INTENDED USE

The AV Avantage™ A/H5N1 Flu Test is intended for the *in vitro* qualitative detection of influenza A/H5N1 virus directly from symptomatic patient nasal or throat swab specimens or in viral cultures for the presumptive laboratory identification of influenza A/H5N1 virus.

Results from testing with the AV Avantage™ A/H5N1 Flu Test should be used in conjunction with other laboratory testing and clinical and epidemiological risk factors for the presumptive identification of patients infected with Influenza H5N1 virus. AV Avantage™ A/H5N1 Flu Test is intended as a Prescription Use device.

Testing should not be performed unless the patient meets the most current U.S. Department of Health and Human Services (HHS) clinical and epidemiologic criteria for testing suspect A/H5N1 specimens. The definitive identification of influenza A/H5N1 either directly from patient specimens or from viral cultures requires additional laboratory testing, along with clinical and epidemiological assessment in consultation with national influenza surveillance experts.

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

SUMMARY AND EXPLANATION

Influenza is one of the most common acute, highly contagious respiratory infections in humans, caused by immunologically diverse single-stranded-RNA viruses. Among the three types of influenza viruses: A, B, and C, type A viruses are the most prevalent and are associated with most serious epidemics. There are many subtypes of type A influenza viruses, distinguished on the basis of different surface proteins (hemagglutinin [HA] and neuraminidase [NA] proteins). The H5N1 subtype of influenza A causes the "Avian Flu" found chiefly in birds. However, confirmed cases of human infection from several subtypes of avian influenza have been reported since 1997, with significant virulence and a high mortality rate. Symptoms of avian influenza in humans have included typical influenza-like symptoms (e.g., fever, sore throat, cough, and muscle aches) as well as more severe respiratory diseases such as pneumonia and acute respiratory distress, eye infections and other severe complications.

Arbor Vita's assay technology specifically detects the avian influenza A/H5N1 virus. The assay is based on detection of the influenza virus nonstructural protein 1 (NS1) in specimens from individuals infected with influenza viruses. The influenza A virus NS1 proteins bind to PDZ domains. PDZ domains are important intracellular receptors that recognize a sequence motif at the carboxy-terminus of their target proteins. The PDZ binding motifs or PDZ ligands (PL) of NS1 are unique, and different in H5N1 isolated from humans as compared to human seasonal influenza isolates[2]. The AV Avantage™ A/H5N1 Flu Test is an immunochromatographic assay, in which the PDZ domain is used as a capture reagent and highly sensitive monoclonal antibodies are used as detection reagents. Line 1 of the test contains PDZ domain 2 from the PSD 95 protein (GI:3318653)[8] and preferentially binds NS1 from H5N1 and Line 2 of the test contains PDZ domain 8 from the INADL protein (GI:3123565)[9] and preferentially binds NS1 from the seasonal influenza A subtypes, H1N1 and H3N2. The AV Avantage™ test has been demonstrated to specifically detect NS1 protein from H5N1 Clades 1, 2.1, 2.2, and 2.3. Other subtypes of influenza A viruses that express NS1 protein with identical binding domain sequence motifs may also be detected.

The test permits a sample to be characterized as H5N1-Positive or H5N1-Negative. This test has been shown to detect H5N1 influenza viruses, originally isolated from infected humans, which have been grown in culture as a low passage and spiked into a human negative sample background. However, the

performance of this test with specimens isolated directly from infected humans is unknown due to their rarity.

PRINCIPLE OF THE TEST

The AV Avantage™ A/H5N1 Flu Test is a rapid diagnostic device that detects the presence of the H5N1 strain from nasal swabs or throat swabs collected from symptomatic patients with flu symptoms. It is an immunoassay, using a combination of monoclonal antibodies and recombinant proteins containing PDZ domains to capture and detect NS1.

The AV Avantage™ A/H5N1 Flu Test begins with the extraction of the influenza A H5N1 NS1 viral antigen. The patient sample is prepared by delivering the swab to the transport medium. Sample is then transferred to the Sample Reaction Tube. Next, the Sample Extraction Buffer is added to lyse and condition the sample. The solution is then added to the sample well of the Cassette, where NS1 in the specimen will react with gold-conjugated monoclonal anti-influenza A antibodies as well as capture reagents immobilized on the membrane of the cassette. The gold-conjugated monoclonal anti-influenza A antibodies recognize a broad range of influenza A subtypes and strains. The results are interpreted visually by observing the presence or absence and relative intensity of the color lines at the indicated locations on the membrane. The final test interpretation is influenza A/H5N1 positive or influenza A/H5N1 negative.

REAGENTS AND MATERIALS SUPPLIED

21-Test Kit, Part Number 1000000, Kit box contains the following:

21 **Sample Reaction Tubes:** tube where the specimen is mixed with the Sample Extraction Buffer.

1 **AV Avantage™ A/H5N1 Flu Test Sample Extraction Buffer** tube (green cap) (PN10000410): detergents, salts, and buffering agents.

21 **AV Avantage™ A/H5N1 Flu Test Cassette** pouches (PN1000100): individually wrapped in heat sealed pouches containing a desiccant**.**

1 **AV Avantage™ A/H5N1 Flu Test Positive Control** vial (PN1000500): H5N1 NS1 recombinant protein from A/Viet Nam/1194/2004 (H5N1) isolate. This reagent is not pathogenic.

1 **Procedure Card**

Note: electronic copy of the Package Insert (SP1001100) available from manufacturer.

