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 As of June 23, 2010, the EUAs have been terminated and this information is no longer current.

D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit

For *In Vitro* Diagnostic Use

I. INTENDED USE

The D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit (D^3 2009 Flu-A ID Kit) uses a blend of 2009 H1 influenza A virus antigen-specific murine monoclonal antibodies that when combined with a fluorescein-labeled conjugate is intended for the detection of 2009 H1N1 influenza A viral antigens present in infected cells directly from nasal and nasopharyngeal swabs and aspirates/washes specimens or cell culture from individuals with signs and symptoms of respiratory infection who have previously tested positive for the presence of influenza A virus-infected cells by a currently available FDA-cleared direct immunofluorescence influenza A antibody device.

Testing for the 2009 H1N1 influenza virus 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 2009 H1N1 influenza virus infection and should not be used as the sole basis for treatment or other patient management decisions.

II. SUMMARY AND EXPLANATION OF THE TEST

With the addition of new antiviral drugs for the treatment of Influenza[1], more rapid and sensitive tests for respiratory virus detection[2,3] and the increasing need to be more discriminating in the use of antibiotics[4], early detection and identification of the infecting viral agent has grown substantially in importance. Viral identification is becoming increasingly important in ruling out bacteria as the cause of respiratory infections. Virus identification by either direct antigen detection or cell culture using fluorescent monoclonal antibodies continues to be the standard method in virology laboratories.

Influenza A and B

Influenza viruses (family *Orthomyxoviridae*) contain a single-stranded RNA genome which is present in 8 separate segments of ribonucleoprotein. This segmentation of the genome is rare among viruses and probably contributes to the rapid development of new influenza strains through interchange of gene segments if two different viruses infect the same cell. There are 3 types of influenza, A, B and C. Type A has counterparts in birds and pigs as well as humans, while types B and C are known only in man.
Influenza infects an estimated 120 million people in the US, Europe and Japan each year and it is estimated that in the US there are 75,000 deaths annually from pneumonia caused by influenza. Primary viral pneumonia or pneumonia from secondary bacterial infections are the primary causes of morbidity of the viral infection.[5] Pandemics of influenza A occur about every 10 to 30 years and epidemics of either influenza A or B occur annually. Infections are seasonal, typically extending from November to April in the northern

hemisphere. Complications tend to occur in the young, elderly and persons with chronic cardio-pulmonary diseases.
The new 2009 H1N1 influenza virus (swine flu) was first identified in the spring of 2009. The symptoms of the 2009 H1N1 are very similar to the seasonal strains of influenza. Since its identification the CDC estimates:

- Between 14 million and 34 million cases of 2009 H1N1 occurred between April and October 17, 2009.
- Between about 63,000 and 153,000 2009 H1N1-related hospitalizations occurred between April and October 17, 2009. The mid-level in this range is about 98,000 H1N1-related hospitalizations.
- Between about 2,500 and 6,000 2009 H1N1-related deaths occurred between April and October 17, 2009. The mid-level in this range is about 3,900 2009 H1N1-related deaths.[6]

III. PRINCIPLE OF THE PROCEDURE

The D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit (D^3 2009 Flu-A ID Kit) uses a blend of 2009 H1 influenza A virus antigen-specific murine monoclonal antibodies that when combined with a fluorescein-labeled conjugate is intended for the identification of 2009 H1N1 influenza A viral antigens present in infected cells directly from nasal and nasopharyngeal swabs and aspirates/washes specimens or cell culture from individuals with signs and symptoms and previously tested positive for the presence of influenza A virus-infected cells by a currently available FDA-cleared direct immunofluorescence influenza A antibody device.

The presence of influenza A virus-infected cells in either the nasopharyngeal specimen or culture must be established using a FDA-cleared device prior to testing with the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit.

The identification of 2009 H1N1 influenza should be performed along with a clinical and epidemiological assessment.

The cells to be tested, derived from a clinical specimen or cell culture, are placed onto a glass slide and allowed to air dry. The cells are fixed in acetone. The D^3 Ultra 2009 Flu-A ID Reagent is added to the cells which are then incubated for 15- to 30-minutes at 35° to 37°C in a humidified chamber or humidified incubator. The stained cells are then washed with the diluted phosphate buffered saline (1X PBS). The D^3 Flu-A ID conjugate is added to the cells which are then incubated for 15- to 30-minutes at 35° to 37°C in a humidified chamber or humidified incubator. The stained cells are then washed with 1X PBS, a drop of the supplied Mounting Fluid is added and a coverslip is placed on the prepared cells. The cells are examined using a fluorescence microscope. If 2009 H1 influenza A virus is present the infected cells will fluoresce apple-green. Non-infected cells and cells infected with influenza A virus non-2009 and will contain no fluorescence but will be stained red by the Evans Blue counter-stain.

