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Development of a Reliable Method to Express and Purify Vaccinia Virus Transcription Factors A7 and D6 in Bacteria

By

Younghoon Park

Submitted in partial fulfillment of the requirements for the degree of Masters of Sciences in Microbiology from the Department of Biological Sciences of Seton Hall University September 2018

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Abstract:

Poxvirus early genes code for viral products involved in host interaction, immune evasion, replication, and transcription of the viral genome. These viral early genes contain a unique and conserved promoter sequence that is recognized by early transcription factors (ETFs). *The working hypothesis is pharmacologically targeting the structure of the poxvirus early promoters can inhibit transcription of poxvirus early genes.* Our group has identified unique secondary structure which deviates from a scrambled control near the Vaccinia Virus (VACV) E9L promoter. Targeting these unique structures, which most likely may be G-quadruplexes, with structure specific ligands may distort local DNA structures at the promoter. In turn, this may affect the ability of viral transcription factors to recognize the target sequence. As the E9L gene codes for the viral DNA polymerase, inhibition of E9L transcription would prevent replication of the VACV DNA during infection. To assess potential antiviral activity of G-quadruplex ligands, the central goal of this thesis is to develop an *in-vitro* system to identify compounds that prevent binding of ETFs to target promoters. Using VACV ETFs (VETFs) as a model system, VETFs, A7L and D6R, were cloned into a bacterial expression vector with a His epitope tag for purification. A7 and D6 have successfully been expressed and detected in bacterial cell lysates via immunoblotting with anti-His antibodies. Currently, protocols are being optimized for large-scale purification of viral proteins to use in an *in vitro binding* assay.

Introduction:

Poxviruses are large enveloped double stranded DNA viruses and amongst the largest of DNA viruses. Poxvirus genomes range from 130-375 kb in size (*ViralZone*). Upon entry, the poxvirus envelope fuses with the host membrane allowing the viral capsid to enter the cytoplasm. Once in the cell, poxviruses initially transcribe viral mRNAs inside the capsid to act as a barrier from the host cell. Unlike other DNA viruses, the entire replication cycle of a poxvirus occurs in the cytoplasm of the host cell. Therefore, the virus does not rely on the host's transcriptional machinery in the nucleus. Instead, a poxvirus utilizes viral transcription factors and a viral RNA polymerase to transcribe genes. As the poxvirus matures, it can take on two different mature virion forms: the intracellular mature virion or the extracellular enveloped virion (Condit et al. 2006).

Vaccinia and *Poxvirus* Life Cycle:

Vaccinia Virus (VACV) is a member of the orthopoxvirus genus. VACV is the prototypical poxvirus that has widely been used in research labs to study poxvirus replication and transcription processes. VACV shares an identical life cycle to the more virulent Variola Virus (VARV), the causative agent of smallpox, making VACV an ideal viral model (Condit et al. 2006). Like all poxviruses, VACV does not utilize the host cell's transcriptional machinery housed within the nucleus of the cell, but instead carries its own necessary transcriptional machinery to regulate and express its genes. The VACV genome contains genes without introns that have their own promoters that code for a variety of products including transcription factors, RNA polymerase, DNA polymerase, structural components, and host range factors (Condit et al. 2006).

VACV regulates the transcription of its genes and life cycle temporally using three classes of transcription factors: Viral/Vaccinia Early Transcription Factors (VETF), Viral/Vaccinia Intermediate Transcription Factors (VITF), and Viral/Vaccinia Late Transcription Factors (VLTF) (Broyles 2003) (Figure 1). The virion core contains VETFs, the RNA polymerase, and a capping enzyme, which allows the VACV mRNA to be recognized for translation by host ribosomes (Condit et al. 2006). The VETFs bind to early gene promoters and recruit the RNA polymerase to express those genes. Some early gene products include the poxvirus DNA polymerase, immune evasion molecules, a host range factors, and VITFs, which bind to intermediate promoters and express Vaccinia intermediate gene products (Broyles and Li 1991; Broyles and Li 1993; Li and Broyles 1993; Condit et al. 2006). Some of these intermediate gene products are VLTFs, which induce the expression of late gene products (Broyles 2003). VACV late genes code for structural components of the virion (Broyles 2003).

The transcriptional regulation and life cycle of VACV are highly conserved in the *orthopoxviridae* genus (Broyles 2003). The transcriptional machinery is close enough to the point where BSC-40 cells transfected with the horsepox genome and infected with VACV can generate a reconstructed recombinant horsepox virus (Noyce et al. 2018). Specific regions on early promoters are highly conserved across orthopoxviruses: the critical region, spacer region, and initiation regions (Davison and Moss 1989). One important early gene promoter is the E9L promoter, which regulates the expression of the VACV DNA polymerase (Condit et al. 2006). The E9L promoter is recognized by the two VETFs: A7 and D6 (Cassetti and Moss 1996). A7 binds the promoter and recruits D6. D6 then recruits the RNA polymerase inducing transcription (Cassetti and Moss 1996, Li and Broyles 1993).

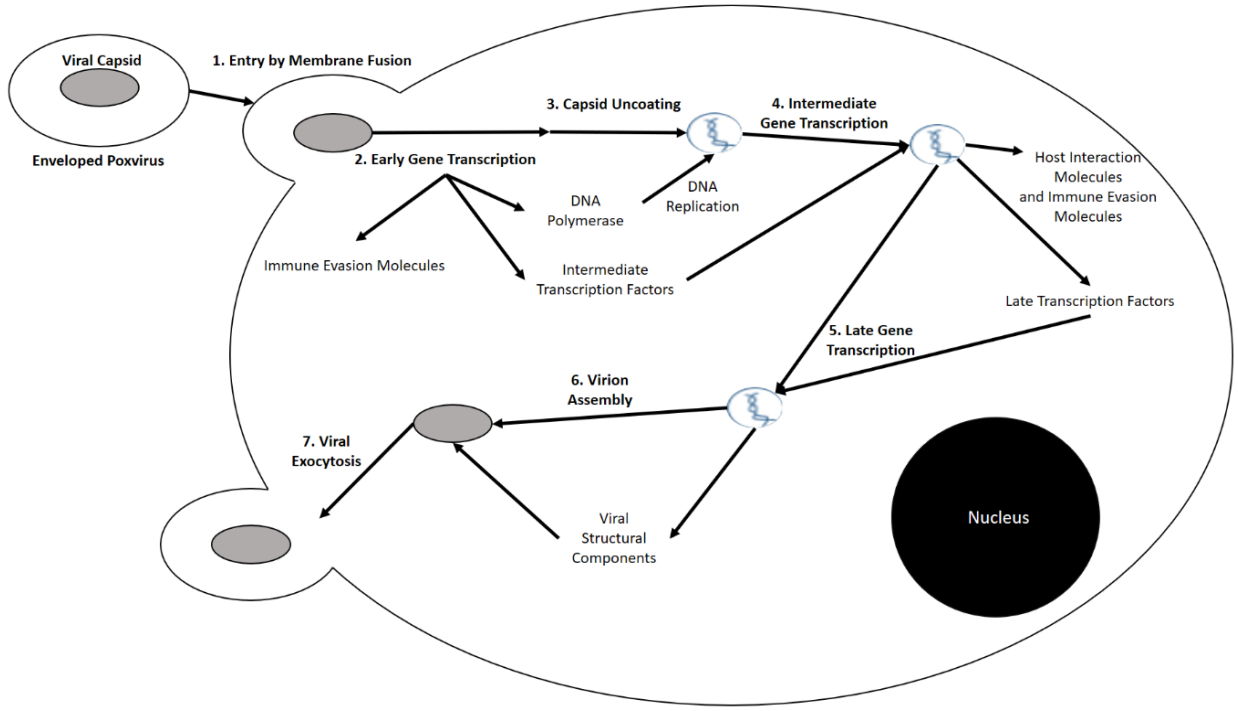


Figure 1. Poxvirus Replication Cycle. Poxvirus replication cycle from entry to viral exocytosis.

This figure was generated by the author.

Poxvirus Diseases:

The most notorious of these poxviruses is the VARV, which is the causative agent of smallpox. VARV is a member of the *orthopoxviridae* genus. VARV alone has killed approximately 300 to 500 million people in just the 20th century alone, making it at the time one of the most common killers of the century (Ist and Sanit 2016). Smallpox plagued people up until a highly successful worldwide vaccination program led to its eradication in the late 1970's. However, poxviruses still remain a topic of interest for public health. After the successful eradication of smallpox, the general population is now no longer vaccinated against the VARV. Theoretically, VARV would not pose a problem as long as all samples of VARV are documented, stored and/or properly disposed. However, undocumented VARV has been found in an FDA facility that was still infectious in 2014 (CDC (2016)). Therefore its possible undocumented VARV exists in other facilities around the world, and the future risk of accidental or intentional exposure and outbreak. Poxviruses like VARV can potentially be used as a bioterrorism weapon for both its transmissibility, severity, and lack of existing countermeasures. VACV, which is used for the VARV vaccine and part of the same *orthopoxviridae* family, is very similar genetically and antigenically to the VARV and can be engineered to be more virulent (Shchelkunova and Shchelkunov 2017).

One of the most common poxviruses is the *Molluscum Contagiosum Virus* (MCV), which infects over 100 million people in the world. With the eradication of smallpox, MCV remains the only poxvirus that exclusively infects humans, with no animal host. MCV is mostly found in school children, adolescents, and immunocompromised individuals. MCV leaves distinct, benign skin lesions on the appendages or trunk of a host causing itchiness and mild discomfort (Nguyen et al. 2014). An MCV infection usually results in mild symptoms. MCV is understudied due

partly to lack of a cell culture or animal model to propagate the virus. This particular limitation forces virologists to study MCV through cloning MCV genes in vectors and studying specific MCV genes and proteins. Currently there are no FDA approved treatment options, nor are there any widely accepted standard of care for MCV (Nguyen et al. 2014).

