

Using CRISPR/Cas9 to screen an *Autographa californica* multiple nucleopolyhedrovirus vector genome for gene essentiality in the production of a HIV-Gag virus like particle

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The baculovirus expression vector system (BEVS) has been widely used for producing recombinant proteins and virus-like particles (VLPs) due to its high protein expression, ability for some complex post translational modifications (PTMs), and high scalability. Of particular interest is the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (*AcMNPV*), which is the most used virus for the BEVS. This baculovirus is amenable to genetic modifications, yet other than replacing or removing a few genes, this baculovirus has not seen major modifications. A hypothesis in the Aucoin lab centers around the idea that more product can be made if less resources are funnelled away from this goal [1]. Recently, a transfection-infection (TI) assay has been described [2], where the baculovirus genome can be scrutinized. Briefly, a stable Sf9 cell line expressing Cas9 (Sf9Cas9) [3], which has been shown to produce similar levels of green fluorescent protein and baculovirus as the native/parental Sf9 cells, is transfected with a plasmid hosting a single guide ribonucleic acid (sgRNA) targeting a specific baculovirus genomic sequence and subsequently infected with a baculovirus vector that is able to express a gene of interest/reporter product. However, given the large genome of the baculovirus, and the number of genes that can be scrutinized, higher throughput methodologies are required. This work addresses some of the engineering challenges related to the scale of the T-I assay and the associated analyses. These changes are then implemented and used to evaluate the essentiality of 21 late or very late genes in *AcMNPV* to produce BV and VLPs. More specifically, the effect of targeting these specific genes is being examined when expressing an enveloped virus-like particle under the control of the *p6.9* promoter.

Moving the T-I assay from a 6-well plate (2.5×10^6 cells/well in 2mL of media), to a 12-well plate required a change in cell seeding density (0.9×10^6 cell/well in 0.8mL of media) to obtain similar infection and production dynamics. Further, using a human immunodeficiency virus (HIV) group specific antigen (Gag) fused with a green fluorescent protein (GFP) virus-like particle (VLP), or Gag-GFP for short, the effect of targeting genes could be assessed on the release of VLPs in the culture supernatant using flow cytometry. Dilutions of cell culture supernatant in a 96-well plate allowed dilutions to be done in parallel, which increased the throughput of VLP quantification using a 96-well plate compatible flow cytometer. Although VLP concentrations were consistently 10%-20% higher when diluted in the plate condition compared to microcentrifuge tubes, this amount was deemed to be acceptable due to the consistent effect. Furthermore, VLP stability over a 7-hour period was tested. It was found that VLPs started degrading 1.5 hours after initial dilution. This was found to be long enough for 15 samples to be run through the flow cytometer at a time and maintain VLP integrity.

Controls were assessed for the T-I assay. One negative control involved only infecting cells with the baculovirus coding for Gag-GFP (infected control). A second negative control involved the use of transfection reagent without any plasmid followed by infection with the baculovirus coding for Gag-GFP (transfection reagent control). A third negative control involved a complete transfection step with a plasmid coding for a sgRNA that was not directed towards any baculovirus sequence. The latter is referred to a scrambled sequence control (or scrambled control for short). A fourth control involved a complete transfection step with a plasmid coding for mKate2, which is a red fluorescent protein (mKate2 control). It was observed that the transfection reagent control, the scrambled control, and the mKate2 control produced higher amounts of budded virus compared to the infected only control. Not only did we determine that that the transfection reagent increased

infectious baculovirus in our assay, we determined that the scrambled control was the most appropriate control for the T-I assay.

Of the 21 genes scrutinized as part of this work, deletion of *lef-2*, *bion*, *lef-1*, *kip* and *lef-11* all lowered budded virus production, which was consistent with previous studies. Deletion of *pif2* lowered budded virus but has been previously reported to be non-essential. Deletion of *ptp*, *Ac4*, *pk1*, and *fgf* showed some indication that budded virus production was impaired, but failed to consistently meet the threshold for significance at a 95% confidence interval across all constructs tested. Deletion of *v-ubi*, *Ac38*, *lef-12*, and *Ac51*, which were previously reported to have lower levels of budded virus upon gene deletion, were found to have no effect in our study. Finally, *Ac19* and *env* displayed no effects on budded virus and VLP production. These final two genes are possible candidates for genes that may be non-essential and should be screened further.

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Dedication

I want to pay my respects to Professor Ray Legge. Although I didn't get to know you outside of the lecture hall, you were one of the reasons why I became interested in this field. You went the extra mile to get to know your students and have helped generations of students within this field. May you rest in peace.

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List of Abbreviations

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| AcMNPV | <i>Autographa californica</i> multiple nucleopolyhedrovirus |
| Au | Arbitrary unit |
| BAC | Bacterial artificial chromosome |
| BCA | Bicinchoninic acid assays |
| BEVS | Baculovirus Expression Vector System |
| BV | Budded virus |
| Cas9 | CRISPR associated protein 9 |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| cVLP | Chimeric virus-like particle |
| CSP | Circumsporozoite protein |
| DLS | Dynamic light scattering |
| DNA | Deoxyribonucleic acid |
| eGFP | Enhanced green fluorescent protein |
| ELISA | Enzyme-linked immunosorbent assays |
| GFP | Green fluorescent protein |
| GV | Granulovirus |

| | |
|--------------|----------------------------------|
| HBV | Hepatitis B virus |
| HIV | Human immunodeficiency virus |
| hpi | Hours post infection |
| HPV | Human papillomavirus |
| MOI | Multiplicity of infection |
| mAG | Monomeric Azami-Green |
| NPV | Nucleopolyhedrovirus |
| NTA | Nanoparticle tracking analysis |
| OB | Occlusion body |
| ODV | Occlusion-derived virion |
| ORF | Open reading frame |
| PBS | Phosphate buffered saline |
| PTM | Post translational modification |
| sgRNA | Single guide ribonucleic acid |
| Sf | <i>Spodoptera frugiperda</i> |
| TEM | Transmission electron microscopy |
| TI | Transfection Infection |
| VLP | Virus-like Particle |

1 Introduction

The baculovirus expression vector system (BEVS) is a platform used for the production of recombinant proteins and has garnered attention for the production of virus-like particles (VLPs) [4]. This system uses a modified baculovirus vector to infect insect cells to produce a desired product. The *Autographa californica* multiple nucleopolyhedrovirus (*AcMNPV*) is a baculovirus with ~150 open reading frames (ORFs) and is commonly used in the BEVS [5]. Despite its use, the *AcMNPV* genome has not been fully characterized and thus there may be genes that are non-essential for the production of budded virus (BV) and recombinant protein production in cell culture. As such, there is potential to streamline the *AcMNPV* genome through the removal of non-essential genes, which can relieve cellular resources towards recombinant protein production, and lower the amount of contaminants thus leading to easier downstream purification processes.

VLPs are particles that resemble their viral counterparts but contain no genetic material. As they do not contain any genetic material, they are non-infectious meaning that they cannot infect healthy cells and self-replicate. Since VLP structures closely resemble that of a viral particle, they can elicit an immune response like that of a native virus but pose minimal threat to the host. As such, VLPs are an interesting field of study and have found use as vaccines, as is the case with Cervarix™ (GlaxoSmithKline) which is used for immunization against the human papillomavirus (HPV) [6].

Screening of genes has generally required the production of individual knockout viruses which are time, resource, and labour intensive, thus making it difficult to probe an entire genome [7]. Due to its relative ease for genetic engineering, the clustered regularly interspaced short palindromic repeats (CRISPR) system with CRISPR associated protein 9 (Cas9) has become a

powerful tool for the insertion and deletion of genes in a variety of genomes. In previous work, the CRISPR/Cas9 system was used to develop a Transfection-Infection (TI) assay to screen the *AcMNPV* genome [2]. In the TI assay, a modified *Spodoptera frugiperda* clonal isolate 9 (Sf9) cell line was developed, wherein the cells constitutively produced Cas9, referred to as Sf9-Cas9 cells. These cells were plated into a 6-well plate then transfected with a single guide ribonucleic acid (sgRNA) that targeted a desired ORF in the *AcMNPV* genome. Cells were then infected with a baculovirus that produced a recombinant protein and the effect of targeting the ORF was assessed. Effects of the gene disruption are quantified through BV and recombinant protein production. This assay decreases the amount of time it takes to screen a gene from months to the span of a few weeks. As well, the assay is scalable, allowing for the testing of multiple genes in parallel. This assay is another tool that displays the versatility of the CRISPR/Cas9 system which allows for the efficient screening of a viral genome.

This thesis is motivated by a need to further optimize and scale the TI assay for more efficient and cost-effective screening of the *AcMNPV* genome. Rather than running the assay as previously described, adjustments to plate type, media volume, and transfection reagent volumes were desired. These adjustments were aimed at decreasing the operational costs of running the TI assay, while maintaining the ability for a high throughput genetic screening tool. Furthermore, with these adjustments being made, the assay was used to probe late and very late genes in the *AcMNPV* genome.

Furthermore, this thesis is motivated by developments made on the quantification of VLPs. In recent developments in the Aucoin lab, it was observed that HIV-Gag VLPs (Gag VLPs) tagged with a green fluorescent protein (GFP) could be quantified using flow cytometry under a $533 \pm 30\text{nm}$ light filter. Previously, VLP quantification was done using methods such as bicinchoninic

acid assays (BCA), enzyme-linked immunosorbent assays (ELISA), nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and dynamic light scattering (DLS) [8], [9]. These assays have various drawbacks such as cost, and the potential inability to discern between VLPs and other types of proteins and cellular products. This development of using flow cytometry to quantify VLPs allowed for a quick and reliable test to determine VLP concentrations. As such, this thesis aims to incorporate this and use the Gag-GFP as a model recombinant protein product.

VLP quantification using flow cytometry must have a linear decrease in particle counts when compared to the dilution to obtain an accurate conversion to concentration. For Gag VLPs produced in Sf9 cells, samples require multiple dilutions (10^{-3} , 10^{-4} , and 10^{-5}) to observe this linear decrease in particle counts. Dilutions have previously been done in microcentrifuge tubes, which are then transferred to polystyrene tubes compatible with the flow cytometer. With the aim of screening all baculovirus genes, this equates to hundreds of samples due to replicates and multiple targets for each gene. As such, individual dilutions in microcentrifuge tubes becomes a significant time and resource sink. To alleviate this, it was proposed to do dilutions in 96-well U-bottom plates which allows for the use of multichannel pipettes, lowers amounts of dilution media thus, and thus increases the overall time and cost efficiency of this screening assay.

The long-term goal of this work was to increase the cost and time efficiency of running the TI assay. It also aimed to study the use of VLPs as a model recombinant product, allowing for a quick and easy way to quantify the effects of a gene knockout on a different class of products (VLPs). Ultimately, these developments were expected to lead to the effective probing of the *AcMNPV* genome, and result in the identification of non-essential genes that can become targets of removal for improved vaccine production.

1.1 Research Objectives and Hypotheses

The overall objective of this thesis is to decrease the resources used in the TI assay, and to show a proof-of-concept that Gag-GFP VLPs can be used as an easily quantifiable recombinant protein product to determine the effects of a gene-knockout. This goal can be divided into four sub-objectives and hypotheses:

1. Determine the conditions in which the 12-well plate produces similar levels of budded virus and protein when compared to the optimized 6-well plate conditions. By maintaining similar cell concentrations between 6- and 12- well plates, infection conditions will remain consistent and thus produce similar levels of budded virus and protein.
2. Increase the throughput of Gag-GFP VLP quantification using flow cytometry. Currently, samples are diluted in tubes which requires a lot of time and resources. By diluting in 96-well plates, a multichannel pipette can be used which allows for dilutions to be done in parallel. These plates are also compatible with the flow cytometer, thus reducing time resource consumption.
3. Determine a control in which gene knockout effects can be compared to. Two controls are proposed: (1) a control where cells are only infected (infected only control) and (2) a control where cells are transfected with a plasmid that contains a scrambled sequence and then infected (scrambled control). Here it is proposed that when a plasmid is transfected into a cell, then the cell has undergone a stressor which will affect budded virus and protein expression.
4. After objectives (1), (2) and (3) are completed, 21 late and very late genes are to be tested for the assay. Here it is proposed that under the new TI assay conditions, positive and

negative effects can be observed on budded virus production. Moreover, the effects on protein production can be quantified through flow cytometry.

1.2 Thesis Outline

This thesis is divided into 8 chapters. The first chapter was a general introduction into the BEVS, VLPs, the TI assay and research objectives. Chapter 2 provides a literature review about the development and properties of the BEVS, usage of VLPs as vaccines, and expressions systems used to produce VLPs. Chapter 3 outlines general materials and methods that overlap throughout the thesis. Chapter 4 delves into the work done to transition the TI assay from being done in 6-well plates to 12-well plates. Chapter 5 presents the work done to determine any differences in VLP concentrations when dilutions were done in tubes vs plates. It also explores the degradation of VLPs at room temperature to ensure no degradation occurs while samples were run through the flow cytometer. Chapter 6 explores the positive controls that are using in the TI assay and whether the transfection reagent has an unintended effect on BV and protein production. Chapter 7 explores the effects of gene disruption on BV and VLP production and chapter 8 provides a unifying discussion, conclusions and recommendations for future work in this area.

2 Literature Review

2.1 Baculovirus

Baculoviruses are a group of enveloped, double stranded DNA (dsDNA) viruses specific toward the infection of arthropods and belong to the *Baculoviridae* virus family. Their characteristics include a rod-shaped nucleocapsid that is 30-60nm in diameter and 250-300nm in length, and contains a circular dsDNA molecule. In general, there are two virion phenotypes of baculoviruses that allow it to propagate: occlusion-derived virions (ODVs) and budded virions (BVs). ODVs are responsible for transmission between insects by initiating the infection in the midgut epithelium of an infected insect. In contrast, BV is responsible for transmission between the cells of an infected insect. As such, after an initial infection by ODV, BVs are produced, thus allowing the virus to propagate through the rest of the host. Upon cell lysis, ODVs are released, thus propagating baculovirus throughout an insect population [10]. Overall, both ODVs and BVs are identical with respect to genetic makeup, however, they differ in their structural characteristics and function [11].

When ODVs are released, they are contained within a crystalline polyhedrin protein matrix, called the occlusion body (OB). The purpose of the OB is to protect the virus from the environment and allow it to propagate between insect hosts. Once the OBs reach the midgut of an insect, the alkaline conditions cause the polyhedrin matrix to dissolve, thus allowing the virions to infect epithelial cells [11]. The structure of the OB allows further classification of baculoviruses: Nucleopolyhedrovirus (NPV), wherein one OB contains multiple ODVs, and Granulovirus (GV), wherein an OB is generally made up of one ODV [12]. Since ODVs require alkaline conditions to become infectious, BVs are used for infection in cell culture for research and commercial purposes.

2.2 *Autographa californica* multiple nucleopolyhedrovirus

The *Autographa californica* multiple nucleopolyhedrovirus (*AcMNPV*) is of the *Alphabaculovirus* genus of *Baculoviridae*. This virus contains a DNA sequence of 133,894 base pairs (bps) and around 150 open-reading frames (ORFs) [5], [13].

Infection of a host cell begins through adsorptive endocytosis which is then followed by the release of the nucleocapsid into the cytoplasm which is mediated by the surface glycoprotein gp64 [14], [15] and the nucleocapsids make their way to the nucleus where they enter via pore complexes [16] and the genetic material is released. The viral genome is then used for viral gene transcription and replication.

The infection cycle can be divided into three phases: early, late, and very late. The early phase can be subcategorized into immediate early and delayed early [17]. The early phase generally spans 0-6 hours post infection (hpi) and is characterized by the expression of genes that are recognized and transcribed by the host cell RNA polymerase II. Specifically, the genes expressed in the early phase encode for transactivators that are required for viral gene expression and suppression of host cell functionality [18]. The late phase generally spans 6-18 hpi and is characterized by genes that are recognized and transcribed by an RNA polymerase derived directly from the viral genome [17]. Moreover, during this phase, viral DNA is replicated and nucleocapsids are produced. The nucleocapsids leave the nucleus and migrate towards gp64 rich areas on the cell membrane where they bud out of the cell, thus producing BVs [19]. The very late phase begins ~18hpi and lasts until cell death [11]. It is characterized by the production of polyhedrin (polh) protein, which is the main component in the matrix of OBs, and p10 protein, which constitutes major fibrillar structures within infected cells that potentially stabilize the cell for the maturation of OBs [20].

2.3 Baculovirus Expression Vector System (BEVS)

The Baculovirus Expression Vector System (BEVS) uses a transfer plasmid that contains a gene that codes for a recombinant protein, a baculovirus vector – in many cases *AcMNPV*, and an insect cell line [21]. Transfer plasmids can be modified depending on the purpose such as containing sequences for signal peptides that increase the efficiency for the secretion of proteins, different promoters for different temporal expression profiles, purification tags to ease recovery, and much more [21]. Useful promoters that have been extensively used in the BEVS system are *polh* and *p10* since they are active in the very late stage of the infection cycle and are shown to result in high recombinant protein yields. Another promoter of note is *p6.9* since it has been shown to produce more recombinant protein than *polh* under specific conditions [22].

The first commercial baculovirus vector was the BacPAK6TM system by Clontech. In this system, the circular DNA of *AcMNPV* is linearized by subjecting it to the restriction enzyme *Bsu36I* to remove a portion of the essential gene *orf1629* (*Ac9*). This DNA is then mixed with a transfer plasmid that contains a foreign gene along with the complementary *orf1629* sequence and the mixture is transfected into insect cells where homologous recombination occurs to insert the transfer plasmid sequence into the baculovirus genome. This homologous recombination restores the *orf1629* gene and re-circularizes the DNA, thus making the viral genome infectious. The infection cycle can then begin, and BV that contains the recombinant genome is produced and harvested. However, since *Bsu36I* digestion is not 100% effective, there is a mix of recombinant and parental baculovirus that is produced. As such, the recombinant baculovirus needs to be isolated via plaque-purification. [21]

Plaque-purification is labor-intensive, and thus there was a desire for a system that removes this step. The Bac-to-BacTM system by Invitrogen did this by introducing bacterial artificial

chromosomes (BACs) into the viral genome which allows recombinant baculovirus to be made and amplified in bacteria. To do this, three genes are inserted into baculovirus DNA: (1) a mini-F replicon (2) an antibiotic resistance gene and (3) a Tn7 transposition site. The transfer plasmids contain a Tn7 transposase sequence allowing for the plasmid to fuse to the baculovirus genome at the *polh* locus. Bacteria containing the recombinant baculovirus can then be screened using agar plates treated with the antibiotic and can then be amplified. The baculovirus DNA, hence known as the bacmid, is then transfected into insect cells where it produces budded virus which can be used to infect other cell cultures [21]. A major drawback of this system is the loss of recombinant gene expression over multiple passages of the virus [23].

Even further, the *flashBAC*TM system removes the requirement for screening, thus simplifying the process for recombinant baculovirus production. In this system, the *AcMNPV* genome is linearized, part of the *orf1629* gene is removed, and a BAC at the *polh* locus is added. This DNA can only be propagated in bacterial cells since the partial removal of *orf1629* prevents replication in insect cells. After a bacterial culture containing this DNA sequence is grown, cells are lysed, and DNA is purified using cesium chloride gradients. This process can be done manually, or the DNA can be purchased directly from Oxford Expression Technologies. The baculovirus DNA can then be transfected into insect cells along with a transfer plasmid that contains the removed *orf1629* sequence. Recombination occurs within the insect cell allowing the DNA to be replicated, and BVs containing the inserted gene are produced. [21]

2.4 Virus-Like Particles (VLPs)

Virus-like particles (VLPs) are nano-scale particles that resemble their viral particle counterparts but contain none of the genetic material. Since they do not contain any DNA or RNA, VLPs are non-infectious, and thus, unlike live-attenuated viruses, cannot evolve into a viral strain

that could cause disease. As well, due to their resemblance to their viral counterparts, VLPs generally elicit a stronger immune response than inactivated and live-attenuated vaccines, making them very useful as vaccine candidates [24], [25], [26].

VLPs vary in size, generally ranging from 20-200nm in diameter. This size allows VLPs to easily enter lymphatic nodes, and allows uptake by antigen-presenting cells, both of which contribute to eliciting a strong immune response [24]. They are mainly comprised of viral coat or envelope proteins and have a high density of viral surface proteins. VLPs can be classified further as either enveloped or non-enveloped, depending on whether the VLP takes part of the plasma membrane upon budding out of the cell.

Non-enveloped VLPs are generally comprised of the viral nucleocapsid proteins of their respective viruses. Non-enveloped VLPs are generally smaller than their enveloped counterparts due to the lack of the lipid layer taken from the plasma membrane. The lipid layer of enveloped VLPs can make downstream purification more difficult due to the host cell proteins existing on both the VLPs, budded viruses, and extracellular vesicles [27], [28], [29].

Another subset of VLPs are chimeric VLPs (cVLPs). These are VLPs that have protein constituents from two or more viruses. One commercial example of a cVLP is the Mosquirix™ (GlaxoSmithKline) vaccine which is used to immunize against malaria. Although malaria is a protozoan disease, VLPs have been found to be an effective tool in vaccination. Initially, the circumsporozoite protein (CSP) antigen was found to be a target that could be used to generate an immune response against malaria. However, it failed to stimulate an effective immune response in a free protein form. Later, the central repeat region of CSP was fused to the hepatitis B surface antigen in a hepatitis B VLP, and a stronger immune response was generated [30]. Overall, cVLPs

have the potential to simultaneously immunize against a variety of viral diseases and have shown potential in immunization of non-viral diseases.

2.5 Expression Systems for VLP Production

One production system that has been used to produce VLPs is bacteria. In particular, *Escherichia.coli* (*E.coli*) is one of the most widely used platforms for recombinant protein production, and has found use in the production of VLPs [4]. This system offers scalability with high growth rates and high cell densities, high protein expression levels, and low production costs which makes them lucrative for large scale production of VLPs [4]. Despite these benefits, VLP production in bacterial systems has been limited with only a few products like Hecolin®, a Hepatitis E vaccine produced by Wantai BioPharm and only approved for use in China and Pakistan [31]. Another example is Cecolin®, which is a human papillomavirus vaccine also produced by Wantai BioPharm. This lack of VLP based vaccines produced in bacterial systems may be due to the limited post translational modifications in bacteria [32]. Despite this, research is ongoing, and multiple products are in the works including vaccines for West Nile virus, Foot-and-Mouth disease virus, and Hepatitis C virus [33].

Yeasts have also been used for VLP production. *Saccharomyces cerevisiae* and *Komagataella pastoris* are popular organisms for VLP production due to high scalability, high protein expression levels, low production costs, and ability to perform simple PTMs [4]. Using this expression system has yielded some VLP vaccines such as the Energix®-B (GlaxoSmithKline) vaccine used against hepatitis B virus (HBV), the Gardasil® (Merck & Co.) vaccine used against HPV, and the Mosquirix™ vaccine used to protect against malaria [4], [30]. The latter is used to protect against *Plasmodium falciparum*, one of five malarial species that can infect humans, thus showing the

ability for VLPs not only able to vaccinate against viruses, but used as an adjuvant to vaccinate against protozoan infections [30]. Although yeasts have shown great promise, they lack more complex PTMs that higher eukaryotes have [4].

The BEVS is among the most popular systems for the production of VLPs, and allows for the production of enveloped VLPs [4]. Common hosts are Sf9, Sf21, and High Five cells, which allow for high protein expression levels, scalability, and the ability to perform complex PTMs [6]. The HPV vaccine CervarixTM is produced using BEVS and consists of both HPV16 and HPV18 L1 proteins [34]. Although this is the most notable FDA approved VLP, there are other recombinant vaccine products that are produced using the BEVS, such as the Flublok® (Sanofi) vaccine which protects against the seasonal flu strains, thus displaying the quick scalability of the BEVS [35]. As well, the Nuvaxovid® (Novavax) vaccine for SARS-CoV2 is of interesting note as the spike protein is produced using BEVS, but the proteins are adjuvanted onto lipid nanoparticles from the Matrix-M formulation [36]. The Nuvaxovid® formulation displays new potential for vaccine development, wherein subunit proteins can be adjuvanted onto lipid nanoparticles, emulating similar effects to that of a VLP.

Mammalian cells are usually used to produce complex biotherapeutics such as monoclonal antibodies. Mammalian cells generally produce less protein compared to other systems, and are more difficult to scale up which is a massive drawback for widespread vaccination efforts [37]. As such, mammalian cells are generally used to produce VLPs that contain multiple structural proteins that require complex and accurate PTMs [37]. Mammalian cell lines that have been used for VLP production include Chinese hamster ovary (CHO) cells, baby hamster kidney-21 (BHK-21) cells, human embryonic kidney 293 (HEK-293) cells, CAP-T cells, and Vero cells [4]. Of particular interest are CHO cells which lower the risk of contamination with human viruses as they are not

derivative of human cells [37]. Due to high production costs and low yields, commercial use is limited, with Sci-B-Vac® (VBI Vaccines), used for HBV immunization, being one of few in production.

2.6 CRISPR/Cas9

The clustered regularly interspaced short palindromic repeats (CRISPR) system was first acknowledged in 1987 while Ishino et al. were studying the *iap* gene of *E.coli* [38]. In this study, it was acknowledged that “five highly homologous sequences of 29 nucleotides were arranged as direct repeats with 32 nucleotides as spacing” [38]. In the coming years, repeat sequences were found in different species of bacteria and archaea that were separated by various nucleotide sequences termed ‘spacers’ [39], [40], [41], [42]. In 2005, Mojica et al. found that the spacer sequences between the repeat regions were strands of DNA found in bacteriophages and conjugative plasmids [43]. Moreover, they found that cells that contained the spacers were unable to be infected with the virus that the spacer belongs to, thus providing a link between CRISPR and phage immunity [43]. Alongside the discovery of the CRISPR sequences in bacteria and archaea genomes, four CRISPR associated proteins (Cas) were identified, with Cas3 showing similarities to a group of DNA-modifying proteins and Cas4 showing similarities to RecB exonucleases [44]. Later it was confirmed that the CRISPR/Cas system was directly responsible for protection against foreign genetic material [45].

The CRISPR/Cas process can be broken down into three steps – adaptation, expression, and interference [46]. In the adaptation phase, a foreign piece of DNA is recognized by the cell. On this DNA, a nucleotide sequence of ~3bp is identified, referred to as the protospacer-adjacent motif (PAM). The PAM is downstream of a protospacer, which is a 20-30bp sequence that is cut from

the foreign DNA by Cas1 and Cas2 [46]. The protospacer sequence is then appended to the 5' end of the CRISPR array on the host cell chromosome, followed by the addition of a repeat region.

In the expression phase, the CRISPR array is transcribed into RNA, generating the pre-CRISPR RNA (pre-crRNA), which is then cleaved at the repeat regions to generate smaller strands of CRISPR RNA (crRNA) [46]. Here, CRISPR/Cas systems can be categorized into Class 1 and Class 2, where Class 1 systems consist of multi-subunit effector complexes and Class 2 systems consist of single-protein effector modules [47]. Each class is subcategorized into 'types', that refer to the Cas proteins that are used to cleave genetic material, where Class 1 consists of type I, III, and IV, whereas Class 2 consists of type II, V, and VI (Koonin et al., 2017). Of particular interest is type II found in *Streptococcus pyogenes* that uses a trans-encoded small RNA (tracrRNA) that binds to the repeat region of the crRNA, thus producing a guide RNA (gRNA) [48]. Cas9 protein then attaches to the gRNA by recognizing the tracrRNA, thus producing a Cas9-gRNA complex. In the interference stage, Cas9-gRNA complex finds foreign DNA, and identifies a PAM sequence. When a PAM sequence is found, the upstream nucleotides are checked against the spacer sequence. If the spacer sequence is complementary, the Cas9 protein cleaves the DNA in the protospacer region [49].

In 2012, Cas9 was purified from *Streptococcus pyogenes*, and determined that three components were required for the cleaving of genetic material to produce double stranded DNA breaks (DSBs) – Cas9, tracrRNA, and crRNA [50]. Moreover, they showed that crRNA and tracrRNA could be combined into one strand of RNA that can be used in conjunction with Cas9 to induce DSBs and showed that the CRISPR/Cas9 system could be used to cleave almost any gene [50]. In higher eukaryotes, DSBs are repaired through non-homologous end joining (NHEJ), or homology-directed repair (HDR) pathways, both of which are useful for the addition, or deletion

of genes [51]. In particular, NHEJ can produce insertion-deletion (indel) mutations wherein a nucleotide is removed or added upon the repair of the DNA strand that disrupt the reading frame [51]. In contrast, the HDR pathway can be used to introduce new genes into a target location [51].

Since these developments, the CRISPR/Cas9 system has been used to modify various genomes. In the BEVS some developments include the targeting of *ie-1* in the *BmNPV* genome [52], virus-induced CRISPR/Cas9 system in transgenic silk worms to protect against *BmNPV* infection [53], transfection of sgRNA and Cas9 to disrupt the *AcMNPV* genome [54], and knock-in of a *gfp* gene in *AcMNPV* [54]. Moreover, the CRISPR/Cas9 system was used to screen the *Bombyx mori* cell line which identified 1006 genes (of 16,571) that are essential for cell viability displaying the effectiveness of the CRISPR/Cas9 system for efficient screening of large genomes [55]. Sf9 cells were also adapted to transiently express Cas9, allowing for the efficient screening of *AcMNPV* genome [7].

3 Materials and Methods

3.1 Cell culture and maintenance

Sf9 and Sf9-Cas9 cells were maintained in suspension culture using Gibco SF900-III serum free media (Fisher Scientific, Whitby, ON). Flasks were seeded at 0.6×10^6 cells/mL or 0.4×10^6 cells/mL corresponding to 3- or 4-day passage cycles respectively. Cells were maintained at 27°C in a non-humidified incubator, and an orbital shaker was kept at 130 rpm. For Sf9-Cas9 cells, Puromycin ($5 \mu\text{g/mL}$; Sigma-Aldrich, Oakville, ON) was added every other passage cycle to maintain the expression of the *cas9* gene.

3.2 Production of Virus Stocks

Parental viral stocks were stored in a -80°C freezer and thawed at room temperature. Sf9 cells were grown in suspension culture to a concentration of $\sim 2 \times 10^6$ cells/mL. 80 μL of the viral stock was added to the Sf9 cells. The infected cells were maintained at 27°C in a non-humidified incubator, and an orbital shaker was kept at 130 rpm. Cell concentration and viability measurements were checked daily using a hemacytometer. Once cells reached $\sim 80\%$ viability, usually 2-3 days post infection, cells were transferred to a 50mL centrifuge tube (Fisher Scientific, Whitby, ON). Cells were centrifuged at 800 g, for 10 minutes at 4°C. The supernatant was transferred to a separate 50mL centrifuge tube (Fisher Scientific, Whitby, ON). The supernatant, now the viral stock used for experimentation, was stored at 4°C. The viral concentration was determined using endpoint dilution assays (Section 3.3)

3.3 Endpoint Dilution Assay (EDPA)

Sf9 cells were grown in suspension culture to a concentration of $\sim 3 \times 10^6$ cells/mL. Sf9 cells were diluted to 0.2×10^6 cells/mL in 50mL centrifuge tubes (Fisher Scientific, Whitby, ON). Seeded centrifuge tubes were inverted to maintain cell suspension and poured into 50mL reservoirs. Using a 12-channel pipette and the reverse pipetting technique, $100 \mu\text{L}$ was placed into every well of a 96-well, flat bottom (F-bottom), treated plate (VWR, Mississauga, ON). Cells were allowed to attach in a humidified incubator at 27°C with no shaking. Cells were left to attach for at least 1 hour prior to infection.

96-well curved bottom (U-bottom) plates (VWR, Mississauga, ON) were used for the serial dilutions. In rows B-H and columns 1-8, $180 \mu\text{L}$ of SF900-III serum free media (Fisher Scientific, Whitby, ON) was added to each well. Typically, row A was reserved for an initial dilution of 10^{-2} , which was achieved by combining $990 \mu\text{L}$ of SF900-III serum free media (Fisher Scientific, Whitby, ON) with $10 \mu\text{L}$ of virus in a 1.7mL microcentrifuge tubes (FroggaBio, Vaughan, ON). $200 \mu\text{L}$ of the 10^{-2} dilution was added to each well in row A. $20 \mu\text{L}$ from all 8 wells in row A was transferred to row B using an electronic multichannel pipette (FroggaBio, Vaughan, ON). This was repeated to obtain dilutions from 10^{-4} to 10^{-8} . Row H was kept virus free to keep as a negative control.

An F-bottom plate containing the cells was taken from the incubator. The electronic multichannel pipette was used to take $120 \mu\text{L}$ of “Virus 1” from rows A-H (All rows). $10 \mu\text{L}$ of each dilution was added to column 1 of the F-bottom plate, then column 2, and so on. The F-bottom plates containing the infected cells were placed back into the humidified incubator at 27°C with no

shaking. The plates were left to sit for 6-7 days and scored under a fluorescent microscope. The resulting score was translated to a concentration value using TCID50.

