

Flu Detect™ Avian Influenza Type A Antigen Test Kit
For the Surveillance of Avian Influenza
Synopsis of Validation Studies
November 4, 2005

I. INTRODUCTION

This document is a report on the findings of various studies evaluating the efficacy of the Flu Detect™ Avian Influenza Type A Antigen Test Kit in the detection of Type A flu antigen. A number of studies performed in Southeast Asia have shown the kit is able to detect the H5N1 virus at the sensitivity of 10^3 EID₅₀/ml, a rate which matches or exceeds the sensitivity of competitive rapid AIV antigen detection test systems. Materials and methods relevant to each study will be discussed within the description of the particular study.

II. ABSTRACT

Novel diagnostic assays have been developed for the rapid and efficient large-scale screening of Avian Influenza (AI) virus and associated antibodies. One of these tests is Synbiotics Corporation's rapid Flu Detect™ Avian Influenza Type A Antigen Test Kit. This assay was developed at Synbiotics Europe in Lyon, France. The test detects all 16 hemagglutinin sub-types of Type A Influenza within 15 minutes. This product can be used on the farm or in the laboratory for the detection of Type A Influenza. The Flu Detect™ Avian Influenza Type A Antigen Test Kit is designed to aid in the qualitative detection of Influenza Type A virus in tracheal or cloacal swabs from symptomatic birds or flocks.

Avian Influenza virus infects domestic and wild birds and is characterized by a full range of responses from almost no signs of the disease to very high mortality. Influenza type A viruses can infect avian, porcine, equine and other species including humans. Sixteen serologically distinct hemagglutinin and nine neuraminidase subtypes of Influenza type A virus have been isolated from avian species. Subtypes H5 and H7 are associated with significant to catastrophic losses. Disease signs in poultry range from only a slight decrease in egg production to a highly fatal fulminating infection. Signs of infection may include respiratory problems, edema of the head and face or diarrhea.

The antigen and antibody surveillance of commercial poultry flocks has been an important element in recent disease control programs worldwide. Virus isolation (VI) and identification is a standard laboratory method for detecting AI; yet, VI is time consuming and costly. This study describes the validation of an antigen capture test strip for Type A Influenza virus.



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III. EVALUATION STUDIES AND RESULTS:

A. A comprehensive panel of Influenza (Types A and B) and non-influenza antigen samples tested by various laboratories using samples from the lab banks is presented in TABLE 1 below. The samples were tested using prototype Flu Detect™ Avian Influenza Type A Antigen Test Kits. The H5N1 sample was tested at Southeast Poultry Research Lab (SEPRL), Athens, Georgia. The H5N2 sample and all other samples of subtype H1 through H10 were tested at the University of Maryland, College Park, Maryland. The samples of subtype H11 through H16 were tested at Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia.

TABLE 1 – Panel Test Results

Sample	Flu Detect Result	Sample	Flu Detect Result
A/Swine/TN/25/77 (H1N1)	Positive	A/Qa/AR/29209-1 (H9N2)	Positive
A/Mallard/Alberta/11(9/98) (H1N1)	Positive	A/Ck/HK/WF2/99 (H9N2)	Positive
VR-897,A/New Jersey/8/76 (H1N1)	Positive	A/Ck/HK/SF3/99 (H9N2)	Positive
VR-97,A/FM/1/47 (H1N1)	Positive	A/Ck/HK/G9/97 (H9N2)	Positive
VR-98,A/Malaya/302/54 (H1N1)	Positive	A/Duck/HK/Y280/97 (H9N2)	Positive
VR-95, A/PR/8/34 (H1N1)	Positive	RG A/Qa/HK/A28945/88 (H9N2)	Positive
A/New Caledonia/20/99 (H1N1)	Positive	10/16/03A/RG (H9N2)	Positive
A/Taiwan/1/86 (H1N1)	Positive	A/Shorebird/DE (9/96) (H9N6)	Positive
A/Beijing/262/95 (H1N1)	Positive	A/Pintail/Alberta/202/00(H10N7)	Positive
A/Mallard/NY/6750/78 (H2N2)	Positive	A/tern/Australia/75 (H11N6)	Positive
A/Mild/Potsdm/178-4/83 (H2N2)	Positive	A/re necked sting/WA/5745/85 (H12N9)	Positive
VR-100, A2/Japan/305/57 (H2N2)	Positive	A/gull/Maryland/704/77 (H13N6)	Positive



