Abstract #2046

Detection of Influenza A Nucleoprotein Antigen and H5-specific Antibodies in Dairy Cow Samples

Laura R.H. Ahlers[1], Nicholas Sammons[1], Brian S. Lane[1], Anu Mathew[1], Ismaila Shittu[2], Gregory C. Gray[2], George Sigal[1], and Jacob N. Wohlstadter[1]

[1]Meso Scale Diagnostics, LLC., Rockville, MD; [2]University of Texas Medical Branch, Galveston, TX

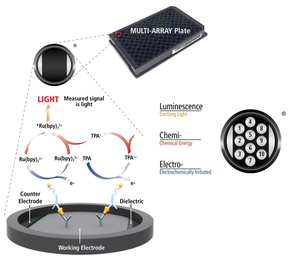
1 Abstract

The highly pathogenic avian influenza (HPAI) H5N1 (clade 2.3.4.4b) is of growing concern to both human and animal health. Outbreaks have been observed in dairy herds and poultry farms across much of the U.S., leading to sporadic infections in human agricultural workers. New influenza H5 subtypes are being identified in dairy herds, in addition to the B3.13 genotype from the initial spillover event to cows, increasing the risk that new strains will develop that are capable of sustained transmission in humans. To provide effective surveillance of flu infections in animal populations, new high-throughput assays are needed to detect H5 infection in a variety of species and sample types, including bovine serum and milk.

We developed high-throughput plate-based immunoassays to measure Influenza A nucleoprotein (NP) antigen and H5 hemagglutinin (HA) specific antibodies. Both assays are animal species-agnostic and use electrochemiluminescence (ECL) technology from Meso Scale Discovery to perform highly specific and sensitive measurements. The Influenza A NP assay is a sandwich-immunoassay for antigen detection. Using this assay, we detected NP antigen in 26 of 50 cow milk samples tested from H5N1-exposed herds, with concentrations ranging from 14 to 486 µg/mL. We measured H5 HA specific antibodies with a quantitative bridging serology assay, in which we incubate antibody-containing samples with labeled constructs of the H5 HA head domain. We detected H5-specific antibodies in 11 of 50 cow milk samples from H5N1-exposed herds. We did not detect NP antigen or H5-specific antibodies in milk samples from a healthy, unexposed herd. The antigen and antibody levels are complementary. Together the assays identify 64% of the H5N1-exposed milk samples as containing NP antigen, H5 antibodies, or both. This percentage is greater than the number of milk samples that tested positive by qRT-PCR. These assays can screen samples from any host species for Influenza A antigen and antibodies against H5 HA.

2 Methods

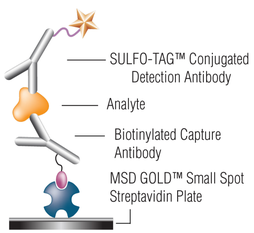
MSD® electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

3a R-PLEX® Assay for Nucleoprotein Antigen

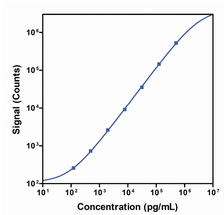


Protocol

1. Incubate streptavidin plates with capture antibody 1 hour at room temperature (RT).
2. Wash and add assay diluent (25 µL per well) plus calibrator, control, or sample (25 µL per well). Incubate 1 hour at RT.
3. Wash and add detection antibody solution (50 µL per well). Incubate 1 hour at RT.
4. Wash and add read buffer (150 µL per well). Analyze with MSD instrument.

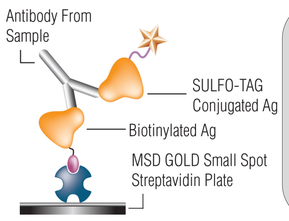
Calibrator Curve

	Influenza A NP	
Conc. (pg/mL)	Average Signal	%CV
500,000	527,258	0.1
125,000	146,635	1.6
31,250	35,545	3.5
7,813	9,141	3.2
1,953	2,640	5.3
488	731	5.0
122	259	1.6
0	99	0.7



Typical standard curve for NP antigen detection assay. Calibrator for the NP antigen assay is native Influenza A nucleoprotein purified from inactive virus.