IV. REAGENTS

A. Kit Components

1. **D^3 Ultra 2009 Flu-A ID Reagent**, 5.0-mL. One dropper bottle containing a mixture of murine monoclonal antibodies directed against 2009 H1 influenza A virus antigen. The buffered, stabilized, aqueous solution contains 0.1% sodium azide as preservative.
2. **D^3 Flu-A ID Conjugate**, 5.0-mL. An aqueous, stabilized, buffered solution containing fluorescein-labeled, affinity purified goat-anti-mouse IgG antibody and Evans Blue with sodium azide as preservative.
3. **40X PBS Concentrate**, 25-mL. One bottle of 40X PBS concentrate containing 4% sodium azide (0.1% sodium azide after dilution to 1X using de-mineralized water).
4. **Mounting Fluid**, 7-mL. One dropper bottle containing an aqueous, buffer-stabilized solution of glycerol with 0.1% sodium azide.
5. **D^3 Ultra 2009 H1N1 Influenza A Virus ID Antigen Control Slides**, 5-slides. Five (5) individually packaged control slides containing 2 wells with cell culture-derived positive and negative control cells. The positive well contains cells infected with 2009 H1N1 influenza A virus. The negative wells contain non-infected cells. Each slide is intended to be stained only one time.

B. Warnings and Precautions

1. For *in vitro* diagnostic use.
2. Cells may have some potential to be hazardous. Personnel working with these cultures must be properly trained in safe handling techniques[7,8,9], and have experience with tissue culture before attempting this procedure.
3. All procedures must be conducted in accordance with the CDC 5th edition Biosafety in Microbiological and Biomedical Laboratories, 2007, and CLSI Approved Guideline M29-A, Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue.
4. Acetone, a reagent that is required for the test but not provided in the kit, is a flammable, volatile organic solvent. Use it in a well-ventilated area and keep away from flames and other sources of ignition.
5. Sodium azide is included in the 40X PBS Concentrate at 4%, and in the other solutions in this kit at 0.1%. A MSDS for sodium azide or for Diagnostic Hybrids, Inc (DHI) reagents containing sodium azide is available by contacting a Diagnostic Hybrids' Technical Service Representative.
    a. Reagents containing sodium azide should be considered a poison. If products containing sodium azide are swallowed, seek medical advice immediately and show product container or label. [Refer to NIOSH, National Institute for Occupational Safety and Health; CAS#: 2628-22-8; and also to GHS, The Globally Harmonized System of Classification and Labeling of Chemicals.]

b. Aqueous solutions of sodium azide, when mixed with acids, may liberate toxic gas (sodium azide in water exists is ionic equilibrium with hydrazoic acid, which when mixed with acid may liberate a toxic gas).
c. Any reagents containing sodium azide should be evaluated for proper disposal. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. If products containing sodium azide are discarded into a drain, flush with a large volume of water to prevent azide build-up. Check with regulatory agencies to determine at what concentration sodium azide may cause a product to be regulated as hazardous waste.

6. Evans Blue counter-stain is a potential carcinogen. If skin contact occurs, flush with water immediately.
7. The ID Reagent and Conjugate are supplied at working strength. Any dilution of the reagents will decrease sensitivity.
8. Reagents should be used prior to their expiration date.
9. Each Antigen Control Slide should be used only once. Do not re-use a Control Slide.
10. Microbial contamination of reagents may cause a decrease in sensitivity.
11. Store 1X PBS in a clean container to prevent contamination.
12. All specimens and materials used to process them should be considered potentially infectious and handled in a manner which prevents infection of laboratory personnel. Decontamination is most effectively accomplished using a solution of sodium hypochlorite (1:10 final dilution of household bleach).
13. Although Antigen Control Slides have been shown to be non-infectious, the same precautions taken in handling and disposing of other infectious materials should be employed in their use.
14. Never pipette reagents or clinical samples by mouth; avoid all contact of clinical samples with broken skin.
15. Avoid splashing and the generation of aerosols with clinical samples.
16. Use aseptic technique and sterile equipment and materials for all tissue culture procedures.
17. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
18. Do not expose reagents to bright light during staining or storage.
19. Use of other reagents than those specified with the components of this kit may lead to erroneous results.

C. Preparation of 1X PBS Solution

1. After storage at 2° to 8°C, some salts in the 40X PBS Concentrate may have crystallized. Warm the solution to ambient temperature to re-dissolve the crystals and mix.
2. Add contents of the fully dissolved 25-mL 40X PBS Concentrate to 975-mL of de-mineralized water.
3. Label the 1X PBS Solution with a sixty (60) day expiration date after reconstitution and store at ambient temperature (20° to 25°C).