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A/Mallard/Alberta/33/01 (H2N4)	Positive	A/mallard/Gurjie/244/82 (H14N6)	Positive
A/Ruddy Turnstone/DE/142/99 (H3N2)	Positive	A/shelduck/WA/1762/79 (H15N9)	Positive
A/HongKong/1/68 (H3N2)	Positive	A/gull/Denmark/68110/02 (H16N3)	Positive
A/Duck/HK/3/75 (H3N2)	Positive	VR-790, B/Russia/69	Negative
A/HK/1/68/Luman (H3N2)	Positive	VR-295, B/Taiwan/2/62	Negative
VR-810,A/Port Chalmers/73 (H3N2)	Positive	VR-102,B/Allen/45	Negative
VR-544, A/HongKong/8/68 (H3N2)	Positive	B/Tokio/53/99	Negative
VR-547,A/Aichi,2/68 (H3N2)	Positive	VR-101, B/Lee/40	Negative
VR-822,A/Victoria/3/75 (H3N2)	Positive	B/Victoria/504/00	Negative
A/Panama/2007/99 (H3N2)	Positive	B/Quingdao/102/91	Negative
A/Kiev/301/94 (H3N2)	Positive	VR-823, B/HongKong/5/72	Negative
A/Shangdong/9/93 (H3N2)	Positive	CAP FluB VR1-04 2004	Negative
A/Texas/1/77 (H3N2)	Positive	B/Maryland/1/59	Negative
A/Mallard/Alberta/31/01 (H3N9)	Positive	RSV VR-1341, Caprine Lot 1W	Negative
CK/AI/75/A (H4N8)	Positive	RSV-VR-14-1, B, Wash/18537	Negative
Duck Meat/AVL/China-1/01 (H5N1)	Positive	RSV Strain A-2, Lot 263504	Negative
AI/Chicken/86 (H5N2)	Positive	RSV VR-1400, B-1 Wild Type	Negative
A/Teal/HongKong/w312/97 (H6N1)	Positive	RSV Vr-26	Negative
A/Mallard/Alberta/20 (6/96) (H6N8)	Positive	Adenovirus 8, 9/23/94	Negative
A/CK/DE/04/297267 (Hobo Farm) (H7N2)	Positive	Adenovirus 40, VR-931, Lot 6W	Negative
A/Mild/Alberta/24/01 (H7N3)	Positive	Adenovirus 11, TH408	Negative
A/Mallard/Alberta/19 (4/92) (H8N4)	Positive	Adenovirus 2, TJ2184	Negative



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III. EVALUATION STUDIES and RESULTS (continued):

B. Newcastle disease virus (NDV), Avian reovirus (REO) and Fowl Laryngotracheitis (ILT) viruses were propagated in primary culture and embryonated eggs. In addition to the virus samples, fecal samples were collected from healthy birds and spiked with virus. These samples were tested at the Georgia Poultry Laboratory for the purpose of evaluating specificity of the prototype Flu Detect™ Avian Influenza Type A Antigen Test Kit. The CK/AI/75/A H4N8 (10^{7.9}/0.1ml) virus was used as a control.

The results of testing various avian viruses in the form of culture fluid and spiked into feces from healthy chickens is presented below in Table 2. The data below exhibits the specificity of the Flu Detect™ Test Strip as compared to a commercially available rapid test. In this study avian viruses were tested. The CK/AI/75/A H4N8 (10^{7.9}/0.1ml) virus was used as a control.

Table 2 – Specificity Testing at Georgia Poultry Lab

	Flu Detect	“Kit A”	Sample Description
A	Negative	Negative	A = NDV CEK Primary Cells, first passage (tissue pool of trachea, eyelid)
B	Negative	Negative	B = NDV 3rd passage in eggs (tissue pool of trachea, lungs)
C	Negative	Negative	C = ILT 1st passage in eggs (tissue pool of trachea, eyelid)
D	Negative	Negative	D = ILT 1st passage in eggs (tissue pool of trachea, eyelid)
E	Negative	Negative	E = NDV CEK Primary Cells, second passage (tissue pool of trachea)
F	Negative	Negative	F = ILT CEK Primary Cells, first passage (tissue pool of trachea, eyelid)
G	Negative	Negative	G = REO CEK Primary Cells, 3rd passage (spleen)
H	Negative	Negative	H = NDV 3rd passage in eggs (tissue pool of trachea, lungs)
I	Negative	Positive	I = Feces spiked with B
J	Negative	Positive	J = Feces spiked with G
K	Negative	Positive	K = Feces spiked with F
L	Positive	Positive	L = Feces spiked with positive control (CK/AI/75/A H4N8)