Zoonotic poxviruses also remain a cause of concern to both humans and livestock. Some zoonotic poxviruses include VACV, cowpox virus, horsepox virus, sheeppox virus and cammelpox virus. Monkeypox has had consistent outbreaks in Central Africa. Like VARV, Monkeypox Virus is a member of the *orthopoxviridae* genus and shows similar signs and symptoms to a VARV infection (Yinka-Ogunleye et al. 2018; Haller et al. 2014). While Monkeypox has a lower mortality rate when compared to the smallpox, Monkeypox still poses a clinical threat both for the severity of its symptoms and transmissibility (Doshi et al. 2018). There have been outbreaks as recent as 2017 in the rural parts of Nigeria of western Africa with 89 cases and in the Democratic Republic of Congo with over 100 cases (Yinka-Ogunleye et al. 2018; Doshi et al. 2018; Durski et al. 2018). The most recent and only outbreak of Monkeypox in the United States occurred in 2003, with 47 confirmed cases in the midwest, which were attributed to a shipment of Monkeypox infected wild animals from West Africa to the United States (Durski et al. 2018). Increasing reports of the reemergence of Monkeypox virus means that surveillance and proper countermeasures are becoming increasingly difficult.

Vaccinia Virus (VACV) is currently the most studied orthopoxvirus and believed to be originated from the cowpox vaccine. However, a recent review suggests that the VACV may have originated from horsepox (Esparza et al. 2017). Vaccinia is nearly identical genetically to VARV from its life and replication cycle, conserved genes, and transcription, which makes VACV used as the current smallpox live attenuated vaccine against smallpox. VACV has had

outbreaks in South America with many of these incidents in rural Brazil (de Oliveira et al. 2017). VACV naturally transmits from cattle to people (de Oliveira et al. 2017). Rodents and other animals can act as potential reservoirs for the VACV, which could become a major health concern if VACV were able to get into a densely populated urban area (de Oliveira et al. 2017). There are multiple strains of VACV with varying degrees of virulence, which can be a cause of concern, since VACV is so similar to VARV.

Additionally, the concern for the emergence of new pathogenic poxvirus remains a public health concern. Recently, a new poxvirus was found in a rodent population in east central Texas in the United States in 2018. The poxvirus was named Brazospox for the nearby Brazos River from where the poxvirus was found (Hodo et al. 2018). Brazospox was found to be closest to the *Chorodopoxvirinae* subfamily with a generated phylogram with other poxviruses (Hodo et al. 2018). Some of its closest predicted relatives were Squirrelpox and MCV (Hodo et al. 2018). With the recent discovery, its pathogenicity towards humans must be assessed since Brazospox can potentially be a human and veterinary public health concern.

Poxvirus Inhibitors:

The development and study of anti-poxviral therapeutics is necessary to control and prevent future and current outbreaks. The smallpox vaccine is available in the event of an orthopoxvirus outbreak or a bioterrorist attack. However, the limited stock of the smallpox vaccine is most likely not sufficient to combat the threat of a potential outbreak, especially since vaccinations have ceased after its eradication in the 1980s. Cidofovir is a broad spectrum nucleotide analog antiviral compound that is commonly used against DNA viruses (Stittelaar et al. 2006). Cidofovir was successful at treating monkeypox during an outbreak (Stittelaar et al. 2006). Cidofovir cannot be administered orally and the drug is known to be nephrotoxic, which

can cause major kidney damage (Foster et al. 2017). Recently, a new similar compound called Brincidofovir is in development to replace Cidofovir. Brincidofovir overcomes major issues of Cidofovir with a lipid moiety HDP conjugated to the phosphonate of Cidofovir (Foster et al 2017). Brincidofovir was shown to be successful as an antipoxviral therapy, but poxviruses have been found to develop resistance similar to what has been reported with cidofovir (Foster et al. 2017).

One of the gold standards of orthopoxvirus inhibitors is the drug ST-246 or more commonly known as Tecovirimat (Mucker et al 2013). Tecovirimat targets and inhibits the VARV p37 protein encoded by the C17L and its homologs of the *orthopoxviridae* (Yang et al. 2005). In Vaccinia, the homolog for p37 is the F13L gene which codes for the F13 protein. F13 acts as a critical protein for the formation of the viral particle (Yang et al. 2005). The molecular mechanism is poorly understood for F13 and other p37 homologs (Moss 2015; Bryk et al. 2018). Bryk et al. recently suggested that F13 was critical to the extracellular virion formation and their rapid entry into cells by enhancing the sensitivity of the membrane to acid induced dissolution (Bryk et al. 2018). Tecovirimat has been proven to be a consistent post exposure and post symptomatic antipoxviral therapy against many orthopoxviruses including Monkeypox, VARV, VACV and Cow Pox (Berhau et al 2015; Yang et al. 2005; Mucker et al 2013). Tecovirimat resistance has been reported highlighting the need for continued research into novel poxvirus inhibitors (Pires et al. 2018; Smith et al. 2009).

Targeting Unique DNA structures in the promoter as a novel antipoxvirus strategy:

G-quadruplex structures consist of four guanines that fold in a planar arrangement through Hoogsteen hydrogen bonds (Metifiot et al. 2014). G-quadruplex structures have been identified in many viruses including HIV, SARS-Coronavirus, Herpes simplex virus 1, and

Poxviruses with functions ranging from genome stability,transcriptional regulation, and translational regulation (Lavezzo et al. 2018; Metifiot et al. 2014). It would not be a surprise if small ligands like meso-Tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMPyP4) and N,N'-(9-(4-(Dimethylamino)phenylamino)acridine-3,6-diyl)bis(3-(pyrrolidin-1-yl)propanamide) hydrochloride (BRACO-19) that bind these structures may represent a new class of anti-viral therapeutics. The G quadruplex binding ligands have already been shown to inhibit HIV-1 replication (Metifiot et al. 2014). TMPyP4 has promise as a candidate for anti-poxviral applications. Utilizing circular dichroism and UV-Vis drug titrations, preliminary studies performed by Dr. Cosimo Antonnacci and Taryn Heiser have suggested the compound has high specificity for the VACV DNA polymerase promoter sequence. Additionally, TMPyP4 showed no significant cytotoxic effect to HeLa and HEK293T cells up to 50μM (Gandbhir 2017). Since the G-quadruplex structure is so prevalent and based on their known function in viruses, the working hypothesis is that TMPyP4 binds to G quadruplex structures found at the VACV E9L promoter. By binding this DNA structure, TMPyP4 may prevents A7 and D6 from binding DNA (Figure 2).

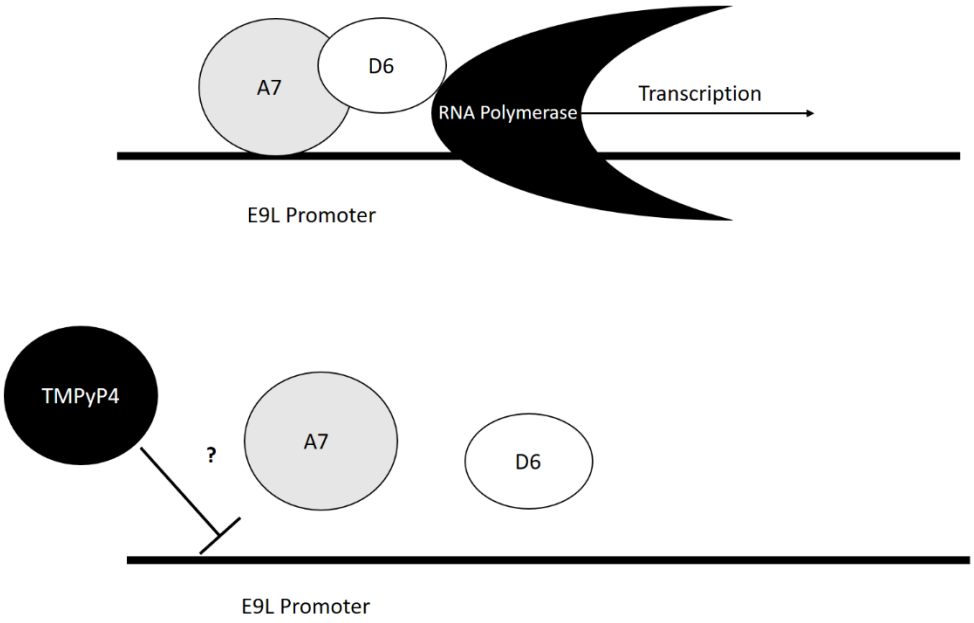


Figure 2: E9L Transcription Illustration with and without TMPyP4. This figure and all subsequent figures were generated by the author.

Direct In-Vitro Study on Viral Early Transcription Factors and Limitations on Poxvirus Studies:

The goal of this study is to develop a direct *in-vitro* screening method to identify compounds that inhibit viral early transcription factors (VETFs) from binding target promoters. Targeting VETFs is attractive target for anti-poxviral drugs. The transcription of the viral DNA could not only be tailored to specific VETFs, but depending on the VETF can halt or stall the viral replication cycle, like the E9L promoter which regulates the poxvirus DNA polymerase(Condit et al. 2006; Cassetti and Moss 1996). Targeting VETFs also has the added benefit of having a high barrier of resistance. The poxvirus would have to overcome two major changes. The promoter critical region that the VETFs bind to would have to be altered. These changes would subsequently force the VETF to undergo alterations to make it capable of binding to that same region and potentially become resistant. Since these regions are often highly conserved across the *orthopoxviruses*, the development of these drugs could also be a broad spectrum anti-poxvirus therapeutic.

