IMPLEMENTATION OF A REVERSE GENETICS METHOD FOR THE GENERATION OF LOW-PATHOGENIC CONTEMPORARY H3N2 INFLUENZA VIRUS

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ABSTRACT

In order to evade host immune pressures, influenza viruses evolve genetically and antigenically. Circulating H3N2 strains display this tendency resulting in increased infections and decreased vaccine efficacy. This variability makes selection of the recommended seasonal vaccine strains increasingly difficult, reducing the time researchers have to manufacture effective vaccines. The inaccessibility of new circulating H3N2 strains in WHO and CDC repositories underscores a need for improvements in the acquisition of virus to test antibody responses from vaccine strains within the clinical setting. Although thousands of the new H3N2 strains are sequenced per year, very few have been isolated with viral stocks made for them by the CDC, because of this, testing vaccine efficacy against these strains is impossible. Implementation of a reassortant reverse genetics platform can provide the recombinant virus samples necessary to enable insight into the effectiveness and range of antibodies produced from recommended vaccine strains. This allows testing the immune response following vaccination by evaluating the ability of serum antibodies to neutralize or inhibit hemagglutination of a wide array of antigenically distinct influenza strains particularly the H3N2 subtype circulating strains.

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LIST OF ABBREVIATIONS

BSA V	BSA fraction V
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
C.O.	Codon optimized
CPE	Cytopathic effect
DPBS	Dulbecco's phosphate buffered saline
DBPST	Dulbecco's phosphate buffered saline with Tween 20
DMEM	Dulbecco's minimal essential medium
EMEM	Eagle's minimum essential medium
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GISAID	Global Initiative on Sharing Avian Influenza Data
HA	Hemagglutinin
HAg	Hemagglutination assay
HAI	Hemagglutination inhibition assay
HAU	Hemagglutination units
HEK293T	Human embryonic kidney 293 cells
HRP	Horseradish peroxidase
IRR	International Reagents Resource
M	Matrix protein
MAb	Monoclonal antibody

MDCK	Madin-Darby Canine Kidney cells
NA	Neuraminidase
NCR	Non-coding region
NIBSC	National Institute for Biological Standards and Controls
NP	Nucleoprotein
NS	Non-structural protein
OD	Optical Density
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Penn/Strep	Pennicillin-streptomycin
RBC	Red blood cells
RBS	Receptor binding site
RDE	Receptor destroying enzyme
RT-PCR	Reverse transcription polymerase chain reaction
SPG	Sucrose Phosphate Glutamate
Sf9	Spodoptera Frugiperda clonal isolate
TCID	Tissue culture infectious dose
TMB	3,3’,5,5’-Tetramethylbenzidine
WHO	World Health Organization

INTRODUCTION

Seasonal influenza is an acute respiratory illness caused by the influenza virus which infects its host through the mucosal membranes of the respiratory tract. It is estimated that 3-5 million people will be affected by seasonal influenza throughout the world each year with an estimated 250,000 to 500,000 respiratory deaths annually according to The World Health Organization (WHO) (Ping et al. 2015). From those affected each year, people over the age of 65 years old and children under 5 years old, especially children under 2 years old are at an increased risk of developing complications from seasonal influenza. This can mostly be attributed from a weakened immune system in the elderly and an immune system that is not yet fully developed in children (Coates et al. 2015). This makes receiving an annual influenza vaccine within these age groups essential.

Today annual influenza vaccination is the best preventative measure for seasonal influenza. Influenza vaccines are formulated to protect against the circulating influenza strains which include both type A and type B influenza viruses. Type A influenza viruses are then further characterized into subtypes dependent on the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), while type B is differentiated based on lineage, B/Victoria or B/Yamagata. Trivalent influenza vaccines from 2018 contain an H1N1 strain, a H3N2 strain, and a B/Victoria lineage strain and quadrivalent vaccines add a B/Yamagata lineage strain (Centers for Disease Control, Nov. 2018). Every year the WHO meets with collaborating centers to discuss influenza surveillance, laboratory and clinical study results, as well as the availability of vaccine viruses to formulate recommendations

on the composition of the influenza vaccine for the upcoming influenza season (Centers for Disease Control, Sep. 2018).

Recently there has been a shift in the prevalence of influenza infection from H3N2 strains coupled with poor H3N2 vaccine efficacy (Allen and Ross, 2018). Since its introduction to humans in 1968, H3N2 has constantly been evolving in order to escape the pressures of our immune system. Mutations near the receptor binding site (RBS) on HA in virus progeny change the antigenicity compared to parental strains which has resulted in the emergence of clades and subclades within H3N2 strains that current vaccines do not protect against. An H3N2 clade can be characterized by amino acid substitutions that ultimately influence the antigenicity of H3N2 lineages. The error prone influenza RNA-dependent RNA polymerase results in a high mutation rate for virus progeny, which coupled with sequence evolution gives rise to antigenic variants with similar properties and to new H3N2 subclades (Fig 1). This antigenic variability makes it difficult to select the best vaccine strain months before the season in order to provide manufacturers with enough time to produce the vaccine (Allen and Ross, 2018; Smith et al., 2004). Unfortunately, most influenza vaccine viruses are still grown in chicken eggs where H3N2 viruses often acquire egg adaptation mutations, changing antigenic sites causing then to grow very poorly. Also the majority of diverse H3N2 viruses are observed by modern gene sequencing methods, making it difficult to obtain the replicating viruses for use in influenza vaccine research. These challenges emphasize the need for improvements in vaccine development and for greater protection against H3N2 strains over other influenza type A or type B strains (Belongia et al., 2016).

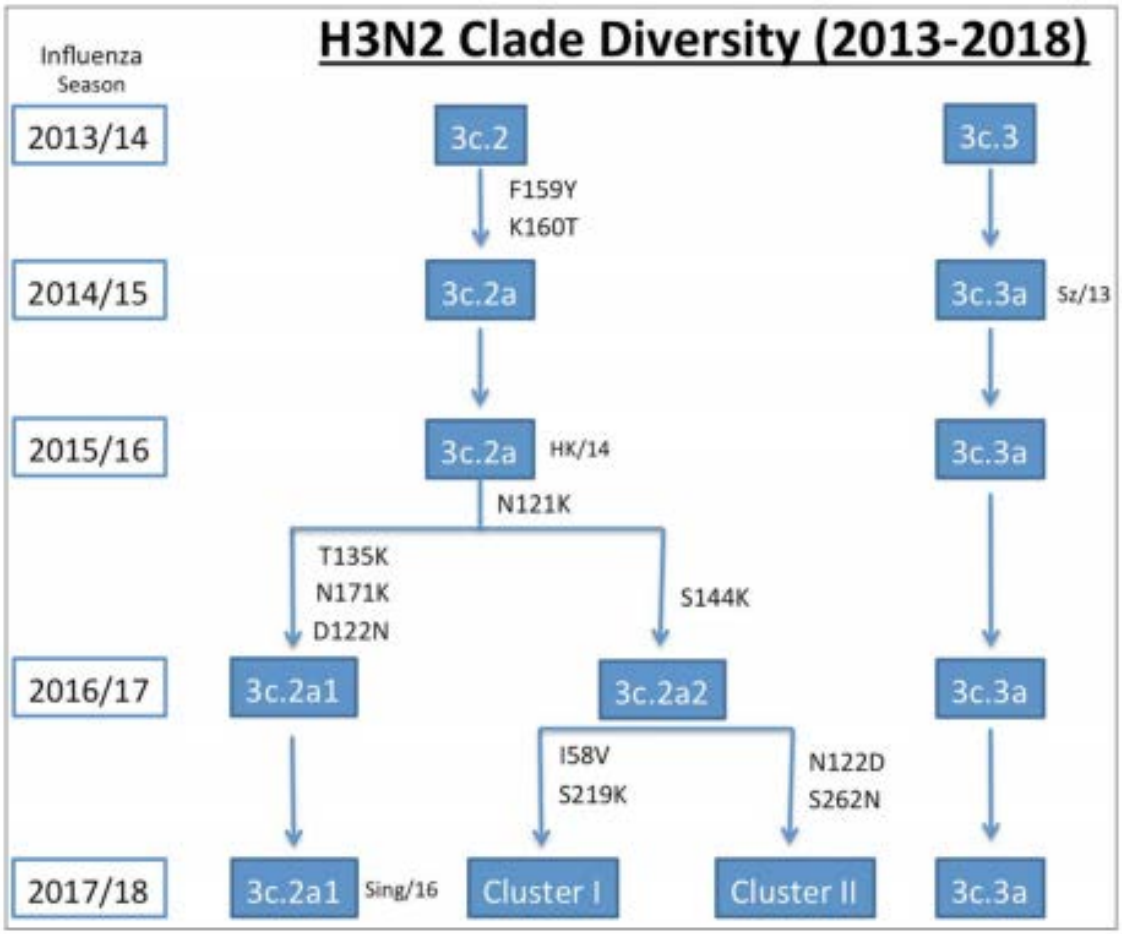

Figure 1. Overview of H3N2 clade diversity from 2013-2018. Amino acid substitutions representing the emergence of new clades. (Allen and Ross, 2018)

To address this, we are investigating a reverse genetics platform that would allow us to generate any H3N2 virus based on HA and NA sequence alone. Influenza is a single stranded negative sense segmented RNA virus. The eight segments are packaged as ribonucleoproteins within the enveloped virus (Kulman et al., 2008). This allows the employment of the 6 + 2 reassortant approach as detailed by Hoffman et al. (2002). This method utilizes DNA transfection of six plasmids containing genes for PB1, PB2, PA, NP, NS, and M from the H1N1 A/Puerto Rico/8/34 strain as well as two plasmids with HA and NA genes from an H3N2 virus. This system exploits the fact that the backbone coming

from an H1N1 laboratory strain provides low pathogenicity while producing high virus yields. The resulting reassortant however will contain the surface viral glycoproteins (HA & NA) of the contemporary circulating H3N2 virus strain, thus enabling its use in laboratory testing. This procedure is currently used by commercial manufacturers to obtain a high yield influenza virus seed for vaccine manufacturing. The plasmid pHW2000 is used as a cloning vector where each segment of influenza virus is inserted between a pol I promoter and a pol I terminator. The pol I transcription unit is flanked by a pol II promoter of the human cytomegalovirus and the polyadenylation signal of bovine growth hormone. This allows both negative-sense vRNA and positive-sense mRNA with a 5'cap and 3' poly(A) tail to be synthesized from one plasmid design as described in Figure 2 (Hoffman et al., 2000).

Nextstrain is a genomic analysis tool used to examine the evolution of specific pathogens through an open-source platform that allows for visualization of pathogen epidemiology to aid in improved outbreak response. Nextstrain uses a real-time tracking system that follows influenza evolution for the sequenced circulating strains which are mostly unavailable from WHO or Centers for Disease Control (CDC) repositories for use in laboratory testing. For influenza H3N2 viruses, this tool enables real-time tracking of accumulated random mutations within the influenza genome and sorts these new strains into a phylogenetic breakdown by clade and can highlight current isolates that have epitope mutations and show which clades are currently predominating as shown in Figure 3. (Hadfield et al., 2018).