C. Quail: Ten 3-week old quail were inoculated with A/quail/Hong/A28945/88 H9N2 at a concentration of 10⁴ EID₅₀/ml. Tracheal swabs were collected at 1, 2, 3, 5, 7, 9, and 11 days post infection. This testing was performed at the University of Maryland.

The data in Table 3 demonstrate the sensitivity of the Synbiotics AIV FLU DETECT™ Test Strip at detecting Type A AIV in quail. The virus isolation was performed in nine day old embryonated eggs from specific pathogen free chickens. The Flu Detect Antigen Capture Test strip results were compared to virus isolation.

Table 3 – FLU DETECT™ Test Strip sensitivity comparison with Virus Isolation

Day	Flu Detect	Virus Isolation
0	0/10	0/10
1	7/10	not tested
3	9/10	10/10
5	10/10	10/10
7	4/10	10/10
9	0/10	6/10
11	0/10	0/10



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III. EVALUATION STUDIES AND RESULTS (continued):

D. Comparison of Antigen Detection Methods: Two hundred forty broiler chickens housed in isolaters were infected with AIV strain low path H7N2 isolate from the Delmarva 2004 outbreak. The broiler chickens were infected at approximately 2 weeks of age by the ocular route with 10^5 EID₅₀/ml per chick. Sixty tracheal samples were collected randomly on day 1, day 3, day 5, and day 14. Individual tracheal swabs were placed in 0.5 ml PBS. The PBS was pooled, 5 birds per pool per treatment and 3 or five birds per pool as controls. The pools were tested by Real Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), Synbiotics' Flu Detect™ Avian Influenza Type A Antigen Test Kit and the Directigen test. The RT-PCR methodology was the USDA approved method for AI detection. This experiment was performed at the University of Delaware.

The data in Table 4 summarizes the results of testing performed to compare various antigen detection methods. "Kit A" is a commercially available rapid test for detection of Type A Influenza antigen. **Appendix A** at the end of this report provides further details on this study conducted at the University of Delaware.

Table 4 – Comparison of Antigen Detection Methods

Day	FLU DETECT	"Kit A"	RT-PCR
1	0/12	0/12	4/12
3	4/12	4/12	5/12
5	6/12	6/12	6/12
14	0/12	0/12	1/12

E. Determination of Flu Detect™ Avian Influenza Type A Antigen Test Kit Detection Limit: This study was conducted to determine the detection limit using known stock virus in allantoic fluid. This study was conducted at the Georgia Poultry Diagnostic Laboratory, Oakwood, Georgia and the University of Maryland, College Park, Maryland. Stock H4N8 CK/A/75/A ($10^{8.3}$ EID₅₀/ml) virus was provided by Dr. David Swayne, SEPRL, USDA, Athens, Georgia. The H7N2 and H2N2 viruses were obtained from the University of Maryland.

The data in Table 5 summarizes the results of testing performed to determine the detection limit of the Flu Detect Antigen Capture Test Strip using stock virus of three different strains of AIV. Kit A and Kit B are two different commercially available Type A Influenza antigen test kits.

Table 5 – Detectability Comparison

Dilution	H4N8 CK/AI/75/A $10^{8.9}$ EID ₅₀ /mL		H7N2 A/CK/DE/04/297267 8×10^9 EID ₅₀ /mL			H2N2 A/Mallard/NY/6750/78 5×10^7 EID ₅₀ /mL		
	Flu Detect	Kit A	Flu Detect	Kit A	Kit B	Flu Detect	Kit A	Kit B
10^{-1}	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
10^{-2}	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
10^{-3}	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative
10^{-4}	Positive	Negative	Positive	Negative	Negative	Positive	Negative	Negative
10^{-5}	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
10^{-6}	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
10^{-7}	Negative	Negative	Negative	Negative	Negative	Negative	Negative	NT
10^{-8}	Negative	Negative	Negative	Negative	Negative	NT	NT	NT
10^{-9}	Negative	Negative	Negative	Negative	Negative	NT	NT	NT