MATERIALS REQUIRED BUT NOT SUPPLIED

- ❑ Liquid transfer device (pipette)
- ❑ Remel Sterile Traditional Flocked Swabs (part of Remel M6™ Kit, Cat. #R12568)
- ❑ M6™ Viral Transport Media (VTM) (part of Remel M6™ Kit, Cat. #R12568)
- ❑ Timer or watch
- ❑ Sterile barrier tips for pipette
- ❑ Tube rack
- ❑ Tool to remove crimp caps (optional)

WARNINGS AND PRECAUTIONS

- For *In Vitro* Diagnostic Use.
- The AV Avantage™ A/H5N1 Flu Test is indicated for use only in high complexity laboratories.
- Although this test has been shown to detect cultured human-derived Influenza A subtype H5N1 virus, the performance characteristics of this test with specimens from humans infected with H5N1 or other avian influenza viruses are unknown.
- May also detect other subtypes of influenza A viruses that express NS1 protein with the following binding domain sequence motifs: ESKV, ESEV, and ESEI.
- It has been reported[2] that binding domains detected by the AV Avantage A/H5N1 Flu Test can be present in viruses infecting swine and equine hosts. The performance of the test with viruses infecting swine and other animal hosts, or specimens from humans infected with swine influenza viruses has not been established.
- Monoclonal antibodies or PDZ's may fail to detect or show lower sensitivity of detection with influenza A viruses that have undergone reassortment or amino acid changes in the target epitope region.
- The performance of the AV Avantage™ A/H5N1 Flu Test with specimens obtained from humans infected with influenza A H7N3, H7N7, and H7N2 viruses has not been established.
- Do not use the kit contents beyond the expiration date printed on the box.
- To obtain accurate results, you must follow the Package Insert.
- Use of Nitrile or Latex gloves is recommended when handling patient samples.
- Do not re-suspend any of the lyophilized reagents in advance of running the test. After the test has been completed, discard any remaining re-suspended Lysis Buffer or Loading Buffer.
- Dispose of containers and used contents in accordance with Federal, State and Local Government requirements.
- Ensure appropriate precautions are taken in the collection, handling, storage, and disposal of patient samples and used kit contents; otherwise the test performance may be compromised.
- The cassette must remain sealed in the protective foil pouch until just before use.
- The Sample Extraction Buffer contains detergents and salts. If the solution contacts the skin or eye, flush with copious amounts of water.
- The Sample Extraction Buffer also contains 0.06% of sodium azide. Sodium azide is a toxic chemical and reagents containing sodium azide should be handled carefully. Care should be taken to avoid contact with skin. Sodium azide is harmful if ingested. Sodium azide may also react with lead and copper plumbing to form explosive compounds. Precaution should be taken when disposing material containing sodium azide by flushing with copious amount of water to prevent build-up in the drainage system.
- Use only the Transport Media recommended in the Package Insert.
- If infection with A/H5N1 influenza virus is suspected, based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions for novel virulent influenza viruses and sent to state or local health departments for testing.
- Specimen processing should be performed in accordance with national biological safety regulations

- Handle all specimens as if infectious using safe laboratory procedures. Refer to Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition for standard biological safety guidelines for all procedures. (htpp://www.cdc.gov/biosafety/)
- Perform all manipulations of live virus samples within a Class II (or higher) Biological Safety Cabinet (BSC).
- Viral culture should not be attempted in cases of suspected A/H5N1 influenza virus infection unless a BSL 3+ facility is available to receive and culture specimens.
- **Note**: **Novel influenza A, re-emergent or mutant human strains of influenza A viruses are new as opposed to those strains commonly circulating in humans that cause common seasonal epidemics and to which human populations have residual or limited immunity (either by vaccination or previous infection).**

Important Public Health and Surveillance Information

Centers for Disease Control and Prevention (CDC) recommends maintaining the enhanced surveillance efforts by state and local health departments, hospitals, and clinicians to identify patients at increased risk for avian influenza A (A/H5N1). Guidelines for enhanced surveillance are as follows:

Testing for avian influenza A/H5N1 is indicated for hospitalized patients with

1. Radiographically confirmed pneumonia, acute respiratory distress syndrome (ARDS), or other severe respiratory illness for which an alternate diagnosis has not been established, **AND**
2. History of travel within 10 days of symptom onset to a country with documented A/H5N1 avian influenza in poultry and/or humans. (For a regularly updated listing of A/H5N1-affected countries, refer to OIE website and WHO website).

Testing for avian influenza A/H5N1 should be considered on a case-by-case basis in consultation with state and local health departments for hospitalized or ambulatory patients with:

1. Documented temperature of >38°C (>100.4°F), **AND**
2. One or more of the following symptoms: cough, sore throat, shortness of breath, **AND**
3. History of contact with poultry (e.g., visited a poultry farm, a household raising poultry, or a bird market) or a known or suspected human case of influenza A (A/H5N1) in an A/H5N1-affected country within 10 days of symptom onset.

The above recommendations are subject to change; please refer to current recommendations posted on the CDC website: http://www.cdc.gov/flu/avian/professional/#keyfacts

KIT STORAGE AND STABILITY

Store the kit at 2°C to 30°C (35.6°F to 86°F), away from direct sunlight. Kit contents are stable prior to the expiration date printed on the outer box. Do not freeze any of the kit components.

SPECIMEN COLLECTION, HANDLING

Proper specimen collection, transport, and storage, are critical to the performance of this test.

Clinical Specimen

Throat Swab Specimen: use Remel M6™ Traditional Flocked Swab Kit 3mL (Cat. # R12568, contains M6™ Viral Transport Media and traditional flocked swabs). Using aseptic technique, hold down the tongue with a sterile tongue depressor to prevent it from contaminating the swab with saliva. Vigorously rub the sterile swab across the tonsillar fossa and posterior pharynx. Count 3 seconds to swab each area using a rolling method for a total of 9 seconds. Do not touch cheeks, teeth, gums or saliva with the swab as you withdraw it from the mouth. Place swab in a vial with 3 mL of Remel M6™ Viral Transport Media. Keep the swab inside the tube and break off the shaft to fit into the transport media vial. The specimen should be kept cold immediately after collection (e.g. on crushed ice for a maximum of 60 minutes) and stored under refrigeration at 4°C for longer periods of time. Test samples within 72 hours after collection.