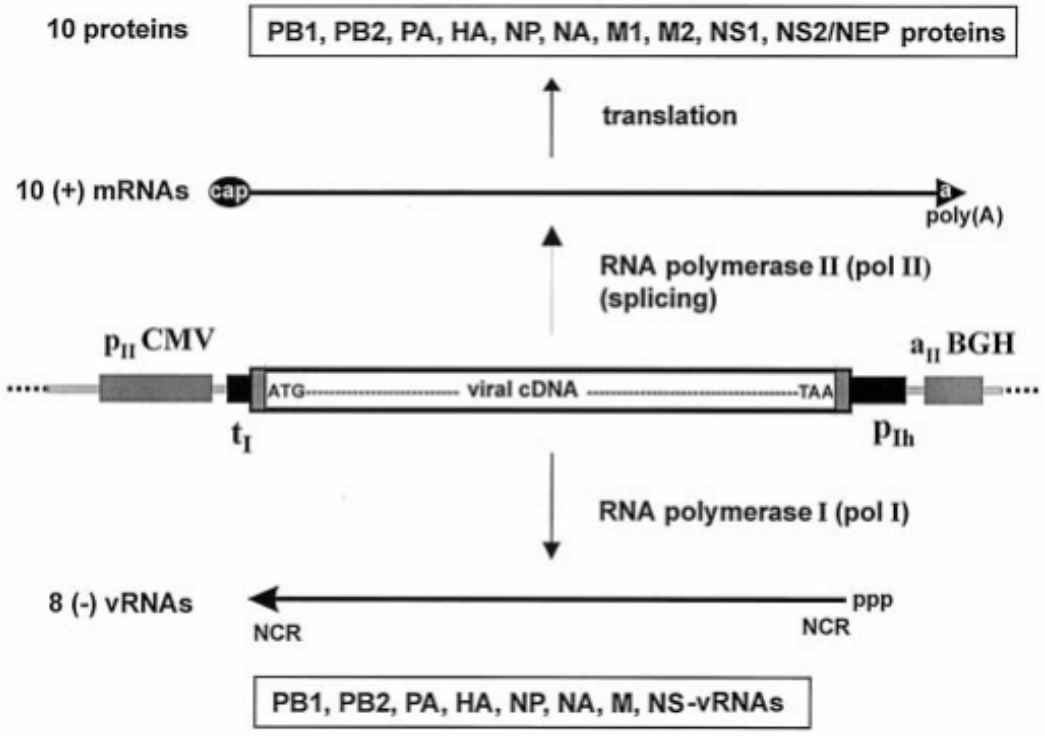

Figure 2. Schematic representation of the transcription system employed by plasmid pHW2000 for the synthesis of both vRNA and mRNA. The cDNA for each of the 8 influenza virus genes are inserted in between a Pol I promoter and a Pol I terminator which is flanked by a Pol II human cytomegalovirus promoter and a Bovine growth hormone polyadenylation signal. Once 8 influenza genes are transfected into cell culture, both mRNA and vRNA are synthesized to allow for the complete packaging of the influenza genome generating functional virus. (Hoffman et al., 2000)

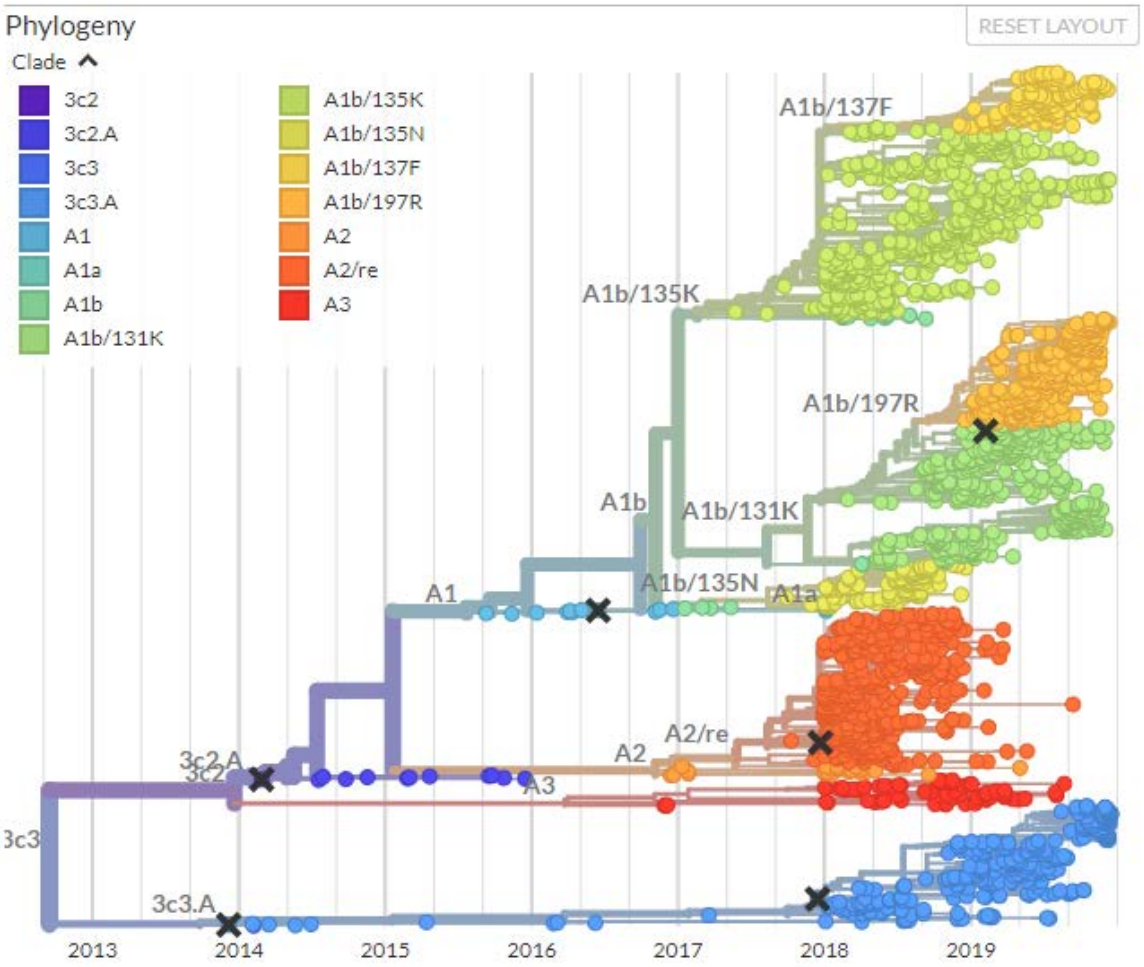

Figure 3. Phylogeny of clade distribution of H3N2 strains. H3N2 strains are distinguished by clade in a phylogenic tree representation. This outlines the evolution of antigenic mutation within hemagglutinin from 2013 to 2019. The X's within the phylogenic tree represent current and previous strains used in commercial vaccines (https://nextstrain.org). Dec 2019.

MATERIALS AND METHODS

A/Puerto Rico/08/1934 Cloning:

Plasmids containing each of the 8 influenza viral segments for A/Puerto Rico/08/1934 were obtained from the laboratory of Dr. Richard Webby (St. Jude Children's Hospital). These eight plasmids were Sanger sequenced by GENEWIZ (South Plainfield, New Jersey) using the universal primers for the T7 promoter, 5'-TAATACGACTCACTATAGGG-3' and the bovine growth hormone (BGH) terminator, 5'-TAGAAGGCACAGTCGAGG-3'. Once each plasmid was sequence confirmed they were amplified using One Shot TOP10 chemically competent *E. coli* (Invitrogen). TOP10 *E. coli* cells were thawed on ice for 8 minutes. One microliter of plasmid DNA diluted to 1ng/μL was added to the thawed cells and mixed gently. The TOP10 *E. coli* cells and plasmid DNA mixture was then incubated on ice for 30 minutes. After this incubation period, the cells were heat shocked for 30 seconds in a 42°C water bath and immediately placed back on ice for 2 minutes. Two hundred and fifty microliters of ambient room temperature S.O.C Medium (Invitrogen) was added to each vial of cells and incubated at 37°C with shaking at 225 RPM. After 1 hour incubation, cells were spread on two pre-warmed LB Agar with Ampicillin plates in 50μL and 200μL volumes using ColiRollers Plating Beads (Millipore Sigma). Plates were incubated inverted at 37°C overnight. Colonies were then picked and prepared for purification by QIAprep Miniprep Kit (Qiagen) as outlined in Figure 5. Colonies from the transformation procedure were sub-cultured in 4.5mL LB broth with 100ug/mL Ampicillin for 16 hours. Four milliliters of the sub-culture was centrifuged in

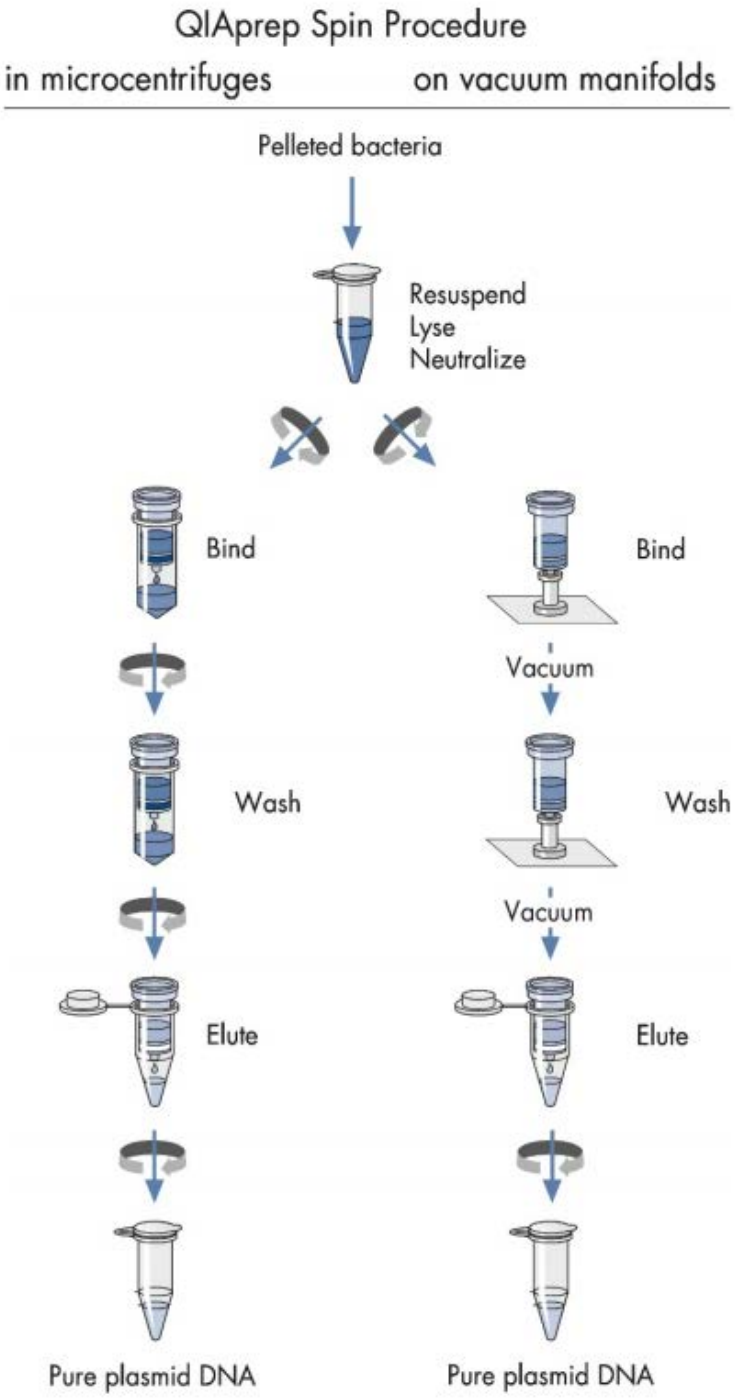

Figure 4. QIAprep Spin Procedure. A schematic representation of Qiagen's spin columns technology outlining the general workflow. (QIAprep Miniprep Handbook, 2020).

2.0 mL microcentrifuge tubes (2x2.0mL) at 8,000 RPM. The remaining 0.5mL sub-culture was added to an equal volume of 50% glycerol and stored at -80°C for later use as a glycerol stock. The pelleted bacterial cells were resuspended in 250μL buffer P1 containing LyseBlue reagent and RNase A. Two hundred and fifty microliters of buffer P2 was added and mixed by inversion until the solution turned blue followed by the addition of 350μL buffer N3 which was mixed by inversion until the solution returned colorless. The solution was centrifuged at 13,000 RPM for 10 minutes and 800μL of the supernatant was added to the provided QIAprep 2.0 spin column and centrifuged for 1 minute at 13,000 RPM. The flow through was discarded and DNA was washed with 750μL buffer PE and centrifuged for 1 minute at 13,000 RPM. The flow through was discarded and empty column was centrifuged for 1 minute at 13,000 RPM to remove excess buffer PE. The QIAprep 2.0 column was placed into a clean 1.5 microcentrifuge tube and 50μL buffer EB was added to the resin, incubated for 5 minutes and eluted by centrifugation for 1 minute at 13,000 RPM. Two microliters of eluted plasmid DNA were analyzed on a NanoDrop One UV-Vis Spectrophotometer (Thermofisher Scientific) to assess DNA concentration and purity. The plasmid DNA was diluted to 50ng/μL and 10μL of sample was added to a 0.2 mL PCR tube for a total of 500ng of DNA and sent to GENEWIZ (South Plainfield, New Jersey) for sequence confirmation by Sanger sequencing using the aforementioned universal primers. Once sequence confirmed, a 200μL pipet tip was used to stab the glycerol stock previously made and placed in 2 mL of LB broth with 100μg/mL ampicillin for sub-culturing. After 8 hours, 1 mL of the subculture was added to 160 mL of LB broth with 100μg/mL ampicillin and incubated at 37°C with shaking at 225 RPM overnight.