D. Reagent Storage Instructions

TABLE 1: Reagent Storage Conditions	
a. D^3 Ultra 2009 Flu-A ID Reagent	Store at 2° to 8°C in the dark.
b. D^3 Flu-A ID Conjugate	
c. Antigen Control Slides	
d. 40X PBS Concentrate NOTE: The Concentrate may crystallize when stored at 2° to 8°C. The crystals will dissolve when the Concentrate is warmed to ambient temperature.	
e. 1X PBS	Store at ambient temperature (20° to 25°C).

E. Stability

Reagents and components will retain their full potency through the expiration date shown on the label of each bottle when stored at recommended temperatures. Light exposure of the reagents should be kept to a minimum.

Discard 1X PBS Solution if it becomes cloudy.

V. PROCEDURE

A. Materials Provided

1. D^3 Ultra 2009 Flu-A ID Reagent
2. D^3 Flu-A ID Conjugate
3. 40X PBS Concentrate
4. Mounting Fluid
5. Antigen Control Slides

B. Materials Required But Not Provided

1. Fluorescence microscope with the correct filter combination for FITC (excitation peak = 490 nm, emission peak = 520nm).
2. Cover slips (22 x 50mm) for Antigen Control Slides and for specimen slides.
3. Reagent-grade acetone (>99% pure) chilled at 2° to 8°C for fixation of direct specimen slides and shell-vials.
   NOTE: Keep the reagent-grade acetone container tightly sealed to avoid hygroscopic absorption of water, which may cause a hazy, non-specific, background fluorescence.
4. Sterile graduated pipettes: 1 0-mL, 5-mL, and 1-mL.
5. Sterile Pasteur pipettes or other “transfer”-type pipettes.
6. Fine-tipped forceps.
7. 200-mL wash bottle.
8. Sodium hypochlorite solution (1:10 final dilution of household bleach).

9. Humid chamber (e.g., covered Petri dish with a damp paper towel placed in the bottom).
10. Acetone-cleaned multi-well glass microscope slides (2-well masked slides).
11. Blotters for multi-well glass microscope slides: Two-well absorbent blotters, used to blot excess liquid from the mask to prevent spread of liquid or stained cells from one well to the other.
12. Incubator, 35° to 37°C.
13. De-mineralized water for dilution of 40x PBS Concentrate.
14. Wash Container: Beaker, wash bottle or Coplin jar for washing slides.
15. Fixing Container: Coplin jar, slide dish or polyethylene holder for slides for use in fixing the cells on the slides.

C. Preliminary Comments and Precautions

1. Adhere to the recommended volumes and times in the following procedure to ensure that accurate results are obtained.
2. When staining with fluorescent reagents and examining cells microscopically for fluorescence, it is very important to include controls, both positive and negative, to monitor the procedure and performance of the reagents. It is recommended that such controls be run with each batch of patient specimens.
3. The closed, humidified container for holding the slides during incubation should be kept in the incubator so it is at incubator temperature when the slides are placed in it. By doing this, the cells and antibody solution will come up to temperature more rapidly, yielding more intense stains in shorter periods of time.

IMMUNOFLUORESCENCE MICROSCOPY:

1. It is good practice to examine the positive and negative controls before examining the test specimens. If one of these fails to perform as expected, review the steps and conditions under which the test was performed to determine the cause(s). Do not report results until controls perform properly.
2. There are three aspects of the fluorescence microscope that must be functioning properly and optimally in order to achieve maximum brightness of fluorescence:
    i. The activation light source has a finite life and as it ages, its output decreases, resulting in lower fluorescence intensity from the DFA.
    ii. The light source is focused by a number of lenses and mirror(s). For maximum intensity, these must be properly aligned.
    iii. The filters used in the light path must be appropriate for the particular fluor, in this case, fluorescein.
3. There are several fluorescent artifacts that may be observed in the cell monolayers being examined:
    i. Cell debris, lint, etc. can non-specifically adsorb DFAs, resulting in highly intense fluorescence. These can be identified by their morphology, i.e., they don't have the appearance of a complete cell and typically do not appear to be a part of the monolayer like the other cells.

ii. A low grade, yellow-green fluorescence may sometimes be seen, particularly in areas that have piled cells or are near holes in the cell monolayer. In both cases, the diffusion of the entrapped DFAs is retarded during the wash step, resulting in the non-specific fluorescence.
iii. Intense fluorescence around the periphery of slide wells is indicative of drying of the DFA Reagent during incubation, suggesting that it was incubated too long or the humidity was not controlled.
iv. Inadequate removal of the mucus from direct specimens can lead to non-specific adsorption of DFAs.
v. Inadequate washing can lead to a general low grade fluorescence due to residual DFAs remaining on the monolayer of cells.
vi. On direct specimens, beware of trapping of fluorescence by leukocytes and monocytes. Likewise, the presence of RBCs in the specimen may leave a green haze on the sample.