Nasal Swab Specimen: collect a single nasal swab specimen from a patient by carefully inserting a sterile Remel traditional flocked swab, (part of the Remel M6 Traditional Flocked Swab Kit 3mL, Cat. # R12568) as far as 1 cm into the nostril. Rotate the swab and let it rest for 10 seconds to absorb secretions. Place swab in a vial with 3 mL of Remel M6™ Viral Transport Media (VTM). If collecting from both nostrils (optional), use separate swabs for each nostril; both swabs may be placed in the same transport media. Break or cut the shaft so that the swab(s) fits into the transport media vial. The specimens should be kept cold immediately after collection (e.g. on crushed ice for up to 60 minutes) and then stored under refrigeration at 4°C until tested. Specimens may be stored at 4°C for up to 72 hours prior to testing.

Viral Culture Specimen

Inoculate MDCK cell culture according to the manufacturer's (e.g. ATCC) instructions and/or standard laboratory protocol. Observe the cells for cytopathic effect (CPE). When 50-75% CPE is observed, prepare the viral stock by collecting the cell growth media and detached cells. Dilute a portion of the sample with equal volume of M6™ VTM and use immediately on AV Avantage™ A/H5N1 Flu Test. Freeze the remaining supernatant in 0.5 mL aliquots at -70°C.

Specimen Transport and Storage

It is recommended to test the patient specimens as soon as possible after collection. Alternatively, specimens may be stored and/or transported at 2–8°C, in a clean, dry, closed container for up to 72 hours prior to testing.

QUALITY CONTROL

BUILT-IN CONTROL FEATURES

Each AV Avantage™ A/H5N1 Flu Test Kit contains a built-in procedural control feature. When running the test, the appearance of a red Control Line (C) in each test indicates proper functioning of the buffer reagents, capillary flow, and functional integrity of the test strip within the cassette. If the Control Line (C) does not appear, the test is considered Invalid.

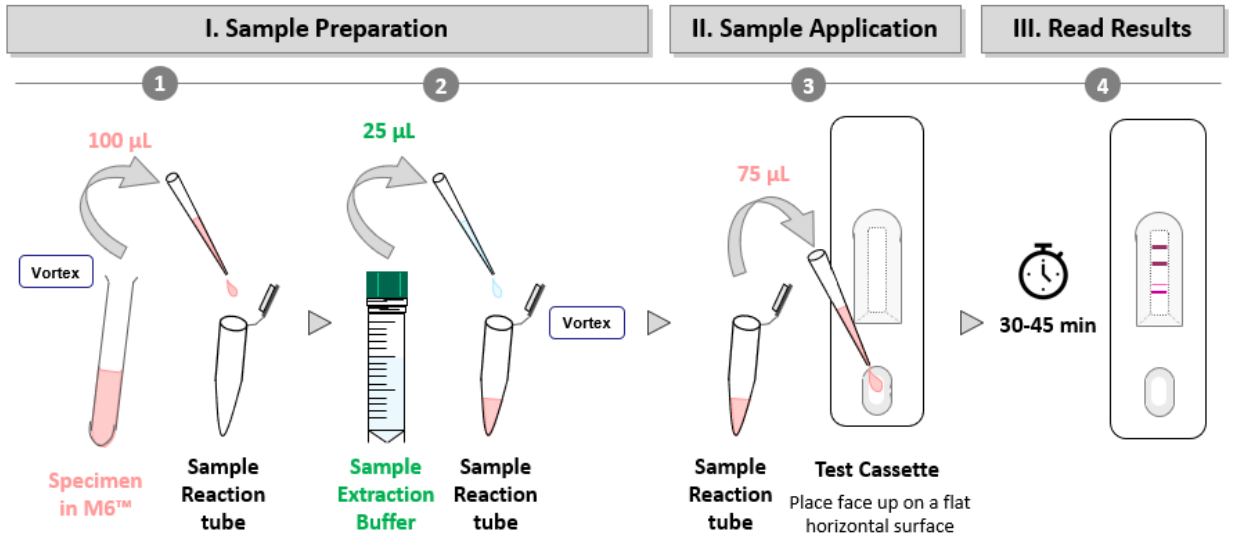
EXTERNAL QUALITY CONTROL

Each AV Avantage™ A/H5N1 Flu Test Kit contains an external **Positive Control**. The positive control contains recombinant influenza A H5N1 NS1 protein in dried form (non-pathogenic), that must be re-suspended before use (see Test Procedure). M6™ VTM should be used as the **Negative Control** (not included in the kit). External positive and negative controls should be performed in conformance with local, state, and federal regulations or accreditation requirements and the user's laboratory's standard quality control procedures. At a minimum, a positive and a negative control test must be performed and the expected results documented prior to using each kit for the first time. The kit should not be used if external control tests do not produce the correct results. Repeat the external control tests as the first step in determining the root cause of the failure. Contact Arbor Vita Corporation Customer Support for assistance when control failures are repeated. The kit should be used for patient specimens only if both the positive and negative controls tests give expected results.

TEST PROCEDURE

Prior to beginning the test, ensure that all clinical specimens and test materials are at room temperature. Check the expiration on each individual reagent vial and outer kit box before using the test. Do not use any tests past the expiration date on the label.

Figure 1. Test Workflow



I. SAMPLE PREPARATION

1.1. Obtain a Sample Reaction Tube and label it as appropriate.

1.2. Obtain the sample in the M6™ VTM tube, gently vortex it for 3 seconds, and transfer 100 µL to the Sample Reaction Tube using a pipette.

1.3. Add 25 µL of AV Avantage™ A/H5N1 Flu Test Sample Extraction Buffer (green cap) to the Sample Reaction Tube.

1.4. Gently vortex the Sample Reaction Tube, at the lowest setting, for 1 second to mix the solution.

Immediately proceed to sample application.

II. SAMPLE APPLICATION

2.1. Remove the AV Avantage™ Flu Test Cassette from its pouch. Do not touch the sample well or read-window openings on the cassette.

