

**INFLUENZA POLYMERASE COMPLEX:  
Novel Antiviral Drugs and Polymerase Fidelity**

A Major Qualifying Project Report

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## **ABSTRACT**

The Influenza-A virus is a highly pathogenic virus with the potential to cause major pandemics and annual outbreaks. The goal of this project was to investigate the highly conserved trimeric polymerase complex of the virus, which is needed for viral replication and transcription. We developed the reagents for a fluorescence polarization assay to help identify small molecules that disrupt the interactions within the complex, and have the potential to be a new anti-viral drug. This project also developed a transcription assay system to test the polymerase fidelity.

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# BACKGROUND

## Influenza

Influenza, also known as the flu or la grippe, is a serious respiratory illness which is caused by infection with influenza virus. The disease is very contagious, can cause symptoms ranging from mild to very severe, and can even cause death, especially in young children, and the elderly.

There have been several devastating influenza outbreaks throughout human history. One of the worst was the Spanish flu outbreak of 1918. At this time, Type A H1N1 influenza infected almost one third of the total world population, killing about 50 million people. This Spanish flu was assumed to be the ancestor to all other Type A infections since; however we have never seen as extreme of a pathogenicity since (Taubenberger and Morens, 2006). Since 1918, there have been a number of other serious outbreaks of the flu, including Russian flu in the late 1800s, Asian flu in the 1950s, and Hong Kong flu in the 1960s. In the more recent past, there have been outbreaks of Avian flu and the Swine flu in 2009-2010, which has killed over 18,000 people worldwide (H1N1, 2010).

In addition to these serious pandemics, influenza is also an annual seasonal disease, occurring during autumn and winter in temperate regions. Worldwide, influenza results in three to five million cases of severe illness a year. The number of deaths from annual influenza varies, but the Center for Disease Control and Prevention estimates between 3,000 and 49,000 people worldwide will die from flu-related causes each year. While anyone can be infected with influenza, the most susceptible to infection are the very young, the elderly, and people with other medical conditions. These groups are also

the most likely to die from influenza – almost 90% of flu deaths occur in these populations (Influenza [Seasonal], 2009).

In humans, influenza infection targets the respiratory tract. The flu can cause many different symptoms, such as fever, chills, cough, sore throat, head and body aches, runny or stuffy nose, fatigue, and vomiting/diarrhea. Affected persons may experience some or all of these symptoms. A typical flu infection lasts anywhere from two days to two weeks, but some people may develop further complications such as pneumonia or bronchitis, especially those who are already at risk (Influenza [Seasonal], 2009).

## **Influenza Virus**

### *Nomenclature*

The influenza virus is divided into three types – A, B and C – based upon the type of nucleocapsid found within the virus. Type A is responsible for the majority of annual flu outbreaks and pandemics. Type B causes sporadic outbreaks, while Type C typically only causes mild illness. Only Type A is then divided into further subtypes based on the different combinations of viral surface proteins that it contains. Types A and B can also be further classified into strains, or grouped by low/high pathogenicity (“Influenza Viruses”, 2005).

There are 16 hemagglutinin (HA) subtypes, and only three are found in humans. Additionally, there are 9 neuraminidase (NA) subtypes, two of which are found in humans (Kimball, 2010). The combination of the two HA and NA proteins is where the influenza strains such as H1N1 or H3N2 get their names from. The different combinations of these glycoproteins allow for infection in specific species, such as

humans or avian species. This species specificity occurs because of the structures of the HA and NA proteins. These proteins bind to species-specific proteins on the host cell surface (Squires and Scheuermann, 2006).

### *Structure*

Influenza A viruses are lipid-enveloped viruses which come in different shapes and sizes – from spherical virions about 100nm in diameter to long filamentous particles. The influenza virus contains a genome of eight strands of negative-sense RNA. This genome, made up of segments ranging from 890 to 2341 nucleotides, encodes ten viral proteins which make up the influenza A virion. Due to this segmented genome, reassortment, recombination of the virus genes, is common. In mixed infections with more than one strain of influenza, combinations of gene segments can be exchanged (Steinhauer and Skehel, 2002). This can lead to antigenic shift. Antigenic shift is very dangerous because if it leads to new combinations of surface proteins, then the immune system or vaccines will not target the virus (Staley et al., 2007).

Each of the eight gene segments encodes one or more viral proteins. The three largest segments encode the polymerase proteins, PA, PB1 and PB2. The polymerase proteins are named for their acidic or basic properties, respectively. The polymerase proteins have a number of functions, including transcribing mRNAs, synthesizing cRNAs, and transcribing cRNAs into viral gene segments for progeny. The fourth gene segment encodes hemagglutinin, or HA, which is involved in membrane fusion with host cells and neutralizing host antibodies. The fifth segment encodes the nucleoprotein, or NP. This protein encapsulates the genetic material of the virus (cRNAs and vRNAs), and

is associated with the three polymerase subunits. Gene segment six encodes neuraminidase, or NA, which is involved with cleavage at the end of the viral life cycle to allow the virions to be released. The seventh segment encodes two different proteins due to RNA splicing. The first is matrix protein M1, which is involved with virus assembly and budding. This is the most abundant protein. The second is transmembrane protein M2, which is involved with virus disassembly. Finally, the eighth gene segment encodes NS1 and NS2. NS1 is a regulator of splicing and translation, while NS2 mediates transport in and out of the nucleus. These two proteins were originally thought to be non-structural proteins (hence the name), but there has since been evidence proving otherwise (Steinhauer and Skehel, 2002).