Plasmid Plus Maxi Kit (Qiagen) was then used to obtain plasmid concentrations high enough for transfection as well as obtain DNA with low endotoxin content. The 160 mL culture was divided into 2 x 0.5 liter plastic bottles and centrifuged at 6,000 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 8 mL buffer P1 containing LyseBlue reagent and RNase A. Once resuspended 8 mL buffer P2 was added and mixed by inversion until the solution turns blue. Eight milliliters of buffer S3 was added and mixed by inversion until the solution returned colorless. The solution was then centrifuged at 6,000 x g for 15 minutes at room temperature. The supernatant was then added to the QIAfilter cartridge and filtered using the provided plunger into a 50 mL conical tube. Five milliliters of buffer BB was added to cleared lysate which was then transferred to the QIAGEN Plasmid Plus spin column with a tube extender attached to a QIAvac 24 plus vacuum manifold with ~300 mbar vacuum applied. Once all lysate has been drawn through the spin column, the DNA was washed with 750μL buffer ETR followed by 750μL buffer PE. The spin column was then placed into a 2.0mL microcentrifuge tube, centrifuged at 10,000 x g for 1 minute, and afterwards the spin column was placed into a new 2.0mL microcentrifuge tube. Four hundred microliters of buffer EB was added, incubated for 5 minutes and centrifuged at 10,000 x g. The purified DNA was then analyzed on a NanoDrop One UV-Vis Spectrophotometer to assess the DNA concentration and purity. The purified DNA was then sequence confirmed by sending 500ng of DNA in 10μL to Genewiz (South Plainfield, New Jersey) for Sanger sequencing using the aforementioned universal primers.

Contemporary HA & NA Plasmid Cloning:

Sequences for the influenza H3N2 drifted strains A/Pennsylvania/44/2017 and A/Iowa/02/18 HA and NA segments were downloaded from the Global Initiative on Sharing Avian Influenza Data (GISAID) EpiFLU database (gisaid.org). The appropriate non-coding region (NCR) consisting of the influenza universal NCR and the HA and NA specific NCR were added for both the 5' and 3' ends of the gene sequence. The gene was then flanked with sequence to include the BsmBI restriction enzyme sites (5'-CGTCTC $(N)_1$-3') which will be needed for cloning in to the pHW2000 backbone. These sequences were scanned for internal BsmBI restriction enzyme sites within the HA and NA gene sequence and removed through the insertion of a silent mutation to avoid unwanted cutting within the gene of interest. HA and NA genes sequences were then sent to GenScript (Piscataway, New Jersey) for synthesis into the pcDNA 3.1 (-) plasmid backbone. Lyophilized plasmid DNA containing the HA and NA genes in pcDNA3.1 (-) were received and resuspended in 20μL molecular grade H_2O to a stock concentration of 200ng/μL and amplified by transformation into One Shot TOP10 chemically competent *E.Coli* (Invitrogen) and purified by Plasmid Plus Maxi Kit (Qiagen) using the same procedure as detailed above. After sequence confirmation, 3μg plasmid DNA for the HA and NA genes of A/Pennsylvania/44/2017 and A/Iowa/02/18 and empty pHW2000 backbone were digested with 3μL BsmBI restriction enzyme in 5μL 10X NEB Cut Smart buffer and brought to a total volume of 50μL with molecular grade H_2O. The digestion reaction was then incubated on a 37°C heat block for 15 minutes. The pHW2000 backbone was then treated with 1U/μg rAPID alkaline phosphatase (Sigma) and incubated for 10 minutes on a 37°C

heat block. This helps to ensure that the pHW2000 backbone does not religate on itself. The digested plasmid DNA was separated on a 1% agarose gel for 30 minutes to separate the backbone from the HA and NA genes. The 3,440bp pHW2000 linearized vector and, 1,791bp HA and the 1,409bp NA genes were excised from the gel using a clean razor blade, placed into a 2.0mL microcentrifuge tube and weighed. The excised gel fragments were then purified by the QIAquick Gel Extraction Kit (Qiagen). Three volumes buffer QC was added to 1 volume of gel (0.1g gel = 100μL buffer QC) and incubated on a 50°C heat block for 10 minutes with intermittent vortexing to help dissolve the gel fragment. One gel volume of 2-propanol was added, mixed by inversion, applied to a QIAquick column and centrifuged for 1 minute at 13,000 RPM with the flow through being discarded. The excised DNA was then washed with 500μL buffer QC and centrifuged for 1 minute at 13,000 RPM with the flow through being discarded. Seven hundred and fifty microliters of buffer PE was added to the QIAquick column and incubated for 5 minutes before centrifugation for 1 minute at 13,000 RPM with the flow through being discarded. The empty QIAquick column was then centrifuged for 1 minute at 13,000 RPM to remove the residual buffer PE, placed into a new 1.5mL microcentrifuge tube and eluted with 30μL buffer EB by incubating for 5 minutes and centrifugation for 1 minute at 13,000 RPM. The purified excised DNA fragments were then analyzed on a NanoDrop One UV-Vis Spectrophotometer to assess the DNA concentration and purity. The resultant DNA fragments were then used for ligation into the pHW2000 vector using the Rapid DNA Ligation Kit (Roche). NEBioCalculator Ligation Calculator (https://nebiocalculator.neb.com/#!/ligation) was used to calculate the mass of insert

DNA to vector ratio. Both a 2:1 and 3:1 ratio were used for ligation of insert DNA into the pHW2000 vector as shown in figure 5. The DNA insert and vector were mixed with 1x DNA Dilution buffer. The T4 DNA Ligation Buffer was thoroughly mixed and 10µL added to diluted DNA. The reaction volume at this point was 19µL. One microliter of T4 DNA Ligase was added to the reaction mixture (Note* Do not pipette up and down) and incubated at room temperature for 30 minutes. Once the ligation reaction has completed the incubation period, the reaction proceeds to transformation as previously outlined above.

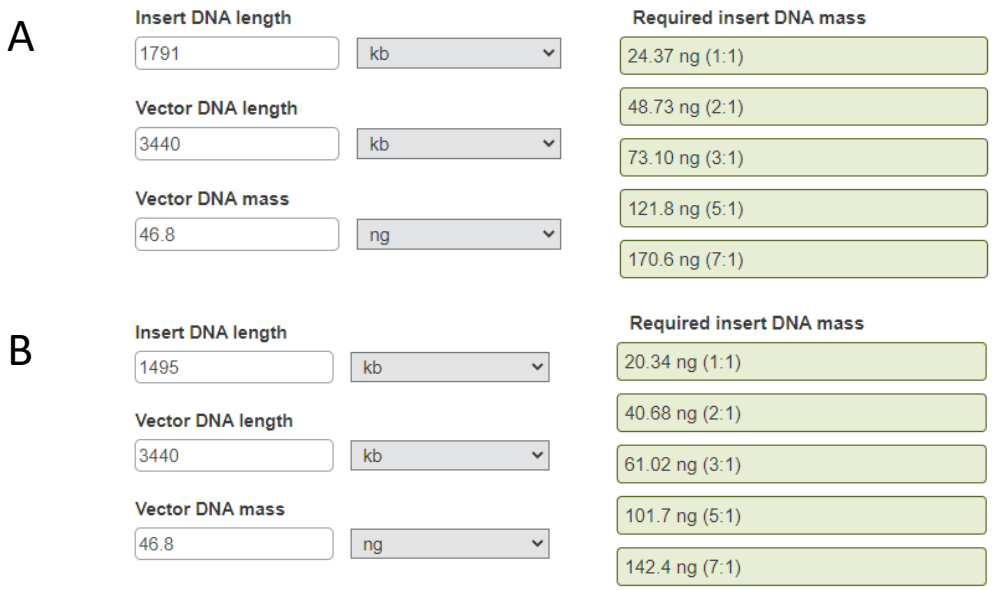

Figure 5. NEBioCalculator Ligation Calculator. A. Ligation reaction calculation for insertion of the HA gene into pHW2000 vector, B. Ligation reaction calculation for insertion of NA gene into pHW2000 vector. (https://nebiocalculator.neb.com/#!/ligation)

Sf9 Codon Optimized Plasmid Cloning:

For use of Spodoptera frugiperda (Sf9) codon optimized (C.O.) HA and NA plasmids from pre-existing A/Iowa/25/2017 plasmids currently used for protein engineering in

insect cell culture expression system, the influenza universal and HA or NA gene specific NCRs need to be added as genes for virus-like particles (VLPs) and nanoparticles do not contain them. Two sets of primers were designed to add an overhang with one set of primers designed to add the necessary NCR and another set of primers to add the BsmBI restriction site for use in the pHW2000 backbone. Table 1 shows the primers used for polymerase chain reaction (PCR) extension of both HA and NA genes to include the required sequence elements. PCR was performed using AccuPrime Taq DNA Polymerase (Invitrogen). Five microliters of 10X AccuPrime PCR Buffer I, 1μL of both forward and reverse primers (10uM), and 1μL of AccuPrime Taq DNA Polymerase was added to 1μL of A/Iowa/25/17 HA plasmid DNA (28ng/μL) and to 2μL of A/Iowa/25/17 NA plasmid DNA (14.8ng/μL). Both reactions are brought to a final total volume of 50μL with molecular grade H_2O. Touchdown PCR was performed using a hot start cycle at 94°C for 3 minutes followed by 10 cycles of 94°C for 30 seconds, 65°C for 30 seconds (-1°C per cycle) and 68°C for 2 minutes followed by 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 2 minutes with a final extension time at 68°C for 5 minutes. Five microliters of PCR products with 2μL Blue Juice Dye (Invitrogen) were then run on a 1% Agarose gel with a1kb NEB DNA ladder at 116V for 30 minutes for confirmation of the PCR reaction. An additional PCR reaction was required to add the BsmBI restriction sites on the 5' and 3' ends of the recently added NCR extensions. The PCR setup and cycle program were exactly the same as previously described with the new PCR products used as template DNA. The PCR fragments were then purified by PCR Cleanup Kit (Qiagen) where 50μL of

Table 1. Primers used for PCR Extension. Two rounds of PCR extension were needed to add the appropriate influenza and HA specific non-coding regions to flanked by BsmBI restriction enzyme sites.

Primer	Sequence 5‘ to 3‘
A/Iowa/25/17 HA F	GCAGGGGATAATTCTATTAACCATGAAGACCATCATCGCTCTG
A/Iowa/25/17 HA R	CAAGGGTGTTTTTAATTAATGCACTTAGATGCAGATGTTGC
A/Iowa/25/17 HA NCR F	TATTCGTCTCAGGGAGCAAAAGCAGGGGATAATTCTATTAAC
A/Iowa/25/17 HA NCR R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTTTAATTAATGC
A/Iowa/25/17 NA F	GCAAAAGCAGGAGTAAAGATGAACCCAAACCAGAAG
A/Iowa/25/17 NA R	GGAGTTTTTTCTAAAATTGCGAAAGCTTAGATCAGCATGAGGTTC
A/Iowa/25/17 NA NCR F	TATTCGTCTCAGGGAGCAAAAGCAGGAGTAAAG
A/Iowa/25/17 NA NCR R	ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTTCTAAAATTGC

PCR product was diluted in 250μL buffer PB, spun through a QIAquick spin column, washed with 750μL of buffer PE and eluted with 35μL buffer EB. The subsequently purified PCR fragment was then used in a BsmBI digest and ligated into pHW2000 and sequence confirmed as previously described.

Cell Culture:

The human embryonic kidney cell line (HEK293T) and Madin-Darby canine kidney cell line (MDCK) were obtained from the American Type Culture Collection (ATCC). HEK293T cells were maintained in Dulbecco’s modified eagle media (DMEM) (Sigma) supplemented with 10% gamma-irradiated fetal bovine serum (FBS) (Sigma), 4mM L-Glutamine (Gibco) and incubated at 37±2°C with 5% CO_2 in a vented flask. MDCK cells were maintained in minimum essential medium with Earle’s balanced salt solution (EMEM) supplemented with 10% FBS, 4mM L-Glutamine and incubated at 37±2°C in a plug flask. General cell maintenance routine was performed when cells grown in T-75 tissue culture flasks reached approximately 70%-95% confluency (Fig. 6). This typically

took HEK293T cells 2-3 days and MDCK cells 4-5 days to reach the desired confluency. Once cells are at the desired confluency for splitting, the media was removed, cells were rinsed with 30mL Hank's Buffered Salt Solution (HBSS) (Gibco) to remove residual (FBS), trypsinized with 5mL 0.5% Trypsin-EDTA, incubated at 37±2°C to allow dissociation of the cells from the tissue culture treated plastic. Trypsinization was stopped by addition of maintenance media containing FBS. HEK293T cells generally took 1 minute to fully dissociate from the flask whereas MDCK cells took between 20-25 minutes to fully dissociate. Cells were then split to an appropriate ratio, 1:6 for HEK293T cells and 1:8 for MDCK cells.