2.2. Place the cassette face up on a flat horizontal surface and label it as appropriate.

2.3. Transfer 75 µL of sample solution from the Sample Reaction Tube into the cassette sample well.

2.4. Incubate for 30-45 minutes.

III. READ AND INTERPRET RESULTS

IMPORTANT: readings beyond the 30-45 minutes window of time may affect accuracy of results.

3.1. Appearance of red line(s) indicates a reaction.

3.2. Lines can occur at any of four distinct positions and only three of them, the Control Line (C), Line 1 and Line 2 are interpreted visually for test interpretation (any line appearing at position 3 is not taken into consideration for the test interpretation) – refer to Figure 2. Depending on the pattern, the output of the test is H5N1 Flu Positive, or Negative, or Invalid.

3.3. A Positive test result can be seen as early as after 5 minutes of incubation.

3.4. A Negative test result can only be confirmed after 45 minutes of incubation.

Figure 2. AV Avantage™ A/H5N1 Flu Test Cassette v1.1 line positions and interpretable lines

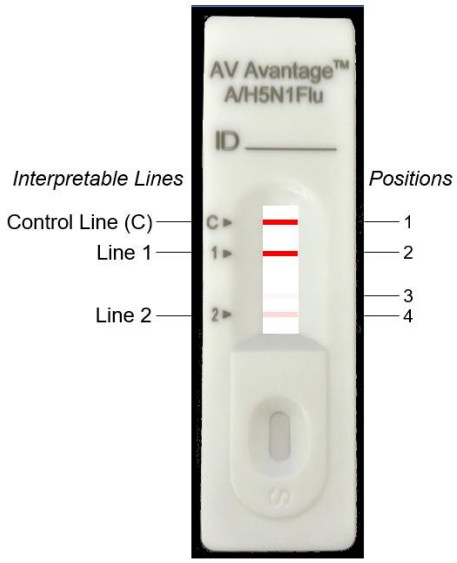
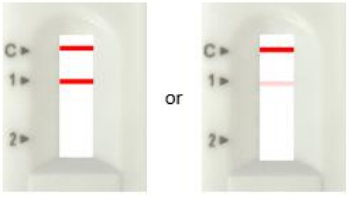
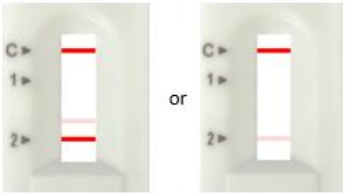
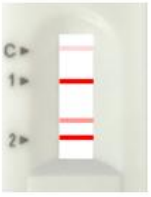


Figure 3. Test Interpretation

Test is **NEGATIVE** when	Test is **POSITIVE** when	Test is **INVALID** when
 Only Control Line (C) is present	 Control line (C) is present as well as Line 1 only	 No control line (C) is seen
 Control Line (C) is present as well as Line 2 only	 Control line (C) is present, and Line 1 intensity is greater than Line 2 intensity	 Both Line 1 and Line 2 intensities are greater than the Control Line (C) intensity Note: in that case, dilute sample 1:10 in M6™ medium and repeat test
 Control Line (C) is present, and Line 2 intensity is greater than or equal to Line 1 intensity		

3.5. **In case of a positive test, report the specimen to be presumptive positive for influenza A/H5N1 virus and notify immediately the appropriate local, state or federal public health authorities to coordinate transfer of the specimen for additional testing.**

3.6. For an invalid test which shows both Line 1 and Line 2 intensities greater than the Control Line (C) intensity, dilute sample 1:10 in M6™ VTM and repeat the AV Avantage™ A/H5N1 Flu Test with the diluted sample. This result may occur with samples having high viral concentrations such as viral culture specimens.

IV. RUNNING CONTROLS

4.1. Positive Control

The AV Avantage™ A/H5N1 Flu Test Positive Control provided with the kit contains a lyophilized pellet of recombinant NS1 protein. This reagent is not pathogenic.

Note: control must be reconstituted in M6™ VTM prior to use. M6™ VTM is not provided with the kit.

4.1.1. Ensure dried Positive Control material is located at the bottom of the vial. If not, tap vial to bring dried material to the bottom.

4.1.2. Uncap the vial.

4.1.3. Add 100 µL M6™ VTM to the Positive Control vial.

4.1.4. Swirl the Positive Control vial on a flat surface, for about 5-10 seconds, to ensure complete resuspension of the dried Positive Control material.

4.1.5. Add 25 µL of AV Avantage™ A/H5N1 Flu Test Sample Extraction Buffer (green cap) to the Positive Control vial and pipette up and down 5 times to mix.

4.1.6. Swirl the Positive Control vial on a flat surface, for about 5-10 seconds, to ensure complete mixing of the reconstituted Positive Control solution.

4.1.7. Remove an AV Avantage™ A/H5N1 Flu Test Cassette from its pouch. Do not touch the sample well or read-window openings on the cassette.

4.1.8. Place the cassette face up on a flat surface and label it as "(+)".

4.1.9. Transfer 75 µL of the reconstituted Positive Control solution into the "(+)" cassette sample well.

4.1.10. Incubate for 30-45 minutes for maximum signal.

4.1.11. Interpret the result following instructions from section III. The test should be H5N1 Positive.

4.2. Negative Control

M6™ VTM should be used as the Negative Control sample (not supplied in the kit).