### *Infection and Replication*

In an influenza infection, the virus first invades cells of respiratory passage. The surface HA molecules bind to sialic acid residues on proteins of the epithelial cells of the respiratory system, and the virus is then engulfed by the host cell. While in the endocytic vessel, there is a drop in pH which causes a change to the HA protein. This change enables the fusion of the viral membrane with the vesicle membrane, allowing the viral contents to be exposed to the cell. The viral RNA is then replicated by the viral polymerase protein (made up of PA, PB1, and PB2, in conjunction with NP). The host cell's machinery is then used for translation to create new viral proteins (Kimball, 2010). The virus particle formation occurs at the cell membrane. Budding occurs from the sections of the membrane where viral glycoproteins hemagglutinin and neuraminidase



have accumulated (Steinhauer and Skehel, 2002). The infection then continues to new cells.

### *Drugs and Vaccines*

Antibodies produced by humans against virus activity are directed against the viral surface proteins. However, the influenza viruses are continually evolving to evade existing human immunity. This allows infection to continue happening. To combat this, medical professionals have developed a number of antiviral drugs (to be taken once infection has already occurred), as well as vaccines.

There are two methods of vaccination against influenza: a trivalent, inactivated vaccine (the “flu shot”), and a live vaccine which comes in the form of a nasal spray (FluMist). The major difference in the vaccinations is the method of delivery. The nasal spray is made of very low titer live virus, which has a non-existent virulence. Both vaccines are made of three current viruses – two A strains and a B strain. The two types of vaccine provide different immune responses. The flu shot elicits good serum antibody response, but poor induction of IgA antibodies and cell mediated immunity. The live nasal spray, on the other hand, produces a more long lasting immune response similar to natural immunity (Cox et al., 2004). However, the flu shot seems to be more effective despite the nasal spray having a better immune response (Monto et al., 2009).

For those who did not get the vaccine, there are also two main types of antiviral drugs. The first antiviral drug was amantadine. Amantadine, along with the related compound rimantadine, was first discovered to have anti-influenza properties in the 1960s. This drug works by two mechanisms. The first mechanism, which takes place at

concentrations of 100 $\mu$ M or greater, is a broad antiviral activity. This occurs due to the compound's weak basic properties. The second, at concentrations of 5 $\mu$ M or less, is a specific activity against several strains of influenza A (no effect on influenza B or C). At this concentration, the compound affects the gene segment responsible for M1 and M2, and causes a change in the pH composition in the cells (Steinhauer and Skehel, 2002). Virtually all strains of influenza A have developed resistance to this drug (Kimball, 2010).

The second main set of antiviral drugs are oseltamivir and zanamivir. These drugs act against the NA protein, preventing it from binding to sialic acid (Steinhauer and Skehel, 2002). This prevents the release and spread of fresh virions. All H1N1 strains circulating in the US by the 2008-2009 flu season were resistant to Tamiflu (brand name oseltamivir) (Kimball, 2010). There has been widespread resistance to these drugs as early as 2007 and it has only gotten worse since then (McKimm-Breschkin, et al., 2007).

Antiviral drugs are difficult to find due to the mechanisms of viruses. Viruses rely heavily on the host cell they are in to supplement its life cycle. There are many agents which could kill off the virus, but they would also kill part of or the entire host cell, thus detrimental to the human (Kimball, 2010). Additionally, it is easiest to make drugs targeting the surface proteins of the virus – but these are the first to be changed and adapt, thus rendering the drugs useless. The goal for new antiviral drugs is to target molecular machinery specific to the virus (Liu, et al., 2007) – something we aim to do with this project.

