps exist.]*

Position		Position		Position		Position	
1	NTC	9	S7	17	S9	25	PTC
2	NTC	10	S7	18	S9	26	PTC
3	NTC	11	S7	19	S9	27	PTC
4	NTC	12	S7	20	S9	28	PTC
5	S6	13	S8	21	S10	29	Pr. Ctl.
6	S6	14	S8	22	S10	30	Pr. Ctl.
7	S6	15	S8	23	S10	31	Pr. Ctl.
8	S6	16	S8	24	S10	32	Pr. Ctl.

Note: Negative template controls (NTC) should be added first before any of the samples are added to check for contamination in the master mix. The negative external processing control should be added next. HSC should be added after the samples have been added to check for cross-contamination during sample preparation or addition. Positive template controls (PTC) should be added last after all samples and NTCs are sealed.

(B) 1 to 4 specimens (1 rotor):

Table 2. Shows the positions for all four of the master mixes: For example, the Influenza A (InfA) master mix should be dispensed into positions 1, 5, 9, 13, 17, 21, 25, & 29.

Position		Position		Position		Position	
1	InfA	9	InfA	17	InfA	25	InfA
2	swInfA	10	swInfA	18	swInfA	26	swInfA
3	swH1	11	swH1	19	swH1	27	swH1
4	RP	12	RP	20	RP	28	RP
5	InfA	13	InfA	21	InfA	29	InfA
6	swInfA	14	swInfA	22	swInfA	30	swInfA
7	swH1	15	swH1	23	swH1	31	swH1
8	RP	16	RP	24	RP	32	RP

Assuming singlet testing, up to 4 specimens can be extracted and batched on 1 rotor. If the user is testing 1 to 4 samples, then only the rotor 1 set up would apply. *[If fewer than 4 specimens, Pr.Ctl, PTC & HSC must be moved to lower numbered positions so that no gaps exist.]*

Rotor 1 (contains 4 specimens plus external negative processing control (Pr Ctl)).

Position		Position		Position		Position	
1	NTC	9	S2	17	S4	25	PTC
2	NTC	10	S2	18	S4	26	PTC
3	NTC	11	S2	19	S4	27	PTC
4	NTC	12	S2	20	S4	28	PTC
5	S1	13	S3	21	Pr. Ctl.	29	HSC
6	S1	14	S3	22	Pr. Ctl.	30	HSC
7	S1	15	S3	23	Pr. Ctl.	31	HSC
8	S1	16	S3	24	Pr. Ctl.	32	HSC

Note: Negative template controls (NTC) should be added first before any of the samples are added to check for contamination in the master mix. The negative external processing control should be added next. HSC should be added after the samples have been added to check for cross-contamination during sample preparation or addition. Positive template controls (PTC) *should be added last after all samples and NTCs are sealed.*

5.3.2.2 Before moving the capillaries to the nucleic acid handling area, set up the NTC reactions in the assay set-up area. As shown above, samples can be added by column.

5.3.2.3 **Pipette 5 µl of nuclease free water into the NTC capillaries.** Cap the NTC capillaries.

5.3.2.4 Close the lid on the capillary box and move the capillaries to the nucleic acid handling area.

5.3.2.5 Change Personal Protective Equipment (PPE) such as gloves and lab coat when moving from the reagent area to the nucleic acid handling area.

5.3.3 Sample Setup

5.3.3.1 Set up the extracted nucleic acid samples in the cold rack (2-8°C).

5.3.3.2 **Pipette 5 µl of nuclease free water into the Processing Control capillaries**. Cap the Processing Control capillaries.

5.3.3.3 **Pipette 5 µl of the first sample** into all the capillaries labeled for that sample (for example, Sample "S1" as shown in Table 1). Change tips after each addition.

5.3.3.4 Cap the capillaries to which the sample has been added. This will help to prevent sample cross contamination and enable you to keep track of where you are in the series.

