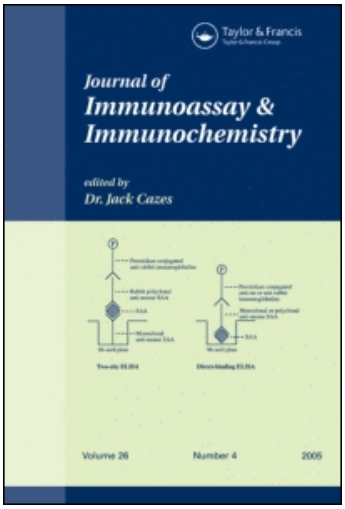


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# Prototype Single Step Lateral Flow Technology for Detection of Avian Influenza Virus and Chicken Antibody to Avian Influenza Virus

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**Abstract:** A rapid and effective lateral flow assay (LFA) for detection of avian influenza virus (AIV) was developed. For antigen capture, the assay used monoclonal antibody specific for a conserved nuclear protein (NP) epitope, immobilized on a cellulose acetate matrix, in conjunction with a second NP monoclonal antibody chemically linked to either coloured latex beads or colloidal gold particles contained in a sample pad for detection. Virus sample added to the sample pad flowed into the trapping antibody to form a visible band as well as a second, control band further along the acetate strip. The control band consisted of recombinant protein A/G, also immobilized on the matrix.

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A second LFA for detection of chicken antibody to AIV was developed where NP antigen was immobilized on the matrix with recombinant protein A/G immobilized as a control band. Latex beads or colloidal gold particles to which monoclonal anti-chicken antibody was attached, were used as the indicator system.

**Keywords:** Lateral flow technology, Avian influenza virus, Chicken antibody



## INTRODUCTION

Human influenza is caused by infection with members of 2 genera of orthomyxoviridae, Influenza viruses A and B (FLUAV and FLUBV). In distinction from FLUBV, FLUAV viruses infect a number of hosts, including a large number of avian species.[1,2] There are 16 hemagglutinin (HA) serotypes and 9 neuraminidase (NA) serotypes of FLUAV. All serotypes are found in wild birds and also often in domestic birds and most cause subclinical infection, except in relatively rare instances where the virus has evolved to high virulence. The symptoms vary considerably from inapparent to systemic disease associated with high mortality. The serotypes are further divided into highly pathogenic viruses (HPAI) and low pathogenic viruses (LPAI), the former being an OIE reportable disease.[3] HPAI strains are almost exclusively variants of serotypes H5 and H7 with the current widespread Eurasian HPAI epidemic being caused by serotype H5N1.

Because of the highly contagious nature of the virus, it is important to diagnose infection as early as possible in order to establish control measures to prevent its spread. Thus, a rapid field test for screening avian FLUAV virus and serum samples is indispensable for identifying influenza outbreaks in humans and animals. In particular, a disposable rapid screening test that requires no auxiliary equipment, (and, thus, has no need of subsequent equipment decontamination) would be ideal for initial determination of the presence of the virus. While further confirmation would require laboratory testing, a preliminary diagnosis would allow control measures to be implemented immediately to limit further spread. Lateral flow tests (LFA) have been developed for detection of a number of pathogens, including HIV,[4] PRRSV,[5] foodborne pathogens,[6] PSA,[7,8] toxins,[9] pregnancy,[10] and for contaminant detection in animal feed.[11] Highly sensitive and specific LFAs have also been developed for detection of influenza virus in respiratory specimen from children.[12–16]

There is one similar assay currently available for detection of AIV in avian species and several that were developed for human testing but used for testing avian samples. In this communication, two prototype lateral flow tests for detection of avian FLUAV and antibody are described. The LFAs for virus detection use two different monoclonal antibodies to detect nucleoprotein (NP) antigen while the LFA for antibody detection uses NP as the antigen and a monoclonal antibody to chicken IgG (IgY) for detection. In

both assays, the detection systems consisted of monoclonal antibodies conjugated with coloured polystyrene beads or colloidal gold developing a visual readout in about 5 minutes.