## **Influenza Polymerase**

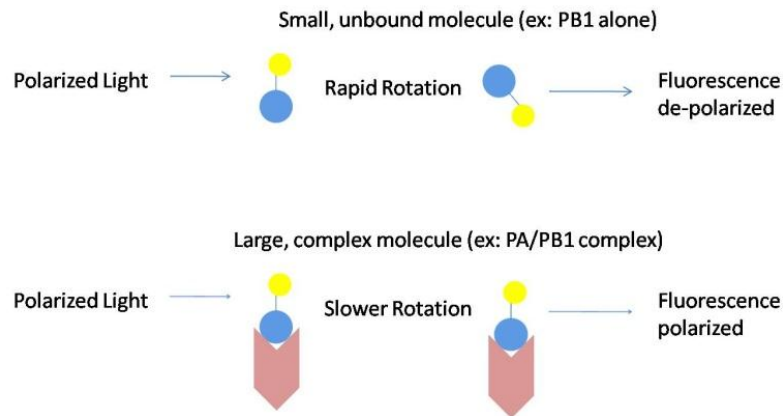
The polymerase complex is comprised of three proteins (PA, PB1 and PB2). All three proteins are required for both viral replication and transcription. PB1 is located at the core of the complex, and the complex is formed by separate interactions between PB1 and PA, and PB1 and PB2. Several of the key protein-protein interactions involved in the formation of the 3P complex have been previously described. They include the binding of the C-terminus of PA to the N-terminal domain of PB1, the binding of the C-terminus of PB1 to the N-terminal domain in PA, and the binding of the C terminus of PB2 to a poorly defined structure of PB1 (Bradel-Tretheway, et al., 2008). These interactions are critical to the formation of the polymerase complex, and thus the viability of the virus as a whole.

## **Polymerase Interaction Targeting**

There is a need for new antiviral drugs that have a low potential of being susceptible to viral resistance. Targeting the interactions within the polymerase complex for drug design is appealing, because the proteins contain highly conserved peptide sequences which are found among most influenza A viruses, including highly pathogenic strains with pandemic potential (Heiny et al., 2007). Among the binding region of PA (composed of 406 residues), there are only 13 which are not conserved between different H1N1 and avian strains. Similarly, the region of PB1 which binds to PA (70 residues), there are only 2 which are not conserved (Obayashi et al., 2008).

To target the interaction between the PA and PB1 proteins, we will be using a Fluorescence Polarization Assay. The theory of the fluorescence polarization assay is that

larger molecules rotate slower than smaller ones, and thus emit more light in a more concentrated direction (more polarized) when polarized light is passed through them (**Figure-1**). Smaller molecules rotate faster, and therefore emit light in different directions (depolarized) when shot with polarized light. In this system, the PB1 peptide alone would rotate more quickly, whereas once it is bound to PA it would rotate more slowly. To perform the assay, PB1 samples are added to a 384-well plate and mixed with PA. The change in fluorescence polarization of the labeled peptide is measured. The chemical library can then be screened for chemical compounds that inhibit the PA-dependent fluorescence polarization. If PA/PB1 binding is interrupted the fluorescence will be depolarized after the chemical compound is added.



**Figure 1: Illustration of the Fluorescence Polarization Assay.** This figure shows the concept of the FP assay. A small molecule (PB1 with a chromophore attached) would rotate rapidly, leading to depolarized light. A larger molecule (PB1 bound to PA) would rotate more slowly, giving polarized light.

## Polymerase Fidelity

The high genetic diversity of the influenza A virus, together with the previously discussed gene reassortment, allows the influenza virus to acquire its efficient host adaptability. One suggested mechanism for this efficient evolutionary process was a

highly error-prone replication process. Every genome copy packaged in a new virus has been synthesized by the influenza polymerase, and thus it has been assumed that the error-prone polymerase was responsible for generating mutations which allowed the virus to evolve. In a similar virus with high mutation rates, the HIV-1 DNA polymerase is well known as an error prone polymerase (Ji and Loeb, 1992). However, in a recent publication, the influenza polymerase was found to have a high fidelity, with a much lower rate of mis-incorporation than the HIV-1 DNA polymerase (Aggarwal et al., 2010). In this report, the PA, PB1 and PB2 genes from the H3N2 strain were cloned into a baculovirus expression vector, and transfected into Sf9 insect cells to produce the recombinant viral proteins. The three recombinant viruses were then used to coinfect Tni insect cells, and the lysates were purified using the TAP purification technique as described previously (Bradel-Tretheway et al., 2008; Puig et al., 2001). The purified polymerase complex was then used in an newly developed ApG initiated transcription assay (see Methods section). This study found that high influenza activity in this assay system. In further mis-incorporation studies, influenza polymerase was found to have a high fidelity, and it was concluded that this must be because the influenza polymerase does all the viral replication itself rather than using host mechanisms. Having a low fidelity (error prone) polymerase would cause catastrophic mutations and inhibit the virus from further infection. In contrast, HIV-1 RT only replicates the viral DNA once and then the host proteins take over. In comparison to past transcription assay systems, this study eliminated the biased NTP pool which was often seen before (Aggarwal et al., 2010).

## **PROJECT PURPOSE**

A need for new antiviral drugs has been demonstrated due to the high viral resistance to current drugs. This project will develop a protocol for preparing reagents (influenza polymerase constructs) to be used in an assay system which will screen libraries of compounds which disrupt the interaction between PA-GST fusion proteins and PB1 peptides. These compounds then can be further tested for their ability to directly inhibit the influenza A virus.

Additionally, the influenza polymerase has always been assumed be a low fidelity enzyme, until recently. The influenza polymerase (strain H3N2) was found to have high fidelity when the proteins are grown in insect cells. The project will also develop new assay conditions to attempt to further characterize the polymerase's fidelity with mammalian host proteins, as well as with different strains of influenza.