NT = Not Tested

III. EVALUATION STUDIES AND RESULTS (continued):

F. Determination of detection limit of Flu Detect using infected samples: Fifteen four week old SPF chickens were purchased and kept in an isolator. The chickens were infected with low path A/CK/De/04 H7N2 virus (5×10^6 EID₅₀/ ml) by the oronasal route. Tracheal swabs were taken from each bird at 1 and 3 days post infection. One tracheal swab collected 3 days PI was placed in 1 ml of Brain Heart Infusion (BHI) broth for later testing by virus isolation. Tracheal swabs collected at 1 and 3 days PI were tested in the Flu Detect assay. Lastly, tracheal swabs from each bird were used to evaluate pooling of samples. Two different pooling methods were evaluated. First, single swabs were extracted into a single tube of 1 ml BHI buffer. The 1 ml extracts were then pooled. Second, multiple swab extractions from up to five different birds were performed in a single tube.

Virus isolation was performed as follows: Individual samples collected 3 days post infection were placed in BHI broth for later testing by virus isolation. These individual samples were further diluted 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} in BHI broth. 200ul of each sample dilution was used to inoculate 9 day-old SPF embryonated eggs. Two eggs per dilution were inoculated. Eggs were held in the egg incubator for 48 to 72 hours. Allantoic fluid was harvested and tested for HA activity using chicken red blood cells (RBC) and the EID₅₀/ml was calculated. The results are tabulated in Tables 7 and 8.

The data in Table 6 summarizes the Virus Isolation test results and the Flu Detect results for the individual samples tested in this study.

Table 6 – Flu Detect compared with virus isolation on individual samples

BIRD ID	Day 1 PI Flu Detect	Day 3 PI Flu Detect	D3 PI Log 10 (EID ₅₀ /ml)
219	-	-	2.7
220	+	+	3.1
221	+	+	3.1
222	+	+	ND
223	+	W+	ND
224	-	-	ND
YB	+	-	2.7
B	+	+	2.1
YY	+	+	2.1
YG	+	+	2.2
G	+	+	2.1
Y	W+	+	3.1
G CaHA+	+	+	3.1
BCaHa	-	+	2.7
YCaHA	+	+	2.7

III. EVALUATION STUDIES AND RESULTS (continued):

Table 7 – Comparative data of Pooling Methods

Bird ID	Pool	Individual Extractions Pooled		Multiple Extractions per Tube	
		Day 1 PI	Day 3 PI	Day 1 PI	Day 3 PI
219	A	positive	positive	positive	positive
220					
221					
222					
223					
224	B	positive	positive	positive	positive
YB					
B					
YY					
YG	C	positive	positive	positive	positive
G					
Y					
G CaHA+	D	positive	positive	positive	positive
BCaHa					
YCaHa					

- G. **Thailand Study: The evaluation of Flu Detect was conducted by the Thai National Institute of Animal Health (NIAH) using field H5N2 isolate.** The FluDetect™ Antigen Capture Test Strip was compared with RT-PCR and a locally available commercial rapid Flu A test (Innova Flu-A). Specificity of the test was evaluated by testing field isolates of the H5N1 subtype as well as other subtypes, including a Type A Swine Influenza virus sample. Sensitivity of the test was evaluated by making serial dilutions of a stock H5N1 field isolate virus and testing the diluted samples with the Flu Detect and the locally available commercial rapid test.

Table 8a summarizes the results of the Flu Detect Antigen Capture Test Strip conducted by the Thai National Institute of Animal Health (NIAH) to determine specificity of the test. Table 8b summarizes the results of testing to determine the sensitivity of the Flu Detect kit as compared to Innova Flu-A, a test that is locally available in Thailand.

