# An In-Depth Look at Repeated Propagation of Multiple Viruses Simultaneously in Insect Cell Culture

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Applied Science
in
Chemical Engineering

Waterloo, Ontario, Canada, 2020

## 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.

I understand that my thesis may be made electronically available to the public.

**Abstract**

It is well known that the insect cell-baculovirus expression vector system (IC-BEVS) can be used for the production of various proteins of interest and virus-like particles (VLPs). In the past, many higher order proteins and protein complexes like antibodies (zu Putlitz et al., 1990), viral vectors (Aucoin et al., 2006) and VLPs (Pushko et al., 2005, 2010) have been produced using this system. For the production of recombinant proteins of interest or VLPs, one or more baculovirus constructs, each carrying one (monocistronic) or more (polycistronic) gene(s) of interest can be introduced to insect cells. Three common expression systems for protein production mainly include infecting insect cells with a number of baculovirus constructs each carrying a gene of interest (coinfection), infecting insect cells with a single baculovirus construct carrying multiple genes of interest (coexpression), or a combination of the two systems (Sokolenko et al., 2012). Although coexpression is the ultimate goal, there are many reasons coinfection is still used. Coinfection allows a degree of flexibility that is not as easily achieved through coexpression. The hypothesis behind this work is that, the virus ratios or the population distribution remains constant upon repeated propagation. Here, we are seeking to avoid coinfection at larger scale by amplifying viruses together at smaller scale. By infecting cells with baculovirus coding for reporter proteins, eGFP and mKate2, at high and low MOIs we can track the virus population being amplified and be confident that the ratios are preserved or will require adjustment upon repeated amplification. We can then use this knowledge

to improve the production of influenza A VLPs, a more industrially relevant system.

Different assays and/or techniques were used to assess the virus population distribution over repeated propagation at high or low MOI, in addition to purification and analysis of influenza VLPs. It was concluded that repeated amplification at high MOI changed the population distribution, and also resulted in significant drops in the infectious virus titer. Amplifications at low MOI improved the virus titer, and not much variation was observed in the distribution over subsequent passaging. That being said, it was found that ultracentrifugation alone did not have the resolution to purify VLPs, and additional purification steps must be employed to produce VLPs for biopharmaceutical applications.

**Acknowledgements**

I would like to thank my supervisor Prof. Marc Gordon Aucoin for giving me the opportunity to be a part of his group. Thank you for having faith in me and being a mentor anyone would ask for. I am really grateful for all the positive criticisms and comments that have helped into the shaping of this work and me as a researcher.

I thank the entire Aucoin Lab; Megan, Mark, Scott, Eduardo, Alex, Sadru, Merve and Meagan C. Thank you Megan and Mark for all the insights you have given into my project and for the meaningful discussions we have had over time. Mark for providing all the virus constructs and GFP inducing Sf9 cell lines used in this project; Megan for training me in microscopy and Eduardo for training me on the Flow and Ultracentrifuge.

I would like to thank the Department of Chemical Engineering, University of Waterloo and NSERC for funding this work. I thank the administrative and technical support staff of the Department of Chemical Engineering- Judy, Ellen, Rose, Liz, Colleen, Ingrid, Ralph, Bert, Rick and Tom.

All my friends back home for always believing in me. Ishita and Ina, you two have been my support system since the day we have crossed paths. My family away from family... Shafaq, Twara, Hiren, Prafful, Waqas (bhaiya), Manas (papa), Daniyal and Mehali, thank you for being there for me and with me at every moment of my life in Canada and bearing my tantrums. I am not sure what I would have done without you guys here. Kavitha, you have been an awesome roommate; thank you for always being patient with

me and guiding me.

I thank my family for their immense support in all my life decisions and encouraging me in every possible way. Last but not the least, my parents and Bibi (granny) for just being who you are, trusting my potential and loving me unconditionally.

## Dedication

This is dedicated to my parents, Manash and Sanchita, and Bibi.