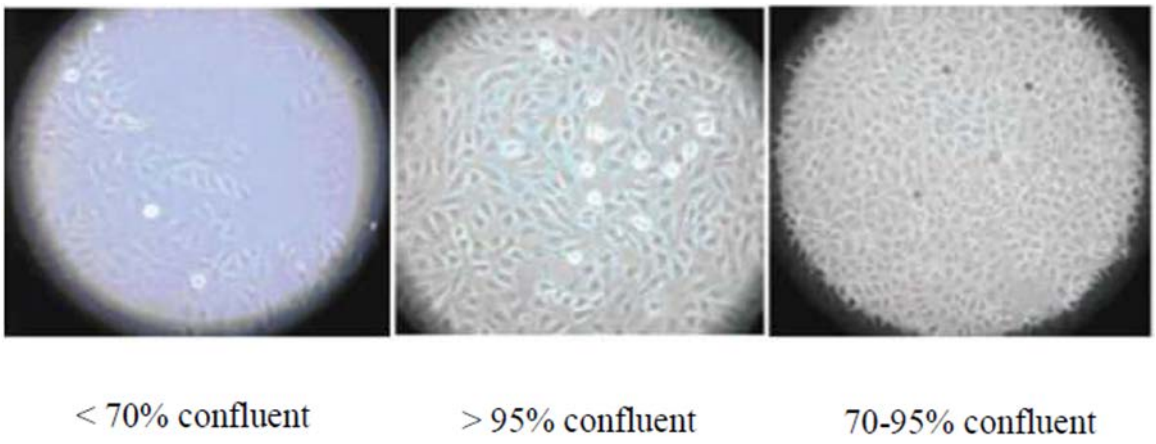

Figure 6. Example of confluency in MDCK monolayer. (WHO Manual for the laboratory diagnosis and virological surveillance of influenza, 2011)

Generation of recombinant H3N2 drifted virus strains:

Recombinant H3N2 influenza virus strains were generated by DNA transfection using Lipofectamine 3000 reagent (Invitrogen). The day before transfection, confluent T-

75 flasks of HEK293T and MDCK cell were trypsinized with 5mL 0.5% Trypsin-EDTA (Gibco) and counted on a Cellometer Auto T4 Bright Field Cell Counter (Nexcelom) with Trypan Blue (Gibco) staining to assess cell viability. Cells were diluted in cell culture media with DMEM and seeded on 6-well plates at a HEK293T:MDCK ratio of 1:1 and a total co-culture mixture of $7x10^5$ cells/well in DMEM supplemented with 10% FBS, 4mM L-Glutamine, and incubated at 37±2°C with 5% CO_2. On the day of transfection, cells were examined and confirmed to be between 70% and 90% confluent. Transfection was performed using a 2:1 ratio of Lipofectamine 3000 reagent to DNA meaning that for every 1μg of DNA 2μL Lipofectamine 3000 reagent is used. Sixty Four microliters of Lipofectamine 3000 reagent were added to a 5mL Falcon tube containing 0.5mL of OptiMEM reduced-serum medium and labeled LPF. Thirty two micrograms of DNA, 4μg for each of the 8 influenza plasmids, were added to a 5mL Falcon tube containing 0.5mL OptiMEM reduced-serum medium and 64μL P3000 reagent from the Lipofectamine 3000 kit which helps in formation of the lipid/DNA complex and labeled DNA. Both tubes were gently mixed and the entire contents of the tube labeled DNA was added to the tube labeled LPF and incubated at room temperature for 20 minutes to allow the Lipofectamine + DNA complex to form. After 20 minutes, 250μL of the transfection complex was pipetted drop-wise into 4 wells of the 6 well plates containing the HEK293T+MDCK co-culture in 3mL of media and incubated at 37±2°C with 5% CO_2. The other two wells are used as non-transfected controls. After 24 hours, the transfection medium was removed, cells gently washed with DMEM and refed with inoculation medium consisting of DMEM supplemented with 4mM L-Glutamine, 1% 2.18M Sucrose 0.11M Phosphate 0.054M Glutamate (SPG), 0.5μg/mL

TPCK-Trypsin and further incubated at 37±2°C with 5% CO_2. SPG was added to the inoculation medium to support viral stability while TPCK-Trypsin is added to support viral replication by cleaving HA into HA1 and HA2 therefore producing a fusion peptide necessary for the virus to fuse with the cellular membrane (Chaipan et al., 2009). Cells were observed for 48-72 hours post transfection for signs of influenza virus mediated cytopathic effect (CPE). Upon observation of at least 50%-100% CPE, media from each of the transfected wells were pooled, centrifuged at 500 x g for 15 minutes and distributed into 1mL aliquots.

Virus Passaging:

MDCK cells at 75%-95% confluency were washed 3-times with either EMEM or DMEM to remove residual FBS that can block trypsin cleavage of the HA, and refed with 30mLs of EMEM supplemented with 4mM L-Glutamine, 1% SPG and 0.5μg/mL trypsin when using a T-75 plug flask and in 3mLs of DMEM supplemented with 4mM L-Glutamine, 1% SPG and 0.5μg/mL trypsin when using a 6-well plate. Rescued virus from the transfection protocol was used for first passage in MDCK cells diluted from $1x10^1$ to $1x10^5$ in Leibovitz's L-15 Medium (L-15) (Gibco) supplemented with 1% SPG. One hundred microliters of diluted virus was used to inoculate each well of a 6 well plate of MDCK cells. The inoculated MDCK cells were then incubated at 37±2°C with 5% CO_2 until at least 50%-100% CPE was observed. Once the desired amount of CPE was observed or 7 days had elapsed since inoculation, 300μL of 10% SPG were added to the virus flask or plates respectively, and one cycle freeze/thaw cycle at -80°C, used to lyse the cells before

distributing the virus in 1mL aliquots. For the second passage of recombinant virus, virus was diluted from $1x10^4$ to $1x10^7$ in L-15 supplemented with 1% SPG. One milliliter of diluted virus is used to inoculate T-75 flasks and incubated at 37±2°C until at least 50%-100% CPE was observed. Once the desired amount of CPE was observed or 7 days had elapsed since inoculation, 3mLs of 10% SPG was added to the virus flask and one cycle of freeze/thaw was used to lyse the cells before distributing the virus in 1mL aliquots.

Viral RNA Extraction:

TRIzol LS reagent (Invitrogen) was used to extract viral RNA from rescued virus. Three hundred microliters of viral supernatant was diluted in 900μL TRIzol (3:1 ratio) and incubated for 5 minutes to allow for complete dissociation of the nucleoproteins complex. Two hundred microliters of chloroform was added and incubated for 3 minutes at room temperature to allow phase separation of the viral RNA from genomic DNA and proteins. The solution was then centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase (400-500μL) was carefully removed without disruption of the interphase, which contains genomic DNA and proteins, and placed into a new RNase-free 1.5mL microcentrifuge tube. The RNA was precipitated using 0.5mL isopropanol, incubated for 10 minutes at room temperature and centrifuged at 12,000 x g for 10 minutes. The supernatant was carefully removed by a micropipettor not to disturb the RNA pellet. The RNA pellet was then washed using 1.0mL of 75% ethanol, vortexed briefly and centrifuged at 7,500 x g for 5 minutes. The supernatant was carefully removed using a micropipettor and the RNA

pellet was allowed to air dry for 5-10 minutes. The RNA pellet was resuspended in 50μL of RNase-free water and incubated at 55°C for 10 minutes to promote resolubilization.

RT-PCR for DNA Amplification:

Reverse transcription polymerase chain reaction (RT-PCR) was used to synthesize complimentary DNA (cDNA) from the viral RNA for amplification of the HA gene using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen). A master mix containing 25μL 2X reaction Buffer, 1μL 25pmol forward primer, 1μL 25pmol of reverse primer, 19μL RNase-free RT-PCR grade H_2O and 1μL Superscript III Taq polymerase was used per 3μL of RNA template for a total reaction volume of 50μL. The primers were designed to be specific to the non-coding region of the HA gene. PCR was sequentially performed using one cycle at 50°C for 30 minutes to allow complete synthesis of cDNA from the RNA template followed by a hot start cycle at 94°C for 2 minutes, 45 cycles of 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1 minute respectively followed by 68°C for 5 minutes. The PCR DNA was then purified by the PCR Cleanup Kit (Qiagen). Two hundred and fifty microliters of buffer PB was added to the 50μL PCR reaction (5:1) and mixed by inversion. The solution was added to a QIAquick spin column and centrifuged for 1 minute at 13,000 RPM. The flow through was discarded and the DNA was washed with 750μL buffer PE and centrifuged for 1 minute at 13,000 RPM. The flow through was discarded and the empty QIAquick spin column was centrifuged for 1 minute at 13,000 RPM to remove residual buffer PE. The QIAquick spin column was placed into a new 1.5mL microcentrifuge tube and the DNA eluted using 50μL

molecular grade H_2O. The purified PCR DNA was then analyzed on a NanoDrop One UV-Vis Spectrophotometer to assess the DNA concentration and purity.

Sanger Sequencing:

For Sanger sequencing, the DNA was diluted to 4ng/μL in molecular grade H_2O for a total concentration of 40ng in 10μL total volume. HA gene specific primers with a working dilution of 100uM (Table. 2) were diluted to 5pmol/μL with 5μL added to the PCR DNA for a total of 25pmol final primer concentration. Each primer requires its own separate sequencing reaction. The PCR DNA was sequence confirmed by Sanger sequencing (GENEWIZ) to confirm the HA gene sequence since all downstream applications assay HA activity.

Table 2. Primers used for RT-PCR and Sequencing. RT-PCR is performed to generate cDNA from an RNA template and the HA gene is amplified using the H3N2 Universal forward and reverse primers. The amplicon is then sequenced using the H3N2 Universal forward and reverse primers as well as the H3N2 forward and reverse primers.

Primer	Sequence 5‘ to 3‘
H3N2 Universal F	AGCAAAAGCAGGGGATAATTC
H3N2 Universal R	AGTAGAAACAAGGGTGTTTTTA
H3N2 Seq F1	TGAGTTGGTTCAGAATTCCTCA
H3N2 Seq F2	ACCAAAAGAAGCCAACAAGC
H3N2 Seq F3	GCACTCAAGCAGCAATCGAT
H3N2 Seq F4	GGCAGCAGATCTCAAAAGCA
H3N2 Seq R1	ATTGCGCCAAATATGCCTCT
H3N2 Seq R2	CCTTGATCTGGAACCGGTTG
H3N2 Seq R3	AGTCACGTTCAATGCTGGAT

Hemagglutination Assay:

Pooled male Guinea Pig Whole Blood in Alsevers solution (BioChemed Services) was washed by filtering 25 mL of whole blood solution using a sterile gauze into a 50mL conical tube containing 25mL of phosphate buffered saline (PBS) pH 7.2 (Gibco). The solution was mixed by inversion and centrifuged at 2,000 RPM for 10 minutes. The supernatant was discarded and the volume brought to 50mL with PBS pH 7.2, gently mixed by inversion and centrifuged at 2,000 RPM for 10 minutes. This process was repeated one more time. The red blood cells (RBCs) were transferred to a 15mL conical tube, the volume brought to 15mL with PBS pH 7.2, gently mixed by inversion and centrifuged at 2,000 RPM for 10 minutes. The packed RBC volume was noted, the supernatant was removed and brought to a final volume so that the final concentration of RBCs is 10% v/v. Twelve 2-fold serial dilutions of influenza virus starting at 1:2 were made in Dulbecco's phosphate buffered saline (DPBS) at 50μL per well, with 12 additional wells containing only DPBS used as a control were made in 96 well round bottom microtiter plates. Fifty microliters of 1.5% Guinea Pig RBC suspension in DPBS was added to all wells and the mixture was incubated at ambient room temperature for 1 hour. The hemagglutination (HAg) titer was calculated as the reciprocal of the highest dilution of influenza virus that resulted in agglutinated lattice formation with the red blood cells.

Determination of TCID:

Twenty four 2-fold serial dilutions of virus starting at 1:100 were made in DMEM supplemented with 1% BSA V, 0.02M HEPES, 4mM L-Glutamine and 1X Penn/Strep (Assay

diluent) in a 96 well round bottom microtiter plate and incubated at 37±2°C with 5% CO_2 for 1hour. All virus samples are titered in duplicate and 50μL of diluted virus was transferred to a 96 well flat bottom microtiter plate. A T-75 flask of MDCK cells at 75-95% confluency was trypsinized, cells counted and resuspended in Assay Diluent. One hundred microliters of trypsinized cells at $1.5x10^5$ cells/mL was added to the 50μL of diluted virus on the 96 well flat bottom microtiter plate as shown in Figure 7. Tissue culture infectious dose (TCID) plates were then incubated for 20 hours ± 2 hours at 36°C+5% CO_2. After incubation, cells were fixed with 100μL/well 80% Acetone in PBS for 12 minutes at ambient room temperature, washed twice with 300μL/well Dulbecco's Phosphate Buffered Saline with Tween 20 (DPBST)(Gibco), air dried and blocked with 200μL/well of StartingBlock (PBS) Blocking Buffer (Invitrogen) for 1 hour. Blocking solution was removed and 100μL/well of anti-NP monoclonal antibody MAB8521 (Millipore) diluted 1:2000 in 1X ELISA diluent (Invitrogen) supplemented with 0.05% Tween 20, and incubated for 2 hours at room temperature. Wells were washed three times with 300μL/well DPBST. Plate was incubated with 100μL/well of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (SeraCare) diluted 1:10,000 in 1X ELISA diluent supplemented with 0.05% Tween 20, and incubated for 1 hour at room temperature. After washing five times with 300μL/well DPBST, 100μL of 3,3',5,5'-Tetramethyl-benzidine (TMB) substrate was added to each well and incubated for 30 minutes at room temperature. The reaction was stopped with the addition of 100μL/well of Stop Buffer (ScyTek) and the virus titer was defined by reading the optical density (OD) at 450nm on a microplate reader. A 4-

		1	2	3	4	5	6	7	8	9	10	11	12
	Dilution Factor (Row A and B)	100	200	400	800	1600	3200	6400	12800	25600	51200	102400	204800
Virus Dilution	A												
	B												
	Dilution Factor (Row C and D)	4.1E5	8.2E5	1.6E6	3.3E6	6.6E6	1.3E7	2.6E7	5.2E7	1.1E8	2.1E8	4.2E8	8.4E8
	C												
	D												
CC	E												
	F												

Figure 7. Plate template for setting up TCID Assay. A representation of the layout used for setting up the TCID assay in 96 well microtiter plates.

parameter logistic curve fit was applied using the SoftMax program. From this curve fit, the titer of the influenza virus (1 TCID in 50µL) was calculated as the reciprocal of the virus dilution where two times the mean OD value of the cell control (background) is reached.

