Development and validation of a recombinant-H5 hemagglutinin-based competitive ELISA for serodiagnosis of avian influenza A subtype H5 antibodies.

By

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Abstract

Hemagglutinin (HA) protein is a major antigen presenter within avian influenza viruses, which in turn triggers a substantial immunogenic response within the infected host. This study set out to exhibit a successfully designed, developed, optimized, and validated a highly sensitive and effective competitive ELISA based on recombinant-HA proteins as antigens from two unique strains of AIV H5 that is capable of detecting a wide range of strains of North American and Eurasia lineages, including clade 2.3.4.4 groups B and C viruses. An important reason behind the development of this assay was to achieve the ability to overcome a glaring drawbacks of needing access to high biocontainment facilities in order to perform the golden standard HI assay internationally recognized as the best characterization assay for AIV within aviary samples. This competitive ELISA is able to perform under a low biocontainment environment due to the stability, non-infectious nature of the reagents and the lack of need for live virus. This assay has the capability to be deployed worldwide to facilities that would not be able to perform HI assays and have the ability to effectively detect AIV-H5 antibodies within samples. An unforeseen obstacle within the HI assay was the necessity to have a homologous or calibrated virus for the detection of new and emerging strains that was discovered while testing this assay, while our cELISA did not have the same hindrance. The cELISA's were based on recombinantly expressed AIV-H5 HA full-length protein from 2 strains: A/Canadagoose/Oregon/AH0012452/2015 and A/Teal/Germany/Wv632/2005. Both cELISA showed high sensitivity and specificity, with low variation and no cross-reactivity to other viruses following calibration and optimization of the assays. This study shows the rec-H5 cELISA was able to perform with great confidence, equally or even outperforming the HI assay results based on our statistical analysis.

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Dedication

I would like to dedicate this dissertation to all those who supported me through this journey. To my partner, Brooke, who was there by myside from the very start and saw me through to the conclusion of my program. To my parents, who always encouraged me throughout.

Table of Contents.

List of Tables.

List of Figures.

1.0. Introduction

A significant issue currently posing an immense burden on our commercial poultry industry, at both global and domestic levels, is Notifiable Avian Influenza (NAI). The causative agents are avian influenza A viruses. These viruses continue to remain endemic and ravage the world's supply chain, leading to the deaths of millions of birds annually, millions of dollars of lost revenue, and possible supply shortages which effects are felt by people around the world. The World Animal Health Organization (WOAH) has put forward subtype and pathotype-specific international standards to control outbreaks of these NAI viral pathogens, which in turn leads to mandatory governmental reporting of such outbreaks (World Organization for Animal Health, 2015). The main industry concern is highly pathogenic avian influenza (HPAI) caused by viruses of H5 and H7 subtypes, which have mortalities of up to 100% (Biswas *et al*., 2011). These outbreaks have occurred throughout the world and have only increased in occurrence due to the ever-increasing demand for poultry products. An effective diagnostic program and surveillance tracking of new and arising strains, including H5 AIV, is of significant importance to maintaining a strong and healthy global poultry industry. Identifying potential outbreak strains and initiating early containment actions before they can establish themselves within commercial flocks is of major emphasis in today's world of poultry production.

Avian influenza virus (AIV) is classified in the family *Orthomyxoviridae*, genus *Influenza A virus*, and contains a negative-sense, single-stranded RNA genome composed of 8 gene segments encoding at least 11 viral proteins (Alexander *et al*., 2007). The AIV proteins can be categorized as 'surface proteins' (hemagglutinin [HA], neuraminidase [NA], and matrix protein 2 [M2]), 'internal proteins' (tripartite polymerase consisting of polymerase basic protein 2 [PB2], polymerase basic protein 1 [PB1], polymerase acidic protein [PA], nucleoprotein [NP], matrix protein 1 [M1] and nuclear export protein [NEP]) and 'nonstructural proteins (NS1 and PB1-F2) (Webster *et al*. 1992; Brown 2000; Cheung and Poon 2007). Avian influenza A viruses in terrestrial poultry evolve rapidly via a complex process that involves the accumulation of mutations over time and the rearrangement of viral RNA segments in cells infected with two (or more) different viruses (known as "reassortment'). The major antigen presenters in AI virus are hemagglutinin (HA) and neuraminidase (NA). In birds, there are 16 HA subtypes and 9 NA subtypes known, which can theoretically be configured into many different presentation

combinations (Abolnik *et al*., 2014). The hemagglutinin protein is a surface protein that binds to the sialic receptor on the host cell membranes and initiates cell-mediated endocytosis (Suzuki., 2005). Epithelial cells are the major entry route for AI viruses in both bird and mammal species, due to these cells heavily expressing many sialic acid receptors. The main route of infection for avian species is through the respiratory or intestinal tract (breathing in contaminated droplets or eating/drinking from contaminated sources), which both contain a high number of susceptible epithelial cells expressing α2,3-linked sialic acid receptors. This residue conformation is the preferential binding receptor that this virus has originally evolved to recognize (Neumann, 2015). Humans and most mammalian species on the other hand primarily express α2,6-linked sialic acids receptors on their epithelial cells of the respiratory tract (the main route of infection is the inhalation of viral infected droplets/aerosols) with only a few highly specific lung tissue cells capable of expressing the preferred receptor conformation (Yao *et al*., 2008). This lack of α2,3-linked sialic acid receptors in mammals explains a key reason why HPAI H5 and H7 do not cross into humans readily (require mutation to recognize α2,6-linked sialic acids receptors) and have extremely low human-human transmission rate (Maines *et al*., 2011).

This ability to bind and initiate entry and thus infection, is why the hemagglutinin (HA) protein is the most significant factor in the pathogenicity of the virus strain. The HA protein is a class 1 fusion protein composed of two subunits, HA1 and HA2, which are then co-translated with a cleavage site (HA0) between them that allows for infectivity (Luczo *et al*., 2018). The amino acid sequence within this cleavage site is the defining factor affecting viral pathogenicity and a key determinate between high pathogenic and low pathogenic AI viruses (Monne *et al*., 2014). This is due to the cleavage process, which is done via host cellular proteases. The amino acid sequence of an AI HA0 cleavage site is PEKQT-/GLF. The -1 position from the cleavage site is the explicit feature that distinguishes it from HPAI and LPAI. Low Pathogenic Avian Influenza contained a mono-basic cleavage site, containing one basic amino acid residue (Illustrated via X) in the -1 position (i.e PEKQTX/GLF). This only allows highly specific host cellular proteases located in the gut and respiratory tract to cleave progeny AI virions, limiting the virus and its mortality to the host. Highly pathogenic avian influenza contains a poly-basic cleavage site, encoding multiple basic amino acids insertions around the cleavage site (PEKQTXXXX/GLF). The addition of more basic amino acids at the cleavage site allows a wider variety of host cellular proteases to cleave AI progeny, allowing viral infection

systemically throughout various systems in the birds' body. HPAI is, therefore, able to affect more major systems of the host and grow more rapidly, overwhelming the immune system and causing significantly higher mortality rates and a major drop in agricultural production (Banks *et al*., 2000; Garcia *et al*., 1996; Perdue *et al*., 2008; Senne *et al*., 1996).

In addition to molecular characteristics of the HA protein, more specifically as changes in the proteolytic cleavage site, the phenotype classification of avian influenza into LPAI and HPAI viruses is based on in vivo testing (i.e., their pathogenicity or their ability to cause mortality in birds). Highly pathogenic avian influenza (HPAI) viruses are characterized by intravenous pathogenicity index (IVPI) greater than 1.2 in 6-8-week-old specific-pathogen-free (SPF) chickens or a mortality rate of over 75% in a poultry population during over an interval of 10 days (World Organization for Animal Health, 2015). Low pathogenic avian influenza (LPAI) are viruses that are characterized by their mild symptoms and low mortality in wild birds or poultry. These viruses are not less infectious but either lack the ability to infect systemically or cause death within the host. Both HPAI and LPAI can still cause disease in poultry and cross into mammals at varying rates (United States Department of Agriculture,2015). Understanding the difference between HPAI and LPAI is important, because low pathogenic strains can mutate and if a few key mechanisms are correctly targeted, LPAI viruses can become highly pathogenic leading to mass mortality and impact on producers and the supply chain. Currently, all known and isolated HPAI virus strains belong to H5 and H7 subtypes. Historically, these viruses originate from LPAI virus precursors that were able to mutate to the current HPAI virus strains containing novel expressed viral proteins compared to the original strain (Monne *et al*., 2014).

Since 1959, most HPAI outbreaks have remained regional and restricted within geographical regions due to a lack of circulation within wild and migratory birds leading to 42 individually distinct epizootics (Lee *et al*., 2017). The main route of spread was farm to neighboring farm transmission, which was able to be controlled via effective measures such as detection-culling programs and farmer compensation systems (Swayne *et al*., 2006). However, this changed in 1996 after the detection of an infected domestic goose in Guangdong, China. This strain, designated the Gs/GD lineage clade 2.3.4, was unique within HPAI viruses due to its ability to cause death across wild and domestic birds along with humans. This lineage, previously uncharacteristically, spread globally across Asia, Europe, Africa, and the Americas

leading to its labeling as panzootic (Swayne *et al*., 2016). Since its discovery, the H5NX Gs/GD lineage has evolved and mutated into 10 clades, all genetically distinct from each other (WHO/OIE/FAO., 2008) with multiple other HPAI subtypes (H5N2, H5N5, and H5N8) bearing the genetic signature of this lineage within their genome due to its involvement in reassortment events (Gu *et al*., 2011; Wu *et al*., 2014; Zhao *et al*., 2012). Amongst these subclades, 2.3.4.4 branched off its precursor and rapidly began genetic reassortment events with other viruses including local LPAI viruses and other clades (Lee *et al*., 2017). Research into this clade has shown it has since evolved into 9 different distinct genetic groups, A-H which continue to circulate and enter poultry farms (Lee *et al*., 2015;Lee at al., 2016).

This is a major reason avian influenza A H5 subtypes were chosen to design an effective diagnostic tool due to its high public health concern along with its agricultural and economic importance in the poultry industry. Both LPAI and HPAI viruses of the H5 lineage can cause widespread outbreaks in countries' poultry flocks leading to bird death, mass culling, and major economic losses (Luczo *et al*., 2018). Effective methods for detection of both types of viruses are needed to ensure proper surveillance is achieved and the ability to detect against a broad range of lineages exists. While low pathogenic AI strains do not have high mortality, they still have the ability to cause mild respiratory disease in birds, depression, and/or a decrease in egg production for laying hens (Fouchier *et al*., 2009). The more widespread infections are within flocks and countries industries, the greater the chance new novel or high pathogenic mutations can occur sparking off a new outbreak. And this concern is not just limited to poultry but is a major concern to human health with the possibility of a pandemic level spillover event into the human population similar to current events such as SARS-Cov-2.

Canada has already felt the brunt of such outbreaks, as one occurred in British Columbia in 2004. This led to the killing of approximately 17 million birds at a cost of $500 million (Kermode-Scott *et al*., 2004). A second major outbreak reoccurred in 2014-2015, when another HPAI event happened again in British Columbia, causing the culling of 240,000 birds across multiple farms in the province. This epizootic event was caused by the emergence of H5N1-like Gs/GD lineage clade 2.3.4.4 group C in North America for the first time (Berhane *et al*., 2016). Just recently in late 2021 to early 2022, a new virus has emerged in Eastern Canada and quickly swept across Canada and the Northern United States spreading rapidly into domestic and wild

bird populations. This HPAI is represented in H5N1-like Gs/GD lineage clade 2.3.4.4 group B of viruses and its devastation to both birds and monetary cost has yet to be fully known. Containment and extermination protocols are being implemented as of early 2022.

The Canadian Notifiable Avian Influenza Surveillance System (CanNAISS) program was created by the government to track the domestic and international incidence of AI and aims to control the spread of NAI. The goal is to detect, contain, and eliminate early NAI within the domestic poultry flocks or guard our domestic flocks against the outside introduction of these viruses. Canada's national goal is to demonstrate the freedom of notifiable avian influenza H5 and H7 subtypes across Canada's domestic poultry flocks. This goal also requires the need for annual surveys of the wild and migratory birds within or traversing our borders to better understand the potential risks our domestic flocks have at exposure to these potential viruses. This identification of potential strains is done through the detection of AIV nucleoprotein (NP) antibodies in collected serum samples of birds, both wild and domestic. Any positive samples are then further analyzed for subtype specificity through an assay called hemagglutination inhibition (HI). The HI tests work on the interaction of sialic acid receptors on the surface of host red blood cells and the HA protein of AIV. If Influenza virions are present, they can attach to multiple red blood cells, cross-linking and causing visible clumping of the erythrocytes on the plate. The addition of neutralizing antibodies to a specific HA subtype (H7 specific antibodies or H5 specific antibodies) can inhibit this cross-linking/clumping of the red blood cells and identify the subtype of the virus. This is the primary gold standard test in identifying HA subtypes of unknown viruses (Webster *et al.*, 2002). The major limiting drawback to this method is the need to maintain an inventory of H1 to H16 subtype viruses on hand, which in turn means all work must be done in a containment level-3 lab (CL-3).

This drawback presents a challenge for early and effective diagnosis, which gives room for assay improvement. We propose to develop a serological test that can quickly identify H5 antibodies in an ELISA format without the need of using a live virus, hence without the need for CL-3 use. This utilization of diagnostic capability allows significant cutting of time (shipping samples from provincial testing labs to the National Centre for Foreign Animal Disease located in Winnipeg, Manitoba, Canada), deduction in staff specialization, and costs. We aim to develop this method as an improved competitive enzyme-linked immunosorbent assay (cELISA) which

can serve as a rapid, sensitive, convenient, and cost-effective tool for large-scale screening and identification of H5 specific antibodies in sera samples independent of animal origin or lineage. Our goal is to develop an assay to detect against a broad spectrum of H5 AIV branches of both LPAI strains HPAI strains, including coverage of clade 2.3.4.4. This includes the group C viruses from the 2014-2015 outbreak and the present group B viruses currently in an epizootic outbreak within North America.

2.0. Literature Review

2.1. Introduction

Avian Influenza (AI) was initially known as Fowl Plague and was first ever reported in the year 1878 (Alexander *et al*., 2009), with the earliest successful isolation of an AI virus in China in 1996 originating from a goose (United States Department of Agriculture., 2015). During the latter half of the 20th century, Avian Influenza outbreaks were localized and loss to flock population was minimal. In the late 1990's and early 2000's, countries poultry populations started rapidly increasing, leading to a rise in the incidence of AI outbreaks. Developed countries poultry stocks increased 23% and developing countries stocks increased 76%, which corresponded to an estimated 11 major outbreaks between 1996-2008 resulting in the loss of millions of birds (Alders *et al*., 2014). Now, outbreaks are extremely common due to the intensive farming practices of many countries and the commercialization of the bird industry.

Avian influenza spreads most often due to the interaction of infected and healthy birds in close space but workers and equipment contaminated with the virus may also spread it (World Organization for Animal Health., 2015). The common mode of transmission is by the nostrils, mouth and eyes via secretions and droplets. The influenza virus is not airborne but spread by the droplets being up-taken by other birds in the proximity of infected birds. Transmission of AI to people is possible during slaughtering, plucking of feathers or other close contact interactions with infected birds (World Organization for Animal Health., 2015). Most transmission is from birds to humans with very little evidence of human to human transmission, besides incidents involving extremely prolonged contact such as family dwellings transmission (World Organization for Animal Health., 2015).

The influenza A virus has a broad host range, including animals such as waterfowl, chickens, turkeys, pigs, bats, humans and horses. While some strains are species-specific such as swine flu, birds are known to have the ability to harbor all known subtypes of influenza A virus (Hiromoto., 2000). Waterfowls and other migratory birds are a major transmitter of AI, as disease rarely results in symptoms and fatality, while their migratory nature can spread it to other wild bird species and domestic stocks over large areas and continents (Hénaux *et al.*, 2011). Common commercial poultry species are at large risk of these transmissions which can explode due to the manmade, highly populated ecosystems these birds live in.

The global economy has routinely felt the consequences of Avian Influenza outbreaks and major culling events to limit its spread. Poultry has recently equalled or surpassed pork consumption in 2018, consuming 120 million tonnes of poultry meat and 1.2x1012 eggs per year equaling a 217-billion-dollar industry according to data published by the Food and Agriculture Organization of the United Nations. Due to the pressure to maintain such supply, extensive farming practices have contributed to AI outbreaks and major economic losses. Between 2003-2005 the global poultry community experienced a pandemic of highly pathogenic H5 virus which devastated countries flocks around the world. Worldwide GDPs dropped, such as China and Vietnam by 1.5% totaling 450 million USD. Up to 140 million birds were culled in 1 year of outbreak containment protocols and the deaths of 175 people were linked to the virus. Domestically in Canada, 17 million birds were culled at a cost of $500 million CAD (Kermode-Scott *et al.*, 2004). The prevention and containment of AI viruses are both extremely agriculturally important due to the high demand for poultry products and economically relevant as many countries' economies are dependent on export and sales.

2.2. Discussion

With the prevalence of AIV throughout the aviary world, understanding its characteristics and intricate genetics of this virus will help in being able to combat these outbreaks and stomp or prevent them from occurring. Our increasing dependence on industrial scale farming practices and the interconnection of global trade and travel makes it extremely important in studying and laying the foundation of knowledge on the virulence, transmission and genomic changes these viruses go through prior to erupting into HPAIV. A large array of studies have been undertaken to achieve this comprehension and linked specific proteins or nucleotide sequences within the AI

genome that are major contributors to virulence or human transmission. These studies have honed into the proteins hemagglutinin surface protein (HA), polymerase PB2 protein subunit and NS1 protein.

Viral hemagglutinin is a surface glycoprotein that binds to sialic acid receptors on the host cells and mediates entry (Suzuki., 2005). Epithelial cells are the major entry route for AI viruses in both bird and mammal species each expressing varying sialic acid receptors. The major route of infection for avian species is through the respiratory or intestinal tract (breathing in contaminated droplets or eating/drinking from contaminated sources), which epithelial cells express an α2,3-linked sialic acid receptor. This residue conformation is the preferential binding receptor that this virus has originally evolved to recognize (Neumann, 2015). Humans and most mammalian species on the other hand primarily express α2,6-linked sialic acids receptors on their epithelial cells of the respiratory tract (main route of infection is inhalation of viral infected droplets/aerosols) with only a few highly specific lung tissue cells capable of expressing the preferred receptor confirmation (Yao *et al*., 2008). This lack of α2,3-linked sialic acid receptors in mammals explains a key reason why HPAI H5 and H7 do not cross into humans readily (require mutation to recognize α2,6-linked sialic acids receptors) and have extremely low human-human transmission rate (Maines *et al*., 2011).

Polymerase PB2 protein subunit is part of a trimeric polymerase complex that replicates and transcribes viral RNA. Key mutations in this protein are linked by various studies to increased infection of mammalian host and present in all HPAI lineage strains. Yet surprisingly these mutations are more relevant in human infections than mortality rates and virulence in avian species. A mutation at position 627 replaced the native residue (glutamic acid) with a lysine. These studies concluded that this mutation allows for replication of AI viral RNA to occur at upper respiratory tract temperature in humans, which the native form is unable to do and hence not properly thrive in a human host (Hatta *et al*., 2001; Subbarao *et al*., 1993). This has been seen in a 2005 outbreak of HPAI H5N1 in Qinghai Lake, China. Isolate viruses were found to have the position 627 lysine mutation in the PB2 protein (Chen *et al*., 2005; Chen *et al*., 2006; Liu *et al*., 2005). Yet, reports out of Indonesia and Vietnam contradict this claim. These countries continue to deal with human infections of HPAI, and genetic studies have shown viral isolates containing the native amino acid sequence which places in doubt the necessity of this

mutation for human infection (Le *et al*., 2013; Nguyen *et al*., 2012). A study in 2013, recently provided evidence of strong selective pressure during replication for this mutation and its ability to out compete the native strain resulting in evidence to why we on average consistently find this mutation in most HPAI human infections (Wright *et al*., 2013).

In this protein, multiple other mutations of been found and provide evidence for increased virulence in hosts. A mutation at position 701 (aspartic acid to asparagine) involved in importin α interaction has been discovered. Importin α is responsible for protein transport from the cytoplasm to the nucleus in host cells. The position 701 mutation has been shown in studies to increase binding to mammalian importin α than to the avian counterpart resulting in the ability for the virus to increase the effectiveness of replication and hence its virulence (Gabriel *et al*., 2005; Gabriel *et al*., 2008; Li *et al*., 2005). While studies have shown other mutations to have occurred in PB2 subunit (i.e. 591K, 271Ala, 147T, 339T and 588T) and have been documented in the Centers for Disease Control and Prevention H5N1 genetic changes inventory, they have not been well studied and are not documented to occur at significant rates in the HAPI lineages or viral isolate population (Bussey *et al*., 2010; Fan *et al*., 2014; Yamada et a., 2010).

NS1 protein is an important factor in blocking stimulation of the innate immune system via several mechanisms outlined in a study by Garcia-Sastre *et al*., 1998 such as IFN-β promoter inactivation, RIG-1 suppression, and inhibition of antiviral factors. IFN is an important signalling protein produced via the host in response to viral stimulus (Parkin *et al*., 2001). Most viruses target this cytokine with strategies for interfering with or downregulating its expression, and avian influenza A is capable of such mechanism. The first evidence linking mutated NS1 protein to increased virulence was confirmed by two studies (Ma *et al*., 2010 ;Seo *et al*., 2002) that found highly pathogenic H5N1 avian influenza that had passed its viral RNA segment containing NS1 protein onto other AI strains (H7N1) became more virulent and pathogenic than its natural state. This can also be seen when a study compared low pathogenic and high pathogenic H5N1 strains which found the NS gene was a major determining factor between the two (Imai *et al*., 2010). Several studies identified a variety of mutations to the amino acid sequence that may play a role in this difference. Mutations such as position 92 with a substitution of a glutamic acid are implicated in the increased virulence of a low pathogenic strain that acquires this gene from HPAI H5N1 (Seo *et al*., 2002). This specific amino acid position has

been found to be directly linked to regulating IFN induction which explains the ability of this gene to allow increased virulence (Li *et al*., 2010).