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# Nomenclature

$APCs$ Antigen presenting cells

$AcMNPV$ *Autographa californica* multicapsid nucleopolyhedrovirus

$BEVS$ Baculovirus Expression Vector System

$BV$ Budded virus

$BmNPV$ *Bombyx mori* nucleopolyhedrovirus

$DIPs$ Defective interfering particles

$DNA$ Deoxyribonucleic acid

$DO$ Dissolved oxygen

$EPDA$ End point dilution assay

$EV$ Extracellular virus

$FBS$ Fetal bovine serum

$FDA$ Food and Drug Administration

$GAG$ Group-specific antigen, Retrovirus

$GAG - GFP$ Retroviral GAG genetically tagged with eGFP

$GFP$ Green fluorescent protein, referring to eGFP

$GFP - Sf9$ GFP inducing Sf9 cell line

$GV$ Granuloviruses

$HA\ assay$ Hemagglutination assay

$HA$ Hemagglutinin, Influenza protein

$HAU$ Hemagglutination units

$HEK293$ Human embryo kidney cells

$HIV$ Human immunodeficiency virus

$IC - BEVS$ Insect cell-baculovirus expression vector system

$M1$ Influenza Matrix 1

$M2$ Influenza Matrix 2

$MDCK$ Madin Darby canine kidney cells

$MOI$ Multiplicity of infection

$NA$ Neuraminidase, Influenza protein

$NOV$ Non-occluded virus

$NPVs$ Nucleopolyhedroviruses

$OB$ Occluded bodies

$OV$ Occluded virus

$PBS$ Phosphate buffered saline

$PER.C6$ Human embryonic retinal cells

$PIBs$ Polyhedral inclusion bodies

$RBCs$ Red blood cells

$RFP$ Red fluorescent protein, referring to mKate2

$RNA$ Ribonucleic acid

$SFM$ Serum free media

$Sf21$ *Spodeptera frugiperda* clonal isolate 21

$Sf9$ *Spodeptera frugiperda* clonal isolate 9

$TCID50$ Tissue culture infectious dose 50

$TOH$ Time of harvest

$TOI$ Time of infection

$VLPs$ Virus-like particles

$Vero$ African green monkey derived kidney cells

$WHO$ World Health Organization

$WIV$ Whole influenza virions

*polh* Poyhedrin gene

$eGFP$ enhanced Green Fluorescent Protein

*hpi* hours post infection

$non-hr\ ori$ Non-homologous repeated origin of replication regions

$p10GFP$ Recombinant baculovirus expressing eGFP under the very late p10 promoter

$p10RFP$ Recombinant baculovirus expressing mKate2 under the very late p10 promoter

$p6.9GAG-GFP$ Recombinant baculovirus expressing GAG-GFP under the late p6.9 promoter

$p6.9HA$ Recombinant baculovirus expressing HA under the late p6.9 promoter

$pfu$ Plaque forming unit

$rHA$ Recombinant hemagglutinin

*rpm* Revolutions per minute

# Chapter 1

# Introduction

The production of recombinant proteins and virus-like particles (VLPs) using the insect cell-baculovirus expression vector system (IC-BEVS) has become a promising platform owing to their ease of use and versatility. Development of a number of transfer vectors, simplified recombinant virus isolation and quantification methods and improvements in cell culture technology have made this system easy to use, safe and readily scalable (Kost et al., 2005). The limited host-range in which these baculovirus can replicate, capability of the baculovirus to accept multiple large insertions and high protein yields makes the system a great choice for recombinant protein production (George, 2016). Ability of insect cells to perform post-translational modifications including glycosylation (James et al., 1996), phosphorylation (Héricourt et al., 2015) and disulfide bond formation (Hodder et al., 1996), makes BEVS a platform of choice for production of foreign protein that requires such mod-

ifications (Reed and Muench, 1938). A complex and sophisticated series of tightly temporally regulated events takes place upon infection of insect cells with baculovirus (George and Aucoin, 2015). IC-BEVS provides a number of advantages including high levels of expression, mammalian cells like post-translational modifications and inherent safety during manufacture and of the final product as reviewed by Sokolenko et al., 2012. Moreover, the lack of human infectitious viruses in insect cells (Summers, 2006) and inability of baculovirus to grow in or infect mammalian cells (Sokolenko et al., 2012) makes it advantageous for production of therapeutic proteins.

On the basis of using multiple baculoviruses each carrying a foreign gene (monocistronic baculovirus) or a single baculovirus carrying multiple foreign genes (polycistronic baculovirus) to infect insect cells, the three most common infection strategies include coinfection (infection with a number of baculovirus constructs each expressing a gene of interest), coexpression (infection with one baculovirus construct expressing multiple genes of interest) and a combination of the two (Sokolenko et al., 2012). The introduction of viruses to cell cultures by an infection strategy, and the subsequent steps comprising of virus entry into cells, viral gene replication and assembly, and virus budding is known as virus amplification/propagation/passaging. It is believed that while coinfection is more suited to determine the levels of individual genes required to make a desired product, coexpression plays a more important role for large scale production. That being said, the flexibility offered by coinfection to control the ratios of different proteins needed to form

a particular product can be successfully exploited at a small scale.