Microneutralization Titer:

Ten 2-fold serial dilutions of anti-influenza ferret serum starting at 1:10 were made by adding 25µL/well Assay Diluent in a 96 well flat bottom microtiter plate, 12 additional wells containing only Assay Diluent were used as a control. Influenza virus was diluted to its 100 TCID per 25µL (2000 TCID per mL) in DPBS and 25µL added to the serially diluted anti-influenza ferret or sheep serum. Plates were then treated the same as the determination of TCID section. The virus neutralization antibody 50% titer is calculated where X = [(Average OD of Virus Control wells)-(Average OD of the Cell Control wells)] / 2 + (Average of the Cell Control wells). Here X is the OD at which 50% of the MDCK cells were infected (50% cut-off). All OD values below or at 50% cut-off are considered positive

for neutralization activity. The reciprocal of the serum dilution from the final well where the OD value is below or at the 50% cut-off is considered the microneutralization titer.

Human sample#	Dilution Factor	10	20	40	80	160	320	640	1280	2560	5120	VC	CC
		1	2	3	4	5	6	7	8	9	10	11	12
1	A												
2	B												
3	C												
4	D												
5	E												
6	F												
7	G												
8	H												

Figure 8. Plate template for Microneutralization Assay. A representation of the layout used for setting up the TCID assay in 96 well microtiter plates.

Serum Sample Treatment:

Serum for use in the hemagglutination inhibition assay (HAI) was received from either the National Institute for Biological Standards and Control (NIBSC) (South Mimms, United Kingdom) or the International Reagent Resource (IRR) (Manassas, Virginia). Anti-A/New Caledonia/20/1999 HA Sheep Serum (NIBSC), Ferret Antisera to Influenza A A/Michigan/45/2015 (H1N1)pdm09 (IRR), Ferret Antisera to Influenza A A/Singapore/INFIMH-16-0019/2016 (H3N2 (IRR), Ferret Antisera to Influenza A A/Hong Kong/4801/2014 (H3N2) (IRR), and Ferret Negative Control Sera (IRR) were treated with Receptor Destroying Enzyme (RDE) (Denka Seiken) to eliminate inhibitors of non-specific hemagglutination in serum during HAI. Three volumes of RDE solution was added to 1 volume of serum sample with mixing by vortex for 10 seconds (300mL RDE to 100mL

serum sample). The solution was then incubated at 37±2°C overnight (18-20 hours) in a 37°C water bath. After incubation at 37±2°C, the solution was incubated at 56±2°C for 30 minutes in a water bath to inactivate RDE. Six volumes of DPBS were added to 1 volume of RDE treated serum sample and mixed by vortex for 10 seconds (600μL DPBS to the 400μL inactivated RDE + RDE treated serum sample) to complete a 1:10 dilution. The RDE treated serum was used in the HAI assay.

Hemagglutination Inhibition Assay:

Ten 2-fold serial dilutions of RDE treated anti-influenza serum was used with a starting dilution of 1:10 in a 96 well round bottom microtiter plate (25μL per well) with the RDE treated Ferret Negative Control Sera used as a control. A murine monoclonal antibody (MAb), rA2.91.3, was also used in the HAI assay with a starting dilution of 3μg/mL. The MAb was found to target the top of the head domain of HA as demonstrated by HAI activity (Smith et al, 2017) and was found to compete with a RBS antibody F005-092 in a separate competitive binding study (Portnoff et al. 2020). Twenty five microliters of influenza virus diluted to 4 hemagglutination units (HAU) in DPBS was added to each well containing 25μL serum or MAb. Eight wells were used as a virus only control and 8 wells were used as a DPBS control as shown in Figure 9. The plates were incubated at room temperature for 1 hour. After a 1 hour room temperature incubation, 50μL of 1.5% Guinea Pig RBC suspension in DPBS was added to each well, mixed by gentle manual agitation by tapping on the side of the plate, and incubated at room temperature for 1

hour. The HAI titer of the sample was determined as the highest sample dilution where Guinea Pig RBCs were not agglutinated.

Sample#	Dilution Factor	10	20	40	80	160	320	640	1280	2560	5120	VC	CC
		1	2	3	4	5	6	7	8	9	10	11	12
1	A												
	B												
2	C												
	D												
3	E												
	F												
4	G												
	H												

Figure 9. Plate template for Hemagglutination Inhibition Assay. A representation of the layout used for setting up the TCID assay in 96 well microtiter plates.

Western Blotting

Western Blotting is a technique for the identification of recombinant protein expression. Proteins are denatured by sodium dodecyl sulphate (SDS) and separated by size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins are transferred onto a nitrocellulose membrane. Primary protein specific antibodies are incubated with the nitrocellulose membrane, recognize the target protein, and bind to them. A secondary antibody which is used to visualize the specific target protein that the antibody binds to. Here, 100µL of each A/Puerto Rico/08/1934, A/Pennsylvania/44/2018, A/Iowa/02/2018 and Sf9 C.O. A/Iowa/25/17 viral supernatants

were mixed with 100μL of 2X NuPage LDS Sample Buffer (Invitrogen) and incubated at 90°C for 10 minutes to denature the proteins. Twenty microliters of viral sample and 8μL of Precision Plus Protein Dual Color Standards (BioRAD) were loaded to the wells of a pre-cast 4-12% Bis-Tris NuPage Gel (Invitrogen) and then run in MOPs running buffer (Invitrogen) at 150V for 1 hour. The resultant gel was then used to transfer the separated proteins to a nitrocellulose membrane (BioRAD) using the Trans-Blot Turbo Transfer System (BioRAD). The nitrocellulose membrane was then blocked with 12mLs of Blocking Buffer (Quality Biological) for 1 hour at room temperature. The blocking buffer was removed, discarded and replaced with 12mL of either rabbit anti-A/Fujan (H3N2) or rabbit anti-A/New Caledonia (H1N1) polyclonal antibody (Novavax) at a 1:500 dilution in blocking buffer (24μL antibody in 12mL blocking buffer) and incubated overnight at 4°C. The next morning, the primary antibody was removed, and the membrane washed 3 times with 12mL of DPBST with 5 minute incubations with each wash. The last wash was removed, replaced with 12mL of Goat anti-rabbit AP secondary antibody (Seracare) at a 1:2000 dilution in blocking buffer (6μL antibody in 12mL blocking buffer) and incubated at room temperature for 1 hour. The nitrocellulose membrane was then washed 3 times with 12mL of DPBST with 5 minute incubations with each wash. Twelve milliliters of BCIP/NCT Alkaline Phosphatase Substrate (Sigma) was added, and incubated at room temperature while protein bands appear. Colored molecular weight markers are used to help calculate the approximate molecular weights of the visualized protein bands.

RESULTS

Recombinant Virus Generation and Optimization

To evaluate whether the implementation of the 6 + 2 reverse genetics platform would yield influenza virus, a co-culture of HEK293T and MDCK cells were transfected using 2 different Lipofectamine 3000 reagent to DNA ratios (2:1 and 4:1) with all 8 segment genes coming from the A/Puerto Rico/08/1934 influenza strain. By using two different Lipofectamine 3000 reagent to DNA ratios the optimal ratio for transfection efficiency for the aforementioned cell-lines used for the co-culture was achieved. Upon virus rescue, HAg and TCID were performed on the viral supernatant to confirm the generation of a functional virus stock. HAg tests the presence of HA activity and to compare how well virus produced from each transfection ratio was able to agglutinate Guinea Pig RBCs and produce a TCID titer by infecting MDCK cells. Results showed no difference in A/Puerto Rico/08/1934's ability to agglutinate Guinea Pig RBCs from rescued virus transfected with either transfection protocol while TCID titers revealed a 10-fold higher virus titer in rescued virus from the 2:1 Lipofectamine 3000 protocol versus the 4:1 Lipofectamine 3000 protocol (Table 3.). Due to these findings, a 2:1 Lipofectamine 3000 reagent to DNA ratio was used for all subsequent transfection protocols.

Table 3. HAg and TCID titers from rescued virus. HAg titer results showed no difference between the two transfection protocols used. However, a 10-fold difference in TCID titers was observed between the two transfection protocols.

Virus Strain	Subtype	HAg	TCID
A/PR/08/1934 Transfection 2:1	H1N1	64	10,000
A/PR/08/1934 Transfection 4:1	H1N1	64	1,000

Generation of H3N2 Contemporary Influenza Strains.

The A/Pennsylvania/44/2018, A/Iowa/02/2018 and the A/Iowa/25/2017 were chosen as the H3N2 drifted strains through the use of the Nextstrain real-time tracking system. The most current H3N2 strains can be displayed by the occurrence of epitope mutations within Nextstrain. Strains with the most mutations sequences were taken and sequence aligned with the previous 2 years recommended vaccine strains which are used as the reference sequences. H3N2 strains that differ in their epitope mutation from the reference sequences were then used for the reverse genetics platform. To test the utility of the 6 + 2 reassortant reverse genetics platform in generating recombinant influenza virus, two wild-type HA and NA genes (A/Pennsylvania/44/2018 and A/Iowa/02/2018) and one Sf9 codon optimized HA and NA gene (A/Iowa/25/2017) were used with plasmids containing the genes from the PB2, PB1, PA, NS, NP, and M genes to transfect a HEK293T/MDCK. Upon successful virus rescue the HA activity was assessed through HAg assay. The results show that both A/Pennsylvania/44/2018 and A/Iowa/02/2018 were able to agglutinate Guinea Pig RBCs with titers of 64 and 16 respectively while the Sf9 C.O. A/Iowa/25/2017 did not show agglutination (Table 4). Each of the rescued viruses

Table 4. HAg and titers from H3N2 Drifted Strains. HAg titer results showed agglutination between A/PA/44/18 and A/IA02/18 and Guinea Pig RBCs, however, no agglutination was observed with Sf9 C.O. A/IA/25/17 and Guinea Pig RBCs from virus rescued during the transfection protocol.

Virus Strain	Subtype	HAg
A/PA/44/18 Transfection 2:1	H3N2	64
A/IA/02/18 Transfection 2:1	H3N2	16
A/IA/25/17 Transfection 2:1	Sf9 C.O. H3N2	<2

including the A/Puerto Rico/08/1934 strain were passaged in 6 well plates of MDCK cells. In order to assess HA activity in the propagated recombinant virus, HAg assay was performed. The HAg titers showed at least an 8 fold increase in HA activity from propagated virus compared to virus rescued post-transfection (Table. 5). The results also showed that propagation of the Sf9 C.O. A/Iowa/25/2017 recombinant virus was unable to agglutinate Guinea Pig RBCs. To test this, we wanted to determine what effect the Sf9 C.O. HA and NA had on virus replication in mammalian cells even in the presence of wild-type PB2, PB1, PA, NS, NP and M genes. Protein engineering using baculovirus in a Sf9 expression system to generate HA nanoparticles and virus-like particles (VLPs) requires having genes C.O. for use in Sf9 cells. The use of these plasmids for the generation of recombinant influenza virus was being investigated to see if they could produce functional virus by reverse genetics in mammalian cell culture. A TCID titer was performed on the Sf9 C.O. A/Iowa/25/2017 transfection to see if any virus replication was occurring. TCID titration of Sf9 C.O. A/Iowa/25/2017 shows that viral replication was not observed when the Sf9 C.O. genes were used for recombinant transfection even though there

appeared to be low levels of CPE observed (Fig. 10). It is possible that this observed CPE was not caused by the virus but by something else. Through western blot analysis, results show that no HA or M proteins were made by Sf9 C.O. A/Iowa/25/17 (Fig. 11). Without either of these proteins, infectious virus cannot be made. These findings suggest that further exploration will be needed to test the use of these plasmids in mammalian expression systems at a later time. Due to this, the Sf9 C.O. A/Iowa/25/17 was removed from testing and was no longer pursued as a viable option for implementation of the 6 + 2 reverse genetics system. The first passage of A/Pennsylvania/44/2018 MD1 10^5 and A/Iowa/02/2018 MD1 10^3 were propagated once more in T-75 flasks of MDCK cells at $1x10^4$ through $1x10^7$ dilutions. The viral dilutions used for propagation were chosen by taking the highest dilution of virus from the MD1 passage that yielded the highest HAg titer of 512 and 128 respectively. Once virus was harvested, HA activity was determined by HAg and infectivity titer by TCID (Table 6). The A/Iowa/02/2018 MD2 10^7 virus was not used as no CPE was observed after 7 days. The A/Pennsylvania/44/2018 MD2 10^4 virus was not used for determination of TCID titer since the 3 proceeding viral dilutions yielded consistent HAg titers. Generally, the acceptable cut-off for determining if a virus is suitable for HAI or TCID testing of clinical samples requires a HAg titer above 8 and a 100TCID above 100. Taking into account the acceptable cut-off for clinical testing of virus samples, A/Pennsylvania/44/2018 MD2 10^7 and A/Iowa/02/2018 MD2 10^5 were chosen for further analysis.