4. Protect stained monolayers from light as much as possible during testing.
   i. Bleaching or fading of the fluorescence of stained cells may occur on exposure to light, particularly light of high intensity.
   ii. This bleaching can occur when a stained cell is microscopically examined for an extended period.

D. Identification Staining

1. Spot 25-µL of the cell suspension on a designated well of a 2-well slide. Repeat this step for each specimen.
   NOTE: The cell suspension must have previously been identified as containing cells expressing influenza A viral antigens via a FDA-cleared device. Please refer to the specific devices product insert for cell suspension processing.
2. Air dry the wells completely.
3. Fix the cells to the slides using fresh acetone for 5- to 10-minutes.
4. Remove the slides from the fixative and allow to air dry.
5. Add one drop of the D^3 Ultra 2009 Flu-A ID Reagent to completely cover the dried, fixed cells on one well of each of the 2-well slides.
6. Add one drop of the D^3 Ultra 2009 Flu-A ID Reagent to each of the wells of a fresh Antigen Control Slide. An Antigen Control Slide should be stained only once.
7. Place the slides in a covered chamber at 35º to 37ºC for 15- to 30-minutes.
8. Rinse the stained cells using the 1X PBS. For only a few slides, this can be done using a beaker of the 1X PBS. For many slides, a slide carrier that holds 10 to 20 slides can be placed in its container of 1X PBS. For effective rinsing, dip the slide(s) up and down a minimum of four times.
9. Discard the used PBS and repeat the washing step using new 1X PBS.
10. Add one drop of the D^3 Flu-A ID Conjugate to completely cover the dried, fixed cells on one well of each of the 2-well slides.
11. Add one drop of the D^3 Flu-A ID Conjugate to each of the wells of a fresh Antigen Control Slide. An Antigen Control Slide should be stained only once.

12. Place the slides in a covered chamber at 35º to 37ºC for 15- to 30-minutes.
13. Rinse the stained cells using the 1X PBS. For only a few slides, this can be done using a beaker of the 1X PBS. For many slides, a slide carrier that holds 10 to 20 slides can be placed in its container of 1X PBS. For effective rinsing, dip the slide(s) up and down a minimum of four times.
14. Discard the used PBS and repeat the washing step using new 1X PBS.
15. Blot the excess PBS, and add a small drop of Mounting Fluid to each cell-containing well and cover the wells with a coverslip.
16. Examine the stained, mounted cells using a fluorescence microscope with magnifications between 200X to 400X. (Refer to Section VII., 'Interpretation of Results').

E. Quality Control

A fresh Antigen Control Slide should be stained each time the staining procedure is performed to ensure proper test performance. The positive wells will show multiple infected cells of bright apple-green fluorescence with non-infected cells staining a dull red due to the included Evans Blue counter-stain. The negative well will show only non-infected cells staining a dull red. Positive and negative controls must demonstrate appropriate fluorescence for specimen results to have validity. Antigen Control Slides may also aid in the interpretation of patient specimens.

VI. INTERPRETATION OF RESULTS

It is recommended that controls be examined first to ensure proper test performance before examination of the specimens. A positive reaction is one in which bright apple-green fluorescence is observed in the infected cells. Non-infected cells will stain dull red due to the Evans Blue counter-stain included in the conjugate. Technologists should not confuse cell clumps which may fluoresce due to entrapment of antibody with virus-specific staining. Occasionally, dead, rounded cells due to specimen toxicity or improper cell storage may non-specifically stain a dull olive green due to trapped antibody. Adequate washing between steps will help to eliminate this type of non-specific staining.

A. Results for Direct Specimen Testing

1. The quality of the specimen with respect to the number of epithelial cells in the sample can be assessed by examining the different fields at a magnification of 200X. A satisfactory specimen should have at least 2 columnar epithelial cells per field. A negative result is indicated by the absence of fluorescence in a minimal sampling of 20 columnar epithelial cells. An inadequate sample is indicated by fewer than 20 columnar epithelial cells present in the sample, in which case the test is considered invalid. The cell suspension should be tested again using a new cell spot.

2. A satisfactory specimen with no fluorescent cells found should be reported as "No 2009 H1N1 influenza A viral antigens detected by direct specimen testing".
3. A positive result is indicated by intact cell exhibiting apple-green fluorescence. Cells expressing the 2009 Flu-A H1 antigen will have a fluorescent outer cell wall. Fluorescence in the cell's cytoplasm or nuclei will rarely be seen.
4. A specimen with fluorescent cells found should be reported as "2009 H1N1 influenza A viral antigens detected by direct specimen testing".