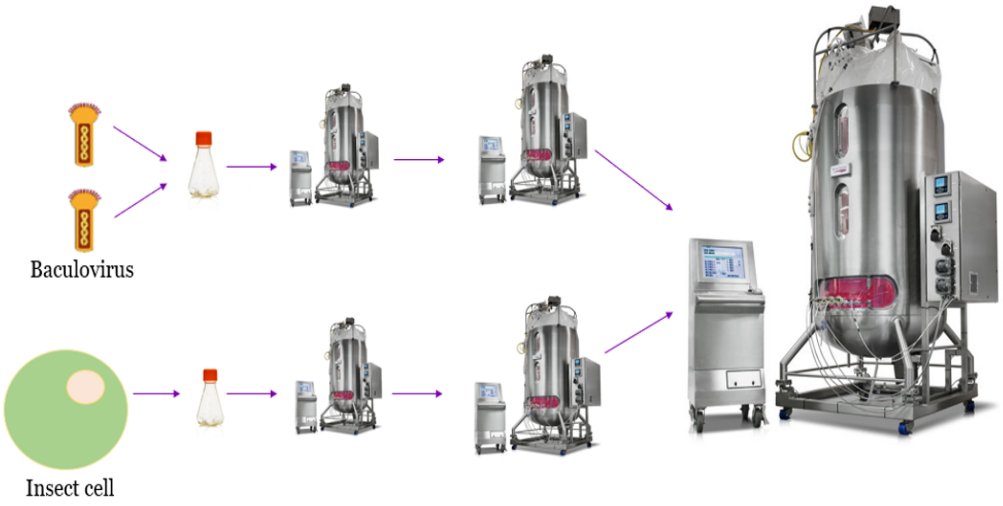


Figure 1.1: **Schematic of a possible scale-up process.**

There are many reports present in literature that exploit the benefits of the IC-BEVS for the production of virus-like particles. These VLPs, which are basically derived from the structural components of a virus of interest, can induce the same immune response in host as that of a virus. In other words, VLPs are similar to a virus except it does not contain the viral genome. Production of VLPs in multiple cell culture systems, such as mammalian cells (Wu et al., 2010; Thompson, 2013) and insect cells using BEVS (Lopez-macias et al., 2011), and their non-replicative nature makes them a safer alternative for many applications including vaccination. According to the World Health Organization (WHO), influenza A virus infection, that results in acute respiratory tract infection, causes around 300,000 to 500,000

deaths annually worldwide (Organization, 2010). Mutation in the major glycoprotein hemagglutinin (HA) is mainly responsible for poor control of the infection with immunization. New vaccine strategies need to include immunization with viral antigens in addition to HA. These antigens should induce a broadly cross-reactive immunity capable of protecting vaccine recipients from antigenic heterovariants by including influenza viral neuraminidase (NA) and/or matrix protein (Johansson and Eichelberger, 2010). Influenza A VLPs provide better immune response as compared to inactivated or live attenuated vaccines (Pushko et al., 2005, 2010; Bright et al., 2007). Also, GAG-based influenza VLPs containing influenza proteins have been reported to exhibit sturdy vaccine protection (Haynes et al., 2009). Although the use of IC-BEVS is quite popular, the production of VLPs using this system needs to be optimized for industrial scale.

The driving hypothesis behind this work is that when two viruses are amplified together in cell culture, the ratio of the two virus populations is maintained over repeated propagation. Although coinfection seems to be the choice for small scale production owing to the additional degree of freedom it provides, for industrial scale its use is usually minimized. In this study we start with coinfection to determine the individual levels of each gene at a smaller scale and try to track the population of viruses after repeated amplification and make sure the ratio of viruses are maintained or if some modifications are needed upon amplification. Using this information we can explore an amplification process which starts with multiple viruses, each

carrying a gene of interest, and then use the virus stocks from the coinfected cultures for subsequent infections. With some modifications, this can then be extended for influenza A VLP production.