Table 5. HAg and from first passage recombinant virus. HAg titer results showed agglutination between A/PR/08/34, A/PA/44/18 and A/IA02/18 and Guinea Pig RBCs, however, no agglutination was observed with Sf9 C.O. A/IA/25/17 and Guinea Pig RBCs from a first passage of virus in MDCK cells.

Virus Strain	Subtype	HAg
A/PR/08/34 MD1 10^1	H1N1	512
A/PR/08/34 MD1 10^2	H1N1	512
A/PR/08/34 MD1 10^3	H1N1	1024
A/PR/08/34 MD1 10^4	H1N1	1024
A/PR/08/34 MD1 10^5	H1N1	1024
A/PA/44/18 MD1 10^1	H3N2	512
A/PA/44/18 MD1 10^2	H3N2	512
A/PA/44/18 MD1 10^3	H3N2	<2
A/PA/44/18 MD1 10^4	H3N2	8
A/PA/44/18 MD1 10^5	H3N2	512
A/IA/02/18 MD1 10^1	H3N2	64
A/IA/02/18 MD1 10^2	H3N2	128
A/IA/02/18 MD1 10^3	H3N2	128
A/IA/02/18 MD1 10^4	H3N2	32
A/IA/02/18 MD1 10^5	H3N2	16
A/IA/02/18 MD1 10^1	Sf9 C.O. H3N2	<2
A/IA/25/17 MD1 10^2	Sf9 C.O. H3N2	<2
A/IA/25/17 MD1 10^3	Sf9 C.O. H3N2	<2
A/IA/25/17 MD1 10^4	Sf9 C.O. H3N2	<2
A/IA/25/17 MD1 10^5	Sf9 C.O. H3N2	<2

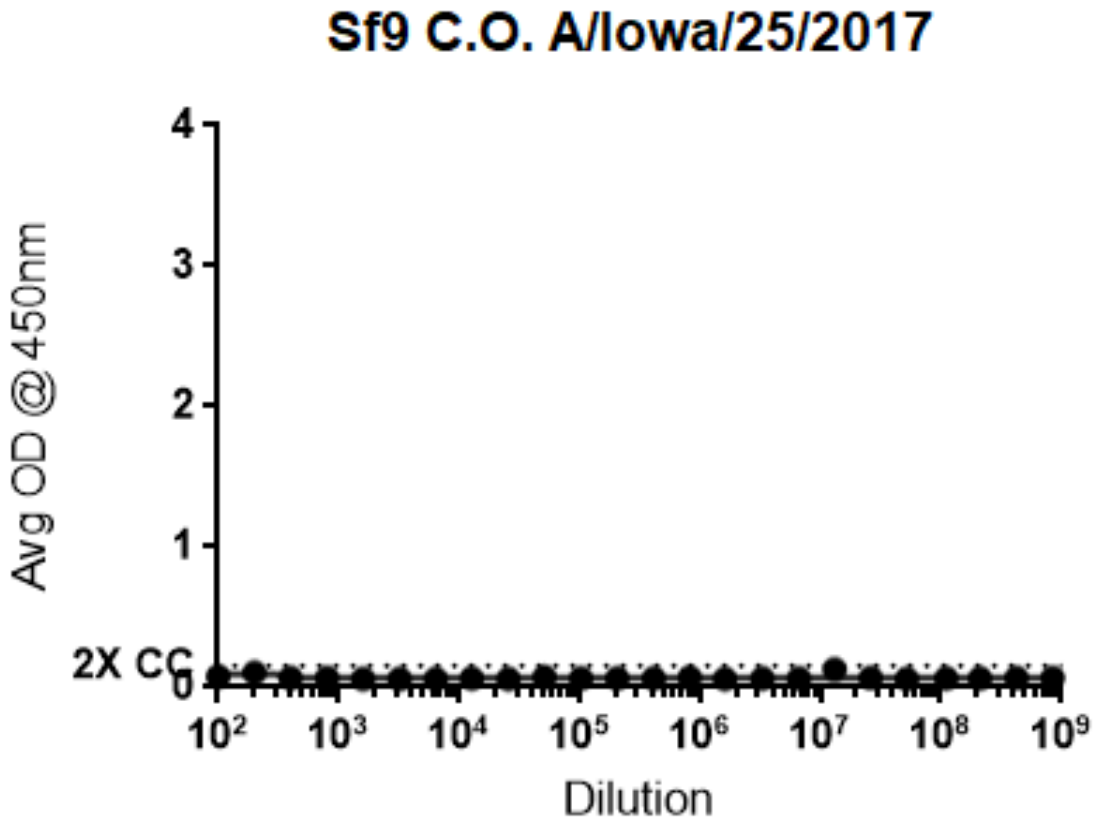

Figure 10. TCID titer for Sf9 C.O. HA and NA recombinant virus. The graph shows that the average OD at 450nm for each dilution of Sf9 C.O. A/IA/25/17 was at or below the average OD at 450nm for the cell only control wells. This indicates that no virus replication occurred and functional Sf9 C.O. A/IA/25/17 influenza virus is not being generated.

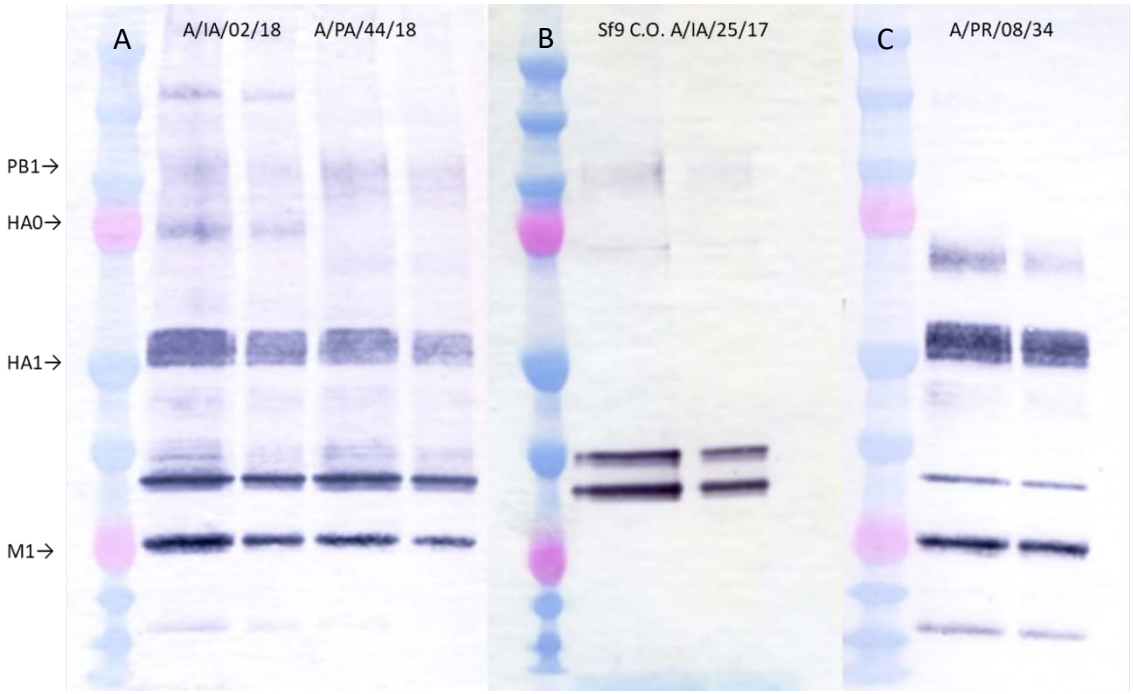

Figure 11. Western Blot Analysis of Recombinant Influenza Virus. Virus samples were analyzed by SDS-PAGE and viral protein detected by western blot using either rabbit anti-A/Fujan (H3N3) for (A and B) or rabbit anti-A/New Caledonia (H1N1) for (C). Arrows on the left identify influenza viral proteins detected.

Table 6. HAg and TCID titer for 2nd passage of H3N2 drifted strains. HAg and TCID titration was performed on second passage of influenza A/PA/44/18 and A/IA/02/18 virus samples. Results indicated below.

Virus Strain	Subtype	HAg	TCID
A/PA/44/18 MD2 10^4	H3N2	512	-
A/PA/44/18 MD2 10^5	H3N2	512	3,743,885
A/PA/44/18 MD2 10^6	H3N2	512	1,630,091
A/PA/44/18 MD2 10^7	H3N2	512	3,833,954
A/IA/02/18 MD2 10^4	H3N2	256	63,619
A/IA/02/18 MD2 10^5	H3N2	256	144,771
A/IA/02/18 MD2 10^6	H3N2	16	89,602

Sequencing

Analysis of the HA sequence was needed to determine that the recombinant H3N2 drifted strain's identity was consistent throughout the implementation of the reverse genetics system. All downstream applications test either the neutralization of HA genes or their ability to agglutinate RBCs. HA is also notorious for mutating in cell culture, because of this, the sequence of the HA gene was confirmed in the reasserted virus made by reverse genetics. Sanger sequencing confirmation of A/Iowa/02/2018 HA showed that the HA sequence remained intact with the exception of a T176K mutation within the antigenic site B (Fig. 12). This is a common mutation found in influenza when propagating in cell culture and the effects this mutation may cause on downstream clinical testing is currently unknown. The red boxes on the sequence alignment outlines the receptor binding domains antigenic sites in HA. Secondary peaks are defined as the fraction of max peak height for calling of 0.2 or larger and deemed ambiguous for the nucleotide where such secondary peaks occur. Once the nucleotide sequence is translated to amino acid, sites where secondary peaks occur are labeled with an X. Within the sequence alignment dots (.) represent amino acids that align while dashes (-) represent amino acids where no sequence coverage was found. The sequence alignment for A/Iowa/02/2018 shows full sequence coverage with at least secondary coverage where secondary peaks were called which validates the sequencing results and allows confidence that the virus population is homogenous. Secondary coverage can be defined as having another sequence that covers the amino acid residue in question, for example, where there is a secondary peak found, having another sequence that does not contain this secondary peak can be used for

secondary coverage. Unfortunately, sequencing of A/Pennsylvania/44/2018 HA has proved troublesome. Several sets of sequencing primers specific for A/Pennsylvania/44/2018 HA were used as well as the H3N2 universal primer which is designed to the influenza specific universal NCR (5’ AGCGAAAGCAGG 3’) as well as the HA specific NCR for the H3N2 subtype (5’GGATAATTCTATTAACC 3’). Both A/Iowa/02/2018 HA and A/Pennsylvania/44/2018 share this region, however, only A/Iowa/02/2018 HA was able to be sequenced.