Since the late 1990's high pathogenic H5N1 viruses have been isolated and sequenced. A common trait in wild type HPAI H5N1 is a deletion of 5 amino acids from positions 80-84 of the NS1 protein. Researchers were able to replicate this increased pathogenicity by causing the same amino acid deletion artificially in LPAI H5 strains leading to a direct correlation to this mutation having a major role and were able to trace the cause back to this gene ability to control IFN activation in the host cells at a more effective rate (Long *et al*., 2008)

To discover how and why these genomic changes have been observed, it is important to know the mechanics of AIV genomic mutation. This can occur via 2 major different mechanisms: Reassortment and recombination events. AIV antigenic shift or mutations play a role in AI genetics but to a lesser extent. Genetic reassortment is defined as the biological process of a whole gene swapping between similar strains to create new combinations (Alberts *et al*., 1997). This process is a common occurrence in viruses, especially influenza A. The avian influenza A genome is made up of 8 individual segments of RNA (genes) and each virion requires 1 copy of each to remain viable. If two AI viruses infect or are carried in the same host/reservoir, they can mix their segments during replication giving rise to new combinations of viral genetic configurations (McHardy *et al*., 2009). In this way, the new virion progeny can share phenotypes of both parent viruses. HA and NA gene switching is a major outcome for such an event.

The hemagglutinin gene is located exclusively on segment 4 and neuraminidase on segment 6. Two influenza A viruses could reassort these genes giving rise to unique combinations of knew HA and NA antigens (Parrish *et al*., 2005). As an example, H5N1 and H7N9 co-infection can cause a reassortment into a variety of new combinations such as H5N9, H7N1 with both parental strains potentially still being maintained with other genes swapping. This is extremely common in species that act as a natural reservoir to AI viruses where many different variants can be isolated from the same population as most do not cause adverse effects until a highly pathogenic combination occurs (Campitelli *et al*.,2004). HA and NA proteins are the main antigens to which neutralizing antibodies of the immune system recognize and can render previously infected or vaccinated hosts who under normal circumstances would have

immunity, vulnerable to infection (Parrish *et al*., 2005). Another example is passing a segment that confers higher virulence to a strain that previously did not cause illness. This was described by two studies (Ma *et al*., 2010; Seo *et al*., 2002) that found highly pathogenic H5N1 avian influenza had passed its NS1 viral RNA segment onto other AI strains (H7N1) and that H7N1 strain became more virulent and pathogenic than its natural state. All segments are capable of reassorting to new viral progeny (Parrish *et al*., 2005; Taubenberger *et al*., 2010). Positive selection can change the dynamic of a viral population (Taubenberger *et al*., 2010).

The other mechanism that affects avian influenza A genetics is the process of recombination. This is defined as the swapping of sections of an RNA segments between viral strains giving a novel combination or introducing unique phenotypes. Although this naturally developed as a way to fix viral genome damage (Barr *et al*., 2010). In a co-infection of a host by two different strains, the viral capsids and membranes are removed during RNA transcription. During this time, matching gene segments may recombine and swap sections of identical segments to form a new variant of the segment. In this way antigenic shifting can occur to change the virus antigen presentation, hence avoid recognition from the host immune system and/or overcoming vaccine-produced antibodies (Bernstein *et al*., 2018).

To combat these events and limit the amount of infection/spread of AIV, hence limiting its chance at genetic changes leading into novel or HPAIV biosecurity and good health practises must be in place globally, including vaccination programs. Vaccination practices in the poultry industry aim to provide an additional level of biosecurity by providing protective immunity to the flocks from infectious diseases. This is done by either providing immunity from infection or less severe outcomes to infections. Vaccination is a proven method for both reducing the risk of exposure, spread and economic loss by AI according to the World Organization for Animal Health. Avian influenza A is very region-specific regarding strain prevalence and a good vaccination program must be tailored towards the strains circulating in both the wild and domestic flocks of the region. Currently, the following vaccines are available for poultry producers to access: Monovalent H5 and H7 vaccines or a bivalent H5/H7 vaccine according to the Food and Agriculture Organization of the United Nations. Each vaccine can be tailored with a specific neuraminidase variant in accordance to the regions outbreak strains. Countries independently decide on national vaccination programs and if that includes AI vaccines. China,

Egypt, Indonesia and Vietnam are the only countries that have developed a national routine AI vaccination program that inoculates all birds (layers, breeders and broilers) against both H7 and H5. These countries use approximately 95% of the world's supply of the vaccine. Mongolia, Kazakhstan, France and the Netherlands undergo a preventative vaccination program on high risk, high density producers. Cote d'ivore, Sudan, North Korea, Israel, Russia and Pakistan approved vaccination programs in only emergency cases. All other countries do not have any national level of vaccination mandates (Swayne *et al*., 2012).

H5 subtype avian influenza is a major viral lineage that affects the poultry industry and has caused massive damage to all parties. There are 9 known H5 viral subtypes: H5N1, H5N2, H5N3, H5N4, H5N5, H5N6, H5N7, H5N8, and H5N9. Most of these viruses are characterized as low pathogenic but they can mutate to a high pathogenic form and some lineages have already, specifically H5N1. These viruses have shown the ability to infect both humans and poultry species (Diederich *et al*., 2015).

Influenza A H5N1 has been shown to both infect birds and mammals and is enzootic (endemic in the wild bird population) specifically in Southeast Asian countries (Li *et al*., 2004). H5N1 family of viruses contain a variety of strains, each harbouring different mutations and hence varying pathogenicity and mortality. One H5N1 strain is currently spreading globally after first appearing in Asian bird flocks. This strain is a highly pathogenic, capable of 100% mortality within 48 hours and has led to multiple outbreaks and the culling of hundreds of millions of birds. The Asian lineage can be broken into two different clades, clade 1 contains isolates that can infect both human and birds from Vietnam, Thailand, Cambodia, Laos and Malaysia. Clade 2 isolates come from China, Indonesia, Japan, South Korea. Clade 2 is the most significant on a global scale, as it's been seen spreading towards Europe, the Middle East and Africa (Robert *et al*.,2006). These viruses that are spreading out from Asia have been identified by the WHO to have caused the massive pandemic like outbreaks in 2005-2006 (World Health Organization, 2006).

This outbreak reached across the globe to all inhabited continents, killed or led to the culling of hundreds of millions of birds and infected humans with a mortality rate of 53% (Wan *et al*., 2012). Besides culling, the best prevention to HPAI H5N1 is vaccination. Multiple countries have routine vaccination practises in high enzootic areas with other countries

approving vaccine for emergency use (Gao *et al*., 2006). The biggest hurdle for HPAI H5N1 vaccine use is the virus's high mutation rate, which gives each developed vaccine a determined length of time that it is effective against the viruses (Shao *et al*.,2017). This has led to constant redevelopment of vaccines and the chance a mutation can led to a human adaptive virus may catch poultry producers by surprise and without an effective treatment.

The main strain circling in North America is a LPAI H5N2, which has caused outbreaks in multiple states and province across the continents (West Virginia 2007, British Columbia 2004/2009, Manitoba 2010) according to the Canadian Food Inspection Agency tracking. This strain is usually very low pathogenic and flocks experience only mild symptoms with low mortality rates (Jhung *et al*., 2015). Since surveillance and tracking of AI viruses in North America began, two instances of HPAI H5N2 North American strain outbreaks have occurred. In 1983 at a few Pennsylvanian chicken farms a HPAI H5N2 began spreading, requiring the culling of 17 million birds to eradicate it. Studies done later involving infecting SPF chickens with wild guinea fowl H5N2 collected that same year, resulting in mild to no symptoms and no mortality. This finding indicated that this virus occurred via mutation within the domesticated flocks instead of wild bird transmission. Genetic analysis determined the HA gene in the chicken strain mutated at a higher rate than the wild bird populations strain, leading to the production of a novel HPAI variant. Extensive hazard control and tough protocols maintained its spread and resulted in its elimination from the poultry population (Wood *et al*,. 1985). The second outbreak occurred in 2004 in Texas, affecting a flock of approximately 7,000 birds. Similar circumstances are presumed to occur as when the genome of this virus was analysed no Eurasian genes were detected and wild bird surveys proved no linkage between the strains (Roos, 2004).

In 2014 British Columbia, a new novel HPAI strain was identified in poultry barns across the province. This strain had a combination of HPAI Eurasian lineage H5N8 hemagglutinin genes and the North American H5N2 neuraminidase genes. This reassortment event produced a HPAI H5N2, a first HPAI strain in the North America wild bird population (Ip *et al*., 2014). The viruses caused severe symptoms in birds with an extremely high mortality rate. A combination of turkey, broiler and laying hen barns were affected. This outbreak lead to quarantining of barns and surrounding farms, the complete culling of flocks, biologically controlled composting, and an enhanced biosecurity for Western Canadian producers under CFIA's containment protocols.

No human associated cases were every reported during these outbreaks and LPAI H5N2 which has been circulating for decades has not caused human illness.

Genetic analysis of this virus determined this strain to contain 5 HPAI H5N8 Eurasian lineage genes and 3 LPAI H5N2 North American Lineage genes (Pasick *et al*., 2014). Outbreaks across the United States have continuously occurred throughout the Pacific, Central and Mississippi flyways (Figure 3) even through extensive culling and biosecurity has been undertaken according to CDC tracking. This strain of HPAI H5N2 is a continuous burden to the poultry industry that has caused the loss of millions of birds. The novelty is a first of its kind where a Eurasian strain has mixed with a North American strain and vaccination has been undertaken, specifically in breeding ground in Mexico and central America to help combat these outbreaks (Bertran *et al*., 2020).

There are 7 other subtypes in the H5 avian influenza virus family, H5N3, H5N4, H5N5, H5N6, H5N7, H5N8, and H5N9. Most of these viruses are low pathogenic, cause low mortality rates in birds and little risk to human transmission. They have also been commonly found in both wild bird population as well as domestic flocks (Centres for Disease Control., 2017). Avian influenza H5N3 was first isolated in Canada and again in Europe in summer of 2005 (Recombinomics., 2005). This virus is commonly found in wild migratory birds and in poultry birds along with H5N1 and H5N2 infections, giving evidence from wild bird transmission into domesticated flocks due to coinfection of multiple viruses usually arise from reservoir spread. A chicken adapted virus has been isolated which has a 20 amino acid deletion in the NA gene that is not present in isolates from the wild bird population. The chicken adapted H5N3 viruses has been documented to cause lesions on multiple organs unlike HPAI H5N1 which only cause massive lesions in the respiratory tract (Mundt *et al*., 2009).

Avian Influenza H5N4 is a low pathogenic virus which has been isolated in wild bird populations, specifically waterfowl, in North America (González-Reiche., 2013). No outbreaks have been discovered in domestic flocks, human transmission has not been associated with H5N4 and mortality in wild birds has not been observed. Avian influenza H5N4 is on a surveillance list for reassortment events with Asian lineage high pathogenic strains (Huang., *et al* 2013). Avian Influenza H5N5 has been associated with high pathogenicity in birds with outbreaks occurring in China and Taiwan domestic production (Gu *et al*., 2011). A study testing

HPAI H5N5 receptor binding found that this virus can bind both avian sialic acid receptor(α-2,3) and human (α-2,6) receptors, giving the possibility of spill over into humans given the necessary genetic reassortments and mutations (Li., *et al* 2015).

Avian influenza H5N6 has been categorized into 3 groups of virus lineages; A Eurasian strain that reassorted its NA gene with a LPAI Eurasian H5N8 virus and two Asiatic strains with one involved with human isolates (Shin *et al*., 2020). These Asiatic strain H5N6 circulate China, Laos, Vietnam and South Korea. A H5N6 outbreak in South Korea led to the culling of over a billion birds from 2014-2018 with 21 laboratory confirmed human cases of transmission (Baek *et al*., 2020).

A new isolated of H5N7 was first discovered in mallard ducks in Denmark. Genetic analysis showed a close relationship between the HA gene of the LPAI H5N7 and a HPAI H5N2, with a slightly modified HA cleavage site more associated with LPAI viruses. In addition, the N7 gene most likely reassorted from an event with a HPAI H7N7 virus that is known to infect humans (Bragstad *et al*., 2006). This is the first reported isolation of a H5N7 virus, and the birds had no clinical signs of disease (Bragstad et a., 2005). The close relationship of this new virus to two HPAI viruses, which can infect humans, gives rise to H5N7 needing to be closely monitored for a potential pandemic level outbreak that may involve human illness.

Outbreaks of avian influenza H5N8 has been first reported in Ireland in 1983 (Swain, 2008), with the most recent ones occurring in 2016-2017 and 2020. These viruses are highly pathogenic, but not a serious threat to human infection, and present all over Eurasia, Middle East and Africa. It is strongly connected with migratory birds and has caused mass culling on poultry farms (Jeong *et al*., 2014). Currently in 2020, an outbreak has occurred in Saudi Arabia eventually spreading into multiple countries including Russia, Kazakhstan, Germany and France.

Lastly, avian influenza H5N9 has been isolated first in Manitoba and Ontario (1966) causing flu like symptoms in turkeys (Ping *et al*., 2012). Even though it is of HPAI designation this virus has not caused serious harm in the poultry industry and its host range is mostly turkey and mallard ducks (Yang *et al*., 2015). A recent study identified AI viruses from a live animal market in China and following genetic analysis linked a new novel H5N9 strain with the reassortment of H5N1, N7N9 and H9N2. They discovered the HA gene was obtained from a HPAI H5 gene and a N9 gene from a H7N9 viruses with known ability to infect humans (Yu *et*

al., 2015). Further research is necessary as this novel strain has shown limited mortality in animal models in this study.

3.0. Methods and Materials

3.1. General Laboratory Methods

3.1.1. Western Blot

Testing samples were reduced in a solution mixture of NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent (Invitrogen, Massachusetts, United States) at 70°C for 10 minutes. Samples were then spun down and a NuPAGE 10% Bis-Tris, 1.5 mm, Protein Gels was set up utilizing NuPAGE MOPS SDS Running Buffer (Invitrogen, Massachusetts, United States). 10µl of sample and size marker (SeeBlue Plus2 Pre-stained Protein Standard, Invitrogen, Massachusetts, United States) was added to the wells of the gel and run at 200 volts for 45 minutes or until the leading line reaches the end . Following electrophoresis, the gel is soaked in dH2O along with the transfer membrane of the iBlot Transfer Stack , nitrocellulose kit (Invitrogen, Massachusetts, United States). The iBlot machine was set up according to operating procedure using the above-mentioned kit and run on a pre-programmed run (P3) for 7 minutes. Following the transfer, the membrane was blocked using 1x Casein Blocking Buffer diluted from 10x concentrate (Milliepore Sigma, Massachusetts, United States) for 1 hour with rocking. The Membrane was then coated with Anti-His HRP-Conjugate (Novagen, Billerica, MA) at 1/1000 dilution for 1 hour at room temperature or with a reference H5 serum (1/50 dilution) overnight at 4°C with rocking. The membrane was then washed 3 times for 5 minutes each with a PBS plus 0.05% tween20 (PBS-T, Milliepore Sigma, Massachusetts, United States) bath with rocking. The membrane coated with reference serum was incubated for 1-hour rocking with an anti-chicken HRP conjugated mAb (1/2000 dilution) followed by another wash cycle. A final wash with dH2O was performed and development was conducted via 3,3'-Diaminobenzidine (DAB) substrate. Following color formation, the reaction was stopped by the addition of dH2O.

3.1.2. Hemagglutination Inhibition (HI)

Performance of HI test was performed under CL-3 containment conditions. Samples were initially tested for non-specific binding with 0.05% chicken red blood cell (CRBC). If binding was observed by the formation of a compact button after a 30 minutes incubation with the

CRBC, samples were hemabsorbed with 5% CRBC and retested. Following confirmation; all samples lacked non-specific binding, and the virus to be used in the HI assay was standardized using the hemagglutination Assay (HA) method. The known virus is diluted 1:1 in the first well (A-H1, depending on the number of viruses being tested) in a v-bottom 96-well microtitre plate with 0.01M Phosphate-buffered saline (PBS). A 2-fold serial dilution was then performed on these test samples across wells 2-12. Following serial dilution, 0.05% CRBC suspension is added. The microtitre plate is covered and shaken briefly before a 30-minute room temperature incubation. Following 30-minutes, the plate is placed on a 45° angled stand for 30 seconds with the reaction being recorded. Compact "buttons" (tight collection of CRBC) should be present prior to tipping. If a "teardrop" or running of CRBC buttons is observed on microtitre plate, the sample is called negative. If CRBC buttons remain tight with no running, the sample is called positive. Viral titre is calculated based on serial dilution, with the highest dilution of positive results representing 1 HA unit. The reciprocal of the dilution is therefore considered the hemagglutination titre of the virus within the sample. The HI assay utilizes a viral dilution of 4HA units.

Testing of unknown sera samples can now be done with the inclusion of a positive control, negative control, and back titration row. 25µl of 0.01M PBS was added to all testing rows wells (1-12). 25µl of the sample was then added to well 1, with a serial dilution of all samples across the testing row performed. 25µl of diluted virus (according to the HA mentioned above) is then added to all test wells, with the plate shaken briefly before incubation at room temperature for 30-minutes. Following incubation, 50µl of 0.05% CRBC is added to all wells and the plate is shaken briefly before another 30-minutes incubation at room temperature. Following this final incubation, the plate is tilted at a 45° angled stand for 30 seconds with the reaction being recorded. If a "teardrop" or running of CRBC buttons is observed on microtitre plate, the sample is called positive, as antibodies present within the sample successfully inhibited viral binding. A compact "button" (tight collection of CRBC) would indicate the sample is negative as no antibodies are present to inhibit viral binding and cross-linking of CRBC occurred. The reciprocal of the highest dilution of test sera which still produced a negative agglutination reaction is considered the hemagglutination inhibition titre of that virus.

3.2. Preparation of viruses

Avian influenza subtype A H5 A/Turkey/ON/6213/66 was acquired from the National Center for Foreign Animal disease (NCFAD) viral depository. Embryonated chicken eggs (9-day-old specific pathogen-free) were inoculated into the allantoic cavity with 0.2 ml of the AIV strain inoculum. Eggs were incubated in a stationary incubator at 37°C, with 55% relative humidity and monitored twice daily for mortality. Embryos that died within 24 h were discarded. Allantoic fluid from embryos that died after 24 h was collected aseptically in a biosafety cabinet and tested via hemagglutination assay (HA). Confirmed Allantoic fluid containing the inoculated H5 strain was pooled and clarified by centrifugation at 4600 × g, using a JLA 10.5 rotor for 30 min at 4°C. The clarified supernatant was collected, and the virus was inactivated using Binary ethylenimine inactivation (BEI) at a final concentration of 0.1M. The suspension was stirred for 2 hours at room temperature and then overnight at 4°C. The inactivated virus supernatant was filtered through a 0.8µm filter and then concentrated via ultracentrifugation with a 25% sucrose cushion at 28,000 rpm for 120 minutes using the Optima XPN-100 ultracentrifuge (Beckman., California, United States). The viral pellet was suspended in PBS and stored at -80˚C for later use. All procedures were performed under containment level 3 conditions.

3.3. Preparation and expression of recombinant HA proteins

For the development of the competitive ELISA, we used recombinantly expressed full-length AIV hemagglutinin protein (Both HA1 and HA2 together) of A H5 A/Teal/Germany/Wv632/2005 (GenBank accession no. CY061885) and H5 A/Canadagoose/Oregon/Ah0012452/2015 (Genbank accession no. KU201744). We performed alignments of all available gene sequences of H5 subtype viruses from GeneBank and analyzed and optimized for consensus encoding nucleotides. These consensus gene sequences for full-length H5 hemagglutinin genes were cloned into a pAB-119 beeTM-FH vector (AB Vector, LLC, San Diego, CA) by GenScript (GenScrip USA Inc, Piscataway, NJ). The vector containing the desired gene sequences was then purified and co-transfected with linearized baculovirus vector DNA and ProFoldTM-ER1 (AB Vector, San Diego, CA) onto Spodoptera frugiperda (Sf9) insect cells to generate recombinant baculovirus containing full-length HA gene for both H5 avian influenza type A strains. This recombinant baculovirus was plaque purified and sequenced to verify the correct nucleotide sequence. Following verification, the recombinant

virus was then infected onto Trichoplusia ni (Tni) insect cells for A/Teal/Germany/Wv632/2015 at an MOI of 10. For strain A/Canadagoose/Oregon/Ah0012452/2015, the recombinant virus was infected on Spodoptera frugiperda (SF9) cells with a similar MOI. The infected insect cells were incubated for 72 hours at 27˚C shaking and harvested via centrifugation (10,000 rpm for 20mins). The cell pellet was lysed using I-PER insect cell protein extraction reagent (Pierce Biotechnology, Rockford, IL) on ice for 10 minutes, then centrifuged again at 15,000 rpm for 20mins. The soluble protein in the supernatant was purified using Ni-NTA bead resin. The recombinant proteins were detected and confirmed via Western blotting using a 6× histidine-specific mAb (Novagen, Billerica, MA). After the determination of the correct protein size, the concentration of both antigens was determined via Qubit 2.0 Flex and bicinchoninic acid assay (BCA). An average between the 2 methods was used to calculate antigen per well concentration.

3.4. Chicken and turkey sera production

Chickens or turkeys of specific-pathogen-free stock were used in the production of avian influenza antisera. All procedures involving experimental animal inoculations and care complied with the Canadian Council of Animal Care guidelines. These animals were allowed to acclimate for 5-7 days prior to inoculations. For LPAI viruses, fresh allantoic fluid was collected that contained an HA of greater than 1:16 was used and diluted 1:10 in PBS. For HPAI viruses, fresh allantoic fluid with a HA of greater than 1:16 was collected and inactivated via BEI or BPL virus inactivation procedures as stated above. The inactivated HPAI viruses were then formulated with a 50µg of QuilA/ml of allantoic fluid prior to inoculation. Before inoculation of the birds, the undiluted virus was plated on blood agar plates and incubated at 37°C ±2°C overnight. If plates present positively for bacterial presence, the inoculum is then filtered through a .45µm syringe filter. If not already diluted, the inoculum is diluted 1:10 in PBS prior to inoculation (LPAI viruses are already previously diluted).

LPAI viruses are inoculated intravenously via the wing vein, in conjunction with 3 drops of inoculum administrated to the oral, nasal, and ocular routes of the bird. HPAI viruses are injected intravenously along with emulsigen adjuvant into different regions of the pectoral muscle. Approximately 0.25ml of HPAI inoculum is administrated in each injection. A pre-determined schedule is made in consultation with veterinarians for a bleeding. These serum bleeds were then collected from each infected bird, and tested for the presence/absence of

antibodies against the inoculated virus via HI assay. Boosters were determined on the HI reactivity results. Once the desired antibody response and titre has been achieved, the animals were anesthetized and exsanguinated via predesignated protocols. Serum pools were then created and tested again for HI reactivity to obtain a greater than 1:16 positive result. Serum was then aliquoted and stored at -20°C for later usage. The viral serum used is listed in the supplemental material.