4 A study to determine the conditions in a 12-well plate that result in desired virus and protein production

4.1 Introduction

The baculovirus expression vector system (BEVS) has been in use for protein production since its inception in 1983 [12]. This system employs the use of a baculovirus, a transfer plasmid and insect cells to produce a recombinant protein product. Common insect cells include Sf9 cells, Sf21, and High Five Cells. A common baculovirus used for the BEVS is the *Autographa californica* multiple nucleopolyhedrovirus (*AcMNPV*) [5]. Inserted genes have generally been under the *polh* or *p10* promoters due to being extensively studied and documented to produce high amounts of recombinant proteins [21].

Previously, the BEVS has struggled to find mainstream acceptance within the biologics industry as prokaryotes and mammalian cells have dominated. Prokaryotes have the distinct advantage of high scalability due to low doubling times and the ability to reach high cell density resulting in high production of desired products. In contrast, mammalian cells are more difficult to reach high-scale protein production, but their complex post translational modifications (PTMs) allow for complex structures to be created which are desirable for specialized drugs [4]. Although the BEVS is not very popular in the biologics industry, there are a few specialized products where it has been used such as the production of the CervarixTM vaccine for HPV, Flublok® and Flublok® Quadrivalent vaccines for influenza, and the Nuvaxovid® vaccine for COVID-19 [56]. As the industry begins to acknowledge the benefits of the BEVS due to its scalability and ability for PTMs, there is a need to better understand the baculoviruses, particularly the *AcMNPV* genome.

In previous work, a high throughput assay for screening the *AcMNPV* genome was developed [2]. For this, a modified Sf9 cell line was developed where the Sf9 cells constitutively produce the Cas9 protein (Sf9-Cas9 cells) allowing for the use of the CRISPR system for genetic engineering. A single guide RNA (sgRNA) that targets an ORF on the *AcMNPV* genome can be transfected into the Sf9-Cas9 cells and targeted genomic changes can be achieved. The cells can then be infected with an *AcMNPV* vector and the effect of specific genes on baculovirus infection can be determined. In work by Bruder, the targeted sequences were in the baculovirus genome, and the effect of targeting these sequences was assessed by using a baculovirus vector that can allow the expression of a green fluorescent protein (GFP). Bruder's findings were consistent with what has previously been reported by others for the genes tested, leading to the conclusion that that this screening assay was an effective tool that could be used to probe the *AcMNPV* genome. Moreover, it reduces the amount of time to probe the genome from the scale of months, to only weeks [2].

The assay described by Bruder was established for use in 6-well plates, however, direct translation of the assay to 12-well plates have resulted in poor amounts of protein and virus production, potentially leading to inaccurate results when probing the *AcMNPV* genome. Although 6-well plates could continue to be used, the benefit of transitioning to 12-well plates cannot be understated due to the reduction of cost associated from probing each gene, particularly due to the reduced amount of transfection reagent required per sample. In this study, it is desired to find conditions for 12-well plates that result in similar protein and virus production to that of its 6-well counterparts.

4.2 Materials and Methods

4.2.1 Assay Setup

Sf9 and Sf9-Cas9 cells were grown in suspension culture to a concentration of $\sim 3 \times 10^6$ cells/mL. Cells were diluted in Gibco SF900-III media to one of four concentrations given in Table 1 in 50mL centrifuge tubes. Cells were transferred to 6- and 12- well, F-bottom, treated plates (VWR, Mississauga, ON). Plated cells were allowed to attach for 1-hour in a humidified incubator at 27°C with no shaking. After 1 hour, plates were checked under a light microscope at 4x magnification to ensure attachment. Cells were then infected with a baculovirus expressing p6.9mAG at an MOI of 3. Cells were placed back in the humidified incubator at 27°C with no shaking for 48 hours.

Table 1 Summary of plate conditions tested for 6- and 12- well plates

| Condition Number | Plate | Liquid Height Condition | Cell Concentration (Cells/mL) | Cell Concentration (Cells/well) |
|-------------------------|--------------|--------------------------------|--------------------------------------|--|
| 1 | 6-Well | Low | 1.25×10^6 cells/mL | 2.5×10^6 cells/well |
| 2 | 6-Well | High | 0.5×10^6 cells/mL | 2.5×10^6 cells/well |
| 3 | 12-Well | Low | 1.125×10^6 cells/mL | 0.9×10^6 cells/well |
| 4 | 12-Well | High | 0.45×10^6 cells/mL | 0.9×10^6 cells/well |

After 48 hours, cells were detached through rigorous pipetting. The contents of the well were then transferred into 1.7 mL microcentrifuge tubes, or 15 mL centrifuge tubes (Fisher Scientific, Whitby, ON) depending on the volume recovered. Cells were centrifuged at room temperature and 800 g for 10 minutes. Supernatant was transferred to 1.7 mL microcentrifuge tubes with excess volume being discarded. The supernatant was stored at 4°C to be tittered through EDPA (section 3.3).

36% paraformaldehyde (Sigma Aldrich, Oakville, ON) was diluted to 2% in D-PBS (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Canada). Centrifuged cells were resuspended in 1mL of 2% formaldehyde to fix the cells. Cells were left to fix at 4°C for at least 30 minutes. After cells were fixed, 1mL of D-PBS was added, resulting in a 2x dilution (1mL fixed cells, 1mL D-PBS) and placed into 5mL polystyrene tubes (VWR, Mississauga, ON). Cells were run through a BD Accuri C6 plus flow cytometer to measure cell fluorescence.

4.3 Results and Discussion

4.3.1 Testing the effectiveness of the Sf9-Cas9 cell line, compared to its parent Sf9 cell line

Table 2 Summary of conditions used to determine if there are any differences between Sf9 and Sf9-Cas9 cells

| Condition | Cell Line | Plate | Media Volume (mL) |
|-----------|-----------|---------|-------------------|
| 1 | Sf9 | 6-Well | 2mL |
| 2 | Sf9 | 12-Well | 2mL |
| 3 | Sf9-Cas9 | 6-Well | 2mL |
| 4 | Sf9-Cas9 | 12-Well | 2mL |

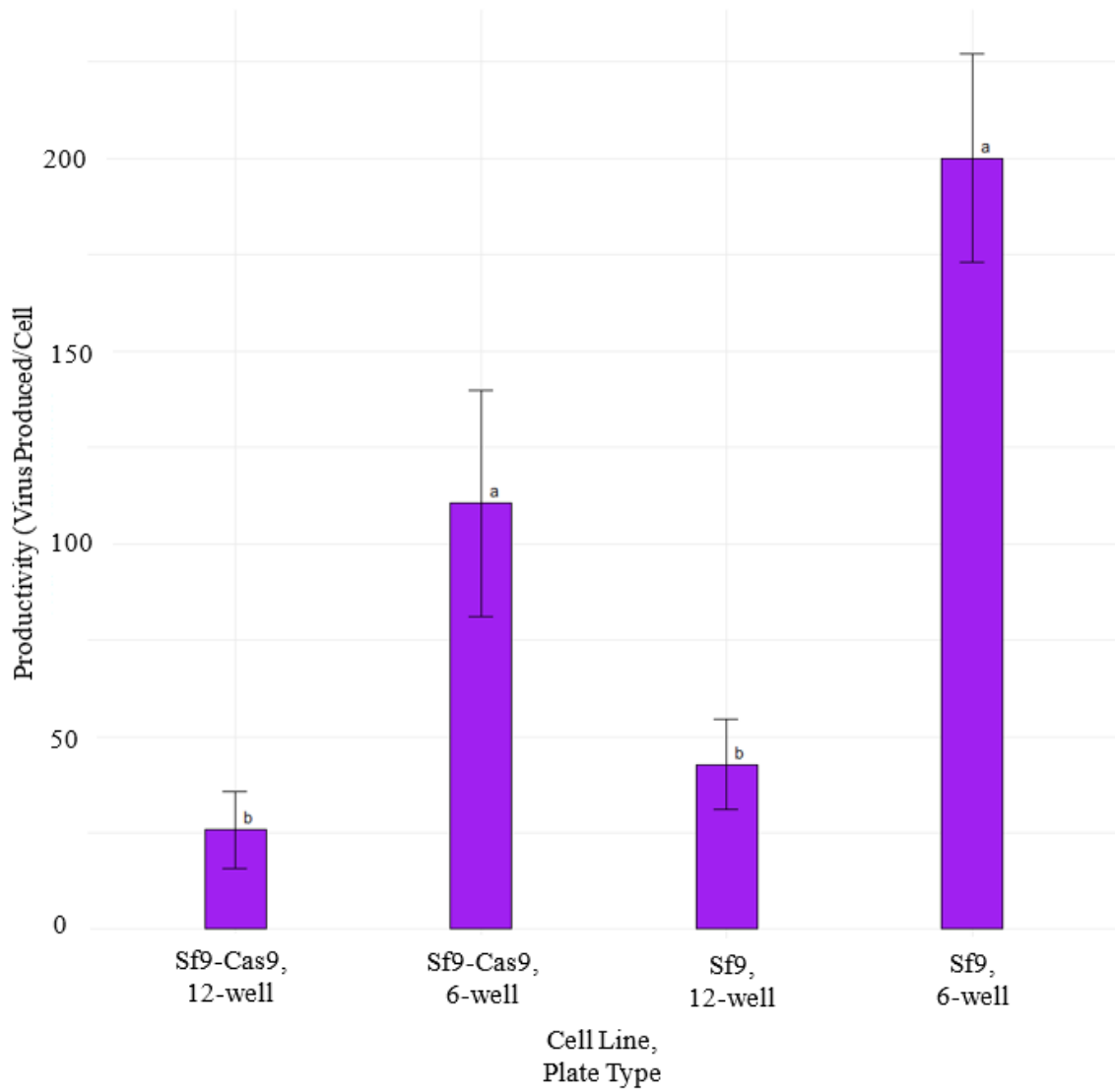


Figure 1 Viral productivity (Virus particles produced/cell) of Sf9 and Sf9-Cas9 cells after 48 hours in 6 and 12 well plates. Both conditions were at a 2mL working volume. Significance was determined after a log transformation and a Tukey test was performed on a 95% confidence interval. Letters indicate which group the mean belongs to. $n = 3$ for each treatment and error bars are one standard deviation.

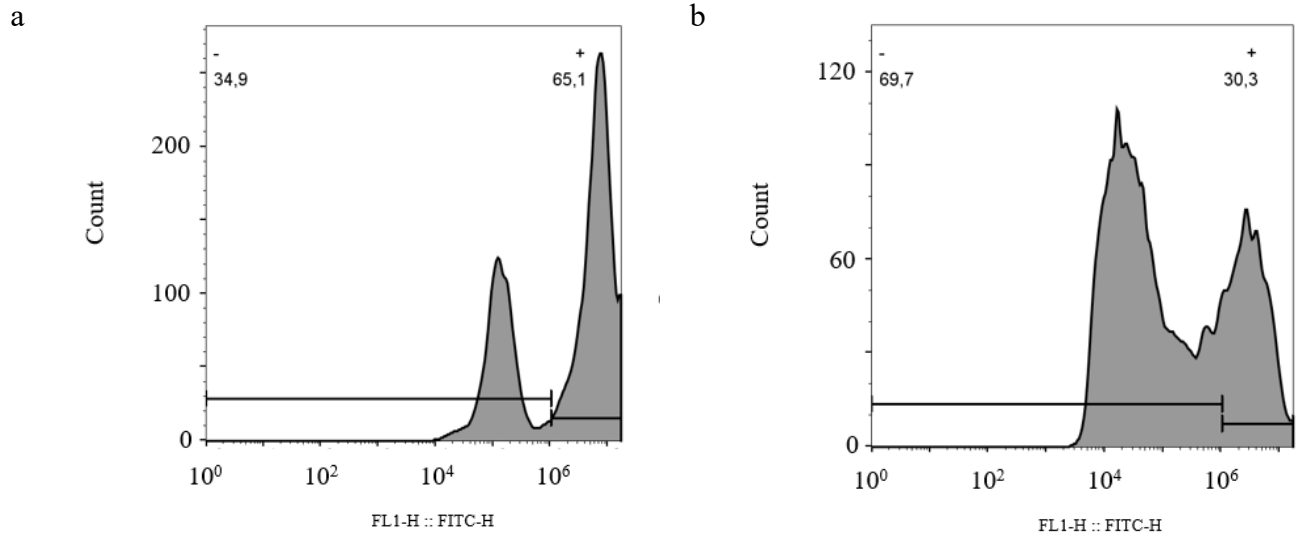


Figure 2 Flow cytometry data for cells infected with p6.9 mAG. The light filter used was at 533nm. a) shows the results for Sf9 cells in 6-well plates and b) shows the results for Sf9 cells in 12-well plates. Both conditions were at 2mL of liquid volume

To ensure that there was no discrepancy in the productivity (defined by the average amount of virus produced per cell) between the Sf9 cells and Sf9-Cas9 cell lines, an experiment was conducted to compare. In this experiment, 6 and 12 well plates were seeded according to the conditions specified in Table 2. Cells were infected with a baculovirus expressing mAG under the p6.9 promoter. The cell productivities are given in Figure 1. The results indicate that there are no significant differences between the productivity in Sf9 and Sf9-Cas9 cell lines because when comparing within the same type of plate, both average productivities are contained in the same group. In other words, Sf9 and Sf9-Cas9 cells in 6 well plates are both part of group 'a' and those in 12-well plates are part of group 'b'. This indicates that the production of the Cas9 protein has an insignificant effect on baculovirus production which is consistent with previous reports [7].

Of note, there was a significant difference in productivity between the 6- and 12-well conditions for both the Sf9 and Sf9-Cas9 cells. Moreover, flow cytometry data indicates lower fluorescence levels for the 12-well plate conditions (Figure 2) which indicates lower amount of

mAG protein produced. The lower production of virus and protein production in the 12-well plate condition indicate sub-optimal conditions for virus and protein production.

Ideally, to reduce costs, and increase throughput, 12-well plates would be preferable for the transfection-infection assay, however, 12-well plate conditions resulted in lower protein production, and lower viral productivity, which may indicate that effects on gene disruption might not be observed. As such, it is important to determine 12-well plate conditions that result in similar viral productivity and protein production that are currently seen in the 6-well plates.

4.3.2 Adjusting liquid heights to help determine desired conditions in a 12-well plate

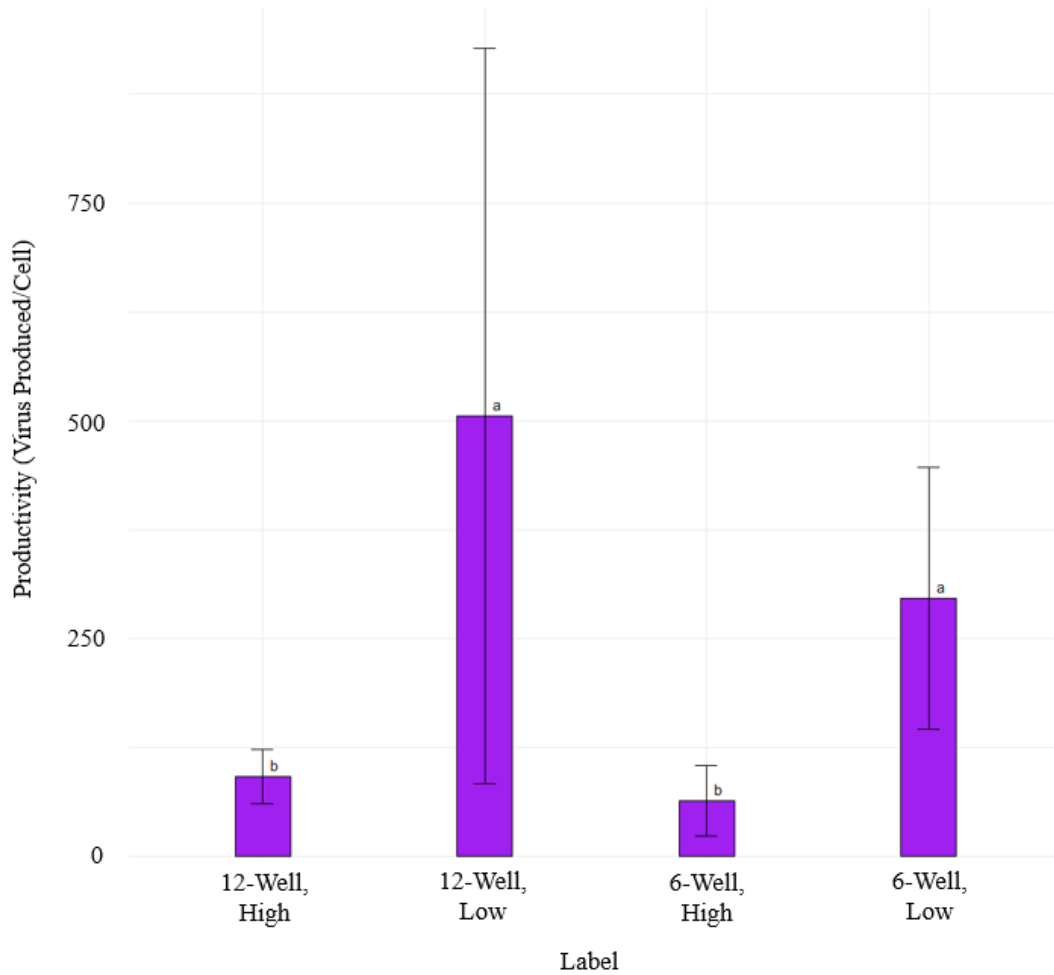


Figure 3 Viral productivity of Sf9 cells at low and high liquid heights in both 6 and 12 well plates. Significance was determined after a log transformation and a Tukey test was performed using a 95% confidence interval. Letters indicate which group the mean belongs to. $n = 6$ for each treatment (exception for 6-well, low where $n=5$ due to contamination issues in the EDPA) and error bars are one standard deviation.

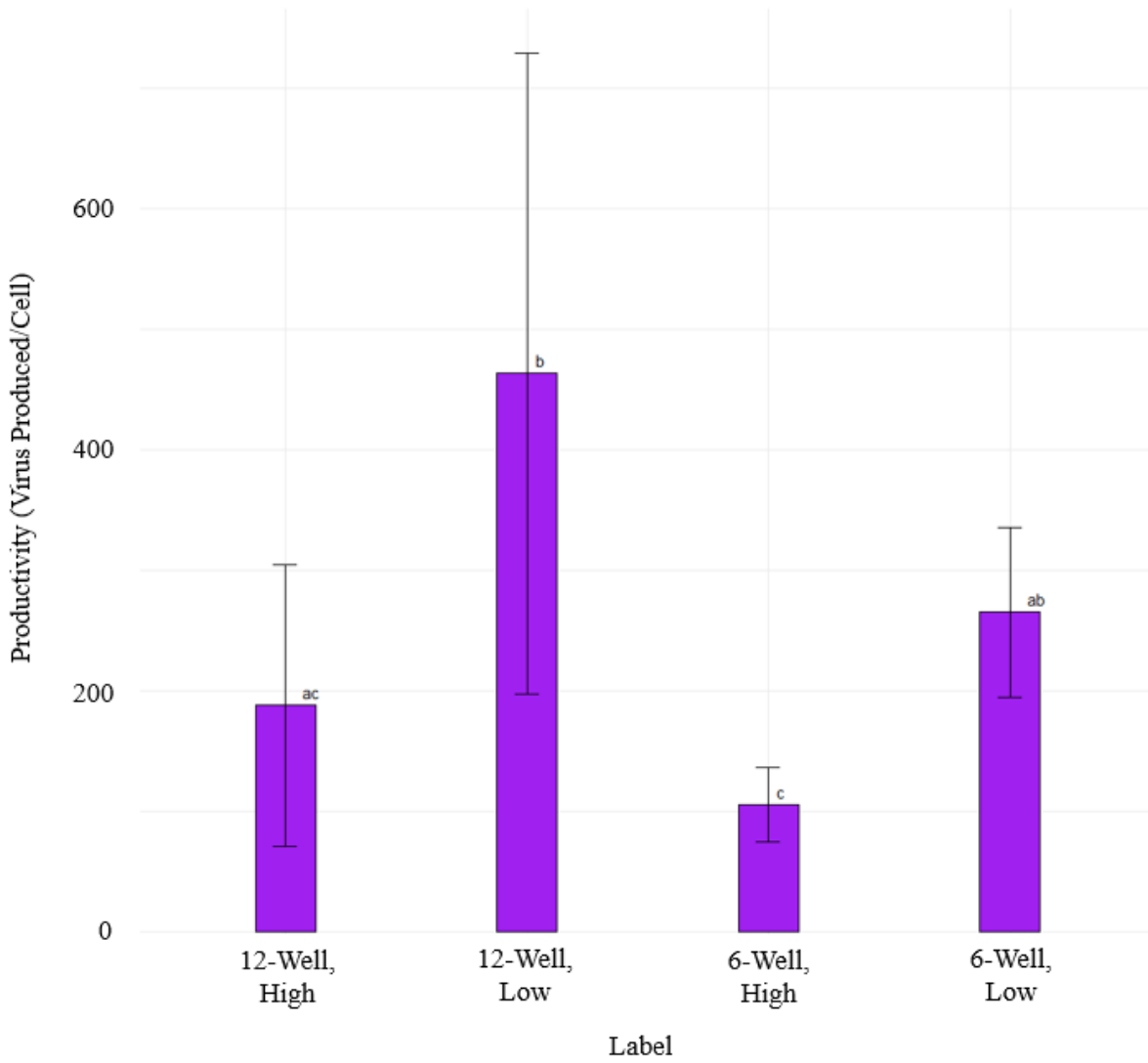


Figure 4 Viral productivity of Sf9-Cas9 cells at low and high liquid heights in both 6 and 12 well plates. Significance was determined after a log transformation and a Tukey test was performed using a 95% confidence interval. Letters indicate which group the mean belongs to. n = 6 for each treatment (exception for 12-well, low where n=5 due to contamination issues in the EDPA) and error bars are one standard deviation.

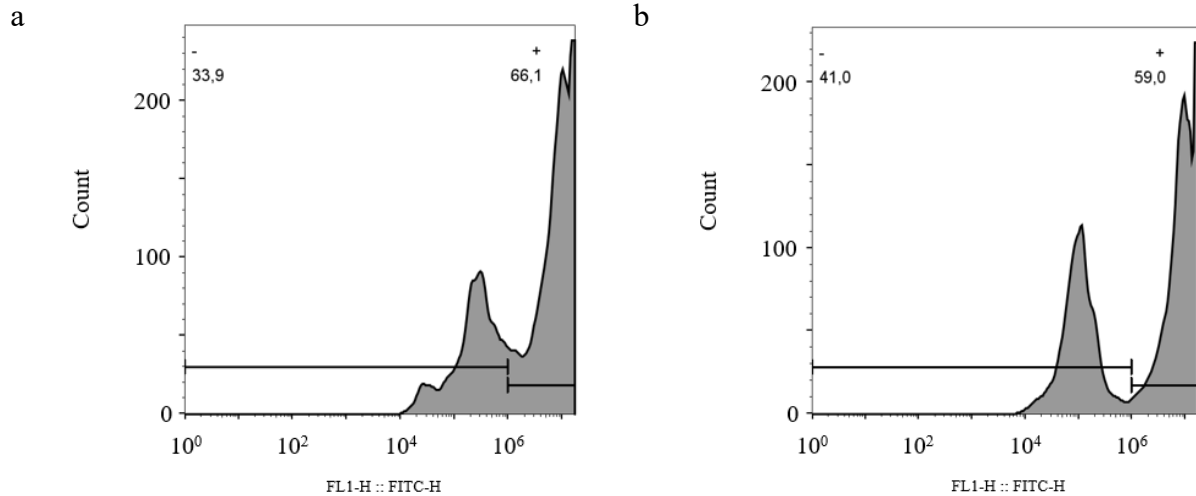


Figure 5 flow cytometry data for Sf9 cells infected with p6.9 mAG. The light filter used was 533nm. a) shows the results for Sf9 cells in 6-well plates at 0.21cm volume height and b) shows the results for Sf9 cells in 12-well plates at 0.21cm volume height

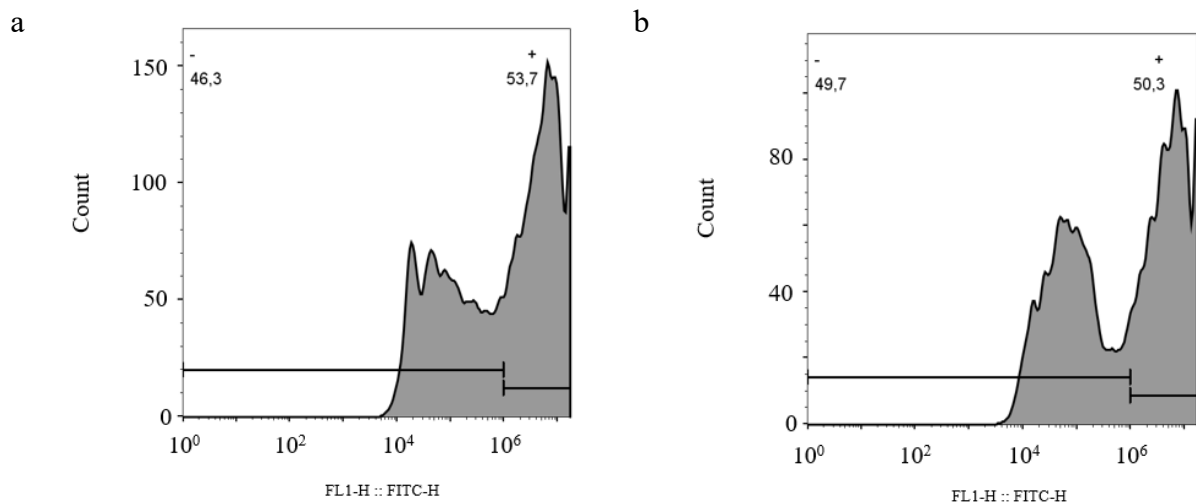


Figure 6 flow cytometry data for Sf9 cells infected with p6.9 mAG. The light filter used was 533nm. a) shows the results for Sf9 cells in 6-well plates at 0.52cm volume height and b) shows the results for Sf9 cells in 12-well plates at 0.52cm volume height

In another experiment, the liquid height in 6- and 12-well plates were adjusted to determine whether this was a contributing factor on the productivity. Through measurements, it was found that the liquid height in the 6- and 12-well plates were 0.21cm and 0.52cm respectively. As such, liquid heights in both plates were ran at 0.21cm and 0.52cm and the results are summarized in Figure 3 and Figure 4 for Sf9 and Sf9-Cas9 cells respectively. For Sf9 cells at the same heights,

the productivity between 6- and 12-well plates were indistinguishable with both conditions at the 0.21cm height having means within group 'a' and both conditions at the 0.52cm height having means within group 'b'. This is further supported when the cells were run through the flow cytometer, where Sf9 cells at the 0.21cm height (Figure 5) display a fluorescence peak at $\sim 10^5$ au that has small variance, as well as another peak at $\sim 10^7$ au that also has small variance, where au is 'arbitrary units' given by the flow cytometer. Sf9 cells at the 0.52cm height (Figure 6) still display a fluorescence peak at $\sim 10^5$ au and $\sim 10^7$ au but have much higher variance as given by the wider peaks.

Some differences were observed when working with the Sf9-Cas9 cells. Firstly, the viral productivity was split into three groups of 'a', 'b', and 'c'. Like the Sf9 cells, there are two groups wherein group 'b' consists of both conditions at low liquid height, and group 'c' consists of both conditions at high liquid heights. However, there is a third group 'a' that encompasses the 12-well at high liquid height, and 6-well at low liquid height. This result was unexpected as the Sf9 cells were able to separate these means into two distinct groupings. However, in this experiment, they were unable to be distinguished. This can be attributed to the high productivity given by 3 of the 6 measurements in the 12-well, high liquid height condition that ranged from 219-354 viral particles/cell compared to the other three ranged from 80-100 viral particles/cell. From the results given by the Sf9 cells, a range of 71-153 viral particles/cell was observed which implies that the higher 3 measurements in the Sf9-Cas9 cells were an abnormality. Removal of the outliers results in two distinct groups that follow those found in the Sf9 cells (data not shown).

It should be noted that the liquid level may not be the factor directly affecting the productivity, and it may be the seeding density used. It is a well-established practice that Sf9 cells should be infected at $\sim 1.5-2 \times 10^6$ cells/mL for optimal production of virus and protein. In the high-

volume conditions, the cell densities are $\sim 0.5 \times 10^6$ cells/mL which is outside of the optimal range. Conversely, the low volume conditions result in cell densities of $\sim 1.25 \times 10^6$ cells/mL which is close to the optimal cell density for virus and protein production. Despite not knowing the exact mechanisms of how the lower liquid level can increase viral and protein production, the low liquid level condition will continue to be used in 12-well plates for the transfection-infection assay.

4.4 Conclusions

The focus of this chapter was to determine experimental conditions wherein Sf9 cells and its derivatives can have high protein and virus production in 12-well plates which will help with lowering costs and increasing throughput in the transfection-infection assay. In this, Sf9 cells and Sf9-Cas9 cells were tested against each other and found that the Cas9 production has no significant impact on the production of recombinant protein under the *p6.9* promoter. Moreover, by adjusting the liquid height in the 12-well plate condition, the protein and virus production matched the output given by the 6-well plate conditions that have been used by Mark Bruder [7]. As such, for future experimentation, 12-well plates will be seeded with 0.9×10^6 cells/plate, with a working volume of 800 μ L.

5 Development of a high throughput assay for HIV-Gag VLPs that are tagged with a green fluorescent protein

5.1 Introduction

Virus-like particles (VLPs) are self-assembling, non-infectious particles that mimic the structure of their viral counterparts, but lack genetic material, rendering them non-infectious [24]. Due to the similar structure of a viral particle, VLPs are highly immunogenic making them an interesting platform for vaccine development. Some notable VLP based vaccines currently on the market include Gardasil® and Cervarix™ for immunization against the human papillomavirus (HPV), as well as Energix®-B and Recombivax HB® (Merck & Co.) for immunization against HBV. Although not self-assembling, the Nuvaxovid® vaccine used against SARS-CoV2 can be grouped in as a VLP as the spike protein is adjuvanted onto lipid nanoparticles, giving a similar size and structure comparable to a viral particle [4].

Despite the growing interest and research involving VLPs, quantifying them remains a challenge. Infectious viral particles can be easily measured through assays such as endpoint dilution assays, or plaque assays. However, since VLPs lack the ability to infect a host and reproduce, various types of assays must be used. Some assays that have been used for VLP quantification include bicinchoninic acid assays (BCA), enzyme-linked immunosorbent assays (ELISA), nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and dynamic light scattering (DLS) [8], [9]. Each of these assays has have limitations such as inability to discern between VLPs and proteins in the case of BCA, cost and availability of antibodies in the case of ELISA, high costs for NTA and TEM, and difficulties in discerning between VLPs and

extracellular vesicles for DLS. All of these make working with VLPs difficult even though they are an important high value product.

Furthermore, the long-term storage and stability of VLPs is important due to their use as vaccines or vaccine candidates. VLPs, particularly eVLPs, are sensitive to factors such as temperature, shear stress, agitation rate, chemical treatment, and dissolved oxygen which can affect VLP stability [8]. In one study, SARS-CoV-2 VLP stability was measured and was found that the VLPs remained stable over the span of a week at around 0°C in a liquid buffer [57]. When these VLPs were dried on a surface at 22°C there was minimal degradation, but degradation occurred rapidly when temperatures were raised to 34°C [57]. Further testing also showed that degradation occurred in a liquid buffer at 34°C, although not as aggressive as the dry condition [57]. Although temperature exposure times were not explicit in this study, the degradation of VLPs at, or close to room temperature is something to be considered. Another study looked at long term storage of Gag VLPs at various temperatures (4°C, -20°C and -70°C) and formulation media (sorbitol, sucrose and trehalose) [58]. Of the conditions tested, sorbitol and sucrose were ineffective at long term storage, whereas 15% trehalose at -70°C allowed the most stable formulation over a 12-month period, [58]. Overall, the storage temperature and liquid buffer are important factors to consider for the short- and long-term stability of VLPs.

In this study, a high throughput screening assay for fluorescent VLPs was studied. In previous work, an HIV-Gag VLP that fluoresced - because the *Gag* gene was fused to *eGFP* - was quantified through flow cytometry. However, future studies within this thesis require hundreds of samples to be run. Currently samples are diluted one by one, using microcentrifuge tubes, and run through the flow cytometer in 5mL polystyrene tubes. With hundreds of samples to be run, a lot of time and resources will be used with current methodologies. Here, a change to 96-well plates is

proposed which allows for multiple dilutions to occur in parallel and plates can be run directly through the flow cytometer leading to increased efficiency of time and resources. Further testing will be done to ensure that VLP degradation does not occur during the time it takes to prepare and run samples the samples.