B. Results from Culture Isolation / Confirmation

1. The quality of the cell culture suspension with respect to the number of cells can be assessed by examining the different fields at a magnification of 200X. A satisfactory suspension should have at least 20 cells per field. An inadequate sample is indicated by fewer than 200 cells present in the cell spot, in which case the test is considered invalid. The cell suspension should be tested again using a new cell spot.
2. A satisfactory suspension with no fluorescent cells found should be reported as "No 2009 H1N1 influenza A viral antigens detected in cell culture".
3. A positive result is indicated by intact cell exhibiting apple-green fluorescence. Cells expressing the 2009 Flu-A H1 antigen will have a fluorescent in the cell's cytoplasm or nuclei.
4. A cell culture suspension with fluorescent cells found should be reported as "2009 H1N1 influenza A viral antigens detected in cell culture".

VIII. LIMITATIONS OF PROCEDURE

A. This test has not been evaluated for direct specimen staining on specimens other than respiratory specimens. It is the user's responsibility to establish assay performance for specimens other than respiratory specimens.
B. 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.
C. The prevalence of infection will affect the test's predictive value.
D. As with other tests, negative results do not rule out Influenza A or 2009 H1N1 influenza infections.
E. As with other tests, false positive results may occur. Repeat testing or testing with a different device may be indicated in some settings
F. This test is a qualitative test and does not provide the quantitative value of detected organism present.
G. This test has not been evaluated for patients without symptoms of influenza infection.
H. This test has not been evaluated for monitoring treatment of Influenza A or 2009 H1N1 influenza infection.

I. This test has not been evaluated for screening of blood or blood product for the presence of Influenza A or 2009 H1N1 influenza.
J. This test cannot rule out diseases caused by other bacterial or viral pathogens.
K. Incubation times or temperatures other than those cited in the test instructions may give erroneous results.
L. Detection of viruses will vary greatly depending upon the specimen quality and subsequent handling. A negative result does not exclude the possibility of virus infection. Results of the test should be interpreted in conjunction with information available from epidemiological studies, clinical evaluation of the patient and other diagnostic procedures.
M. The effects of antiviral therapy on the performance of this kit have not been established.
N. The monoclonal antibodies used in this kit are from hybridomas created using inactivated purified virus as the immunogen. Since the monoclonal antibodies have been prepared using defined virus strains, they may not detect all antigenic variants or new strains of the viruses, should they arise. Monoclonal antibodies may fail to detect strains of viruses which have undergone minor amino acid changes in the target epitope region.
O. The viral antigens detected in some direct specimens may be from non-viable virus and cannot be isolated by culture.
P. A negative *direct* specimen should be inoculated into an appropriate cell culture and incubated to isolate and identify any respiratory virus that may be present in the specimen.
Q. A negative result on a direct or cultured specimen does not rule out the presence of virus.
R. Performance of the kit can only be assured when components used in the assay are those supplied by Diagnostic Hybrids.
S. Prolonged storage of the reagents under bright light will decrease the staining intensity.
T. Light background staining may occur with specimens contaminated with *Staphylococcus aureus* strains containing large amounts of protein A. Protein A will non-specifically bind to the Fc portions of conjugated antibodies. Such binding can be distinguished from viral antigen binding on the basis of morphology, i.e., *S. aureus*-bound fluorescence appears as small (~1 micron diameter), bright dots. Results from cell cultures with bacterial contamination must, therefore, be interpreted with caution.

X. SPECIFIC PERFORMANCE CHARACTERISTICS

A. Clinical Studies

1. Direct Specimen

A study was performed using 53 nasopharyngeal swab specimens and the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit. All specimens were identified as containing cells positive for influenza A viral antigens by the D^3 Ultra DFA Respiratory Virus Screening & ID Kit (k092300). Cell spots

were prepared and stained with the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit. The specimens were also tested with the EUA device CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel (according to the device's PI).

TABLE 2: Comparison of the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit and the EUA device CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel in Direct Specimens

Fresh nasal/nasopharyngeal Swab	EUA device swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel (2009 H1N1Result)			
D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit	**Positive**	**Negative**	**Indeterminate***	**Total**
Positive	45	0	4	45
Negative	0	1†	0	1
Total	45	1	4	46
				95% CI
Sensitivity	45/45	100%		92.1-100%
Specificity	1/1	100%		N/A

* Indeterminate – Specimens were positive for InfA and Univ SW only. The four specimens failed to produce Ct values with the primers for the 2009 H1 gene suggesting that the target gene sequence was not amplified**.** Gel electrophoresis of the PCR products revealed a band of the appropriate size for the 2009 H1N1 primer set. PCR amplicon sequencing analysis confirmed the four samples did contain the 2009 H1N1 virus (data not shown). Single nucleotide polymorphisms identified in the probe-binding region likely resulted in the negative rRT-PCR results observed.