VACV provides a strong model for this study, as it is the most well studied of the *orthopoxviruses* and shares replication and transcription processes similar to other poxviruses. The long term goal of the project is to establish a system to perform in-vitro studies in a BSL-1 facility A BSL-1 model would provide academic and nonacademic research labs a better opportunity to study and screen potential VETF anti-poxviral compounds, by utilizing molecular cloning in a bacterial model. This model would cut costs from doing live virus anti-poxviral drug studies in a BSL-2 or higher facility. This approach can also be applied to study binding of VETFs to promoters of poxviruses with BSL-3 or higher level precautions such as monkeypox and VARV. Additionally, transcription factors from poxviruses that are difficult to culture, such as MCV, could be studied, overcoming the hurdle of not being culturable in labs (Shisler 2015).

The important first step of these goals is developing a reliable system to generate large quantities of VETF proteins using bacteria. Purifying VETFs in bacteria has proven challenging in the past as both A7 and D6 are difficult to solubilize when overexpressed. Here, the process to express, solubilize and purify the VACV VETFs for subsequent analysis is described.

Methods

Plasmid DNA Extraction:

DNA plasmids were extracted using the PureYield Plasmid Miniprep System protocol by Promega. *E. coli* strain DH5α transformed with either A7/pET303 (A7:pET303CT-His) or D6/pET303 (D6:pET303NT-His) were grown for 16-18 hours at 37ºC in LB broth supplemented with 100 µg/mL ampicillin. Bacteria were centrifuged for 14,000 rpm for 30 seconds and then resuspended in 600 µL Tris EDTA Buffer. The plasmid DNA was extracted as per the manufacturer's instruction and eluted with either Nuclease Free Water or Tris EDTA Buffer. The concentration and purity of each sample was evaluated using the NanoVue Plus Spectrophotometer by GE Healthcare or the BioDrop.

Protein Expression

A7:pET303CT-His and D6:pET303NT-His plasmid as described above and transformed into BL21 (DE3) *E.coli*. Plasmids with A260/A280 of 1.8-2 were used for the transformation. The transformed BL21 (DE3) were then plated on LB plates with 100 µg/mL of ampicillin. Isolated colonies were then taken to make glycerol stocks for preservation of culture for future growth and inductions.

A starter culture of BL21 (DE3) was inoculated in 5 mL of LB broth with 100 µg/mL of ampicillin for 18-20 hours at 37°C and shaking at 225 rpm. This starter BL21 (DE3) culture was then placed into 50 mL of LB broth with 100µg/mL of ampicillin. This was also scaled up to10 mL of starter culture into 100 mL of LB broth with 100 µg/mL of ampicillin. Once the OD_{600}=0.8, D6 and A7/D6 expression was induced through addition of Isopropyl β-D-1thiogalactopyranoside (IPTG) stimulation at 1mM for 4 hours. BL21 (DE3) cells were

centrifuged at 5000 xg for 10 minutes and the supernatant was discarded. The pellet was stored at -80°C prior to lysing.

Immunoblotting and Commassie Staining.

The expression of A7 and D6 was visualized through immunoblot and Coomassie staining. Samples were lysed and prepped by boiling for 5 minutes with 5% 2-mercaptoethanol in SDS sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue). Samples were resolved in a 10% SDS polyacrylamide gel electrophoresis to confirm expression, solubilization, and purification. These polyacrylamide gels were run at 60mA for two gels simultaneously or 30mA for a single gel with a buffer dam in a 1x SDS buffer containing, 0.1% SDS, 190 mM glycine, and 25 mM Tris-Base. Polyacrylamide transferred to a Polyvinylidene (PVDF) membrane or Nitrocellulose membrane made by Amersham Protran for antibody probing with Transfer Buffer (20% Methanol, 80% water, 25.3 mM Tris Base, 19 mM Glycine) at 90V for 1 hour. The nitrocellulose or PVDF membranes were blocked overnight in 5% milk (carnation non fat dry milk) 1x TBST (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% Tween-20) and probed with antiHis (mouse, *Thermofischer Scientific)* diluted to 0.5 µg/mL in 0.5% milk 1xTBST for 1 hour. Blots were washed in three 10 minute cycles in 0.5% milk TBST. The membranes were then probed with secondary antibody with Goat-α-mouse HRP by and diluted by 1:10,000 in 0.5% milk 1xTBST. Membranes were then washed similarly after the primary antibody. Bands corresponding to either His-tagged A7 or D6 were visualized with SuperSignal West Femto chemiluminescent reagent by Thermo Scientific for 5 minutes prior to using a FluorChem E imaging system.

Alternatively, polyacrylamide gels for coomassie were stained with either Comassie blue (40% distilled water, 50% methanol, 10% glacial acetic acid and 0.1 g Brilliant Blue/100mL

solvent) and destained, or treated with SimplyBlue SafeStain by Invitrogen. The SimplyBlue SafeStain, the gel was rinsed for three 5 minute washes with distilled water at room temperature and treated with the stain for 1 hour at room temperature and shaking. After the stain was discarded and washed in distilled water for 1 hour, discarded and washed again in distilled water for 1 hour. Coomassie gels were visualized through the same FluorChem E imaging system.

Bacterial Lysis:

BL21 (DE3) *E. coli* were lysed using two different methods labeled as 6.5 pH Lysis Buffer and BPer method (*Thermofisher Scientific*). In the 6.5pH Lysis Buffer method, the pelleted BL21 (DE3) was resuspended into the 6.5 pH Lysis Buffer, which constituted: distilled water set at the pH of 6.5, 1 mg/mL of chicken egg white lysozyme, and protease cocktail inhibitor. The resuspended BL21 (DE3) in 6.5 pH Lysis Buffer was incubated for 30 minutes on ice at a ratio of 1 mL of bacterial culture into 160 µL of lysis buffer. The lysate was then sonicated in six 10 to 30 second bursts and intermittent 10-30 second rests, followed by centrifugation at 4°C at 3000 xg. Longer sonication bursts were necessary with larger volume lysates. The supernatant and debris were separated for analysis by immunoblot and coomassie staining.

In the BPer method, the pelleted BL21 (DE3) samples were resuspended and lysed using the BPer buffer at a ratio of 4 mL of BPer buffer per 1 gram of bacterial pellet at room temperature for 15 minutes. The BPer buffer was supplemented with protease cocktail inhibitor by ThermoScientific, DNAse and lysosome provided by the BPer buffer lysis system ThermoScientific. The lysates were then sonicated similarly to the normal method at six 10 to 30 second bursts and intermittent 10-30 second rests. The lysates were then centrifuged at 15,000 xg

for 5 minutes. The debris pellet and supernatant were separated for analysis by immunoblot and coomassie staining.

Protein Gravity Column Purification:

The gravity column consisted of 1 mL of settled HisPur Ni-NTA Resin purchased from ThermoScientific. The resin's storage buffer was drained by gravity flow. Then 2 mL of Equilibration Buffer (Table 1) was used to treat the column and allowed to flow through. The same Equilibration buffer was then used to treat the crude lysate in a 1:1 v/v ratio. This crude lysate was run through the column twice to ensure maximal protein binding to the column and both the crude lysate and flow through were saved as samples. The column was then washed with 12.5 mL of Wash (Table 1). Another wash buffer was used later with the same conditions supplemented with 50 mM of imidazole (Table 1). After the wash 5 mL of Elution buffer was then placed into the column and collected (Table 1). The Elution Buffer was also left with the resin overnight and collected to allow for more equilibration. A gradient elution was also done, in which 1.5 mL of elution buffer containing various imidazole concentrations (200 mM, 250 mM, 300 mM, 400 mM, 450 mM and 500 mM) flowed through the column from lowest to highest imidazole concentration (Table 1). HisPur Ni-NTA Resin was sampled by resuspending the resin with Wash Buffer, pipetting the resin and transferring the resin sample into a sterile tube. The Resin sample resuspended in Wash Buffer was then prepped with the same method as the samples for the Coomassie Staining and Immunoblotting.