4.2.1. Obtain a Sample Reaction Tube and label it as "(–)".

4.2.2. Add 100 µL of the M6™ VTM to the "(–)" Sample Reaction Tube.

4.2.3. Follow the procedure starting at step 1.3 of Section I.

LIMITATIONS

- For *in vitro* diagnostic use only.
- Although this test has been shown to detect cultured human-derived Influenza A subtype H5N1 virus, the performance characteristics of this test with specimens from humans infected with H5N1 or other avian influenza viruses are unknown.
- The performance of the AV Avantage™ A/H5N1 Flu Test with human specimens positive for swine influenza viruses has not been evaluated.
- The AV Avantage™ A/H5N1 Flu Test does not detect avian or human viruses with the binding domain containing the EPEV motif. The EPEV PL does not bind to the PDZ in Line 1 of the test to any measurable extent. The EPEV binding domain was characteristic in clade 0 avian viruses and human derived viruses[3]. No clade 0 infections have been reported since 2002.
- Failure to follow the Test Procedure and Instructions on Interpretations of Test Results may adversely affect test performance and/or invalidate the Test Result.
- If the level of antigen in a sample is below the detection limit of the test, a negative test result may occur.
- Results obtained with this test must be evaluated in conjunction with other laboratory testing and clinical and epidemiological assessments in consultation with influenza surveillance experts.
- Test results do not provide subtyping of influenza A or B infections.
- Negative test results do not exclude the presence of other influenza or non-influenza viral infections.
- Positive test results do not exclude co-infections with other viral or bacterial pathogens.
- The performance of this assay has not been determined in individuals who received an H5N1 vaccine.
- Sensitivity studies of the test included only Clade 2.2 H5N1 viral culture specimens.
- Epstein Barr Virus (EBV) and human Metapneumovirus (hMPV) have not been evaluated for cross-reactivity with the AV Avantage A/H5N1 Flu Test.
- Monoclonal antibodies or PDZ's may fail to detect or show lower sensitivity of detection, with influenza A viruses that have undergone reassortment or amino acid changes in the target epitope region.

CONFIRMED HUMAN CASES OF AVIAN INFLUENZA

Outbreaks of Avian influenza infections are very rare, but localized cases of the Avian Flu (H5N1 subtype) have been documented in various regions of the world, including the Middle East (Egypt, Iraq), Asia and Indonesia. As of January 2011, 518 cases of human H5N1 infection have been observed world-wide, primarily in Indonesia, Vietnam, Egypt, China, and Thailand. The mortality rate has been over 55% among those infected with the virus[10].

The definitive identification of influenza A/H5N1, either directly from patient specimens or from viral cultures, requires additional laboratory testing along with clinical and epidemiological assessment in consultation with national influenza surveillance experts.

PERFORMANCE CHARACTERISTICS

CLINICAL STUDIES

I. Retrospective Study of the AV Avantage™ A/H5N1 Flu Test in Human Derived H5N1 Viral Culture Specimens

Due to the rare occurrence of H5N1 infection and the absence of infection in the United States, sensitivity studies of the AV Avantage™ A/H5N1 Flu Test were performed using H5N1 isolates from infected individuals, collected in the course of WHO/NAMRU-3 (World Health Organization; Naval Medical Research Unit-3) pandemic surveillance and response activities. The 24 H5N1 viruses studied were collected primarily in Egypt, with two from Azerbaijan, and one from Iraq. All isolates studied herein were classified as Clade 2.2 and are part of the CDC global H5N1 repository.

The 24 human-derived H5N1 first or second passage viral culture specimens were grown in MDCK cells or eggs. Included in the study were three H5N1 negative samples. Study personnel were blinded to the true H5N1 status. The reference methods used to verify H5N1-positive status of the viral culture specimens were hemagglutinin inhibition (HAI). Eleven of these specimens were from first passage cultures, and 13 of the specimens were from second passage cultures. The study was conducted in BSL-3 labs at NAMRU-3 by NAMRU-3 personnel. The AV Avantage™ A/H5N1 Flu Test used in this study was performed according to the AVC Test Instructions for Use.

AV Avantage™ A/H5N1 Flu Test results showed 100% positive agreement for all 24 H5N1-positive samples. The three H5N1-negative specimens were reported as H5N1-negative by the AV Avantage™ A/H5N1 Flu Test.

Table 1. Performance Summary - AV Avantage™ A/H5N1 Testing with Viral Culture Samples

NAMRU-3 Comparison Results	Virus Culture (Gold Standard) Results		Performance
	H5N1 (+)	H5N1 (-)	
AV Avantage™ A/H5N1 Flu Test Positive	24	0	100% Positive Agreement* 95% CI = (86.2 %, 100 %)
AV Avantage™ A/H5N1 Flu Test Negative	0	3	100% Negative Agreement 95% CI = (43.8 %, 100 %)
Total	24	3	

*4/24 samples (17%) produced invalid results due to high signal intensities in the initial test. The samples were diluted and retested yielding positive results. See Test Procedure, Section VI.

II. Prospective Study of the AV Avantage A/H5N1 Flu Test in Specimens from Symptomatic Patients

The performance of the AV Avantage A/H5N1Flu Test was assessed in a prospective clinical study during the 2007-2008 U.S influenza season. Symptomatic subjects with a history of fever and (sore throat, rhinorrhea, or cough) were recruited from four clinics at three sites into a respiratory illness surveillance study conducted by the Naval Health Research Center (NHRC). Subjects enrolled on Monday, Tuesday, or Wednesday were included in the AV Avantage testing protocol. This schedule allowed for timely completion of laboratory testing on all samples. Swab specimen collection was attempted from two anatomical sites, nasal and throat, for each subject. For twenty subjects only one specimen was obtained

(15 nasal only, 5 throat only), resulting in a total of 908 specimens for analysis. Of these 464 symptomatic subjects 110 had an influenza infection, 73 subjects had Influenza A (113 positive specimens) and 37 subjects had Influenza B (55 positive specimens). Of the 908 samples tested, invalid results were reported for 13 samples (1.4%) and were not included in the analyses in Table 2. A total of 895 sample results were included in the study.

Reference Method Results:

A portion of each clinical specimen was used to inoculate Madin-Darby Canine Kidney (MDCK) cells and tested for the appearance of cytopathic effect (CPE). Infected cells were recovered from tissue culture and confirmed for influenza A or B by indirect fluorescent antibody (IFA) staining. Low titer influenza A positive samples (eight) were encountered for which subtyping results were not obtained. Further RT-PCR testing demonstrated that all eight samples were positive for H3N2 viruses.