3.5. Monoclonal Antibody Production

3.5.1. Immunization of mice and fusion of hybridoma

Immunization of mice was performed based on the Canadian Science Centre for Human & Animal Health standard operating procedures under ISO 17025. Female BALB/C mice of 5–6 weeks old were immunized with 20 mg of BEI inactivated H5N1 whole virus (A/Turkey/ON/6213/66) in an equal volume of TiterMax Gold (TiterMax USA Inc., Norcross, GA) subcutaneously. Identical boosters emulsified in incomplete Freund's or Emulsigen adjuvant were given at 4 weeks interval. Mice were boosted with the same antigen in PBS by intraperitoneal injection 3–4 days before fusion. The immunized mice used for each fusion were sacrificed by overdose anesthesia. A single-cell splenocyte suspension was obtained for fusion. BD Cell Quantum Yield medium (BD Biosciences, Franklin Lakes, NJ) with 10% fetal bovine serum was used for fusion and subcloning. Immunized spleen cells were fused with myeloma cells (P3X63 Ag8.653) at a 5–10:1 ratio in the presence of 50% polyethylene glycol (Roche., Basel, Switzerland). The cells were plated out in semisolid medium (Stem Cell., Vancouver, Canada) and incubated at 37 °C in humidified 5% CO2 atmosphere (Davis *et al*., 1982). After 2 weeks, single colonies were transferred to 96-well culture plates.

3.5.2. Primary screening of hybridoma monoclonal antibodies for reactivity

Hybridoma supernatants containing monoclonal antibodies were screened by indirect ELISA (iELISA) for specificity to the H5 recombinant hemagglutinin gene and whole virus in parallel testing. Nunc F 96-well microtiter plates (Thermo Fisher Scientific., Rochester, NY) were coated with purified, recombinantly expressed H5 full-length protein or whole virus to both strains (A/Teal/Germany/Wv632/2015 and H5 A/Canadagoose/Oregon/Ah0012452/2015). The optimum dilution of the recombinant protein and secondary detection antibody, horseradish

peroxidase (HRP)-labeled goat anti-mouse IgG (H+L) conjugate (Jackson Immunoresearch Laboratories., West Grove, PA), were determined via a checkerboard titration that showed the required signal to noise ratio before screening took place. Recombinant proteins were diluted in 0.06 M carbonate buffer, pH 9.6 (45 mM 188 sodium bicarbonate and 18mM sodium carbonate) and used as a coating antigen in a volume of 100 µL per well. The plates were incubated overnight at 4˚C, then washed 5X with PBS plus 0.05% tween20 (PBS-T). Each well was then blocked with 100 µL of PBS-T plus 3% Fetal Bovine Serum (FBS, Gibco, Waltham, MA) and incubated for 1 hour at 37˚C shaking. Testing hybridoma supernatant was added at a volume of 100 µL to each well. Plates were incubated for 1-hour at 37˚C shaking, and then washed 5X with PBS-T. Next, 100 µL of HRP-goat-anti-mouse IgG was added at a dilution of 1/2000 with PBS-T, incubated for 1 hour at 37˚C shaking, then washed and developed with 3,3',5,5'-tetramethylbenzidine, peroxidase substrate (TMB) (Sigma-Aldrich, St. Louis, MO) for 15 minutes at room temperature with shaking. Following the 15-minute incubation, 50µl of 200M H2SO4 was added to each well to neutralize the reaction. Colorimetric development was quantified spectrophotometrically at 450 nm with a Molecular Devices EMax precision microplate reader 202 (Molecular Devices, San Jose, CA). An optical density (OD) that was 2x higher than a negative control was considered as a positive clone and would be used for further analysis. The positive hybridomas were subcloned using the limiting dilution technique. The mAb isotyping was performed using a mouse monoclonal antibody isotyping kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

3.5.3. Secondary screening of hybridoma monoclonals for competitiveness

Hybridoma supernatants containing monoclonal antibodies with specificity to H5 were screened via a competitive ELISA (cELISA) to assess their ability to compete with AIV serum. The optimum dilutions of the recombinant protein and monoclonal antibody were determined via a checkerboard titration that showed the required signal-to-noise ratio before screening took place. Recombinant H5 Full-length proteins were diluted in 0.06 M carbonate buffer, pH 9.6 (45 mM sodium bicarbonate and 18 mM sodium carbonate), and used as a coating antigen in a volume of 100µL per well on the NUNC F plate. The plates were incubated overnight at 4 °C. They were then washed 5X with PBS plus 0.05 % tween20 (PBS-T) followed by the addition of 100µL of PBS-T plus 3 % Fetal Bovine Serum in each well as a blocked agent and incubated for

1 h at 37°C. Following the incubation, the plates were washed 5x with PBS-T and patted dry. AIV positive test sera were diluted 1/10 in dilution buffer mix and 50µL of the solution was added to each test well. 50µL of dilution buffer without any serum was added to control wells. The monoclonal antibody was diluted in PBS-T 3% FBS buffer according to the checkboard titration described above and 50µL was added to every well on the plate. Plates were then incubated for 1 h at 37°C and washed 5X with PBS-T. 100 µL of HRP-donkey- anti-mouse IgG (0.4 mg/mL) detection antibody was added at a dilution of 1/2000 with PBS-T, incubated for 1 h at 37°C, then washed and developed with 3,3′,5,5′- tetramethylbenzidine, peroxidase substrate (TMB) (Sigma-Aldrich, St. Louis, MO). The reaction was carried out for 15 min at room temperature with shaking followed by adding 50 µL of 2 M H2SO4 to each well to stop the reaction. Colorimetric development was quantified spectrophotometrically at 450 nm with a Molecular Devices EMax precision microplate reader (Molecular Devices, San Jose, CA). Sample to Positive (S/P) ratios were calculated using the following formula: S/P = (optical density (OD) of the sample – OD of buffer)/ (OD of positive control – OD of buffer).

3.5.4. Candidate monoclonal antibody H5 testing

A list of Monoclonal antibodies that exhibited binding and competitive fitness towards H5 AIV proteins and poly-clonal chicken serum are shown in Table 1., and were designated as candidate mAbs. These candidate mAbs were tested across a variety of H5 AIV strains to determine the best candidate with the broadest range of coverage that would confer the highest diagnostic assay application. These strains included LPAI and HPAI from both North American and Eurasian lineages. A complete list of H5 AIV strains tested in this trial is listed in Table 2.

3.6. cELISA optimization of conditions

The optimum conditions of the cELISA were assessed by various factors, i.e. microtitre plate type, blocking/diluent buffer, pre-assay blocking, incubation temperatures and/or agitation, coating antigen/mAb, and testing serum concentration. Monoclonal antibody F37#10 was the sole mAb used in the optimization process due to its complete coverage of strains and ability to cover both antigens. Statistical analysis was performed in each method based on 45 identical replicates in duplicate on 2 separate plates.

3.7. Validation methods for the determination of diagnostic sensitivity, specificity, repeatability, and threshold cut-off level

To assess the diagnostic sensitivity and specificity of the assay, chicken and turkey samples of known serostatus, confirmed by diagnostic assays performed at the National Center for Foreign Animal Disease (NCFAD), an AIV reference lab accredited by the OIE, were used. This included experimental chicken and turkey serum samples produced at the NCFAD. For validating the assay, confirmed negative serum samples and positive serum samples were used with in a receiver operating characteristic (ROC) analysis and calculated to assess diagnostic performance, which included the determination of sensitivity, specificity, and threshold cut-off using SigmaPlot version 14.0 (Systat Software, San Jose, CA).

The repeatability of the assay was assessed by running the reference sera in multiple replicates within the same run or between runs. The intra-assay repeatability was calculated for 45 replicates on 2 separate plates and then repeated over 2 days for inter-assay repeatability assessment. The values were expressed as a mean, standard deviation, and percent coefficient of variation (CV%) for the repeated measure.

3.8. Statistical analysis

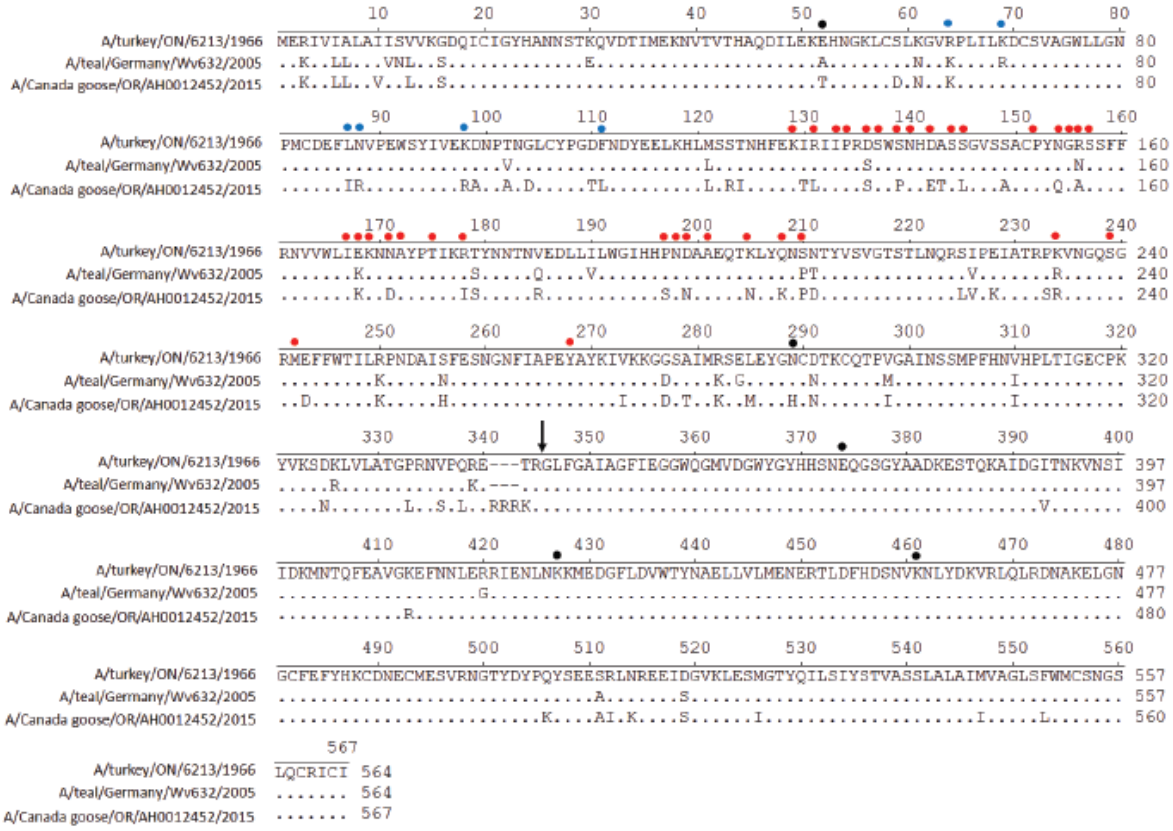
Both rec-H5 antigen-based cELISA was validated using ROC analysis and calculated diagnostic performance, which included determination of sensitivity, specificity, threshold cut-off, and AUC using SigmaPlot version 14.0 for statistical analysis. The measurement of agreement between the gold standard HI test and both cELISA tests was assessed using the cohen's kappa (κ) test. Interpretation of the given κ value utilized the criteria laid out by Landis and Koch (Landis *et al*., 1977). $\kappa \leq 0.00$ was designated as poor agreement, $0.00 < \kappa \leq 0.20$ slight agreement, $0.21 < \kappa \leq 0.40$ fair agreement, $0.41 < \kappa \leq 0.60$ moderate agreement, $0.61 < \kappa \leq 0.80$ substantial agreement, and $0.81 < \kappa < 1.00$ almost perfect agreement.

4.0. Results

4.1. Amino acid alignments of H5 HA proteins

The Antigen strain for generating H5 mAbs is A/turkey/ON/6213/1966 (H5N1) and its HA gene encodes 564 amino acids (aa). The H5 HA genes for generating two recombinant H5 proteins are derived from A/teal/Germany/Wv632/2005 (H5N1) and A/Canada goose/Oregon/AH0012452/2015 (H5N8) and encode 564 aa and 567 aa, respectively in **Figure. 1**. Comparisons of the HA proteins of three strains showed amino acid sequence identity of 85.7%-93.2%. The highest aa identity (93.2%) was found between A/turkey/ON/6213/1966 and A/teal/Germany/Wv632/2005 and the lower aa identity (85.7%) was between A/turkey/ON/6213/1966 and A/Canada goose/Oregon/AH0012452/2015. A major genomic sequence change is in the cleavage site (represented with a black arrow), with the rec-H5 A/Canadagoose/Oregon/AH0012452/2015 sequence exhibiting the trademark poly-basic site common to HPAIVs. Rec-H5 A/Teal/Germany/Wv632/2005 cleavage site has a very high similarity to A/turkey/ON/6213/1966, as both strains represent LPAIVs. Out of the reported 45 critical antigen-antibody binding residues, 5 residues are located in the stalk region (shown in black dots), 6 residues are in the vestigial esterase domain (VED, shown in blue dots), and 34 residues reside in the receptor-binding domain (RBD, shown in red dots), this distribution is visualized in **Figure. 1**.

Figure 1. Amino acid alignment of rec-AIV H5 strains HA protein region. Small dashes within sequences of A/Teal/Germany/Wv632/2005 and H5 A/Canada goose/Oregon/Ah0012452/2015 represent the same amino acid as the inoculant strain. Black dots above amino acids represent antigenic sites located in the stalk region, blue dots represent antigenic sites found in the VED region and red dots represent antigenic in the RBD region. The cleavage site is indicated by a black arrow.

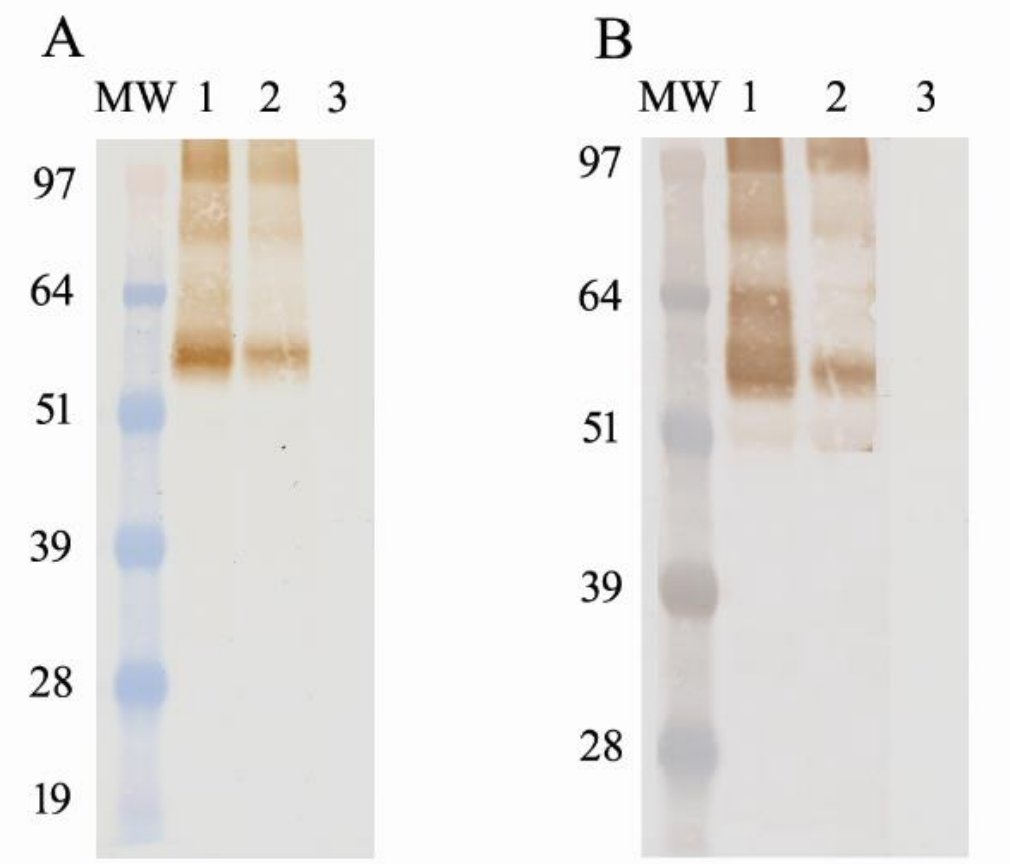


4.2. Expression of recombinant Avian Influenza A subtype H5 Hemagglutinin protein

The desired sequences of our rec-H5 HA proteins were cloned into a pAB-beeTM-FH vector by GeneScript, which were tagged with 8 histidine residues. Each rec-H5 HA plasmid DNA was co-transfected with linearized baculovirus vector DNA into Sf9 insect cells to generate a recombinant baculovirus containing an H5 HA gene. Correct H5 HA nucleotide sequence was confirmed by sequence analysis. The rec-H5 protein was expressed in Tni insect cells and the infected cells were harvested at 72 hours post-infection. Following expression, harvesting, and purification of rec-H5 HA proteins from insect cell culture, proteins were confirmed via Western blotting using a 6x histidine-specific mAb for detection of the inserted HIS-tag present in the recombinant. Detection of recombinant proteins was also shown via binding to reference H5 chicken serum (A/Chicken/Pennsylvania/1370/1983). **Figure. 2** showed a visualization of both

the expressed recombinant-H5 HA proteins and uninfected insect cell lysate along with a size ladder marker. The calculated molecular weight of the proteins (A/Teal/Germany/Wv632/2005=68.67 kDa, A/Canadagoose/Oregon/Ah0012452/2015=69.39 kDa) relates to the seen band sizes of the proteins present on the Western blot membrane. Colour visualization is only present in lanes 1 and 2 representing the rec-H5 HA proteins and absent in lane 3 representing insect cell lysate indicating correct band identification. It is understood that due to glycosylation of the proteins during expression, the molecular weight of the proteins may show up slightly different than the calculated weight.

Figure 2. Western blot analysis showing the detection of recombinantly expressed H5 HA protein. (A) Detected with anti-polyhistidine mAb. (B) Detected with reference serum produced from A/chicken/Pennsylvania/1370/1983 (H5N2). MW, molecular weight; 1, recombinant H5 HA protein derived from HA gene of A/teal/Germany/Wv632/ 2005 (H5N1); 2, recombinant H5 HA protein derived from HA gene of A/Canada goose/OR/AH0012452/2015 (H5N8); 3, uninfected cell lysates.



After determination of the correct protein size, both antigen concentrations were determined via Qubit 2.0 Flex and bicinchoninic acid assay (BCA). The concentration of A/Teal/Germany/Wv632/2005 full-length HA protein was determined to be 455 µg/ml and 460.2

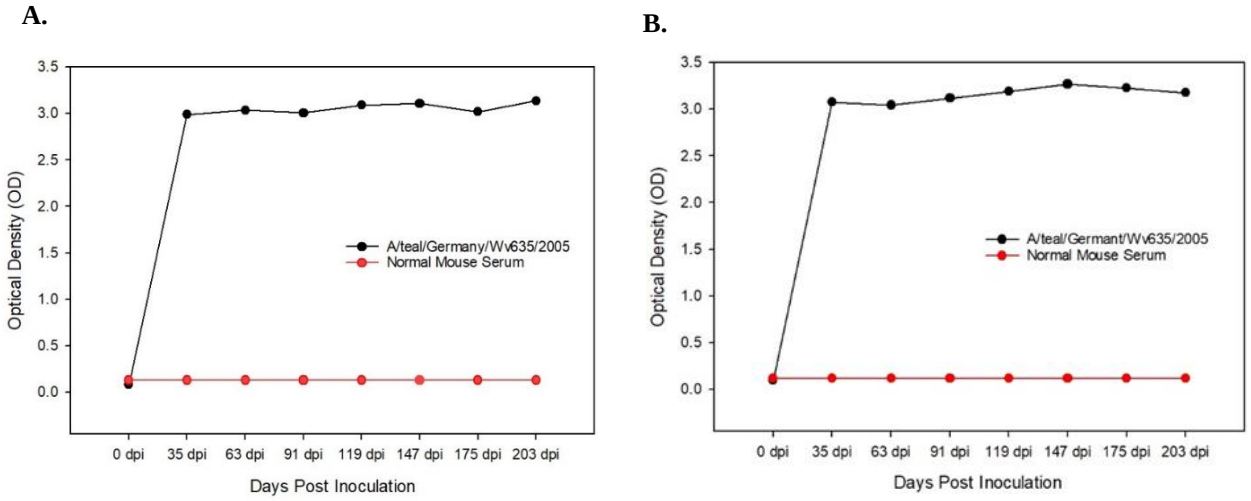
µg/ml respectively on the 2 different protein concentration methods. Whereas for A/Canadagoose/Oregon/Ah0012452/2015, the protein concentration was determined to be 390 µg/ml and 370 µg/ml respectively. These concentrations were used to calculate antigen per well concentration.

4.3. Monoclonal Antibody Production

4.3.1. Kinetic time course antibody evaluation in inoculated mice

Female BALB/C mice were inoculated with 20 mg of BEI inactivated H5N1 whole virus (A/Turkey/ON/6213/66). The mice antibody response was monitored via indirect ELISA on recombinant full-length HA protein antigen and the inactivated whole virus from strain A/teal/Germany/Wv632/2005. This response was used as a guide to determine if the mice were actively producing an antigenic response to the inoculant. As shown in **Figure. 3**, the inoculated mice immediately exhibited an antigenic response to the AI virus via the production of an antibody response detected by iELISA. This antigenic response was detected on the first bleeding that took place at 35 days post-inoculation (dpi) and maintained a high level of continuing response through subsequent bleedings at dpi 63, 91, 119, 147,175, and 203.

Figure 3. Kinetic time course antibody evaluation in inoculated mice prior to monoclonal antibody fusion. Antibody time course kinetics were calculated for whole virus **(A.)** and recombinant-H5 **(B.)** A/teal/Germany/Wv632/2005 antigen using serial bleeds from inoculated mice collected at 4 week intervals compared to normal un-inoculated mouse serum.



The response as shown above in **Figure. 3**, displays a characteristic curve with a high antigenic response from the host animal followed by a plateauing effect as the dpi increase. The response was observed both in iELISAs coated with the whole virus (**Fig. 3A**) as well as recombinant-H5 HA protein (**Fig. 3B**). These results obtained from this curve indicated that the mice were both producing a large titer of antibody and ready for hybridoma fusion, which is observed with the high OD results, and that the antibodies can both recognize the whole virus and recombinant HA protein of A/Teal/Germany/Wv632/2005 as an antigen.