Table 1. Lysis and Purification Buffers

	Buffer Name	**Components**
1	6.5 pH Buffer	6.5 pH, 1mg/mL Chicken Egg Whie Lysozyme, Distilled Water
2	Bper Buffer	7.5 pH, Proprietary Nonionic Detergent, 20mM Tris-HCl
3	PBS	20mM Sodium Phosphate, 300 mM Sodium Chloride, Distilled Water
4	Equilibration Buffer	7.4 pH, 10mM Imidazole, PBS
5	Wash Buffer	7.4 pH, 25mM or 50mM Imidazole, PBS
6	Elution Buffer	7.4 pH, 250mM Imidazole, PBS
7	Gradient Elution Buffer 1	7.4 pH, 200 mM Imidazole, PBS
8	Gradient Elution Buffer 2	7.4 pH, 250 mM Imidazole, PBS
9	Gradient Elution Buffer 3	7.4 pH, 300 mM Imidazole, PBS
10	Gradient Elution Buffer 4	7.4 pH, 350 mM Imidazole, PBS
11	Gradient Elution Buffer 5	7.4 pH, 400 mM Imidazole, PBS
12	Gradient Elution Buffer 6	7.4 pH, 450 mM Imidazole, PBS
13	Gradient Elution Buffer 7	7.4 pH, 500 mM Imidazole, PBS

Results:

A7 VETF Expression and Solubilization:

A7 is one of two VETFs (viral early transcription factors) that bind to the E9L promoter, which contributes to the transcriptional regulation of VACV early genes (E9). A7 is notoriously difficult to purify and to our knowledge has never been attempted in a bacterial system. BL21 (DE3) *E. coli* was transformed with the pET vector with A7L gene cloned into the vector (BL21+A7:pET303CT-His) or with the empty pET vector without A7L (BL21+pET303CT-His) (Gandbhir 2017). A7L is the notation of the gene for the large VETF A7 protein. The "L" denotes the direction of transcription of the VACV dsDNA genome. The pET303CT-His vector contains an in frame C-terminus his tag epitope for later detection and purification. BL21 (DE3) cells contain the T7 RNA polymerase to drive transcription of the insert via a T7 promoter. The promoter on the vector is controlled via the *lac* operon at the LacI binding site, which prevents the transcription in the absence of IPTG. BL21+A7:pET303CT-His was inoculated into Luria Broth (LB) with 100µg/mL of ampicillin overnight. BL21+A7:pET303CT-His starter culture was then transferred to fresh LB with 100µg/mL of ampicillin that was 10 times the volume of the original starter culture. The BL21+A7:pET303CT-His was induced 1 mM IPTG when OD_{600} reached 0.8, which usually required 4 hours. After the 4 hour induction, BL21+A7:pET303CT-His was lysed using 6.5 pH Lysis Buffer with 1mg/mL Chicken Egg White Lysozyme. This process was repeated with the BL21+pET303CT-His as a control. The BL21+pET303CT-His and BL21+A7:pET303CT-His were then compared and analyzed with a His immunoblot. A band corresponding to the predicted molecular weight of A7 at 83 kDa was observable in BL21+A7:pET303CT-His and only present in the BL21+A7:pET303CT-His with a 1mM IPTG treatment of 4 hours at OD_{600}=0.8 (Figure 3). There was no observable band for

BL21+pET303CT-His with and without IPTG, and BL21+A7:pET303CT-His without IPTG (Figure 3).

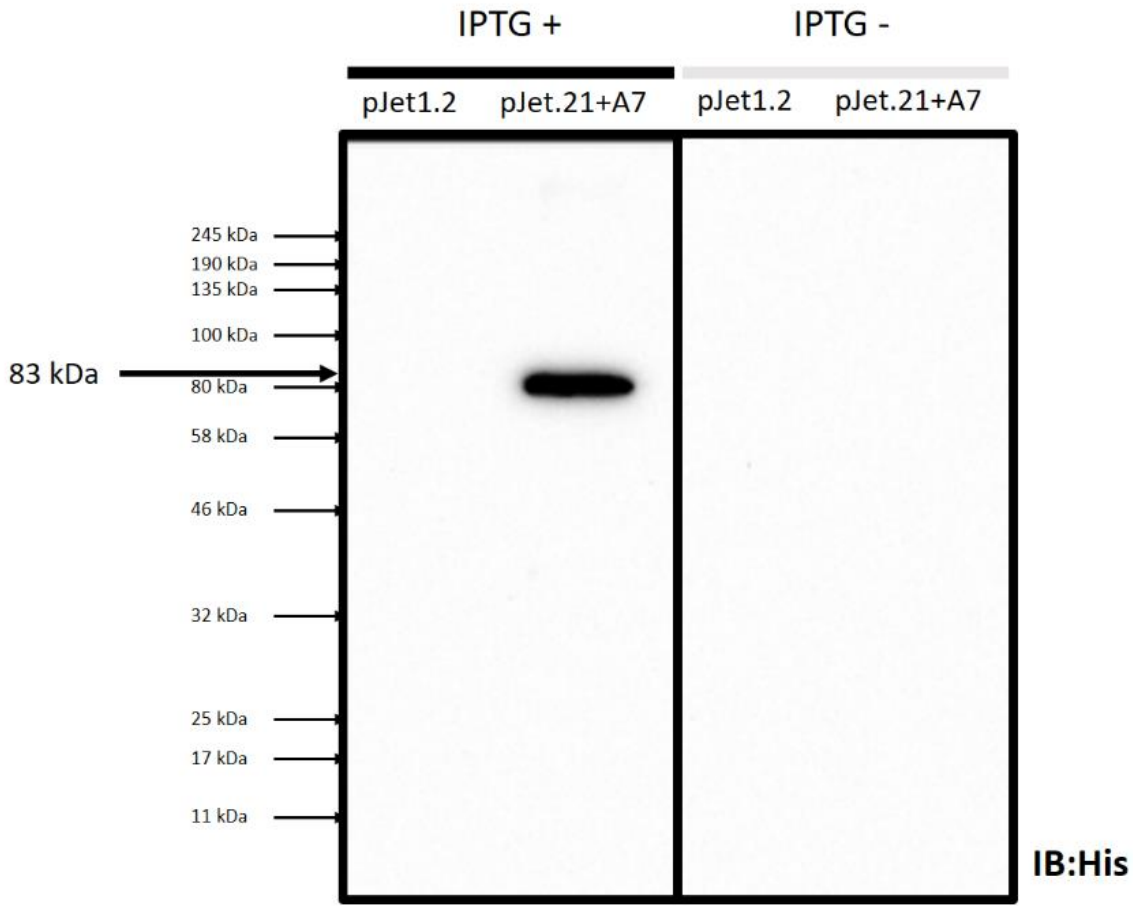


Figure 3: His immunoblot of BL21+pET303CT-His and BL21+A7:pET303CT-His lysates in the presence and absence of 1mM IPTG for 4 hours.

A7 First Purification Attempt:

Once the A7 protein was visualized in the soluble portion of the lysate, these samples were purified via a nickel resin column. During the first A7 purification, no band corresponding to the predicted molecular weight (83kDa) of A7 was detected in the Elution. Instead, a band corresponding to the A7 was visualized in the flow through and the crude (Figure 4A, Figure 4B). To determine if A7-His epitope tagged proteins were bound to the column, a sample of the resin in the column was analyzed with the corresponding A7 Purification method. The column's HisPur Ni-NTA Resin was sampled and resolved by 10% SDS-PAGE for analysis via immunoblot and coomassie staining. An intense band corresponding to the molecular weight of A7 was highly detectable in the HisPur Ni-NTA Resin samples by both His immunoblot and Coomassie staining (Figure 4C, Figure 4D). Only the crude and flowthrough exhibited a visible band for the corresponding band to A7 that was detectable, but was much weaker relative to the HisPur Ni-NTA Resin (Figure 4C, Figure 4D). The purification attempt suggests that the elution was not optimized to pull off the predicted A7 from the column, however the data suggests it was bound to the resin.

A7 Purification:

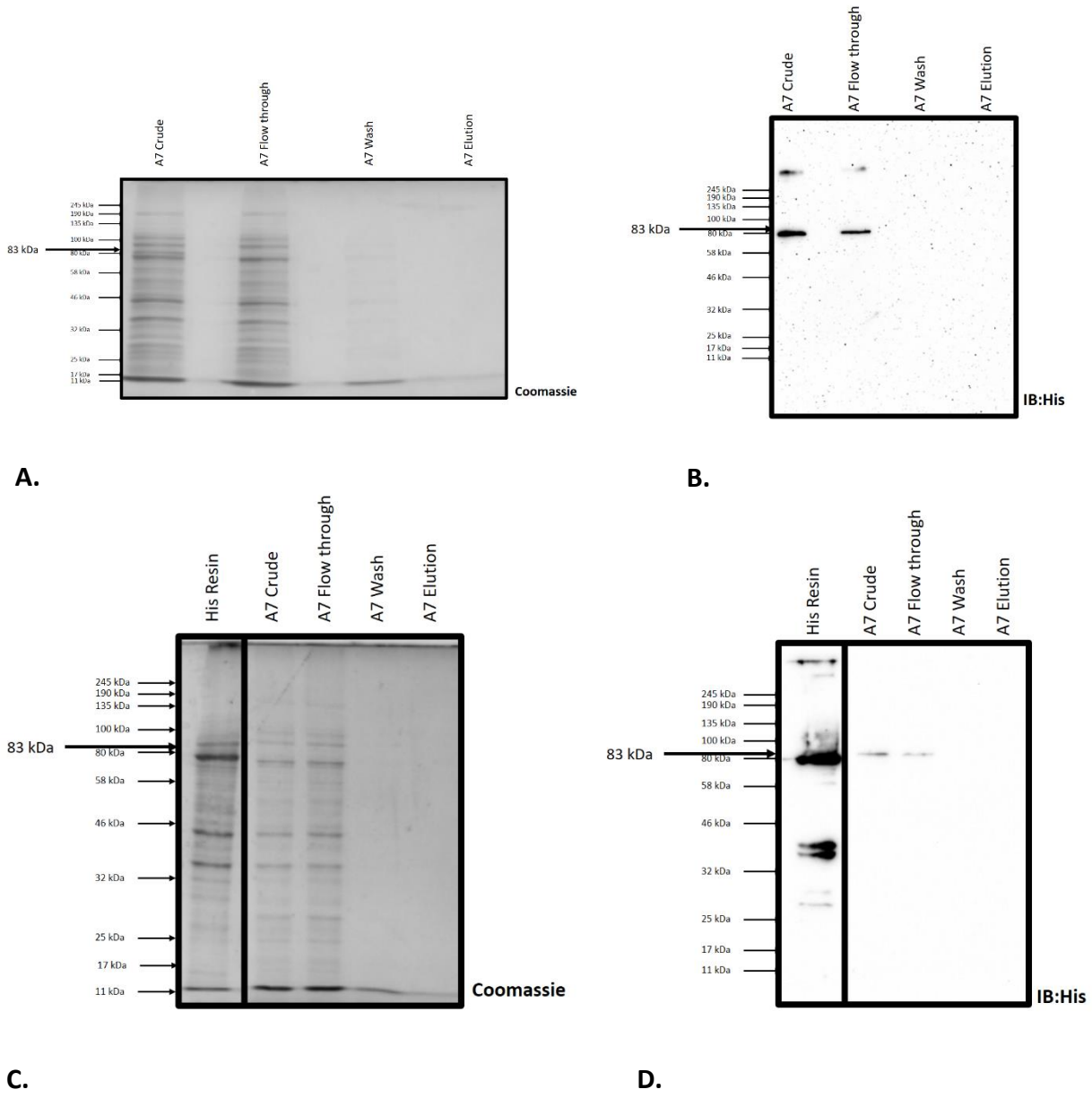


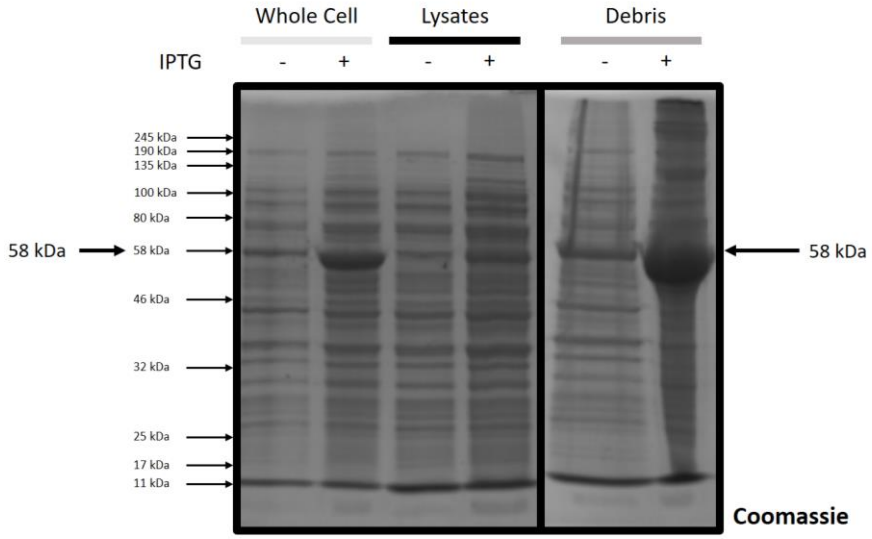
Figure 4: A7 First Purification. A) Coomassie Staining of A7 Purification. B) His Immunoblot of A7 Purification. C) Coomassie Staining of A7 Purification with the HisPur Ni-NTA Resin post Elution Buffer. D) Coomassie Staining of A7 Purification with the HisPur Ni-NTA Resin post Elution Buffer.

Rac Kinase Expression and Solubilization:

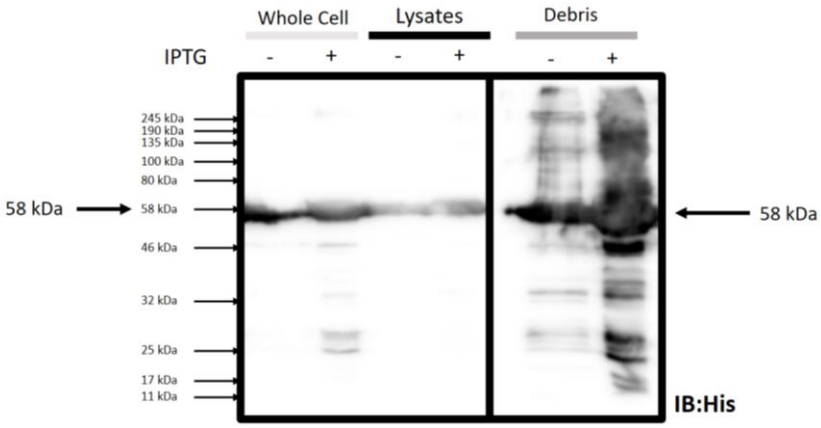
Given that A7 was not successfully eluted from the column, one concern was that the His epitope tagged A7 was not soluble under column conditions. Therefore, a His epitope tagged Rac Kinase protein, which was approximately 58 kDa, was expressed in BL21 (DE3) cells as a positive control. Rac Kinase is a very soluble and easily expressed in a bacterial vector system, which makes Rac Kinase an ideal positive expression control for the developing a bacterial expression system. The BL21 (DE3) cells were grown with the same bacterial induction protocol with 1mM of IPTG. Whole cell lysates were prepared for the SDS PAGE, by resuspending the bacterial pellet in an equal volume to the lysate volume of the 6.5 pH lysis buffer, which was then immediately prepped with SDS loading dye and 2.5% β-mercaptoethanol.

Rac Kinase showed highly visible expression with a significant portion found in the lysate (Figure 5). The Debris held the highest amount of Rac Kinase, but a clear band was still visible in the lysate (Figure 5). The Lysate showed a weaker intensity of the Rac Kinase band in the absence of IPTG (Figure 5).

Rac Kinase Expression and Solubilization:



A.



B.

Figure 5: Rac Kinase Expression and Solubilization with and without 1mM IPTG. A) Coomassie Staining of Rac Kinase Expression and Solubilization. B) His Immunoblot of Rac Kinase Expression and Solubilization.

Rac Kinase Purification:

The purification of Rac Kinase utilized the same HisPur Ni-NTA resin and followed the same protocol. The purification of Rac Kinase showed a visible band for the elution, however much of the detectable Rac Kinase was still found to be bound to the column (Figure 6). This suggests that the elution buffer conditions were not optimized to successfully purify our proteins of interest.

Rac Kinase Purification:

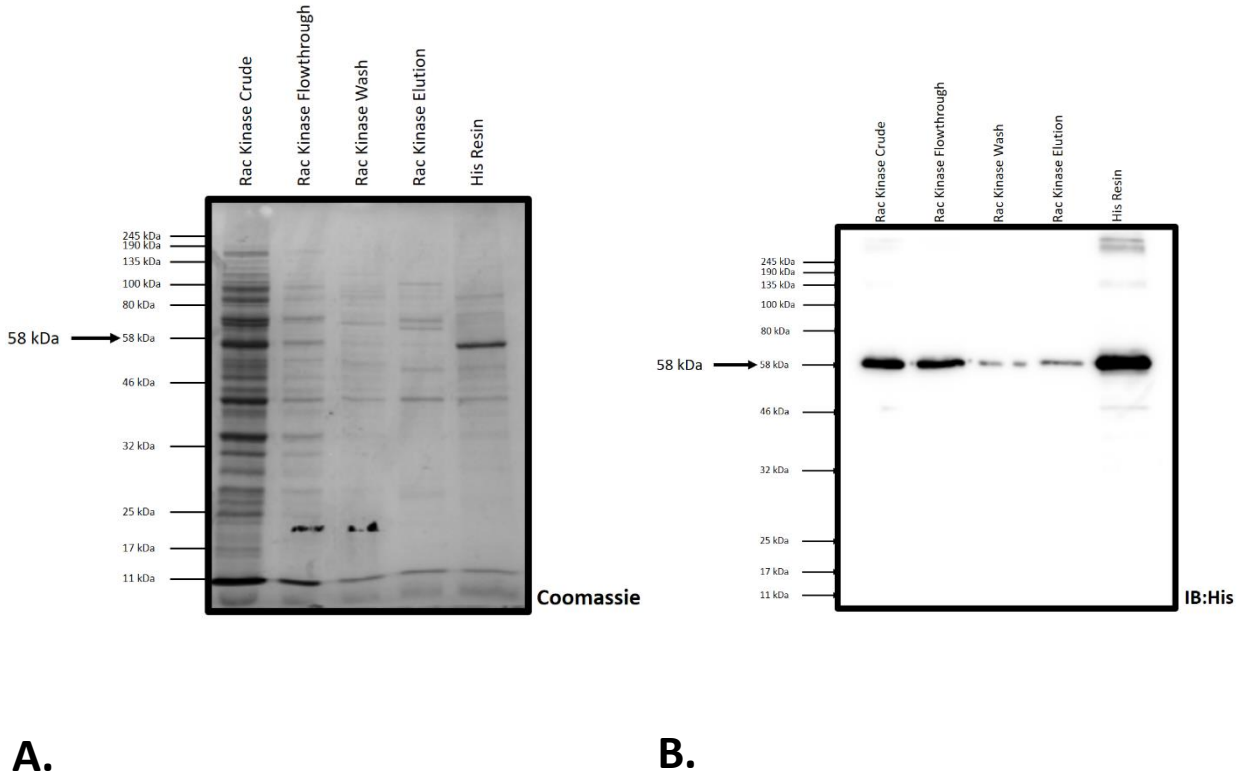


Figure 6: Rac Kinase Gravity Column Purification. A) Rac Kinase Purification Coomassie Staining. B) His immunoblot of Rac Kinase Purification.

