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

Laura R.H. Ahlers<sup>1</sup>, Nicholas Sammons<sup>1</sup>, Brian S. Lane<sup>1</sup>, Anu Mathew<sup>1</sup>, Salvia Misaghian<sup>1</sup>, Ismaila Shittu<sup>2</sup>, Gregory C. Gray<sup>2</sup>, George Sigal<sup>1</sup>, and Jacob N. Wohlstadter<sup>1</sup> 1: Meso Scale Discovery, Rockville, Maryland, USA 2: University of Texas Medical Branch, Galveston, Texas, USA

### **Abstract**

The highly pathogenic avian influenza (HPAI) H5N1 (clade 2.3.4.4b) is of growing concern to both human and animal health. Infections are currently considered widespread in dairy herds and sporadic in poultry farms: as of February 26, 2025, the USDA reported 973 affected dairies across 17 states and 1,604 affected flocks, representing approximately 166 million domestic birds. The risk to human health is apparent, as there are 70 confirmed human cases in the U.S., some caused by an unknown animal exposure. The concern is growing, as additional influenza H5 subtypes besides the B3.13 genotype from the initial spillover event to cows are being identified. For example, H5N9 was discovered on a duck farm in California, and a second spillover event from birds to cattle was discovered with the emergence of the D1.1 genotype. Influenza A H5 infections in agriculture have led to cow milk recalls, quarantines of herds, and culling of flocks. New high-throughput species-independent assays are needed to detect H5 from a variety of sample types.

# **4** Dairy Cow Sample Testing

Dairy cow milk samples were collected from herds of healthy cows and cows suffering from H5N1 infections. These samples were sourced by academic institutions (Purdue and UTMB) during various times in 2024-2025 from multiple farms in Indiana, New Mexico, South Dakota, and Texas. The H5N1-exposed group of samples came from a mix of individual cows that were exposed to virus in these states. Some were acutely ill with H5N1, and some had recovered from H5N1 illness.

Among the 30 healthy cow specimens (all collected in Indiana), no individual had clinical evidence of disease. Among the 50 H5N1-exposed cow specimens, 23 (46%) had molecular evidence of influenza A infection (qRT-PCR assay targeting the influenza A matrix gene of H5N1 virus).

We developed high-throughput plate-based immunoassays to measure both Influenza A nucleoprotein (NP) antigen and H5 hemagglutinin (HA) specific antibodies. Both assays are species-agnostic and use electrochemiluminescence (ECL) technology from Meso Scale Discovery to perform highly specific and sensitive measurements. Cow milk samples from unexposed and H5N1-exposed herds were evaluated for specific detection of Influenza A NP antigen and H5-specific antibodies. Together, these assays quantify antigen and distinguish samples with H5-specific antibodies from naïve controls. Moreover, these assays can screen samples from any host species for Influenza A antigen and antibodies against H5 HA.

### **2** Methods

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



#### **Electrochemiluminescence Technology**

- Minimal non-specific background and strong responses to analyte yield high signal-to-
- 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
- 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.

### **5** Nucleoprotein Antigen in Cow Milk



#### of Influenza A nucleoprotein (NP) using MSD's R-PLEX platform. Samples were diluted 1:10, 1:500, or 1:2500 to measure signal within the linear range. None of the 30 healthy cows had detectable antigen in the milk; however, 26 of 50 (52%) of the exposed cows contained detectable levels of Influenza A antigen. This is greater than the number of samples positive by qRT-PCR (23, or 46%).

Dairy cow milk samples were analyzed for the presence

Samples that quantified at or below the lower limit of detection (LLOD) were set to the LLOD value. The solid horizontal line represents the median concentration for each group, and the dotted horizontal line represents the cut-point defining a positive result. This cut-point was set at 2 times the LLOD.

# **6** H5-Specific Antibodies in Cow Milk



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

Samples that quantified at or below the lower limit of detection (LLOD) were set to the LLOD value. The solid horizontal line represents the median concentration for each group, and the dotted horizontal line represents the cut-point defining a positive sample. This cut-point was set at 2 times the LLOD.