Table 2. Performance Summary - AV Avantage A/H5N1 Testing of Prospectively Collected Samples

NHRC Comparison Results	Virus Culture (Gold Standard) Results				Performance
	H5N1 (+)	Influenza A (+) H5N1 (-)	Influenza B (+) H5N1 (-)	Influenza A&B (-) H5N1 (-)	
AV Avantage™ A/H5N1 Flu Test Positive	0	0	0	0	N/A*
AV Avantage™ A/H5N1 Flu Test Negative	0	113	55	727	100% Specificity 95% CI = (99.57%; 100%)
Total	0	113**	55	727	

Sample status was confirmed by IFA and hemagglutination-inhibition test (HAI).

** No true positive samples were identified by Gold Standard methods.*

*** 8 samples were not subtyped by IFA or HAI, but were determined to be H3 by Lightcycler RT-PCR using primers developed by the Air Force Institute of Operational Health.*

ANALYTICAL PERFORMANCE

Analytical Sensitivity, Reproducibility, and Interference testing were conducted with a full-length recombinant protein H5N1 NS1 analyte (A/Viet Nam/1194/2004 (H5N1) isolate). In addition, the Limit of Detection (LoD) was also established from human-derived H5N1 culture samples.

I. Precision

The precision/repeatability of the AV Avantage™ A/H5N1 Flu Test was demonstrated by conducting within-laboratory testing of a range of recombinant H5N1 NS1 protein analyte concentrations over twelve consecutive days. Precision studies were conducted using recombinant H5N1 NS1 protein spiked into M4™ VTM at levels of 0.4 x LoD (4 pg/100 µL, high negative), at LoD (9 pg/100 µL, low positive), and 2.7 x LoD (24 pg/100 µL, moderate positive). A Negative Control was included in each run. Performance of the assay was consistent with the Negative Control yielding 100% negative results, the High Negative sample

yielding 8 % positive results while the Low Positive and Moderate Positive samples respectively yielded 96 % and 100 % positive results.

Table 3. Summary of Within Lab Precision Results

Sample Type	Protein Concentration (LoD)	Recombinant H5N1 NS1 Positive
High Negative	4 pg/100 µL (0.4 x LoD)	4+/48
Low Positive	9 pg/100 µL (LoD)	46+/48
Moderate Positive	24 pg/100 µL (2.7 x LoD)	48+/48
Negative Control	N/A	0+/24

II. Reproducibility

The reproducibility of the AV Avantage™ A/H5N1 Flu Test was determined by measuring the consistency of assay performance using the Negative Control, High Negative, Moderate Positive, and High Positive recombinant protein H5N1 NS1 samples over five days at three sites with two operators at each site. Since the nonstructural protein 1 (NS1 protein) is not a part of the intact virion but rather is expressed in infected cells and released into secretions and/or media, recombinant NS1 protein expressed in mammalian cells was used for the reproducibility study. Due to safety concerns a reproducibility study with live H5N1 virus infected cells was not performed.

Reproducibility studies were conducted using recombinant H5N1 NS1 protein spiked into M4™ VTM at levels of 0.4 x LoD (3.8 pg/100 µL, High Negative), 2.7 x LoD (24 pg/100 µL, Moderate Positive), and 4.0 x LoD (36 pg/100 µL, High Positive). On the fifth day an additional Challenge Sample was tested at each site. This sample had no H5N1 NS1 protein but did include 250 pg/100 µL of recombinant H1N1 NS1 protein (A/Taiwan/112/1996(H1N1)). The results showed reproducible performance across days, sites and operators (see **Table 4**). Results of interpretations at each site and for each sample type are combined for each operator across the five different days of testing. Visual interpretations are designated as negative (-) or positive (+).

Table 4. Test Reproducibility

Site	Operator	Negative Control	High Negative	Moderate Positive	High Positive	Challenge Sample
1	1	15-/15	15-/15	15+/15	15+/15	3-/3
	2	14-/15	15-/15	15+/15	14+/15	3-/3
2	1	15-/15	15-/15	15+/15	15+/15	3-/3
	2	15-/15	15-/15	15+/15	15+/15	3-/3
3	1	15-/15	15-/15	15+/15	15+/15	3-/3
	2	15-/15	15-/15	15+/15	15+/15	3-/3
Total		89-/90	90-/90	90+/90	89+/90	18-/18

III. Analytical Sensitivity/ Limit of Detection

The Analytical Limit of Detection (LoD) was determined using recombinant NS1 protein from Influenza A virus (A/Viet Nam/1194/2004 (H5N1), (Clade 1)). Serial dilutions of the recombinant H5N1 NS1 protein in M4™ medium were prepared and tested in the AV Avantage A/H5N1 Flu Test assay in quadruplicate. The lowest dilution (12 pg/100µL) expected to yield 4/4 positive results from this titration was then chosen as the nominal (N) and three solutions with concentrations N, 0.5N and 0.75N were prepared. Each of these concentrations and a negative control were tested in replicates of 20 with the AV Avantage A/H5N1 Flu Test. The LoD was determined to be 9 pg/100 µL. The results are summarized in Table 5.

Table 5. AV Avantage A/H5N1 Flu Test LoD Determination with Recombinant NS1 Protein

Concentration H5N1 NS1 pg NS1/100 µL	Visual Interpretation Results
0	0+/5
6	7+/20
9	20+/20
12	20+/20

The limit of detection (LoD) was determined for two H5N1 human-derived viral culture specimens, (both clade 2.2). These specimens were serially diluted in a background of Negative Clinical Pool by a factor of 10 to obtain an initial approximation of the LoD. Additional 1/100 and 1/200 dilutions were performed and tested in replicates of 20 to confirm the LoD. The results are summarized in Table 6.

Table 6. AV Avantage A/H5N1 Flu Test LoD Determination with H5N1 Viral Culture Specimens in a Negative Clinical Pool

Influenza A Virus Isolate Name	Dilution 1:100	Viral Titer, $TCID_{50}$/mL
A/Egypt/14724-NAMRU3/2006(H5N1)	20+/20	36
A/Egypt/3158-NAMRU3/2008(H5N1)	20+/20	134

Note: *Sample A/Egypt/14724-NAMRU3/2006(H5N1) with LoD of 36 $TCID_{50}$/mL corresponds to a NS1 protein concentration of 30 pg NS1/100 µL.*

Sample A/Egypt/3158-NAMRU3/2008(H5N1) with LoD of 134 $TCID_{50}$/mL corresponds to an NS1 protein concentration of 27 pg/100 µL.