A7 Gradient Elution Purification:

A second purification attempt on the same A7 column (Figure 4) was attempted with a gradient elution with varying concentrations of imidazole at 200mM, 250mM, 300mM, 400mM, 450mM, and 500mM sequentially (Figure 7). Rac Kinase that was previously expressed and solubilized was used as a positive control marker (Figure 7). Under these conditions, a band corresponding to the expected size of A7 was detectable in all elution concentrations at increasing imidazole concentrations (Figure 7A, Figure 7B). Less A7-His protein was found bound to the resin post elution than pre-elution further indicating a successful elution (Figure 7B, compare lanes 3 and 4) The same column used in Figure 4 was used for this gradient elution, which suggests that kinetics of the elutions and column need to be optimzed. One question that arose at this time was whether a fraction of the protein on the column was rendered insoluble due to lysis conditions. Every protein is different, therefore a specific protein can have different interactions with the solutes and can react differently based on what buffer was used. The column was stored away at 4°C prior to the gradient elution, which may have also contributed to these results. At 4°C the lower temperature can alter the structure of the protein, while also affecting the kinetics of the column as well.

A7 Gradient Elution: Purification Attempt 2

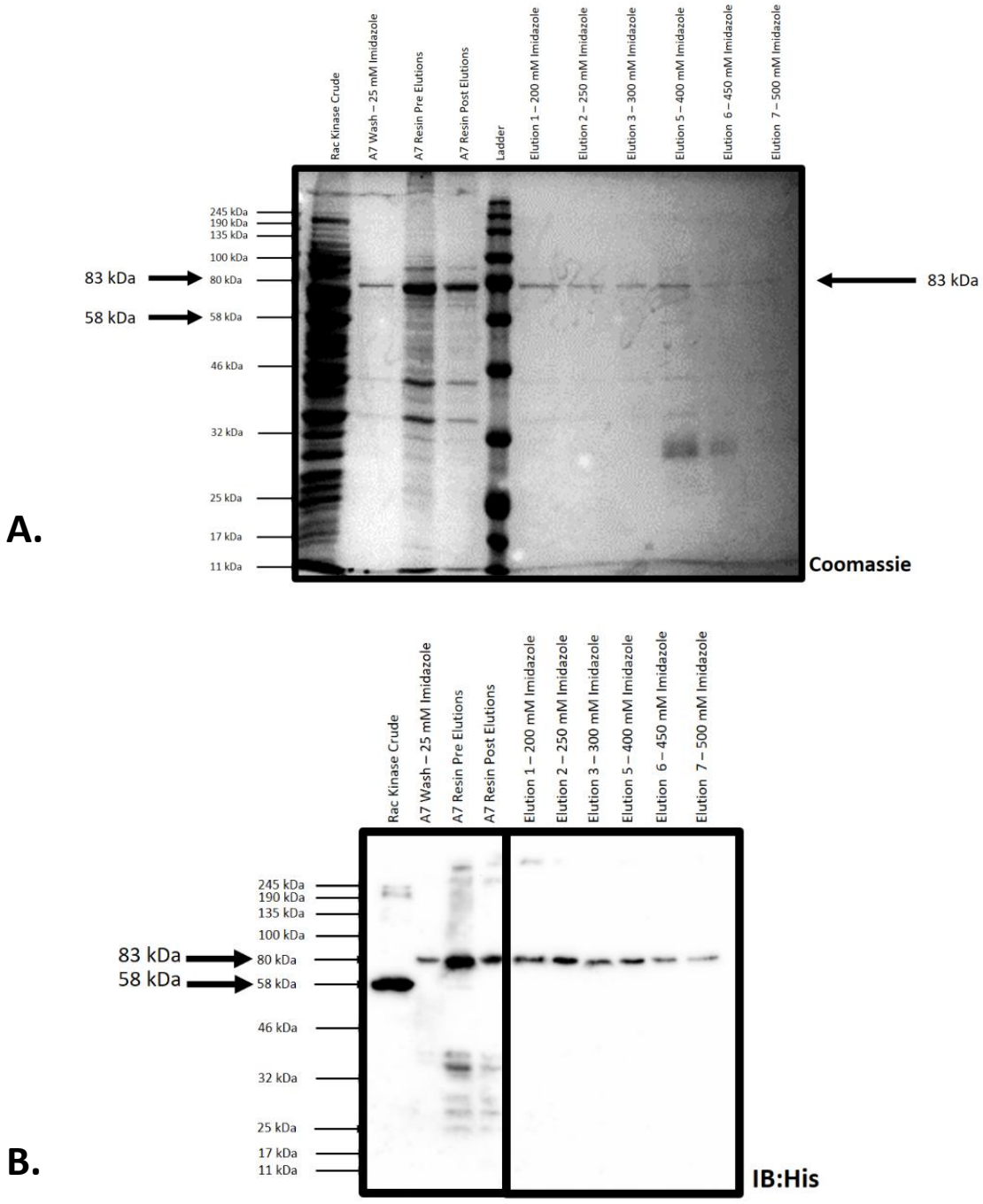


Figure 7: Second A7 Purification Attempt: Gradient Elution Purification was done sequentially. A) A7 Gradient Elution Purification Coomassie Staining. B) His immunoblot of A7 Gradient Elution Purification.

A7 Expression and Solubilization by BPer Lysis Conditions:

The HisPur Ni-NTA resin may be optimized at 7.4 pH similar to Equilibration Buffer, Wash Buffer, and Elution Buffers. At the time, we postulated that an acidic pH (lower than 7.4) for the lysis buffer would effect the secondary structure, His tag access and solubility, which in turn could negatively affect the resin's ability to bind to the His epitope tag, or release A7. 6.5 pH lysis buffer was used because it was the optimal pH condition for the chicken egg white lysozyme. Therefore, BPer Buffer purchased from Thermoscientific was tested as a potentially more optimal lysis buffer. BPer Lysis Buffer was designed to lysis bacterial cells specifically. BPer Lysis Buffer's pH ranges from 7.4 to 7.6. The BPer lysis protocol and buffer was compared to the 6.5 pH lysis buffer for the solubilization of A7 (Figure 8). Whole cell lysates were prepared similarly to conditions used on the Rac Kinase BL21 (DE3) samples (Figure 5).

The band corresponding to A7, showed a visibly more intense band with the BPer Lysis Buffer conditions in comparison to the 6.5 pH Lysis Buffer (Figure 8). Since BPer was found to solubilize a greater proportion of the predicted A7, the BPer Buffer lysed BL21+A7:pET303CT-His was then purified through the same HisPur Ni-NTA column and compared with the 6.5 pH Lysis Buffer lysed BL21+A7:pET303CT-His (Figure 8). The HisPur Ni-NTA column was eluted from the column with 5 mL of 400mM imidazole Elution Buffer for both the BPer Buffer and 6.5 pH Lysis Buffer (Figure 9). 400mM imidazole Elution Buffer was used because the previous gradient elution showed the most intense band for both the immunoblot and coomassie stain at this concentration (Figure 7). The BPer Buffer Lysis method, with the purification showed both a more intense band for the predicted A7 (Figure 8; Figure 9). In addition, virtually all the A7 was removed from the resin as indicated by reduction of band intensity when comparing resin pre elution to resin post elution (Figure 9)

A7 Expression and Solubilization Optimzation with BPer:

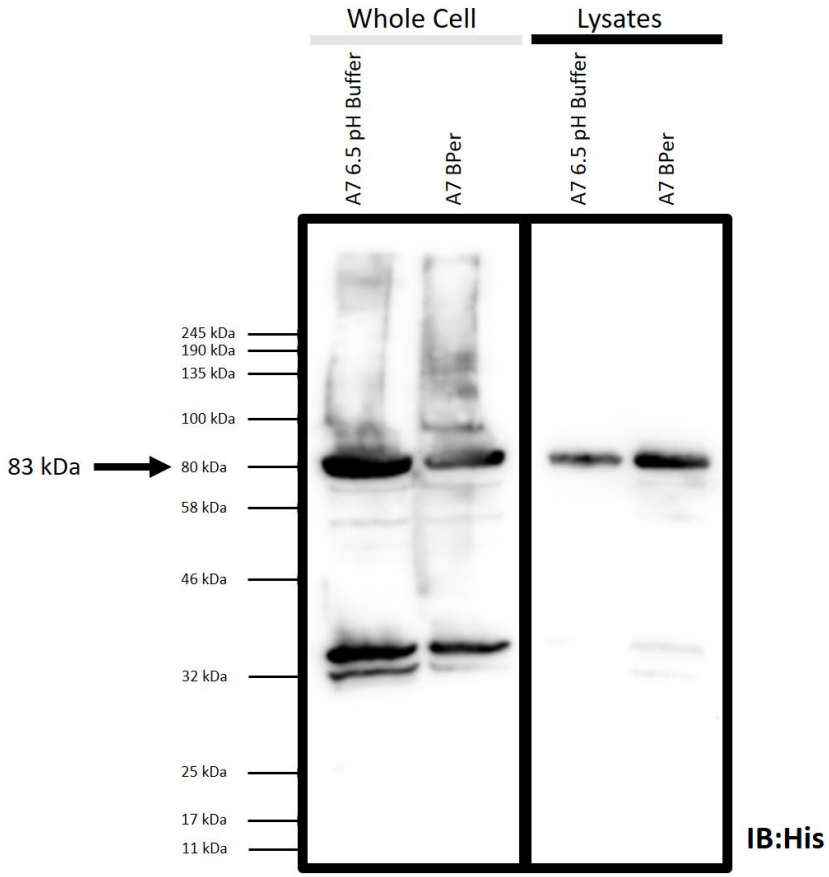


Figure 8: A7 Expression and Solubilization Optimization of A7 with the BPer buffer. A) His immunoblot of whole cell lysates 6.5 pH Lysis Buffer and BPer Buffer of A7. B) His immunoblot of lysates of 6.5 pH Lysis Buffer and BPer Buffer of A7.