4.3.2. Primary and secondary screening of hybridoma monoclonal antibodies for reactivity and competitiveness

Following the fusion of myeloma and spleenocyte cells together, the newly formed hybridoma cells were screened via iELISA to determine if the monoclonal antibody produced reacted on either of our designed recombinant antigens, A/teal/Germany/Wv632/2005 full-length HA protein and/or A/Canada goose/Oregon/AH0012452/2015 full-length HA protein antigen. A positive reaction on any of the recombinant antigens was designated when that mAb had an optical density (OD) that was 2x higher than that of negative control, which would then be used for further analysis. A total of 14 mAbs were called as positive, either reacting on one of the antigens or across multiple. Of the14 mAbs that showed reactivity, all reacted on the recombinant A/teal/Germany/Wv632/2005 full-length HA protein, with 1 also reacting on A/Canada goose/Oregon/AH0012452/2015 full-length HA protein. Monoclonal antibody F37 #10 reacted on both recombinant antigens of interest and was the only mAb to react to A/Canadagoose/Oregon/AH0012452/2015 full-length HA protein. Full results are visualized in **Table 1.**

Table 1. Summary of candidate H5 mAbs and their reactivity on specific recombinant H5 hemagglutinin proteins.

	Recombinant H5 Full-Length HA Proteins Used as Antigens			
Monoclonal Antibodies	**A/Teal/Germany/ Wv632/2005**	**A/Canada goose/Oregon/AH0012452/2015**	**Monoclonal Isotype**	**Binding Epitope**
F73 #1-2-1	+	-	N/A	Conformational
F73 #3	+	-	N/A	Conformational
F73 #9	+	-	N/A	Conformational
F73 #12-2-1	+	-	N/A	Conformational
F73 #13	+	-	N/A	Conformational
F73 #15	+	-	N/A	Conformational
F73 #16	+	-	N/A	Conformational
F73 #19	+	-	N/A	Conformational
F37 #2	+	-	N/A	Conformational
F37 #4	+	-	N/A	Conformational
F37 #5	+	-	N/A	Conformational
F37 #7	+	-	IgG2a/k	Linear
F37 #10	+	+	IgG2b/k	Conformational
F37 #12	+	-	IgG1/k	Conformational

Following primary screening for reactivity as shown in the above table, positive hybridomas actively producing mAbs were passaged and regrown for secondary testing. This testing was conducted under competitive conditions against a common reference poly-clonal chicken serum with affinity to both H5 recombinant antigens (confirmed via iELISA testing, data not shown). This was done to observe if competition between the common reference chicken serum and mAb to the recombinant-H5 HA antigens was present indicating prime candidates for diagnostic application in a cELISA method. All mAbs showed varying levels of competitiveness (PI above 30%) to this common reference serum. Further testing was done to distinguish the breadth of coverage against a wide variety of AIV H5 serum strains.

Understanding the binding epitope type was undertaken on all 14 potential mAbs by reducing the rec-H5 HA proteins in a reducing agent for 10 minutes at 70°C. Following this, the reduced antigen was coated and run against the mAbs on an iELISA. All mAbs except F37 #7 had a conformational epitope, as they did not react to the reduced (linearized) recombinant antigen. F37 #7 mAb continued to react the same on the reduced antigen as compared to the naïve rec-protein indicating its epitope was linear. The full results are listed in **Table 1.**

[a]Host animal virus was grown in for experimental use
[b]Sister Sequence to early HPAI A/Ck/Scotland/1959

4.3.3. Candidate H5 mAb Testing

To understand the breadth of H5 AIV strain coverage each mAb possessed, a variety of different lineage and pathogenic type reference sera were used to determine the dynamic nature of the interaction of each mAb with these different strains. Serum binding was confirmed prior on both antigens via iELISA and positive competition was designated with a PI above 30% and an OD above 1.0. A full list of reference sera used is located in **Table 2.**

Reference Serum Strain Name	Subtype	Host[a]	Geography	Lineage/Clade	Pathogenicity
A/Ck/Vietnam/14/2005	H5N1	Chicken	Eurasian	Gs/GD 2.3.2	HPAI
A/Tk/BC/Fav2/2015	H5N1	Goose	Eurasian	Gs/GD 2.3.4.4	HPAI
A/Tk/ONY/6213/1966	H5N1	Chicken	N. American	nonGs/GD	HPAI
A/Swan/Germany/R065/2006	H5N1	Chicken	Eurasian	Gs/GD 2.2	HPAI
A/Tk/BC/Fav10/2014	H5N2	Chicken	Eurasian	Gs/GD 2.3.4.4	HPAI
A/Dk/BC/CN26-6/2005	H5N2	Chicken	N. American	nonGs/GD	LPAI
A/Quail/Oregon/20719/1986	H5N2	Chicken	N. American	nonGs/GD	LPAI
A/Ck/Penn/1370/1983	H5N2	Chicken	N. American	nonGs/GD	HPAI
A/Tk/MN/3689-1551/1981	H5N2	Chicken	N. American	nonGs/GD	LPAI
A/Ck/WA/13413/1984	H5N2	Chicken	N. American	nonGs/GD	HPAI
A/Tk/CA/35621/1984	H5N3	Chicken	N. American	nonGs/GD	LPAI
A/Ck/Tern/SouthAfrica/1961	H5N3	Chicken	African	Outgroup[b]	HPAI
A/Tk/Ireland/1378/1983	H5N8	Chicken	N. American	nonGs/GD	HPAI
A/Tk/Wis/1/1968	H5N9	Chicken	N. American	nonGs/GD	LPAI

Monoclonal antibodies designated as F37 #4, #5, and #7 along with F73 #12-2-1, #13, and #16 showed no competitive feature against almost all tested reference serum. The mAbs designated as F73 #1-2-1, #9, #15, and #19 all showed a better range of competitive nature with reference serum as compared to the previously mentioned antibodies but still failed to either cover key serum strains or had larger gaps of coverage when compared to other mAbs. F37 #2 monoclonal antibody reacted with a wide range of reference serum but exhibited a low OD (below 1.0) that disqualified it. Monoclonal antibodies F37 #10 and #12 showed the broadest coverage of competitiveness. F37#12 covered almost all reference strain sera except strain A/TK/BC/FAV10/2014 from clade 2-3-4-4. F37 #10 was able to effectively cover all reference serum strains used along with its ability to cross-react to both antigens made it our prime candidate and focus for this study. All other mAbs were frozen down and discontinued, with full results shown in **Table. 3 and Table. 4.**

Table 3. Results for mAb #F37 testing against H5-AIV reference serum for competitiveness interaction

Reference Serum Strain Name	F37 #2	F37 #4	F37 #5	F37 #7	F37 #10	F37 #12
A/Ck/Vietnam/14/2005	+	-	-	-	+	+
A/Tk/BC/Fav2/2015	-	-	-	-	+	+
A/Tk/ONY/6213/1966	-	-	-	-	+	+
A/Swan/Germany/R065/2006	+	-	-	-	+	+
A/Tk/BC/Fav10/2014	-	-	-	-	+	-
A/Dk/BC/CN26-6/2005	+	-	-	-	+	+
A/Quail/Oregon/20719/1986	+	-	-	-	+	+
A/Ck/Penn/1370/1983	+	+	-	+	+	+
A/Tk/MN/3689-1551/1981	+	-	-	-	+	+
A/Ck/WA/13413/1984	+	-	-	-	+	+
A/Tk/CA/35621/1984	+	-	-	-	+	+
A/Ck/Tern/SouthAfrica/1961	+	-	+	-	+	+
A/Tk/Ireland/1378/1983	+	-	-	-	+	+
A/Tk/Wis/1/1968	+	-	-	-	+	+
Total	**11/14**	**1/14**	**1/14**	**1/14**	**14/14**	**13/14**

Table 4. Results for mAb #F73 testing against H5-AIV reference serum for competitiveness interaction

Reference Serum Strain Name	F73 #1-2-1	F73 #3	F73 #9	F73 #12-2-1	F73 #13	F73 #15	F73 #16	F73 #19
A/Ck/Vietnam/14/2005	+	+	+	-	+	+	-	+
A/Tk/BC/Fav2/2015	-	-	-	-	-	-	-	-
A/Tk/ONY/6213/1966	-	+	-	-	-	-	-	-
A/Swan/Germany/R065/2006	+	+	+	-	-	+	-	+
A/Tk/BC/Fav10/2014	-	-	-	-	-	-	-	-
A/Dk/BC/CN26-6/2005	+	+	+	+	+	+	-	+
A/Quail/Oregon/20719/1986	+	-	-	-	-	-	-	+
A/Ck/Penn/1370/1983	+	+	+	+	+	+	+	+
A/Tk/MN/3689-1551/1981	+	+	+	+	-	+	-	-
A/Ck/WA/13413/1984	+	+	+	-	-	+	-	-
A/Tk/CA/35621/1984	+	+	+	-	+	+	-	-
A/Ck/Tern/SouthAfrica/1961	+	+	+	-	-	-	-	+
A/Tk/Ireland/1378/1983	+	+	+	-	-	-	-	-
A/Tk/Wis/1/1968	+	-	+	-	-	-	-	-
Total	**11/14**	**10/14**	**10/14**	**3/14**	**4/14**	**7/14**	**1/14**	**6/14**

Monoclonal antibody isotype was determined via a commercially available typing kit on mAbs of interest. Results are referenced in **Table 1**.

4.4. cELISA Condition Parameters

4.4.1. Coating Antigen Concentration

The determination of the optimal amount of coating antigen per well was achieved via utilizing a checkerboard style titration by indirect ELISA. Wells with an OD of between 1.0-2.0 were designated of interest visualized in **Table 5**.

Table 5. Checkerboard titration of mAb F37#10 on rec-H5 HA antigens **(A.)** A/Canadagoose/Oregon/Ah0012452/2015 and **(B.)** A/Teal/Germany/Wv632/2005 protein

A.

	Rec-H5 HA Antigen Dilutions from Stock Concentration									
mAb Dilution	**1/50**	**1/100**	**1/200**	**1/400**	**1/800**	**1/1600**	**1/3200**	**1/6400**	**1/12800**	**No Ag**
1/50	2.8	2.7	2.4	**2.0***	**1.7***	**1.1***	0.5	0.3	0.2	0.1
1/100	2.9	2.7	2.3	**2.0***	**1.5***	0.9	0.5	0.2	0.1	0.0
1/200	2.8	2.7	2.4	**2.0***	**1.4***	0.8	0.4	0.2	0.1	0.0
1/400	2.8	2.6	2.3	**1.8***	**1.1***	0.6	0.3	0.1	0.1	0.0
1/800	2.7	2.6	**2.0***	**1.5ǂ**	0.9	0.5	0.2	0.1	0.1	0.0
1/1600	2.6	2.4	**1.8***	**1.3***	0.8	0.4	0.2	0.1	0.1	0.1
1/3200	2.6	2.5	**1.9***	**1.3***	0.8	0.4	0.2	0.1	0.1	0.0
No mAb	0.6	0.2	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
A/Canadagoose/Oregon/AH0012452/2015										

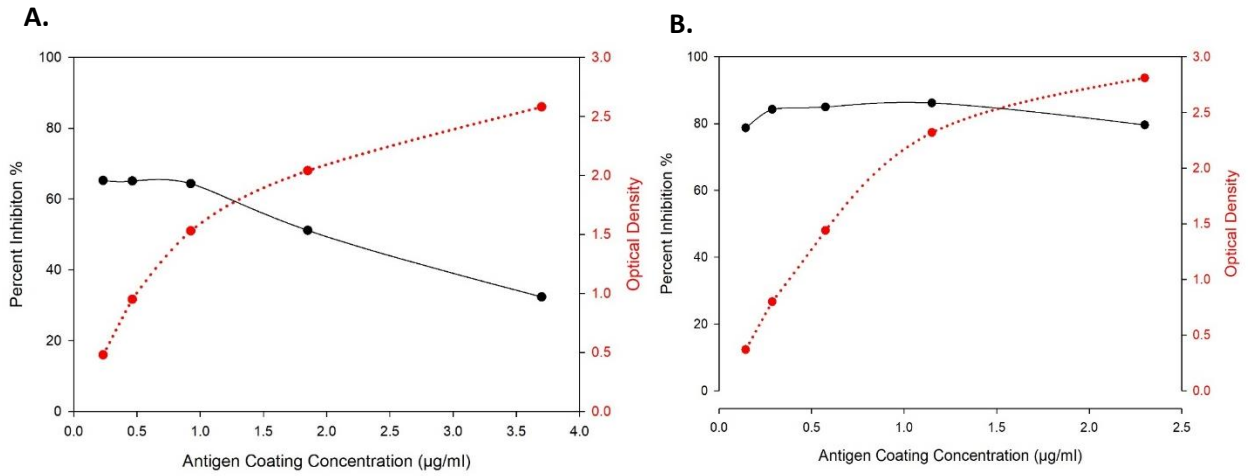
B.

	Rec-H5 HA Antigen Dilutions from Stock Concentration									
mAb Dilution	**1/50**	**1/100**	**1/200**	**1/400**	**1/800**	**1/1600**	**1/3200**	**1/6400**	**1/12800**	**No Ag**
1/50	3.26	3.26	3.05	2.95	2.66	2.21	**1.28***	0.65	0.35	0.04
1/100	3.28	3.27	3.01	2.90	2.76	2.18	**1.33***	0.65	0.30	0.04
1/200	3.26	3.26	3.14	2.97	2.74	2.15	**1.24***	0.58	0.28	0.04
1/400	3.31	3.19	3.08	2.90	2.43	**1.81***	0.89	0.41	0.19	0.04
1/800	3.14	3.06	3.00	2.68	2.05	**1.26***	0.50	0.23	0.09	0.04
1/1600	3.02	2.98	2.81	2.32	**1.44ǂ**	0.80	0.37	0.17	0.09	0.15
1/3200	2.85	2.71	2.58	2.01	**1.21***	0.61	0.29	0.14	0.08	0.06
No mAb	0.15	0.07	0.05	0.04	0.04	0.04	0.04	0.04	0.06	0.05
A/Teal/Germany/Wv632/2005										

Both antigen and mAb diluted from original stock concentration
Potential conditions chosen based on OD between 1.0 and 2.0.
***Potential Antigen Concentration Candidate**
ǂ Chosen Optimal Antigen Dilution and Optimal mAb Dilution based off optical density results

The amount of coating antigen per well is reported in µg/ml and was calculated from stock protein concentration determined via the average between the Qubit 2.0 and BCA methods. A/Teal/Germany/Wv632/2005 rec-H5 antigen had a stock protein concentration of 457.6 µg/ml and A/Canadagoose/Oregon/Ah0012452/2015 rec-H5 antigen was 380 µg/ml. As shown in **Figure 4**., a titration curve was compiled comparing coating antigen µg/ml with both optical density(OD) and percent inhibition(PI).

Figure. 4. Coating antigen curve comparing antigen concentration to optical density (OD) and percent inhibition (PI). Antigen concentration reported in µg/ml and calculated from stock protein concentration. Monoclonal antibody F37 #10 was used on both antigen to determine OD, along with the same reference H5 AIV serum to determine PI. A 2-fold dilution series was chosen centered around the previously stated (**Table. 5**) optimal antigen dilution. Final coating antigen concentration was based off a OD of between 1.0-2.0 along with the highest PI. **(A.)** A/Canada goose/Oregon/Ah0012452/2015 rec-H5 antigen **(B.)** A/teal/Germany/Wv632/2005 rec-H5 antigen.



As shown in **Figure 4(A.)**, A/Canada goose/Oregon/AH0012452/2015 rec-H5 protein coating antigen was affected in both percent inhibition and optical density significantly in a 2-fold dilution series. It was observed that the greater the antigen concentration, the rapid lowering in the percent inhibition that was displayed in the assay, with the inverse being true for optical density results. Appropriate antigen coating concentration was determined to be 0.950 µg/ml per well due to its optimal OD value in conjunction with a high percent inhibition. As seen from **Figure 4(B.)**, A/Teal/Germany/Wv632/2005 rec-H5 protein antigen did not significantly improve or impair percent inhibition of the assay throughout the dilution series but significantly affected optical density. Appropriate antigen coating concentration was determined to be 0.575 µg/ml per well due to its high percent inhibition and optimal OD value.

4.4.2. Monoclonal and Secondary Antibody Concentration

As shown in **Table 5.**, the concentration of mAb F37 #10 was calculated based on checkerboard titration with the different antigens. The A/Canadagoose/Oregon/Ah0012452/2015 antigen-based cELISA used a mAb dilution of 1:800 whereas the A/Teal/Germany/Wv632/2005 antigen-based cELISA used a dilution of 1:1600. Antibody was diluted in diluent/blocking buffer. The amount of mAb per well was calculated based on a qubit 2.0 value reading of stock mAb concentration. F37 #10 stock mAb supernatant had a concentration of 902 µg/ml. For the

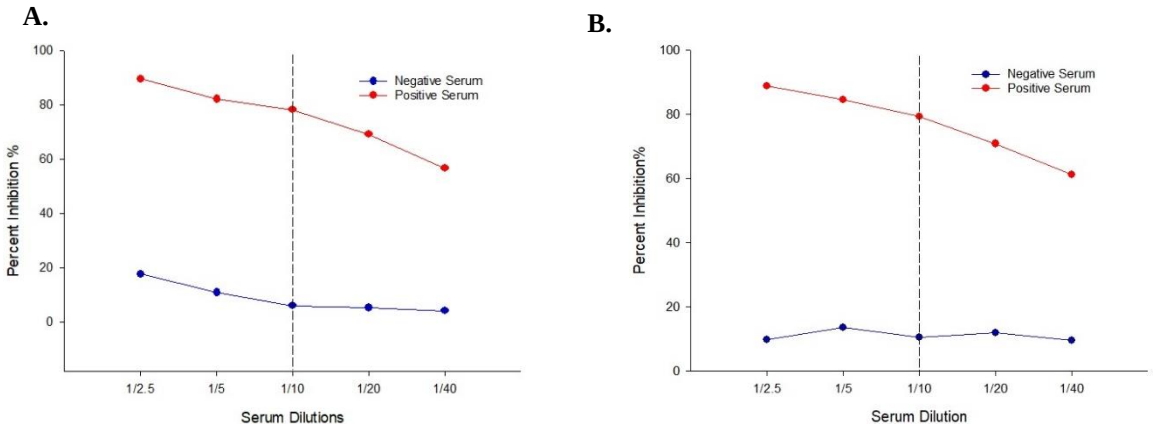
antigen, A/Canada goose/Oregon/Ah0012452/2015, the assay was calculated to require the addition of 1.13 µg/ml per well for effective reading at the chosen antigen coating concentration. Using the A/Teal/Germany/Wv632/2005 antigen, the addition of 0.56 µg/ml per well was needed for the chosen antigen coating concentration.

Dilution of stock secondary antibody was 1:2000 dilution in diluent/blocking buffer and chosen for continuity with other ELISA protocols currently used in the laboratory. The concentration of secondary antibody was confirmed as acceptable based on checkerboard titration, viewed in **Table5.,** which presents appropriate OD value readings. The stock concentration of protein in the anti-mouse-HRP conjugate was 864 µg/ml and calculated to need the addition of 0.432 µg/ml per well for effective reaction.

4.4.3. Antisera Working Dilution

To ascertain the proper working dilution of serum for efficient assay development, a serial dilution of positive H5 AIV serum and negative normal chicken serum was conducted. A 2-fold dilution series of neat serum from 1:2.5-1:40 ratio was undertaken and compared. As visualized in **Figure 5.**, the antisera diluted at 1:10 in dilution buffer provided the optimum conditions, with the lowest normal serum background interference and the highest PI value. Using A/Teal/Germany/Wv632/2005 antigen, serum dilution of 1:2.5 provided similar results with a slightly higher PI but with an insignificant difference in either background suppression or PI increase, it was decided to maintain continuity between the assays.

Figure. 5. Optimal antisera working dilution for testing determined via 2-fold serial dilution from 1:2.5-1:40. The working dilution condition that provided the highest PI values on positive H5 AIV serum along with the lowest PI values on normal chicken serum was determined to be 1:10 dilution. The black dotted vertical line indicates the working dilution condition. cELISA was performed on both **(A.)** A/Canadagoose/Oregon/Ah0012452/2015 and **(B.)** A/Teal/Germany/Wv632/2005 using their appropriate mAb and coating antigen conditions.



4.5. cELISA Optimization

4.5.1. 96-well Plate Type: Treated Maxisorp plate vs Non-Treated Surface Plate

Two different variants of 96-well microtitre plates were used to determine the best platform for the cELISA test. A non-treated, flat bottom, clear polystyrene plate that has a naturally hydrophobic surface property was tested in parallel with a specially treated, flat bottom, polystyrene plate that is designed to have a high protein binding surface property for maximum capture (Maxisorp, Thermofisher). As the results show in **Table 6**., the treated maxisorp 96-well microtitre plate performed significantly weaker in every category when compared to using a non-treated microtitre plate in identically performed testing. Assays with the plate types were run in parallel using 14 reference sera, in duplicate, to determine differences in resulting outcomes using the identical protocol and lot numbered reagents. This was done for both rec-H5 antigen strains using mAb F37#10. The percent inhibition was significantly weakened on both antigens using the treated maxisorp plate. In most cases, confirmed positive reference serum resulted in a negative or significantly lower outcome while using the treated plates when directly compared to the accompanying results on the non-treated microtitre plate. Reference H5 sera H5N1 A/Tk/ONY/6213/1966, H5N3 A/Ck/Tern/SouthAfrica/1961, and H5N9 A/Tk/Wis/1/1968 when run on A/Canada goose/Oregon/AH0012452/2015 antigen, were the only sera to show a slightly better PI% (difference of 1.40%, 5.90%, and 1.50% respectively) when running on the maxisorp treated plates compared to the non-treated ones. Statistical analysis of the intra-repeatability of each assay analyzing microtitre plate performance showed the cELISA performed noticeably better on the non-treated microtitre plate, having a lower coefficient of variation for both antigens. The CV% for the maxisorp microtitre plate utilizing A/Canadagoose/Oregon/Ah0012452/2015 antigen was calculated at 37.0 which would fail by a large margin the recognized standard threshold of 10%. Non-treated microtitre plates had a CV% of 11.0 which is significantly better than the maxisorp plate. Rec-H5 A/teal/Germany/Wv632/2005 antigen had a CV% of 9.7 and 7.1 on the treated maxisorp and non-treated microtitre plate respectively. Our results show the use of non-treated microtitre plates performed better than treated maxisorp plates for both antigen types.

Table 6. Comparison of 96-well microtitre plate platform surface performance for cELISA optimization.