IV. Analytical Inclusivity and Reactivity

Clinical reactivity of the AV Avantage A/H5N1 Flu Test with H5N1 viruses from Clade 2.2 was demonstrated by testing 24 independent viral culture samples all identified as Clade 2.2. The NS1 protein PDZ Ligand (or PL) from Clade 2.2 H5N1 viruses is predominantly ESKV (93%) with occasional occurrence of ESEV PL (7%). The results are summarized in Table 7.

Table 7. Confirmed A/H5N1 Specimens Tested with AV Avantage A/H5N1 Flu Test

Passage #	WHO ID #	Isolate Name	Reference
1	2008902289	Influenza A virus (A/Egypt/2289-NAMRU3/2008(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2006902782	Influenza A virus (A/Egypt/2782-NAMRU3/2006(H5N1))	http://www.who.int/csr/disease/influenza/tree_large.pdf
	2008903401	Influenza A virus (A/Egypt/3401-NAMRU3/2008(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2008902546	Influenza A virus (A/Egypt/2546-NAMRU3/2008(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2006903458	Influenza A virus (A/Egypt/3458-NAMRU3/2006(H5N1))	http://www.who.int/csr/disease/influenza/tree_large.pdf
	2006902786	Influenza A virus (A/Egypt/2786-NAMRU3/2006(H5N1))	http://www.who.int/csr/disease/influenza/tree_large.pdf
	2007904082	Influenza A virus (A/Egypt/4082-NAMRU3/2007(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2007910211	Influenza A virus (A/Egypt/10211-NAMRU3/2007(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2006902838	Influenza A virus (A/Azerbaijan/008-208/2006(H5N1))	http://www.who.int/csr/disease/influenza/tree_large.pdf
	2006902834	Influenza A virus (A/Azerbaijan/006-207/2006(H5N1))	http://www.who.int/csr/disease/influenza/tree_large.pdf
	2007904226	Influenza A virus (A/Egypt/4226-NAMRU3/2007(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
2	2006902991	Influenza A virus (A/Egypt/2991-NAMRU3/2006(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2006902992	Influenza A virus (A/Egypt/2992-NAMRU3/2006(H5N1))	http://h5n1.flugenome.org/show_subtypes.php

Table 7. Confirmed A/H5N1 Specimens Tested with AV Avantage A/H5N1 Flu Test

Passage #	WHO ID #	Isolate Name	Reference
	2006914724	Influenza A virus (A/Egypt/14724-NAMRU3/2006(H5N1))	http://www.who.int/csr/disease/influenza/tree_large.pdf http://h5n1.flugenome.org/show_subtypes.php
	2006914725	Influenza A virus (A/Egypt/14725-NAMRU3/2006(H5N1))	http://www.who.int/csr/disease/influenza/tree_large.pdf http://h5n1.flugenome.org/show_subtypes.php
	2008901980	Influenza A virus (A/Egypt/1980-NAMRU3/2008(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2007901902	Influenza A virus (A/Egypt/1902-NAMRU3/2007(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2007902321	Influenza A virus (A/Egypt/2321-NAMRU3/2007(H5N1))	http://www.who.int/csr/disease/avian_influenza/guidelines/H5VaccineVirusUpdate20080214.pdf
	2008903300	Influenza A virus (A/Egypt/3300-NAMRU3/2008(H5N1))	http://www.who.int/csr/disease/avian_influenza/guidelines/200809_H5VaccineVirusUpdate.pdf
	2008903158	Influenza A virus (A/Egypt/3158-NAMRU3/2008(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2007910216	Influenza A virus (A/Egypt/10216-NAMRU3/2007(H5N1))	http://h5n1.flugenome.org/show_subtypes.php
	2007910217	Influenza A virus (A/Egypt/10217-NAMRU3/2007(H5N1))	Sequence not yet on GenBank
	2006900207	Influenza A virus (A/human/Iraq/207-NAMRU3/2006(H5N1))	http://www.who.int/csr/disease/avian_influenza/guidelines/recommendationvaccine.pdf http://h5n1.flugenome.org/show_subtypes.php
	2007910215	Influenza A virus (A/Egypt/10215-NAMRU3/2007(H5N1))	http://h5n1.flugenome.org/show_subtypes.php

Analytical reactivity of the AV Avantage A/H5N1 Flu Test with H5N1 viruses from Clades 1, 2.1, 2.2, and 2.3 was further demonstrated by testing full length recombinant NS1 proteins containing the PLs ESKV, ESEV, and ESEI.

V. Cross Reactivity

The AV Avantage A/H5N1 Flu Test was evaluated for potential cross-reactivity with a total of 49 bacterial and viral isolates. The bacterial isolates were tested at concentrations of approximately $1.5x10^8$ cfu/mL. The viral isolates were used at concentrations of 10^4 – 10^9 $TCID_{50}$/mL, or 10^2 – 10^4 $CEID_{50}$/mL. Testing for each organism was performed in triplicate. None of the pathogens tested showed cross-reactivity with the assay.