## METHODS

### Cloning and Expression of PA-H5N1 protein constructs

Two separate constructs were created: H5N1-PA<sub>239-716</sub> and H5N1-PA<sub>657-716</sub>. The larger protein has been recognized as the main binding segment of PA to PB1, while the smaller one includes the sites required for binding (Obayashi et al., 2008). DNA fragments encoding portions of the Influenza A PA protein were amplified by polymerase chain reaction (PCR) using full length PA as a template and a set of primers: (5'-CCATTCCATGGGAATGTGCAAAACCCCGAG-3' and 5'-CATGAGAATTCTCAAGGGCAGGGTTTGTA-3' (PA<sub>239-716</sub>) or 5'-TACAGATAAGGATTCCCGACAGTTGTAA-3' (PA<sub>657-716</sub>)). The PCR conditions were 94 °C for 3 min followed by 30 cycles (94 °C for 1 min, 60°C for 1 min and 72°C for 1 min) and finally 72 °C for a 5-min extension. Primers were synthesized through the University of Rochester Functional Genomics center. The DNA was amplified using a routine Deep Vent PCR Reaction (New England Biolabs), using Deep Vent DNA polymerase. The PCR products were then gel purified and the amplified fragments were then digested with BamHI and XhoI restriction enzymes and ligated into pGEX-5x-1 vector (Novagen) in frame with the N-terminally located glutathione S-transferase (GST). The plasmid was ligated using T4 DNA Ligase in a 6:1 insert:vector ratio for 2hr at room temperature. The sequence of the pGEX-PA plasmids were verified by DNA sequence analysis, and the ligation was verified through gel electrophoresis.

The plasmids were then transformed into *E. coli* BL21-Codon Plus competent cells using electrotransformation. 1µL DNA was added to 25µL competent cells. The electroporation apparatus was set to 1.8kV, 25µF, with a pulse of 200 ohms. The cells

were grown with 500mL of S.O.C. and the resulting suspension was placed in a floor shaker at 225rpm at 37°C for one hour to allow expression of the ampicillin gene. 20µL and 200µL samples were then spread on an LB+ampicillin plate overnight at 37°C.

The next day, colonies were picked and placed in 10 ml LB Broth and grown for 2 hours at 37°C in the floor shaker. After incubation, the suspension was used to inoculate 1L of LB broth, and grew for another 3 hours. The cultures were then induced overnight with 0.5mM IPTG at room temperature. The following day, the GST-purification of the proteins could be completed (see Results).

The PB1 binding region of the peptide (residues 1-25) was purchased already labeled with a Rhodamine chromophore (Invitrogen) for use in the Fluorescence Polarization Assay. Mutations to the PA construct were introduced with site-directed mutagenesis (Quick-Change mutagenesis kit, Stratagene).

## **Cell Culture**

Human lung endothelial cells (A549) were grown at 37°C and maintained in DMEM solution supplemented with 10% fetal bovine serum, 2mM glutamine, 50 IU penicillin ml<sup>-1</sup>, and 50µg streptomycin ml<sup>-1</sup>.

## **Adenovirus production**

Previously, recombinant adenoviruses expressing PA, PB1 and PB2 were created from pShCMV by homologous recombination into AdEasy and propagation in HEK293A cells (AdEasy system, Stratagene) (Luo, et al., 2007). Viral titres were determined by quantitative real-time PCR. Viruses were purified by CsCl density



gradient centrifugation as well as dialysis. PA was tagged with a tandem-affinity purification tag (TAP tag) (Bradel-Tretheway, et al., 2008).

### **Polymerase purification**

A549 cells (7-8 x 10<sup>6</sup> cells in a T225 flask) were transduced with each of the three adenoviruses (rAd5-PA-TAP, PB1, PB2) at an m.o.i. of 1000, except the H5N1 strain for which a m.o.i. of 100 was used. Recombinant adenoviruses from the following strains were used: H5N1 (VN1203), 1918, H1N1 (A/California/04/09), and WSN (A/WSN/33). Lysates were prepared 48 hours post infection and were purified using the TAP method as described previously (Bradel-Tretheway, et al., 2008; Puig et al., 2001 ).