5.2 Materials and Methods

5.2.1 Production of VLP Stock

A virus stock containing Gag-GFP VLPs produced under the *p6.9* promoter was used. See section ‘Production of Virus Stocks’.

5.2.2 Experimental Procedure for Testing

In a 96-well, U-bottom, untreated plate, 180 μ L of D-PBS (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Canada) was added to columns 2-4, and rows B-H using a multichannel pipette. Note that row A should be empty to allow for flow-set fluorospheres (Beckman Coulter, Mississauga, ON) to be run. These control particle solutions are used to calibrate particle counts. In 1.7mL microcentrifuge tubes (FroggaBio, Vaughan, ON), 990 μ L of D-PBS was added. 10 μ L of supernatant was added to the microcentrifuge tube to create a 10⁻² dilution. 200 μ L of the 10⁻² dilution was added to a well in row B, column 1 in the 96-well plate. This procedure was completed for 6 other samples within the same plate for rows C-H. Using an electronic multichannel pipette (FroggaBio, Vaughan, ON), 20 μ L of 10⁻² dilution (column 1) was added to column 2 to create the 10⁻³ dilution. Column 2 was mixed using the electronic multichannel pipette using the settings of 75 μ L x 5 repeats. This procedure was repeated until column 4 to create 10⁻⁴ and 10⁻⁵ dilutions.

For tube dilutions, 900 μL of D-PBS was added to 3 microcentrifuge tubes. 100 μL of the 10^{-2} dilution was taken and added to one of the microcentrifuge tubes containing 900 μL of D-PBS to create a 10^{-3} dilution. This mixture was mixed by pipetting up and down. 100 μL of the 10^{-3} solution was subsequently added to another microcentrifuge tube containing 900 μL of D-PBS to create a 10^{-4} dilution, and the same was done for the 10^{-5} dilution. The 10^{-3} , 10^{-4} , and 10^{-5} dilutions were added to columns 6, 7, and 8 of the 96-well plate respectively.

To create the flow-set solutions, 3 microcentrifuge tubes were filled with 950 μL of D-PBS. The flow-set fluorospheres were taken from the 4°C refrigerator and shook by hand to homogenize the mixture. ~300 μL of the flow-set fluorospheres were squeezed into a separate microcentrifuge tube. Using a pipette, 50 μL of the flow-set fluorospheres were added to the microcentrifuge tubes containing the 950 μL of D-PBS. The D-PBS + flow-set fluorospheres mixture was mixed by pipetting up and down. 200 μL of each flowset mixture was added to row A, columns 1,2, and 3. The samples were run on a BD accuri C6 Plus flow cytometer.

5.2.3 Converting Particle Counts to Concentrations

To convert the particle counts given by the flow cytometer into concentrations, Equation 1 was used. An average concentrations given by the various dilutions was then calculated.

$$VLP \text{ Concentration} \left(\frac{\text{Particles}}{\text{mL}} \right) = C_v * D * \frac{50000}{C_f} \quad \text{Equation 1}$$

Where:

C_v : Particle counts of the VLP

C_f : Average particle count for the flowsets

D : Dilution rate of the sample (e.g. $10^{-3} = 1\ 000$, $10^{-4} = 10\ 000$)

5.3 Results and discussion

5.3.1 Initial testing of U-bottom dilutions vs tube dilutions

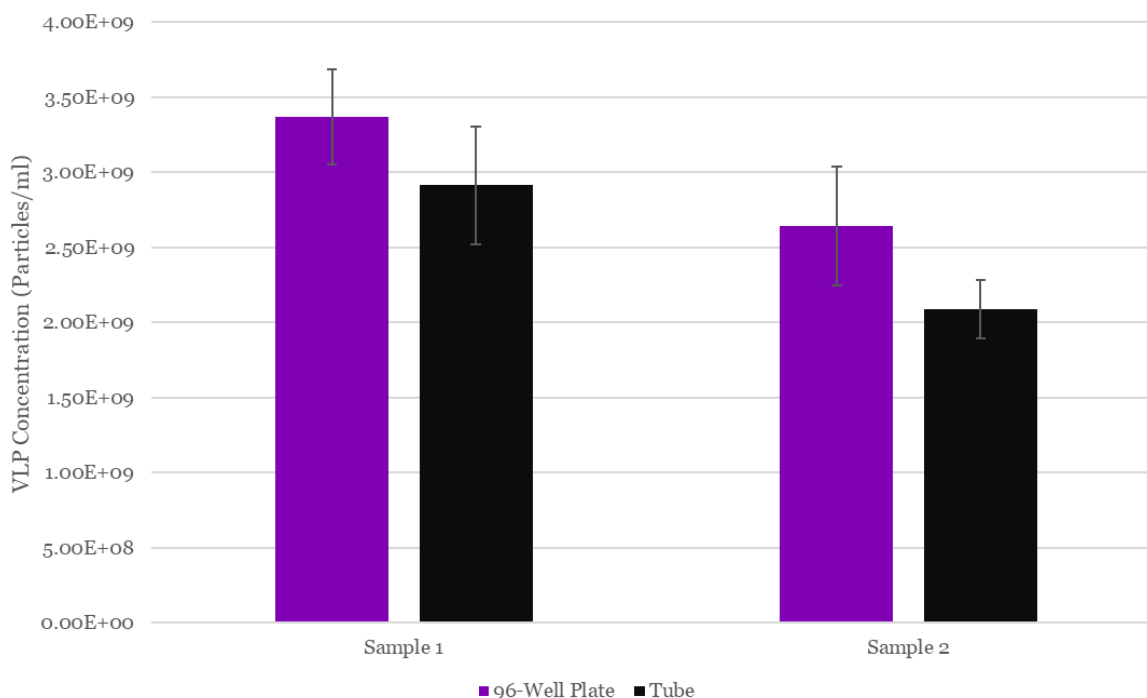


Figure 7 VLP concentrations of dilutions done in the U-bottom plate compared to those done in microcentrifuge tubes. Values were converted from the particle counts by averaging the particle counts given by 10^{-3} and 10^{-4} dilutions. Error bars represent one standard deviation; $n=3$

To determine if dilutions in a U-bottom plate resulted in any differences in particle concentrations, VLP samples were diluted in U-bottom plates along with tubes. Both started with the same 10^{-2} dilution in a microcentrifuge tube, where $200\mu\text{L}$ was then aliquoted into the U-bottom plate to be serially diluted. The rest of the 10^{-2} dilution was used to make the 10^{-3} dilution which was then diluted to 10^{-4} in microcentrifuge tubes. Results are found in Figure 7. Through this study, it can be seen that the dilutions occurring in the U-bottom plate were ~15-30% higher in their particle concentrations when compared to the tube dilutions. This could be the result of liquid carryover on the surface of the pipette tips. This effect would be more pronounced in the U-

bottom dilutions as the diluting volume is lower (200 μ L for U-bottom dilution vs 1000 μ L for tube dilution).

5.3.2 Testing of VLP concentrations over time

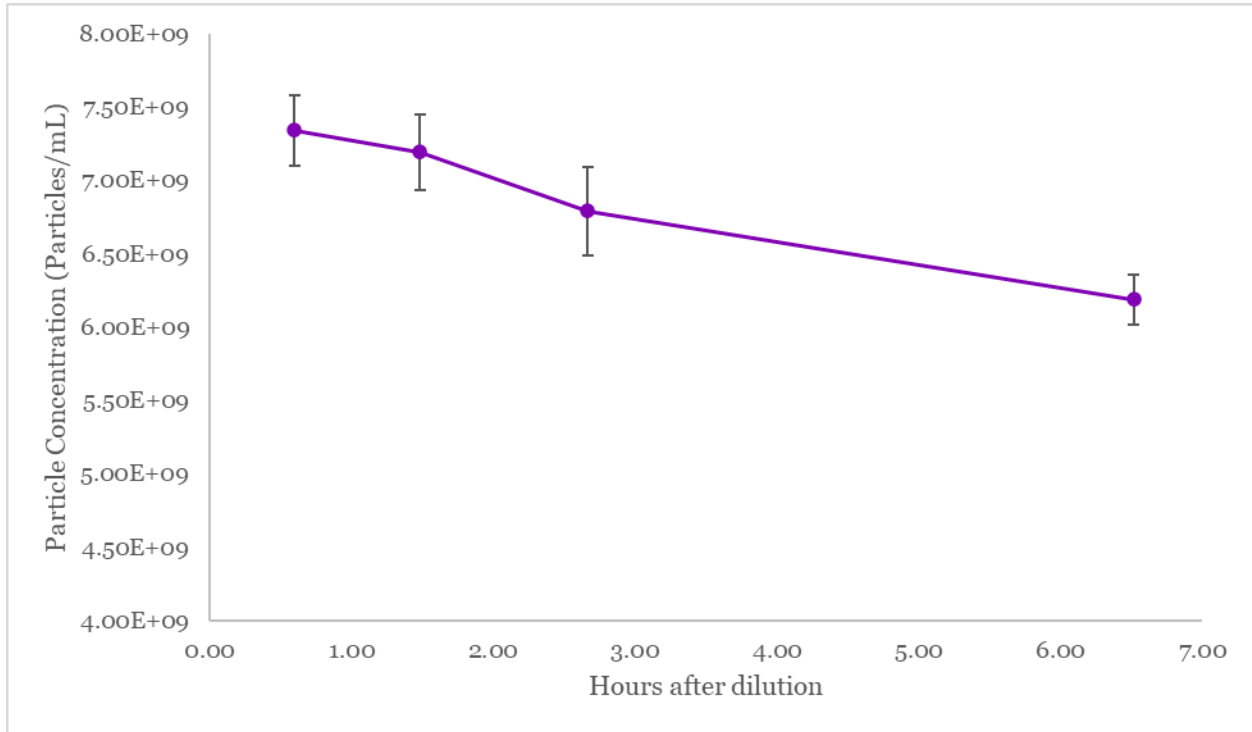


Figure 8 VLP concentrations observed over ~7-hour period, where particle counts were averaged for 10^{-3} 10^{-4} and 10^{-5} and converted to concentrations. Time zero was when samples were initially diluted. Error bars represent one standard deviation; $n=5$

VLP concentrations were tracked over a ~7-hour period to determine particle concentration over time and results can be seen in Figure 8. From the collected data, it does appear that there is an observable drop-off in particle concentration over 7-hours, indicating some particle degradation or aggregation; however, within the first 1.5 hours after samples were initially diluted, there was little change observed. This would indicate that there is ~1.5 hour period wherein the particle counts can be assumed to be accurate. This is within the time it would take to prepare and run each plate of 15 samples, where sample prep takes ~30 minutes and flow cytometry takes ~45 minutes, thus being within the 1.5 hour time period.

5.4 Conclusions

This study looked at HIV-Gag VLPs tagged with a green fluorescent protein using flow cytometry. VLPs were diluted in a 96-well plate and compared to samples diluted in microcentrifuge tubes. Particle concentrations in U-bottom plates were consistently 10-20% higher than those found using tube dilutions. As this was a consistent effect across all samples tested, it was deemed acceptable to use 96-well U-bottom plates for the purposes of genetic screening in future experiments. The stability of HIV-Gag VLPs was observed for 7 hours. Here it was observed that VLP concentrations remained stable for ~1.5 hours after dilution, with particle concentrations steadily dropping after this point. For the purposes of VLP quantification, this timeframe was acceptable given 15 samples per plate, wherein the dilutions for each plate would take ~30 minutes, and running the samples through the flow cytometer takes ~45 minutes, well within the 1.5 hours before VLP degradation begins.

6 A study into the use of a plasmid containing a scrambled sequence as a positive control

6.1 Introduction

The transfection-infection (T-I) assay developed to probe the effect of disrupting genes using CRISPR/Cas9 [2] compares the production of a transgene and the production of virus between a negative control and the experimental condition. However, what should be the positive control? In this chapter, we investigate different controls to determine the best control. The assay involves three steps: seeding cells to a plate, transfection of a plasmid and then infection with a reporter baculovirus. The plasmid transfection step is crucial, as it delivers the targeting sgRNA that disrupts a gene of interest. For this reason, the following controls are investigated: 1) infection of cells without doing the transfection step; 2) transfection of a plasmid

6.2 Materials and Methods

6.2.1 Plasmid amplification and purification

Chemically competent cells were previously made to generate the sgRNA of interest. In 50mL of terrific broth (Thermo Fisher Scientific, Mississauga, ON) competent cells were added. Competent cells were grown for ~19 hours. Contents of the flasks were transferred to 50mL centrifuge tubes (Fisher Scientific, Whitby, ON). sgRNA was purified using the GeneJET Plasmid Midiprep Kit (Thermo Fisher Scientific, Mississauga, ON) according to manufacturer's instruction.

6.2.2 Transfection Infection Assay

Sf9-Cas9 cells were seeded in a flask at 0.6×10^6 cells/mL using Gibco SF-900 III serum free media (Fisher Scientific, Whitby, ON). They were grown in a non-humidified incubator at 27°C, on an orbital shaker at 130 rpm for 2 days. Cells were diluted down to 1.12×10^6 cells/mL in 50mL centrifuge tubes (Fisher Scientific, Whitby, ON). The centrifuge tubes were inverted to mix cells and maintain suspension. 0.8mL of the cells was added to each well of a 12-well F-bottom treated plate (VWR, Mississauga, ON). Plates were placed in a humidified incubator at 27°C with no shaking for 1 hour to allow cells to attach.

While cells attached, the transfection mixture was made. The FuGENE® HD Transfection Reagent (Promega, Madison, WI, USA) was removed from the 4°C refrigerator and allowed to come up to room temperature. Calculations were done to determine the volume of Gibco SF-900 III serum free media and plasmid needed for the recommended ratios of 1pg DNA:cell and 4:1 Transfection Reagent:DNA ratio. Each well required 60µL of solution, and each plasmid was run in triplicate meaning 180µL of each plasmid mixture was made. Firstly, the Gibco SF-900 III serum free media was added to the 1.7mL microcentrifuge tubes (FroggaBio, Vaughan, ON). Next, the sgRNA was removed from the -20°C freezer and thawed at room temperature. After thawing, the sgRNA was added to the mixture, and the mixture was homogenized by pipetting up and down. Finally, the transfection reagent was added to each mixture and homogenized by pipetting up and down. The mixture was left to incubate for 10 minutes at room temperature. Plated cells were removed from the incubator and 60µL of the mixture was pipetted into its respective well in a dropwise manner. After all wells were inoculated with the transfection mixture, the plates were put back into the humidified incubator at 27°C with no shaking for 18-24hours.

After 18-24 hours, p6.9Gag-GFP viral stock was diluted down to 3.38×10^6 pfu/mL in 50mL centrifuge tubes using Gibco SF-900 III serum free media. The plates were removed from the incubator. The liquid from each well was removed using a pipette, and the liquid was disposed of in bleach. 0.8mL of the diluted p6.9Gag-GFP mixture was added to each of the wells for infection at an MOI of 3, with the exception of the negative control. The plates were then placed back into the humidified incubator at 27°C with no shaking for 48 hours.

After 48 hours, plates were removed from the incubator. Cells from each well were detached by rigorous pipetting until the bottom surface of the well was clear to indicate most cells have been removed. The contents of each well were placed into a 1.7mL microcentrifuge tube. The cells were centrifuged for 10 minutes at 800 g and room temperature. The supernatant was pipetted into a separate microcentrifuge tube and stored at 4°C for later EDPA and VLP quantification.

36% paraformaldehyde (Sigma Aldrich, Oakville, ON) was diluted to 2% in D-PBS (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Canada). The cell pellet was resuspended using 0.8mL of the 2% formaldehyde solution and placed in the 4°C refrigerator for 30 minutes to fix. 0.8mL of D-PBS was added to 5mL polystyrene flow tubes (VWR, Mississauga, ON). After cells were fixed, the contents were added to the polystyrene flow tubes and run through the BD Accuri C6 plus flow cytometer under a 510nm wavelength filter to measure the fluorescence emitted by the cells.

6.2.3 VLP Quantification

Supernatant was diluted to 10^{-5} in U-bottom plates then run through a BD Accuri C6 plus flow cytometer under a 533 ± 30 nm wavelength filter to measure the fluorescence emitted by the cells. Detailed steps can be found in 5.2.2.

6.3 Results and Discussion

6.3.1 Verification of a scrambled sequence as a positive control

Table 3 Determination of significant differences between the scrambled control and infected only controls used in experiment 1. Here, the virus groupings and VLP groupings were determined using a t-test, $n=3$ for the scrambled control, $n=2$ for the infected only control, and $\alpha = 0.05$. Virus concentrations and VLP concentrations underwent a log transformation prior to significance testing due to the variance being a function of the mean. A grouping of "a" means that there is a significant difference from a grouping of "b". Significance was shown this way due to future experiments including a third control to compare to, which used a tukey test to determine significance.

| Gene | Average Virus Concentration (pfu/mL) | Standard Error for Virus Concentration | Virus Group | Average VLP Concentration | Standard Error for VLP Concentration | VLP Group |
|----------------------|--------------------------------------|--|-------------|---------------------------|--------------------------------------|-----------|
| Scrambled | 2.82E+08 | 5.06E+07 | a | 2.74E+09 | 1.60E+08 | a |
| Infected Only | 2.46E+08 | 2.31E+07 | b | 1.71E+09 | 1.72E+08 | b |

Table 4 Determination of significant differences between the scrambled control and infected only controls used in experiment 2. Here, the virus groupings and VLP groupings were determined using a tukey test, $n=3$ for all treatments, and $\alpha = 0.05$. Virus concentrations and VLP concentrations underwent a log transformation prior to significance testing due to the variance being a function of the mean. A grouping of "a" means that there is a significant difference from a grouping of "b".

| Gene | Average Virus Concentration (pfu/mL) | Standard Error for Virus Concentration | Virus Group | Average VLP Concentration | Standard Error for VLP Concentration | VLP Group |
|------------------------|--------------------------------------|--|-------------|---------------------------|--------------------------------------|-----------|
| Scrambled | 2.57E+08 | 5.93E+07 | a | 4.94E+09 | 3.80E+08 | a |
| Infected Only | 9.71E+07 | 1.41E+07 | b | 2.42E+09 | 5.77E+08 | b |
| mKate2 Infected | 1.92E+08 | 2.63E+07 | ab | 4.76E+09 | 1.31E+08 | a |

Table 5 Determination of significant differences between the scrambled control and infected only controls used in experiment 3. Here, the virus groupings and VLP groupings were determined using a tukey test, $n=3$ for all treatments, and $\alpha = 0.05$. Virus concentrations and VLP concentrations underwent a log transformation prior to significance testing due to the variance being a function of the mean. A grouping of "a" means that there is a significant difference from a grouping of "b".

| Gene | Average Virus Concentration (pfu/mL) | Standard Error for Virus Concentration | Virus Group | Average VLP Concentration | Standard Error for VLP Concentration | VLP Group |
|------------------------|--------------------------------------|--|-------------|---------------------------|--------------------------------------|-----------|
| Scrambled | 3.16E+08 | 5.64E+07 | a | 3.87E+09 | 7.19E+07 | a |
| Infected Only | 1.49E+08 | 9.12E+06 | b | 2.65E+09 | 1.09E+08 | b |
| mKate2 Infected | 3.06E+08 | 1.46E+07 | a | 3.88E+09 | 2.24E+08 | a |

Table 6 Determination of significant differences between the scrambled control and infected only controls used in experiment 4. Here, the virus groupings and VLP groupings were determined using a tukey test, $n=3$ for all treatments, and $\alpha = 0.05$. Virus concentrations and VLP concentrations underwent a log transformation prior to significance testing due to the variance being a function of the mean. A grouping of “a” means that there is a significant difference from a grouping of “b”.

| Gene | Average Virus Concentration (pfu/mL) | Standard Error for Virus Concentration | Virus Group | Average VLP Concentration | Standard Error for VLP Concentration | VLP Group |
|------------------------|--------------------------------------|--|-------------|---------------------------|--------------------------------------|-----------|
| Scrambled | 1.68E+08 | 3.69E+07 | a | 3.33E+09 | 2.61E+08 | a |
| Infected Only | 1.00E+08 | 2.55E+07 | a | 2.00E+09 | 1.05E+08 | b |
| mKate2 Infected | 2.47E+08 | 4.24E+07 | a | 3.92E+09 | 2.79E+08 | a |

Table 7 Determination of significant differences between the scrambled control and infected only controls used in experiment 5. Here, the virus groupings and VLP groupings were determined using a tukey test, $n=3$ for all treatments, and $\alpha = 0.05$. Virus concentrations and VLP concentrations underwent a log transformation prior to significance testing due to the variance being a function of the mean. A grouping of “a” means that there is a significant difference from a grouping of “b”.

| Gene | Average Virus Concentration (pfu/mL) | Standard Error for Virus Concentration | Virus Group | Average VLP Concentration | Standard Error for VLP Concentration | VLP Group |
|------------------------|--------------------------------------|--|-------------|---------------------------|--------------------------------------|-----------|
| Scrambled | 1.72E+08 | 1.67E+07 | a | 3.94E+09 | 3.54E+08 | a |
| Infected_Only | 2.25E+07 | 6.01E+06 | b | 1.31E+09 | 1.33E+08 | b |
| mKate2_Infected | 1.95E+08 | 6.57E+07 | a | 5.24E+09 | 2.21E+08 | a |

In previous studies, a control that transfected a plasmid consisting of a scrambled sequence then underwent infection was used as a positive control (hereby referred to the ‘scrambled control’) [7]. This contrasts with a control where cells were subjected only to infection (hereby referred to the ‘infected only control’). In this study, it was determined that the scrambled was the correct choice because there was a significant effect of the transfection on virus and VLP concentrations across various experiments (Table 3 - Table 7).

Comparisons between controls were done with samples harvested at the same time due to variability between experiments. In experiment 1 (Table 3), the scrambled control was compared only against an infected only control. A t-test was completed with $\alpha = 0.05$ after a log transformation for the virus and VLP concentrations. This test determined that there was a

significant difference in virus and VLP concentrations between the scrambled and infected controls.

In experiments 2-5 (Table 4 - Table 7), a control was added that transfected a plasmid that produces the mKate2 fluorescent protein and was subsequently infected by the p6.9Gag-GFP virus (hereby referred to the 'mKate2 Infected control'). The mKate2 plasmid is predicted to act as a relatively inert species in the infection phase and thus produce similar levels of budded virus and VLPs as the scrambled control. A Tukey test was completed with $\alpha = 0.05$ after a log transformation for the virus and VLP concentrations. The results are summarized in Table 4 - Table 7 where the budded virus and VLP averages were grouped. In other words, group 'a' implies that it is significantly different from group 'b'. A grouping of 'ab' implies that the mean is in both groups 'a' and 'b', which would require further testing to determine significant differences. In all experiments, the VLP concentrations between the scrambled control and mKate2 infected control were indistinguishable from each other, signified by both treatments being part of group 'a'. A similar trend can be found for the virus concentrations. Note that for experiment 2 (Table 4), the virus grouping of mKate2 was 'ab' which indicates that it was similar enough to both the scrambled and infected only controls. Alone, this result would require further testing to determine significance. However, with the data gathered from the other experiments, there was strong evidence that indicates that the scrambled and mKate2 controls are indistinguishable from each other. Overall, there is strong evidence indicating that the transfection of the mKate2 plasmid does not significantly affect the production of virus and Gag-VLPs under the *p6.9* promoter.

As for the infected only controls, experiments 2-5 grouped VLP production in group 'b'. In contrast, scrambled and mKate2 controls were grouped in 'a'. A similar trend is found for the virus concentrations. However, experiment 4 (Table 6) indicated that the infected only control

came from the same group as the scrambled and mKate2 infected controls. Nevertheless, there was a decrease in viral concentrations in this experiment, similar to the other experiments. The extent of the decrease was not detected at the 95% significance level, but it was on the cusp at $p = 0.0835$ (where significance would be defined at $p \leq 0.05$). Overall, most instances have a significant decrease in VLP and virus production for the infected only control when compared to the scrambled and mKate2 controls. This would imply that the transfection of a plasmid into the cells has a significant effect on the virus and VLP production. Overall, this provides strong evidence that the scrambled control should be used as the point of comparison.

6.3.2 Effect of transfection reagent

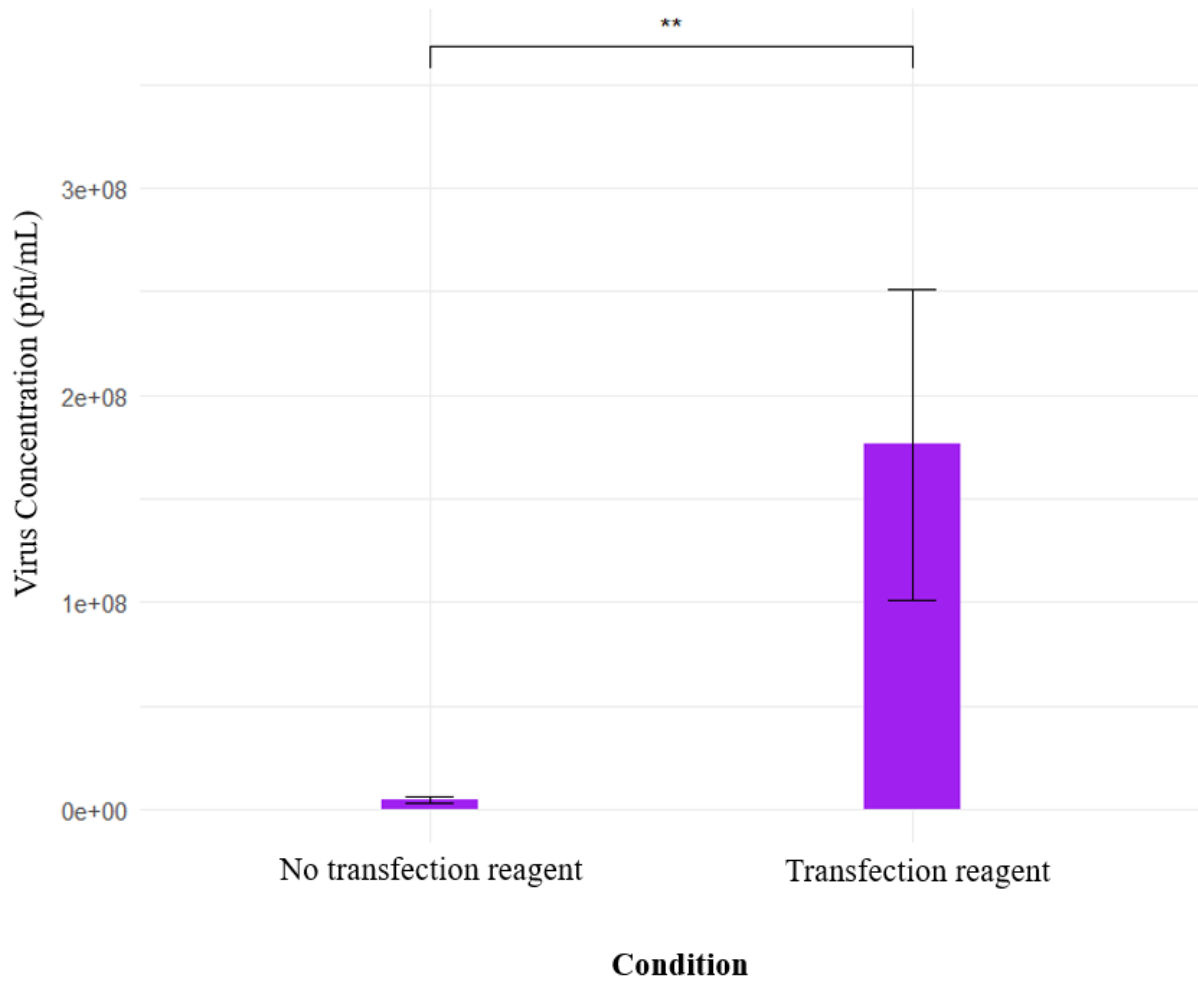


Figure 9 Viral concentrations (pfu/mL) taken from p6.9Gag-GFP samples. 6 replicates were done for samples with no transfection reagent. Those requiring transfection reagent were done in triplicate. A t-test that compares the virus concentrations of the two groups was done after a log transformation. The bars of the graph display the average virus concentrations. The error bars represent the individual treatment standard error. “*” indicates $p \leq 0.05$ | “**” indicates $p \leq 0.01$ | “***” indicates $p \leq 0.001$ | “NS” indicates no significance

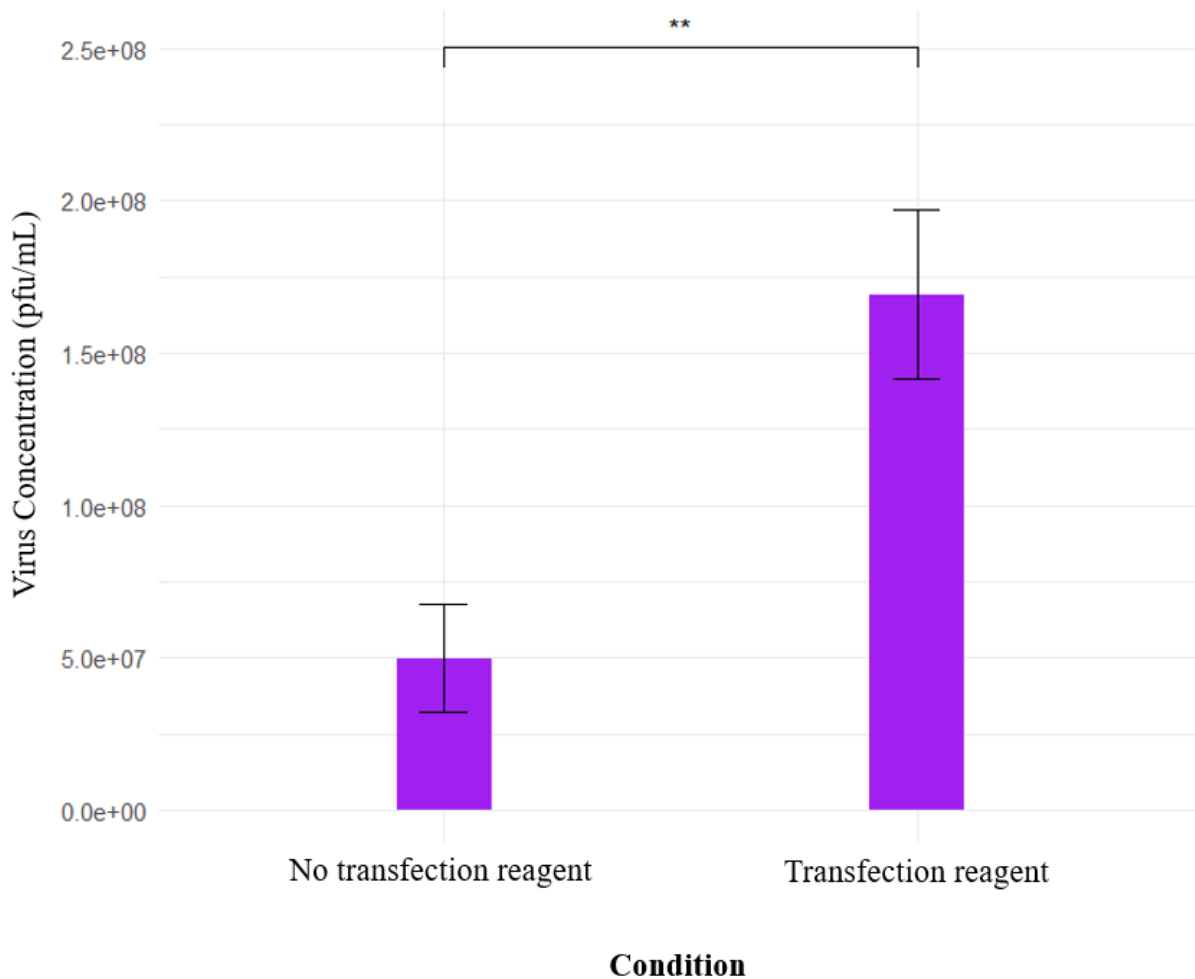


Figure 10 Viral concentrations taken from p6.9mAG samples. 6 replicates were done for samples with no transfection reagent. Those requiring transfection reagent were done in triplicate. A t-test that compares the virus concentrations of the two groups was done after a log transformation. The bars of the graph display the average virus concentrations. The error bars represent the standard error. “*” indicates $p \leq 0.05$ | “**” indicates $p \leq 0.01$ | “***” indicates $p \leq 0.001$ | “NS” indicates no significance

From the results of the various experiments, it was observed that the scrambled control and mKate2 controls had higher virus and VLP concentrations when compared to the infected only controls. This would imply that the scrambled control influences virus and protein production. However, it is possible that the transfection reagent could be responsible for this effect. To test this hypothesis, an experiment was run where Sf9-Cas9 cells were subjected to transfection reagent for 18-24 hours, then media was swapped with media containing p6.9Gag-GFP at an MOI of 3. This was compared against a control where cells were left in clean media for 18-24 hours, which was

then replaced with media containing p6.9Gag-GFP at an MOI of 3. The progeny virus concentrations were recorded. In parallel, p6.9mAG was also tested.