Bacterial Panel

Bacteroides fragilis
Bordetella pertussis
Corynebacterium xerosis
Escherichia coli
Haemophilus influenzae
Lactobacillus casei
Legionella pneumonphila
Moraxella catarrhalis
Mycoplasma pneumoniae
Neisseria meningitidis
Neisseria mucosa
Peptostreptococcus anaerobius
Porphyromonas asaccharolyticus
Pseudomonas aeruginosa
Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus pneumoniae
Streptococcus pyogenes Group.A
Streptococcus salivarius
Streptococcus sp. Group B
Streptococcus sp. Group C

Viral Panel

Adenovirus, Type 2
Adenovirus Type 3
Adenovirus Type 7
Adenovirus Type 14
Coronavirus OC 43
Coronavirus 299E
Coxsackievirus Type A9
Coxsackievirus Type B5
Cytomegalovirus
Echovirus Type 2
Echovirus Type 3
Echovirus Type 6
Enterovirus
Herpes simplex virus Type 1
Measles virus
Mumps virus
Parainfluenza virus Type 1
Parainfluenza virus Type 2
Parainfluenza virus Type 3
Rhinovirus Type 1A
Respiratory Syncytial virus Type A
Respiratory Syncytial virus Type B
A2/Wisconsin/67/2005 (H3N2-like)
A/Hiroshima/52/2005 (H3N2-like)
A/Port Chalmers/1/73 (H3N2)
A/PR8/34 (H1N1)
A1/Denver/1/57
B/Hong Kong/5/72

Bioinformatic Analysis and AV Avantage™ A/H5N1 Flu Test Inclusivity and Cross Reactivity

There have been reported cases of humans infected with pathogenic influenza A H7N3, H7N7 and H7N2 subtypes. Based on bioinformatics analysis of the full length NS1 sequences from H7 viruses isolated from humans, the AV Avantage A/H5N1 Flu Test will likely detect samples from such cases as positive. Out of four NS1 PL sequences available in GenBank, two H7N7 NS1 PLs are ESEV, one H7N3 NS1 PL is ESEV, and one H7N2 NS1 PL is ESEI[25] . The performance of the AV Avantage™ A/H5N1 Flu Test with specimens obtained from humans infected with these viruses has not been established.

The AV Avantage™ A/H5N1 Flu Test detects the H5N1 influenza virus NS1 protein by its C-terminal PDZ-ligand sequence (or PL). The PL sequence distribution among influenza A subtypes supports high specificity for all tested H5N1 with no cross reactivity with other subtypes. As shown in the tables, for each subtype there is a predominant NS1 PL sequence in isolates found throughout the world and over time. In addition, these sequences include many strains, suggesting that the PL is highly invariant. The PLs found in H1 and H3 are biochemically distinct from those observed in H5N1. The H1/H3 PLs have been demonstrated to bind to PDZs with different affinities relative to H5N1. Also, in the clinical study 168 patient samples positive for seasonal influenza were tested using the AV Avantage™ A/H5N1 Flu Test without any false positive results for H5N1.

Table 8. Temporal Distribution of PLs

(Analysis of 644* H1N1 and 1647** H3N2 NS1 Sequences).

Year	Subtype (# seqs)	RSEV	RSEI	RSKV	ESEV	RSKI
1918-1954	**H1N1 (25)**	**100%**	0%	0%	0%	0%
	H3N2 (0)	NA	NA	NA	NA	NA
1970-1994	**H1N1 (6)**	**100%**	0%	0%	0%	0%
	H3N2 (140)	0%	0%	**100%**	0%	0%
1995-2005	**H1N1 (304)**	**100%**	0%	0%	0%	0%
	H3N2 (1358)	0%	0%	**99%**	0%	**1%**
2006	**H1N1 (13)**	**85%**	**15%**	0%	0%	0%
	H3N2 (29)	0%	0%	**100%**	0%	0%
2007	**H1N1 (299)**	**78%**	**22%**	0%	0%	0%
	H3N2 (108)	0%	0%	**99%**	0%	**1%**
2008	**H1N1 (0)**	NA	NA	NA	NA	NA
	H3N2 (12)	0%	0%	**100%**	0%	0%

* includes 96 different H1N1 strains

** includes 161 different H3N2 strains

Table 9. Geographical Distribution of PLs

(Analysis of 644* H1N1 and 1647** H3N2 NS1 Sequences).

Region	Subtype	RSEV	RSEI	RSKV	ESEV	RSKI
	H1N1	**100%**	0%	0%	0%	0%
Asia	**H3N2**	0%	0%	**100%**	0%	0%
	H1N1	**100%**	0%	0%	0%	0%
Europe	**H3N2**	0%	0%	**100%**	0%	0%
	H1N1	**84%**	**16%**	0%	0%	0%
N. America	**H3N2**	0%	0%	**98%**	0%	**2%**
	H1N1	**100%**	0%	0%	0%	0%
Oceania	**H3N2**	0%	0%	**100%**	0%	0%*

* includes 96 different H1N1 strains

** includes 161 different H3N2 strains

VI. Interference

Substances commonly encountered in nasal and throat specimens were tested for their potential inhibitory effect on the performance of the AV Avantage A/H5N1 Flu Test. Listed below are the substances and concentrations at which they were tested.

Potentially Interfering Substances

Whole blood (2%)

Mucin (500 μg/mL)

Mouthwash (Scope®) (25%)

Dextromethoraphan (Watussin®) (5 mg/mL)

Acetaminophen (Tylenol®) (10 mg/mL)

Throat lozenge (Cepacol® - cetypyridium chloride, benzocaine and menthol) (25%)

Oxymetazoline (Afrin®) (10%)

Erythromycin (20 μg/mL)

Nasal corticosteroids (triamcinolone) (25 mg/mL)

Zanamivir (Relenza®) (1 mg/mL)

Phenyephrine (Neosynephrine®) (100 mg/mL)

Diphenhydramine (Benadryl®) (1 mg/mL)

Luffa operculata, Galphimia glauca, Histaminum hydrochloricum and sulfur (Zicam®) (1%)

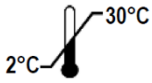
Rimantadine (250 ng/mL)

None of the substances tested had any inhibitory effect on assay performance.

ASSISTANCE

If you have any questions regarding the use of this product, please call Arbor Vita Corporation Customer Support at 650.713.7932, in the U.S.

SYMBOLS

Symbol	Definition
	Catalog Number or Part Number
	*In vitro* diagnostic medical device
	Consult instructions for use
	Do not re-use
	Contains sufficient for 21 tests
	Storage Temperature
	Lot Number
	Use-by Date
	Manufacturer

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