5.3.3.5 Change gloves when necessary to avoid contamination, especially between samples. Repeat previous steps for the remaining samples.

5.3.3.7 **Add 5 µl of HSC extracted sample to the HSC capillaries.** Cap the HSC tubes.

5.3.3.8 Finally, **pipette 5 µl of positive template control RNA into all PTC tubes**. (NOTE: The PTC is the SIPC that has been extracted and diluted 1:10 (v/v) in water.) Cap the PTC tubes. All capillaries should now be capped.

5.3.3.9 **Briefly centrifuge capillaries for 10-15 seconds** to ensure complete reaction mixture is in the bottom of the capillary. Return capillaries to cold rack (2-8°C).

5.3.3.10 Go to the Samples page by clicking the 'Samples' icon in the left panel. Refer to Table 1 and Rotor 1 for placement of the capillaries.

5.3.3.11 Ensure that the 'samples count' at the top of the page correctly corresponds to the number of samples in the run.

5.3.3.12 Confirm that you have loaded the carousel with the reaction capillaries according to the pattern you have entered on the screen. Start the run and wait for the instrument to detect all of the capillaries.

5.3.4 RT-PCR Amplification Conditions

5.3.4.1 **The reaction** volume is 20 µl. Program the thermocycler as follows:

Reverse Transcription	50°C for 30 minutes
Taq inhibitor inactivation	95°C for 2 minutes
PCR amplification (45 cycles)	95°C for 15 seconds 55°C for 30 seconds

5.3.4.2 Fluorescence data (FAM) is collected during the 55°C incubation step.

5.3.5 PCR Analysis Using a Software Macro

5.3.5.1 Use a software Macro to accomplish the analytical run. The amplification conditions are stated above, and they have been programmed into a JBAIDS macro that is distributed via CD/DVD with the swH1N1 testing materials. The JBAIDS System Manual and JBAIDS Macro Usage Document on the DVD provide guidance for importing a macro and analyzing a PCR run.

Manual entry of amplification conditions is available and acceptable in the JBAIDS software; however, the use of macros is preferred.

5.3.5.2 When the JBAIDS run is complete select 'Finish'. Perform an analysis by selecting 'Analysis' then 'Qualitative Detection'. Print a copy of the JBAIDS Report Choose 'No' when asked to set up sample settings prior to analysis.

6. INTERPRETATION OF RESULTS

6.1 Test Run is Valid when all Controls Meet Stated Standards

6.1.1 In order for a test run to be valid, all NTC reactions must be negative, based on the instrument call. Control reactions should not be interpreted subjectively based on displayed amplification curves. If one or more NTC fails, the entire run is invalid and potential sources of contamination should be identified and corrected. Retest the purified sample and controls and re-analyze.

6.1.2 Failure to achieve a Positive PTC reaction for any of the assays invalidates entire run. Obtain new PTC reagents and repeat run.

6.1.3 The Human Specimen Control (HSC) should be positive for the RNase P (RP) assay and negative for all of the Influenza target assays (InfA, swInfA, and swH1 targets).

6.1.3.1 The Human Specimen Control (HSC) serves as a positive control for the RP target (RNase P), and also serves as a control for the extraction and recovery of RNA from the specimen, and for inhibition of the PCR reaction by the specimen. Positive HSC for any influenza target assay suggests that contamination has occurred.

6.1.4 All clinical specimens should exhibit a positive RNase P (RP) reaction indicating sufficient recovery of RNA of acceptable quality from the specimen. A negative RP reaction could indicate improper RNA extraction, low recovery of RNA, improper reaction setup, reagent or equipment malfunction, PCR inhibition, or absence of sufficient human cellular material in the specimen to enable detection.