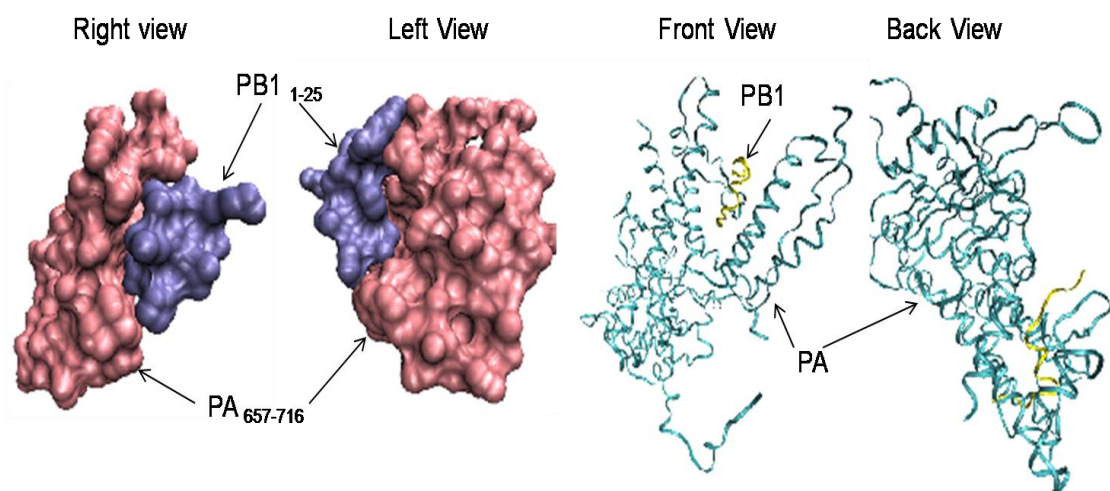
### **ApG-primed transcription assay**

The transcriptional activity of the viral polymerase can be tested using an ApG transcription assay where the 3' and 5' RNA and the dinucleotide primer ApG (Biosynthesis) are provided. ApG is extended using the 3' RNA template by the polymerase. This gives an end result of a RNA fragment. The 5' vRNA template (5'-AGUAGAAACAAGGCC-3') and the 14-nucleotide (nt) 3' vRNA template (5'-GGCCUGCUUUUGCU-3') from the PA gene of the influenza virus were purchased from Dharmacon. Other 3' templates used were 50-nt long and 137-nt long, and these encode the sequences from the 3' end of the Nanchang PA gene. The 50-nt template was purchased from Integrated DNA Technologies, while the 137-nt RNA template was prepared by *in vitro* transcription (Aggarwal, et al., 2010).

The transcription reactions were performed as elsewhere (Aggarwal, et al., 2010; Fodor, et al., 2002). The reactions were 10 $\mu$ L reaction mixtures at 37°C containing transcription buffer (25mM Tris-Cl, pH 7.5, 100mM KCl, 5mM Mg(OAc)<sub>2</sub>, 0.1mM EDTA, 2mM DTT, 0.25 $\mu$ L Nonidet P-40, 12.5% glycerol), IAV polymerase complex, 0.25 U/ $\mu$ l RNasin, 0.3mM ApG, 1.6 $\mu$ M 14-nt 3'vRNA, 1.6 $\mu$ M 5' vRNA, 0.08 $\mu$ M radioactive  $\alpha$ -<sup>32</sup>P-GTP /UTP/ATP (3000 Ci/mmole, Perkin Elmer), and 500mM non-radioactive ATP, UTP, GTP and CTP. The reaction was stopped after 1 hour with the addition of 5 $\mu$ l trichloroacetic acid (TCA) dye, and was then denatured for 3 min at 95°C. Transcription products were separated by 8M urea denaturing 18-20% polyacrylamide gel electrophoresis. The dried gels were analyzed with autoradiography using a PhosphorImager (Molecular Dynamics) and the intensity of the products was quantified using OptiQuant (Version 3.10, Packard Instrument).

## RESULTS

The first part of this project involved optimizing the purification process of the PA<sub>239-716</sub>-GST and PA<sub>657-716</sub>-GST constructs. This was necessary because when following the procedure as described by Amersham in the GST fusion kit, we got insoluble expression and low protein yields. First, we used molecular imaging software Visual Molecular Dynamics (VMD) to input the protein sequence and visualize the interactions between the PA constructs and PB1 binding regions (**Figure-2**). This software reads Protein Data Bank files and then displays the structure in a variety of ways. It allowed us to visualize the structure of the proteins and see how they fit together.