† Specimen was positive for InfA only.

2. Culture Identification

A study was performed using 68 clinical isolates that were identified as influenza A virus by the D^3 Ultra DFA Respiratory Virus Screening & ID Kit (k092300). Each master stock virus preparation was diluted to yield an inoculum of ~50-$TCID_{50}$/mL. The virus was inoculated into 2-wells of a 96-well R-Mix cell culture plate. The plates were centrifuged at 700 x g for 60-minutes, and then incubated at 35°C to 37°C for 17-hours. A well from each inoculated isolate was stained with either the D^3 Ultra DFA Respiratory Virus Screening & ID Kit or the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit, and then examined at 200X magnification for the presence of fluorescent staining cells. The isolates were also tested with the EUA device CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel (according to the device's PI).

TABLE 3: Comparison of the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit and the EUA device CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel			
Fresh nasal/nasopharyngeal Swab	EUA device CDC swH1N1 (swine) Influenza Virus Real-time RT-PCR Detection Panel (2009 H1N1Result)		
D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit	**Positive**	**Negative**	**Total**
Positive	34	0	34
Negative	0	34*	34
Total	34	34	68
			95% CI
Sensitivity	34/34	100%	89.7-100%
Specificity	34/34	100%	89.7-100%

* Specimens were positive for InfA only

B. Analytical Sensitivity

1. Limit of Detection Study

Detection limit of the D^3 Ultra 2009 Flu-A ID Reagent was established using the D^3 Ultra DFA Respiratory Virus Screening & ID Kit as a comparator. Analytical detection limits for four 2009 H1N1 isolates were addressed with results reported in numbers of fluorescent staining cells per cell monolayer. Each master stock (~3e5-$TCID_{50}$ per mL) virus preparation was diluted in a ten-fold manner. Twelve wells of a 96-well R-Mix cell culture plate were inoculated with increasing dilution levels. The plates were centrifuged at 700xg for 60-minutes, and then incubated at 35°C to 37°C for 17-hours. Each well was stained with the each device then examined at 200X magnification and the number of fluorescent staining cells counted. The level of detection for the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit was similar to the D^3 Ultra DFA Respiratory Virus Screening & ID Kit (k092300). The table below lists the virus along with each well's cell count.

TABLE 4: Limit of Detection for D^3 Ultra 2009 Flu-A ID Reagent compared with D^3 Ultra Influenza A DFA Reagent			
Virus strain	**Virus per Inoculum**	**Fluorescent Staining Cells per Well**	
		D^3 2009 Ultra Flu-A ID Reagent Subject Device	**D^3 Ultra Influenza A DFA Reagent Predicate Device**
Influenza A A/California/07/2009	11-$TCID_{50}$	7, 4, 6, 7, 7, 7, 4	15, 16, 12, 5, 6, 3
	1.1-$TCID_{50}$	2, 1, 2, 1, 3, 0	2, 2, 4, 1, 1, 2
	0.11-$TCID_{50}$	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
	0.011-$TCID_{50}$	0, 0, 0, 0, 0, 0	0, 0, 1, 0, 0, 0

TABLE 4: Limit of Detection for D^3 Ultra 2009 Flu-A ID Reagent compared with D^3 Ultra Influenza A DFA Reagent			
	0.0011-$TCID_{50}$	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
Influenza A A/Mexico/4108/2009	17-$TCID_{50}$	5, 6, 10, 15, 12, 8	13, 17, 15, 13, 14, 15
	1.7-$TCID_{50}$	1, 0, 2, 1, 2, 0	2, 1, 2, 0, 1, 1
	0.17-$TCID_{50}$	0, 0, 0, 0, 0, 1	2, 1, 0, 0, 1, 0
	0.017-$TCID_{50}$	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
	0.0017-$TCID_{50}$	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
Influenza A 2009 Clinical Isolate C10	24-$TCID_{50}$	17, 14, 15, 13, 5, 24	16, 26, 19, 26, 8, 17
	2.4-$TCID_{50}$	5, 5, 4, 3, 4, 3	1, 2, 0, 3, 5, 0
	0.24-$TCID_{50}$	3, 0, 2, 0, 0, 0	0, 1, 0, 0, 0, 1
	0.024-$TCID_{50}$	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
	0.0024-$TCID_{50}$	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
Influenza A 2009 Clinical Isolate 859	11-$TCID_{50}$	7, 4, 3, 10, 4, 10	3, 10, 8, 11, 11, 6
	1.1-$TCID_{50}$	3, 0, 0, 2, 0, 0	2, 1, 1, 0, 0, 0
	0.11-$TCID_{50}$	0, 0, 0, 1, 0, 1	1, 0, 0, 0, 0, 0
	0.011-$TCID_{50}$	0, 0, 0, 0, 1, 0	0, 0, 0, 0, 0, 0
	0.0011-$TCID_{50}$	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0