6.1.5 Positive results for any of the influenza target assays (InfA, swInfA, and swH1) should override a failed (negative or uncertain) Human Sample Control (RNase P) assay result for that clinical specimen. A failed RNase P Control assay reaction that accompanies a negative or uncertain target result (InfA, swInfA, and/or swH1) requires the purified sample to be retested undiluted and at a 1:10 dilution (using nuclease-free water) with all assays (InfA, swInfA, and swH1 and RNase P). Valid positive or negative retest results for the undiluted sample will be taken as the final result. Valid positive target assay results for the 1:10 dilution will also be

considered the final result. Negative or uncertain results for the target assays or another failure of the Control assay suggest possible PCR inhibition and require the original clinical specimens to be re-purified and retested.

6.1.6 The external negative processing control associated with each extraction batch should be called negative by the instrument software. If the external sample processing negative control for a set of extracted samples is positive, all results for those specimens are invalid. Retest the purified sample and controls and re-analyze. If the retest for the processing control results is positive, then re-extract the samples and controls.

6.2 Unknown Sample Interpretation

6.2.1 When all controls meet stated requirements (above), the run is valid.

6.2.2 A specimen is considered positive for Influenza A virus if the JBAIDS instrument call for the InfA assay is Positive, and the sub-typing assays (swInfA and swH1) are all called negative by the JBAIDS instrument. The RP assay may be called either positive or negative by the JBAIDS instrument as described above.

6.2.3 A specimen is considered positive for 2009 H1N1 Influenza if InfA and BOTH swine influenza A/H1 (swInf or swH1) assays are called Positive by the JBAIDS instrument. The RP assay may be called either positive or negative by the JBAIDS instrument as described above.

6.2.4 If a specimen is positive for InfA and only one of the sub-typing assays (swInfA and swH1) is called positive by the JBAIDS instrument (Note: The RP assay may be called either positive or negative by the JBAIDS instrument as described above), retest the sample using a new aliquot of the specimen. If the retest results are still InfA positive and only one of the sub-typing assays (swInfA and swH1) positive, the sample is considered positive for Influenza A virus, and the user should contact JBAIDS Technical Assistance for guidance.

6.2.5 If a sample is called InfA negative by the JBAIDS instrument, and one or both of the sub-typing assays (swInfA and swH1) is/are called positive by the JBAIDS instrument (Note: The RP assay may be called either positive or negative by the JBAIDS instrument as described above), retest the sample using a new aliquot of the specimen. If the retest results are still InfA negative and one or both of the sub-typing assays (swInfA and swH1) positive, the user should contact JBAIDS Technical Assistance for guidance.

6.2.6 A specimen is considered negative for Influenza A virus if the InfA and the subtyping assays (swInfA and swH1) are called Negative by the JBAIDS instrument, and the RNase P (RP) assay is called positive by the JBAIDS instrument.

Interpretation of Control & Sample Calls

Test Results			Reported Result
InfA	**swFluA**	**swH1**	
Negative	Negative	Negative	Negative for Influenza A
Negative	Positive	Negative	Retest the sample using a new aliquot of the specimen**.** Seek guidance* if retest generates the same result.
Negative	Negative	Positive	Retest the sample using a new aliquot of the specimen**.** Seek guidance* if retest generates the same result.
Negative	Positive	Positive	Retest the sample using a new aliquot of the specimen**.** Seek guidance* if retest generates the same result.
Positive	Negative	Negative	Positive for Influenza A.
Positive	Positive	Negative	Positive for Influenza A. Retest the sample using a new aliquot of the specimen**.** Seek guidance* if retest generates the same result.
Positive	Negative	Positive	Positive for Influenza A. Retest the sample using a new aliquot of the specimen**.** Seek guidance* if retest generates the same result.
Positive	Positive	Positive	Positive for 2009 H1N1 Influenza

* Contact JBAIDS Technical Assistance/SME for guidance at 210-536-3248/3254 (DSN 240).

.