Table 8a – Specificity Evaluation

Sample	RT-PCR	Flu Detect	HA	Subtype	Innova Flu-A
P1 8860/1	positive	positive	N/A	H5N1	positive
P1 8855/9	negative	negative	N/A	N/A	negative
P1 8858/7	negative	negative	N/A	N/A	negative
P1 8861/3	negative	negative	N/A	N/A	negative
P1 8861/4	negative	negative	N/A	N/A	negative
P1 8862/6	negative	negative	N/A	N/A	negative
9241/12-A	N/A	positive	1:32	N/A	positive
9241/12-B	N/A	positive	1:256	N/A	positive
9241/12-C	N/A	positive	1:512	N/A	N/A
SIV (swine influenza)	positive	positive	1:512	N/A	N/A



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II. EVALUATION STUDIES AND RESULTS (continued):

Table 8b summarizes the results of the titration of the stock virus (H5N1 Field Isolate). The stock virus concentration was $10^{7.8}$ EID₅₀/mL. One-hundred microliters of the virus dilutions were tested in the assays.

Table 8b – Sensitivity Evaluation

Dilution Factor	Virus titer	Flu Detect Test Strip	Innova Flu-A
10^{-1}	$10^{6.8}$	Positive	Positive
10^{-2}	$10^{5.8}$	Positive	Positive
10^{-3}	$10^{4.8}$	Positive	Positive
10^{-4}	$10^{3.8}$	Negative	Negative
10^{-5}	$10^{2.8}$	Negative	Negative
10^{-6}	$10^{1.8}$	Negative	Negative

III. DISCUSSION:

Studies “A”, “B” and “G” all provide information regarding the specificity of the Flu Detect. Flu Detect did not produce any false positive results on the Type B and the non-Influenza virus samples tested in Study “A”. Flu Detect did not produce any false positive results on the non-influenza virus samples or spiked-fecal samples described in Study “B”; although the human test, “KIT A” did produce false positive results on the non-influenza spiked fecal samples. This is important to note as cloacal swabs may be the sample taken from birds tested for presence of AI antigen. No false positive results were observed in the Thai study (Study “G”).

Detectability of Flu Detect is superior to other commercially available Type A Influenza Antigen Tests. These tests are licensed by the Food and Drug Administration for use in detecting Type A Influenza in humans. Study “E” compared the detectability of the three rapid tests on serial dilutions of three different Flu A strains. In all cases, Flu Detect produced a positive reaction on samples of lower concentration than the two human tests. The increased detectability of Flu Detect ranged from one log to three logs for the viruses tested. Study “F” was designed to evaluate the performance of Flu Detect versus Virus Isolation and to determine the sensitivity of the test strip on multi-bird extractions. In this study, compared to VI, Flu Detect demonstrated sensitivities of 80% at one and three days post infection, respectively. Detectability in this study was as low as $10^{2.1}$ EID₅₀/mL. Consistent positive results were seen at concentrations of $10^{3.0}$ EID₅₀/mL. Regarding the pooling of samples, it was observed that the individual extracts pooled together prior to testing produced a positive result. The multiple extractions in a single tube also produced positive results; the observed reaction was faster and more intense than that observed for the single extract pools. Study “G” conducted in Thailand demonstrated Flu Detect’s equivalent detectability as compared to the Innova Flu-A test which is available in Thailand. The ease of use of Flu Detect was superior to the Innova Flu-A test. Flu Detect test strips were clear and easy to read with no background and the assay is quick to complete. The Innova Flu-A is reported to have high background and high variability from lot to lot.

Sensitivity of Synbiotics’ Flu Detect Antigen Capture Test Strip has been demonstrated by various methods. Study “A”, the testing of the comprehensive panel of Type A influenza viruses demonstrated that the test produced a positive result on all Type A influenza virus samples tested, representing all 16 Hemagglutinin types. Study “C” indicates that Flu Detect has a 90% - 100% correlation with Virus Isolation from 3 to 5 days post-infection. Flu Detect provides a useful tool for rapid and early identification of active or acute infection. Similarly, Study “D” indicates that Flu Detect has an 80-100% correlation with RT-PCR from 3 to 5 days post infection; further supporting the usefulness of Flu Detect in the field.

IV. CONCLUSION:

The threat of an Avian Influenza Virus “Bird Flu” pandemic is an international concern. Political and scientific leaders around the globe are focusing on how to prevent the continued spread to avert a human health crises. Synbiotics’ Flu Detect Antigen Capture Test Strip is currently in use around the world for the detection of Type A Influenza virus in avian species. The data presented here proves that Flu Detect is efficacious to use in the field. Synbiotics’ Flu Detect™ has demonstrated greater sensitivity and specificity compared to the other commercial Influenza Type A antigen test kits.



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