2. Cross-Reactivity

The Cross-reactivity of the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit was evaluated using 14 non-2009 H1N1 isolates (8- influenza A subtype H1N1, 6- influenza A subtype H3N2). Each viral strain was grown in duplicate R-Mix cell cultures. Individual cultures were stained concurrently with the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit and the D^3 Ultra DFA Respiratory Virus Screening & ID Kit (k092300). The D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit was non-reactive with all of the cultures.

TABLE 5: D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit Analytical Specificity			
Organism	**Strain or Type (1000-$TCID_{50}$)**	**D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit**	**D^3 Ultra DFA Respiratory Virus Screening & ID Kit (k092300)**
Influenza A	PR/8/34 VR-95 (H1N1)	Negative	Positive
	Denver/1/57 VR-546 (H1N1)	Negative	Positive
	N J/8/76 VR-897 (HSWN1)	Negative	Positive
	WS/33 VR-1520 (H1N1)	Negative	Positive
	Mal/302/54 VR-98 (H1N1)	Negative	Positive
	NWS/33 VR-219 (H1N1)	Negative	Positive
	Brisbane/59/2007 (H1N1)	Negative	Positive
	Solomon Islands (H1N1)	Negative	Positive
	A/Port Chalmers/1/73 VR-810 (H3N2)	Negative	Positive
	A/Wisconsin/67/2005 (H3N2)	Negative	Positive
	A/Hong Kong/8/68 VR-544 (H3N2)	Negative	Positive
	A/Victoria/3/75 VR-822 (H3N2)	Negative	Positive
	A/Aichi/2/68 VR-547 (H3N2)	Negative	Positive
	Uruguay (H3N2)	Negative	Positive

D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit was tested for cross-reactivity against a variety of microorganisms. Stringent conditions for cross-reactivity

testing were achieved by using both the 2 X concentration of D3 Ultra 2009 H1N1 ID reagent and relatively high titers of microorganisms. No cross-reactivity was observed for 29 non-influenza A virus strains. Twenty-one (21) bacterial strains, one yeast, and one *Chlamydia spp.* were also evaluated for cross-reactivity, including *Staphylococcus aureus,* a protein-A-producing bacterium. Except for *Staphylococcus aureus*, which was cross-reactive with the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit, all other microorganisms tested negative.

- Twenty-nine (29) non-influenza A virus strains ATCC derived respiratory viruses were inoculated into R-Mix cultures at 1000-$TCID_{50}$ for a 24-hour incubation time at 37°C. The infected cultures were acetone-fixed and stained with a 2X concentration of the D3 Ultra 2009 H1N1 ID reagent for 1-hour at 37°C. The cultures were stained again using the Flu-A conjugate (i.e. goat anti-mouse counter-stain) for 15 minutes. Afterwards, the stained cultures examined at 200X magnification for any fluorescent staining cells. All infections were verified using the D^3 *Ultra* Respiratory Typing reagents except for parainfluenza 4a and b. An in-house RUO MAb was used as the positive control. The results are summarized in the Table 6 below:

TABLE 6: Respiratory Virus Cross Reactivity Testing

Organism	**Strain or Type**	**[Positive (+) or Negative (-) for Reactivity]**	**Concentrations of targets (viruses: $TCID_{50}$ inoculum level)**
Viruses			
Adenovirus	Type 1	-	1000-$TCID_{50}$
	Type 3	-	1000-$TCID_{50}$
	Type 5	-	1000-$TCID_{50}$
	Type 6	-	1000-$TCID_{50}$
	Type 7	-	1000-$TCID_{50}$
	Type 8	-	1000-$TCID_{50}$
	Type 10	-	1000-$TCID_{50}$
	Type 13	-	1000-$TCID_{50}$
	Type 14	-	1000-$TCID_{50}$
	Type 18	-	1000-$TCID_{50}$
	Type 31	-	1000-$TCID_{50}$
Influenza B	Hong Kong	-	1000-$TCID_{50}$
	Maryland	-	1000-$TCID_{50}$
	Mass	-	1000-$TCID_{50}$
	GL	-	1000-$TCID_{50}$
	Taiwan	-	1000-$TCID_{50}$
	JH-001 Isolate	-	1000-$TCID_{50}$
	Russia	-	1000-$TCID_{50}$
RSV	Long	-	1000-$TCID_{50}$
	Wash	-	1000-$TCID_{50}$