3b Bridging Serology Assay for H5 Antibody Detection



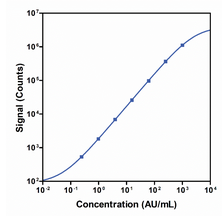
Protocol

1. In a polypropylene plate, incubate calibrator, controls, and diluted samples with 1:1 molar ratio of biotinylated capture antigen and SULFO-TAG conjugated detection antigen for 1 hour at RT.
2. Transfer solution with formed complexes from incubation plate to a pre-blocked MSD streptavidin plate (50 µL per well). Incubate 1 hour at RT.
3. Wash and add read buffer (150 µL per well). Analyze with MSD instrument.

The antigen used in the assay is a recombinant construct of the Influenza virus HA head domain (amino acids 48-326) of the clade 2.3.4.4b H5N1 strain A/Ghana/AVL-763_21VIR7050-39/21.

Calibrator Curve

	Antibodies to H5	
Conc. (AU/mL)	Average Signal	%CV
1,000	1,120,564	1.3
250	367,340	6.1
62.5	97,228	5.3
15.6	26,038	2.7
3.9	6,942	6.9
0.98	1,830	4.5
0.24	535	4.5
0	84	9.9



Typical standard curve for H5 bridging serology assay. Calibrator for the assay was made with polyclonal serum from rabbits immunized with H5 A/Ghana HA antigen. The calibrator was assigned a concentration in arbitrary units (AU).

4 Dairy Cow Sample Testing

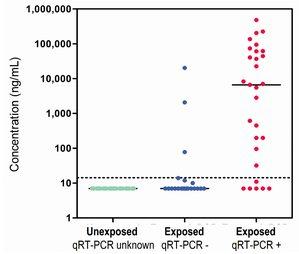
Dairy cow milk and serum samples were collected from herds of healthy cows and cows suffering from H5N1 infections. These samples were sourced by academic institutions [Purdue, Oklahoma State, and University of Texas Medical Branch (UTMB)]. The H5N1-exposed group of samples came from a mix of individual cows from farms in New Mexico, South Dakota, and Texas. These cows were exposed to the virus at various times in 2024-2025. Samples came from acutely ill or recovered cows. Among the 50 H5N1-exposed cow milk specimens, 29 (58%) had molecular evidence of influenza A RNA in their milk (Ct <38 cycles by qRT-PCR assay targeting the Influenza A matrix gene). Of the 60 serum samples from H5N1-exposed cows, 11 (18%) were positive for Influenza by microneutralization (MN) assay carried out at UTMB using a recombinant H5N1 virus (rg-A/bald eagle/Florida/ W22-134-OP/2022 of clade 2.3.4.4b) (MN titer ≥1:40).

Among the healthy cow specimens (collected in Indiana and Oklahoma), no animal showed symptoms of viral infection. Thirty cow milk and 30 serum specimens (unpaired) were collected in Indiana from healthy animals in 2025. Eight pre-2024 archived serum samples from an unrelated study in Oklahoma were also tested. These samples were collected from 4 crossbred female dairy calves prior to and 28 days post infection with the Cooper strain of bovine herpes virus 1 (BoHV-1) in June-July 2020 (El-Mayet, et. al. *mSphere* 2022). BoHV-1 is a DNA virus unrelated to the RNA virus influenza A.

Sample Data

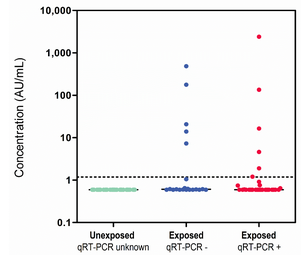
For both R-PLEX and H5 Bridging Serology data sets, samples that quantified at or below the lower limit of detection (LLOD) were set to the LLOD value. Dots represent individual samples, and the solid horizontal line within a sample cluster represents the median concentration for that group. Dotted lines represent the cut-point concentration, above which are deemed positive. The cut-points were set to 2 times the LLOD.