The results indicate that cells that have undergone treatment with transfection reagent produce significantly more virus 48-hours post infection. Looking through the literature, the interaction between transfection reagent and subsequent infection is not well documented. However, these results indicate a noticeable effect where the transfection reagent improved the virus production at the time of harvest. Due to the lack of research on this interaction, any mechanisms or causes are simply speculation. One could postulate that the active ingredients in the transfection reagent puts additional strain on cells and allows for the virus to hijack the cellular machinery at an earlier point in time. This could shorten the infection timeline, thus producing the progeny virus and desired protein at an earlier point in time. Alternatively, the ethanol medium used for the transfection reagent could be the culprit for cellular stress. Assuming the transfection reagent is 80% ethanol [59], and $3.33\mu\text{L}$ of transfection reagent is put into $800\mu\text{L}$ of media, the ethanol concentration would be in the range of 50mM. For a future study, it may be worthwhile to conduct an experiment where the effect of adding ethanol is compared to the addition of transfection reagent, with budded virus and protein production being compared.

This study only tested viral concentrations. This was done since this study was only looking for an effect, and the virus concentration was of particular interest. However, in future studies, cell fluorescence data through flow cytometry should be gathered, and, if applicable, VLP concentrations should be measured which will allow for a holistic view of the system.

Another experiment that could be run is testing the transfection reagent within flask culture. For the purposes of a high throughput assay, and cost effectiveness, all experiments have been run in plates. Flask culture is generally more popular for the Sf9 cell line, and thus, running

experiments in plates may not be representative of what would be found in flasks. As such, testing the transfection reagent effect in flask culture would be an interesting follow up. However, the cost of the transfection reagent makes this a difficult prospect to run replicates to determine significance.

Finally, the last experiment that could be conducted is varying the time of harvesting. In all experiments, the time of harvesting was 48 hours post infection, which was enough time to reach the very late stage of infection. It is possible that virus and VLP production was incomplete at the time of harvesting. As such, it may be worthwhile to test samples at different time points, such as 24, 72, and 96 hours post infection. With this data, it could be seen how the virus and VLP production changes over time when subjected to transfection reagent. It would also be interesting to see if cells that are not treated with transfection reagent approach the same concentrations of virus and VLPs given a longer time frame.

6.4 Conclusions

In this chapter, the effect of transfecting a plasmid that contains a scrambled sequence and then infected was explored. The scrambled control was compared to a treatment where cells were only infected. In this, it was found that the addition of the scrambled sequence increased VLP and virus production when Sf9-Cas9 cells were infected in plates. Moreover, the addition of a relatively inert plasmid that produced mKate2 fluorescent protein was tested. It was determined that the transfection of the mKate2 plasmid was indistinguishable from the scrambled sequence when virus and VLPs were compared. These results provide strong evidence that the scrambled control is a good condition to compare gene disruption results to. As well, due to the mKate2 and scrambled controls being indistinguishable from each other, it was hypothesised that the transfection reagent

may play a role in the increased virus and VLP production. In a subsequent experiment, this hypothesis was tested for virus production, and it was found that exposure to the transfection reagent did increase virus production.

7 Probing of the *AcMNPV* genome using CRISPR-Cas9 to determine essential and non-essential genes

7.1 Introduction

The BEVS is a powerful platform used for recombinant protein and VLP production. The baculovirus *AcMNPV* is a popular viral vector in the BEVS, but it has not been fully characterized meaning that there is much unknown about the function and need for every open reading frame that exists in the *AcMNPV* genome. It is a running hypothesis in the Aucoin lab that the removal of non-essential genes can allocate more cellular resources to produce higher amounts of recombinant protein [1].

In the past, screening of genes has involved the production of knockout viruses through the creation of individual bacmids that are then transfected into host cells. This process is time, resource and labour intensive which makes it difficult to screen an entire genome [7]. Despite the difficulties, another baculovirus, *Bombyx mori* Nuclear Polyhedrosis Virus (*BmNPV*) that contains 141 ORFs, was screened [60] but no such study has been completed for *AcMNPV*. In recent developments, a TI assay was developed wherein an Sf9 cell line, that constitutively expresses Cas9, is transfected with a sgRNA that targets a specific ORF on the *AcMNPV* genome. It is then infected with a modified baculovirus that produces a recombinant protein product, then budded virus and recombinant protein production is quantified to determine the effect of the gene knockout [2]. The use of this assay cut the time to screen a desired gene from the span of months down to weeks, showing its potential as a high-throughput genetic screening tool.

Furthermore, recombinant protein production is generally under the *polh* or *p10* promoters due to their high expression levels in the very late phase of baculovirus infection [61], [62]. Despite

their high expression levels, their activity in the very late phase of infection means that the host cell machinery are compromised and high levels of proteases are present in the supernatant [63], [64], [65]. Due to these concerns, promoters that produce high amounts of protein earlier in the infection cycle are desired. One such promoter is *p6.9*, which is active in the late phase of infection [5], has shown potential as it can produce similar levels of recombinant protein under certain conditions [22]

This chapter screens 21 late and very late genes in the *AcMNPV* genome using the TI assay, with a baculovirus that produces Gag-GFP under the *p6.9* promoter as the model product. In these experiments, the removal of certain genes should result in a measurable effect on the virus and VLP concentrations. As such, any gene cuts that result in a lowering of virus and/or VLP concentrations would be classed as essential genes. Conversely, any gene cuts that result in an insignificant effect on virus and VLP concentrations would be classified as non-essential genes.

7.2 Materials and Methods

Experimental methods have previously been discussed in section 6.2. Here the determination of genes to be tested will be discussed.

7.2.1 Screening of genes to test

In this experiment, the *AcMNPV* genome was assessed. 150 ORFs were evaluated to identify genes that are predicted to, or have been evaluated, to be involved in the late or very late stages of the viral infection. From this pool, a subset of 21 genes were chosen for knockouts, and single guide RNAs were designed and are given by Table 15.

7.2.2 Analytical Methods

Baculovirus and VLP concentrations of each gene target were compared against the scrambled control using a Dunnett test. In short, a log transform was applied to the baculovirus and VLP concentrations for a normal distribution of data. ANOVA was completed on the log transformed data and a Dunnett test was performed each target was compared against the scrambled control using a 95% confidence interval.

7.3 Results

Table 8 Results for gene knockouts of 21 AcMNPV genes

| ORF | Gene | Construct | p-Value for Virus Effect | Virus Effect From Experimental Results | Virus Effect From Literature | p-Value for VLP effect | VLP Effect | Effect on Late Gene Expression in Literature |
|------|-------------|-----------|--------------------------|--|---|------------------------|------------------------|--|
| Ac1 | <i>ptp</i> | 1 | 0.4939 | Non-Significant Effect | No Effect in Sf9 Cells [66] | 0.0000 | Negative Effect | |
| Ac1 | <i>ptp</i> | 2 | 0.0047 | Negative Effect | | 0.0000 | Negative Effect | |
| Ac4 | Ac4 | 1 | 0.1194 | Non-Significant Effect | No Effect in Sf9 Cells [67] | 0.0004 | Negative Effect | |
| Ac4 | Ac4 | 2 | 0.2786 | Non-Significant Effect | | 0.0118 | Negative Effect | |
| Ac4 | Ac4 | 3 | 0.0441 | Negative Effect | | 0.6817 | Non-Significant Effect | |
| Ac6 | <i>lef2</i> | 1 | 0.0000 | Negative Effect | Negative Effect in Sf21 Cells [68] | 0.0000 | Negative Effect | |
| Ac6 | <i>lef2</i> | 2 | 0.0162 | Negative Effect | | 0.0000 | Negative Effect | |
| Ac6 | <i>lef2</i> | 3 | 0.0000 | Negative Effect | | 0.0000 | Negative Effect | |
| Ac10 | <i>pk1</i> | 1 | 0.0714 | Non-Significant Effect | Negative Effect in Sf9 Cells [69], [70] | 0.0000 | Negative Effect | No Effect [70] |
| Ac10 | <i>pk1</i> | 2 | 0.9959 | Non-Significant Effect | | 0.0000 | Negative Effect | |

| | | | | | | | | |
|------|--------------|---|--------|------------------------|---|--------|------------------------|--|
| Ac10 | <i>pk1</i> | 3 | 0.0000 | Negative Effect | | 0.0000 | Negative Effect | |
| Ac13 | <i>bion</i> | 1 | 0.0000 | Negative Effect | Negative Effect in Sf9 Cells [71] | 0.0000 | Negative Effect | |
| Ac13 | <i>bion</i> | 2 | 0.0000 | Negative Effect | | 0.0000 | Negative Effect | |
| Ac14 | <i>lef1</i> | 1 | 0.0048 | Negative Effect | Negative Effect in Sf9 Cells [72] | 0.0000 | Negative Effect | |
| Ac14 | <i>lef1</i> | 3 | 0.0000 | Negative Effect | | 0.0000 | Negative Effect | |
| Ac19 | <i>orf19</i> | 1 | 0.1189 | Non-Significant Effect | No Effect in BmNPV and BmN-4 Cells [73] | 0.0017 | Positive effect | |
| Ac19 | <i>orf19</i> | 2 | 0.4918 | Non-Significant Effect | | 0.5241 | Non-Significant Effect | |
| Ac22 | <i>pif2</i> | 1 | 0.0000 | Negative Effect | No Effect in Sf9 Cells [74] | 0.0000 | Negative Effect | |
| Ac22 | <i>pif2</i> | 2 | 0.0000 | Negative Effect | | 0.0000 | Negative Effect | |
| Ac22 | <i>pif2</i> | 3 | 0.0263 | Negative Effect | | 0.0000 | Negative Effect | |
| Ac23 | <i>env</i> | 1 | 0.3151 | Non-Significant Effect | No Effect in Sf9 Cells [75] | 1.0000 | Non-Significant Effect | |
| Ac23 | <i>env</i> | 3 | 0.3727 | Non-Significant Effect | | 0.1106 | Non-Significant Effect | |
| Ac24 | <i>pkip</i> | 1 | 0.0179 | Negative Effect | Negative Effect in Sf9 Cells [76] | 0.3870 | Non-Significant Effect | |
| Ac24 | <i>pkip</i> | 2 | 0.0281 | Negative Effect | | 0.0000 | Negative Effect | |

| | | | | | | | | |
|------|--------------|---|--------|------------------------|-----------------------------------|--------|-----------------|--|
| Ac31 | <i>sod</i> | 1 | 0.8854 | Non-Significant Effect | No Effect in Sf21 Cells [77] | 0.0003 | Negative Effect | |
| Ac31 | <i>sod</i> | 2 | 0.9985 | Non-Significant Effect | | 0.0013 | Negative Effect | |
| Ac32 | <i>fgf</i> | 2 | 0.9301 | Non-Significant Effect | No Effect in Sf21 Cells [78] | 0.0000 | Negative Effect | |
| Ac32 | <i>fgf</i> | 3 | 0.0002 | Negative Effect | | 0.0005 | Negative Effect | |
| Ac35 | <i>v-ubi</i> | 1 | 0.6539 | Non-Significant Effect | Negative Effect in Sf9 Cells [79] | 0.0017 | Negative Effect | |
| Ac35 | <i>v-ubi</i> | 2 | 0.1325 | Non-Significant Effect | | 0.0000 | Negative Effect | |
| Ac37 | <i>lef11</i> | 1 | 0.0488 | Negative Effect | Negative Effect in Sf9 Cells [80] | 0.0000 | Negative Effect | |
| Ac37 | <i>lef11</i> | 2 | 0.0105 | Negative Effect | | 0.0000 | Negative Effect | |
| Ac38 | <i>orf38</i> | 1 | 1.0000 | Non-Significant Effect | Negative Effect in Sf9 Cells [81] | 0.0000 | Negative Effect | |
| Ac38 | <i>orf38</i> | 2 | 0.9518 | Non-Significant Effect | | 0.0000 | Negative Effect | |
| Ac38 | <i>orf38</i> | 3 | 0.7122 | Non-Significant Effect | | 0.0000 | Negative Effect | |
| Ac40 | <i>p47</i> | 2 | 0.8528 | Non-Significant Effect | Negative Effect in BmNPV and | 0.0000 | Negative Effect | |

| | | | | | | | | |
|------|---------------|---|--------|------------------------|-----------------------------------|--------|------------------------|--|
| Ac40 | <i>p47</i> | 3 | 0.9523 | Non-Significant Effect | BmN Cells [60] | 0.7960 | Non-Significant Effect | |
| Ac41 | <i>lef-12</i> | 1 | 0.9322 | Non-Significant Effect | Negative Effect in Sf9 Cells [82] | 0.0000 | Negative Effect | |
| Ac41 | <i>lef-12</i> | 2 | 1.0000 | Non-Significant Effect | | 0.0000 | Negative Effect | |
| Ac41 | <i>lef-12</i> | 3 | 1.0000 | Non-Significant Effect | | 0.0000 | Negative Effect | |
| Ac43 | <i>orf43</i> | 1 | 0.9996 | Non-Significant Effect | No Effect in Sf9 Cells [83] | 0.0000 | Negative Effect | |
| Ac43 | <i>orf43</i> | 2 | 0.9935 | Non-Significant Effect | | 1.0000 | Non-Significant Effect | |
| Ac51 | <i>orf51</i> | 1 | 0.2505 | Non-Significant Effect | Negative Effect in Sf9 Cells [84] | 0.0000 | Negative Effect | |
| Ac51 | <i>orf51</i> | 2 | 0.9322 | Non-Significant Effect | | 0.0000 | Negative Effect | |
| Ac77 | <i>vlf1</i> | 1 | 0.2328 | Non-Significant Effect | Negative Effect in Sf9 Cells [85] | 0.5402 | Non-Significant Effect | |
| Ac77 | <i>vlf1</i> | 2 | 0.3702 | Non-Significant Effect | | 0.0000 | Positive effect | |
| Ac77 | <i>vlf1</i> | 3 | 0.1465 | Non-Significant Effect | | 0.1788 | Non-Significant Effect | |

| | | | | | | | | |
|-------|-------------|---|--------|------------------------|-----------------------------------|--------|------------------------|--|
| Ac128 | <i>gp64</i> | 1 | 0.7944 | Non-Significant Effect | Negative Effect in Sf9 Cells [86] | 0.0972 | Non-Significant Effect | |
| Ac128 | <i>gp64</i> | 2 | 0.9386 | Non-Significant Effect | | 0.0000 | Positive effect | |
| Ac128 | <i>gp64</i> | 3 | 0.9698 | Non-Significant Effect | | 0.0000 | Positive effect | |
| Ac128 | <i>gp64</i> | 4 | 0.0361 | Negative Effect | | 0.0569 | Non-Significant Effect | |
| Ac128 | <i>gp64</i> | 5 | 0.1834 | Non-Significant Effect | | 0.0002 | Positive effect | |
| Ac128 | <i>gp64</i> | 6 | 0.0739 | Non-Significant Effect | | 0.0000 | Positive effect | |

7.3.1 Ac1 (*ptp*)

Ac1 encodes for protein tyrosine phosphatase (PTP). In one study using Sf9 cells, a double mutant removed *ptp* along with *lef-4* and indicated no change in budded virus formation, but lowered polyhedrin formation [66]. In contrast, our data indicates that *ptp* disruption has a noticeable effect on budded virus and protein production under the p6.9 promoter in Sf9 cells. With construct one, a 41% decrease of viral titers was observed, but was determined to be not significant; this construct had a 59% decrease in VLP formation which was determined to be significant. With this construct, it is possible that the small sample size resulted in a false negative with respect to budded virus formation and further testing may be required. This is further supported as the second construct displayed a 71% decrease in viral titers and was determined to be a significant effect; this construct had a 61% decrease in VLP formation which was determined to be significant. Although previously reported as a non-essential gene, our results indicate that *ptp* is required for budded virus and protein production in Sf9 cells.

7.3.2 Ac4

Ac4 is involved in the nuclear localization of actin, but has been shown to be nonessential for baculovirus production in Sf9 cells [67]. For two of our constructs, a comparison between the Ac4 knockout and control indicated no significant difference in baculovirus production, even though a 46% and 35% reduction in baculovirus titers were observed. However, the third knockout produced a 53% reduction in baculovirus titer which reached the threshold for a significant difference based on a 95% confidence interval. Interestingly, the first two constructs displayed a significant reduction of VLPs of 47% and 30% respectively. However, the third construct that showed a significant difference in baculovirus titers produced VLP amounts that were indistinguishable from the control. For the first two constructs, the small sample size may be resulting in a false negative

and further testing of those constructs may indicate an effect as there was a consistent reduction in budded virus production when looking at all three constructs. The third construct is particularly interesting as VLP production was undisturbed while significantly decreasing the budded virus production. Again, further testing of this construct would be interesting to see if this result is replicable.

7.3.3 Ac6 (*lef-2*)

Ac6 (*lef-2*) is a DNA primase accessory factor and is required for transient DNA replication and involved in late and very late gene expression [5], [68]. In one study using Sf21 cells, it was observed that removal of the *lef-2* gene in the *AcMNPV* genome completely removed the infectivity of the baculovirus, and greatly inhibited *vp39* and *p10* gene expression [68]. These results are consistent with what we observed, wherein the deletion of *lef2* reduced budded virus by 66%-87% depending on the construct. Moreover, it consistently reduced VLP production by ~78% under the *p6.9* promoter. Overall, there is strong evidence indicating that *lef2* is an essential gene for budded virus production as well as late and very late gene expression.

7.3.4 Ac10 (*pk1*)

Ac10 (*pk1*) encodes for a serine-threonine protein kinase that has uses in phosphorylation and regulation of very late genes [69], [70]. In a study by Liang et al. in Sf9 cells, deletion of *pk1* resulted in no formation of budded virus and polyhedrin protein [69]. In a follow up study by Liang et al., the same *pk-1* knockout bacmid was used to observe the effects on the gene expression of *ie1*, *ie2*, *lef-2*, *p6.9*, *vp39*, *p10*, and *polh* where an *egfp* gene was used as a model protein when expressed under each gene's promoters. In this study, it was found that that the deletion of *pk1* had little to no effect on the expression of *ie1*, *ie2*, *lef2*, *p6.9*, and *vp39*. It did have a significant effect

on *polh* and *p10* expression, wherein the deletion of *pk-1* lowers the production of *egfp* when under the *polh* and *p10* promoters [70]. Our results are in alignment where *pk1* may be essential for budded virus formation where one construct significantly reduced baculovirus production by 94%, and another construct was close to the threshold for significance at $p = 0.07$ (60% reduction of budded virus). A third construct did not reach the threshold for significance but still showed a ~26% decrease in budded virus production. Overall, our results indicate a correlation between *pk1* disruption and a reduction in budded virus production. For protein production, Liang et al. determined that the *p6.9* expression was unaffected by the knockout of *pk1*, but our results indicate that *pk1* does affect it as shown by a reduction in VLP concentrations between 76%-85%.

7.3.5 Ac13 (*bion*)

Ac13 is known as baculovirus protein associated with the inner and outer nuclear membrane (BION) [87]. In one study, removal of this gene in *BmNPV* was shown to inhibit polyhedra formation used for occlusion bodies [87]. In another study using Sf9 cells, it was found that the removal of Ac13 had no effect on viral genome replication, formation of nucleocapsids, or creation of occlusion bodies, but reduced budded virus formation by a factor of 400 [71]. Another study also found that the removal of Ac13 resulted in lower production of budded virus in Sf9 cells [72]. Our results are in line with what was previously found wherein the removal of Ac13 reduced baculovirus production between 84-87%. As well, there was a significant effect on VLP production between 72-76%, which may indicate that the gene may have uses in late gene expression.

7.3.6 Ac14 (*lef-1*)

Ac14 (*lef-1*) is a gene involved in DNA replication [88], [89] and has also been linked to late and very late gene expression [90]. It has been classified as essential with respect to budded virus

production in Sf9 cells [72]. This is in alignment with our results as the disruption of *lef-1* reduced budded virus titers between 69%-90%, and a 70-72% in VLP production was observed.

7.3.7 Ac19

Ac19 is a gene that has been scarcely studied, particularly in the *AcMNPV* genome. In studies with *BmNPV* and *BmN-4* cells, the equivalent gene was found to be non-essential for budded virus production [73]. In our study, this gene was determined to be non-essential for budded virus production in Sf9 cells. Moreover, disruption of it had no negative effects on VLP production, and with one construct, a statistically significant increase of 37% of VLPs was observed. This was one of few gene disruptions that had no negative impacts on budded virus and VLP production. As such, it is an interesting gene to study, and a possible candidate that can be removed from the *AcMNPV* genome.

7.3.8 Ac22 (*pif2*)

Ac22 is a per os infectivity factor (PIF) and is required for oral infectivity of an insect but have been observed to be non-essential for cell culture [5], [74]. Interestingly, our data shows that knockout of *pif2* results in a significant drop-off in budded virus production from anywhere from 64% to 90% when this gene was disrupted. As well, there was a 70-75% decrease in VLP production. Our results would indicate that this gene has some effects in budded virus formation and may be essential for infectivity within cell culture conditions.

7.3.9 Ac23 (*env*)

Ac23 is a viral envelope fusion protein for Group II lepidopteran NPVs but are inactive in the *AcMNPV* genome as it is part of Group I NPVs [5]. Instead, the fusion protein found in Group I viruses is GP64. The knockout of this gene has shown to be non-essential for budded virus

formation in Sf9 cells [75]. Our results are consistent with this where there is no significant drop-off in budded virus and VLP production. However, it should be noted that there was a 42%-48% drop-off in budded virus production relative to the control. Significance testing determined that this result was insignificant, but this may be due to the high variability and low sample size resulting in a false negative. As for VLP production, it is unlikely that there was an impact due to the minor drop-offs between 2%-17%. Overall, this gene is interesting because its removal may have no impact on virus and protein production. However, more testing should be conducted to determine whether an effect on budded virus formation exists. Moreover, it was found that removal of this gene accelerated cell death in infected cells [75], which would be something to verify in further experiments.

7.3.10 Ac24 (*pkip*)

Ac24 (*pkip*) produces a protein that interacts with PK-1, which is essential in the *AcMNPV* genome [5]. In various studies involving Sf9 cells, deletion or suppression of *pkip* resulted in a reduction in budded virus production due to its role in nucleocapsid assembly [76]. Furthermore, it has roles in the transcription of very late genes, including the hyperphosphorylation of P6.9, which could be explained by the interaction of PKIP and PK-1 [76], [91]. Our data shows that there is a significant drop-off of budded virus production between 61%-65% relative to the control verifying the gene's importance for budded virus production. However, it is unclear whether VLP production was affected as one construct displayed a nonsignificant reduction in VLP production of 13%, whereas another construct displayed a significant reduction of 55%. Further testing would be interesting for this gene as the knockout effects on protein production may be target dependent.

7.3.11 Ac31 (*sod*)

Ac31 produces super oxide dismutase (SOD), which neutralizes superoxide produced by insect hemocytes by converting it to hydrogen peroxide, which can then be broken down to water and oxygen [5]. In Sf21 cells, the deletion of *sod* showed that this gene is likely non-essential for budded virus formation [77]. Our results provide further evidence for this gene being non-essential for budded virus formation as there was no significant effect found upon knockout of this gene. However, there was a significant drop-off of VLP production of 26%-29%. Relative to other gene knockout effects, the impact on VLP production was much smaller, which may indicate a false positive.

7.3.12 Ac32 (*fgf*)

Ac32 is used to produce a fibroblast growth factor (FGF). This is a secreted protein, and has use in increasing the motility of insect cells, which is useful for infection throughout an insect host [5]. In cell culture of Sf21 and TN-368 cells, the knockout of this gene yielded similar levels of budded virus production as the control [78]. Interestingly, in our study there was a varied response for budded virus production, with one construct showing a non-significant decrease, whereas the other showed a significant decrease of 81%. Both showed a significant decrease in VLP production between 28%-47% relative to the control but still producing levels of $\sim 2 \times 10^9$ particles/mL.

7.3.13 Ac35 (*v-ubi*)

Ac35 encodes for the production of viral-ubiquitin (V-UBI), which is a protein that exists on the inner surface of the viral envelope [92]. In one study that involved Sf9 cells, a frameshift mutation was introduced to *v-ubi* at amino acid 26 to suppress this gene. Results indicated that budded virus was produced, but was lower by a factor of 5 to 10 [79]. Interestingly, our results

indicate that there was an insignificant effect on the production of budded virus. As for VLP production, a reduction of 26%-34% was observed. Similar to *sod*, this decrease in VLP production is lower than most.

7.3.14 Ac37 (*lef11*)

Ac37 is another late expression factor gene. Knockout of this gene has been shown to eliminate infectious budded virions from Sf9 cells [80]. In our experiments, progeny virus was produced, but there was a significant reduction in budded virus production of 63%-69%. Similarly, VLP production was reduced by 47%-61%. These results indicate that *lef11* is an essential gene for both budded virus formation, and protein production.

7.3.15 Ac38

Ac38 has homology to ADP-ribose pyrophosphatase (ADPRase) [81]. A study has found that removal of this gene resulted in budded virus yields of <1% when compared to a control, and significantly reduced the production of GFP when under the *polh* promoter in Sf9 cells, implying that the gene has uses in very late gene expression [81]. Interestingly, our results show that there was no significant impact on budded virus production upon disruption of this gene which goes against what was previously found. However, VLP production was disrupted by 49%-57% when compared to the control, showing that this gene influences late gene expression as the VLPs were produced under the *p6.9* promoter.

7.3.16 Ac40 (*p47*)

Ac40 is a late expression factor gene that forms an RNA polymerase along with *lef4*, *lef8*, and *lef9* [93]. Its role in the formation of the RNA polymerase likely makes this an essential gene and knockout of this gene in *BmNPV* eliminates virus production in *BmN* cells [60]. Interestingly,

our results show that there were no significant effects on budded virus production. This is interesting as this provides some evidence that *p47* may not be essential for budded virus production in *AcMNPV* when infecting Sf9 cells. As for VLP production, one construct showed no significant impacts, whereas the other displayed a 60% reduction in VLP production.

7.3.17 Ac41 (*lef-12*)

Ac41 is another late expression factor used for transient late gene transcription in *S. frugiperda* cells, but is not used in other cell lines such as *T. ni* cells [5], [82]. In a study looking at Sf9 cells, deletion of the *lef-12* gene reduced baculovirus production by a factor of five compared to a control, signifying that deletion of this gene produces viable budded virus, but at lower yields. This study also looked at late gene expression, where levels of P39 and P24 were significantly lower, but PP31 and GP64 levels were similar to that of the control. As well, polyhedra levels when *lef-12* was removed were significantly lower than the control [82]. In contrast, our data shows that there were no significant effects on budded virus production. Late gene effects were found as there was a significant reduction in VLP production of 48%-64% under the *p6.9* promoter.

7.3.18 Ac43

Ac43 is involved in late and very late gene expression [5]. When the gene was removed, there was no effect on budded virus production in Sf9 cells. However, there was a reduction in the number of occlusion bodies formed, but larger polyhedra were produced [83]. From this, it appears that deletion of this gene should not affect budded virus production but has the ability to affect late and very late gene expression. Our results show that there were no significant effects found on the production of budded virus. For VLP production, one construct displayed a 58% reduction in VLPs, whereas the other construct showing no significant effects.

7.3.19 Ac51

Ac51 is a DNAJ domain protein that is required for the nuclear egress of nucleocapsids in Sf9 cells. As such, removal of this gene reduced the amount of budded virus by a factor of 1000. However, no effects on viral DNA synthesis, or production of occlusion derived virus were found [84]. Interestingly, our results indicate there were no significant effects with regards to budded virus production. It should be noted that one construct displayed a 55% reduction in viral titers, but did not meet the threshold for significance. As for VLPs, there was a significant decrease in VLP production between 52%-64%.

7.3.20 Ac77 (*vlf1*)

Ac77 is a very late expression factor (*vlf*) and is a lambda integrase protein [5]. It binds to the ends of nucleocapsids and disruption of this gene has shown that this gene is essential for nucleocapsid formation [94]. Also, disruption of this gene showed that DNA replication was impaired, only producing one third of the amount of viral DNA when compared to a control. Moreover, no nucleocapsids budded out of the Sf9 cells, and there was a negative effect on the production of P10 [85], [95]. Interestingly, our results indicate that there were no significant effects found with regards to budded virus production. As for VLP production, there was no significant decreases, and in one case, VLP production was found to increase by 46% when compared to the control. Disruption of this gene is interesting because it is possible that VLPs can be produced, and budded virus could be limited thus making downstream purification less strenuous.

7.3.21 Ac128 (*gp64*)

Ac128 (*gp64*) codes for a fatty acid acylated glycoprotein which is a low pH activated envelope fusion protein. This protein is a key characteristic for the envelope of Group I viruses

and is responsible for the entry of budded virus into a cell [5]. Disruption of this gene results in non-infectious budded virus from Sf9 cells, therefore has been deemed as essential [86]. Disruption of this gene in the TI assay reduced GP64 production to ~1% when compared to a control. Budded virus production was also reduced by a factor of 66 and total particle concentration was reduced by ~90%. However, protein expression under the *p6.9* promoter was unaffected [96]. Here the *gp64* gene was targeted in different locations. In all cases budded virus production was hindered but only two targets met the threshold for significance. As for protein production under the *p6.9* promoter, no negative effects were observed, and in fact, four constructs displayed a significant increase in VLP production between 37%-78%. Overall, *gp64* is an interesting gene as it is essential for budded virus formation but does not affect protein production for the *p6.9* promoter.

7.4 Limitations

This work screens for potential genes that can aid in the production of budded VLPs. Although no genetic sequencing was completed on progeny virus to determine whether the cuts to the *AcMNPV* genome were completed, sequencing might not even reveal that the cut occurred [97]. Furthermore, only budded virus and VLPs were quantified, but another measure that could have been done is of total particles, which would include defective viral particles.

7.5 Conclusions and Recommendations

In this study, 21 late and very late genes in the *AcMNPV* genome were disrupted in Sf9 cells using CRISPR-Cas9. Effects of gene disruption on budded virus production were measured through EPDA. Effects on late gene expression were measured under the *p6.9* promoter using HIV-Gag VLPs tagged with eGFP as a model protein. VLP concentrations were measured using flow cytometry.

The deletion of *lef-2*, *bion*, *lef-1*, *pif2*, *pkip*, and *lef-11* all lowered budded virus production in Sf9-Cas9 cells indicating potential for essentiality. For *lef-2*, *bion*, *lef-1*, *pkip*, and *lef-11*, this result was consistent with what was previously reported [68], [71], [72], [76], [80]. Of note, *pif2* was previously reported to be non-essential in cell culture [74], but our results have shown otherwise, with a 64%-90% reduction in budded virus production upon disruption of this gene. For *lef-2*, *bion*, *lef-1*, *pif2*, and *lef-11*, a decrease in VLP production was observed, which indicates that they have roles in late and very late gene expression, particularly for the expression of *p6.9*. *pkip* had one construct that displayed a nonsignificant decrease in VLP production of 13%, whereas the second construct displayed a significant decrease in VLP production of 55%.

The deletion of *gp64* lowered budded virus production in Sf9-Cas9 cells. This is consistent with previous studies, as it was shown that the deletion of this gene completely inhibits budded virus production [86]. Of interest, there was no reduction in VLP production in our experiments under the *p6.9* promoter, and in some cases, showed the potential to increase protein production.

Deletion of *ptp*, *Ac4*, *pk1*, and *fgf* showed decreases in budded virus production in Sf9-Cas9 cells, but some targets failed to meet the threshold for significance at a 95% confidence interval. Of these, disruption of *ptp*, *Ac4*, and *fgf* were previously reported to have no effect on budded virus formation in Sf9 cells, with the exception of *fgf* being done in Sf21 cells [66], [67], [78]. However, in our experiments, at least one target in each displayed a significant reduction of budded virus, with the other constructs displaying reduction that did not meet the criteria for significance. All these genes displayed a significant reduction in VLPs under the *p6.9* promoter. For *pk1*, it has previously been reported to produce no progeny virus upon deletion [69], [70], and one of our targets displayed a 94% reduction in budded virus formation, and another at 60% reduction (though this one did not meet the criteria for significance), which provides further evidence that *pk1* is an

essential gene. Interestingly, *pk1* was reported to have no effect on the gene expression of *p6.9* [70], but our results indicate otherwise with VLP concentrations being reduced by 76%-85%. Overall, these genes show evidence towards having roles in budded virus production.

Deletion of *v-ubi*, *Ac38*, *lef-12*, and *Ac51* are interesting because they displayed no reduction in budded virus production in our assay. In previous studies that used a bacmid to study these knockouts, progeny virus was produced, but in significantly less quantities [79], [81], [82], [84]. Future studies into increasing the sensitivity of this assay could be tested. One such study could be the testing of these gene knockouts at lower MOIs, wherein if there was a lower production of budded virus, secondary infections would proceed at a slower rate or not at all when compared to a control.