H5 AIV Reference Serum	Treated Maxisorp[a]	Non-treated[a]*	Difference‡
H5N1, A/CK/Vietnam/14/2005	2.90	86.5	**+83.6%**
H5N1 A/Tk/BC/Fav2/2015	84.4	93.1	**+8.70%**
H5N1A/Swan/Germany/R065/2006	31.7	91.1	**+59.4%**
H5N1 A/Tk/ONY/6213/1966	47.3	45.9	**-1.40%**
H5N2 A/Tk/BC/Fav10/2014	19.6	91.0	**+71.4%**
H5N2 A/Dk/BC/CN26-6/2005	12.3	62.6	**+50.3%**
H5N2 A/Quail/Oregon/20719/1986	61.6	69.9	**+8.30%**
H5N2 A/Ck/Penn/1370/1983	81.0	95.5	**+14.5%**
H5N2 A/Tk/MN/3689-1551/1981	6.50	47.5	**+41.0%**
H5N2 A/Ck/WA/13413/1984	58.6	74.9	**+16.3%**
H5N3 A/Tk/CA/35621/1984	19.7	45.0	**+25.3%**
H5N3 A/Ck/Tern/SouthAfrica/1961	65.2	59.3	**-5.90%**
H5N8 A/Tk/Ireland/1378/1983	77.4	81.5	**+4.10%**
H5N9 A/Tk/Wis/1/1968	63.6	62.1	**-1.50%**
Mean of Means[b]	1.20	0.90	
Standard Deviation[b]	0.45	0.10	
%CV[b]	37.7	11.0	
A/Canadagoose/Oregon/AH0012452/2015			

H5 AIV Reference Serum	Treated Maxisorp[a]	Non-treated[a]*	Difference‡
H5N1, A/CK/Vietnam/14/2005	21.3	60.8	**+39.5%**
H5N1 A/Tk/BC/Fav2/2015	57.3	85.0	**+27.7%**
H5N1A/Swan/Germany/R065/2006	51.5	92.5	**+41.0%**
H5N1 A/Tk/ONY/6213/1966	41.9	53.1	**+11.2%**
H5N2 A/Tk/BC/Fav10/2014	16.0	54.8	**+38.8%**
H5N2 A/Dk/BC/CN26-6/2005	32.4	77.5	**+45.1%**
H5N2 A/Quail/Oregon/20719/1986	54.4	81.5	**+27.1%**
H5N2 A/Ck/Penn/1370/1983	77.7	95.1	**+17.4%**
H5N2 A/Tk/MN/3689-1551/1981	13.7	67.8	**+54.1%**
H5N2 A/Ck/WA/13413/1984	64.2	79.6	**+15.4%**
H5N3 A/Tk/CA/35621/1984	40.1	54.0	**+13.9%**
H5N3 A/Ck/Tern/SouthAfrica/1961	65.3	79.2	**+13.9%**
H5N8 A/Tk/Ireland/1378/1983	68.6	88.0	**+19.4%**
H5N9 A/Tk/Wis/1/1968	56.1	68.1	**+12.0%**
Mean of Means[b]	2.00	1.80	
Standard Deviation[b]	0.19	0.13	
%CV[b]	9.40	7.10	
A/Teal/Germany/Wv632/2005			

a Reported values represent mean PI% of duplicate reference serum results
b Statistical results based off of 45 identical replicates in duplicate under each condition
‡ Average difference between the PI% of the same reference serum run on non-treat microtitre plate vs treated
*** Non-treated microtitre plate designated as preferred choice for assay development**

4.5.2. Blocking vs Non-Blocking: Pre-assay treatment

Utilizing a blocking buffer as a pre-step treatment was studied to determine its effects on the assay. This pre-treatment is to determine if using a blocking buffer can reduce background noise by blocking non-specific binding sites from interacting with the monoclonal antibody. This procedure was done on non-treated 96-well microtitre plates, using both antigens with 3% heat inactivated-FBS in PBS-T as a blocking buffer. A mixture of H5 AIV reference serum was used for each different antigen, (High PI serum=>90%, Medium PI Serum= 60-80%, and low PI serum 40-50%) to evaluate changes in results. The full results are listed in **Table 7.**

Table 7. Comparison of pre-assay blocking treatment vs non-treatment

H5 AIV Reference Serum	Non-Blocked Assay[a]	Blocking Treatment[a*]	Difference‡
H5N1 A/Tk/ONY/6213/1966	38.7	41.7	**+3.00**
H5N2 A/Dk/BC/CN26-6/2005	41.4	42.5	**+1.10**
H5N2 A/Ck/Penn/1370/1983	95.8	96.4	**+0.70**
H5N2 A/Tk/MN/3689-1551/1981	48.7	46.2	**-2.40**
H5N3 A/Ck/Tern/SouthAfrica/1961	60.1	64.2	**+4.20**
Mean of Means[b]	1.70	1.60	
Standard Deviation[b]	0.08	0.09	
%CV[b]	4.82	8.50	
A/Canadagoose/Oregon/AH0012452/2015			

H5 AIV Reference Serum	Non-Blocked Assay[a]	Blocking Treatment[a*]	Difference‡
H5N1 A/Tk/ONY/6213/1966	42.7	55.9	**+13.2**
H5N1, A/Ck/Vietnam/14/2005	59.1	71.4	**+12.3**
H5N2 A/Dk/BC/CN26-6/2005	63.1	74.1	**+11.0**
H5N2 A/Ck/Penn/1370/1983	91.7	93.1	**+1.40**
H5N3 A/Tk/CA/35621/1984	50.3	62.0	**+11.7**
H5N9 A/Tk/Wis/1/1968	66.3	77.4	**+11.1**
Mean of Means[b]	1.70	1.60	
Standard Deviation[b]	0.04	0.10	
%CV[b]	2.60	3.80	
A/Teal/Germany/Wv632/2005			

a Reported values represent mean PI% of duplicate reference serum results
b Statistical results based off of 45 identical replicates in duplicate under each condition
‡ Average difference between the PI% of the same reference serum run on an assay with blocking vs unblocked
*** Pre-assay blocking treatment designated as preferred choice for assay development**

The use of a pre-assay blocking treatment while utilizing the A/Canada goose/Oregon/AH0012452/2015 H5 rec-antigen showed negligible effects. Across the 5 reference sera used, 4 sera had slightly improved PI% results when compared to unblocked assay testing and 1 serum with a slightly decreased PI%. The overall change in PI% was meniscal with a 0-5% difference across reference sera. Whereas using A/Teal/Germany/Wv632/2005 antigen, a

major improvement of PI% was noticed with a pre-assay blocking treatment than when compared to a non-blocked assay. All 6 reference sera used saw an increase in percent inhibition, with the improvement of results being on average over 10% greater. Our results show that utilizing a pre-assay blocking step on this antigen significantly improved results when compared to the same assay without plate blocking. Following statistical analysis, no significant variation was observed between the different treatments in regards to observed means, standard deviation, or the coefficient of variation for either antigen-based cELISA.

4.5.3. Blocking/Diluent buffer determination

A commonly available blocking buffer was compared to determine which would be most beneficial to enhance assay effectiveness. The ability to lower background (blocking non-specific binding) and enhance assays percent inhibition (PI) results were assessed. Buffers tested consisted of 3% heat inactivated-Fetal Bovine Serum (FBS) in PBS-T, 5% skim milk in PBS-T, and 2% Bovine + 2% Rabbit serum in PBS-T. These buffers were tested on both antigens against the same 7 H5 chicken-antiserum in duplicate to observe any changes the buffers may provide in the assay. All other features were maintained. The full results are listed in **Table 8.** For both antigens using the same mAb (F37 #10), the cELISA utilizing 3% FBS in PBS-T as a blocking and/or a diluent buffer provided observably higher PI% when compared to the other buffers using the same reference serum. This observation was consistent across all reference sera used while performing the cELISA on both recombinant antigens. Statistical analysis from the buffer tested showed 3% FBS in PBS-T had a slightly greater standard deviation between the means of 45 identical replicates run in duplicate on both antigens but the coefficient of variation fell below the recognized threshold of variation in an ELISA of 10% (8.5 for A/Canada goose/Oregon/Ah0012452/2015 antigen and 6.4 for A/teal/Germany/Wv632/2005 antigen respectively). Blocking/diluent buffer consisting of heat inactivated-3% FBS in PBS-T showed the best results at providing the highest PI% along with a strong ability to block non-specific binding (low background).

Table 8. Comparison of commonly available blocking/diluent buffer.

H5 AIV Reference Serum	5% Skim Milk[a]	2% Serum Mixture[a]	3% FBS[a*]	Difference‡
H5N1 A/Tk/ONY/6213/1966	44.0	35.8	46.2	**+2.2-10.4**
H5N2 A/Ck/Penn/1370/1983	96.2	94.5	96.4	**+0.2-1.9%**
H5N2 A/Tk/BC/Fav10/2014	31.7	37.1	41.7	**+4.6-10%**
H5N2 A/Dk/BC/CN26-6/2005	55.0	45.0	57.7	**+2.7-12.7%**
H5N3 A/Tk/CA/35621/1984	57.3	38.7	64.2	**+6.9-25.5%**
H5N9 A/Tk/Wis/1/1968	45.5	40.3	53.5	**+8.0-13.2%**
Mean of Means[b]	1.50	1.60	1.50	
Standard Deviation[b]	0.05	0.07	0.13	
%CV[b]	3.48	4.63	8.50	
A/Canadagoose/Oregon/AH0012452/2015				

H5 AIV Reference Serum	5% Skim Milk[a]	2% Serum Mixture[a]	3% FBS[a*]	Difference‡
H5N1 A/Tk/ONY/6213/1966	47.3	39.3	55.9	**+8.6-16.6%**
H5N2 A/Ck/Penn/1370/1983	92.1	83.1	93.1	**+1.0-10%**
H5N2 A/Tk/BC/Fav10/2014	84.4	74.8	86.4	**+2.0-11.6%**
H5N2 A/Dk/BC/CN26-6/2005	69.5	64.1	74.1	**+4.6-10%**
H5N3 A/Tk/CA/35621/1984	53.9	52.7	62.0	**+8.1-9.3%**
H5N9 A/Tk/Wis/1/1968	64.7	60.6	77.4	**+12.7-16.8%**
Mean of Means[b]	1.60	1.60	1.60	
Standard Deviation[b]	0.08	0.09	0.10	
%CV[b]	4.90	6.30	6.40	
A/Teal/Germany/Wv632/2005				

a Reported values represent mean PI% of duplicate reference serum results
b Statistical results based off of 45 identical replicates in duplicate under each condition
‡ Average difference between the PI% of the same reference serum run on 3% FBS vs 2% serum and 5% skim milk
*** 3% heat inactivated FBS in PBST designated as preferred choice for assay development**

4.5.4. Incubation Temperature: 37°C vs Ambient Temperature

To determine the effects of incubation temperature on test serum binding within the assay, we compared reference H5 AI serum at both ambient temperature and 37°C. All other aspects of the assay were kept the same. Using A/teal/Germany/Wv632/2005 rec-H5 protein as the coating antigen, showed increased binding and inhibition when incubated at 37°C than when compared with the same serum at ambient temperature. While two sera (A/Dk/BC/26-6/05, A/Tk/BC/Fav10/2014) showed a significant increase in PI when incubated at 37°C than when incubated at ambient temperature, most reference sera only gained a slight improvement in average PI 37°C compared to ambient temperature.

The assay using rec-H5 A/Canada goose/Oregon/AH0012452/2015 antigen displayed opposite but similar results. A majority of reference sera tested on this antigen showed a very

slight increase in the assays PI when run at ambient temperature rather than at 37°C. While some sera also ran better at the higher incubation temperature, the differences in either scenario were miniscule at best, with the assay effectively identifying all reference sera at a justifiable range. Both methodologies have shown to be acceptable conditions for the cELISA to accurately maintain diagnostic integrity and effectiveness. The full results are listed in **Table 9.**

Table 9. Comparison of assay incubation temperature

H5 AIV Reference Serum	Ambient Temperature[a]	37°C[a*]	Difference‡
H5N1 A/Ck/Vietnam/14/2005	89.3	86.7	**-2.60**
H5N1 A/Tk/BC/Fav2/2015	94.0	93.7	**-0.30**
H5N1 A/Tk/ONY/6213/1966	51.0	40.0	**-11.0**
H5N1 A/Swan/Germany/R065/2006	92.5	93.6	**+1.10**
H5N2 A/Tk/BC/Fav10/2014	90.9	91.2	**+0.30**
H5N2 A/Dk/BC/CN26-6/2005	52.9	52.1	**-0.90**
H5N2 A/Quail/Oregon/20719/1986	70.2	64.0	**-6.20**
H5N2 A/Ck/Penn/1370/1983	96.2	96.7	**0.50**
H5N2 A/Tk/MN/3689-1551/1981	53.9	47.7	**-6.20**
H5N2 A/Ck/WA/13413/1984	79.3	72.0	**-7.30**
H5N8 A/Tk/Ireland/1378/1983	85.1	81.3	**-3.80**
H5N9 A/Tk/Wis/1/1968	61.3	57.2	**-4.10**
Mean of Means[b]	1.40	1.70	
Standard Deviation[b]	0.07	0.08	
%CV[b]	5.31	4.79	
A/Canada goose/Oregon/AH0012452/2015			

H5 AIV Reference Serum	Ambient Temperature[a]	37°C[a*]	Difference‡
H5N1 A/Ck/Vietnam/14/2005	58.0	60.0	**+2.10**
H5N1 A/Tk/BC/Fav2/2015	84.6	85.9	**+1.30**
H5N1 A/Tk/ONY/6213/1966	56.0	59.4	**+3.40**
H5N1 A/Swan/Germany/R065/2006	90.5	96.8	**+6.30**
H5N2 A/Tk/BC/Fav10/2014	42.1	56.0	**+13.9**
H5N2 A/Dk/BC/CN26-6/2005	72.9	96.2	**+23.4**
H5N2 A/Quail/Oregon/20719/1986	78.3	75.3	**+3.00**
H5N2 A/Ck/Penn/1370/1983	91.6	93.5	**+1.90**
H5N2 A/Tk/MN/3689-1551/1981	61.1	65.7	**+4.60**
H5N2 A/Ck/WA/13413/1984	81.8	84.1	**+2.30**
H5N8 A/Tk/Ireland/1378/1983	84.3	87.7	**+3.40**
H5N9 A/Tk/Wis/1/1968	71.0	72.8	**+1.80**
Mean of Means[b]	1.30	1.90	
Standard Deviation[b]	0.15	0.17	
%CV[b]	12.0	8.78	
A/Teal/Germany/Wv632/2005			

a Reported values represent mean PI% of duplicate reference serum results
b Statistical results based off of 45 identical replicates in duplicate under each condition
‡ Average difference between the PI% of the same reference serum run on an assay at ambient temperature vs 37°C
*** Incubation at 37°C designated as preferred choice for assay development.**

Statistical analysis shows a significant decline in optical density means when incubated at room temperature instead of 37°C but still above 1.0. This was common for both antigen-based cELISAs. Using A/Canada goose/Oregon/AH0012452/2015 based cELISA, variability was similar between the conditions but when using A/Teal/Germany/Wv632/2005 antigen, a large variability was observed in the ELISAs with an incubation period at room temperature. Limited variability was observed in the A/Canada goose/Oregon/AH0012452/2015 based cELISA. For assay development, 37°C was chosen as the preferred choice for optimum assay development.

4.5.5. Incubation Conditions: Shaking vs Stationary Incubation

The effect of agitation during the incubation periods of the cELISA was examined both at 37°C and ambient temperature for both rec-H5 antigens. For both antigens, no real advantage was observed in regards to agitation vs stationary incubation in either. Only slight variations were observed within the parameters of the conditions with no significant improvement patterns observed across all reference sera tested. Results are listed in **Table 10.**

Table 10. Comparison of agitation affects at different temperatures on the assay.

	Ambient Temperature			**37°C**		
H5 AIV Reference Serum	**Shaking[a]**	**No Shaking[a]**	**Difference**	**Shaking[a*]**	**No Shaking[a]**	**Difference‡**
A/Ck/Vietnam/14/05	89.3	88.2	**-1.10**	86.7	89.2	**-2.50**
A/Tk/BC/Fav2-1202	94.0	92.0	**+2.00**	93.7	94.4	**-0.70**
A/Tk/ONY/6213/66	51.0	52.0	**+1.00**	40.0	49.3	**-9.30**
A/Swan/Germany/R065/2006	92.5	91.6	**+0.90**	93.6	92.9	**+0.70**
A/Tk/BC/Fav10/2014	90.9	88.4	**+2.50**	91.2	90.2	**+1.00**
A/Dk/BC/26-6/05	52.9	53.7	**-0.80**	52.1	60.3	**-8.20**
A/Quail/Oregon/20719/86	71.6	72.0	**-0.40**	65.7	72.4	**-6.70**
A/Ck/Penn/1370/83	96.2	95.6	**+0.60**	96.7	96.9	**-0.20**
A/Tk/MN/3689-1551/81	53.9	57.0	**-3.10**	47.7	59.3	**-11.6**
A/Ck/WA/13413/84	79.3	81.4	**+2.10**	72.0	80.4	**-8.40**
A/Tk/Ire/83	85.1	84.0	**+0.90**	81.3	85.2	**-3.90**
A/Tk/Wis/68	61.3	67.2	**-5.90**	57.2	68.6	**-11.4**
Mean of Means[b]	1.40	1.10		1.70	1.60	
Standard Deviation[b]	0.07	0.04		0.08	0.09	
%CV[b]	5.31	3.89		4.79	5.28	
A/Canadagoose/Oregon/AH0012452/2015						

	Ambient Temperature			37°C		
H5 AIV Reference Serum	**Shaking[a]**	**No Shaking[a]**	**Difference**	**Shaking[a*]**	**No Shaking[a]**	**Difference[‡]**
A/Ck/Vietnam/14/05	58.0	60.9	**-0.20**	60.0	61.0	**-1.00**
A/Tk/BC/Fav2-1202	84.6	58.8	**+25.8**	85.9	89.0	**-3.10**
A/Tk/ONY/6213/66	56.0	63.2	**-7.20**	59.4	57.9	**+1.50**
A/Swan/Germany/R065/2006	90.5	84.4	**+6.10**	96.8	88.9	**+7.90**
A/Tk/BC/Fav10/2014	42.1	55.3	**-13.2**	56.0	54.6	**+1.40**
A/Dk/BC/26-6/05	72.9	75.7	**-2.90**	96.2	77.0	**+19.2**
A/Quail/Oregon/20719/86	78.3	81.5	**-3.20**	75.3	83.3	**-8.00**
A/Ck/Penn/1370/83	91.6	92.4	**+0.80**	93.5	94.7	**-1.20**
A/Tk/MN/3689-1551/81	61.1	67.8	**-6.70**	65.7	63.5	**+2.20**
A/Ck/WA/13413/84	81.8	81.5	**+0.30**	84.1	82.7	**+1.70**
A/Tk/Ire/83	84.3	88.3	**-4.00**	87.7	88.6	**+0.90**
A/Tk/Wis/68	71.0	72.9	**-1.90**	72.8	70.7	**+2.10**
Mean of Means[b]	1.30	1.30		1.90	1.90	
Standard Deviation[b]	0.16	0.11		0.17	0.12	
%CV[b]	12.1	8.93		8.78	6.51	
A/Teal/Germany/Wv632/2005						

a Reported values represent mean PI% of duplicate reference serum results
b Statistical results based off of 45 identical replicates in duplicate under each condition
‡ Average difference between the PI% of the same reference serum run on an assay with Shaking vs non-shaking
*** Shaking agitation during incubation at 37°C designated as preferred choice for assay development.**

Statistical analysis further confirmed the lesser quality of results at ambient temperature, as both antigens showed again decreased OD results and high CV% when run compared to 37°C. No significant effect was observably calculated when comparing the two treatments at 37°C.

4.5.6. Antigen Coating Time: Overnight at 4°C vs 2 hours at 37°C vs 1 hour at 37°C

The effects of antigen coating time were assessed to determine if the overnight coating was necessary, due to its time restraint in testing delay and planning, or if coating antigen at a higher temperature for a shorter time would yield similar results. Antigen coated overnight at 4°C is the acknowledged standard operating procedure for in-house ELISA protocol was used as the base point to compare the other conditions effects too. Antigen coating overnight at 4°C for both rec-H5 protein was far superior to any other coating temperature and time combination. While positive results were able to be detected on both 2- and 1-hour incubations at 37°C, the optical density was diminished so significantly that a proper interpretation of results would yield poor diagnostic sensitivity. Overnight coating of antigen at 4°C yielded significantly stronger and more reliable results when compared to other forms of antigen coating time. As seen in

Table 11., our rec-H5 AIV cELISA required an overnight antigen coating incubation at 4°C prior to testing to achieve reliable results.

Table 11. Comparison of antigen coating incubation time.