A7 Purification with BPer Lysis Condition:

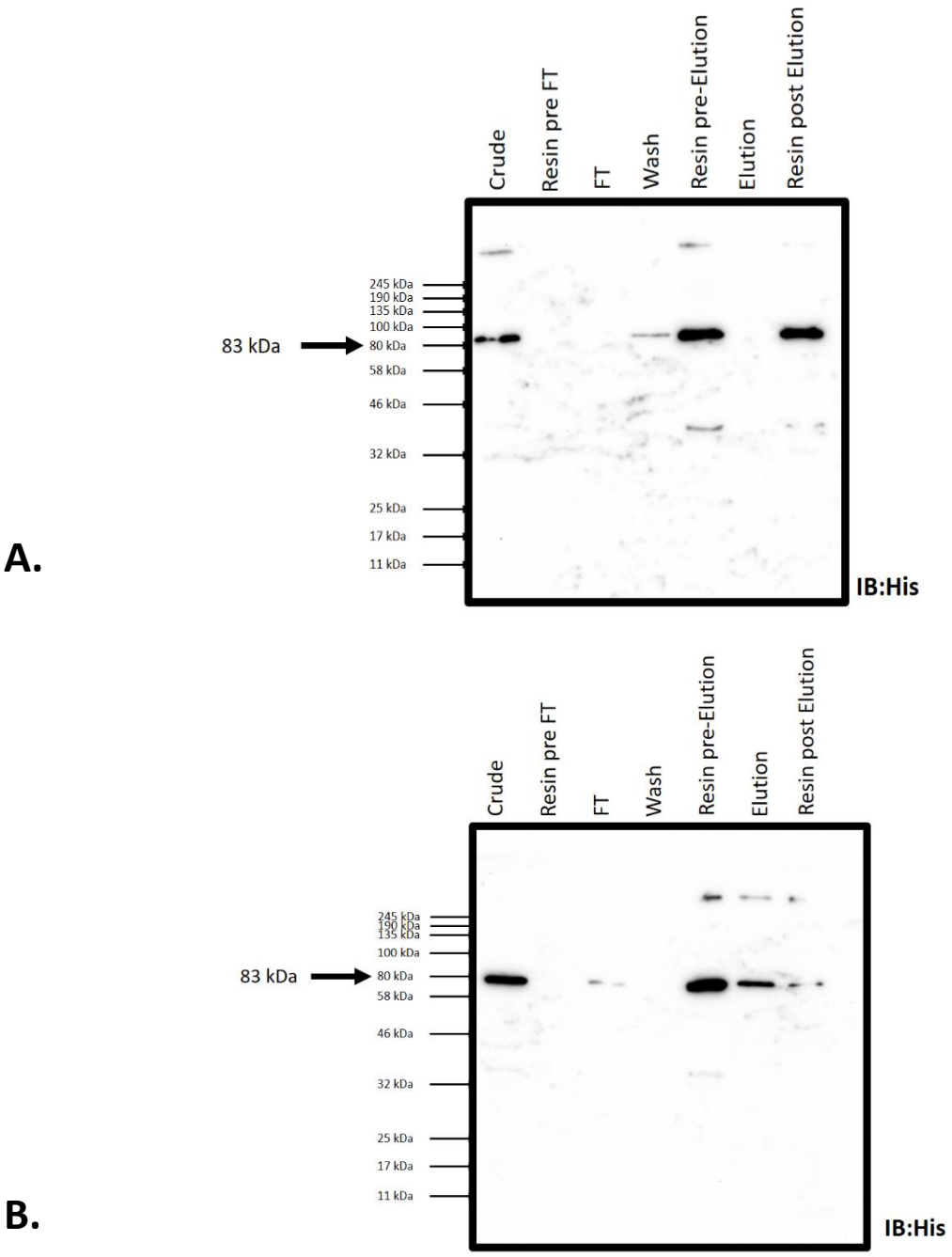


Figure 9: A7 Purification with BPer Lysis Condition. A) A7 purification lysed by 6.5 pH Lysis Buffer. B) A7 Purification lysed by BPer Buffer.

D6 Expression and Solubilization:

Since BPer more efficiently lysed and produced better purification results for the A7 protein (Figure 8, Figure 9), the second VETF D6 was expressed and lysed using the BPer lysis buffer. BL21 (DE3) *E.coli* were transformed with either BL21+D6:pET303NT-HispET303CT-His. The BL21+D6:pET303NT-His was inoculated and induced with IPTG, following the same protocol used for the BL21+A7:pET303CT-His (Figure 3). The BL21+D6:pET303NT-His was lysed using the BPer Buffer and analyzed by an immunoblot. There was only a single visible band for the predicted size of D6 at 72kDa, from the BL21+D6:pET303NT-His lysate with 1mM of IPTG (Figure 10). There was no visible band in the absence of IPTG for BL21+D6:pET303NT-His lysate and BL21+pET303CT-His lysates both in the presence and absence of IPTG (Figure 10).

D6 Expression and Solubilization:

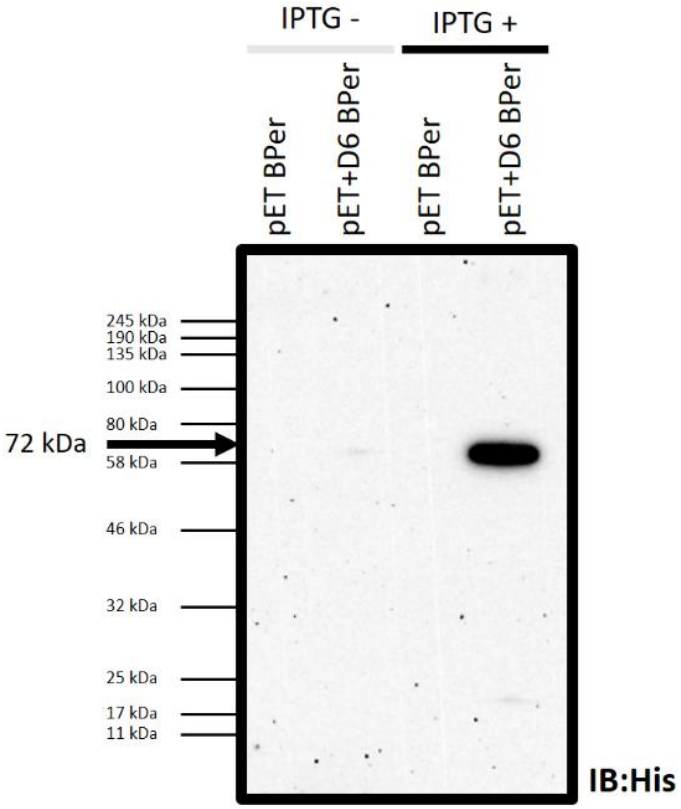
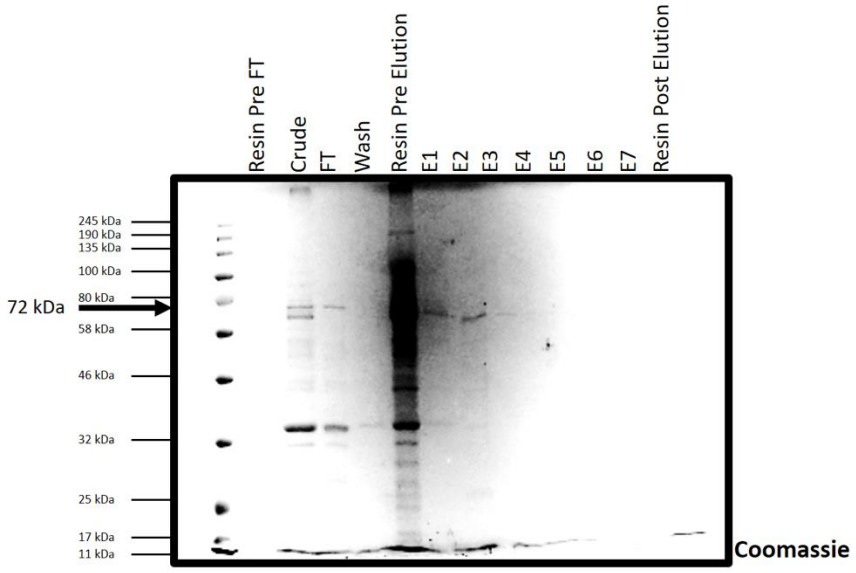


Figure 10: BL21+D6:pET303NT-His and BL21+pET303CT-His Lysates Expression and Solubilization of D6 His Immunoblot.