## EXPERIMENTAL

### Construction of Protein Expression Vectors

Prokaryotic expression vector pETcoco-2 (Novagen. EMD Biosciences, Madison, WI., U.S.A) expressing a conserved fragment of the NP protein derived from influenza virus was prepared. Briefly, the full coding sequence of the NP gene of the Turkey/Wis/66 isolate (H6N2 strain) was amplified by reverse transcription-PCR. The resulting 1560 bp was cut with KpnI and Sal I and cloned in the pQE31 vector (Qiagen Inc. Mississauga, ON., Canada). The desired subfragment of the NP gene was amplified with primers that included appropriate restriction enzymes and was directionally subcloned into the pETcoco-2 vector (Novagen). The coding sequence of the NP gene was confirmed by sequencing. The pETcoco-2 vector (Novagen) containing the NP fragment was transformed into NovaF$^-$ cells which were cultured in LB broth containing Ampicillin. Plasmid DNA was extracted and expressed in *E. coli* strain Tuner$^{TM}$ cells.

### Expression of Recombinant NP Protein

*E. coli* strain Tuner$^{TM}$ cells containing the expression construct were induced to express the recombinant nucleoprotein with 1 mM IPTG. The expressed NP fragment was purified using a Ni-NTA column as previously described.[17] The whole cell and purified proteins were analysed by SDS-PAGE[18] and Western blotting. For Western blotting, the proteins were transferred onto a nitrocellulose membrane using a Bio-Rad Trans-Blot SD semi-dry transfer cell. The blots were probed with a mouse anti-histidine tag monoclonal antibody or with antisera against influenza virus derived from vaccinated, as well as experimentally or naturally infected chickens, ducks, pigs, horses and humans. Bound antibodies were detected with horseradish peroxidase (HRPO)-conjugated goat anti-mouse, rabbit anti-chicken or Protein A/G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. USA) and a 4-chloro-1-naphthol-$H_2O_2$ substrate kit (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada) using the manufacturer's instructions.

### Monoclonal Antibodies

The recombinant NP protein or chicken IgY were used to immunize ND4 and BALB/c mice to generate monoclonal antibodies. The resulting monoclonal

antibodies were analyzed for relative affinity using an IELISA test with increasing concentrations of sodium chloride.[19] From the data, two monoclonal antibodies of high affinity for NP, AIV2455 and AIV2454, were selected for lateral flow assays. Monoclonal antibodies specific for the Fc region of chicken IgY were selected and used in the lateral flow test for AIV antibody from chickens.

## Immunization of Llamas

Llamas (*Lama glama*) have naturally occurring heavy chain antibodies. These unique antibodies can be manipulated into stable fragments containing basically only the variable part of the molecule, $V_H$Hs.

Llamas were initially immunized intramuscularly with 100 ug recombinant NP incorporated into 0.25 mL Freund's complete adjuvant. One month later, the animals began receiving weekly injections of 100 μg recombinant NP for 10 weeks. The animals were bled periodically and the serum was tested for the presence of low molecular weight antibody by reverse western blotting using recombinant NP labelled with HRPO for detection of antibody bands. When low molecular weight antibody was detected, the llamas were bled into EDTA and the lymphocytes processed for recombinant $V_H$Hs development.