H5 AIV Reference Serum	2 Hours 37°C[a]	1 Hour 37°C[a]	Overnight 4°C[a*]	Difference‡
H5N1 A/Ck/Vietnam/14/2005	85.7	75.0	92.2	**+6.50-17.2**
H5N1 A/Tk/BC/Fav2/2015	85.0	84.3	93.7	**+9.40-8.70**
H5N1 A/Tk/ONY/6213/1966	39.5	43.1	61.0	**+17.9-21.5**
H5N1 A/Swan/Germany/R065/2006	77.5	83.8	91.0	**+7.20-13.5**
H5N2 A/Tk/BC/Fav10/2014	82.6	85.0	87.0	**+2.00-4.40**
H5N2 A/Dk/BC/CN26-6/2005	46.2	50.5	68.1	**+17.6-21.9**
H5N2 A/Quail/Oregon/20719/1986	60.3	63.1	79.7	**+16.6-19.4**
H5N2 A/Ck/Penn/1370/1983	89.0	86.4	97.0	**+8.00-10.6**
H5N2 A/Tk/MN/3689-1551/1981	43.4	50.4	65.5	**+15.1-22.1**
H5N2 A/Ck/WA/13413/1984	52.1	61.8	76.8	**+15.0-24.7**
H5N3 A/Tk/CA/35621/1984	12.8	28.6	59.6	**+31.0-46.8**
H5N8 A/Tk/Ireland/1378/1983	75.4	75.6	83.6	**+8.00-8.20**
H5N9 A/Tk/Wis/1/1968	35.9	56.4	67.7	**+11.3-31.8**
Mean of Means[b]	0.50	0.40	1.50	
Standard Deviation[b]	0.04	0.02	0.03	
%CV[b]	8.06	5.98	2.67	
A/Canadagoose/Oregon/AH0012452/2015				

H5 AIV Reference Serum	2 Hours 37°C[a]	1 Hour 37°C[a]	Overnight 4°C[a*]	Difference‡
H5N1 A/Ck/Vietnam/14/2005	50.8	36.9	65.8	**+15.0-28.9**
H5N1 A/Tk/BC/Fav2/2015	70.8	73.5	84.9	**+11.4-14.1**
H5N1 A/Tk/ONY/6213/1966	46.0	48.1	64.0	**+15.9-18.0**
H5N1 A/Swan/Germany/R065/2006	59.7	85.6	92.4	**+6.80-32.7**
H5N2 A/Tk/BC/Fav10/2014	53.7	46.0	60.7	**+7.00-14.7**
H5N2 A/Dk/BC/CN26-6/2005	61.7	66.8	69.5	**+2.70-7.80**
H5N2 A/Quail/Oregon/20719/1986	69.0	71.2	78.3	**+7.10-9.30**
H5N2 A/Ck/Penn/1370/1983	85.1	87.9	95.1	**+7.20-10.0**
H5N2 A/Tk/MN/3689-1551/1981	55.3	52.7	64.1	**+8.80-11.4**
H5N2 A/Ck/WA/13413/1984	69.5	71.1	79.9	**+8.80-10.4**
H5N3 A/Tk/CA/35621/1984	51.3	48.1	52.4	**+1.10-4.30**
H5N8 A/Tk/Ireland/1378/1983	77.5	78.2	87.5	**+9.30-10.0**
H5N9 A/Tk/Wis/1/1968	61.0	60.6	64.3	**+3.30-3.70**
Mean of Means[b]	0.90	0.70	1.70	
Standard Deviation[b]	0.13	0.05	0.03	
%CV[b]	14.7	7.83	2.70	
A/Teal/Germany/Wv632/2005				

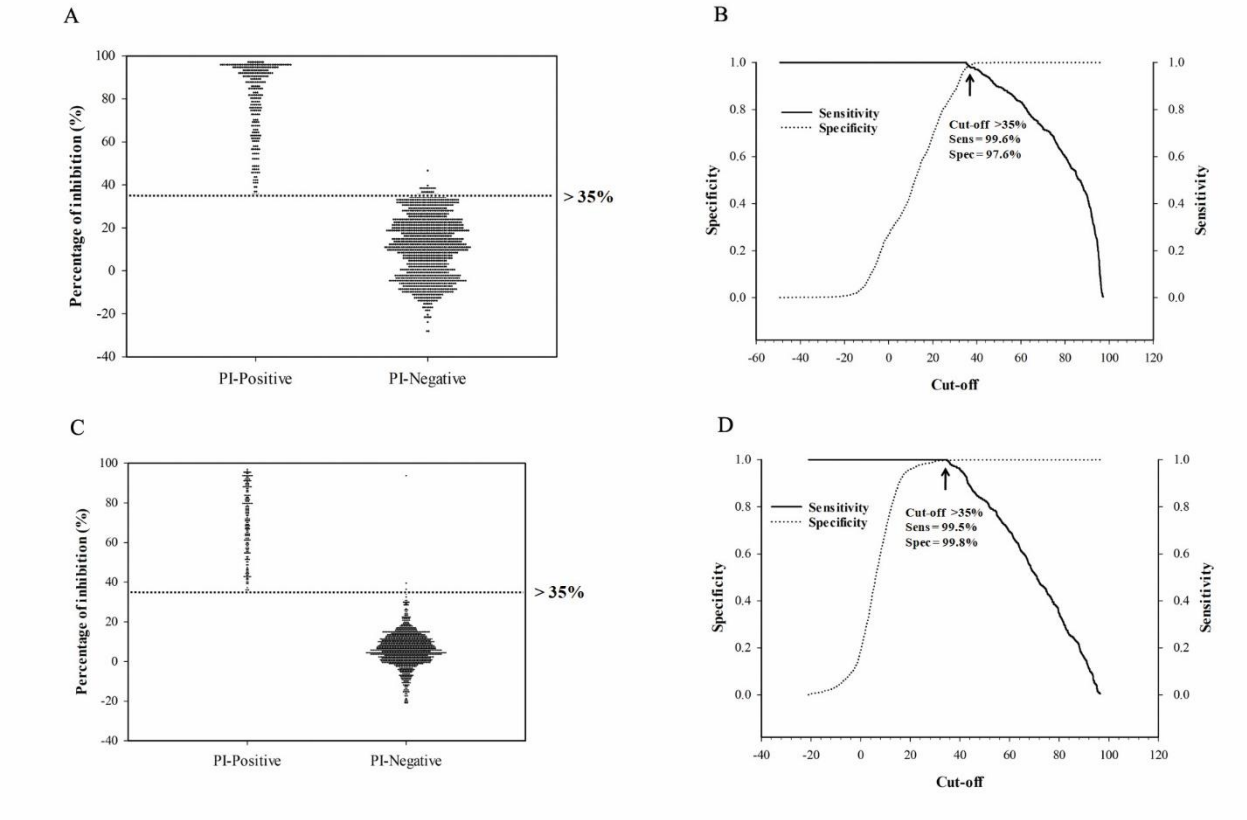
a Reported values represent mean PI% of duplicate reference serum results
b Statistical results based off of 45 identical replicates in duplicate under each condition
‡ Average difference between the PI% of the same reference serum run on an assay with different antigen coating conditions.
***Antigen incubation at 4°C overnight was designated as preferred choice for assay development.**

Following statistical analysis of the antigen coating conditions, a significant difference was observed between the shortened coating time at a higher temperature when compared to overnight coating at 4°C. Both antigen-based cELISAs saw a major decrease in reactivity means which exhibited below acceptable threshold in OD with the overnight coating treatment consistently outputting results within an acceptable OD range. The coefficient of variation for both antigens showed a major deviation for the high-temperature coating treatment than compared to the overnight, that in conjunction with the poor OD results, displaced those treatments as acceptable options. It was chosen for optimal cELISA development to coat protein antigen overnight at 4°C.

4.6. Validation methods for the determination of diagnostic sensitivity, specificity and, threshold cut-off level

To determine sensitivity, specificity, and threshold cut-off level, an ROC analysis was performed using antisera from a multitude of avian species (domestic or experimentally produced chicken and turkey sera along with wild bird sera) of known serostatus. All antisera serostatus was evaluated by AIV-NP ELISA along with HI if a positive result on NP was obtained, to determine if serum was H5 positive. A total of 275 AIV-H5 experimental positive sera and 1204 AIV-NP negative sera were used for validation of the rec-H5 cELISA with the coating antigen of rec-H5 full-length protein derived from strain A/teal/Germany/Wv632/2005 (H5N1), while a total of 200 AIV-H5 experimental positive sera and 1227 AIV-NP negative sera were used for validation of the rec-H5 cELISA with the coating antigen of rec-H5 full-length protein derived from strain A/Canada goose/Oregon/AH0012452/2015 (H5N8). The optimal cut-off value and corresponding sensitivity and specificity of the rec-H5 AIV cELISA are presented in **Figure 6**. The rec-H5 A/Canada goose/Oregon/AH0012452/2015 cELISA demonstrated a sensitivity of 99.5% and a specificity of 99.8% at a cut-off value of 35%. Hence, a sample run on the rec-H5 A/Canada goose/Oregon/Ah0012452/2015 cELISA with a PI% value greater or equal to 35% was considered positive. The rec-H5 cELISA A/Teal/Germany/Wv632/2005 demonstrated a sensitivity 99.6% and a specificity of 97.6% at a cut-of value of 35%. Hence, a sample run on the rec-H5 cELISA A/Teal/Germany/Wv632/2005 with a PI% value greater or equal to 35% was considered positive.

Figure 6. Receiver operator characteristic (ROC) validation and determination of diagnostic sensitivity and specificity of the rec-H5 cELISA. (A) Dot histogram pair for the rec-H5 cELISA using rec-H5 antigen derived from A/teal/Germany/Wv632/2005. The horizontal line bisecting the dot plots represents the cut-off value that gives the optimal diagnostic sensitivity and specificity. (B) Graph of sensitivity and specificity vs. cut-off using data generated from ROC analysis. Rec-H5 antigen is derived from A/teal/Germany/Wv632/2005. The arrow indicating the cut-off is set at the intersection of the sensitivity and specificity plots, where optimal diagnostic sensitivity and specificity are achieved at 99.6% and 97.6%, respectively. (C) Dot histogram pair for the rec-H5 cELISA using rec-H5 antigen derived from A/Canada goose/Oregon/AH0012452/2015. The horizontal line represents the cut-off value. (D) Graph of sensitivity and specificity vs. cut-off using rec-H5 coating antigen derived from A/Canada goose/Oregon/AH0012452/2015. The arrow Indicates the cut-off of >35%, where the diagnostic sensitivity and specificity are 99.5% and 99.8%, respectively.



4.7. Assessment of repeatability

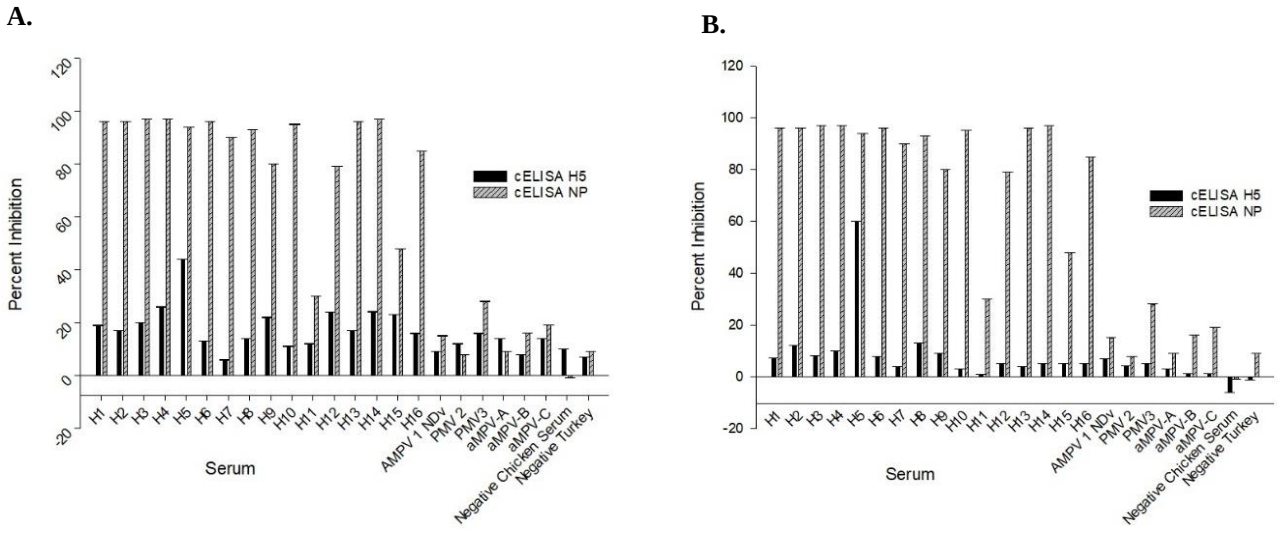
Assessment of repeatability of both rec-H5 antigen-based cELISA was assessed by testing reference sera in duplicate within the same run or between runs. The intra-assay repeatability was calculated with 45 replicates on 2 separate plates and then repeated over 2 days for inter-assay repeatability assessment. For rec-H5 A/Canada goose/Oregon/AH0012452/2015 antigen-based cELISA, an intra-assay repeatability was assessed to be 6.8% with an inter-assay

repeatability of 3.6%. For rec-H5 A/Teal/Germany/Wv632/2005 antigen-based cELISA, an intra-assay repeatability was assessed to be 7.1% with an inter-assay repeatability of 5.5%.

4.8. Assessment of potential for cross-reactivity to other avian based viruses

The potential for the rec-H5 AIV-based HA cELISA to cross-react with other avian-based viruses was assessed. Representative antiserum from AIV H1-H16, *Avian metapneuomviruses subtypes-A,B,C,* and paramyxovirus types 1,2, and 3 were used on both antigens to determine about of reactivity. As shown in **Figure 7.**, no cross-reactivity was observed across all viruses tested on either of the rec-H5 antigens. Our rec-H5 HA-based cELISA is AIV H5 specific with no cross-reactivity against other avian-based viruses.

Figure. 7. Assessment of potential for cross reactivity to other avian based viruses. Representative antiserum from viruses H1N1 A/Sw/Iowa/31, H2N9 A/Pintail/AB/293/1977, H3N8 A/BW TE/Sk/112-15/5/2013, H4N6 A/DK/QC/2323-14, H5N2 A/TK/BC/FAV5-52/09, H6N8 A/TY/On/63, H7N1 A/TY/ON/18-2/2000, H8N4 A/TY/ON/6118/67, H9N9 A/Pheasant/WA/37349/85, H10N8 A/TY/FAV-19/14/2011, H11N9 A/DK/Memphis/546/74, H12N1 A/DK/AB/60/76, H13N6 A/Gull/MD/704/1977, H14N8 A/BW TE/Sk/112-15/5/2013, H15N8 A/Dk/Aust/341/83, H16N3 A/Dk/PEI274.1/2006, APMV 1 NDV Lasota, PMV 2 YUCAIPA III, PMV 3 TY6661, Avian metapneumovirus subtype A strain 14/1 UK, subtype B strain Hungary/657/4, and subtype C strain Colorado.Negative normal chicken and turkey sera were used as negative controls. Cross reactivity was performed on both **(A.)** A/Canada goose/Oregon/Ah0012452/2015 and **(B.)** A/teal/Germany/Wv632/2005 using their appropriate mAb and coating antigen conditions.

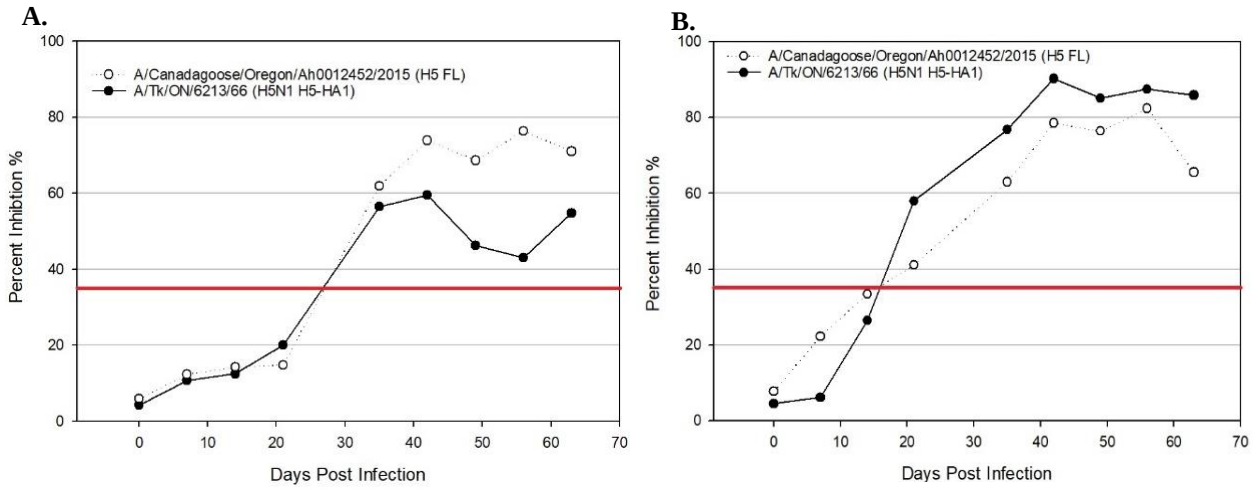


4.9. Evaluation of a Kinetic AIV H5 Antibody Response in Poultry Chickens

As shown in **Figure 8.** below, experimentally challenged chickens with rec-H5 AIV protein of 2 different viral strains could be detected on both antigens. Detection of an antigenic

response varied between the antigens, with A/teal/Germany/Wv632/2005 antigen-based cELISA detecting antibody response at dpi 21 whereas A/Canada goose/Oregon/AH0012452/2015 antigen-based cELISA detected antibody response at 35 dpi. Based on these results, the chicken antibody immune response either begins to plateau, as seen in A/teal/Germany/Wv632/2005 antigen-based cELISA, or fall, as shown in A/Canada goose/Oregon/AH0012452/2015 antigen-based cELISA, at approximately dpi 49. This was consistent amongst both AIV H5 challenge

Figure 8. Evaluation of a kinetic AIV H5 antibody response in poultry chickens. Antibody time course kinetics was calculated for rec-H5 using serum samples from experimentally infected chickens collected at dpi intervals of 0,7,14,21,35,42,49,56, and 63. The solid red horizontal line indicates the diagnostic cut-off for the test. Serum samples were tested on both **(A.)** A/Canada goose/Oregon/Ah0012452/2015 and **(B.)** A/teal/Germany/Wv632/2005 cELISA.

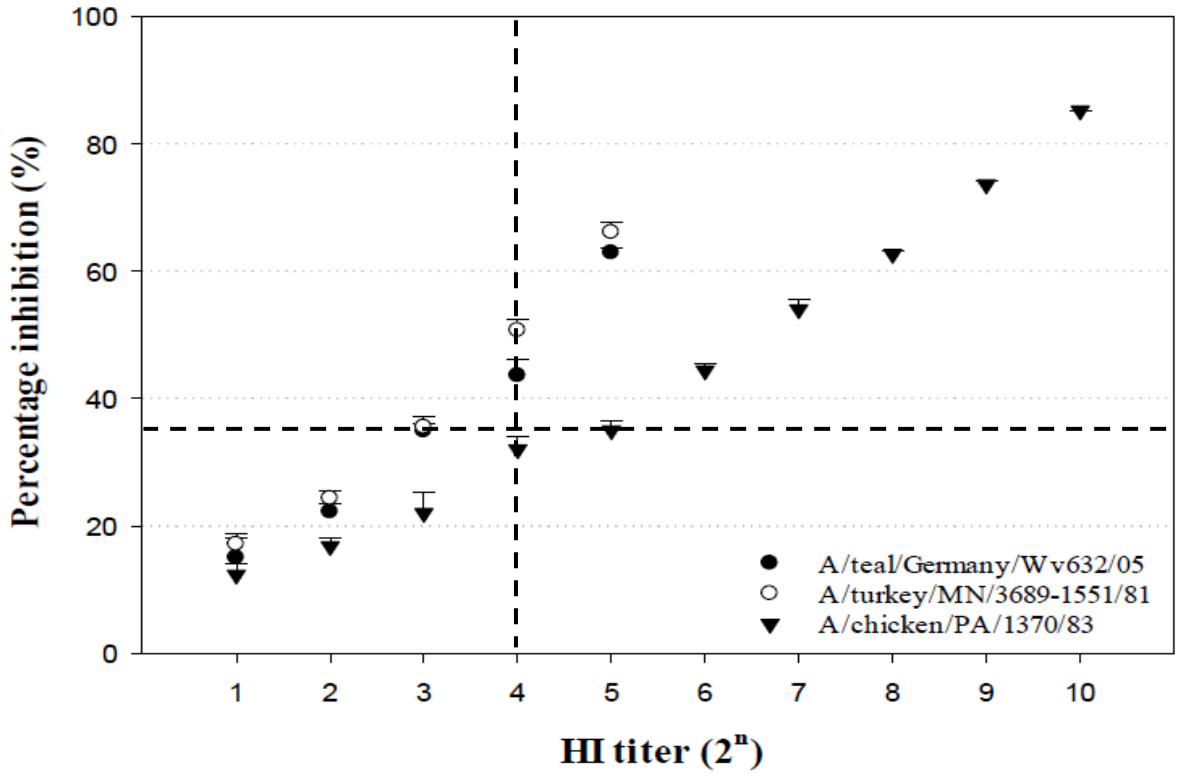


4.10. Relations of subtype-specific H5 cELISA and hemagglutination inhibition (HI) assay

The relationship between the two diagnostic testing assays was compared using three strains of known AIV-H5 positive serum in a dilution series on both platforms. This study was only carried out on the A/teal/Germany/Wv632/2005 based cELISA as the homologous virus is of LPAI origin, whereas the homologous virus for A/Canada goose/Oregon/AH0012452/2015 is an HPAIV and potentially zoonotic, hence was excided the scope of this study. A total of 3 serums were used: A/teal/Germany/Wv632/2005, A/turkey/MN/3689-1551/81, and A/chicken/PA/1370/83. These sera were diluted in a dilution series and run on both platforms. As shown in **Figure 9.,** of the five dilutions using A/teal/Germany/Wv632/2005 serum, the HI was only able to detect 2 of the dilutions compared to the cELISA which was able to detect 3. This same detection level was observed when using A/turkey/MN/3689-1551/81 serum. For the A/chicken/PA/1370/83 serum, 10 dilutions were used. The HI was capable of detecting 7

dilutions compared to the cELISA's 6 detected sera. These observations showed a very similar detection level between the 2 diagnostic platforms and the ability to effectively ascertain roughly the same level of diagnostic capabilities.

Figure 9. Relations of subtype-specific H5 cELISA and hemagglutination inhibition assay. The coating antigen for H5 cELISA is rec-H5 full-length protein derived from A/teal/Germany/Wv632/2005, AIV-H5 reference positive serum (A/teal/Germany/Wv632/05, A/turkey/MN/3689-1551/81, or A/chicken/PA/1370/83) was two-fold diluted and subjected to the cELISA and HI for antibody detection. Values represent the means of absorbance of duplicate wells from two independent tests. The dotted horizontal line indicates the cut-off value of cELISA according to the ROC analysis. Samples above the horizontal line are considered cELISA positive. The dotted vertical line indicates a homologous HI titer of 16. The HI titer of 16 or higher is judged positive.



Further statistical analysis was performed using the interpretation of Cohen's kappa, which based on the measurement one can interpret the agreement between the assays. Cohen's kappa between the cELISA based A/Teal/Germany/Wv632/05 and HI assay was shown to be 0.898 based on the results listed above. This interpretation indicated the assays have an almost perfect (81-100% data reliability) between the results of the assays.

4.11. Field serum trial

In the March of 2022, a submission of 49 samples from Newfoundland and Labrador was sent for AIV testing, consisting of sera bleeds from a variety of wild birds (ABDU=American Black Duck; MALL=Mallard; NOPI=Northern Pintail; AMWI=American Wigeon; EUWI=Eurasian Wigeon; CANG=Canada Goose). The field sera were first tested on a previously developed and validated cELISA detecting the Nucleoprotein (NP), to detect the presence/absence of AIV antibodies but this assay cannot identify viral subtypes (determine HA subtypes). Of the 49 samples, 34 samples tested positive for NP-antibody presence and 15 samples tested negative. All samples were run on both AIV-H5 rec-HA protein-based cELISAs for comparison. The samples found to be negative on the NP-cELISA were similarly found to be negative on both rec-HA cELISAs. A subsection of NP positive samples (10) was run on both rec-HA cELISAs and resulted in negative competition, hinting at infection via another AIV subtype which was later confirmed via HI assay. The A/Canada goose/Oregon/AH0012452/2015 based cELISA was able to detect 15 positive H5 samples with the A/teal/Germany/Wv632/05 cELISA detecting 23 positive H5 samples. A/teal/Germany/Wv632/05 based cELISA was able to detect more samples being H5 positive than the A/Canadagoose/Oregon/Ah0012452/2015, with it detecting 8 samples that were not identified on the other. A/Canada goose/Oregon/AH0012452/2015 based cELISA was able to detect 1 sample that failed to be detected on the other. All samples but 1, were negative on the HI assay utilizing A/teal/Germany/Wv632/05 virus. Compared to the cELISA assays which were able to detect 23 or 15 samples out of 34 possible positives as AIV-H5 positive as described above, whereas HI assay was only able to detect 1 sample (Which was positive on both rec-H5 cELISAs) as AIV-H5 positive. This is a serious decrease in detection between the assays.