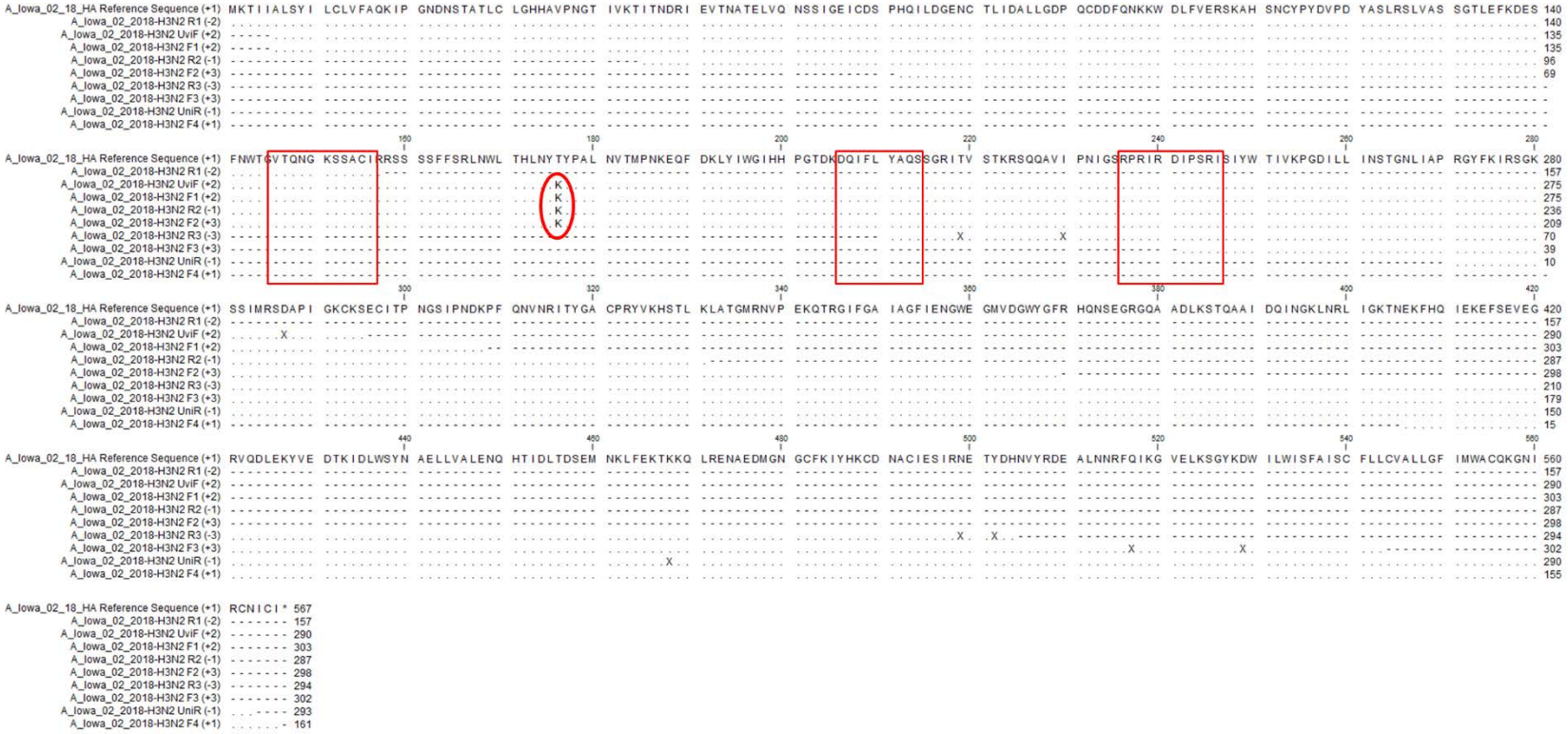

Figure 12. Sequence alignment of A/Iowa/02/2018. The sequence alignment of A/IA/02/18 recombinant virus shows the generation of virus with intact HA. The red boxes show the receptor binding domains within the HA. The T176K mutation (circled) shown here is a common cell-based mutation that frequently occurs in influenza HA.

Hemagglutination Inhibition

The hemagglutination inhibition assay is an extremely reliable test for assessing antibodies in serum's ability to bind to the RBS on the HA protein and block its binding capacity to sialic acid receptor on the surface of RBCs causing agglutination (WHO, 2011). Recombinant influenza A/Pennsylvania/44/2018 MD2 10^7 and A/Iowa/02/2018 MD2 10^5 were used to assess their effectiveness in HAI. Virus samples were diluted to 4 HAU/25μL and incubated with either anti-influenza serum or an anti-influenza (rA2.91.3) MAb. The ferret negative control serum plates showed agglutination inhibition in both the A/Pennsylvania/44/2018 and the A/Puerto Rico/08/1934 virus samples, however, no inhibition of agglutination was seen for A/Iowa/02/2018 (Fig. 13). This would suggest the presence of nonspecific agglutinins in the ferret sera. The presence of alpha, beta, and gamma inhibitors have been found in sera that present varying degrees of HA activity from different influenza strains. These non-antibody particles have the ability to bind to HA causing inhibition and potential false positives in the resultant data (WHO, 2011). Due to the non-antibody induced inhibitory effect seen with the ferret negative control serum and the A/Puerto Rico/08/1934 and A/Pennsylvania/44/2018 samples, it would be impossible to confirm the anti-H3N2 and anti-H1N1 serums ability to bind these HAs and inhibit agglutination to RBCs (Fig. 14, 15).

In the case of A/Iowa/02/2018 however (Fig. 16), there was no non-specificity seen between the ferret negative control sera and virus. Thus, the results show that the ferret anti-(H3N2) sera used inhibited HA's ability to agglutinate RBCs with an HAI titer of

1280. When analyzing the results of the Novavax rA2.91.3 Mab's ability to inhibit agglutination, data showed (Fig. 17) that rA2.91.3 MAb can inhibit agglutination as seen with the ferret and sheep reference sera. Again, agglutination was not inhibited with A/Puerto Rico/08/1934 and A/Pennsylvania/44/2018 but was inhibited down to 0.047μg/mL of rA2.91.3 MAb with A/Iowa/02/2018. In this regard, rA2.91.3 MAb shows that the RBS defines the receptor binding site on HA from A/Iowa/02/2018 and how it binds sialic acid receptors on cells (Portnoff et al, 2020).

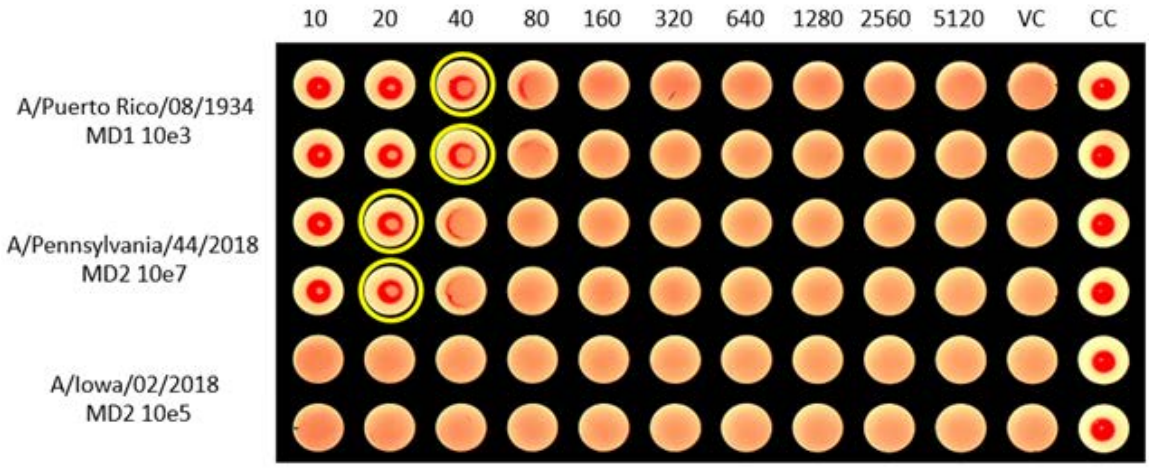

Figure 13. HAI titers of Ferret negative control serum against A/PR/08/34, A/PA/44/18, and A/IA/02/18 virus. The negative control serum showed inhibition of agglutination to both A/PR/08/34 and A/PA/44/18 but no inhibition with A/IA/02/18. This data suggests that the ferret negative control serum contains nonspecific inhibitors that prevent hemagglutination of A/PR/08/34 and A/PA/44/18 inhibiting their ability to agglutinate Guinea Pig RBCs.

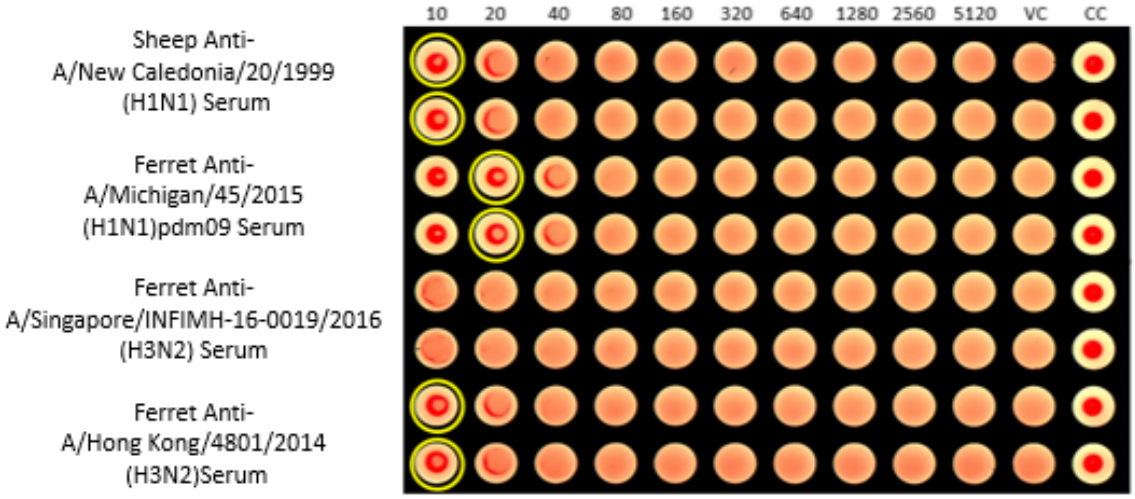

Figure 14. HAI titers of H1N1 and H3N2 reference serum against A/Puerto Rico/08/1934. H1N1 and H3N2 reference serum samples were used to test their ability to inhibit virus agglutination of Guinea Pig RBCs. Since the ferret negative control serum contains nonspecific inhibitors that prevent HAg of A/PR/08/34, confirmation of HAI cannot be concluded.

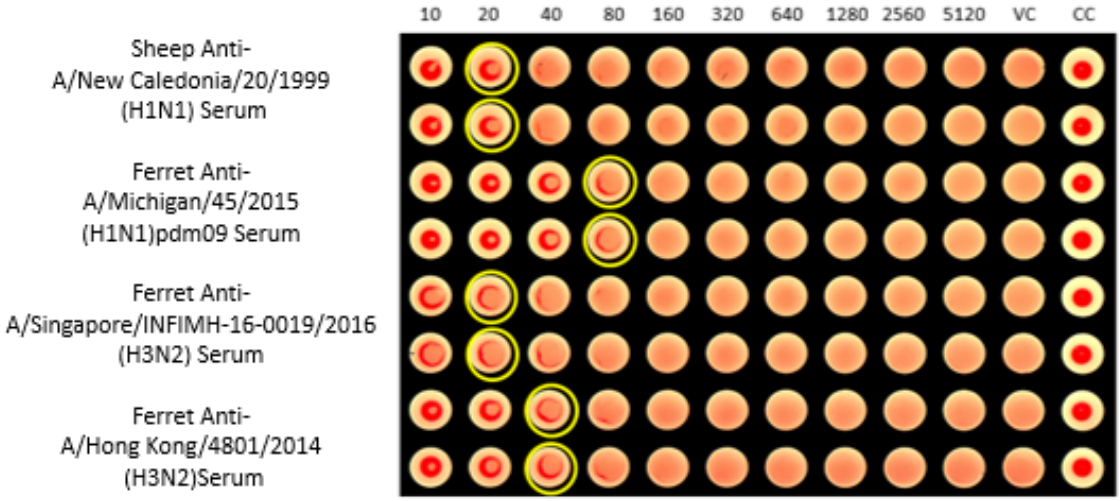

Figure 15. HAI titers of H1N1 and H3N2 reference serum against A/Pennsylvania/44/2018. H1N1 and H3N2 reference serum samples were used to test their ability to inhibit virus agglutination of Guinea Pig RBCs. Since the ferret negative control serum contains nonspecific inhibitors that prevent HAg of A/PA/44/17, confirmation of HAI cannot be concluded.

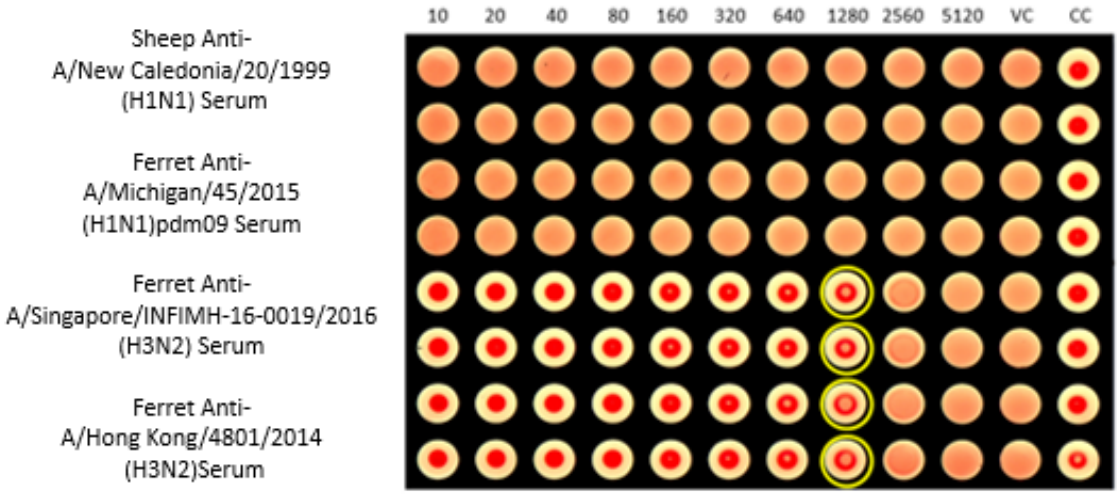

Figure 16. HAI titers of H1N1 and H3N2 reference serum against A/Iowa/02/2018. H1N1 and H3N2 reference serum samples were used to test their ability to inhibit virus agglutination of Guinea Pig RBCs. H3N2 reference serum samples showed inhibition of virus agglutination as indicated in the results.