Deletion of *Ac19* and *env* displayed no effects on budded virus and VLP production. *Ac19* has been seldom studied with *AcMNPV* infecting *Spodoptera frugiperda* cells, but has shown to have no effect on budded virus production with *BmNPV* in *Bmn-4* cells [73]. Here, VLP production showed one target with no significant effects, and the other showed a positive effect. Hence, *Ac19* has shown potential as a non-essential gene for budded virus, and protein production under the *p6.9* promoter and should be further studied. *env* is another gene that has been reported to be non-essential for budded virus formation [75], and our results have shown no negative effects on VLP production signifying that it is another potential gene to explore further.

Deletion of *sod* and *Ac43* both showed no significant effects on budded virus production which is consistent with what has previously been reported [77], [83]. However, there were negative effects on the VLP production when these genes were disrupted making them less desirable to explore further.

Deletion of *p47* and *vlf-1* displayed no significant effects on budded virus production in our assay. However, these genes have previously been reported to be essential in budded virus production [60], [85], [95]. However, for *p47*, essentiality was reported for *BmNPV* when infecting *BmN* cells, which may signify the virus-cell interaction [60]. As for *vlf-1*, our results show no effects when this gene was disrupted, but the threshold for significance was high as reductions of 50%-67% for budded virus were not enough to meet significance.

8 Conclusions and Recommendations

The aim of this thesis was to adjust the conditions for the TI assay to reduce resource consumption. The thesis also explored Gag-GFP VLPs as a model recombinant protein product that can be efficiently quantified to observe the effects of gene knockouts. After these areas were explored, a proof-of-concept experiment was run where 21 late and very late genes in the *AcMNPV* genome were tested to see the effects of their gene knockouts.

The TI assay was adjusted from 6-well plates to 12-well plates. In 6-well plates, a seeding density of 2.5×10^6 cells/well and a media volume of 2mL was used to observe the effects of gene knockouts on BV and protein production [7]. Initial trials to convert to 12-well plates used a seeding density of 0.9×10^6 cells/well and 2mL of media which resulted in lowered BV and protein production, signifying the need for adjustments. Liquid heights were adjusted and tested in 6- and 12- well plates. Sf9 and Sf9-Cas9 cells were infected with a baculovirus expressing *mAG* under the *p6.9* promoter. It was found that the liquid height influenced BV and protein production in both plate types. To maintain equivalence between 6- and 12- well plate results, the 12-well plate conditions were adjusted to 0.9×10^6 cells/well and 0.8mL of liquid volume. These conditions were used for future experiments. These conditions lowered resource consumption by lowering media consumption per sample, lowering transfection reagent per sample, and allowing more samples per plate.

Gag-GFP can be quantified using flow cytometry with a 533 ± 30 nm light filter. This allowed for a quick analysis method to quantify VLP production. As such, it was desired to use a Gag-GFP baculovirus for the TI assay to quantify the effects of a gene knockout. Despite being a relatively quick quantification method, individual dilutions were done in tubes which becomes

time and resource intensive when 10^{-3} , 10^{-4} , and 10^{-5} dilutions are required for hundreds of samples. As such, there was a need to increase throughput. It was desired to do these dilutions in 96-well U-bottom plates as the flow cytometer was compatible with this plate type. Moreover, these plates allow for dilutions to be done in parallel by using a multichannel pipette. To ensure that there were no major discrepancies on VLP concentrations from being diluted in a plate, samples were diluted in plates and tubes, both starting from the same 10^{-2} dilution stock. It was found that samples diluted in plates consistently resulted in 10-20% higher VLP concentrations as opposed to the tube dilutions. As this was a consistent result, it was determined that it was acceptable for use for the TI assay. This is because gene knockout results were compared to a control, so relative differences were more important than accurate particle counts.

Moreover, Gag-GFP stability was monitored over a 7-hour period. This was to ensure that VLP degradation would not skew results. In these tests, it was observed that particle counts were stable for ~1.5 hours after the initial dilution, after which particle counts steadily dropped. This time frame allowed for ~15 samples to be run in a 96-well plate. With 15 samples, dilutions would take ~30 minutes, and the flow cytometer would run for ~45 minutes.

Next, the control for which gene knockouts would be compared to was evaluated. Here the scrambled control was compared to an infected only control. The scrambled control had Sf9-Cas9 cells transfected with a plasmid containing a scrambled sequence, and subsequently infected with a *p6.9Gag-GFP* expressing baculovirus. The infected only control was only infected with a *p6.9Gag-GFP* expressing baculovirus. Here it was found that the scrambled control and infected only control showed a statistical difference at a 95% confidence interval. That is, the scrambled control produced higher amounts of BV and VLPs compared to the infected only control. Also, a plasmid containing a gene pertaining to the production of mKate2 protein was transfected in the

Sf9-Cas9 cells and subsequently infected. This plasmid should act as an inert species and should not interact with *AcMNPV*. Here it was found that the mKate2 control was statistically indistinguishable from the scrambled control with respect to BV and VLP production. Finally, it was shown that the addition of transfection reagent, with no plasmid, resulted in higher BV production when compared to the infected only control. This displays that that the transfection reagent itself influences BV production. These results indicated that the scrambled control should be used to compare gene knockouts as it is the most representative of the conditions that the experimental treatments underwent. It should be noted that the scrambled control was not compared to the transfection reagent only control. A future experiment should be conducted comparing the two as the addition of an inert plasmid may have undocumented effects on BV and VLP production.

Finally, 21 late and very late genes were disrupted using the TI assay using Gag-GFP produced under the *p6.9* promoter as the recombinant protein product. The deletion of *lef-2*, *bion*, *lef-1*, *pif2*, *pkip*, and *lef-11* resulted in lowered BV and VLP production, indicating that the reproduction of *AcMNPV* is dependent on these genes, and have roles in gene expression of *p6.9*. Due to negative effects across the board further exploration into these genes is not required. Disruption of *gp64* lowered budded virus production but had no effect on VLP production. These effects are consistent with what has previously been published [86]. *ptp*, *Ac4*, *pk1*, and *fgf* deletions showed signs of reduced BV production but did not always meet the threshold for significance under a 95% confidence interval, however, deletion of these genes displayed a significant reduction in VLP production, indicating their roles in the gene expression of *p6.9*.

Deletion of *v-ubi*, *Ac38*, *lef-12* and *Ac51* displayed no reduction in BV production. These results go against what was previously published where knockouts of these genes resulted in

reductions of BV [79], [81], [82], [84]. This shows the lack of sensitivity of the assay, wherein less pronounced effects on BV production may be undetectable. Here, a future study to increase the assay sensitivity is proposed. Using *v-ubi*, *Ac38*, *lef-12* and *Ac51* as the knockouts, lower MOIs should be used to induce an asynchronous infection condition in the TI assay. If there is an effect on BV production there should be a lower percentage of infected cells when compared to a control, as secondary infections should proceed at a slower rate.

Deletion of *sod* and *ac43* displayed no significant effects on BV production but did impact VLP production making them undesirable to explore further. Deletion of *p47* and *vlf-1* displayed no significant effects on BV production which goes against what was previously reported [60], [85], [95]. Here it is recommended to run these gene knockouts again to see if these results can be replicated.

Finally, deletion of *Ac19* and *env* were observed to have no effects on BV and VLP production. These indicate two genes that should be further studied with knockout viruses. If complete knockout of these genes displays no negative effects on BV and protein production, it would indicate two genes that are non-essential and can be removed from the *AcMNPV* genome.

The results of these 21 gene knockouts are the result of the work completed in chapters 4-6. In the span of a few months, 21 of these genes were screened, and two were found to be candidates that could be further investigated to be removed from the *AcMNPV* genome. This shows the high throughput of this TI assay and showing its potential to be used a tool for the screening of viral genomes.

References

- [1] S. George, “Use and Control of Co-Expression in the Baculovirus-Insect Cell System for the Production of Multiple Proteins and Complex Biologics,” Aug. 2016, Accessed: Apr. 22, 2025. [Online]. Available: <http://hdl.handle.net/10012/10660>
- [2] M. R. Bruder and M. G. Aucoin, “A sensitive assay for scrutiny of *Autographa californica* multiple nucleopolyhedrovirus genes using CRISPR-Cas9,” *Appl Microbiol Biotechnol*, vol. 107, no. 13, pp. 4323–4335, Jul. 2023, doi: 10.1007/s00253-023-12462-y.
- [3] M. R. Bruder, S.-D. Walji, and M. G. Aucoin, “Comparison of CRISPR–Cas9 Tools for Transcriptional Repression and Gene Disruption in the BEVS,” *Viruses*, vol. 13, no. 10, Art. no. 10, Oct. 2021, doi: 10.3390/v13101925.
- [4] S. Nooraei *et al.*, “Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers,” *Journal of Nanobiotechnology*, vol. 19, no. 1, p. 59, Feb. 2021, doi: 10.1186/s12951-021-00806-7.
- [5] G. F. Rohrmann, “The AcMNPV genome: Gene content, conservation, and function,” in *Baculovirus Molecular Biology [Internet]. 4th edition*, National Center for Biotechnology Information (US), 2019. Accessed: Mar. 03, 2025. [Online]. Available: <https://www.ncbi.nlm.nih.gov/books/NBK543457/>
- [6] B. Donaldson, F. Al-Barwani, V. Young, S. Scullion, V. Ward, and S. Young, “Virus-Like Particles, a Versatile Subunit Vaccine Platform,” in *Subunit Vaccine Delivery*, C. Foged, T. Rades, Y. Perrie, and S. Hook, Eds., New York, NY: Springer, 2015, pp. 159–180. doi: 10.1007/978-1-4939-1417-3_9.

- [7] M. Bruder, “Toward Optimization of the Baculovirus Expression Vector System - Development of Genetic Tools to Improve Biologics Production,” Aug. 2021, Accessed: Mar. 19, 2025. [Online]. Available: <http://hdl.handle.net/10012/17245>
- [8] R. Gupta *et al.*, “Platforms, advances, and technical challenges in virus-like particles-based vaccines,” *Front. Immunol.*, vol. 14, Feb. 2023, doi: 10.3389/fimmu.2023.1123805.
- [9] P. Steppert, D. Burgstaller, M. Klausberger, A. Tover, E. Berger, and A. Jungbauer, “Quantification and characterization of virus-like particles by size-exclusion chromatography and nanoparticle tracking analysis,” *Journal of Chromatography A*, vol. 1487, pp. 89–99, Mar. 2017, doi: 10.1016/j.chroma.2016.12.085.
- [10] J. A. Jehle *et al.*, “On the classification and nomenclature of baculoviruses: A proposal for revision,” *Arch Virol*, vol. 151, no. 7, pp. 1257–1266, Jul. 2006, doi: 10.1007/s00705-006-0763-6.
- [11] F. Monteiro, N. Carinhas, M. J. Carrondo, V. Bernal, and P. M. Alves, “Toward system-level understanding of baculovirus–host cell interactions: from molecular fundamental studies to large-scale proteomics approaches,” *Front. Microbiol.*, vol. 3, Nov. 2012, doi: 10.3389/fmicb.2012.00391.
- [12] H.-W. Ackermann and W. A. Smirnoff, “A morphological investigation of 23 baculoviruses,” *Journal of Invertebrate Pathology*, vol. 41, no. 3, pp. 269–280, May 1983, doi: 10.1016/0022-2011(83)90244-6.
- [13] M. D. Ayres, S. C. Howard, J. Kuzio, M. Lopez-Ferber, and R. D. Possee, “The Complete DNA Sequence of *Autographa californica* Nuclear Polyhedrosis Virus,” *Virology*, vol. 202, no. 2, pp. 586–605, Aug. 1994, doi: 10.1006/viro.1994.1380.

- [14] G. W. Blissard and J. R. Wenz, “Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion,” *J Virol*, vol. 66, no. 11, pp. 6829–6835, Nov. 1992, doi: 10.1128/JVI.66.11.6829-6835.1992.
- [15] L. E. Volkman and P. A. Goldsmith, “Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: Inhibition of entry by adsorptive endocytosis,” *Virology*, vol. 143, no. 1, pp. 185–195, May 1985, doi: 10.1016/0042-6822(85)90107-2.
- [16] T. Ohkawa, L. E. Volkman, and M. D. Welch, “Actin-based motility drives baculovirus transit to the nucleus and cell surface,” *J Cell Biol*, vol. 190, no. 2, pp. 187–195, Jul. 2010, doi: 10.1083/jcb.201001162.
- [17] Y.-R. Chen *et al.*, “The Transcriptome of the Baculovirus *Autographa californica* Multiple Nucleopolyhedrovirus in *Trichoplusia ni* Cells,” *J Virol*, vol. 87, no. 11, pp. 6391–6405, Jun. 2013, doi: 10.1128/JVI.00194-13.
- [18] A. L. Passarelli and L. K. Miller, “Three baculovirus genes involved in late and very late gene expression: ie-1, ie-n, and lef-2,” *Journal of Virology*, vol. 67, no. 4, pp. 2149–2158, Apr. 1993, doi: 10.1128/jvi.67.4.2149-2158.1993.
- [19] A. L. Passarelli, “Barriers to success: How baculoviruses establish efficient systemic infections,” *Virology*, vol. 411, no. 2, pp. 383–392, Mar. 2011, doi: 10.1016/j.virol.2011.01.009.
- [20] D. C. J. Carpentier, C. M. Griffiths, and L. A. King, “The baculovirus P10 protein of *Autographa californica* nucleopolyhedrovirus forms two distinct cytoskeletal-like structures and associates with polyhedral occlusion bodies during infection,” *Virology*, vol. 371, no. 2, pp. 278–291, Feb. 2008, doi: 10.1016/j.virol.2007.09.043.

- [21] R. B. Hitchman, E. Locanto, R. D. Possee, and L. A. King, “Optimizing the baculovirus expression vector system,” *Methods*, vol. 55, no. 1, pp. 52–57, Sep. 2011, doi: 10.1016/j.ymeth.2011.06.011.
- [22] M. R. Bruder and M. G. Aucoin, “Utility of Alternative Promoters for Foreign Gene Expression Using the Baculovirus Expression Vector System,” *Viruses*, vol. 14, no. 12, Art. no. 12, Dec. 2022, doi: 10.3390/v14122670.
- [23] E. Kohlbrenner *et al.*, “Successful Production of Pseudotyped rAAV Vectors Using a Modified Baculovirus Expression System,” *Molecular Therapy*, vol. 12, no. 6, pp. 1217–1225, Dec. 2005, doi: 10.1016/j.ymthe.2005.08.018.
- [24] M. O. Mohsen, A. C. Gomes, M. Vogel, and M. F. Bachmann, “Interaction of Viral Capsid-Derived Virus-Like Particles (VLPs) with the Innate Immune System,” *Vaccines*, vol. 6, no. 3, Art. no. 3, Sep. 2018, doi: 10.3390/vaccines6030037.
- [25] P. Pechsrichuang, S. Namwongnao, and A. Jacquet, “Bioengineering of Virus-like Particles for the Prevention or Treatment of Allergic Diseases,” *Allergy Asthma Immunol Res*, vol. 13, no. 1, pp. 23–41, Aug. 2020, doi: 10.4168/aair.2021.13.1.23.
- [26] A. Urakami *et al.*, “Development of a Novel Virus-Like Particle Vaccine Platform That Mimics the Immature Form of Alphavirus,” *Clin Vaccine Immunol*, vol. 24, no. 7, pp. e00090-17, Jul. 2017, doi: 10.1128/CVI.00090-17.
- [27] K. Reiter, P. P. Aguilar, V. Wetter, P. Steppert, A. Tover, and A. Jungbauer, “Separation of virus-like particles and extracellular vesicles by flow-through and heparin affinity chromatography,” *Journal of Chromatography A*, vol. 1588, pp. 77–84, Mar. 2019, doi: 10.1016/j.chroma.2018.12.035.

- [28] P. Steppert *et al.*, “Separation of HIV-1 gag virus-like particles from vesicular particles impurities by hydroxyl-functionalized monoliths,” *Journal of Separation Science*, vol. 40, no. 4, pp. 979–990, 2017, doi: 10.1002/jssc.201600765.
- [29] A. Venereo-Sanchez *et al.*, “Hemagglutinin and neuraminidase containing virus-like particles produced in HEK-293 suspension culture: An effective influenza vaccine candidate,” *Vaccine*, vol. 34, no. 29, pp. 3371–3380, Jun. 2016, doi: 10.1016/j.vaccine.2016.04.089.
- [30] M. B. Laurens, “RTS,S/AS01 vaccine (Mosquirix™): an overview,” *Hum Vaccin Immunother*, vol. 16, no. 3, pp. 480–489, Oct. 2019, doi: 10.1080/21645515.2019.1669415.
- [31] C. Hartley, P. Wasuwanich, T. Van, and W. Karnsakul, “Hepatitis E Vaccines Updates,” *Vaccines (Basel)*, vol. 12, no. 7, p. 722, Jun. 2024, doi: 10.3390/vaccines12070722.
- [32] A. Bhatwa, W. Wang, Y. I. Hassan, N. Abraham, X.-Z. Li, and T. Zhou, “Challenges Associated With the Formation of Recombinant Protein Inclusion Bodies in Escherichia coli and Strategies to Address Them for Industrial Applications,” *Front Bioeng Biotechnol*, vol. 9, p. 630551, Feb. 2021, doi: 10.3389/fbioe.2021.630551.
- [33] X. Huang, X. Wang, J. Zhang, N. Xia, and Q. Zhao, “Escherichia coli-derived virus-like particles in vaccine development,” *npj Vaccines*, vol. 2, no. 1, pp. 1–9, Feb. 2017, doi: 10.1038/s41541-017-0006-8.
- [34] A. Monie, C.-F. Hung, R. Roden, and T.-C. Wu, “Cervarix™: a vaccine for the prevention of HPV 16, 18-associated cervical cancer,” *Biologics*, vol. 2, no. 1, pp. 107–113, Mar. 2008.

- [35] M. M. J. Cox and J. R. Hollister, “FluBlok, a next generation influenza vaccine manufactured in insect cells,” *Biologicals*, vol. 37, no. 3, pp. 182–189, Jun. 2009, doi: 10.1016/j.biologicals.2009.02.014.
- [36] L. Stertman *et al.*, “The Matrix-MTM adjuvant: A critical component of vaccines for the 21st century,” *Hum Vaccin Immunother*, vol. 19, no. 1, p. 2189885, Apr. 2023, doi: 10.1080/21645515.2023.2189885.
- [37] J. Fuenmayor, F. Gòdia, and L. Cervera, “Production of virus-like particles for vaccines,” *New Biotechnology*, vol. 39, pp. 174–180, Oct. 2017, doi: 10.1016/j.nbt.2017.07.010.
- [38] Y. Ishino, H. Shinagawa, K. Makino, M. Amemura, and A. Nakata, “Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product.,” *J Bacteriol*, vol. 169, no. 12, pp. 5429–5433, Dec. 1987.
- [39] P. M. A. Groenen, A. E. Bunschoten, D. van Soolingen, and J. D. A. van Erftbden, “Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method,” *Molecular Microbiology*, vol. 10, no. 5, pp. 1057–1065, 1993, doi: 10.1111/j.1365-2958.1993.tb00976.x.
- [40] B. Masepohl, K. Görlitz, and H. Böhme, “Long tandemly repeated repetitive (LTRR) sequences in the filamentous cyanobacterium *Anabaena* sp. PCC 7120,” *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*, vol. 1307, no. 1, pp. 26–30, Jun. 1996, doi: 10.1016/0167-4781(96)00040-1.
- [41] F. j. m. Mojica, C. Ferrer, G. Juez, and F. Rodríguez-Valera, “Long stretches of short tandem repeats are present in the largest replicons of the Archaea *Haloferax mediterranei* and

Haloferax volcanii and could be involved in replicon partitioning,” *Molecular Microbiology*, vol. 17, no. 1, pp. 85–93, 1995, doi: 10.1111/j.1365-2958.1995.mmi_17010085.x.

[42] F. J. M. Mojica, C. Díez-Villaseñor, E. Soria, and G. Juez, “Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria,” *Molecular Microbiology*, vol. 36, no. 1, pp. 244–246, 2000, doi: 10.1046/j.1365-2958.2000.01838.x.

[43] F. J. M. Mojica, C. Díez-Villaseñor, J. García-Martínez, and E. Soria, “Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements,” *J Mol Evol*, vol. 60, no. 2, pp. 174–182, Feb. 2005, doi: 10.1007/s00239-004-0046-3.

[44] Ruud. Jansen, Jan. D. A. van Embden, Wim. Gastra, and Leo. M. Schouls, “Identification of genes that are associated with DNA repeats in prokaryotes,” *Molecular Microbiology*, vol. 43, no. 6, pp. 1565–1575, 2002, doi: 10.1046/j.1365-2958.2002.02839.x.

[45] R. Barrangou *et al.*, “CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes,” *Science*, vol. 315, no. 5819, pp. 1709–1712, Mar. 2007, doi: 10.1126/science.1138140.

[46] K. S. Makarova *et al.*, “Evolution and classification of the CRISPR–Cas systems,” *Nat Rev Microbiol*, vol. 9, no. 6, pp. 467–477, Jun. 2011, doi: 10.1038/nrmicro2577.

[47] E. V. Koonin, K. S. Makarova, and F. Zhang, “Diversity, classification and evolution of CRISPR–Cas systems,” *Curr Opin Microbiol*, vol. 37, pp. 67–78, Jun. 2017, doi: 10.1016/j.mib.2017.05.008.

- [48] T. Li *et al.*, “CRISPR/Cas9 therapeutics: progress and prospects,” *Sig Transduct Target Ther*, vol. 8, no. 1, pp. 1–23, Jan. 2023, doi: 10.1038/s41392-023-01309-7.
- [49] J. van der Oost, M. M. Jore, E. R. Westra, M. Lundgren, and S. J. J. Brouns, “CRISPR-based adaptive and heritable immunity in prokaryotes,” *Trends in Biochemical Sciences*, vol. 34, no. 8, pp. 401–407, Aug. 2009, doi: 10.1016/j.tibs.2009.05.002.
- [50] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier, “A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity,” *Science*, vol. 337, no. 6096, pp. 816–821, Aug. 2012, doi: 10.1126/science.1225829.
- [51] J. D. Sander and J. K. Joung, “CRISPR-Cas systems for editing, regulating and targeting genomes,” *Nat Biotechnol*, vol. 32, no. 4, pp. 347–355, Apr. 2014, doi: 10.1038/nbt.2842.
- [52] Z.-Q. Dong *et al.*, “Establishment of a highly efficient virus-inducible CRISPR/Cas9 system in insect cells,” *Antiviral Research*, vol. 130, pp. 50–57, Jun. 2016, doi: 10.1016/j.antiviral.2016.03.009.
- [53] Z. Dong *et al.*, “Establishment of a baculovirus-inducible CRISPR/Cas9 system for antiviral research in transgenic silkworms,” *Appl Microbiol Biotechnol*, vol. 102, no. 21, pp. 9255–9265, Nov. 2018, doi: 10.1007/s00253-018-9295-8.
- [54] V. Pazmiño-Ibarra, A. Mengual-Martí, A. M. Targovnik, and S. Herrero, “Improvement of baculovirus as protein expression vector and as biopesticide by CRISPR/Cas9 editing,” *Biotechnology and Bioengineering*, vol. 116, no. 11, pp. 2823–2833, 2019, doi: 10.1002/bit.27139.

- [55] J. Chang *et al.*, “Genome-wide CRISPR screening reveals genes essential for cell viability and resistance to abiotic and biotic stresses in *Bombyx mori*,” *Genome Res*, vol. 30, no. 5, pp. 757–767, May 2020, doi: 10.1101/gr.249045.119.
- [56] Q. Hong, J. Liu, Y. Wei, and X. Wei, “Application of Baculovirus Expression Vector System (BEVS) in Vaccine Development,” *Vaccines (Basel)*, vol. 11, no. 7, p. 1218, Jul. 2023, doi: 10.3390/vaccines11071218.
- [57] A. Sharma *et al.*, “Structural stability of SARS-CoV-2 virus like particles degrades with temperature,” *Biochemical and Biophysical Research Communications*, vol. 534, pp. 343–346, Jan. 2021, doi: 10.1016/j.bbrc.2020.11.080.
- [58] A. Lynch, A. E. Meyers, A.-L. Williamson, and E. P. Rybicki, “Stability studies of HIV-1 Pr55gagvirus-like particles made in insect cells after storage in various formulation media,” *Virology Journal*, vol. 9, no. 1, p. 210, Sep. 2012, doi: 10.1186/1743-422X-9-210.
- [59] “FuGENE® HD Transfection Reagent, 1ml.” Accessed: Mar. 24, 2025. [Online]. Available: <https://www.promega.ca/resources/msds/msdss/e2000/e2311/>
- [60] C. Ono, T. Kamagata, H. Taka, K. Sahara, S. Asano, and H. Bando, “Phenotypic grouping of 141 BmNPVs lacking viral gene sequences,” *Virus Res*, vol. 165, no. 2, pp. 197–206, May 2012, doi: 10.1016/j.virusres.2012.02.016.
- [61] H. Chaabihi, M. H. Ogliastro, M. Martin, C. Giraud, G. Devauchelle, and M. Cerutti, “Competition between baculovirus polyhedrin and p10 gene expression during infection of insect cells,” *J Virol*, vol. 67, no. 5, pp. 2664–2671, May 1993.

- [62] G. E. Smith, M. D. Summers, and M. J. Fraser, “Production of human beta interferon in insect cells infected with a baculovirus expression vector,” *Mol Cell Biol*, vol. 3, no. 12, pp. 2156–2165, Dec. 1983.
- [63] R. Assenberg, P. T. Wan, S. Geisse, and L. M. Mayr, “Advances in recombinant protein expression for use in pharmaceutical research,” *Current Opinion in Structural Biology*, vol. 23, no. 3, pp. 393–402, Jun. 2013, doi: 10.1016/j.sbi.2013.03.008.
- [64] L. Ikonomidou, Y.-J. Schneider, and S. N. Agathos, “Insect cell culture for industrial production of recombinant proteins,” *Appl Microbiol Biotechnol*, vol. 62, no. 1, pp. 1–20, Jul. 2003, doi: 10.1007/s00253-003-1223-9.
- [65] I. Nobiron, D. R. O’Reilly, and J. A. Olszewski, “Autographa californica nucleopolyhedrovirus infection of Spodoptera frugiperda cells: a global analysis of host gene regulation during infection, using a differential display approach,” *Journal of General Virology*, vol. 84, no. 11, pp. 3029–3039, 2003, doi: 10.1099/vir.0.19270-0.
- [66] Y. Li and L. A. Guarino, “Roles of LEF-4 and PTP/BVP RNA Triphosphatases in Processing of Baculovirus Late mRNAs,” *Journal of Virology*, vol. 82, no. 11, pp. 5573–5583, Jun. 2008, doi: 10.1128/jvi.00058-08.
- [67] K. M. Gandhi, T. Ohkawa, M. D. Welch, and L. E. Volkman, “Nuclear localization of actin requires AC102 in Autographa californica multiple nucleopolyhedrovirus-infected cells,” *J Gen Virol*, vol. 93, no. Pt 8, pp. 1795–1803, Aug. 2012, doi: 10.1099/vir.0.041848-0.
- [68] C. P. Wu *et al.*, “Autographa californica Multiple Nucleopolyhedrovirus LEF-2 Is a Capsid Protein Required for Amplification but Not Initiation of Viral DNA Replication,” *J Virol*, vol. 84, no. 10, pp. 5015–5024, May 2010, doi: 10.1128/JVI.02423-09.

- [69] C. Liang *et al.*, “*Autographa californica* multiple nucleopolyhedrovirus PK-1 is essential for nucleocapsid assembly,” *Virology*, vol. 443, no. 2, pp. 349–357, Sep. 2013, doi: 10.1016/j.virol.2013.05.025.
- [70] C. Liang, X. Su, G. Xu, X. Dai, and S. Zhao, “*Autographa californica* multiple nucleopolyhedrovirus PK1 is a factor that regulates high-level expression of very late genes in viral infection,” *Virology*, vol. 512, pp. 56–65, Dec. 2017, doi: 10.1016/j.virol.2017.09.004.
- [71] X. Chen, X. Yang, C. Lei, F. Qin, X. Sun, and J. Hu, “*Autographa Californica* Multiple Nucleopolyhedrovirus orf13 Is Required for Efficient Nuclear Egress of Nucleocapsids,” *Virol Sin*, vol. 36, no. 5, pp. 968–980, Mar. 2021, doi: 10.1007/s12250-021-00353-3.
- [72] T. Chen *et al.*, “Systematic Analysis of 42 *Autographa Californica* Multiple Nucleopolyhedrovirus Genes Identifies An Additional Six Genes Involved in the Production of Infectious Budded Virus,” *Virol Sin*, vol. 36, no. 4, pp. 762–773, Aug. 2021, doi: 10.1007/s12250-021-00355-1.
- [73] S. Katsuma, J. Kobayashi, Y. Koyano, N. Matsuda-Imai, W. Kang, and T. Shimada, “Baculovirus-Encoded Protein BV/ODV-E26 Determines Tissue Tropism and Virulence in Lepidopteran Insects,” *J Virol*, vol. 86, no. 5, pp. 2545–2555, Mar. 2012, doi: 10.1128/JVI.06308-11.
- [74] T. Ohkawa, J. O. Washburn, R. Sitapara, E. Sid, and L. E. Volkman, “Specific binding of *Autographa californica* M nucleopolyhedrovirus occlusion-derived virus to midgut cells of *Heliothis virescens* larvae is mediated by products of pif genes Ac119 and Ac022 but not by Ac115,” *J Virol*, vol. 79, no. 24, pp. 15258–15264, Dec. 2005, doi: 10.1128/JVI.79.24.15258-15264.2005.

- [75] O. Y. Lung, M. Cruz-Alvarez, and G. W. Blissard, “Ac23, an Envelope Fusion Protein Homolog in the Baculovirus *Autographa californica* Multicapsid Nucleopolyhedrovirus, Is a Viral Pathogenicity Factor,” *J Virol*, vol. 77, no. 1, pp. 328–339, Jan. 2003, doi: 10.1128/JVI.77.1.328-339.2003.
- [76] Q. Lai *et al.*, “AcMNPV PKIP is required for hyperexpression of very late genes and involved in the hyperphosphorylation of the viral basic protein P6.9,” *Virus Research*, vol. 279, p. 197889, Apr. 2020, doi: 10.1016/j.virusres.2020.197889.
- [77] M. D. Tomalski, R. Eldridge, and L. K. Miller, “A baculovirus homolog of aCu/Znsuperoxide dismutase gene,” *Virology*, vol. 184, no. 1, pp. 149–161, Sep. 1991, doi: 10.1016/0042-6822(91)90831-U.
- [78] C. Detvisitsakun, E. L. Cain, and A. L. Passarelli, “The *Autographa californica* M nucleopolyhedrovirus fibroblast growth factor accelerates host mortality,” *Virology*, vol. 365, no. 1, pp. 70–78, Aug. 2007, doi: 10.1016/j.virol.2007.03.027.
- [79] L. M. Reilly and L. A. Guarino, “The Viral Ubiquitin Gene of *Autographa californica* Nuclear Polyhedrosis Virus Is Not Essential for Viral Replication,” *Virology*, vol. 218, no. 1, pp. 243–247, Apr. 1996, doi: 10.1006/viro.1996.0185.
- [80] G. Lin and G. W. Blissard, “Analysis of an *Autographa californica* Nucleopolyhedrovirus *lef-11* Knockout: LEF-11 Is Essential for Viral DNA Replication,” *J Virol*, vol. 76, no. 6, pp. 2770–2779, Mar. 2002, doi: 10.1128/JVI.76.6.2770-2779.2002.
- [81] J. Ge, Z. Wei, Y. Huang, J. Yin, Z. Zhou, and J. Zhong, “AcMNPV ORF38 protein has the activity of ADP-ribose pyrophosphatase and is important for virus replication,” *Virology*, vol. 361, no. 1, pp. 204–211, Apr. 2007, doi: 10.1016/j.virol.2006.11.017.