Test is considered valid if the software makes the following calls:

Controls	InfA	swFluA	swH1	RNase-P	Action
NTC	-	-	-	-	Any failures invalidate the run. Retest from purified sample.
Pr. Ctl	-	-	-	-	Any failures invalidate the run. Re-purify sample and retest.
PTC or SIPC	+	+	+	+	Any failures invalidate the run. Retest from purified sample. If PTC fails again, obtain a new aliquot from freezer.
HSC	-	-	-	+	A failed RNase P call with a neg. unknown requires that purified sample be tested undiluted and at a 1:10 dilution with all assays. If HSC is positive for InfA, swFluA, swH1, then retest the purified sample.
Unknown samples	+	+	+	+	If all target assays come up + then sample is positive for 2009 H1N1 Influenza.
Unknown samples	+	+	-	+	If a specimen is positive for InfA and only one of the subtype reactions, retest the sample using a new aliquot of the specimen. Contact JBAIDS Technical Assistance for guidance if retest generates the same result.
Unknown samples	+	-	+	+	If a specimen is positive for InfA and only one of the subtype reactions, retest the sample using a new aliquot of the specimen. Contact JBAIDS Technical Assistance for guidance if retest generates the same result.

Refer to the full explanation of calls in Interpretation of Results section. Samples with result of "uncertain" must be retested from the purified sample. Contact JBAIDS Technical Assistance/SME for guidance at 210-536-3248/3254 (DSN 240).

7. ANALYTICAL PERFORMANCE CHARACTERISTICS

7.1 Analytical Sensitivity/ Limit of Detection (LoD)

Analytical sensitivity LoD studies estimate, refined estimate and confirm the lowest detectable concentration range of influenza viruses ($TCID_{50}$/mL) at which approximately 95% of all replicates were tested positive. The Limit of Detection was estimated, refined estimated and confirmed for each relevant primer and probe set in the CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS instrument by limiting dilution studies using characterized samples.

Nasopharyngeal swab specimens (NPS) were collected from healthy volunteers. Ten-fold serial dilutions of the characterized influenza viruses were made from quantified ($TCID_{50}$ /mL) influenza virus stocks. The NPS specimens collected from healthy volunteers were spiked with the serial dilutions. The spiked specimen replicates were extracted using the Qiagen QIAamp Viral RNA Mini kit, and analyzed on the JBAIDS instrument using the relevant primers and probe set(s) (i.e. InfA, swInfA, and swH1 reagents) from the CDC Swine Influenza Virus Real-time RT-PCR Detection Panel. Bovine serum albumin (0.25 mg/mL final concentration) was added to each reaction to prevent adsorption of nucleic acid to the sides of the glass capillaries. Three well-characterized influenza A strains, the Influenza A/California/04/2009 2009 H1N1 influenza A strain, the Influenza A/Hawaii/15/2001 H1N1 influenza A strain, and the Influenza A/Texas/71/2007 H3N2 influenza A strain, were used for the LoD estimation, refined estimation and confirmation studies. The results summaries are presented in Table 3 to Table 11.

Table 3. Influenza A/California/04/2009 (H1N1) Virus Initial LoD Estimation

Assay	$TCID_{50}$/mL	Results	Ct			Mean C_t (n=3)	SD
InfA	10000	3/3 Pos	27.11	27.81	27.52	27.48	0.35
	1000	3/3 Pos	31.71	30.80	29.27	30.59	1.23
	100	3/3 Pos	34.51	35.29	34.59	34.80	0.43
	10	2/3 Pos	36.38	40.00	Neg	38.19	2.56
swInfA	10000	3/3 Pos	28.58	29.81	29.14	29.18	0.62
	1000	2/3 Pos	Neg	30.10	28.49	29.30	1.14
	100	2/3 Pos	32.70	Neg	34.08	33.39	0.98
	10	0/3 Pos	Neg	Neg	Neg	--	--
swH1	10000	3/3 Pos	28.91	28.82	29.64	29.12	0.45
	1000	3/3 Pos	32.49	31.65	30.79	31.64	0.85
	100	3/3 Pos	34.14	34.08	34.95	34.39	0.49
	10	0/3 Pos	Neg	Neg	Neg	--	--