## Library Construction, Panning and Phage ELISA

Phage display library construction from the lymphocytes of the immunized llamas, library size determination and library phage production were carried out as described elsewhere.[20] Panning was performed as described with some modifications.[20] Briefly, 100 μL of 100 μg/mL recombinant NP in PBS was added to a Maxisorp™ well (Nunc A/S, Roskilde, Denmark). The well was sealed with parafilm and incubated overnight at 4°C before the solution was discarded and the well was blotted on a paper towel and blocked by adding 300 μL freshly-made 2%MPBS (2% w/v skim milk in PBS) and incubating at 37°C for 2 h, sealed with parafilm. The blocking solution was removed and 100 μl $10^{12}$ phage colony forming units in 2%MPBS was added. The well was sealed and incubated at room temperature for 1.5 h. The unbound phage in the supernatant was discarded and the panning was continued as described.[20] Following panning, clones were screened by standard enzyme-linked immunosorbent assay (ELISA) procedures using a horse radish peroxidase/anti-M13 monoclonal antibody conjugate (GE Healthcare, Baie d'Urfé, QC, Canada) as described for "Phagemid Vectors."[21]

## Production and BIACORE Analysis of $V_H$Hs

$V_H$Hs antibodies were expressed in *E. coli* and purified by immobilized metal affinity chromatography.[20] To remove aggregates, $V_H$Hs were subjected to

Superdex 75 gel filtration before BIACORE analysis. The binding of $V_HHs$ to recombinant NP was determined by surface plasmon resonance using a BIACORE 3000 biosensor system (BIACORE Inc., Piscataway, NJ). Immobilizations were carried out at an antigen concentration of $\sim$25 μg/ml in 10 mM acetate buffer pH 4.5, using the amine coupling kit supplied by the manufacturer. As a reference surface, blood group A-specific scFv was immobilized at the same surface density of 700 RUs under identical conditions. In all instances, analyses were carried out at 25°C in 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% P20 at a flow rate of 20 μL/min. No regeneration was performed except washing with the running buffer. Data were evaluated using the BIA evaluation 4.1 software (BIACORE).

## Labelling of Polystyrene Beads

An aliquot of 300 ug of monoclonal antibody AIV2455 was used to label blue polystyrene microparticles by the glutaraldehyde method following the manufacturer's instructions (Polysciences, Inc. Warrington, PA, USA). The beads were stored at 4°C in PBS, pH 7.4, containing 10 mg/mL bovine serum albumin (BSA) and 0.1% sodium azide. Colloidal gold particles were labelled using 80 ug of monoclonal antibody AIV 2455 and 10 mL of colloidal gold particles (40 nm) following the manufacturer's instructions (Diagnostic Consulting Network LLC, Irvine, CA USA). The beads were washed once with 5% BSA, 1% PEG 20,000 and 0.1% sodium azide. The supernatant solution was discarded, the beads were reconstituted in 2 mL of the above buffer and stored at 4°C.

## Conjugate Pads

Conjugate pads were prepared by loading an aliquot of 4.5 μL of AIV2455 labelled blue beads or 20 μL of AIV2455 labelled gold beads onto a membrane (Cat. No. 10537259, Whatman, Clifton, NJ 07014, USA) to be used as a conjugate pad. The bead complex was dried at room temperature overnight; then sealed in plastic film and stored at room temperature until used.

## Preparation of Strips

Protein A/G and monoclonal antibody AIV2454 were dialyzed overnight against 0.02 M PBS, pH 7.4. Concentrations of Protein A/G (1.3 mg/mL) and AIV2454 (2.8 mg/mL) were applied to the nitrocellulose membrane (Hi-flow Plus 120 membrane, Millipore, Danvers, MA, USA) as control line

and test line, respectively, using a Bio-Dot air-brush device (Bio-Dot, Irvine, CA, USA). The membranes were dried at 30°C for 30 min, left at room temperature overnight and were then stored in sealed foil sachets until required for use.

## Assemblage of the Strip Test Device

The conjugate pad was overlaid onto the base of the nitrocellulose membrane, parallel to the control and test lines. The sample pad (0.48 cm, Surewick CO48 cellulose pad, Millipore) was overlaid on the conjugate pad. An absorbent pad (SA3Jo71V04, Surewick cellulose pads, Millipore) was applied downstream at the opposite end of the membrane.