- [82] L. A. Guarino, T.-A. Mistretta, and W. Dong, “Baculovirus lef-12 Is Not Required for Viral Replication,” *Journal of Virology*, vol. 76, no. 23, pp. 12032–12043, Dec. 2002, doi: 10.1128/jvi.76.23.12032-12043.2002.
- [83] X. Y. Tao *et al.*, “Functional characterization of *Autographa californica* multiple nucleopolyhedrovirus ORF43 and phenotypic changes of ORF43-knockout mutant,” *J Microbiol.*, vol. 51, no. 4, pp. 515–521, Aug. 2013, doi: 10.1007/s12275-013-3058-0.
- [84] J. Qiu, Z. Tang, Y. Cai, W. Wu, M. Yuan, and K. Yang, “The *Autographa californica* Multiple Nucleopolyhedrovirus ac51 Gene Is Required for Efficient Nuclear Egress of Nucleocapsids and Is Essential for In Vivo Virulence,” *J Virol*, vol. 93, no. 3, pp. e01923-18, Jan. 2019, doi: 10.1128/JVI.01923-18.
- [85] Y. Li, J. Wang, R. Deng, Q. Zhang, K. Yang, and X. Wang, “vlf-1 deletion brought AcMNPV to defect in nucleocapsid formation,” *Virus Genes*, vol. 31, no. 3, pp. 275–284, Dec. 2005, doi: 10.1007/s11262-005-3242-3.
- [86] S. A. Monsma, A. G. Oomens, and G. W. Blissard, “The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection.,” *J Virol*, vol. 70, no. 7, pp. 4607–4616, Jul. 1996.
- [87] T. Nagamine, T. Inaba, and Y. Sako, “A nuclear envelop-associated baculovirus protein promotes intranuclear lipid accumulation during infection,” *Virology*, vol. 532, pp. 108–117, Jun. 2019, doi: 10.1016/j.virol.2019.04.006.
- [88] M. Kool, C. H. Ahrens, R. W. Goldbach, G. F. Rohrmann, and J. M. Vlak, “Identification of genes involved in DNA replication of the *Autographa californica* baculovirus.,” *Proc Natl Acad Sci U S A*, vol. 91, no. 23, pp. 11212–11216, Nov. 1994.

- [89] A. Lu and L. K. Miller, “The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication.,” *J Virol*, vol. 69, no. 2, pp. 975–982, Feb. 1995.
- [90] A. L. Passarelli and L. K. Miller, “Identification and characterization of lef-1, a baculovirus gene involved in late and very late gene expression.,” *J Virol*, vol. 67, no. 6, pp. 3481–3488, Jun. 1993.
- [91] Q. Lai, L. Zhu, L. Xu, M. Yuan, W. Wu, and K. Yang, “AcMNPV PKIP is associated with nucleocapsid of budded virions and involved in nucleocapsid assembly,” *Virus Research*, vol. 268, pp. 27–37, Jul. 2019, doi: 10.1016/j.virusres.2019.05.014.
- [92] L. A. Guarino, G. Smith, and W. Dong, “Ubiquitin is attached to membranes of baculovirus particles by a novel type of phospholipid anchor,” *Cell*, vol. 80, no. 2, pp. 301–309, Jan. 1995, doi: 10.1016/0092-8674(95)90413-1.
- [93] M. M. van Oers and J. M. Vlak, “Baculovirus genomics,” *Curr Drug Targets*, vol. 8, no. 10, pp. 1051–1068, Oct. 2007, doi: 10.2174/138945007782151333.
- [94] A. L. Vanarsdall, K. Okano, and G. F. Rohrmann, “Characterization of the role of very late expression factor 1 in baculovirus capsid structure and DNA processing,” *J Virol*, vol. 80, no. 4, pp. 1724–1733, Feb. 2006, doi: 10.1128/JVI.80.4.1724-1733.2006.
- [95] A. L. Vanarsdall, K. Okano, and G. F. Rohrmann, “Characterization of a baculovirus with a deletion of *vlf-1*,” *Virology*, vol. 326, no. 1, pp. 191–201, Aug. 2004, doi: 10.1016/j.virol.2004.06.003.

- [96] M. R. Bruder and M. G. Aucoin, “Evaluation of Virus-Free Manufacture of Recombinant Proteins Using CRISPR-Mediated Gene Disruption in Baculovirus-Infected Insect Cells,” *Vaccines*, vol. 11, no. 2, Art. no. 2, Feb. 2023, doi: 10.3390/vaccines11020225.
- [97] M. Chakraborty, L. Nielsen, D. Nash, J. I. Nissimov, T. C. Charles, and M. G. Aucoin, “Adapting Next-Generation Sequencing to in Process CRISPR-Cas9 Genome Editing of Recombinant AcMNPV Vectors: From Shotgun to Tiled-Amplicon Sequencing,” *Viruses*, vol. 17, no. 3, p. 437, Mar. 2025, doi: 10.3390/v17030437.

Appendix A Chapter 4 Raw Data

Table 9 Raw data used for Figure 1

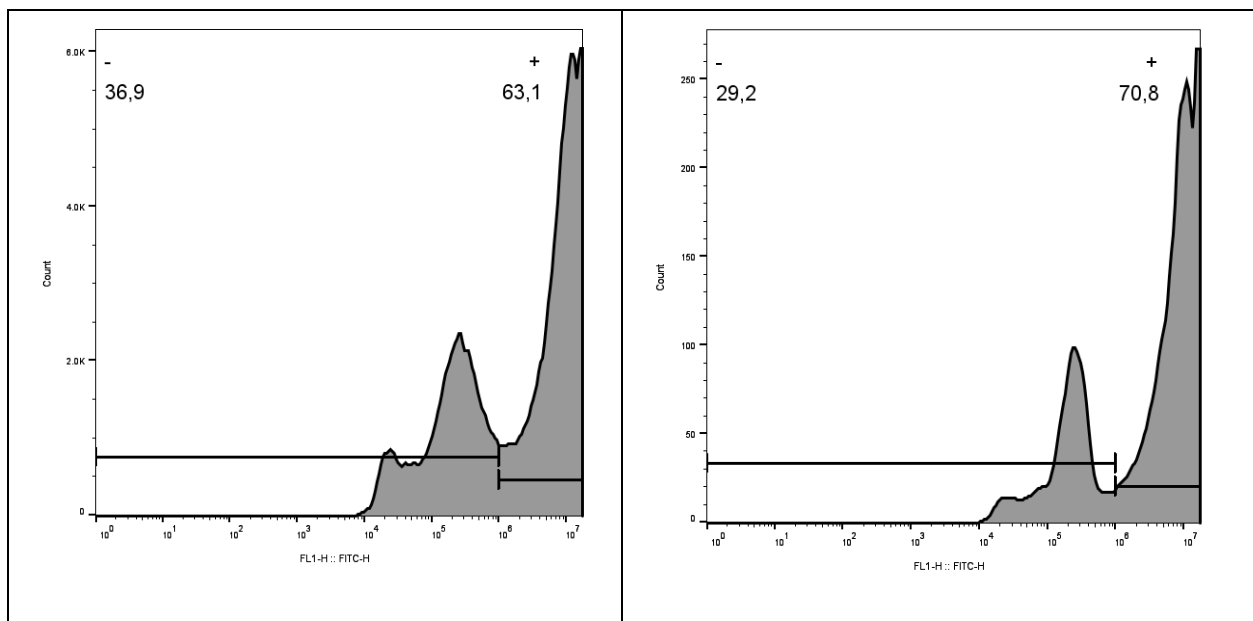
| Label | Cell Line | Plate | Volume (mL) | Titer |
|-------|-----------|---------|-------------|----------|
| 1 | Sf9-Cas9 | 6-Well | 2 | 9.59E+07 |
| 1 | Sf9-Cas9 | 6-Well | 2 | 1.59E+08 |
| 1 | Sf9-Cas9 | 6-Well | 2 | 1.59E+08 |
| 2 | Sf9 | 6-Well | 2 | 2.86E+08 |
| 2 | Sf9 | 6-Well | 2 | 2.47E+08 |
| 2 | Sf9 | 6-Well | 2 | 2.18E+08 |
| 3 | Sf9-Cas9 | 12-Well | 2 | 9.86E+06 |
| 3 | Sf9-Cas9 | 12-Well | 2 | 8.28E+06 |
| 3 | Sf9-Cas9 | 12-Well | 2 | 1.67E+07 |
| 4 | Sf9 | 12-Well | 2 | 2.45E+07 |
| 4 | Sf9 | 12-Well | 2 | 1.39E+07 |
| 4 | Sf9 | 12-Well | 2 | 1.93E+07 |

Table 10 Raw data used for Figure 3

| Label | Cell Line | Plate Type | Volume (mL) | Titer (Virus/mL) |
|-------|-----------|------------|-------------|------------------|
| 1 | Sf9 | 6-Well | 2 | 2.52E+08 |
| 1 | Sf9 | 6-Well | 2 | 2.47E+08 |
| 1 | Sf9 | 6-Well | 2 | 6.90E+08 |
| 1 | Sf9 | 6-Well | 2 | 2.75E+08 |
| 1 | Sf9 | 6-Well | 2 | 3.88E+08 |
| 1 | Sf9 | 6-Well | 2 | 0.00E+00 |
| 2 | Sf9 | 12-Well | 2 | 3.24E+07 |
| 2 | Sf9 | 12-Well | 2 | 4.14E+07 |
| 2 | Sf9 | 12-Well | 2 | 3.88E+07 |
| 2 | Sf9 | 12-Well | 2 | 6.90E+07 |
| 2 | Sf9 | 12-Well | 2 | 3.08E+07 |
| 2 | Sf9 | 12-Well | 2 | 3.43E+07 |
| 3 | Sf9 | 6-Well | 5 | 6.90E+07 |
| 3 | Sf9 | 6-Well | 5 | 1.93E+07 |
| 3 | Sf9 | 6-Well | 5 | 3.42E+07 |
| 3 | Sf9 | 6-Well | 5 | 1.23E+07 |
| 3 | Sf9 | 6-Well | 5 | 2.18E+07 |
| 3 | Sf9 | 6-Well | 5 | 3.42E+07 |
| 4 | Sf9 | 12-Well | 0.802 | 2.75E+08 |
| 4 | Sf9 | 12-Well | 0.802 | 1.39E+09 |
| 4 | Sf9 | 12-Well | 0.802 | 4.97E+08 |
| 4 | Sf9 | 12-Well | 0.802 | 2.42E+08 |
| 4 | Sf9 | 12-Well | 0.802 | 0.00E+00 |
| 4 | Sf9 | 12-Well | 0.802 | 4.28E+08 |

Table 11 Raw data used for Figure 4

| Label | Cell Line | Plate | Volume (mL) | Titer |
|-------|-----------|---------|-------------|--------------|
| 1 | Sf9-Cas9 | 6-Well | 2 | 2.47E+08 |
| 1 | Sf9-Cas9 | 6-Well | 2 | 2.75E+08 |
| 1 | Sf9-Cas9 | 6-Well | 2 | 3.20E+08 |
| 1 | Sf9-Cas9 | 6-Well | 2 | 3.42E+08 |
| 1 | Sf9-Cas9 | 6-Well | 2 | 3.08E+08 |
| 1 | Sf9-Cas9 | 6-Well | 2 | 4.97E+08 |
| 2 | Sf9-Cas9 | 12-Well | 2 | 1.59E+08 |
| 2 | Sf9-Cas9 | 12-Well | 2 | 3.88E+07 |
| 2 | Sf9-Cas9 | 12-Well | 2 | 1.29E+08 |
| 2 | Sf9-Cas9 | 12-Well | 2 | 9.86E+07 |
| 2 | Sf9-Cas9 | 12-Well | 2 | 3.64E+07 |
| 2 | Sf9-Cas9 | 12-Well | 2 | 4.48E+07 |
| 3 | Sf9-Cas9 | 6-Well | 5 | 3.88E+07 |
| 3 | Sf9-Cas9 | 6-Well | 5 | 6.90E+07 |
| 3 | Sf9-Cas9 | 6-Well | 5 | 5.85E+07 |
| 3 | Sf9-Cas9 | 6-Well | 5 | 6.90E+07 |
| 3 | Sf9-Cas9 | 6-Well | 5 | 3.20E+07 |
| 3 | Sf9-Cas9 | 6-Well | 5 | 4.97E+07 |
| 4 | Sf9-Cas9 | 12-Well | 0.802 | 3.20E+08 |
| 4 | Sf9-Cas9 | 12-Well | 0.802 | 2.42E+08 |
| 4 | Sf9-Cas9 | 12-Well | 0.802 | Contaminated |
| 4 | Sf9-Cas9 | 12-Well | 0.802 | 6.90E+08 |
| 4 | Sf9-Cas9 | 12-Well | 0.802 | 9.59E+08 |
| 4 | Sf9-Cas9 | 12-Well | 0.802 | 3.88E+08 |



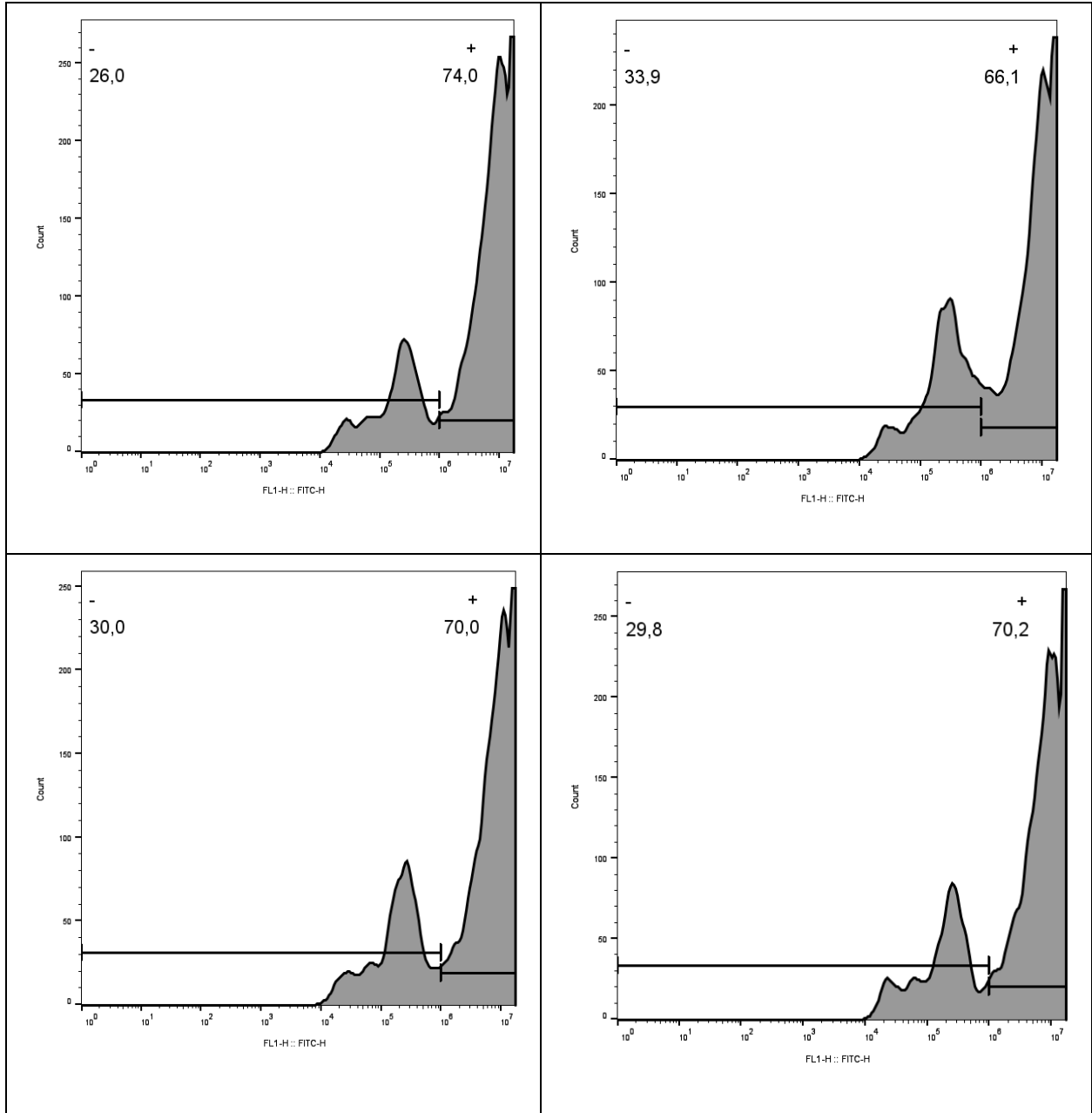
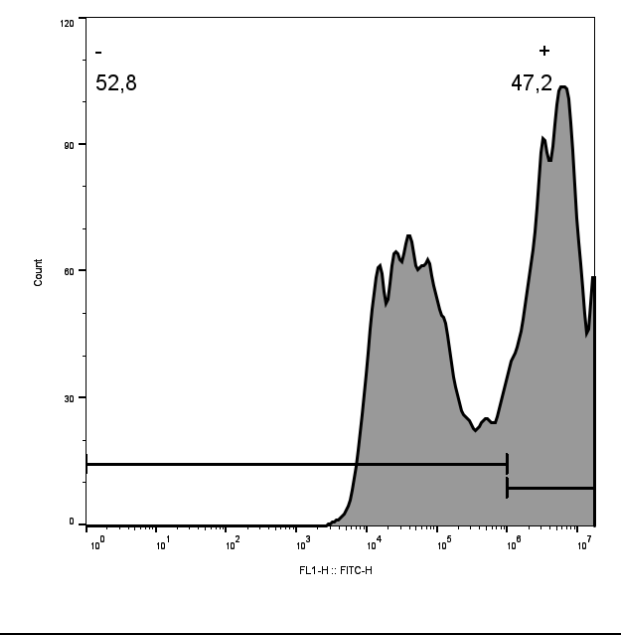
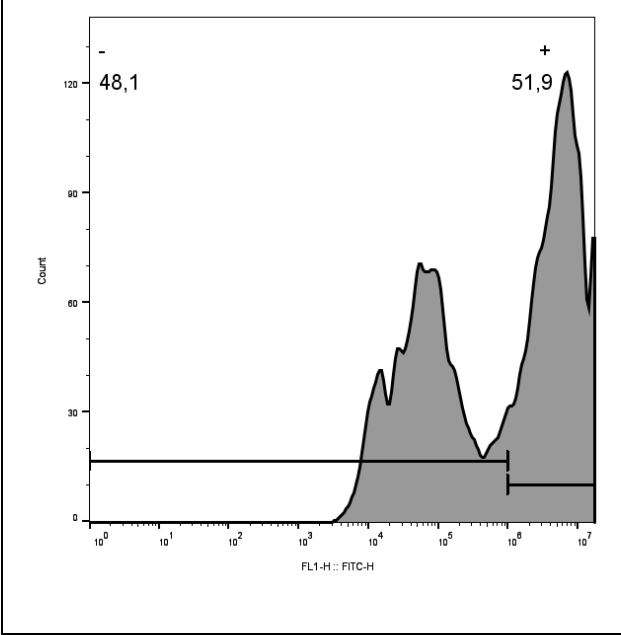
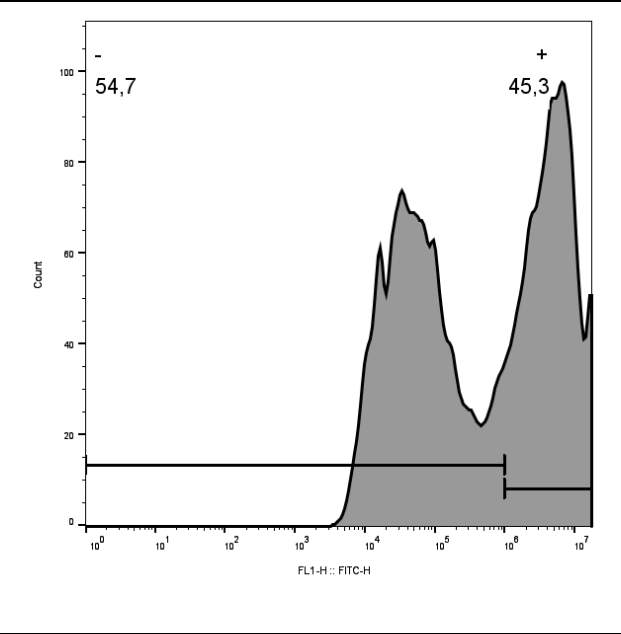
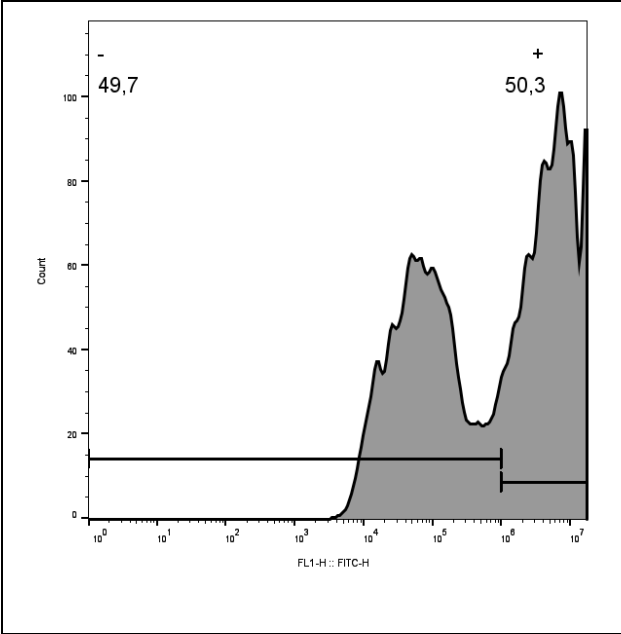


Figure 11 Flow cytometry data for Sf9 cells for the 6-well, low liquid height condition



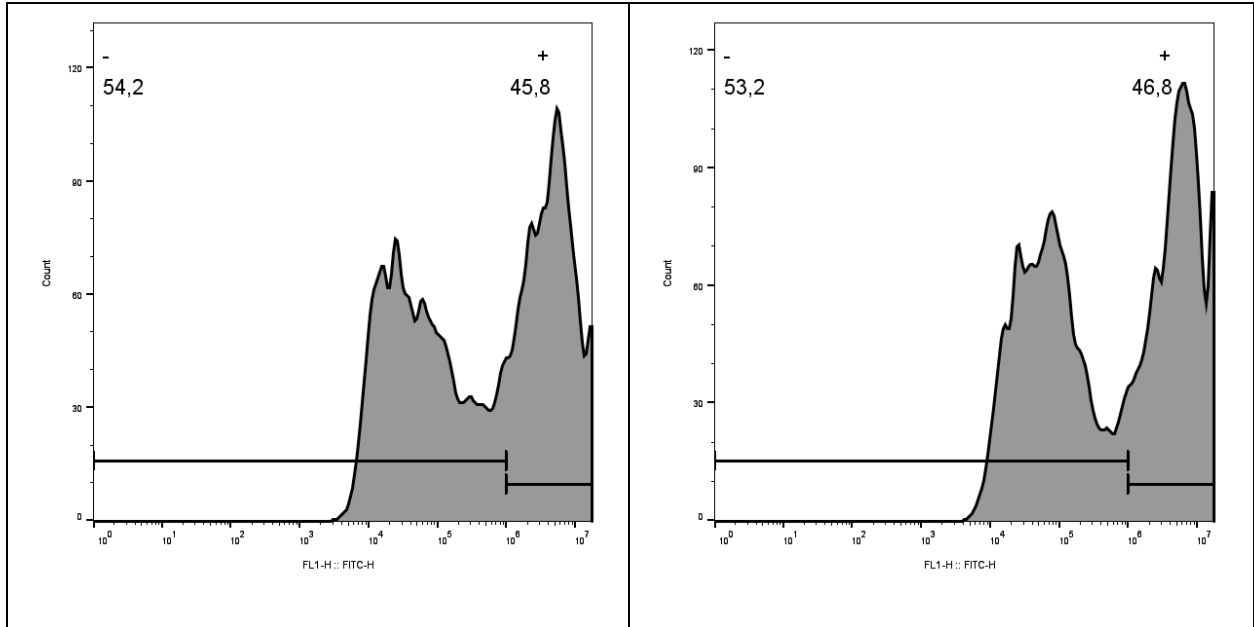
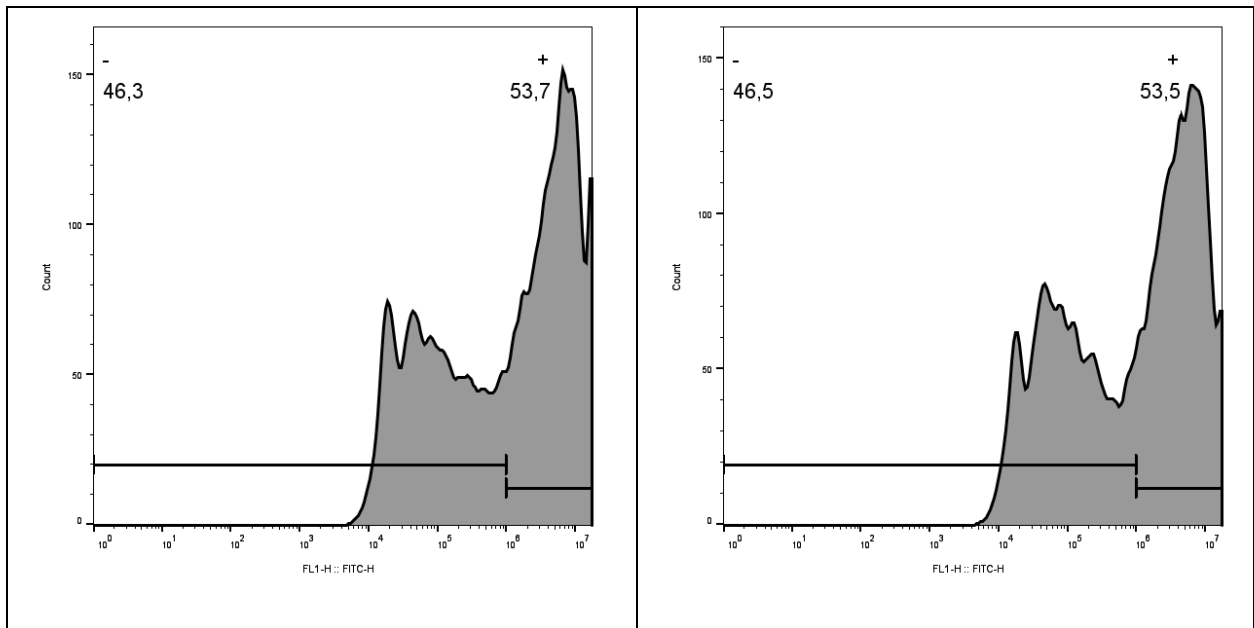


Figure 12 Flow cytometry data for Sf9 cells for the 12-well, high liquid height condition



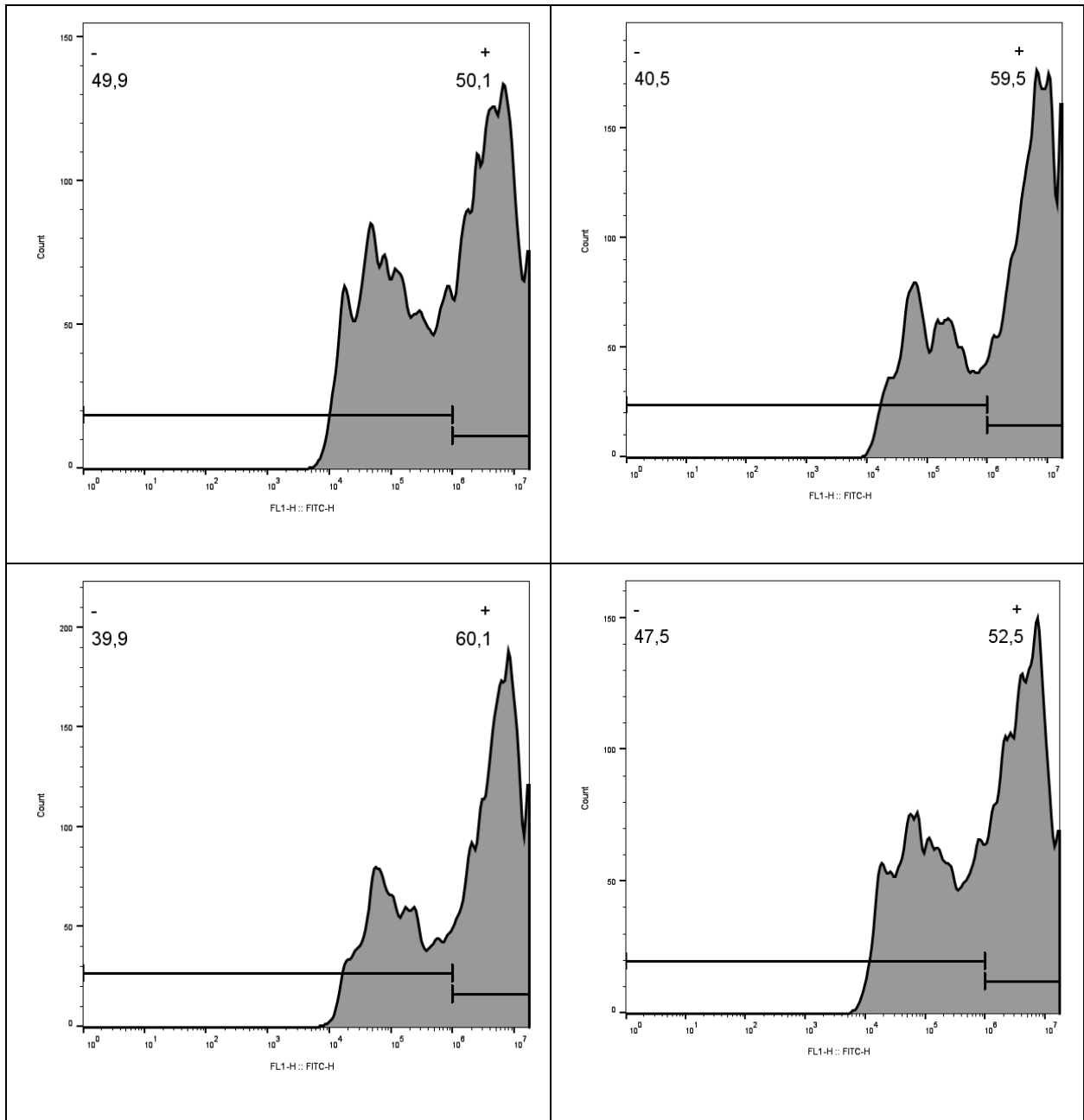
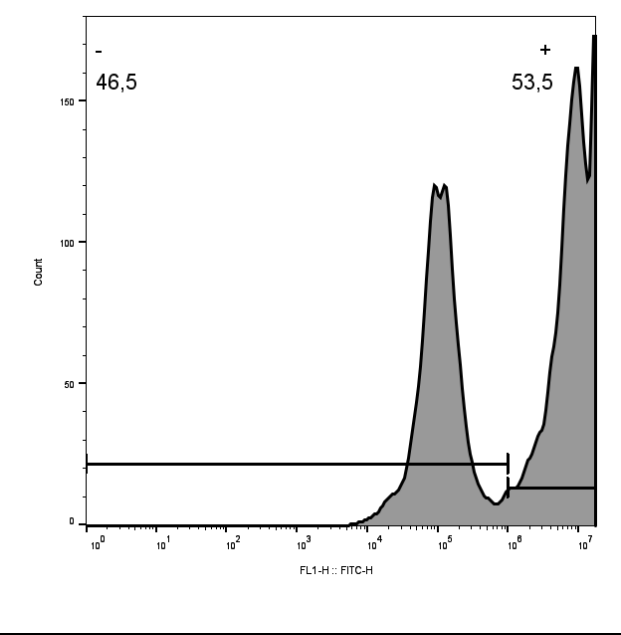
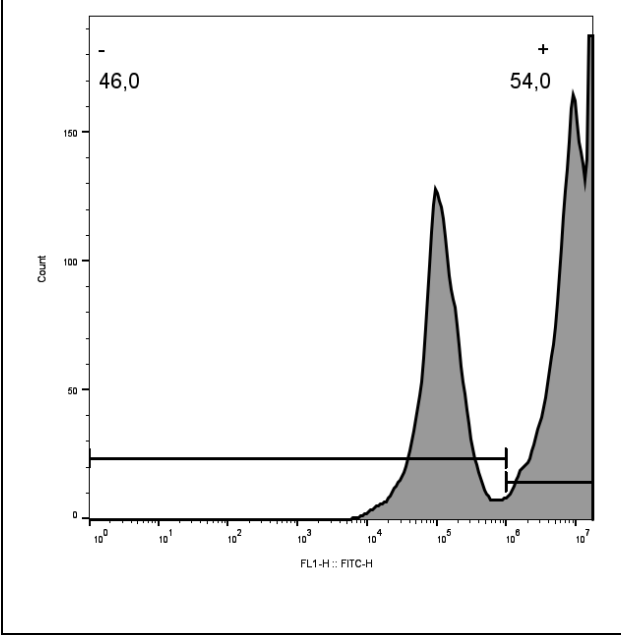
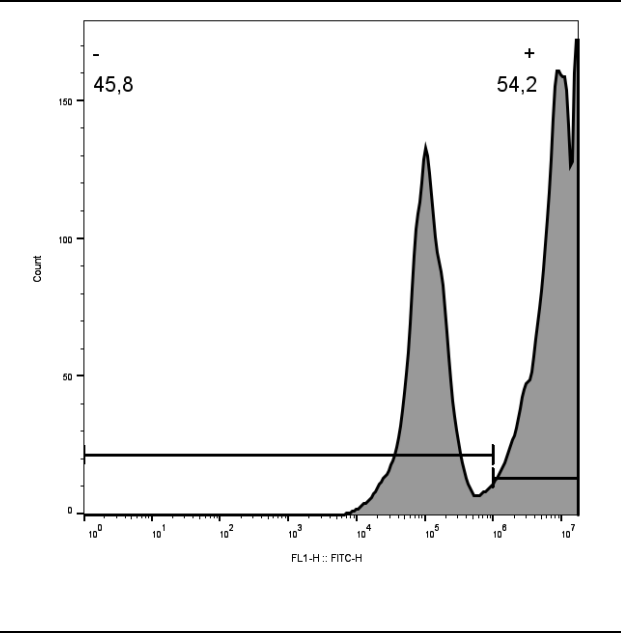
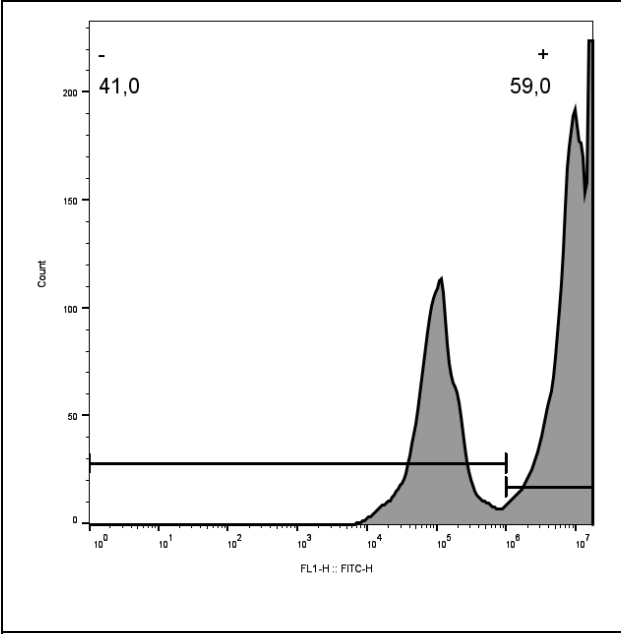