Table 4. Influenza A/California/04/2009 (H1N1) Virus Refined LoD Estimation								
Assay	**$TCID_{50}$/mL**	**Results**	**C_t**				**Mean C_t (n=4)**	**SD**
InfA	10000	4/4 Pos	27.29	27.40	27.19	27.39	27.32	0.10
	5000	4/4 Pos	28.17	27.93	28.03	27.93	28.02	0.11
	1000	4/4 Pos	30.09	30.25	31.81	30.16	30.58	0.82
swInfA	10000	4/4 Pos	25.74	25.63	25.87	26.18	25.86	0.24
	5000	4/4 Pos	26.83	26.52	27.37	27.00	26.93	0.35
	1000	3/4 Pos	29.32	29.81	Neg	30.06	29.73	0.38
swH1	10000	4/4 Pos	27.27	27.82	27.95	28.43	27.87	0.48
	5000	4/4 Pos	29.05	28.68	29.08	29.22	29.01	0.23
	1000	4/4 Pos	30.76	30.81	30.02	29.93	30.38	0.47

Table 5. Influenza A/California/04/2009 (H1N1) Virus LoD Confirmation				
Virus Concentration = 5,000 TCID50/mL				
		C_t		
Sample	**Pos/Neg**	**InfA**	**swFluA**	**swH1**
1	Pos	28.82	29.19	29.55
2	Pos	28.97	28.83	30.28
3	Pos	29.12	29.98	29.61
4	Pos	28.38	28.84	29.27
5	Neg	Neg	Neg	Neg
6	Pos	27.48	28.91	28.94
7	Pos	28.80	28.33	29.73
8	Pos	30.14	28.67	29.84
9	Pos	28.97	28.14	29.37
10	Pos	28.75	28.39	29.81
11	Pos	28.76	29.27	30.21
12	Pos	29.35	30.99	30.42
13	Pos	28.78	30.16	29.75
14	Pos	29.54	30.81	30.25
15	Pos	29.52	30.62	30.42
16	Pos	29.30	30.66	30.21
17	Pos	28.89	29.68	29.94
18	Pos	31.61	29.60	30.04
19	Pos	28.89	28.32	30.17
20	Pos	28.54	27.99	29.97
Mean C_t (n=20)		29.08	29.34	29.88
SD		0.81	0.97	0.41
CV (%)		2.8	3.3	1.4

Table 5. Influenza A/California/04/2009 (H1N1) Virus LoD Confirmation				
Virus Concentration = 5,000 TCID50/mL				
		C_t		
Sample	Pos/Neg	InfA	swFluA	swH1
Results	19/20			

Table 6. Influenza A/Hawaii/15/2001 (H1N1) Virus Initial LoD Estimation							
Assay	$TCID_{50}$/mL	Results	C_t			Mean C_t (n=3)	SD
InfA	1000	3/3 Pos	26.18	25.80	26.23	26.07	0.24
	100	3/3 Pos	29.32	30.19	29.73	29.75	0.44
	10	3/3 Pos	32.60	32.93	33.47	33.00	0.44
	1	3/3 Pos	36.95	35.05	35.46	35.82	1.00

Table 7. Influenza A/Hawaii/15/2001 (H1N1) Virus Refined LoD Estimation								
Assay	$TCID_{50}$/mL	Results	C_t				Mean C_t (n=4)	SD
InfA	10	4/4 Pos	33.18	32.88	32.95	32.94	32.99	0.13
	1	4/4 Pos	36.34	36.77	36.23	36.13	36.37	0.28
	0.1	3/4 Pos	37.33	36.81	Neg	38.04	37.39	0.62