## Test Procedures

A 50 μL sample solution was added to the sample pad and was allowed to react for about 1 min, followed by 100 μL of running buffer (0.13% SDS-PBST-T, pH 7.5), added on the sample pad and allowed to flow through the conjugate pad. An additional 100 μL of running buffer was added to the sample pad. If influenza viruses were present in the sample, NP protein would bind to the antibody test line on the strip and react with monoclonal–blue-bead conjugate, resulting in the formation of a blue line. Unbound monoclonal antibody conjugated beads continued to flow along the membrane to produce a blue line with the Protein A/G control line (Figure 1).

The same protocol as for the blue bead was used with conjugated gold beads except the running buffer was 0.01 M PBS, pH 7.4. A 50 μL sample

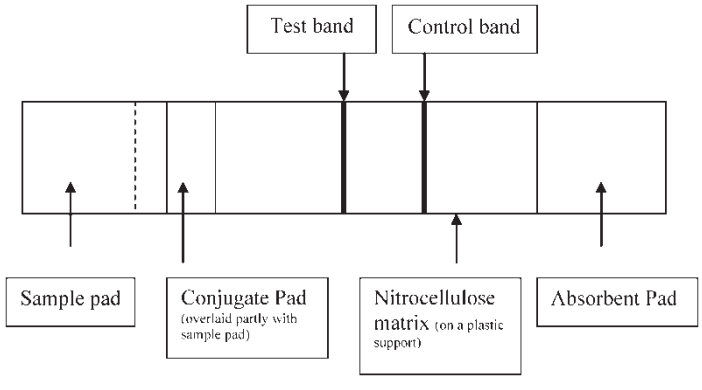


***Figure 1.*** Schematic representation of the lateral flow assay assemblage.

solution was added to the sample pad and allowed to react for about 1 min; this was followed by the sequential application of 100 μL of running buffer to flow through the conjugate pad, followed by the addition of 50 μL of 70% ethanol and another 50 μL of running buffer at about 90 second intervals. If influenza virus were present in the sample, a dark pink line would form at the test line position. Unbound conjugated gold particles would continue to flow along the membrane to produce a dark pink line with protein A/G at the control line position. The lateral flow time was approximately 5 minutes. Only control lines were present with samples containing no virus.

The second format used 200 μL of buffer plus various volumes of virus dispensed into a 10 × 75 mm borosilicate glass tube. The test LFA strip was dipped by the conjugate pad end into the test buffer and allowed to incubate at ambient temperature for 10 to 15 minutes.

A commercially available LFA for AIV was purchased ('FluDetect', Synbiotics Corp., San Diego, CA, USA) for test comparison.



## Preparation of AIV

All strains of the AIV were propagated in 9-day-old embryonated eggs. After inoculation into the allantoic cavity, eggs were incubated at 34°C for 3 days followed by harvesting of the allantoic fluid. Virus infectious titers were not performed, however, the number of virus particles routinely gave hemagglutination inhibition titers of approximately 1:200 as assayed using chicken erythrocytes.

## Lateral Flow Assay for AIV

A total of 18 low pathogenic influenza virus strains and 2 human vaccines for (2004-5 and 2005-6 ID Biomedical Corp, Quebec City, PQ, Canada, containing A/NEW CALEDONIA/20/99, A/NEW YORK/55/04 and B/JIANGSU/10/03) were tested.

The18 low pathogenic influenza virus strains were:

A/WS/33 (H1N1),
A/SINGAPORE/1/57 (N2N2),
A/PHILLIPINES/2/82 (H3N2),
A/DK/CZECKOSLOVAKIA/56 (H4N6),
A/TURKEY/CALIFORNIA/35621/84 (H5N3),
A/TURKEY/WISCONSIN/68 (H5N9),
Rg A/HONG KONG/213/03 (H5N1 (LP)),
A/TURKEY/MASS/3740/65 (H6N2),
A/CHICKEN/BRITISH COLUMBIA/514/04 (H7N3 (LP)),
A/EQ/PRAGUE/1/56 (H7N7),

A/TURKEY/ONT/6118/68 (H8N4),
A/TURKEY/WISCONSIN/1/66 (H9N2),
A/QUAIL/ITALY/1117/65 (H10N8),
A/CHICKEN/GERMANY/N/49 (H10N7),
A/DK/ENGLAND/56 (H11N6),
ADK/WISCONSIN/480/79 (H12N6),
A/DUCK/ALBERTA/60/76 (H12N5),
A/GULL/MARYLAND/704/77 (H13N6).