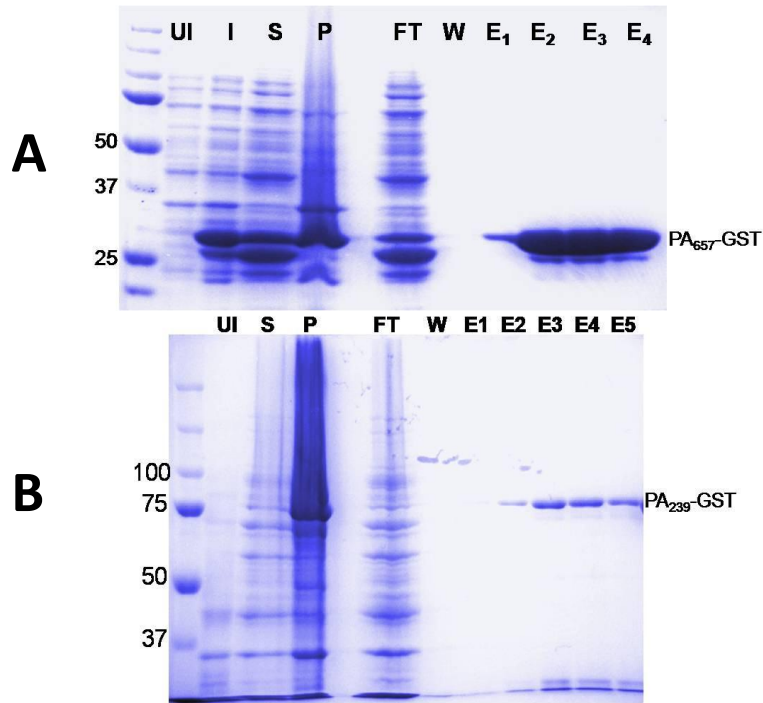


**Figure 2: Visual Representations of the PA/PB1 Interactions.** Shown here is a visualization of how the PA and PB1 molecules fit together using the VMD software. On the left is the sphere representation with the PA<sub>657-716</sub> peptide in red and the binding region of PB1(1-25) in blue. On the right is the ribbon and tube representation of the whole PA molecule (blue) and the PB1 peptide (yellow).

Using this software, we were able to determine an ideal location for a mutation which would prevent binding between the PA and PB1 peptides, and could serve as our negative control for the Fluorescence Polarization Assay. For the PA<sub>239-716</sub>, the mutation

was L640D. For the PA<sub>657-716</sub> protein, the mutation was L666D. The addition of the aspartic acid in exchange for the leucine changed the structure of the protein just enough to prevent binding to the PB1 peptide.

With the constructs and mutants created, we were able to begin purifying the proteins needed for our assay system. Once the proteins were induced overnight, the cells were lysed open with lysis buffer [20mM Tris-Cl pH 8, 500mM NaCl, 500mM urea]. The lysis buffer was refined for optimal lysing of the cells. 1% Triton X, a detergent, and 1x Protease Inhibitor (Roche) were then added. Finally, rLysozyme (Novagen) was added. We used 150µL for the larger protein (PA<sub>239-716</sub>-GST), and only 2µL for the smaller protein (PA<sub>657-716</sub>-GST). The larger PA<sub>239-716</sub>-GST cell suspension was then sonicated 4 times for 30 seconds each time. For the smaller protein, the sonication disrupted the proteins, so a 3 freeze-thaw cycles were performed in an ethanol-dry ice bath for that suspension. The suspension was then spun down, and applied to a column with Glutathione Sepharose beads (which bind to the GST protein) and incubated 2 hours at 4°C. The incubation time was optimized to allow for enough binding time for the GST, but to prevent non-specific binding. Finally, the column was washed with 1x GST wash/bind buffer (Amersham), and eluted 5x with 1x Reduced Glutathione (Amersham). The wash and elution steps were adjusted for purity of the final product. The resulting proteins, as well as samples taken at each step, were run on a gel to observe the purification (**Figure 3**).

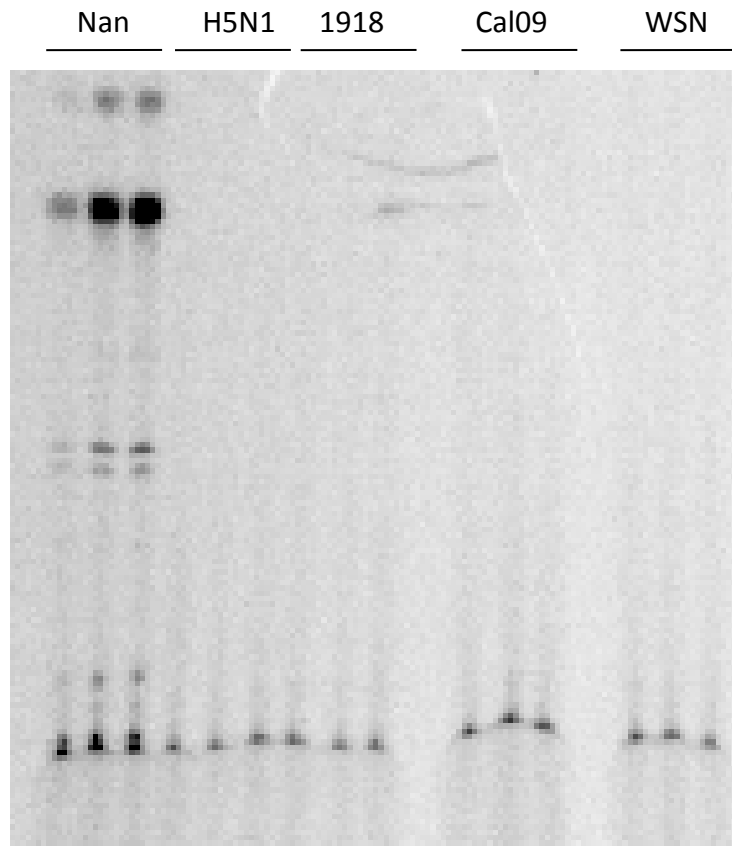


**Figure 3: Purification of PA Constructs Using GST Fusion System.** The gels shown are of the optimized conditions for each protein, run on a 10% SDS-PAGE gel which was Coomassie stained. In A is the PA<sub>657-716</sub> protein, while in B is the PA<sub>239-716</sub> protein. In addition, the mutant proteins for each construct were each purified in the same way (gels not shown).