Table 8. Influenza A/Hawaii/15/2001 (H1N1) Virus LoD Confirmation				
Virus Concentration = 10 $TCID_{50}$/mL				
		C_t		
Sample	Pos/Neg	InfA	swInfA	swH1
1	Pos	35.51	N/A	N/A
2	Pos	35.76	N/A	N/A
3	Pos	34.48	N/A	N/A
4	Pos	34.77	N/A	N/A
5	Pos	33.71	N/A	N/A
6	Pos	35.17	N/A	N/A
7	Pos	34.71	N/A	N/A
8	Pos	34.28	N/A	N/A
9	Pos	34.70	N/A	N/A
10	Pos	33.38	N/A	N/A
11	Pos	32.91	N/A	N/A

Table 8. Influenza A/Hawaii/15/2001 (H1N1) Virus LoD Confirmation				
Virus Concentration = 10 $TCID_{50}$/mL				
		C_t		
Sample	Pos/Neg	InfA	swInfA	swH1
12	Pos	33.64	N/A	N/A
13	Pos	33.35	N/A	N/A
14	Pos	32.88	N/A	N/A
15	Pos	33.76	N/A	N/A
16	Pos	33.70	N/A	N/A
17	Pos	33.14	N/A	N/A
18	Pos	33.38	N/A	N/A
19	Pos	33.33	N/A	N/A
20	Pos	34.68	N/A	N/A
Mean C_t (n=20)		34.06	N/A	N/A
SD		0.86	N/A	N/A
CV (%)		2.5	N/A	N/A
Results	20/20			

Table 9. Influenza A/Texas/71/2007 (H3N2) Virus Initial LoD Estimation							
Assay	$TCID_{50}$/mL	Results	C_t			Mean C_t (n=3)	SD
InfA	10000	3/3 Pos	31.09	30.02	30.28	30.46	0.56
	1000	3/3 Pos	34.08	33.71	33.69	33.83	0.22
	100	3/3 Pos	36.72	36.15	35.11	35.99	0.82
	10	2/3 Pos	37.88	35.70	Neg	36.79	1.54

Table 10. Influenza A/Texas/71/2007 (H3N2) Virus Refined LoD Estimation								
Assay	$TCID_{50}$/mL	Results	C_t				Mean C_t (n=4)	SD
InfA	1000	4/4 Pos	29.22	29.25	29.26	29.21	29.24	0.02
	100	4/4 Pos	32.20	33.30	32.87	32.96	32.83	0.46
	10	4/4 Pos	36.25	36.18	35.68	35.53	35.91	0.36

Table 11. Influenza A/Texas/71/2007 (H3N2) Virus LoD Confirmation				
Virus Concentration = 100 $TCID_{50}$/mL				
		C_t		
Sample	Pos/Neg	InfA	swFluA	swH1
1	Pos	34.88	N/A	N/A
2	Pos	34.05	N/A	N/A
3	Pos	35.90	N/A	N/A
4	Pos	35.35	N/A	N/A
5	Pos	35.33	N/A	N/A
6	Pos	35.48	N/A	N/A
7	Pos	35.22	N/A	N/A
8	Pos	36.83	N/A	N/A
9	Pos	36.30	N/A	N/A
10	Pos	37.91	N/A	N/A
11	Pos	34.90	N/A	N/A
12	Pos	34.98	N/A	N/A
13	Pos	35.75	N/A	N/A
14	Pos	35.03	N/A	N/A
15	Pos	34.95	N/A	N/A
16	Pos	35.62	N/A	N/A
17	Pos	35.70	N/A	N/A
18	Pos	35.59	N/A	N/A
19	Pos	35.91	N/A	N/A
20	Pos	36.94	N/A	N/A
Mean C_t (n=20)		35.63	N/A	N/A
SD		0.86	N/A	N/A
CV (%)		2.4	N/A	N/A
Results	20/20			