5 Nucleoprotein Antigen in Cow Milk



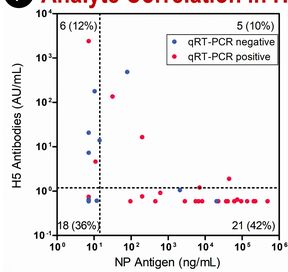
Dairy cow milk samples were analyzed for the presence of NP using samples diluted 1:10, 1:500, or 1:2500 to measure signal within the linear range. Reported concentrations are corrected for dilution. None of the 30 healthy cows had detectable antigen in their milk; however, 26 of 50 (52%) of the exposed cows contained detectable levels of Influenza A antigen.

6 H5-Specific Antibodies in Cow Milk



Dairy cow milk samples were analyzed for the presence of H5-specific antibodies using samples diluted 1:10 in assay diluent before testing. None of the 30 unexposed cows had any measurable antibody response, while 11/50 (22%) exposed cows, including convalescent animals, contained H5-specific antibodies.

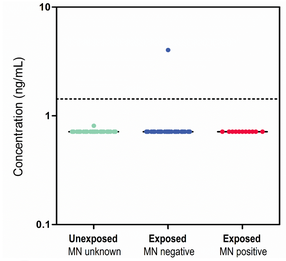
7 Analyte Correlation in H5N1-Exposed Cows' Milk



Antigen and antibody levels for the same samples were compared to identify correlations. Dashed vertical and horizontal lines represent the cut-points established for the Influenza A NP antigen quantification and bridging serology assays.
The number of samples that fall into each quadrant is indicated in its respective corner, with the percentage of samples from the H5N1-exposed herd in parenthesis.

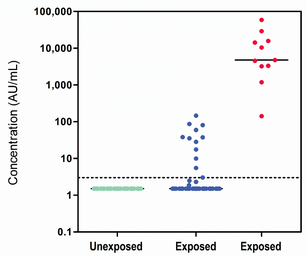
Few milk samples (n=5) test positive for both NP antigen and H5 antibodies. Rather, we see a complementarity between the assays where measuring antigen and antibodies in milk identifies 64% of the samples as being positive by one or both assays.

8 Nucleoprotein Antigen in Cow Serum



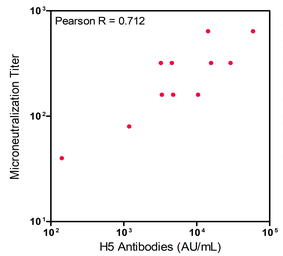
Dairy cow serum samples were analyzed for the presence of NP using undiluted samples. None of the 38 healthy cow samples had measurable antigen in their sera; and only 1 of the 60 exposed cows' sera contained detectable levels of Influenza A antigen. Samples with MN titers ≥1:40 are indicated in red.

9 H5-Specific Antibodies in Cow Serum



Cow serum samples were analyzed for the presence of H5-specific antibodies using samples diluted 1:100 before testing. None of the 38 unexposed cows had a measurable antibody response, while 23/60 (38%) exposed cows' sera contained H5-specific antibodies at levels above the cut-point. Samples with MN titers ≥1:40 are indicated in red. This assay detects low levels of H5-specific antibodies that are not captured by an MN assay.

10 Correlation of MN Titers and H5 Serology Measurements



All serum samples from H5N1-exposed cows were tested by MN assay (UTMB). The MN titers of the 11 samples with a titer ≥1:40 are plotted against the H5-antibody concentration (AU/mL). The data indicate a positive and significant correlation between MN titer and H5 bridging serology concentration.

11 Summary and Conclusions

- Influenza A NP was measured in 52% of cow milk and 1 of 60 serum samples from exposed herds. The milk samples contained 14 to 486 µg/mL of Influenza A NP, indicating a wide range of viral load in these animals. The presence of antigen and viral RNA in milk did not show a strong correlation (data not shown).
- H5-specific antibodies were detected in 22% and 38% of exposed cow milk and serum samples, respectively. This assay can detect previous exposure in milk samples, which is a non-invasive method to collect samples.
- The antigen and antibody levels in milk are complementary. Together, the two assays identify 64% of samples as containing Influenza A antigen and/or H5-specific antibodies in exposed animals.
- The MN titer and H5-bridging serology concentrations in serum have a significant correlation, suggesting that potent antibodies can be detected by both assay formats.
- These assays are species-agnostic and can be used to analyze samples from humans, birds, and other animals.

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