The first chapter of this thesis is a general introduction to IC-BEVS, infection strategies and influenza VLPs. This is followed by a literature review relevant to the work presented in the thesis and a chapter outlining all the materials and methods used in this work. **Chapter 4** presents a simple system where two baculovirus constructs carrying the gene for eGFP (a green fluorescent protein, GFP) or mKate2 (a red fluorescent protein, RFP) were used to establish a baseline. The information gathered from the GFP/RFP work was then expanded to a more industrially relevant system of proteins that can be used for influenza A VLP production, and the results are presented in **Chapter 5**. An overall conclusion of this work and some recommendations based on the data obtained have been summarized in **Chapter 6**.

# Chapter 2

# Literature Review

## 2.1 Baculovirus

Baculoviruses are enveloped DNA viruses with a very narrow host range within arthropod invertebrates (Lu and Miller, 1997). They are divided into the nucleopolyhedroviruses (NPVs), which can form occlusion bodies composed of one or more virions encapsulated in a polyhedrin protein matrix, and the granuloviruses (GV), which can form occlusion bodies containing a single virion encased in a granular protein matrix (Funk et al., 1997). Baculoviruses can exist in one of two forms during their life cycle: occluded form (responsible for transmission between hosts) and budded form (responsible for transmission within a host). Baculoviruses are 300 nm in length, 50 nm in diameter, with a circular supercoiled double stranded DNA genome (Rohrmann, 2019). *Autographa californica* multiple nucleopolyhedrovirus

(AcMNPV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV) are the two most widely studied baculoviruses for production of recombinant proteins and VLPs.

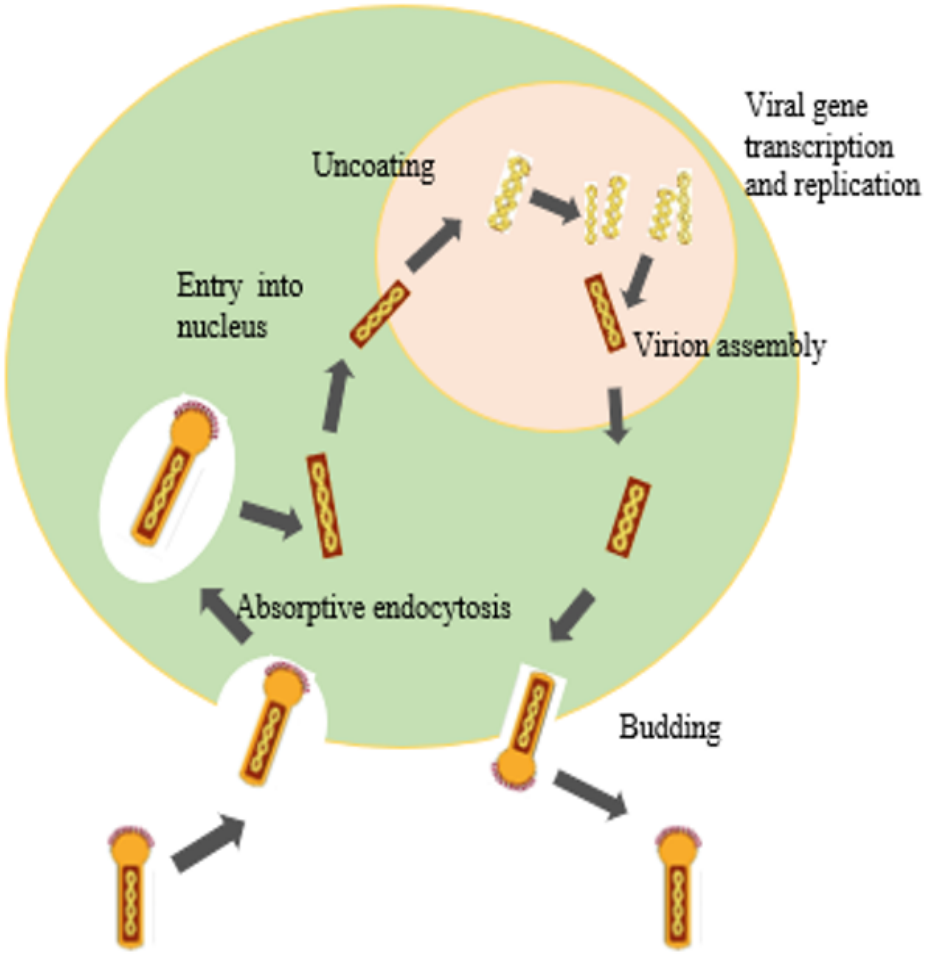


Figure 2.1: **Baculovirus life cycle.**
(Adapted from (George, 2016))