Figure 13 Flow cytometry data for Sf9 cells for the 6-well, high liquid height condition



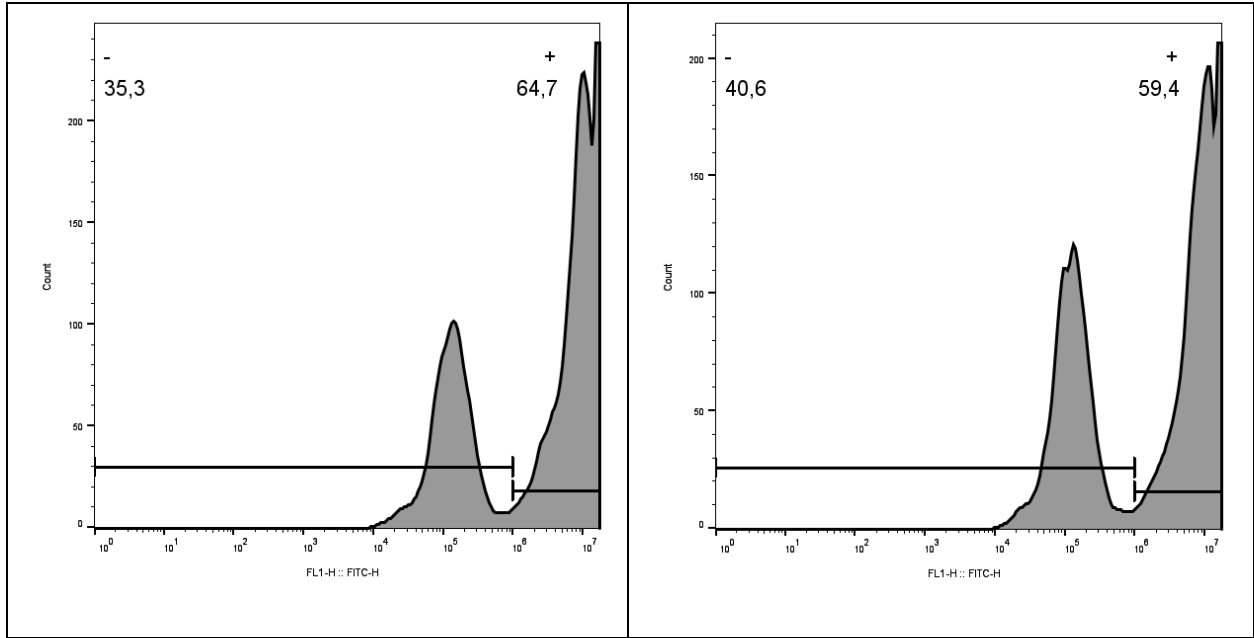
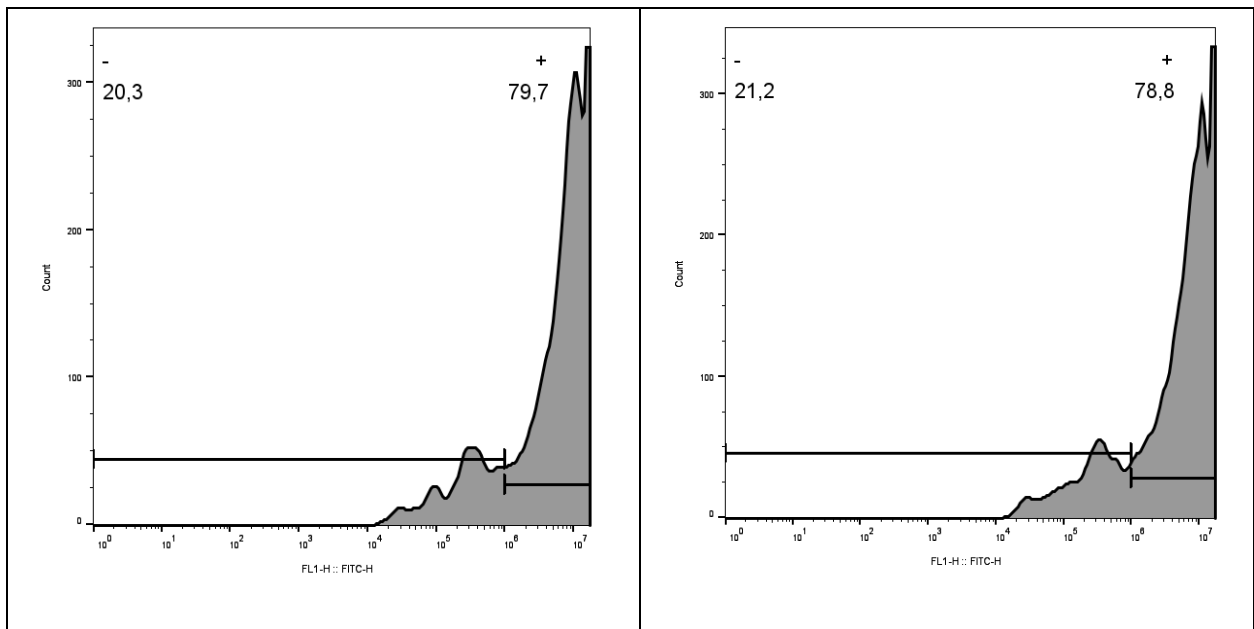


Figure 14 Flow cytometry data for Sf9 cells for the 12-well, low liquid height condition



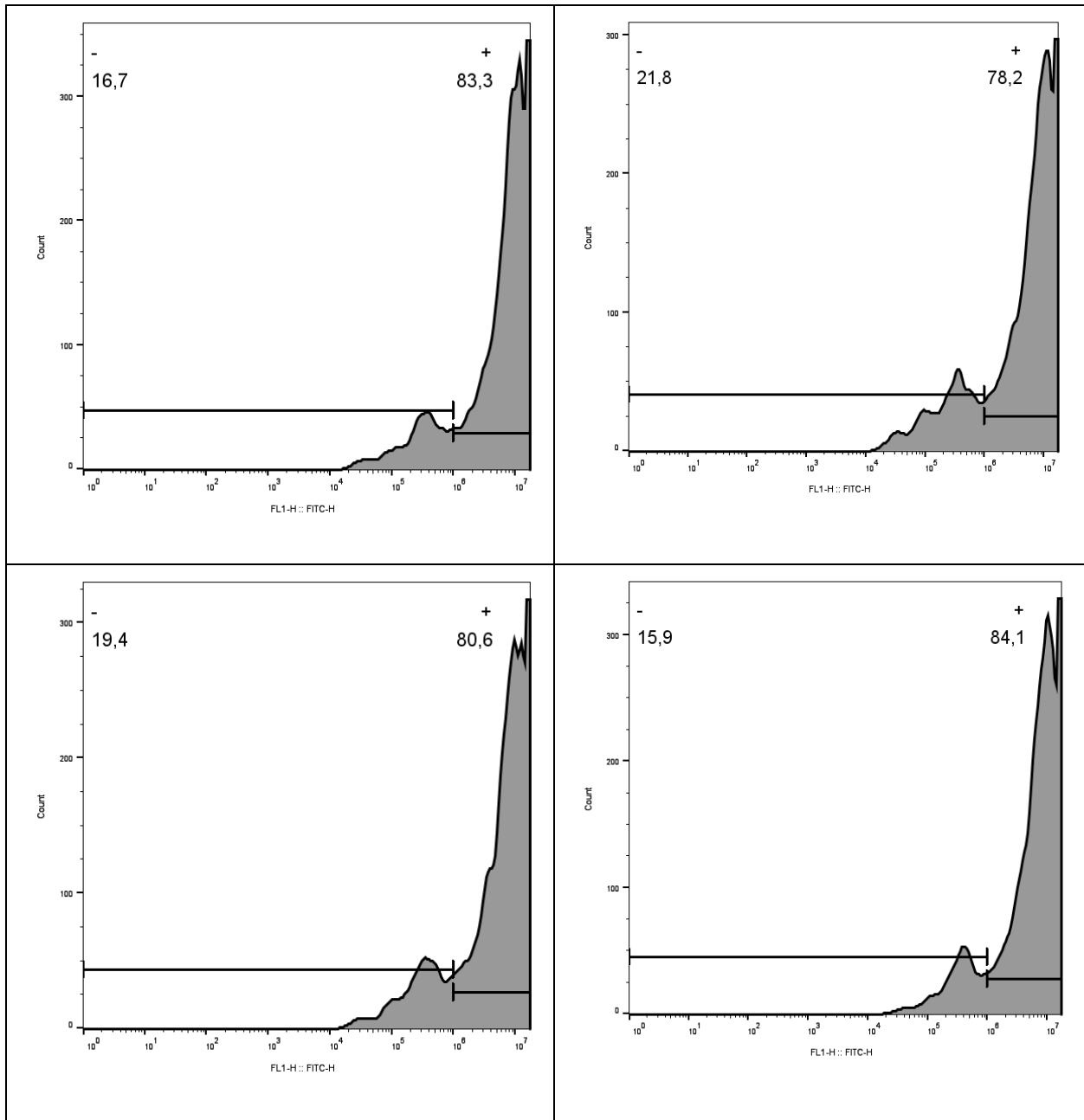
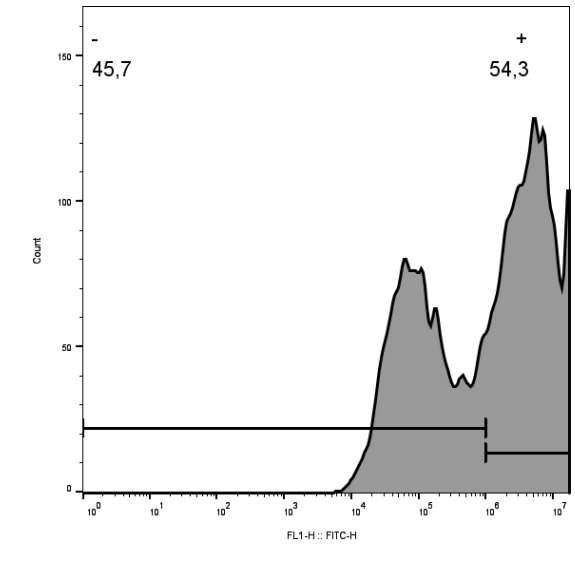
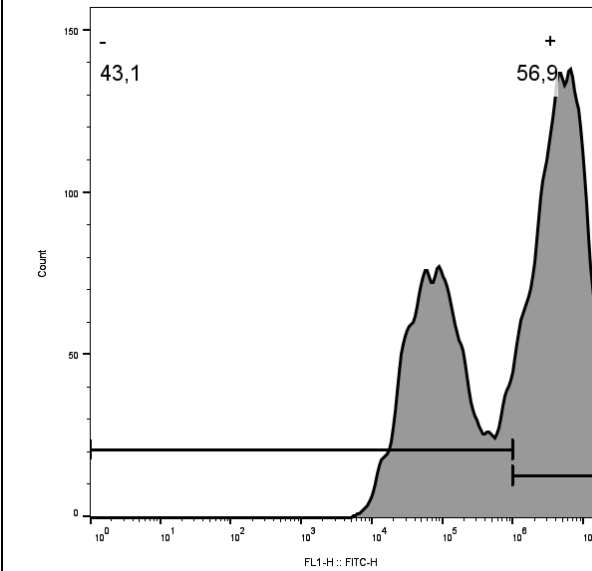
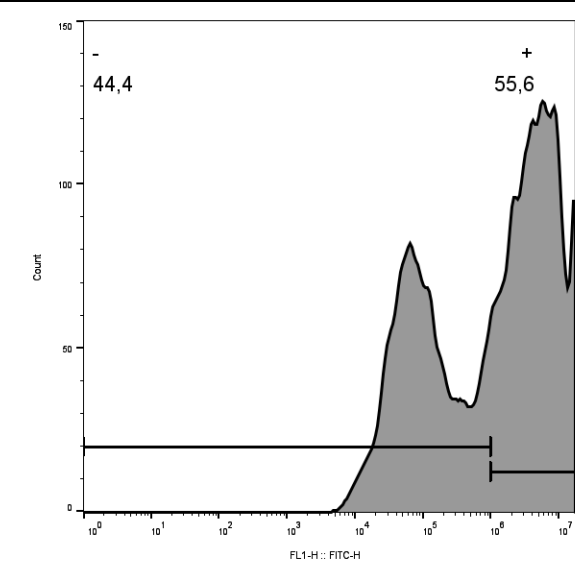
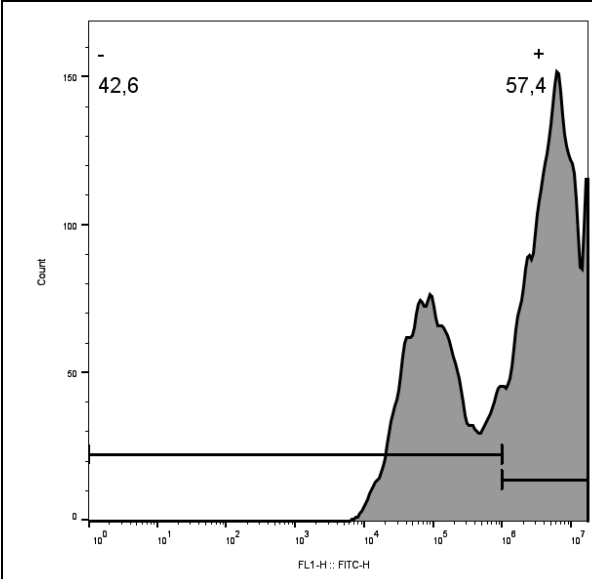


Figure 15 Flow cytometry data for Sf9-Cas9 cells for the 6-well, low liquid height condition



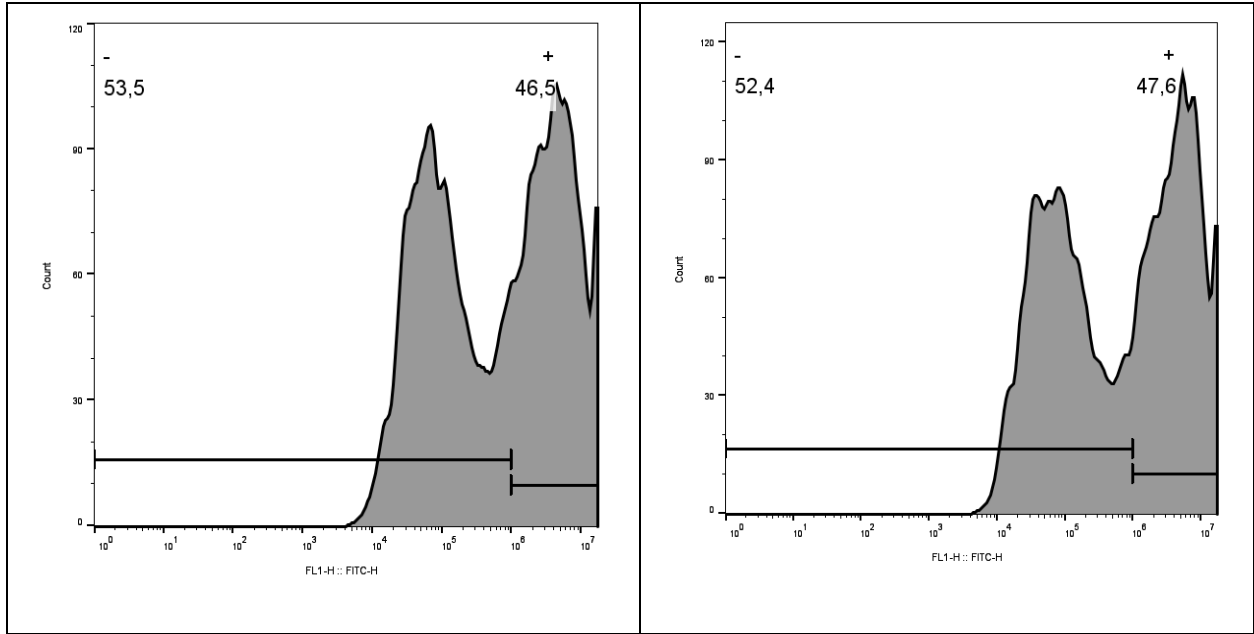
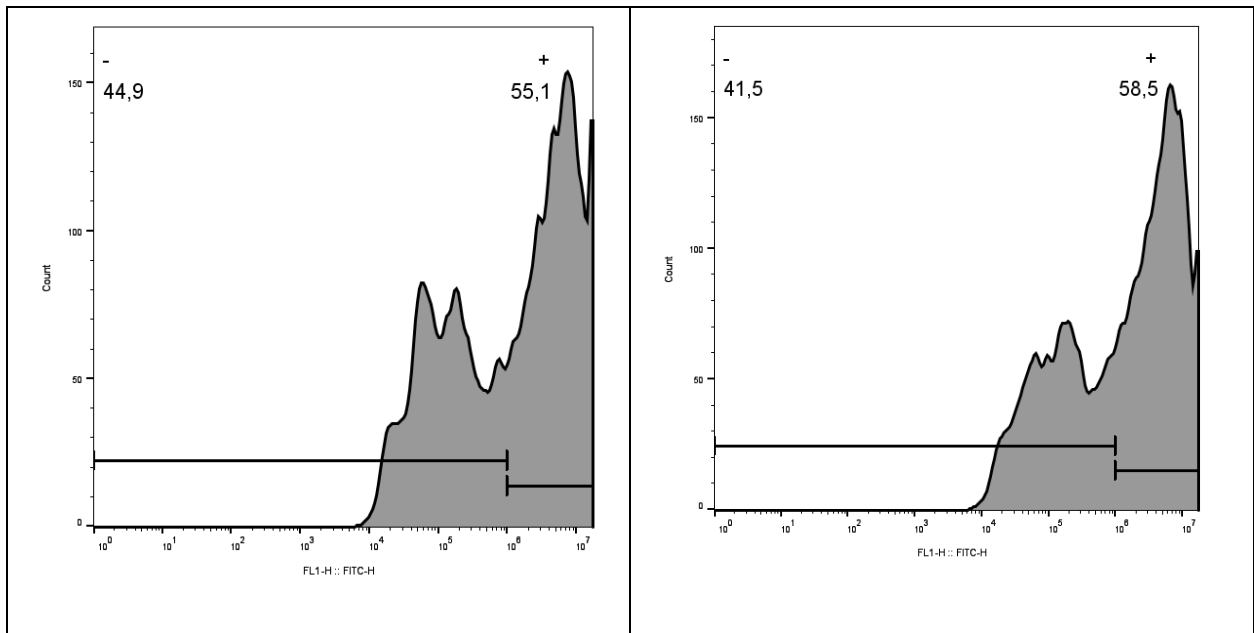


Figure 16 Flow cytometry data for Sf9-Cas9 cells for the 12-well, high liquid height condition



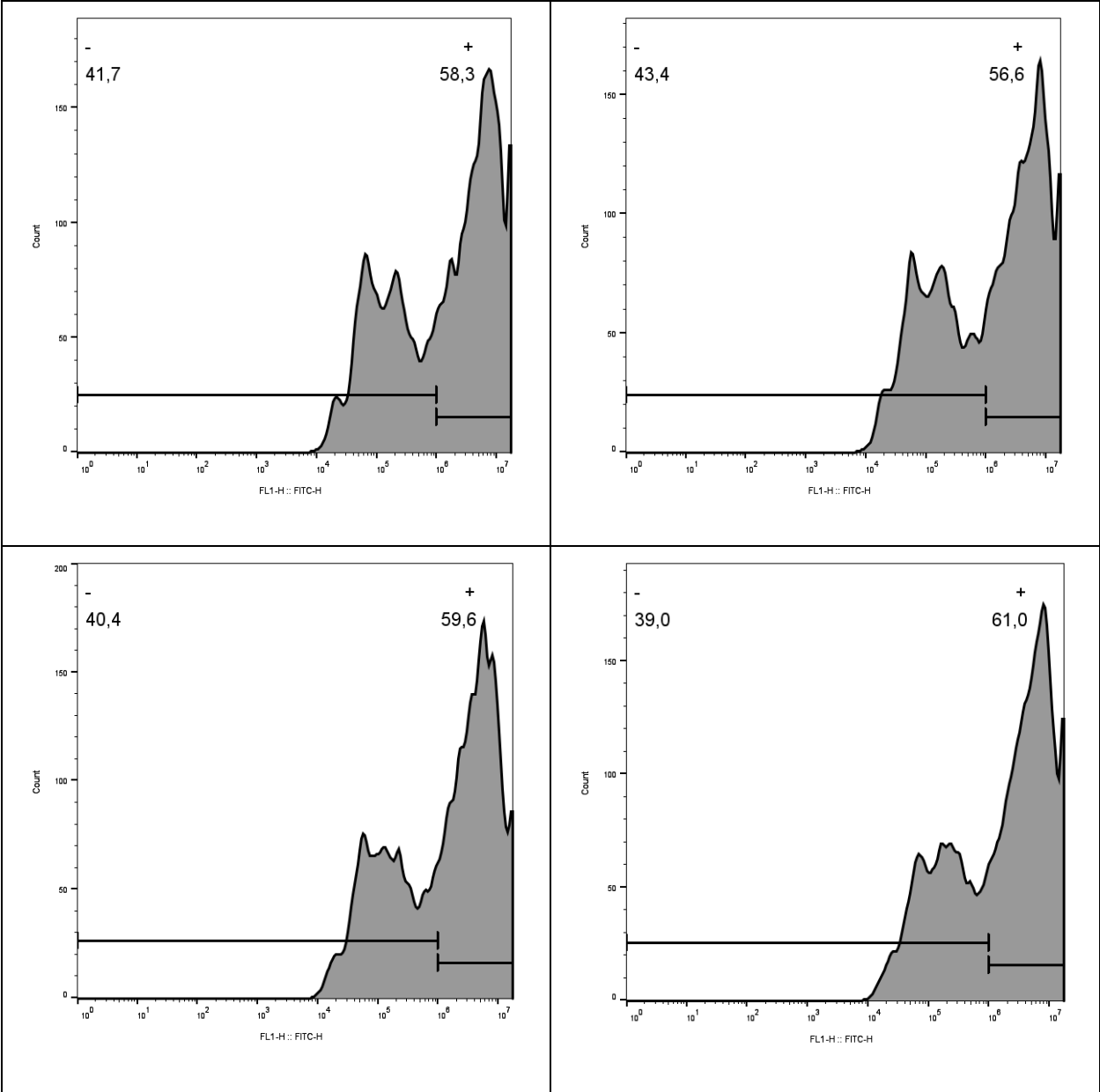
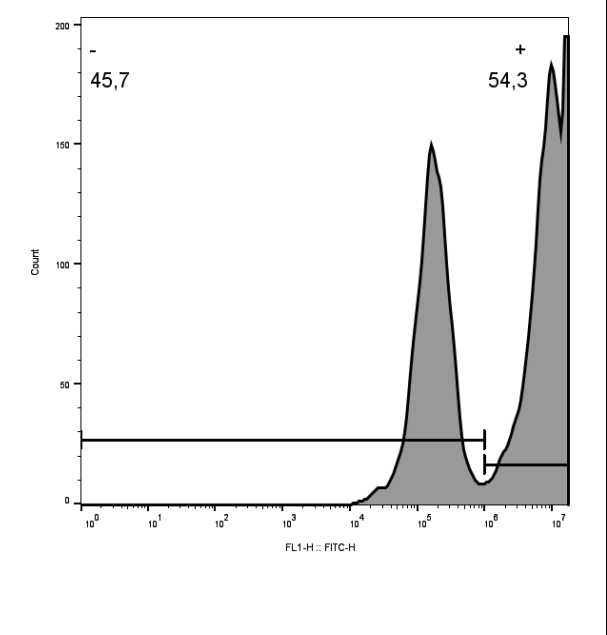
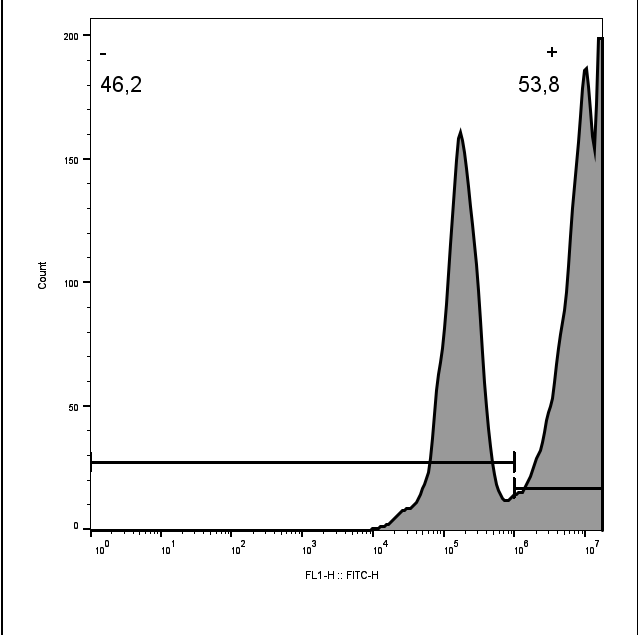
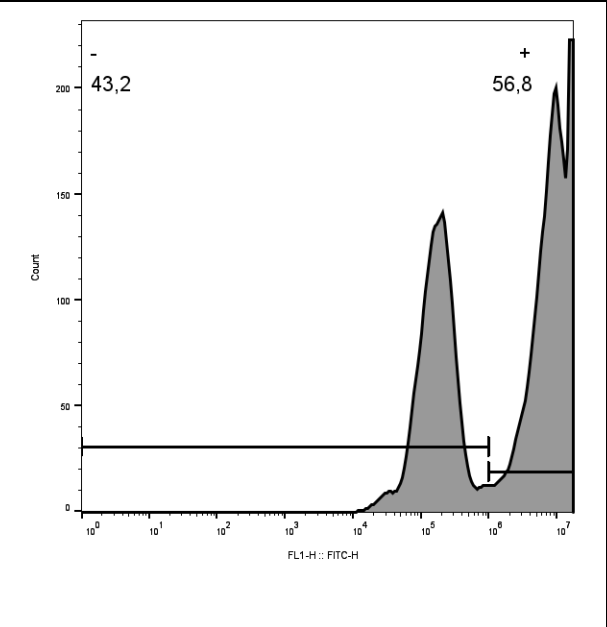
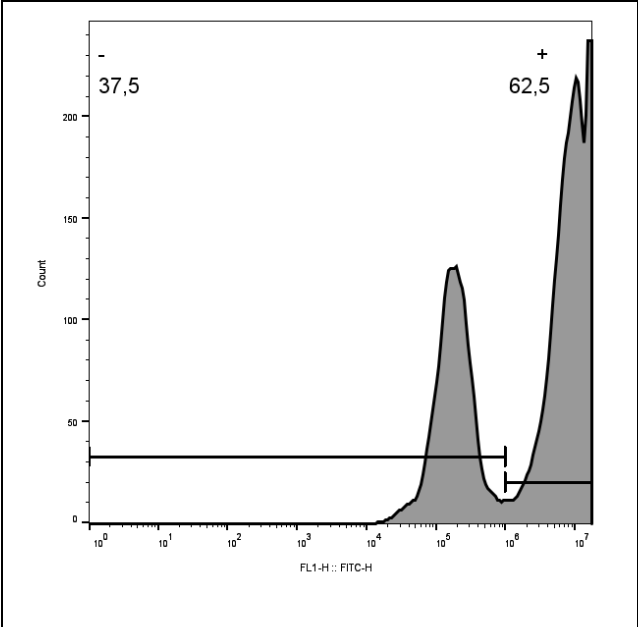


Figure 17 Flow cytometry data for Sf9-Cas9 cells for the 6-well, high liquid height condition



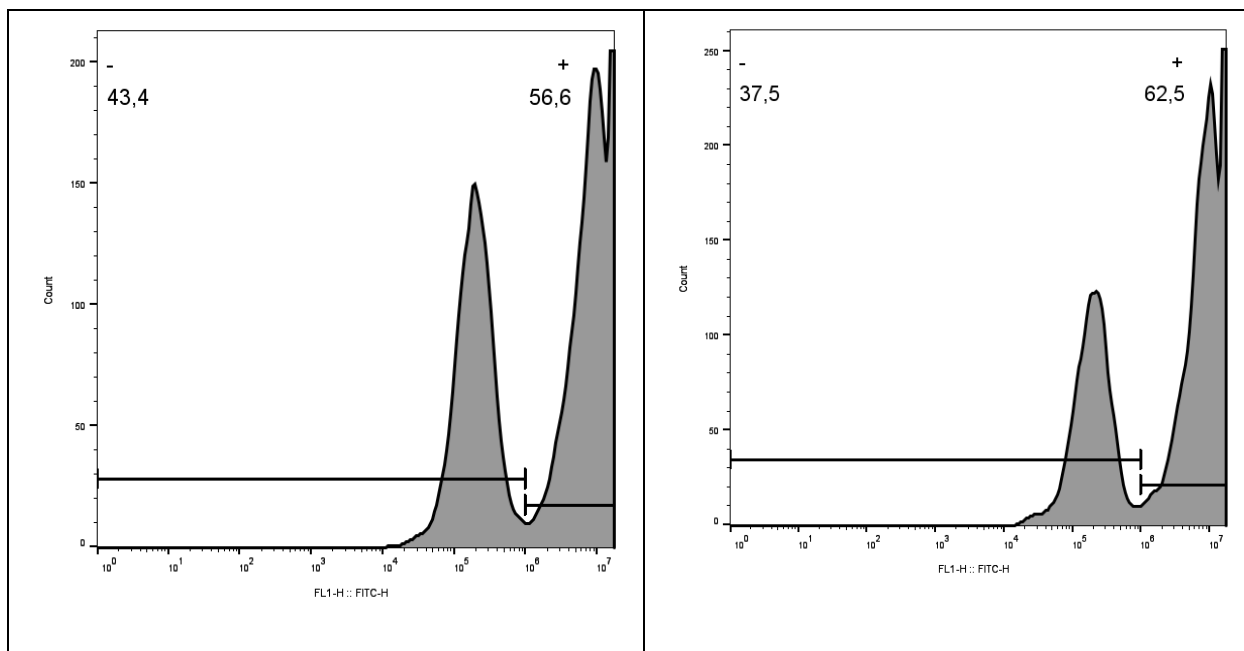


Figure 18 Flow cytometry data for Sf9-Cas9 cells for the 12-wwell, low liquid height condition

Appendix B Chapter 5 Raw Data

Table 12 Raw data used for Figure 7

| Sample | Tubes Concentration (VLPs/mL) | U-Bottom Concentration (VLPs/mL) |
|--------|-------------------------------|----------------------------------|
| 1 | 3.85E+09 | 4.18E+09 |
| 1 | 3.55E+09 | 4.03E+09 |
| 1 | 2.91E+09 | 3.38E+09 |
| 2 | 2.79E+09 | 3.48E+09 |
| 2 | 2.47E+09 | 3.18E+09 |
| 2 | 2.30E+09 | 2.63E+09 |