7.2 Analytical Specificity

The analytical specificity of the swInfA and swH1 primer and probe sets of the CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS instrument was evaluated with respect to potential cross-reactivity with seasonal human influenza A viruses. Analytical specificity (cross-reactivity) was demonstrated by testing each swine primer and probe set (swInfA and swH1) with nucleic acids extracted from two well-characterized seasonal influenza A strains, the Influenza A/Hawaii/15/2001 H1N1 influenza A strain, and the Influenza A/Texas/71/2007 H3N2 influenza A strain. Each virus strain was diluted to 1,000 $TCID_{50}$/mL in viral transport medium. RNA was extracted using the Qiagen QIAamp Viral RNA Mini kit, and analyzed on the JBAIDS instrument. The results are presented in Table 12:

Table 12. Analytical Specificity of the swInfA and swH1 Primer and Probe Sets of the CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS Instrument (Cross-Reactivity)				
Influenza A/Hawaii/15/2001 (H1N1) Virus				
Assay	**$TCID_{50}$/mL**	**Ct**		
		Replicate 1	**Replicate 2**	**Replicate 3**
InfA	1000	28.01	28.01	28.18
swInfA	1000	Neg	Neg	Neg
swH1	1000	Neg	Neg	Neg
Influenza A/Texas/71/2007 (H3N2) Virus				
Assay	**$TCID_{50}$/mL**	**Ct**		
		Replicate 1	**Replicate 2**	**Replicate 3**
InfA	1000	31.29	31.11	31.21
swInfA	1000	Neg	Neg	Neg
swH1	1000	Neg	Neg	Neg

8. CLINICAL PERFORMANCE CHARACTERISTICS

8.1 Clinical Studies

Performance characteristics of the CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS Instrument were established in retrospective studies testing well-characterized banked clinical specimens in a blinded fashion at two testing sites.

8.1.1 Clinical Testing Site 1

A total of 100 clinical NPS specimens (provided by the Utah State Health Department) tested at site 1 (the 9th Area Medical Lab), including 20 2009 H1N1 Influenza positive, 9 seasonal Influenza A/H1 positive, 5 seasonal Influenza A/H3 positive, 6 Influenza B positive, and 60 Influenza negative specimens, were previously characterized using the CDC Human Influenza Virus Real-time RT-PCR Detection and Characterization Panel (FDA cleared IVD device) and the CDC rRT-PCR Swine Flu Panel (currently under an EUA). No sample was excluded from the performance analysis due to protocol deviation.

The results are presented in Table 13 to Table 16.

Table 13. Results from Retrospective Testing of Banked Clinical Specimens Provided by the Utah State Health Department at Site 1 – InfA

Banked Nasopharyngeal Swabs	Comparator Assays		
CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS Instrument	**Positive**	**Negative**	**Total**
Positive	33	0	33
Negative	1	66	67
Total	34	66	100
			95% CI
Positive Percent Agreement	33/34	97.1%	84.7-99.9%
Negative Percent Agreement	66/66	100.0%	94.6-100%

Table 14. Results from Retrospective Testing of Banked Clinical Specimens Provided by the Utah State Health Department at Site 1 – swInfA

Banked Nasopharyngeal Swabs	Comparator Assays		
CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS Instrument	**Positive**	**Negative**	**Total**
Positive	17	0	17
Negative	3	80	83
Total	20	80	100
			95% CI
Positive Percent Agreement	17/20	85.0%	62.1- 96.8%
Negative Percent Agreement	80/80	100.0%	95.5-100%

Table 15. Results from Retrospective Testing of Banked Clinical Specimens Provided by the Utah State Health Department at Site 1 – swH1

Banked Nasopharyngeal Swabs	Comparator Assays		
CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS Instrument	**Positive**	**Negative**	**Total**
Positive	17	0	17
Negative	3	80	83
Total	20	80	100
			95% CI
Positive Percent Agreement	17/20	85.0%	62.1- 96.8%
Negative Percent Agreement	80/80	100.0%	95.5-100%