5.0. Discussion

Avian influenza will always be an endemic problem throughout the world with an ever-increasing demand for poultry production. It's essential in handling these viral incursions with effective surveillance and diagnostic programs. Development and improvement in those tools will allow the global community to maintain a high readiness for inevitable outbreaks and new strains. A clear drawback for early identification of AIV is the need for a high biosecurity containment laboratory, capable of handling live viruses to perform the gold standard HI assay. This need requiring access to those biocontainment facilities is a factor in increased costs and delaying results. Along with those burdens, not every country has access to such a laboratory, while still being tasked with handling AIV outbreaks. The best route for effective control of AIV is a globally reaching program that has access to tools to detect and limit spread early before international outbreaks occur. An alternative to such testing (HI assay) is the development of a serological test that can quickly identify AIV positive samples and with the ability to serotype such viruses without the need for bio-secure facilities. Our goal was to develop an assay to detect against a broad spectrum of H5 AIV strains of both LPAI strains and HPAI strains, including coverage of clade 2.3.4.4. This includes the group C viruses from the 2014-2015 outbreak and the present group B viruses currently in an epizootic outbreak within North America.

The choice of viral strain rec-protein development was very important to the outcome of the study. Our aim of the study was to develop a wide-ranging assay to detect both North American and Eurasian strains that may afflict poultry producers. The majority of diagnostic/surveillance we encounter currently is centered on North American producers. Choosing an N. American viral strain to base the rec-H5 HA protein would have easily been effective at most strains presently encountered by our laboratory. But the possibility of coverage of potential novel Eurasian strains, which have made incursions in N. America and caused devastating outbreaks, has a chance of not being recognized on a solely North American-centered assay. This leaves the possibility that our assay would not be useful to either detect novel Eurasian strains that may have recently spread into our poultry/avian populations and due to the majority of HPAIV being of Eurasian origin leaves major drawbacks. This is why an LPAI Eurasian strain, A/Teal/Germany/Wv632/2005, was chosen to be the basis of our rec-H5 HA protein. This would allow us to see if the developed monoclonal antibody, formed via

inoculation by an N. American AIV strain, could be used with this Eurasian strain and provide the maximum lineage coverage. The second rec-protein was designed based on the HPAI Eurasian strain virus that just recently caused a massive epidemic within North America and is closely related to the current H5 AIV outbreak strain presently afflicting global avian populations. This is important for a variety of reasons. Firstly, having a strain from the Gs/Gd (Guangdong) lineage is important due to its concentration of HPAI clades of viruses and its ability to cause massive epizootic events. This specific strain was also, besides the recent 2021/2022 outbreak, the last major North American outbreak caused by the introduction of an HPAI Eurasian virus. Utilizing these 2 strains as the blueprint for our rec-H5 HA protein provides us with the best chance to have an assay that covers the widest range of strains and the ability to identify current HPAI outbreak strains.

The next major objective was the characterization of the monoclonal antibody, which is key to the production of a properly working and effective cELISA. This meant understanding the basic needs of our assay and what the outcome would be under various conditions. These needs included competitiveness with a wide range of H5 AIV strains including clade 2.3.4.4 and an effective OD range between 1.0-2.0 for proper evaluation of samples (allows sufficient variation from background and buffer between true and false positives).

A kinetic time course antibody evaluation in the inoculated mice was performed to understand the kinetic potential of the antibodies being produced prior to fusion (**Figure 3.)**. The testing of this potential was done using a coating antigen (A/Teal/Germany/Wv632/2005) that was different than that of the inoculant strain (A/Turkey/ON/6213/66) as our previously stated goal was a broad range of AIV H5 recognition. These strains are similar but distinctly different H5 AIV strains, both being LPAI while one is of Eurasian origin, and the other is a North American strain. It is expected that all H5 antibodies will inherently recognize the strain it was inoculated with as this strain acts as the adjuvant for an immune response and this testing style allows us to visualize the potential of our monoclonal production process to successfully achieve an antibody, that while recognizes the original North American strain, contains an epitope to also react to a common Eurasian strain. As seen in the figure, a very high antigenic response was exhibited in the mice to both the whole virus of the Eurasian strain A/Teal/Germany/Wv632/2005 and the recombinantly produced H5-HA protein of the same

strain. These results represent the initial evidence that the monoclonal antibody production has a significant chance of a successful outcome due to its cross-reactivity to the Eurasian-based rec-H5 protein.

This result was important as the next step in our process of developing a competitive ELISA against H5 AIV was to produce hybridoma cells in a fusion process to develop cells that can be grown up and continually produce this desired mAb. A primary screening process was undertaken on the hundreds of potential mAb produced in the hybridoma fusions via an indirect ELISA on both of our produced rec-H5 HA proteins. A requirement for successful screening was the optical density of the potential mAb must be 2x higher than that of negative control to maintain a low background noise variability as sera have lots of background proteins and other interfering components. Following the primary screening, 14 candidates were initially observed with positive binding to rec-H5 A/Teal/Germany/Wv632/2005 HA protein with an OD between 1.0 and 2.0, and of these possible mAbs, and only 1 of those 14 had shown any reactivity to the rec-H5 HA protein of the rec-HPAI strain (A/Canada goose/Oregon/Ah0012452/2015). F37 #10 mAb was found to bind to both antigens, this observation led it to be designated the leading candidate due to its ability to seemingly recognize and bind to both a low pathogenic and high pathogenic HA protein, possibly allowing it to be utilized across a wide breadth of different strains and cover the clade of interest, 2.3.4.4. All 14 mAbs were regrown and retested for competitiveness to a common reference H5 strain. All mAbs competed for binding to the respective rec-H5 antigens with this reference strain providing proof that these candidates were suitable for further testing. To distinguish which mAb was the best to move forward with, a test in the breadth of strain competitiveness was undertaken to evaluate which mAb effectively covered the most strains and where the mAb may lack in coverage.

This breadth of coverage was tested against a variety of AIV strains from LPAI to HPAI of both North American and Eurasian lineages. Positive competitive coverage was designated at a percent inhibition of 40% based on the study published by Yang *et al* (ref). Monoclonal antibodies designated as F37 #4, #5, and #7 along with F73 #12-2-1, #13, and #16 showed no competitive feature against almost all tested serum. These mAbs were discarded as they represented the lowest candidate tier and failed at the goal of having some breadth of coverage. The mAbs designated as F73 #1-2-1, #9, #15, and #19 results indicated they were able to

effectively compete with a range of AIV strains but only resulted in partial coverage of used reference strains and lacked the coverage of clade 2.3.4.4 strains. These mAbs were designated as a middle tier of interest as better candidates existed. F37 #2 mAb failed to be further considered as the OD of this candidate was unable to be replicated from its initial screening and was discontinued due to this fundamental failure. The two prime candidates that were discovered following this test were monoclonal antibodies F37 #10 and #12. F37 #12 covered all AIV sera except for the crucial clade 2.3.4.4 serum, whereas F37 #10 covered all the AIV sera including clade 2.3.4.4 serum hence leading us to designate this mAb as the prime candidate and was the only one continued for full assay development.

F37 #10 represents the best option of mAb for our assay for several reasons, first, it can be readily regrown multiple times without losing reactivity or changing the epitope (hybridoma line proven to be pure and monoclonal as a result of limiting dilution series steps), providing us with the ability to produce repeatable results and a consistent supply of mAb that outcome and characteristics are known and expected. Secondly, this mAb was able to bind to both the LPAI A/teal/Germany/Wv632/2005 and the HPAI A/Canada goose/Oregon/Ah0012452/2015 meaning we could utilize the same mAb on either antigen, simplifying development and usage of this assay. Lastly, based on our trials, this mAb can cover a very wide and useful range of AIV lineages including the important HPAI clade 2.3.4.4 group C which has previously caused epizootic outbreaks across the globe.

With the choice of mAb complete and the successful expression of both rec-H5 HA proteins, the next step in assay development is analyzing the cELISA's baseline parameters and conditions. The first parameter to decide is the concentration of coating antigen applied to the microtitre plate during each assay run. As stated earlier the optimal optical density (OD) is between a value of 1.0-2.0, as this gives enough buffer between background noise and provides enough range for competitiveness inhibition to be visualized properly. The stock concentration of each purified recombinant protein was taken via Qubit 2.0 and BCA protein concentration methods. These stock concentrations were as follows, A/teal/Germany/Wv632/2005 rec-H5 antigen had a stock protein concentration of 457.6 µg/ml and A/Canadagoose/Oregon/Ah0012452/2015 rec-H5 antigen was 380 µg/ml. Dilutions series was performed on both recombinant proteins and the mAb F37#10 to visualize the OD per dilution

combination. These results can be seen in **Table 5.**, and show the dilution combinations that exhibit the ideal OD interaction with both protein and mAb. This dilution combination was chosen based on its ability to initially result in an OD between 1.0-2.0, and its surrounding dilutions to be similar or still within that range to allow for possible long-term storage degradation not significantly affecting assay results. The chosen dilution of recombinant protein for A/Canada goose/Oregon/AH0012452/2015 was 1:400 dilution of stock concentration; for A/teal/Germany/Wv632/2005 a dilution of 1:800 from stock concentration was chosen.

Optical density is not the only factor that must be accounted for when choosing protein concentration, as the percent inhibition (PI%) is a key component of a cELISA. As shown in **Figure 4.**, a titration curve comparing a dilution series of the coating antigen concentration around the chosen dilutions of stock concentrations was performed. When studying this parameter using A/Canadagoose/Oregon/Ah0012452/2015 rec-H5 protein, both OD and PI were significantly affected by protein dilutions. At a low protein concentration, OD was impacted the most compared to PI. The OD of these low concentrations was insufficient and significantly hindered by the low abundance of proteins yet the PI% against a positive H5 reference serum remained relatively unchanged initially. As the amount of coating antigen increased from our low starting protein concentration point, OD naturally increased as more available binding targets became available, yet the PI observed remained stable until coating antigen concentration reach over 1.0 µg/ml per well. At this point, the PI of the assay significantly fell as the OD continued its increase in output and exceeded our testing range. This observed pattern showed when using this rec-H5 coating protein a delicate balance must be used to achieve a proper outcome to satisfy both OD and PI criteria. Based on these observations, it was calculated the optimal coating protein concentration for A/Canadagoose/Oregon/Ah0012452/2015 based cELISA was 0.950 µg/ml per microtitre well.

The cELISA based on A/teal/Germany/Wv632/2005 coating antigen concentration was studied in the same way. This time, the rec-H5 antigen did not react similarly compared to the previous recombinant protein. At a low concentration, an expected low OD was observed, and with the increase of protein abundance per well the OD increased expectedly as well. With the increase in protein concentration, surprisingly the PI% was not significantly affected and stayed relatively unchanged. This is a major deviation from the pattern observed with the previous rec-

H5 protein. The protein concentration ranged between 0.154 µg/ml to 2.3 µg/ml per well but all scenarios (with the same concentration of mAb and positive serum) produced similar PI% (~80%). This observation could indicate a large abundance of available binding spots or efficient attachment to mAb/Serum epitopes. As the PI component is not affected by protein concentration, the limiting factor is the OD outcome. In this way, the appropriate coating antigen concentration that exhibits the chosen OD value we previously discussed was found to be 0.575 µg/ml per microtitre well.

Based on the above-mentioned checkered board titration (**Table 5.**), the dilution of stock monoclonal antibody F37#10 that corresponded with the optimal antigen coating concentration was used to calculate the concentration of mAb necessary per microtitre well for optimal assay performance. This stock concentration was calculated on the Qubit 2.0 and was found with a protein concentration of 902 µg/ml. For the protein A/Canada goose/Oregon/AH0012452/2015, a dilution of 1:800 from the stock concentration of mAb was found to exhibit the results for optimal OD assay outcome in conjunction with the corresponding antigen concentration. Based on the stock concentration, the optimal amount of mAb per well for this antigen-based cELISA was 1.13 µg/ml. Meanwhile, protein A/teal/Germany/Wv632/2005 based cELSIA was shown to require a dilution of 1:1600 from stock concentration to exhibit optimal OD outcome. As calculated above, this assay required the addition of 0.56 µg/ml per well. This mAb concentration was used in the coating antigen curve (**Figure 4.**) and based on those results, the concentration of mAb was deemed acceptable and provided the appropriate parameters in both OD and PI results for further assay development.

When performing the checkered board titration mentioned above and the antigen coating curve, a standard dilution of 1:2000 of secondary HRP-conjugated antibody was used to ascertain the above results. The stock concentration of this secondary antibody was 864 µg/ml and calculated to be 0.432 µg/ml per well. This dilution was used across both rec-protein assays, no titration of different concentrations was undertaken as positive results were achieved with this dilution. The main factor in not undertaking studying of different concentrations was due to the need for assay protocol continuity with other pre-existing assays that utilize the same dilution or concentration of secondary antibody. In this way, we standardize this use of secondary antibodies across all assays while still achieving optimal results as the secondary antibody only

plays a role in reporting or creating colorization already present and not kinetics like the above studies.

The last parameter studied for the development of a rec-H5 cELISA was the working dilution of the testing antiserum samples. This was done via 2-fold dilution of positive serum in parallel with negative chicken serum. The reason behind this was to eliminate or dilute out background noise that is present in all sera while still maintaining a high degree of sensitivity. Serum, whether positive or negative for AIV, contains many different proteins (some of which contain sticky properties i.e. Albumin) which could potentially unwantedly interact non-specifically with our antigen. These interactions would unnaturally raise the level of inhibition we observe later in the assay, potentially providing false-positive results that would otherwise not have been observed if the serum was diluted to a point when these proteins could not interfere at an observable level. This is the reason both positive and negative sera were diluted in parallel. We intended to observe at which dilution the positive serum continued to exhibit a high PI result (competition with mAb for binding with the rec-protein) and when the negative serum, containing all the naturally occurring proteins except AIV antibodies, showed the lowest PI (little to no binding to the rec-protein, blocking mAb from binding and reacting). Both recombinant antigens were tested using the same positive H5 serum and a commercially available negative chicken serum (Milliepore Sigma, Massachusetts, United States) with dilutions from 1:2.5 to 1:40. An outside factor was the previously mentioned, want for continuity between assays either between the H5 antigens or with the other inter-laboratory assays. As seen in **Figure 5.**, rec-H5 protein derived from A/Canada goose/Oregon/AH0012452/2015 had the lowest background result in negative chicken serum from a 1:10 dilution while maintaining a very high PI result (~80%). This dilution is advantageous for multiple reasons. Firstly, dilution of the original sample is ideal as the limited sample is available in applied real-world scenarios and this dilution, while providing low background, preserves the original sample for either further downstream testing or repetition. Secondly, this is a commonly used dilution of sera used for many similar assays including at the National Center for Foreign Animal disease, where this assay was developed.

The assay utilizing the rec-H5 protein derived from A/teal/Germany/Wv632/2005 showed the ability to use this same dilution of working antisera effectively. With this assay, very

little variation in results was observed in the negative serum dilution, maintaining a stable output and not showing significant background noise from the initial dilution to the end. Naturally, the least dilute sample of positive AIV serum would exhibit the highest PI result. When comparing this least dilute sample to the 1:10 diluted sample, the PI% difference was negligible at best and, while providing continuity with the other assay and sample preservation, still exhibited near identical results. Both assays use a dilution of 1:10 in blocking/diluent duffer for antiserum testing samples before assay application.

With the parameters of the competitive ELISA set, the next obstacle in development is the optimization of the assay for the most efficient and effective outcome. In this way, all variables were tested and compared against each other with a number of positive AIV H5 sera under multiple replicates. A successful optimization for each variable was dependent on maintaining OD operating range and producing the strongest PI result. A secondary feature of this testing is the determination of how or if the assays are variable under these different testing conditions. This is a valuable piece of information, as potentially sharing the assay with other laboratories that may have limitations, is key in order to be able to effectively create a working protocol to overcome these possible limitations. Such limitations could be reagent/material inaccessibility, stable electrical grid access, or cost. To effectively determine which variable optimized the assay and to avoid any variation bias, each reference serum was run in replicates of 45 individual assays within the same microtitre plate. The mean OD and PI were used for statistical analysis.

The first variable tested for assay optimization was the type of physical microtitre plate used within the assay. Two different 96-well microtitre plates were used to determine which is the most effective platform for cELISA testing. Both types are common microtitre plates used within ELISA tests and were procured from the same company to maintain variation in external company variability. The first is a non-treated, flat bottom, clear polystyrene plate with a naturally occurring hydrophobic surface. The second was a specially treated, flat bottom, polystyrene designed to have a high protein binding surface property for maximum protein capture (Maxisorp plates). These results can be found in **Table 6.** The microtitre Maxisorp plates performed significantly worse than the non-treated plate on all tested reference sera on both rec-H5 antigens. Comparing the PI of the same reference serum for both antigens across both plate

types, it is easy to see a significant reduction in PI result in most reference sera when run on Maxisorp plates. This results in major issues which render this special plate option non-viable. Firstly, the plates are significantly more costly which already adds a hindrance to the assay's accessibility to outside use. Secondly, the majority of reference serum exhibited a much-reduced ability to detect experimentally derived positive serum that would otherwise have been detected on the non-treated plate, providing a significant amount of error and false-negative results devaluing the assay's sensitivity. The reason behind this reduction is within the design of the plate's protein binding property. There are a lot of different proteins being used or introduced within the microtitre well and the plate lacks the ability to identify or have specificity towards a certain protein. High binding of coating antigen could, in theory, be beneficial, allowing the use of lower concentration and preservation of recombinant protein as a larger amount is trapped and not washed off. However, the ideal outcome is when the mAb and serum (which may or may not contain antibodies) are introduced together in the well they compete for the limited binding on the antigen. Instead, it seems higher OD and lower PI on the Maxisorp plates compared to the non-treated plate showed that the mAb was not just binding to the antigen but as able to bind to the plate itself with its high protein binding affinity, artificially raising the OD and canceling out any inhibition results that would normally occur with the presence of other competing antibodies. In this instance we are able to accomplish both needs, lowering the need for specialized material and reducing cost plus optimizing which platform performs the best and most effective diagnostic results.

With the microtitre platform chosen for the recombinant H5 cELISA, the next step is to determine if the plates require a pre-blocking treatment step before applying the mAb and testing serum. This pre-blocking treatment is used to block sites naturally within the plate (due to its non-treatment from the manufacturer) that may cause non-specific binding to occur to our mAb or testing serum. These sites would cause reactivity to appear that is not indicative of the presence or absence of antibodies for AIV. To this extent, it was decided to observe how our assay results would be with or without the blocking of these sites against a variety of positive H5 serums. Interestingly, the recombinant antigens showed very different results when compared together in **Table 7**. Rec-H5 HA antigen-based off of A/Canadagoose/Oregon/Ah0012452/2015 showed very little variation between PI% of the different H5 positive serums when comparing pre-blocked vs non-blocked plates. All other facets of the assay remained the same. This would

indicate to us that non-specific binding does not significantly alter the outcome of the assay and could potentially be removed from the operating procedure if the need or want arose for it, allowing the assay to become faster in overall time. However, improvements in PI% of the positive sera over the replicates were observed overall. On the other hand, the cELISA based on the rec-H5 A/teal/Germany/Wv632/2005 HA antigen showed major improvement in resulting PI% when assays had undergone a pre-blocking treatment vs when it was done without. All reference serum showed an increase in PI%, with most of them on average having a 10% greater resulting PI than when run without. This is a significant improvement in the outcome of the assay which would indicate that this treatment massively improves the sensitivity of the assay and outweighs the burden of an additional step, reagent preparation, and incubation time. Additionally, due to this blocking treatment positive attribute for both antigens, even though one may not require the need for it, for this study it was determined to incorporate it to both rec-H5 antigens for continuity between the 2 assays and ease of parallel testing for other possible optimization factors.

After deciding on both the plate and the need for a blocking treatment in the optimization processes, the next variable that was studied was the differences between different blocking/diluent buffers. The data from this would serve two purposes, firstly to understand which is the best regardless of other variables. Secondly, to report how the assay would react under each buffer condition for other laboratories that may either not have access to certain buffers via physical or economic factors. Three different buffers were used, 5% skim milk, 3% FBS, and 2% Bovine and Rabbit serum mixture in PBS-T. These buffers were used for both antigens as the initial blocking buffer and the diluent buffer, keeping the type of buffer consistent throughout the assay. The first objective was to determine which buffer lowered the background best (blocking treatment for non-specific binding) and enhanced the assay's PI%. 7 positive H5 reference sera were used to determine how each buffer performed. Both recombinant antigen-based cELISAs were observed to have noticeable improvements in PI% for all reference sera when the 3% FBS buffer was used within the assay, as shown in **Table 8**. This would indicate the best protein-based block/diluent buffer for this ELISA is to use the 3% FBS mixture to produce the most optimal results. Of the other buffers tested, 5% skim milk performed adequately coming second to the above-mentioned buffer, with the 2% serum mixture resulting in the lowest average PI% of the reference sera. After statistical analysis, it was seen that the

assays using 3% FBS did show slightly more standard deviation and coefficient of variation between the replicates but well within normal limitations. These results indicate for the most optimal performance 3% FBS in PBS-T is the best candidate buffer to be used with both antigen-based cELISAs, while 5% skim milk is an optional replacement. The buffer utilizing a 2% serum mixture is not recommended for use within the assay, as the poor PI% results could significantly hinder effectiveness.

The next factor tested for assay optimization was incubation temperature. Two different temperatures were tested to see how the assay reacted, one at ambient temperature and one at 37°C. This was done to understand if heating the assay and its contents helped increase sensitivity or if it was able to remain effective at normal room temperature. Agitation was included in both categories. Both recombinant antigen-based cELISAs showed similar results indicating the ability to use either incubation temperature for an effective assay. The major observation that was an identical pattern across both assays was the noticeable reduction in mean ODs when run at ambient temperature. While the means were still maintained in the stated working range, it was a significant difference between ambient and 37°C. The rec-H5 A/Canada goose/Oregon/AH0012452/2015 based antigen assay showed a very slight increase in PI% on the reference sera when run at ambient temperature whereas the exact opposite was observed on the rec-H5 A/teal/Germany/Wv632/2005 based cELISA. An observed increased variation was detected in the A/teal/Germany/Wv632/2005 based antigen when done at ambient temperature compared to 37°C (over the normal 10% cutoff), whereas the other antigen didn't show much observable variation between the conditions. With this information, we can see that both conditions may be usable within the context of effective assay use but for optimal performance incubation at 37°C was the most beneficial.