Table 13 Raw data used for Figure 8. Values are VLP particle concentrations in particles/mL

| Sample | Hours Post Infection | | | |
|--------|----------------------|----------|----------|----------|
| | 0.60 | 1.48 | 2.67 | 6.52 |
| 1 | 7.65E+09 | 7.60E+09 | 7.11E+09 | 6.43E+09 |
| 2 | 7.29E+09 | 7.25E+09 | 7.04E+09 | 6.16E+09 |
| 3 | 7.52E+09 | 7.01E+09 | 6.63E+09 | 6.27E+09 |
| 4 | 7.06E+09 | 6.94E+09 | 6.38E+09 | 6.11E+09 |
| 5 | 7.19E+09 | 7.18E+09 | 6.80E+09 | 5.97E+09 |

Appendix C Chapter 6 and 7 Raw Data

Table 14 Raw data used for chapter 5 and 6. Samples are grouped in 5 different groups to differentiate the scrambled control that should be used for significance testing.

| Group | Gene | VLP_Concentration | Baculovirus_Concentration |
|-------|---------------|-------------------|---------------------------|
| 1 | orf4_1 | 1.35E+09 | 1.67E+08 |
| 1 | orf4_1 | 1.43E+09 | 1.67E+08 |
| 1 | orf4_1 | 1.57E+09 | 1.23E+08 |
| 1 | orf4_2 | 1.69E+09 | 1.58E+08 |
| 1 | orf4_2 | 1.80E+09 | 2.66E+08 |
| 1 | orf4_2 | 2.26E+09 | 1.29E+08 |
| 1 | orf4_3 | 2.30E+09 | 1.89E+08 |
| 1 | orf4_3 | 2.38E+09 | 9.59E+07 |
| 1 | orf4_3 | 2.85E+09 | 1.11E+08 |
| 1 | Scrambled | 3.03E+09 | 1.93E+08 |
| 1 | Scrambled | 2.70E+09 | 3.68E+08 |
| 1 | Scrambled | 2.48E+09 | 2.86E+08 |
| 1 | Infected_Only | 1.51E+09 | 2.18E+08 |
| 1 | Infected_Only | 1.57E+09 | 2.75E+08 |
| 1 | Infected_Only | 2.05E+09 | |
| 2 | ptp_1 | 1.89E+09 | 8.29E+07 |
| 2 | ptp_1 | 1.84E+09 | 1.28E+08 |
| 2 | ptp_1 | 1.92E+09 | 2.39E+08 |
| 2 | ptp_2 | 2.30E+09 | 5.17E+07 |
| 2 | ptp_2 | 2.19E+09 | 1.23E+08 |
| 2 | ptp_2 | 1.54E+09 | 4.97E+07 |
| 2 | lef2_1 | 1.02E+09 | 3.20E+07 |
| 2 | lef2_1 | 1.09E+09 | 3.88E+07 |
| 2 | lef2_1 | 1.23E+09 | 5.85E+07 |
| 2 | lef2_2 | 1.37E+09 | 1.23E+08 |
| 2 | lef2_2 | 1.11E+09 | 9.59E+07 |
| 2 | lef2_2 | 1.06E+09 | 4.31E+07 |
| 2 | lef2_3 | 1.06E+09 | 3.42E+07 |
| 2 | lef2_3 | 1.01E+09 | 3.20E+07 |
| 2 | lef2_3 | 9.69E+08 | 3.43E+07 |
| 2 | pk1_1 | 7.20E+08 | 9.86E+07 |
| 2 | pk1_1 | 7.07E+08 | 1.36E+08 |
| 2 | pk1_1 | 8.78E+08 | 6.90E+07 |
| 2 | pk1_2 | 1.17E+09 | 2.18E+08 |
| 2 | pk1_2 | 1.12E+09 | 1.67E+08 |
| 2 | pk1_2 | 1.19E+09 | 1.87E+08 |
| 2 | pk1_3 | 8.09E+08 | 1.67E+07 |
| 2 | pk1_3 | 8.02E+08 | 1.11E+07 |

| | | | |
|---|-----------------|----------|----------|
| 2 | pk1_3 | 6.78E+08 | 1.11E+07 |
| 2 | orf13_1 | 1.06E+09 | 4.48E+07 |
| 2 | orf13_1 | 8.71E+08 | 3.88E+07 |
| 2 | orf13_1 | 1.49E+09 | 3.88E+07 |
| 2 | orf13_2 | 9.04E+08 | 2.52E+07 |
| 2 | orf13_2 | 1.68E+09 | 3.64E+07 |
| 2 | orf13_2 | 1.47E+09 | 3.43E+07 |
| 2 | lef1_1 | 1.57E+09 | 8.29E+07 |
| 2 | lef1_1 | 1.54E+09 | 1.23E+08 |
| 2 | lef1_1 | 1.31E+09 | 3.20E+07 |
| 2 | lef1_3 | 1.53E+09 | 3.42E+07 |
| 2 | lef1_3 | 1.30E+09 | 2.52E+07 |
| 2 | lef1_3 | 1.20E+09 | 1.23E+07 |
| 2 | orf22_1 | 1.12E+09 | 2.18E+07 |
| 2 | orf22_1 | 1.10E+09 | 2.48E+07 |
| 2 | orf22_1 | 1.37E+09 | 3.20E+07 |
| 2 | orf22_2 | 1.33E+09 | 2.18E+07 |
| 2 | orf22_2 | 1.40E+09 | 3.20E+07 |
| 2 | orf22_2 | 1.62E+09 | 3.88E+07 |
| 2 | orf22_3 | 1.32E+09 | 8.47E+07 |
| 2 | orf22_3 | 1.11E+09 | 5.17E+07 |
| 2 | orf22_3 | 1.65E+09 | 1.39E+08 |
| 2 | Scrambled | 5.41E+09 | 3.64E+08 |
| 2 | Scrambled | 5.23E+09 | 2.47E+08 |
| 2 | Scrambled | 4.19E+09 | 1.59E+08 |
| 2 | Infected Only | 2.08E+09 | 1.11E+08 |
| 2 | Infected Only | 1.63E+09 | 6.90E+07 |
| 2 | Infected Only | 3.55E+09 | 1.11E+08 |
| 2 | mKate2_Infected | 4.52E+09 | 2.18E+08 |
| 2 | mKate2_Infected | 4.80E+09 | 2.18E+08 |
| 2 | mKate2_Infected | 4.97E+09 | 1.39E+08 |
| 3 | orf19_1 | 5.03E+09 | 1.59E+08 |
| 3 | orf19_1 | 6.14E+09 | 9.59E+07 |
| 3 | orf19_1 | 4.72E+09 | 1.49E+08 |
| 3 | orf19_2 | 3.46E+09 | 1.89E+08 |
| 3 | orf19_2 | 3.67E+09 | 1.23E+08 |
| 3 | orf19_2 | 3.15E+09 | 2.18E+08 |
| 3 | env_1 | 3.57E+09 | 6.90E+07 |
| 3 | env_1 | 3.43E+09 | 1.93E+08 |
| 3 | env_1 | 4.31E+09 | 2.85E+08 |
| 3 | env_3 | 3.55E+09 | 1.39E+08 |
| 3 | env_3 | 3.30E+09 | 1.81E+08 |
| 3 | env_3 | 2.82E+09 | 1.67E+08 |
| 3 | pkip_1 | 3.47E+09 | 1.73E+08 |
| 3 | pkip_1 | 3.60E+09 | 5.85E+07 |

| | | | |
|---|-----------------|----------|----------|
| 3 | pkip_1 | 3.06E+09 | 9.86E+07 |
| 3 | pkip_2 | 1.75E+09 | 2.15E+08 |
| 3 | pkip_2 | 1.66E+09 | 5.85E+07 |
| 3 | pkip_2 | 1.84E+09 | 9.59E+07 |
| 3 | sod_1 | 3.02E+09 | 2.18E+08 |
| 3 | sod_1 | 2.61E+09 | 1.89E+08 |
| 3 | sod_1 | 2.53E+09 | 2.18E+08 |
| 3 | sod_2 | 2.64E+09 | 1.93E+08 |
| 3 | sod_2 | 2.90E+09 | 3.10E+08 |
| 3 | sod_2 | 2.93E+09 | 2.52E+08 |
| 3 | fgf_2 | 1.93E+09 | 2.18E+08 |
| 3 | fgf_2 | 2.09E+09 | 2.18E+08 |
| 3 | fgf_2 | 2.02E+09 | 2.09E+08 |
| 3 | fgf_3 | 2.41E+09 | 5.85E+07 |
| 3 | fgf_3 | 2.76E+09 | 4.97E+07 |
| 3 | fgf_3 | 3.14E+09 | 6.90E+07 |
| 3 | ubi_1 | 3.13E+09 | 2.42E+08 |
| 3 | ubi_1 | 2.78E+09 | 3.15E+08 |
| 3 | ubi_1 | 2.65E+09 | 8.29E+07 |
| 3 | ubi_2 | 2.66E+09 | 8.29E+07 |
| 3 | ubi_2 | 2.31E+09 | 1.73E+08 |
| 3 | ubi_2 | 2.63E+09 | 1.67E+08 |
| 3 | lef11_1 | 1.45E+09 | 9.59E+07 |
| 3 | lef11_1 | 1.55E+09 | 1.23E+08 |
| 3 | lef11_1 | 1.50E+09 | 1.29E+08 |
| 3 | lef11_2 | 2.10E+09 | 9.59E+07 |
| 3 | lef11_2 | 2.07E+09 | 1.23E+08 |
| 3 | lef11_2 | 1.88E+09 | 6.90E+07 |
| 3 | mKate2_Infected | 4.33E+09 | 2.76E+08 |
| 3 | mKate2_Infected | 3.62E+09 | 3.20E+08 |
| 3 | mKate2_Infected | 3.69E+09 | 3.20E+08 |
| 3 | Scrambled | 3.82E+09 | 4.28E+08 |
| 3 | Scrambled | 3.78E+09 | 2.47E+08 |
| 3 | Scrambled | 4.01E+09 | 2.75E+08 |
| 3 | Infected_Only | 2.83E+09 | 1.39E+08 |
| 3 | Infected_Only | 2.46E+09 | 1.39E+08 |
| 3 | Infected_Only | 2.66E+09 | 1.67E+08 |
| 4 | orf38_1 | 1.53E+09 | 9.59E+07 |
| 4 | orf38_1 | 1.28E+09 | 1.93E+08 |
| 4 | orf38_1 | 1.46E+09 | 1.73E+08 |
| 4 | orf38_2 | 1.53E+09 | 1.11E+08 |
| 4 | orf38_2 | 1.77E+09 | 9.59E+07 |
| 4 | orf38_2 | 1.78E+09 | 1.11E+08 |
| 4 | orf38_3 | 2.24E+09 | 1.67E+08 |
| 4 | orf38_3 | 1.61E+09 | 9.86E+07 |

| | | | |
|---|-----------------|----------|----------|
| 4 | orf38_3 | 1.86E+09 | 4.14E+07 |
| 4 | p47_2 | 1.54E+09 | 1.11E+08 |
| 4 | p47_2 | 1.11E+09 | 1.79E+08 |
| 4 | p47_2 | 1.32E+09 | 4.48E+07 |
| 4 | p47_3 | 3.10E+09 | 1.29E+08 |
| 4 | p47_3 | 2.76E+09 | 2.99E+08 |
| 4 | p47_3 | 3.06E+09 | 3.42E+08 |
| 4 | orf41_1 | 1.84E+09 | 1.67E+08 |
| 4 | orf41_1 | 1.61E+09 | 6.90E+07 |
| 4 | orf41_1 | 1.68E+09 | 9.59E+07 |
| 4 | orf41_2 | 1.25E+09 | 8.29E+07 |
| 4 | orf41_2 | 1.20E+09 | 2.05E+08 |
| 4 | orf41_2 | 1.50E+09 | 1.67E+08 |
| 4 | orf41_3 | 1.32E+09 | 1.17E+08 |
| 4 | orf41_3 | 1.06E+09 | 1.49E+08 |
| 4 | orf41_3 | 1.15E+09 | 1.67E+08 |
| 4 | orf51_1 | 1.13E+09 | 2.75E+07 |
| 4 | orf51_1 | 1.24E+09 | 1.23E+08 |
| 4 | orf51_2 | 1.54E+09 | 1.67E+08 |
| 4 | orf51_2 | 1.59E+09 | 6.90E+07 |
| 4 | orf51_2 | 1.64E+09 | 9.59E+07 |
| 4 | orf43_1 | 1.23E+09 | 1.73E+08 |
| 4 | orf43_1 | 1.42E+09 | 1.39E+08 |
| 4 | orf43_1 | 1.48E+09 | 8.47E+07 |
| 4 | orf43_2 | 3.35E+09 | 1.39E+08 |
| 4 | orf43_2 | 3.53E+09 | 1.67E+08 |
| 4 | orf43_2 | 3.17E+09 | 6.90E+07 |
| 4 | Infected_Only | 2.13E+09 | 4.97E+07 |
| 4 | Infected_Only | 1.79E+09 | 1.29E+08 |
| 4 | Infected_Only | 2.07E+09 | 1.23E+08 |
| 4 | mKate2_Infected | 3.47E+09 | 2.47E+08 |
| 4 | mKate2_Infected | 3.84E+09 | 3.20E+08 |
| 4 | mKate2_Infected | 4.43E+09 | 1.73E+08 |
| 4 | Scrambled | 3.17E+09 | 2.18E+08 |
| 4 | Scrambled | 2.99E+09 | 9.59E+07 |
| 4 | Scrambled | 3.85E+09 | 1.89E+08 |
| 5 | gp64_1 | 4.90E+09 | 1.29E+08 |
| 5 | gp64_1 | 4.48E+09 | 1.23E+08 |
| 5 | gp64_1 | 4.32E+09 | 5.85E+07 |
| 5 | gp64_2 | 7.17E+09 | 1.49E+08 |
| 5 | gp64_2 | 6.83E+09 | 4.97E+07 |
| 5 | gp64_2 | 7.05E+09 | 1.89E+08 |
| 5 | gp64_3 | 6.01E+09 | 1.59E+08 |
| 5 | gp64_3 | 5.87E+09 | 1.49E+08 |
| 5 | gp64_3 | 5.19E+09 | 6.90E+07 |

| | | | |
|---|-----------------|----------|----------|
| 5 | gp64_4 | 5.17E+09 | 2.86E+07 |
| 5 | gp64_4 | 4.46E+09 | 6.90E+07 |
| 5 | gp64_4 | 4.31E+09 | 3.20E+07 |
| 5 | gp64_5 | 5.38E+09 | 2.42E+07 |
| 5 | gp64_5 | 5.39E+09 | 6.90E+07 |
| 5 | gp64_5 | 5.41E+09 | 1.21E+08 |
| 5 | gp64_6 | 6.03E+09 | 9.49E+07 |
| 5 | gp64_6 | 5.73E+09 | 4.48E+07 |
| 5 | gp64_6 | 6.09E+09 | 2.42E+07 |
| 5 | vlf1_1 | 4.19E+09 | 6.90E+07 |
| 5 | vlf1_1 | 4.16E+09 | 3.20E+07 |
| 5 | vlf1_1 | 4.53E+09 | 1.11E+08 |
| 5 | vlf1_2 | 6.06E+09 | 8.29E+07 |
| 5 | vlf1_2 | 5.81E+09 | 1.39E+08 |
| 5 | vlf1_2 | 5.45E+09 | 3.20E+07 |
| 5 | vlf1_3 | 4.25E+09 | 4.97E+07 |
| 5 | vlf1_3 | 4.58E+09 | 6.90E+07 |
| 5 | vlf1_3 | 4.60E+09 | 4.97E+07 |
| 5 | mKate2 Infected | 5.47E+09 | 1.67E+08 |
| 5 | mKate2 Infected | 4.79E+09 | 9.82E+07 |
| 5 | mKate2 Infected | 5.44E+09 | 3.20E+08 |
| 5 | Scrambled | 4.64E+09 | 1.39E+08 |
| 5 | Scrambled | 3.70E+09 | 1.93E+08 |
| 5 | Scrambled | 3.49E+09 | 1.85E+08 |
| 5 | Infected Only | 1.16E+09 | 1.93E+07 |
| 5 | Infected Only | 1.19E+09 | 1.41E+07 |
| 5 | Infected Only | 1.57E+09 | 3.42E+07 |

Appendix D Chapter 6 Genetic Targets

Table 15 Genes targets

| AcMNP V ORF | Gene Target | Label | Target location | Spacer sequence (5'-3') | PAM (5'-3') | Strand |
|-------------|-------------|--------|-----------------|----------------------------------|-------------|------------|
| AcOrf-1 | <i>ptp</i> | ptp_1 | 85 | GTCACGTACGCAAACA ACT C | GGG | Anti-sense |
| | | ptp_2 | -204 | TGGTGTGCATTTTT TGC GG G | CGG | Sense |
| AcOrf-4 | Ac4 | orf4_1 | -78 | CGAGTCAAGTGATCAA AGT G | TGG | Sense |

| | | | | | | |
|----------|--------------|---------|------|-------------------------------|-----|------------|
| | | orf4_2 | 116 | CGACGGCATGATTA AAAAGC G | AGG | Anti-sense |
| | | orf4_3 | -223 | TTGTTTGC GCATATCTATCG | TGG | Sense |
| AcOrf-6 | <i>lef-2</i> | lef2_1 | 28 | AAACATGACGCTCTAATGA G | CGG | Anti-sense |
| | | lef2_2 | -99 | ATTGACCCTAACTCCATAC A | CGG | Sense |
| | | lef2_3 | -162 | GCGATTGTACATGCTGTTA A | CGG | Sense |
| AcOrf-10 | <i>pk-1</i> | pk1_1 | -73 | AAAATAATCAACGGGCGCT T | TGG | Sense |
| | | pk1_2 | 192 | TTGGGTGGTCGCTCATTA A | TGG | Anti-sense |
| | | pk1_3 | -249 | CAACAACCAAGTGATCGTG A | TGG | Sense |
| AcOrf-13 | <i>bion</i> | orf13_1 | -61 | TGCATTGAACCGATCTTCG G | CGG | Sense |
| | | orf13_2 | 329 | CAAACGCGATCTCAACTCG T | TGG | Anti-sense |
| | | orf13_3 | 355 | CATTGTTCGCGACCAAGCA C | GGG | Anti-sense |
| AcOrf-14 | <i>lef-1</i> | lef1_1 | -68 | CCGTCATGAACGCGTACTT G | CGG | Sense |
| | | lef1_2 | 191 | GTTGGACGACGGCGGCGG CA | GGG | Anti-sense |
| | | lef1_3 | 269 | GAAAATTTACATTGGCGCC A | CGG | Anti-sense |
| AcOrf-19 | Ac19 | orf19_1 | 104 | CAATGTGCTTACAAACGCG A | TGG | Anti-sense |
| | | orf19_2 | 155 | CGAGTTGATCAAATTGCAC G | CGG | Anti-sense |
| AcOrf-22 | <i>pif2</i> | orf22_1 | 140 | GTAGTGCCTTCTACGCATTA | CGG | Anti-sense |
| | | orf22_2 | 195 | TGATACTAATCTCGGCACG T | TGG | Sense |
| | | orf22_3 | -294 | GATATGCGATAACCCGTCT G | CGG | Sense |
| AcOrf-23 | <i>env</i> | env_1 | -85 | TCGACGACGAGTACAGTTG T | CGG | Sense |
| | | env_2 | 530 | TCGGAGCAAAGCTTGCGC GT | CGG | Anti-sense |
| | | env_3 | 556 | AACATTACTCTGTCTTTGG G | CGG | Anti-sense |
| AcOrf-24 | <i>pkip</i> | pkip_1 | 116 | CGACAAAATGTTGTGCATA G | CGG | Anti-sense |

| | | | | | | |
|----------|---------------|---------|------|---------------------------|-----|------------|
| | | pkip_2 | 137 | GGCCGATATCAAAGGCCAA G | TGG | Anti-sense |
| AcOrf-31 | <i>sod</i> | sod_1 | 97 | AATTTGCCTCGAGGTTTGC A | CGG | Sense |
| | | sod_2 | 167 | GGTGGGATTAAGTGCTCA C | CGG | Anti-sense |
| AcOrf-32 | <i>fgf</i> | fgf_1 | 66 | ATCACGGGCACGTCTATTC C | CGG | Anti-sense |
| | | fgf_2 | -89 | ACTGCCGGTTAATAAACAG C | TGG | Sense |
| | | fgf_3 | -190 | GACGATGCGATTTCTGTCA A | CGG | Sense |
| AcOrf-35 | <i>v-ubi</i> | ubi_1 | -27 | CAAACCATTACCGCCGAA A | CGG | Sense |
| | | ubi_2 | 89 | TACGGGCACACCTTCTTTAT | CGG | Anti-sense |
| AcOrf-37 | <i>lef-11</i> | lef11_1 | -4 | TAAGTGC GTGCAATTTTGG G | GGG | Sense |
| | | lef11_2 | 59 | CGAAATACAAGCGCTGTTC A | GGG | Anti-sense |
| AcOrf-38 | Ac38 | orf38_1 | 23 | TATGATAATCGAGCCGGAC A | AGG | Anti-sense |
| | | orf38_2 | -94 | AAAAGTGTCGTTTCATGTCG G | CGG | Sense |
| | | orf38_3 | -209 | TGAACGCGCTGTCAAAAA AC | CGG | Sense |
| AcOrf-40 | <i>p47</i> | p47_1 | -114 | ACTTCGCGCTTTACAAAAC G | AGG | Sense |
| | | p47_2 | 295 | TACAGCTTGTGTGCCGAGA T | CGG | Anti-sense |
| AcOrf-41 | <i>lef-12</i> | orf41_1 | -76 | TTGAACGTTTTACGACAGC A | GGG | Sense |
| | | orf41_2 | -126 | TTTGTGCGTGTCAGACGAC A | CGG | Sense |
| | | orf41_3 | 176 | GCGGAACGATACAAAATTG C | AGG | Anti-sense |
| AcOrf-51 | Ac51 | orf51_1 | 203 | AACTTGTTTCGTCACCACG T | TGG | Anti-sense |
| | | orf51_2 | -246 | CGAAAAGCACCATTACAAC A | CGG | Sense |
| AcOrf-77 | <i>vlf-1</i> | vlf1_1 | -79 | ATCGGTGGCCAAATCGAAC A | CGG | Sense |
| | | vlf1_2 | 254 | CATGTCG TACTCGTTGGAC A | AGG | Anti-sense |
| | | vlf1_3 | 248 | | | |

| | | | | | | |
|-----------|-------------|--------|---------|---|-------------|------------|
| AcOrf-128 | <i>gp64</i> | gp64_1 | 131 | GGAAACGCTGCAAAAGGA CG | TGG | Anti-sense |
| | | gp64_2 | -160 | GTTGTAGTCCGTCTCCACG A | TGG | Sense |
| | | gp64_3 | 131/384 | GGAAACGCTGCAAAAGGA CG/ TTTCGCGACAACGAGGGCC G | TGG/CG G | Anti-sense |
| | | gp64_4 | 278 | AACGCTGAATGTGGGCAA AG | AGG | Anti-sense |
| | | gp64_5 | 378 | GACTGTTTTTCGCGACAACG A | GGG | Anti-sense |
| | | gp64_6 | 418 | AAGGCAAAGAGTTGGTGA AG | CGG | Anti-sense |

Appendix E Chapter 6 Data Analysis Code

```
# Load necessary libraries
library(ggsignif)
library(ggplot2)
library(multcomp)
library(readxl)
library(dplyr)
library(openxlsx)

set.seed(20250213) #Set seed to avoid variability in p-values

#This code is for Tukey testing on the VIRUS Concentrations for each experiment

expnum <- 1

# Load the dataset (using the actual file name "Summary Sheet.xlsx")
all_data <- read_excel("(Original) Summary Sheet.xlsx")

# Filter for genes of "Scrambled, "Infected_Only" and "mKate2_Infected" then filter out
experiment number
data <- all_data %>% #Filter out for the experiment you want
  filter(Gene %in% c("Scrambled", "Infected_Only", "mKate2_Infected")) %>%
  filter(Experiment == expnum)

# Convert 'Gene' to a factor to ensure it's treated correctly
data$Gene <- as.factor(data$Gene)
```

```

# Reorder the 'Gene' factor to specify the desired order of the bars
data$Gene <- factor(data$Gene, levels = c("Scrambled", "Infected_Only", "mKate2_Infected",
                                         setdiff(unique(data$Gene), c("Scrambled", "Infected_Only",
"mKate2_Infected"))))
#Log transform the virus concentrations
data <- data %>%
  mutate(Log_Virus_Concentration = log10(Baculovirus_Concentration)) %>%
  mutate(Log_VLP_Concentration = log10(VLP_Concentration))

# Calculate the average VLP_Concentration for each Gene
group_means <- data %>%
  group_by(Gene) %>%
  summarize(
    avg_Virus = mean(Baculovirus_Concentration, na.rm = TRUE),
    se_Virus = sd(Baculovirus_Concentration, na.rm = TRUE) / sqrt(n()),
    avg_VLP = mean(VLP_Concentration, na.rm = TRUE),
    se_VLP = sd(VLP_Concentration, na.rm = TRUE) / sqrt(n()))

##### Baculovirus Testing #####

# Perform the ANOVA model on the log transform
virus_anova_model <- aov(Log_Virus_Concentration ~ Gene, data = data)

# Perform Tukey's test with "Scrambled" as the control group
virus_tukey_result <- glht(virus_anova_model, linfct = mcp(Gene = "Tukey"))

#Summarize results of the Tukey Test
virus_tukey_summary <- summary(virus_tukey_result)

# Extract p-values from the Tukey test
virus_p_values <- virus_tukey_summary$test$pvalues

# Convert p-values to significance levels (e.g., "*" for p < 0.05, "***" for p < 0.01, "****" for p <
0.001)
virus_significance <- ifelse(virus_p_values < 0.001, "****",
  ifelse(virus_p_values < 0.01, "***",
    ifelse(virus_p_values < 0.05, "*",
      "NS")))

#Extract names from the Tukey test
virus_comparisons <- names(virus_tukey_summary$test$coefficient) # Get the names of
comparisons (e.g., "Scrambled - Infected_Only")

#Get the groups that each treatment belongs to

```

```

virus_cld_result <- cld(virus_tukey_result, level = 0.05)
virus_group <- virus_cld_result$mcletters$Letters

# Split the comparisons into two columns
virus_comparison_df <- data.frame(Gene1 = sapply(strsplit(virus_comparisons, " - "), `[`, 1),
  Gene2 = sapply(strsplit(virus_comparisons, " - "), `[`, 2),
  Experiment = expnum,
  "p_value" = virus_p_values,
  "Significance" = virus_significance)

#Add the group that was decided by the tukey test to the group_means dataframe for graphing
group_means = mutate(group_means, "Virus Group" = virus_group)

#####VLP Testing #####

# Perform the ANOVA model on the log transform
VLP_anova_model <- aov(Log_VLP_Concentration ~ Gene, data = data)

# Perform Tukey's test with "Scrambled" as the control group
VLP_tukey_result <- glht(VLP_anova_model, linfct = mcp(Gene = "Tukey"))

#Summarize results of the Tukey Test
VLP_tukey_summary <- summary(VLP_tukey_result)

# Extract p-values from the Tukey test
VLP_p_values <- VLP_tukey_summary$test$pvalues

# Convert p-values to significance levels (e.g., "*" for p < 0.05, "***" for p < 0.01, "****" for p <
0.001)
VLP_significance <- ifelse(VLP_p_values < 0.001, "****",
  ifelse(VLP_p_values < 0.01, "***",
    ifelse(VLP_p_values < 0.05, "*",
      "NS")))

#Extract names from the Tukey test
VLP_comparisons <- names(VLP_tukey_summary$test$coefficient) # Get the names of
comparisons (e.g., "Scrambled - Infected_Only")

#Get the groups that each treatment belongs to
VLP_cld_result <- cld(VLP_tukey_result, level = 0.05)
VLP_group <- VLP_cld_result$mcletters$Letters

# Split the comparisons into two columns
VLP_comparison_df <- data.frame(Gene1 = sapply(strsplit(VLP_comparisons, " - "), `[`, 1),
  Gene2 = sapply(strsplit(VLP_comparisons, " - "), `[`, 2),

```

```

Experiment = expnum,
"p_value" = VLP_p_values,
"Significance" = VLP_significance)

#Add the group that was decided by the tukey test to the group_means dataframe for graphing
group_means = mutate(group_means, "VLP Group" = VLP_group)
group_means = mutate(group_means, Experiment = expnum)

##### Summarize Useful Data and Export #####

data_summary <- group_means

# Define the file path where you want to save the Excel file
file_name <- paste0("(Experiment ", expnum, ") Tukey Data.xlsx")

# Export the data frame to an Excel file
write.xlsx(data_summary, file_name)

```

Appendix F Chapter 7 Data Analysis Code

```

# Load necessary libraries
library(ggsignif)
library(ggplot2)
library(multcomp)
library(readxl)
library(dplyr)
library(openxlsx)

set.seed(20250213) #Set seed to avoid variability in p-values

expnum <- 5 #Set experiment number
all_data <- read_excel("(Original) Summary Sheet.xlsx") # Load the dataset (using the actual file
name "Summary Sheet.xlsx")

data <- all_data %>% #Filter out for the experiment you want
  filter(Experiment == expnum)

data$Gene <- as.factor(data$Gene) # Convert 'Gene' to a factor to ensure it's treated correctly

data <- data %>%
  filter (!Gene %in% c("Infected_Only", "mKate2_Infected"))

#Log transform the data
data <- data %>%

```

```

mutate(Log_Virus_Concentration = log10(Baculovirus_Concentration)) %>%
mutate (Log_VLP_Concentration = log10(VLP_Concentration))

## Reorder the 'Gene' factor to specify the desired order of the bars
# data$Gene <- factor(data$Gene, levels = c("Scrambled", "Infected_Only", "mKate2_Infected",
#
#           setdiff(unique(data$Gene), c("Scrambled", "Infected_Only",
"mKate2_Infected"))))

# Relevel the factor to set 'Scrambled' as the reference group
data$Gene <- relevel(data$Gene, ref = "Scrambled")

# Calculate the average Concentration for each Gene
Virus_means <- data %>%
  group_by(Gene) %>%
  summarize(avg_Virus = mean(Baculovirus_Concentration, na.rm = TRUE))

VLP_means <- data %>%
  group_by(Gene) %>%
  summarize(avg_VLP = mean(VLP_Concentration, na.rm = TRUE))

#####
#####

# Perform the ANOVA model on the log transform of Virus
Virus_anova <- aov(Log_Virus_Concentration ~ Gene, data = data)

# Perform Dunnett's test with "Scrambled" as the control group for the Virus
Virus_dunnett <- glht(Virus_anova, linfct = mcp(Gene = "Dunnett"))

# Extract p-values from the Dunnett test
Virus_p_values <- summary(Virus_dunnett)$test$pvalues

#Extract names from the Dunnett test
names <- rownames(Virus_dunnett$linfct)

#Remove the " - Scrambled" from the names which allows for applying significance lines on the
bar graph later
clean_name <- gsub(" - Scrambled", "", names)

# Convert p-values to significance levels (e.g., "*" for p < 0.05, "***" for p < 0.01, "****" for p <
0.001)
Virus_significance <- ifelse(Virus_p_values < 0.001, "****",
  ifelse(Virus_p_values < 0.01, "***",
    ifelse(Virus_p_values < 0.05, "*",

```

```

"NS"))))

#####
#####

# Perform the ANOVA model on the log transform of VLP
VLP_anova <- aov(Log_VLP_Concentration ~ Gene, data = data)

# Perform Dunnett's test with "Scrambled" as the control group for the VLP
VLP_dunnett <- glht(VLP_anova, linfct = mcp(Gene = "Dunnett"))

# Extract p-values from the Dunnett test
VLP_p_values <- summary(VLP_dunnett)$test$pvalues

# Convert p-values to significance levels (e.g., "*" for p < 0.05, "***" for p < 0.01, "****" for p <
0.001)
VLP_significance <- ifelse(VLP_p_values < 0.001, "****",
                           ifelse(VLP_p_values < 0.01, "***",
                                   ifelse(VLP_p_values < 0.05, "*",
                                           "NS")))

#####
#####

#Summarize the useful data
data_summary <- data.frame (
  Experiment = expnum,
  clean_name = c("Scrambled", clean_name),
  Virus_p_values = c(NA, Virus_p_values),
  Virus_significance = c("NS", Virus_significance),
  Virus_means$avg_Virus,
  VLP_p_values = c(NA, VLP_p_values),
  VLP_significance = c("NS", VLP_significance),
  VLP_means$avg_VLP
)

# Define the file path where you want to save the Excel file
file_name <- paste0("C:/Users/Chris/OneDrive/School/Grad School/5.) Thesis Project/Thesis
Writing/(Chapter 5) Data Summary for Gene Experiments/Dunett Testing/Virus Concentration
Graphs",
  "(Experiment ", expnum, ") Dunnett Data.xlsx")

# Export the data frame to an Excel file
write.xlsx(data_summary, file = file_name)

```