TABLE 6: Respiratory Virus Cross Reactivity Testing			
Organism	**Strain or Type**	**[Positive (+) or Negative (-) for Reactivity]**	**Concentrations of targets (viruses: $TCID_{50}$ inoculum level)**
	9320	-	1000-$TCID_{50}$
Parainfluenza 1	C-35	-	1000-$TCID_{50}$
Parainfluenza 2	Greer	-	1000-$TCID_{50}$
Parainfluenza 3	C-243	-	1000-$TCID_{50}$
Parainfluenza 4	M-25	-	1000-$TCID_{50}$
Parainfluenza 4a	CH-19503	-	1000-$TCID_{50}$
Metapneumovirus	A1	-	1000-$TCID_{50}$
Metapneumovirus	B1	-	1000-$TCID_{50}$
Metapneumovirus	B2	-	1000-$TCID_{50}$

- Twenty three (23) microorganisms, including 21 bacterial, 1 yeast, and 1 *Chlamydia spp.* were tested for cross-reactivity. Bacteria were cultured, processed as suspensions, then 10-µL of the suspension dried and fixed onto a glass slide at levels (as CFUs, colony-forming units) ranging from 2.3×10^7 to 5.2×10^{12} CFUs depending on the bacterium. These bacterial cell spots were then stained with a 2X concentration of the D3 Ultra 2009 H1N1 ID reagent for 1-hour at 37°C. The spots were rinsed and stained again using the Flu-A conjugate (i.e. goat anti-mouse counter-stain) for 15 minutes. Afterwards, the stained bacteria were examined at 200X magnification for any fluorescent staining cells. Except for *Staphylococcus aureus*, which was cross-reactive with the D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit, all other microorganisms tested negative. Reactivity with *Staphylococcus aureus* is more than likely due to binding the protein A produced by *Staphylococcus aureus*. Microorganisms tested are listed in Table 7 below:

TABLE 7: Microorganisms Tested for Cross-Reactivity with D^3 FastPoint L-DFA Respiratory Virus Identification Kit			
	Organism	**D^3 Ultra 2009 H1N1 Influenza A Virus ID Kit**	**CFU tested**
Bacteria	*Acinetobacter calcoaceticus*	-	4.17e12 CFU
Bacteria	*Bordetella bronchiseptica*	-	3.10e7 CFU
Bacteria	*Bordetella pertussis*	-	3.30e12 CFU
Bacteria	*Corynebacterium diphtheriae*	-	2.30e7 CFU
Bacteria	*Escherichia coli*	-	1.71e12 CFU
Bacteria	*Gardnerella vaginalis*	-	5.60e11 CFU
Bacteria	*Haemophilis influenzae type A*	-	7.50e11 CFU
Bacteria	*Klebsiella pneumoniae*	-	2.01e12 CFU
Bacteria	*Moraxella cartarrhalis*	-	1.50e12 CFU
Bacteria	*Mycoplasma hominis*	-	In culture
Bacteria	*Mycoplasma orale*	-	In culture
Bacteria	*Mycoplasma pneumoniae*	-	In culture
Bacteria	*Proteus mirabilis*	-	2.67e12 CFU
Bacteria	*Pseudomonas aeruginosa*	-	5.20e12 CFU
Bacteria	*Salmonella enteriditis*	-	1.27e12 CFU

Bacteria	*Salmonella typhimurium*	-	1.65e12 CFU
Bacteria	*Staphylococcus aureus*	+*	3.20e7 CFU
Bacteria	*Streptococcus agalactiae*	-	3.99e12 CFU
Bacteria	*Streptococcus pneumoniae*	-	4.10e7 CFU
Bacteria	*Streptococcus pyogenes*	-	3.10e12 CFU
Bacteria	*Ureaplasma urealyticum*	-	In culture
Chlamydia sp.	*Chlamydia trachomatis*	-	Control slide
Yeast	*Candida glabrata*	-	2.00e12 CFU

XI. BIBLIOGRAPHY

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* Staining of *S. aureus* appeared as small points of fluorescence while all other cultures were negative. This has been noted in labeling in the section "Limitations of the Assay": The Protein A produced by the bacterium, *Staphylococcus aureus*, will bind the Fc portion of some of the fluorescein-labeled monoclonal antibodies used in this kit. Such binding can be distinguished from viral antigen binding on the basis of morphology, i.e. *S. aureus*-bound fluorescence appears as small (~1 micron diameter), bright dots. Results from cell cultures with bacterial contamination must, therefore, be interpreted with caution.