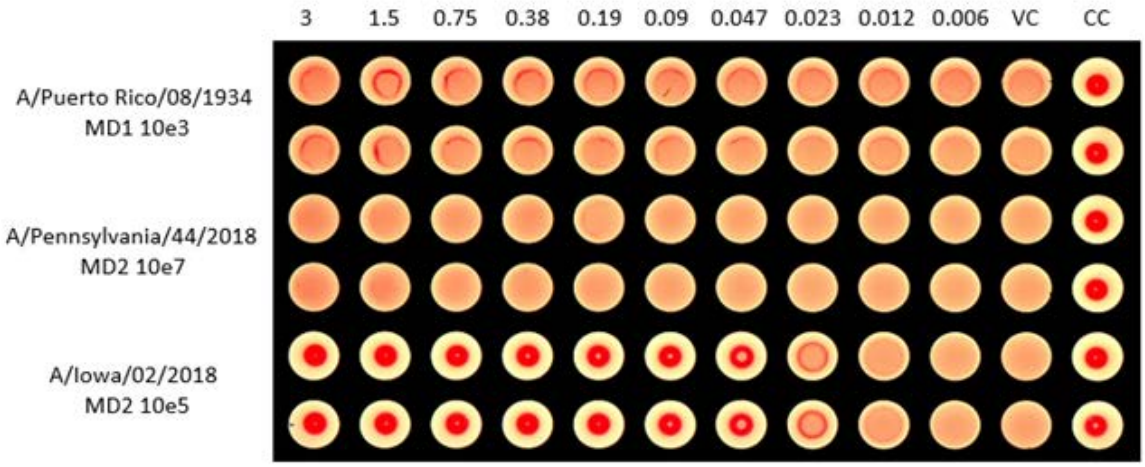

Figure 17. rA2.91.3 anti-H3N2 Murine Monoclonal Antibody HAI Results. Anti-H3N2 rA2.91.3 monoclonal antibody was used to test its ability to inhibit virus agglutination of Guinea Pig RBCs. The rA2.91.3 H3N2 MAb showed inhibition of A/Iowa/02/2018 virus agglutination as indicated in the results.

Microneutralization

The microneutralization assay is a sensitive and specific assay used to measure the relative amount of neutralizing antibodies present in a serum sample pre- and post-vaccination as well as a way to identify influenza virus strain specific neutralizing antibodies. Microneutralization assay is a very common test in the clinical setting as it can easily detect the presence of functional antibodies specific for HA present within sera, and is a relatively quick assay to run requiring no more than 2 days (WHO, 2011). Recombinant influenza virus was diluted to 100 TCID in Assay Diluent and incubated with either sheep or ferret reference serum to allow the antibodies present in the sera to bind HA and block the virus from infection of MDCK cells thereby neutralizing the influenza virus. Since this assay does not include RBCs, there is no need to treat the sera with RDE beforehand. Most results remained consistent with those found in the hemagglutination inhibition assay. With A/Pennsylvania/44/2018 no neutralization activity was observed with the anti-H1N1 nor the anti-H3N2 reference sera. Sera used with A/Puerto Rico/08/1934 on the other hand had neutralization activity against Anti-A/New Caledonia/20/1999 (H1N1) HA serum where its microneutralization titer was 40 and showed mild neutralization activity against the H1N1pdm09 sera (10). As expected, H3N2 sera showed no neutralization activity when tested with A/Puerto Rico/08/1934. The H3N2 strain A/Iowa/02/2018 was neutralized by both anti-H3N2 reference sera (160) as well as some neutralization activity with the H1N1pdm09 reference serum (10) (Table. 7). This suggests that the antibodies in the ferret anti-A/Michigan/45/2015 H1N1pdm serum cross-reacts to both H1N1 and H3N2 influenza subtypes.

Table 7. Evaluation of the serum's neutralization activity with viruses. Microneutralization assay was used to determine the 50% neutralization titer of H1N1 and H3N2 reference serum against generated influenza virus as shown in the table.

Virus Strain	Virus Subtype	Serum Sample	50% Neutralization Titer
A/IA/02/18 MD2 10^5	H3N2	Anti-A/New Caledonia/20/99 (H1N1)	<10
A/IA/02/18 MD2 10^5	H3N2	Anti-A/Michigan/45/2015 (H1N1)pdm09	20
A/IA/02/18 MD2 10^5	H3N2	Anti-A/Singapore/INFIMH-16-0019/2016 (H3N2)	160
A/IA/02/18 MD2 10^5	H3N2	Anti-A/Hong Kong/4801/2014 (H3N2	160
A/PR/08/34 MD1 10^3	H1N1	Anti-A/New Caledonia/20/99 (H1N1)	40
A/PR/08/34 MD1 10^3	H1N1	Anti-A/Michigan/45/2015 (H1N1)pdm09	10
A/PR/08/34 MD1 10^3	H1N1	Anti-A/Singapore/INFIMH-16-0019/2016 (H3N2)	<10
A/PR/08/34 MD1 10^3	H1N1	Anti-A/Hong Kong/4801/2014 (H3N2	<10
A/PA/44/18 MD2 10^7	H3N2	Anti-A/New Caledonia/20/99 (H1N1)	<10
A/PA/44/18 MD2 10^7	H3N2	Anti-A/Michigan/45/2015 (H1N1)pdm09	<10
A/PA/44/18 MD2 10^7	H3N2	Anti-A/Singapore/INFIMH-16-0019/2016 (H3N2)	<10
A/PA/44/18 MD2 10^7	H3N2	Anti-A/Hong Kong/4801/2014 (H3N2	<10

DISCUSSION

This thesis outlines the implementation of a reverse genetics system to generate 6 + 2 reassortant H3N2 and H1N1 recombinant viruses with the potential for use in downstream clinical sample testing such as hemagglutination inhibition and microneutralization assays. The reassortant H3N2 recombinant viruses were generated through transfection of a co-culture consisting of HEK293T and MDCK cells. Plasmid DNA for the H3N2 HA and NA genes from strains of interest were used with the remaining PB2, PB1, PA, NS, NP and M genes from an A/Puerto Rico/08/1934 H1N1 backbone using a pHW2000 vector system. This can be valuable in testing the ability of antibodies induced by seasonal influenza vaccination to neutralize WHO and CDC recommended influenza strains as well as drifted strains where the virus is not available.

It has been demonstrated that both A/Puerto Rico/08/1934 (H1N1), A/Pennsylvania/44/2018 (H3N2) and A/Iowa/02/2018 (H3N2) reassortants can be generated through this reverse genetics system and used to propagate infectious influenza virus in cell culture to moderately high HAg and TCID titers in-line with the expected levels seen when propagating wild-type influenza virus. Reassortant H3N2 recombinant virus showed functional activity in both HAI and microneutralization assays when tested with reference serum. The monoclonal antibody rA2.91.3 showed HAI activity to influenza A/Iowa/02/2018 and neutralizing responses against both influenza A/Puerto Rico/08/1934 and A/Iowa/02/2018. However, it should be noted that generating reassortant virus with high HAg and TCID titers does not validate them for use

in clinical sample testing such as HAI and microneutralization assays as seen from the A/Pennsylvania/44/2018 (H3N2) data. Influenza A/Pennsylvania/44/2018 showed the highest HAg and TCID titers among the reassortant viruses generated including A/Iowa/02/2018 which was from the same 3c.2a1 H3N2 clade and shares more than 98% sequence homology with only 11 differences seen in the amino acid sequence (Fig.18). However, when assaying with antibodies against H3N2 only antibodies subjected to A/Iowa/02/2018 HA showed any functional response. More interestingly, when the RBS MAb r2.91.3 which binds to the antigenic site B was used to test HAI response, it only showed any inhibition of HA activity with A/Iowa/02/2018 H3N2. The sequence starting at amino acid 234 (-DQIFLYAQS-) of the antigenic site B is exactly the same for A/Iowa/02/2018 as it is for A/Pennsylvania/44/2018. Since this is an antibody and not serum, there are no nonspecific molecules that are known to inhibit agglutination present which would suggest that something else is going on with A/Pennsylvania/44/2018 HA. The rA2.91.3 MAb was also previously shown to bind to A/Pennsylvania/44/2018 HA nanoparticle produced in Sf9 expression system through fluorescence-activated cell sorting (FACS) analysis as shown in Portnoff et al, 2020. Furthermore, the fact that A/Iowa/02/2018 HA contains a T176K mutation within the antigenic site B could also be a potential reason why rA2.91.3 MAb was able to bind to A/Iowa/02/2018 HA and not A/Pennsylvania/44/2018. The T176K mutation removes the N-linked glycosylation motif (Asn-X-Ser/Thr), and therefore removes possible blocking of the RBS by N-linked

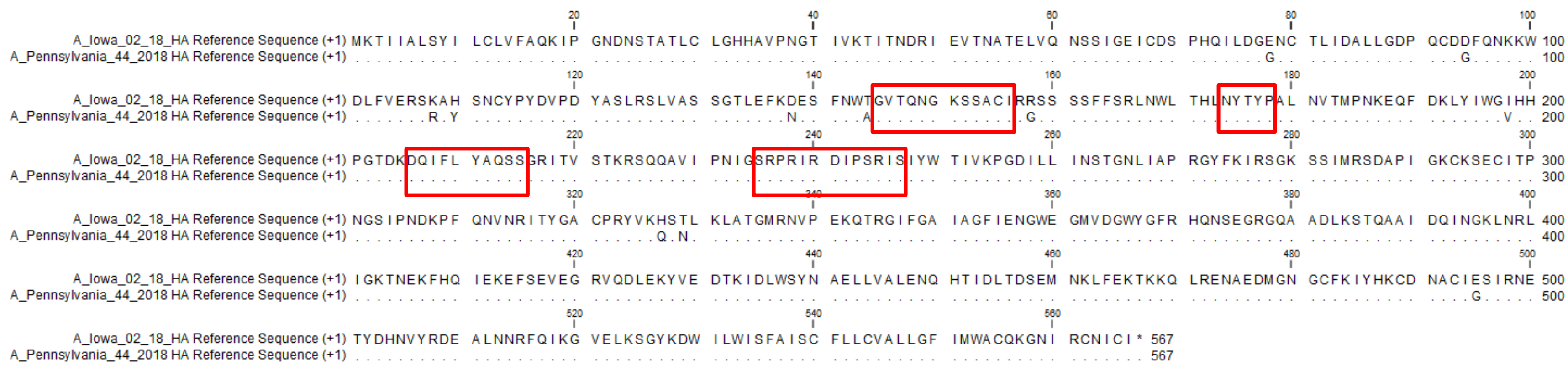

Figure 18. Sequence alignment of A/Iowa/02/2018 to A/Pennsylvania/44/2018. The sequence alignment of A/IA/02/18 to A/PA/44/18 shows that the 2 sequences are more than 98% homologous with only 11 amino acid differences between them. The red boxes point out the antigenic regions within HA. The sequence alignment shows that no major differences are observed between these two H3N2 strains.

glycosylation. Insect cells glycosylate proteins similar to mammalian cells except the resultant glycans are generally shorter and contain less complex carbohydrates (Shi and Jarvis, 2007). If A/Pennsylvania/44/2018 were to contain the intact N-linked glycosylation motif, this could be a reason for why the rA2.91.3 MAb was unable to bind the HA and inhibit agglutination. However, without successful sequencing of the A/Pennsylvania/44/2018, it is impossible to delineate any real cause for the aforementioned results.

The implementation of this reverse genetics system has been proven successful. The generation of 6 + 2 reassortants has progressed in the virology laboratory at Novavax with five other reassortants having been generated and two of them, A/Cardiff/0508/2019 and A/California/94/2019 being used for qualification for phase III clinical trial testing. The implementation of this system has also generated G4 swine influenza virus reassortants and currently the design of four H3N2 drifted strains for 2020 HA and NA plasmids are being conducted. The selection for H1N1 strains, B/Victoria-like and B/Yamagata-like lineages are being analyzed from Nextstrain for potential use currently. The insight gained from this thesis project has shed light on the requirements needed to bring viable reassortants from design to application for use in a clinical setting.

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