With the GST fusion purification process optimized, the process could be scaled up in order to put the proteins to use in the new assay system. Up to 6L of initial culture could be purified at one time yielding high quality protein at around 2mg/mL.

The second part of the project involved optimizing a transcription assay system for use in a mammalian cell system. Another lab member had previously published findings showing the Nanchang influenza polymerase complex was a high fidelity protein. The polymerase in this study was purified from insect cells, and only one strain was tested. This project meant to test polymerase complexes which were purified from a mammalian lung cell line, as well as compare the fidelity and activity of the polymerase between different strains.

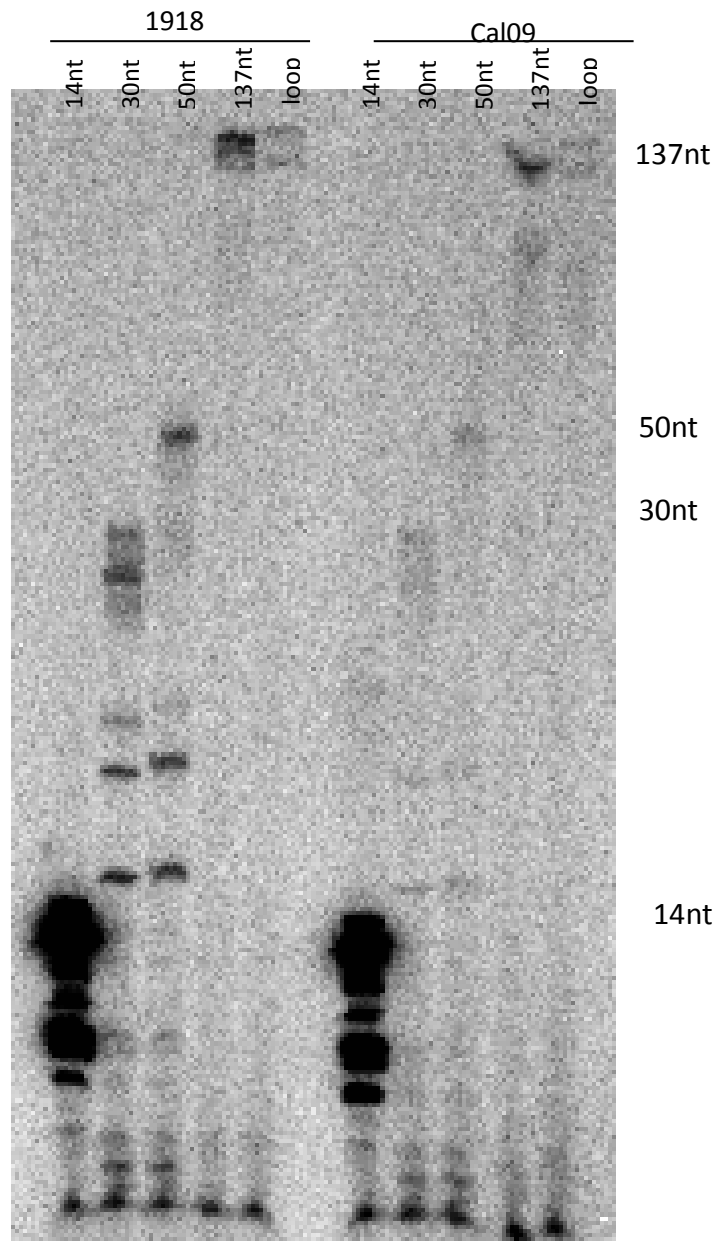
Using the previously described system, there was no activity from any of the purified polymerase complexes. This can be seen in **Figure 4**. The negative control (previously tested Nanchang polymerase) showed high activity (seen in lanes one through three). However, none of the other polymerase complexes showed any activity at all.



**Figure 4: Initial Transcription Assay.** The 3 bands on the left are the purified Nanchang virus used previously in this assay system, serving as a control. In the rest of the rows, no activity can be seen from any of the purified complexes. Along the top designates which strain was used, and within each strain three different concentrations of polymerase were tried.

After the initial attempt did not work, a different assay system was tried. It had all the same components as described above, except 1mM ATP/CTP/UTP, 2 $\mu$ M GTP, and 0.08 $\mu$ M radioactive  $\alpha$ -<sup>32</sup>P-GTP. In this system, unlike the other one, there was a NTP bias, with 1000 fold less GTP in the reaction than the rest of the nucleotides. However,

this new system with the bias gave much better results when used with the polymerase complex from the 1918 strain (which was selected for its high activity). This assay with the nucleotide bias was then tried with the different templates (14-nt, 30-nt, 50-nt, and 137-nt) to see if the template made a difference. The results of this test can be seen below in Figure 5. With this test of different templates, the 137-nt template would work the best for future use in a misincorporation assay. There were no secondary bands which would hinder the visualization of the stop sites in the misincorporation assay. This template also was long enough to utilize in the misincorporation assay.