Table 16. Results from Retrospective Testing of Banked Clinical Specimens Provided by the Utah State Health Department at Site 1 – by Specimen Characteristics					
Specimen	**Sample Number**	**JBAIDS Correct**	**JBAIDS Incorrect**	**Percent Agreement (%)**	**95% CI**
2009 H1N1 Influenza	20	17	3	85.0	62.1-96.8%
Seasonal Influenza A/H1	9	9	0	100.0	66.4-100.0%
Seasonal Influenza A/H3	5	5	0	100.0	47.8-100.0%
Influenza B	6	6	0	100.0	54.1-100.0%
Influenza Negative	60	60	0	100.0	94.0-100.0%
Total	100	97	3		

8.1.2 Clinical Testing Site 2

A total of 99 clinical specimens provided by the University of Nebraska Medical Center tested at site 2 (Navy Health Research Center) to supplement the primary testing data generated from Site 1, including 24 2009 H1N1 Influenza positive (all NPS), 17 seasonal Influenza A/H1 positive (16 NPS and 1 NW), 2 seasonal Influenza A/H3 positive (all NPS), 10 Influenza B positive (all NPS), and 46 Influenza negative (42 NPS and 4 NW) specimens, were previously characterized using the Luminex Respiratory Virus Panel (FDA cleared IVD device) and the CDC rRT-PCR Swine Flu Panel (currently under an EUA). Six (6) samples (all NPS) were excluded from performance analysis due to protocol deviation (PTC failure). Results are presented in Table 17 to Table 20.

Table 17. Results from Retrospective Testing of Banked Clinical Specimens Provided by the University of Nebraska Medical Center at Site 2 – InfA			
Banked Clinical Specimens	**Comparator Assays**		
CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS Instrument	**Positive**	**Negative**	**Total**
Positive	40	3	43
Negative	1	50	51
Total	41	53	94
			95% CI
Positive Percent Agreement	40/41	97.6%	87.1-99.9%
Negative Percent Agreement	50/53	94.3%	84.3-98.8%

Table 18. Results from Retrospective Testing of Banked Clinical Specimens Provided by the University of Nebraska Medical Center at Site 2– swInfA			
Banked Clinical Specimens	**Comparator Assays**		
CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS Instrument	**Positive**	**Negative**	**Total**
Positive	22	0	22
Negative	1	71	72
Total	23	71	94
			95% CI
Positive Percent Agreement	22/23	95.7%	78.0-99.9%
Negative Percent Agreement	71/71	100.0%	94.9-100.0%

Table 19. Results from Retrospective Testing of Banked Clinical Specimens Provided by the University of Nebraska Medical Center at Site 2 – swH1			
Banked Clinical Specimens	**Comparator Assays**		
CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel on the JBAIDS Instrument	**Positive**	**Negative**	**Total**
Positive	20	0	22
Negative	3	71	72
Total	23	71	94
			95% CI
Positive Percent Agreement	20/23	87.0%	66.4-97.2%
Negative Percent Agreement	71/71	100.0%	94.9-100.0%

Table 20. Results from Retrospective Testing of Banked Clinical Specimens Provided by the University of Nebraska Medical Center at Site 2 – by Specimen Characteristics					
Specimen (NPS)	**Sample Number**	**JBAIDS Correct**	**JBAIDS Incorrect**	**Percent Agreement (%)**	**95% CI**
2009 H1N1 Influenza	23	20	3	87.0	66.4-97.2%
Seasonal Influenza A/H1	15	14	1	93.3	68.0-99.8%
Seasonal Influenza A/H3	2	2	0	100.0	15.8-100.0%
Influenza B	9	8	1	88.9	51.8-99.7%
Influenza Negative	39	37	2	94.9	82.7-99.4%
Total	88	81	7		
Specimen (NW)	**Sample Number**	**JBAIDS Correct**	**JBAIDS Incorrect**	**Percent Agreement (%)**	**95% CI**
2009 H1N1 Influenza	0	0	0		
Seasonal Influenza A/H1	1	1	0	100.0	N/A
Seasonal Influenza A/H3	0	0	0		
Influenza B	0	0	0		
Influenza Negative	4	4	0	100.0	39.8-100.0%
Total	5	5	0		