Serial dilutions of 4 virus preparations were tested to give an indication of relative sensitivity. Human vaccines were also diluted and used to evaluate sensitivity of the lateral flow assay. Both sets of dilutions were compared to the commercially available AIV LFA for sensitivity assessment.

### Lateral Flow Assay for Chicken Antibody to AIV

Twenty five 8-week-old white leghorn chickens were bled and then immunized intramuscularly with the equivalent of 15 μg H antigen of human influenza vaccine incorporated into 0.2 mL Freund's complete adjuvant. A month later, each chicken received a further 15 μg total H antigen equivalent in saline, also intramuscularly. A week later, all chickens were bled and the serum frozen until use.



## RESULTS

### Lateral Flow Test for AIV

Initial attempts employed llama $V_HHs$ anti-AIV recombinant single chain antibody labelled with either polystyrene or gold beads. These reagents did not function well in the test because in both cases the beads aggregated, limiting their ability to diffuse in the LFA. Several attempts using various minor buffer modifications to the conjugation procedure failed to produce usable beads. Subsequent attempts, therefore, focused on the use of purified anti-NP monoclonal antibodies for conjugation to polystyrene and gold beads and in both cases the resulting conjugates diffused well in the LFA. The assay was established using the human vaccines as the test antigen. Both vaccine lots gave strong bands with both the blue beads and the gold particles (Figure 2). Negative controls using PBS as a replacement for the vaccine were included. We sporadically found that, blue bead conjugated monoclonal antibody gave non-specific lines whereas this was not observed with gold particle conjugated antibody. As a result, subsequent development was done using gold conjugated monoclonal antibody. This assay, using the vaccine as a source of virus, provided a useful quality control procedure. In

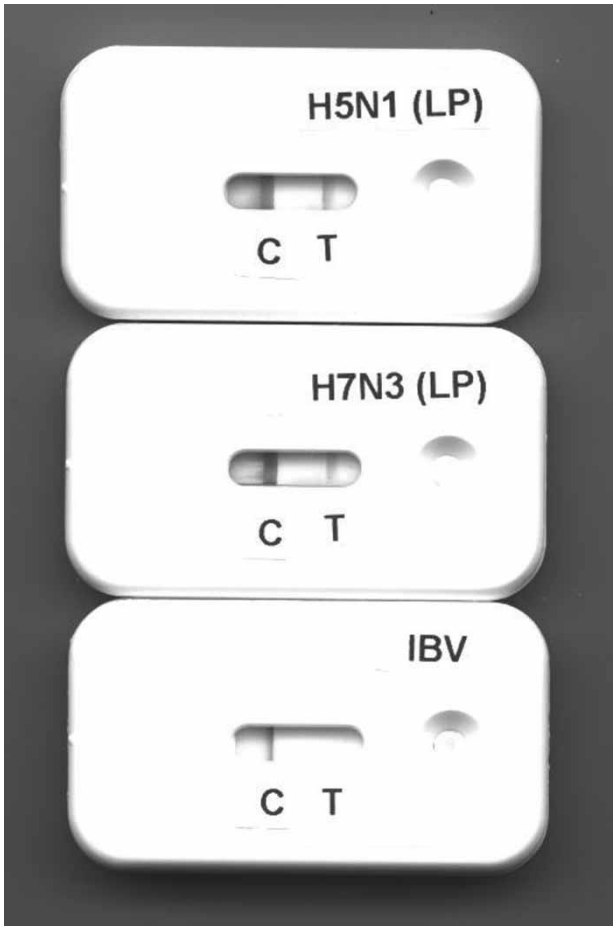


***Figure 2.*** Lateral flow cassettes in which avian influenza virus subtypes H5N1 (top) and H7N3 (middle) were tested while an unrelated virus was added to the bottom cassette. C refers to the positive control band and T indicated reaction with the NP antigen of the virus.

addition, a control band using immobilized protein A/G reacting with the conjugated monoclonal antibody, was used. In this case, a positive reaction was necessary for the individual assays to be accepted as valid.