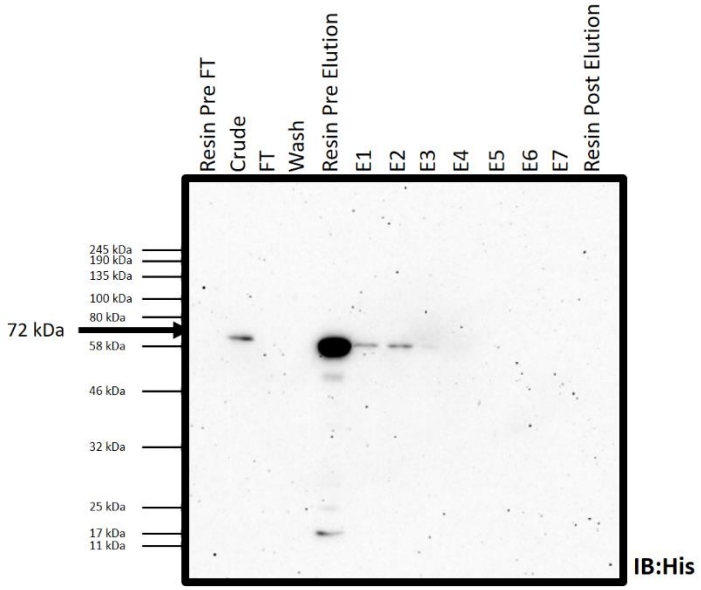
D6 Purification:

Since the predicted band for D6 was observed, the next step was to purify it with the HisPur Ni-NTA column. D6 was purified with an elution gradient using Elution Buffer supplemented with increasing concentrations of imidazole (200 mM (E1), 250 mM (E2), 300 mM (E3), 350 mM (E4), 400 mM (E5), 450 mM (E6), and 500 mM (E7)). Each buffer had a pH balanced to 7.4. The predicted D6 band showed clear visible bands in the crude, resin Pre Elution, Elution Fraction 1(E1), and E2 (Figure 11). There was a very faint band also visible for E3 that matches the predicted size of D6. No band was detected when the resin was run after all elutions were complete indicating that the majority of the His-D6 protein was eluted from the column (Figure 11B). D6 was also observed in the flow through. This may result because of the limited capacity of the column, where we may have just overloaded the column with D6.

D6 Purification:



A.



B.

Figure 11: D6 Purification. A) Coomassie staining of D6 Purification. B) His immunoblot of D6 Purification.

Discussion:

To our knowledge, this approach represents the first attempt to utilize bacteria to express, solubilize and purify full-length VETFs. A truncated version of D6 was produced in a similar bacterial system to generate an antibody against D6 (Broyles and Fesler 1990). Both A7 and D6 are well known to be difficult to solubilize and purify. Therefore, the methods generated herein are critical for the ultimate goal of testing bacterial purified D6 and A7 for the ability to bind viral early promoters such as the E9L promoter. Once established, this system will allow the *in vitro* testing of scaffolds with an affinity for the unique sequences and structures of poxvirus promoters that hypothetically could inhibit binding of the VETFs. This method and these steps will allow a direct *in vitro* screen to be done safely in a BSL-1 facility, because a live virus is not necessary to run and reproduce these experiments. This method is cost effective method because BL21 (DE3) lines are much easier to maintain, select, and expression studies can be done overnight. Further, not all poxviruses can be cultured in mammalian tissue culture. Many poxviruses have a significant risk of infection and can only be studied in facilities with BSL-3 level containment or higher (i.e. Monkeypox Virus, Variola Virus, etc..). Once complete, our system will provide a cost effective and safe model to study transcription factors from these high-risk viruses.

After the successful purification of A7 and D6, these VETFs will then be directly assessed for their ability to bind to the E9L promoter. The E9L promoter has been cloned into a plasmid construct of pJet1.2 by dual restriction enzyme digest with HindIII and XbaI. The pJet1.2 vector also holds a single off target restriction cut site for PvuI opposite from the E9L promote insert. Thus, cutting with PvuI allows the linearization of the vector. This linearized construct will be tested for binding to A7 and D6 and compared to the empty pJet1.2 vector and

a scrambled E9L promoter sequence. To test for binding, Circular Dichroism (C.D.), Ultraviolet Visible Spectra (UV-Vis), and Electrophoretic Mobility Shift Assays (EMSAs) will be employed to determine if VETFs, A7 and D6, produced in a bacterial cell system maintain their function to bind viral DNA. If A7 and D6 maintain their function display visible shifts in the plasmid constructs withthe transcription factors, then A7 and D6 would be suggested for binding to the plasmid constructs. Drug binding studies can then be performed to further compare shifts in the spectra and assays. The plasmid constructs, A7 and D6 should have its own unique UV-Vis and C.D. spectras. The spectras of the A7 and D6 with the plasmid constructs should exhibit differences in the UV-Vis and C.D. spectra compared to the plasmid constructs alone and the A7 and D6 alone. EMSA's would allow us to visualize transcription factor binding, by cross linking the A7 and D6 onto the plasmid constructs. Running the crosslinked plasmid constructs to our A7 and D6 on a Western Blot and Coomassie would show larger band sizes in comparison to the A7 and D6 without the plasmid constructs.

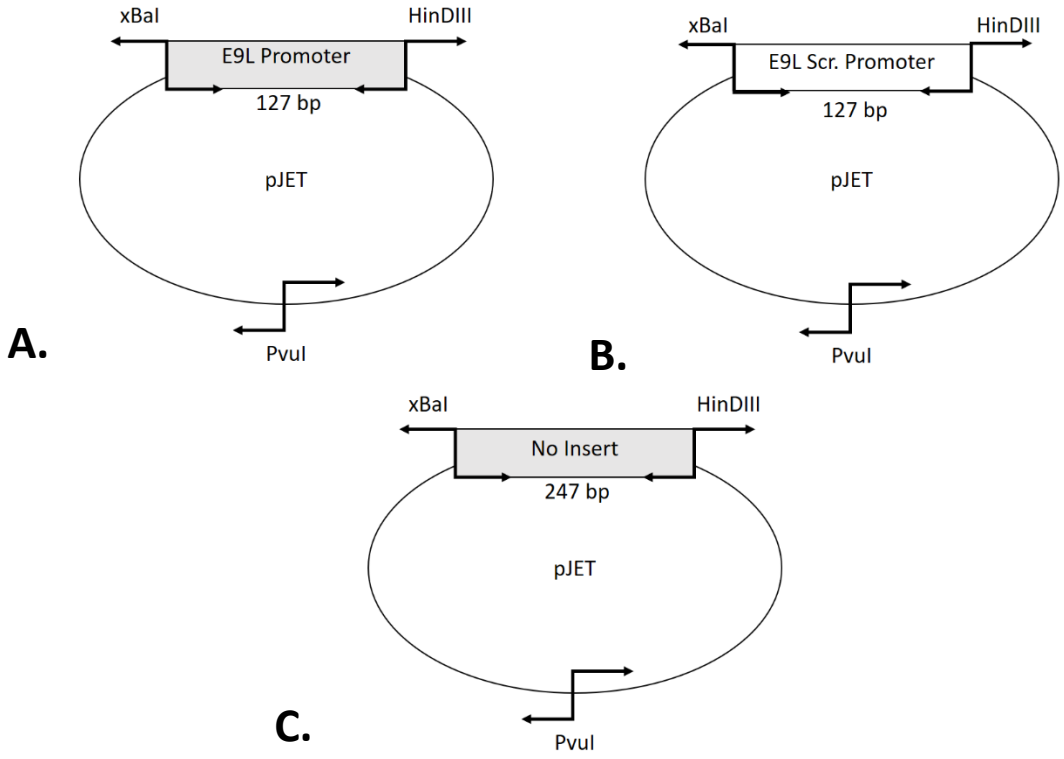


Figure 12: E9L Promoter Constructs. A) E9L promoter construct cloned into pJet1.2 with HindII, xBaI, and PvuI restriction cut sites illustrated. B) E9L scrambled sequence promoter construct cloned into pJet1.2 with HindII, xBaI, and PvuI restriction cut sites illustrated. C) pJet1.2 vector with no E9L promoter insert with HindII, xBaI, and PvuI restriction cut sites illustrated.

It is important to note A7 and D6 produced in bacteria may not fold properly due to possible translational and post-translational modification that A7 and D6 may require that can only be done in a eukaryotic cell line. Currently, we do not anticipate post translational modifications to be a problem as there is no report of either A7 or D6 requiring post translational modification such as glycosylation. However, as an alternative approach A7 and D6 can be produced in an in-vitro system with eukaryotic cells. Eukaryotic cells can be transfected to overexpress A7 and D6 in eukaryotic cell culture line or be infected with live VACV to obtain A7 and D6. One potential cell line for expression and purification studies is the insect cell line Sf9 cells. Insect cell lines provide a happy median between mammalian cells and bacteria since insect cell culture is easier and cheaper to maintain than most mammalian cell culture produces proteins with similar post-translational modification as would be obtained from mammalian cell culture (McKenzie and Abbott 2018). Also insect cell lines can be transfected with plasmids to express signaling sequences to make A7 or D6, and then export it extracellularly. Sf9 cells have been previously used as well to produce viral proteins successfully like viral decoy tumor necrosis factor receptor and influenza nucleoproteins (Shen et al. 2014; Yoon et al. 2018).

Through this study, A7 and D6 were expressed and purified in a bacterial system with BL21 (DE3) bacteria. The research described herein overcame numerous technical challenges and resulted in the creation of a system to overexpress and study the VETFs using bacteria. A7 and D6 produced and purified through this system can safely be done in a BSL-1 facility without the need for a live virus. This system could provide a safe and responsible screening method for drug design and discovery in the future. Further, the approach can be further adapted to study binding of poxvirus transcription factors to target promoters. When successful, this assay will provide a low cost and safer means in which to screen compounds for the ability to bind early

promoter sequences and prevent binding of VETFs, which can be later validated for antiviral activity in cell cultures with live virus. The assay being developed does not require the initial use of live virus and can be safely performed in a BSL-1 level facility. This system would potentially enable us to develop a new anti-poxviral therapy that targets the VACV DNA polymerase, E9L, early gene transcription in Poxviruses, which would halt or delay the Poxviral replication cycle. Because the E9L promoter is highly conserved, this drug may potentially be a broad spectrum anti-poxviral drug.

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