**Figure 5: Transcription Assay with Different Templates.** The left five lanes are the 1918 polymerase, while the right five used the California 09 Swine polymerase. Each polymerase had a reaction with the 14-nt template, the 30-nt template, the 50-nt template, the 137-nt template, and finally a loop template.

However, even though the 137-nt template worked, there was nowhere near the activity that can be seen with the 14-nt template. Additionally, the NTP pool was still biased, which is believed to lead to an inaccurate measure of true activity of the



polymerase, because the GTP pool is a limiting factor compared to the other nucleotides. It is unclear why the unbiased NTP system worked for the polymerase proteins purified from the insect cells and not from the human cells. When a further study of the effect of concentration of NTPs was performed, it was found that the ratio of the cold GTP to the radioactive labeled GTP had to be similar for there to be activity (**Figure 6**). Additionally, as long as there were similar amounts of hot and cold GTP, the amount of the other three NTPs did not matter as much (until it was in the 10,000 times higher range).

<b>Component</b>	<b>1.</b>	<b>2.</b>	<b>3.</b>	<b>4.</b>	<b>5.</b>	<b>6.</b>	<b>7.</b>	<b>8.</b>
<b>ATP/UTP/CTP</b>	20 $\mu$ M	100 $\mu$ M	500 $\mu$ M	1000 $\mu$ M	20 $\mu$ M	100 $\mu$ M	500 $\mu$ M	1000 $\mu$ M
<b>GTP</b>	.1 $\mu$ M	.1 $\mu$ M	.1 $\mu$ M	.1 $\mu$ M	20 $\mu$ M	100 $\mu$ M	500 $\mu$ M	1000 $\mu$ M
<b>*GTP</b>	.08 $\mu$ M	.08 $\mu$ M	.08 $\mu$ M	.08 $\mu$ M	.08 $\mu$ M	.08 $\mu$ M	.08 $\mu$ M	.08 $\mu$ M
<b>Activity (1-5 scale)</b>	3	4	5	1	0	0	0	0

**Figure 6: Result of NTP Concentration Test.** Each column is a separate reaction, as described above. The activity was ranked on a 0-5 scale with 0 being no activity and 5 being large amounts of activity. The effects varying concentrations of the ATP/CTP/UTP was tested, as well as matching the cold GTP concentration to the other NTP concentrations.

From the tests performed above, we can conclude that the assay conditions described in Aggarwal, et al., 2010, do not work for the purified proteins from the mammalian cell line. We speculate that in the insect system, some other host proteins are playing a role in NTP sequestering or aiding the polymerase in some unknown way.

## DISCUSSION

This project optimized the preparation of the PA-GST fusion proteins to eventually be used in the Fluorescence Polarization assay along with the PB1 peptide which was pre-labeled with a Rhodamine chromophore. Optimizing the purification process took much trial and error. Each purification step took multiple days to complete, so it was a slow process.

However, in the end, a system was created for each of the new proteins which worked well and could be scaled up to create the large quantities of PA which were needed for the Fluorescence Polarization assay. The resulting protein was of high quality with few imperfections and a relatively concentrated protein. The production of the PA protein has been the limiting factor in the FP assay, so if there were ways to further improve the process that would be helpful.

To date, numerous chemical compounds have been screened from a chemical library at the University of Rochester High Throughput Screening (HTS) Core Facility. The fluorescence polarization assay has already been optimized for binding and there have even been a few hits on chemical compounds which have been found to disrupt the binding between the PB1 peptide and the PA constructs. One of these compounds is ribavirin, an antiviral drug used to treat RSV. This compound is undergoing further *in vitro* tests to investigate the extent of the binding disruption. If chemical compounds are found, they could eventually turn into new antiviral drugs, for which there is a strong need (McKimm-Breschkin et al., 2007). These drugs would have limited resistance because the polymerase is a highly conserved structure (Obayashi, 2008).

The second portion of the project began to improve upon a transcription assay system that was created to test the fidelity of influenza polymerase. However, the original assay was developed for a polymerase complex that was purified out of insect cells (Aggarwal et al., 2010), and it did not work with our mammalian system. It is important to use the mammalian system because a more accurate picture is seen of what the transcription rates are actually like during a human infection.

This portion of the project was difficult because there really was not much headway made in improving the assay system. The polymerase complex proteins were purified from A549 cells successfully and are ready to be used in assays. Some polymerase activity was gained using the two different assay systems that were tried in this project.

In the future, there needs to be further testing to determine what the ideal NTP concentration is for the transcription assays. Once that is determined, the activity of all the polymerase complexes from different strains can be compared and quantitated in the assay. This will give insight into whether the polymerase complex has anything to do with the virulence of the influenza virus. Additionally, mis-incorporation assays can be performed with the polymerase complexes to determine their level of fidelity, or accuracy. This will also give researchers new information about the influenza virus which someday could be helpful in preventing pandemics or developing vaccines.

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