### **R-PLEX®** Assay for Nucleoprotein Antigen Detection



#### **Protocol**

- 1. Incubate streptavidin plates with capture antibody 1 hour at room temperature (RT).
- 2. Wash and add calibrator, control, or sample (25  $\mu$ L/per well). Incubate 1 hour at RT.
- 3. Wash and add detection antibody solution (50  $\mu$ L per well). Incubate 1 hour at RT.
- 4. Wash and add read buffer (150 µL per well). Analyze with MSD<sup>®</sup> instrument.

# **Bridging Serology Assay for H5 Antibody Detection**

### Antibody From Sample SULFO-TAG Conjugated Ag Biotinylated Ag MSD GOLD Small Spot Streptavidin Plate

#### **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 1 hour at room temperature (RT).

2. Transfer solution with formed complexes from off-line 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 antigens here are recombinant constructs with the HA head domain (amino acids 48-326) of the clade 2.3.4.4b H5N1 strain A/Ghana/AVL-763\_21VIR7050-39/21.

# Analyte Correlations in H5N1-Exposed Cows



Antigen and antibody quantities 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 the corner of each quadrant, with the percentage of samples from the H5N1-exposed herd in parenthesis.

Few milk samples (n=4) 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 having influenza antigens and H5-specific antibodies by one or both assays.

### **8 Summary and Conclusions**

Influenza A NP antigen was measured in 26 of 50 cow milk samples from exposed herds. The milk samples contained 14 ng/mL to 486 µg/mL of Influenza A NP antigen, indicating a wide range of viral load in these animals.

### **Calibrator Curves**



Typical standard curves for (A) NP antigen detection and (B) H5 bridging serology. Calibrator for the NP antigen assay was made with native Influenza A nucleoprotein purified from inactive virus. The dynamic range of the assay is 714-500,000 pg/mL. Calibrator for the H5 bridging serology assay was made with polyclonal serum from rabbits immunized with H5 A/Ghana HA antigen. The top calibrator was assigned 1,000 arbitrary units (AU). The lower limit of detection (LLOD) was calculated as the signal 2.5 standard deviations above background.

- H5-specific antibodies were measured in 11 of 50 exposed cow milk samples, suggesting these cows are convalescent to H5N1 virus.
- The antigen and antibody levels are complementary, indicating a group of cows that are actively infected, but seronegative, and a group of cows that lack viral antigen, but are seropositive (convalescent or recovered). Together, the two assays identify 64% of samples as containing Influenza A antigens and H5-specific antibodies in exposed animals.
- These assays are species-agnostic and can be used to analyze samples from humans, birds, and other animals.

#### Acknowledgements 9



Meso Scale Discovery

A division of Meso Scale Diagnostics, LLC.

www.mesoscale.com®

We thank Dr. Jacquelyn Boerman at Purdue University, Agriculture/Animal Science, for providing healthy control milk samples from dairy cows. Research reported in this publication was supported by the National Institute of Allergy

and Infectious Diseases of the National Institutes of Health under Award Number UH2AI176136. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

MESO SCALE DISCOVERY, MESO SCALE DIAGNOSTICS, MSD, mesoscale.com, www.mesoscale.com, methodicalmind.com, www.methodicalmind.com, Booster Pack, DISCOVERY WORKBENCH. MesoSphere, Methodical Mind, MSD GOLD, MULTI-ARRAY, MULTI-SPOT, QuickPlex, QuickPlex Ultra, ProductLink, SECTOR, SECTOR HTS, SECTOR PR, SULFO-TAG, TeamLink, TrueSensitivity, TURBO-BOOST TURBO-TAG, N-PLEX, R-PLEX, S-PLEX, T-PLEX, U-PLEX, V-PLEX, MSD (design), MSD (luminous design), Methodical Mind (head logo), 96 WELL SMALL-SPOT (design), 96 WELL 1-, 4-, 7-, 9-, & 10-SPOT (designs), 384 WELL 1- & 4-SPOT (designs), N-PLEX (design), R-PLEX (design), S-PLEX (design), T-PLEX (design), U-PLEX (design), V-PLEX (design), It's All About U, Spot the Difference, The Biomarker Company, and The Methodical Mind Experience are trademarks and/or service marks owned by or licensed to Meso Scale Diagnostics, LLC. All other trademarks and service marks are the property of their respective owners. ©2025 Meso Scale Diagnostics, LLC. All rights reserved

For Research Use Only. Not for use in diagnostic procedures.



DOWNLOAD POSTER