Along with incubation temperature, the presence or absence of agitation was studied to understand the role shaking or stationary incubation may have on the kinetics of the assay. This was done for both temperature conditions mentioned above. No major improvement or hindrance was observed between the temperature incubation conditions whether agitation was present or absent during that time. Kinetically, ambient temperature (whether with shaking or not) showed a lower overall mean OD when compared to 37°C but no major advantage in PI% performance of positive H5 serum was observed, the rec-H5 A/teal/Germany/Wv632/2005 based antigen did

show slightly better results with agitation when compared without. While shaking during incubation did not show to be a major factor in assay success or failure, it is a common practice within ELISA protocols and did show some improvement on one antigen. Agitation during incubation is chosen to be included for best optimal results but may be excluded if deemed necessary for protocol adaptation.

The last optimization factor studied was the length of incubation times for the coating antigen. Antigen coating times may vary between different assay times with the most common being overnight at 4°C or at 37°C for either 1 or 2 hours. Our baseline coating time was overnight at 4°C, with the other conditions used as comparisons if the overnight coating was necessary. We attempted to understand this necessity as overnight coating requires pre-planning before testing and can add time to reporting results. The data in **Table 11** clearly shows the superiority of antigen coating overnight at 4°C. Inhibition and positive results were able to be reported at the higher temperature/shorter time incubations but the mean OD of the assay fell so significantly that it made the protocol unusable. This data definitively showed overnight coating was necessary to gain proper results for the most effective and reliable reporting. This requirement, based on this data, cannot be replaced and must be present within the protocol for basic assay performance.

After all optimizations were completed, the most effective protocol for the highest optimal performance was determined to be as follows. Using a non-treated 96-well microtitre plate, coat the calculated amount of antigen overnight at 4°C. After incubation, a blocking treatment, with 3% FBS in PBS-T should be done to eliminate non-specific binding and reduce background. Following blocking and addition of testing serum/mAb, incubation should be performed at 37°C with agitation if possible. This set of protocols will allow the assay to perform at its highest standard and was used based on all following statistical analyses. Alternatively, data collected from the above optimization conditions can allow facilities to tailor and customize the protocol to fit their needs, reagent accessibility, or facility obstacles that may be encountered while maintaining confidence in the assay's ability to perform.

To determine both assay's sensitivity, specificity, and threshold cut-off, a statistical analysis called Receiver Operator Curve was performed. This analysis required the use of a large sample size of positive and negative samples to accurately assess the parameters mentioned

above. These samples came from a wide variety of different avian species and had known serotypes confirmed via AIV-NP ELISA along with HI (if the sample was positive to confirm). These results are shown in **Figure 6.** After analysis from the data of over 1200 negative samples and 200 positive samples, we were able to determine with great accuracy how sensitive and specific each recombinant protein-based cELISA was at successful identification (positive or negative) of each sample and at which threshold a sample would be declared positive based on PI%. The sensitivity of the assay is based upon true positives or how good is the cELISA at successfully detecting AIV H5 antibodies within the sample. Specificity, on the other hand, is based upon true negatives or how good is the cELISA at successfully rejecting a sample due to a lack of AIV H5 antibodies. For the cELISA using antigen-based off of A/Canadagoose/Oregon/Ah0012452/2015, the sensitivity of this assay was determined to be 99.5% with a specificity of 99.8%. Likewise, the cELISA using antigen-based off of A/teal/Germany/Wv632/2005 had a sensitivity of 99.6% and a specificity of 97.6%. These values represent a very accurate and very selective assay that is able to recognize an extremely high-level sample truly containing AIV-H5 antibodies with the likely hood of false positives slim to none. As well, both antigens showed a high degree of ability to correctly label a sample negative or lacking AIV-H5 antibodies, minimizing false negative presence in reporting. The antigen-based off of A/Canadagoose/Oregon/Ah0012452/2015 cELISA did have a slightly higher degree of specificity than the other but both were more than acceptable in having confidence in reporting results.

The next step is to calculate the assay's threshold-cut off, or when do we label a sample positive and when it should be declared negative based on the resulting PI%. This is done by finding the intersection of the sensitivity and specificity curves respectively with the X-value showing the threshold PI cutoff. For both cELISAs, the threshold cut-off was found to be 35% PI. This means any sample above a PI of 35% for either antigen-based cELISA would signal a positive result or the presence of AIV-H5 antibodies within the sample. A sample having a PI below 35% would indicate a negative sample or the lack of AIV-H5 antibodies within the samples.

These results can be fully contextualized within **Figure 6 A, C** histograms. All resulting PI values from the ~1200 known negatives and ~200 known positives were placed on the

histogram. Following this, a threshold line at 35% is placed across the graph. This histogram visually shows the level of sensitivity and specificity based on this threshold. All known positive samples (true positives) are above the threshold as our sensitivity% predicated, meaning with almost complete confidence a true positive sample will almost always be labeled a positive at this threshold. Conversely, the negative column of the histogram explains the specificity of the assay. At the threshold of 35%, which correctly distinguish all known positives, the mass majority of samples are under the threshold line. This shows that the assay, in accordance with the above-stated specificity results, was able to correctly reject a sample for lack of AIV-H5 antibodies and call it negative. Some of the resulting dots did land above the threshold line for both assays, with a higher amount seen on the A/teal/Germany/Wv632/2005 antigen-based cELISA. These results and their corresponding histogram accurately portray the calculated specificity% of each cELISA. A very small amount of true negatives, when run on these cELISA were incorrectly identified as having AIV-H5 antibodies resulting in false-positive reporters. A total of 2 out of 1227 samples for A/Canada goose/Oregon/AH0012452/2015 based cELISA and 29 out of 1204 samples for A/teal/Germany/Wv632/2005 based cELISA were incorrectly labeled positive. Overall, these small amounts are negligible given the high degree of specificity seen for both assays and provide strong confidence in all results obtained on the cELISAs respectively.

The next statistical analysis performed was the degree of repeatability within the assays. Two different types of repeatability were assessed, intra-assay repeatability (how repeatable is the same test within an assay) and inter-repeatability (how repeatable is the same test repeated over several different assays). For these studies, the same sample was run with 45 replicates for each rec-antigen and repeated the next day to calculate both repeatabilities. An internationally used standard for intra-assay repeatability is under 10% with inter-repeatability being under 15%. For the assay using A/Canadagoose/Oregon/AH0012452/2015 based antigen, an intra-repeatability was calculated to be 6.8% and inter-repeatability to be 3.6%. These results show the possible variation between the same sample both repeated within the assay depending on where on the microtitre plate it is located or the possible variation between different assay runs. All parameters are well below the threshold for acceptable variability, showing this cELISA has relatively stable and consistent reporting of results within the assay and when repeated. For the assay using A/teal/Germany/Wv632/2005 based antigen, the intra-repeatability was found to be 7.1% and the inter-repeatability to be 5.5%. The same conclusion can be drawn for this antigen-

based cELISA having consistent results whether run within the assay or on another performed assay. However, slightly more variation was observed using this antigen when compared to the other rec-H5 antigen but well within the limits we previously stated.

Both rec-H5 HA cELISAs are extremely effective at detecting and identifying AIV H5 antibodies in experimental serum. The next task for this assay is to both determine if it is H5 specific or if it crosses with other AIV subtypes leading to further testing needed. This would be a great hindrance which adds uncertainty to our results and the need for further specific testing to elicit the true identity of the unknown sample. Representative antiserum from AIV subtypes 1-16, AMPV-1, PMV-2, and PMV-3, and aMPV-A, B, and C to see if any cross-reactivity would occur. As seen in **Figure 7.**, using the threshold-cut off calculated above no cross-reactivity was observed amongst any of the other avian viruses on either rec-antigen-based cELISAs. This provides evidence that both of our assays are specific for only reporting reactivity to AIV H5 antibodies solely without the worry of any cross-reactivity. This allows these assays to become a serotyping test, which while may not be able to be used in the large screening of unknown samples containing a variety of AIV subtypes, can distinguish positives samples of unknown HA-type and determine whether it is H5 or not.

This understanding provides us with the scenario where this assay could potentially be deployed. If the goal is to screen a large batch of surveillance wild avian samples that may or may not contain AIV antibodies, these assays would only be able to detect AIV-H5 positive samples with all other (whether truly negative or positive for other AIV-subtypes) samples returning a negative result. On the other hand, this assay could be mobilized in two different ways. Firstly, following a broader screening of samples (i.e. NP-cELISA), this cELISA could detect from these true positive (but unknown serotype) which is H5 positive. This information of confirmation of either presence or absence of AIV-H5 antibodies is extremely useful and important. AIV-H5 is one of the leading viral subtypes that cause major outbreaks within the poultry production systems and detection within wild bird population surveys could help identify potential hotspots and lead to risk assessment and prevention in the geographic area the virus was detected in. The H5 LPAIV is also capable of becoming HPAI, leading to its detection being the most important whether it is in surveillance of avian populations or confirmation testing in the poultry industry for either import/export or veterinary purposes. Secondly, following a major

epizootic outbreak and confirmation of AIV-H5 virus causation, this cELISA could be used as a quick screening to detect the major concerning virus that is known to be circulating and being of the highest risk of viruses. It is unnecessary to test with an assay that could only detect AIV without serotyping as more time is needed to confirm it is H5 AIV leading to slower reaction in biocontainment and epidemiological assessment. In this way, the rec-H5 based cELISA is versatile enough to fill the role of a serotyping assay that can characterize known AIV positive samples and as a mass screening assay capable of being deployed in epizootic H5 outbreak scenarios for quick and effective determination of infection and transmission of virus between avian populations and farms. Both are useful for surveillance and tracking of AIV-H5 presence and spread, whether it is survey-based or large-scale epidemiological/biocontainment-based tasks.

Now that we are aware of the specificity of the assay for AIV-H5 antibodies, we need to understand at which point of infection are these cELISAs capable of detecting antibodies. Poultry Leghorn chickens were experimentally inoculated with rec-H5 AIV HA proteins from 2 different strains, with bleeds were taken every 7 days to observe for antibody detection of both ELISAs. A/teal/Germany/Wv632/2005 based rec-antigen cELISA was able to detect the presence of H5 antibodies earliest between the assays at a day post-inoculation (DPI) of 21 days. A/Canada goose/Oregon/Ah0012452/2015 based cELISA was able to detect H5 antibodies at dpi 35. This data suggests that, at these initial antibody response detection, our cELISAs would be able to detect the HA antibodies of an H5 AIV infection 21 days post-infection on the A/teal/Germany/Wv632/2005 based assay, and 35 days post-infection on the A/Canadagoose/Oregon/Ah0012452/2015 based assay. Birds sampled earlier than these dpis may not be able to be detected effectively. Peak response was found to be present on both antigen-based cELISAs at 42 dpi, followed by plateauing or decline in response on the ELISAs. This response detection was not unexpected, as this assay required the immune response upon infection prior to being able to detect it. Antibody response is not an immediate response, requiring the body to recognize the infection and respond adequately. A delayed ability to detect antibodies was fully expected, and following this kinetic study we now can estimate approximately the time frame of infection the samples may be at adding to the epidemiological data provided from these assays.

The final step of validation when assessing these cELISAs is to compare the assay to the gold industry-standard test, the HI assay. This was done solely on the A/teal/Germany/Wv632/2005 based cELISA. The reason behind this is the need for a homologous virus to be used, and the growth of an HPAIV would be above the level of biocontainment possible for this study, which also demonstrates a drawback to this test. All HI assays were done with the homologous virus to the used positive serum. The data in **Figure 9.**, showing the comparison of sensitivity between the different assays. When comparing serum from A/teal/Germany/Wv632/2005 infected birds on both assays, the sensitivity for antibody detection was identical in the detection of 3 samples out of 5 dilutions. This pattern is similarly observed on positive serums A/turkey/MN/3689-1551/81 (both assays detecting 3 out of 5 dilutions) and A/chicken/PA/1370/83 (6 out of 10 dilutions for the cELISA and 7 out of 10 dilutions for the HI assay). These patterns across different AIV-H5 positive sera present a strong initial argument for roughly the same diagnostic capabilities between the assays, which may not be viewed as an upgraded protocol. But being able to almost match identical results of the internationally recognized gold standard, while providing the flexibility in not needing live virus or limitation in virus chosen due to HPAI restriction is a major addition to any laboratories repertoire of assays. Likewise, another piece of evidence of the rec-H5 cELISA's ability to equally match that of the HI assay results is the Cohen's kappa co-efficient. This measures the amount of agreeability the assays have to one another. This was found to be 0.898 or interpreted as almost perfect, meaning that the reliability of the data based on the cELISA is between 82-100% reliable when compared to the data based on the HI assay. This ability accomplishes the stated goal of this study, to replace the HI assay with an equal or better protocol that is able to effectively identify AIV-H5 antibodies, including clade 2.3.4.4, in a cost-effective and highly accurate way without the need for a live virus.

This comparability was tested on field samples from a wild bird submission from Newfoundland and Labrador, Canada. These samples were collected from a variety of wild bird species within the location of the recent major outbreak of AIV-H5 clade 2.3.4.4 group B. All 49 samples were tested on the initial screening test for the presence of NP antibodies. Of the 49 samples, 34 tested positive AIV antibodies. Of these 34 positive samples, the rec-H5 A/teal/Germany/Wv632/2005 based cELISA detected 23 positive samples out of those 34 positive samples, whereas the A/Canadagoose/Oregon/Ah0012452/2015 based cELISA detected

15 out of those 34 positive samples. 8 unique individual samples were detected on A/teal/Germany/Wv632/2005 based cELISA and not the other assay; whereas the A/Canadagoose/Oregon AH0012452/2015 based cELISA detected 1 unique individual sample not detected on the other. The A/teal/Germany/Wv632/2005 based cELISA showed the capability to detect more H5 positive samples of both North American and Eurasia origins than that of the A/Canada goose/Oregon/AH0012452/2015 based cELISA. This is attributed to 1) higher H5 HA amino acid identity (93.2%) observed between mAb origin strain (A/turkey/ON/6213/1966) and rec-H5 strain (A/teal/Germany/Wv632/2005); 2) of 45 reported antigenic residues, the H5 HA gene of A/Canada goose/Oregon/AH0012452/2015 (23 substitutions, 2 in stalk, 16 in RED, and 5 in VED) exhibited more alterations than that of A/teal/Germany/Wv632/2005 (8 substitutions, 1 in stalk, 2 in VED, and 5 in RED) when compared to H5 HA gene of A/turkey/ON/6213/1966.

All NP-positive samples were detected on HI for confirmation using virus A/teal/Germany/Wv632/2005. All but one sample (that was found positive for H5 antibodies on both cELISAs) were found to be negative.

It is expected to have NP positive but H5 negative samples as wild birds can contain a plethora of different AIVs that will not be detected on a serotype-specific assay like the cELISA or HI. It was unexpected to have such a large discrepancy in the detection of AIV-H5 positive between the HI and cELISA in the number of positives reported, as the comparison reported above showed similar abilities. This difference may be due to the virus used within the HI assay not being homologous enough to be bound by the present antibodies but homologous enough within the cELISA to be recognized. This shines a light on another advantage the cELISA has against the HI assay, the ability to easily cover a broad range of strains without the need to evaluate the homology or relatedness of the virus within the HI assay to the present or suspected strain. This advantage saves time, both in result reporting and in technician labor, by having an assay that does not need to be calibrated constantly for the most up to date strain that is being tested or worry the negative result is not due to a lack of antibodies but due to poor homology affecting the assay. In this way the cELISA proves to be a far more superior protocol in that is able to provide equal to greater sensitivity and detectability of a wider range of strains of AIV

H5, while unobstructed by the need to determine the homologous virus needed to effectively perform.

6.0. Conclusion

In summary, this study shows that we designed, developed, optimized, and validated a highly sensitive and effective competitive ELISA based on recombinant-HA proteins as antigens from two unique strains of AIV H5 that is capable of detecting a wide range of strains of North American and Eurasia lineages, including clade 2.3.4.4 groups B and C viruses. This assay has shown the ability to overcome the clear drawbacks of needing access to high biocontainment facilities in order to perform the golden standard HI assay internationally recognized as the best characterization assay. Our cELISAs are able to be performed in a low biocontainment environment due to the stability and non-infectious nature of the reagents. In essence, our assay could be deployed across the globe to a wide range of regions with different facilities that can still perform this assay, giving all countries equal access to effective diagnostic and surveillance tools. We also were able to effectively show that another unforeseen hindrance of the HI assay was the need to have a homologous or calibrated virus for the detection of new and emerging strains. This leaves a massive gap in diagnostic or surveillance effectiveness as the need to either calibrate or ensure effective reporting will be significantly decreased by this obstacle. Our cELISA has shown the ability to overcome this obstacle, by continuing to effectively detect and accurately report results, even with newly arisen AIV H5 strains. Following initial calibration of parameters and optimization of the protocol, both rec-H5 cELISAs have shown to be highly sensitive and specific assays, with low variation and no cross-reactivity to other viruses. In this sense, the assays are able to operate with great confidence, equally or even outperforming the HI assay results based on our statistical analysis. In closing, our HA-based rec-H5 cELISA is able to be easily deployed to a variety of facilities and performed straightforwardly with highly effective detection and repeatable results, and is able to match the internationally recognized golden standard assay (HI). These qualities allow our assay to be used in lieu of the HI assay when being used to detect or confirm against avian influenza A subtype H5 antibodies in a wide range of avian species.

Nevertheless, the rec-H5 cELISA described in this study should be continuously evaluated for its capability to detect current circulating H5 positive serum samples as well as newly emerging H5 positive serum samples of different avian species.

7.0. General Discussion and Conclusion

7.1. General Discussion

Avian influenza outbreaks have and will continue to increase in frequency with the ever growing demand for poultry products. These intense farming practise has caused AIV to undergo a genetic explosion in new and novel strains due to circulating infection of poultry flocks with LPAIV. Every new infection gives the virus a host for viral replication, that during said process, can create mutation/recombination or re-assortment events leading to the possibility of conversion to a HPAI strain.

While the majority of commercial poultry flocks that continue to experience LPAI circulation are in developing countries, these viruses are not stationary. Migratory birds, that do not adhere to nation borders, act as reservoir that pick up viruses from one geography location and spread it over the flyway to their destination usually in another country. This mode of transmission is the keystone to deploying effective AIV control, it must be undertaken as a global program to eradicate circulating LPAI prior to its evolution to HPAI, and biosecurity along with effective surveillance of migratory birds. This surveillance and detection aspect is an incredibly important to part of understanding the epidemiological picture of AIV spread. While it is only a piece of the overall need for controlling AIV, it is massively important. This study was built on 2 key overall objectives. First, to produce an effective competitive ELISA that has the ability to detect AIV subtype H5 antibodies. Secondly, to effectively show its ability to match the recognized standard test for H5 detection within avian samples.

As mentioned previously, currently circulating avian influenza characterized as high pathogenic belong to subtypes H5 and H7. These are recognized internationally as the subtypes of greatest concern and have been the focus of global efforts in monitoring and vaccination programs. This gives our study merit in the pursuit of attaining an ELISA based on the detection of AIV H5 antibodies. This was achieved by utilizing a recombinantly expressed protein based off 2 unique strains of H5 AIV hemagglutinin protein sequences. A monoclonal antibody was characterized using these rec-H5 proteins and challenged against a wide spectrum off different H5 AIV strains.

The necessity for having a mAb capable of detecting as broad of H5 strains is an important characteristic of the assay developed in this study. Use in surveillance activities requires the assay to be able to detect viruses that can be of any lineage or clade or even a mixture of strains. If the assay was only specific to a certain lineage/clade then deployment for detection of new or novel strains that may have just been introduced into the testing area or that have changed/re-assorted would significantly hamper the results. Our study showed this ability, as both of these rec-H5 assays were capable of covering all tested AIV H5 strains including 2 groupings from clade 2.3.4.4. This aspect gives us great confidence from the results above, that when challenged with unknown serum from either migratory or commercial birds, we will be able to detect all H5 positive strains including HPAIV strains. While this assay is unable to report specific H5 strains from these samples, its main task is to effectively and in short time detect positives that may then be further analyzed for more specific characterization.

The second objective was to be able to show the assays sensitivity and specificity was equal to or greater than the HI assay, which is the standard characterization test used in laboratories around the world today. The reason behind this objective was to be able to develop a test with equal efficiency that contains no infectious reagents necessary to perform this protocol (live virus). This is the major drawback to the HI assay which limits its use to specialized laboratories and hinders globalized testing especially in developing nations. That aspect is significantly important as these nations usually struggle with high outbreaks of AIV, where these types of diagnostic ability would significantly help a nations poultry sector. As shown above, with the application of a ROC and Cohens coefficient, both rec-H5 HA based cELISA's exhibited extremely high sensitivity and specificity to H5 AIV antibodies (with no cross-reactivity) along with an almost perfect agreement when compared to the HI assay. These statistics support the notion that our developed rec-based cELISA's can provide extremely accurate results that if run in parallel to the HI assay, would conclude with almost identical results. This understanding of our assays efficiency gives us great confidence to conclude our assay succeeded in its objective in matching or outperforming the HI assay in the detection of AIV H5 antibodies within a variety of unknown avian species samples.

Along with assessing our study against these two objectives, during the initial optimization we were able to gather valuable data on the flexibility of the assays within both

protocol or reagent use. This data, along with the above mentioned outcomes, can provide outside users of these assays key understanding on how they may implement this diagnostic tool within their laboratories around the world regardless of reagent or facility constraints.

7.2. General Conclusion

In conclusion, our study provided concrete data showing the effectiveness of our designed recombinant AIV-H5 HA protein based cELISA in detection of AIV H5 antibodies, include from clade 2.3.4.4 group B and C, in a variety of avian species samples. Along with this, our study shows equal effectiveness of AIV H5 detection in these samples compared with the recognized standard diagnostic testing used.

7.3. Future Directions

Further consideration from this study include continuing testing in pre-coating the antigens into already coated-ready to use immediately kits for quicker overall testing times. This helps in overcoming need for antigen coating at 4°C overnight. Another consideration would be to include a possible mixed mAb assay that can detect both H5 and H7 AIV antibodies within samples, removing additional testing for other high importance AIV detection.

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