All 18 AIV strains tested, representing 13 serotypes, gave positive results in the LFA. The intensity of the coloured band varied, presumably due to variations in the virus content of each preparation. Bands were generally visible within 5 min of addition of virus to the sample well. All assays were repeated at least 5 times with similar outcomes thereby demonstrating the reproducibility of the test results. Of the 4 virus samples that were diluted

and tested, 2 (H5N3 and H7N3) gave visible bands when diluted $10^3$ fold while the other 2 (H10N7 and H12N5) gave visible bands when diluted $10^5$ fold suggesting that these tests are sensitive enough to detect lower levels of virus expected in test samples.

For further assay sensitivity assessment, the original cassette format was found to be able to detect one log of diluted virus less that a commercially available AIV LFA. When the format was changed to a tube format in which the diluted virus preparations were added directly to 200 μL of test buffer, the sensitivity equalled that of the commercial LFA, the equivalent of 18 ng of NA antigen.

### Lateral Flow Test for AIV Antibody

The pre- and post-immunization sera from the 25 chickens immunized with human trivalent influenza vaccine were tested. None of the pre-immunization sera gave visible bands in the LFA while all post-immunization sera gave strong bands approximately 10 minutes after addition of the sera to the sample wells. All assays were repeated at least 3 times.



## DISCUSSION

Llama $V_HH$ recombinant antibody fragments attached to polystyrene beads or gold particles in a manner that resulted in aggregation of the particles in such a way that diffusion was not possible in the LFA. It was also observed that polystyrene beads would occasionally give a positive control reaction in the absence of virus. It was, therefore, preferable to only use gold particles, to which anti-NP monoclonal antibodies were attached, for LFA development.

The prototype LFA for detection of AIV in amniotic fluid of chick embryos was found to be rapid, repeatable and easy to perform. It provided a simple solution to testing for the presence of all AIV serotypes included in the study. It is assumed that the assay would be directly applicable to field use by testing tracheal or cloacal swabs from chickens or other avian species but this must be confirmed by further testing of infected birds. The LFA was found to detect virus over a wide range of dilutions, indicating a relatively high sensitivity and comparable to a commercially available AIV LFA. According to the manufacturer's specifications, the commercial test is capable of detecting $10^3$ to $10^4$ $EID_{50}$/ml and it can detect infection 3–5 days after exposure (www.synbiotics.com).

It is realized that this type of assay is not as sensitive as virus isolation or nucleic acid detection; however, it is advantageous in terms of speed, cost and mobility without the requirement for laboratory equipment. A positive reaction would require laboratory confirmation; however, a positive field assay would provide a preliminary or presumptive indication of the

presence of the virus. Thus, a positive field test, combined with clinical observations would allow officials to rapidly initiate control. Field trials with laboratory testing are needed to assess test specificity and sensitivity under actual field conditions.

The LFA for detection of antibody may prove useful for epidemiological studies, passive surveillance and for determination of post-vaccination protection. It was found to detect antibody in 100% of the immunized chickens tested while no reactions were observed with sera from non-exposed chickens.

While these assays are in their infancy, this preliminary evaluation provides a starting point for further evaluation, preferably in a field setting and with a protocol for comparison with other, established tests. Future refinements will be directed to the design of LFAs with serotyping capabilities. The implementation of an effective field test will be a great asset to both local and international efforts to control emerging influenza outbreaks and epidemics.



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