The baculovirus infection cycle is divided into three phases: the early phase, the late phase and the very late phase. The early phase, also known

as virus synthesis phase, occurs from 0.5 to 6 hours post infection (Chisholm and Henner, 1988) and includes two sub-phases (immediate early and delayed early) during which the virus prepares the infected cells for viral DNA replication. The cells start to produce extracellular virus (EV), also called non-occluded virus (NOV) or budded virus (BV) in the late phase or the viral structural phase, between 6 to 12 hours post infection. Late genes coding for viral DNA replication and viral assembly are expressed with the peak release of EV between 18 to 36 hours after infection. The very-late phase, also known as the viral occlusion protein phase, marks the expression of polyhedrin and p10 genes, formation of occluded virus (OV) or occluded bodies (OB) or polyhedral inclusion bodies (PIBs) and beginning of cell lysis (Kelly et al., 2007). Although the origin of baculovirus DNA replication is not known clearly, some reports suggests that the homologous regions are the DNA replication initiation sites (Kool et al., 1993), while others claim that initiation can occur at other sites (Habib and Hasnain, 2000). The DNA replication in baculovirus occurs either by rolling circle mechanism (Volkman and Oppenheimer, 1997) or by recombination (Okano et al., 2007) and requires several baculovirus gene products.

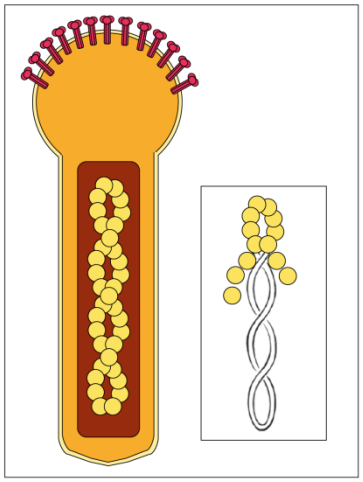

Figure 2.2: **Morphology of budded baculovirus.**

When a baculovirus is used as an expression vector for *in vitro* infection, the naturally occurring polyhedrin (*polh*) gene in a wild type baculovirus is usually replaced with recombinant gene(s) of interest. However, other early and late promoters are being investigated for foreign gene expression (George, 2016). The polyhedrin and p10 promoters are associated with large production of recombinant protein(s) in the very-late phase of baculovirus infection. The metabolic condition of culture and growth medium used as well as the insect cell line and baculovirus strain used, can control the timing of baculovirus infection cycle.

## 2.2 Insect cell culture

As early as the 1930s insect cell lines were initiated and used for replication of insect baculoviruses and arboviruses (Arif and Pavlik, 2013). Insect cells usually do not carry any infectious human virus and especially retrovirus (Summers, 2006). Many continuous cell lines such as *Spodoptera frugiperda* cells (Sf9 or Sf21 clones) and *Trichoplusia ni* cells (High Five clone) have been isolated and characterized for the production of biologics (Mena and Kamen, 2011). The cell lines grow well in suspension thus allowing for recombinant protein production in large scale bioreactors, and were rapidly developed for suspension culture and serum free media to minimize production costs (Maranga et al., 2003).

Grace's supplemented medium (TNM-FH) developed in the 1960s has been a traditional medium of choice for *in vitro* insect cell culture (Grace, 1962). Other serum/hemolymph dependent media such as, IPL-41 and TC-100, and serum free media such as Sf-900 II SFM, Sf-900 III SFM and EX-PRESS FIVE SFM have been developed thereafter (Chan and Reid, 2016; Bauer and Schnapp, 2007). Optimized second generation serum free media such as Sf-900 II and III SFM with lot-to-lot consistency, reduced down-stream processing complexity and low cost, have facilitated large scale production of recombinant proteins (Zhang et al., 1992).

The three most common insect cell lines, Sf9, Sf21 and High Five, used in research are derived from lepidopteran insects (Arunkarthick et al., 2017)

with Sf9 and Sf21 cells being derived from pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda* (Wickham et al., 1992) and High five cells from the ovarian tissues of the cabbage looper, *Trichoplusia ni* (Hong et al., 2015). The main components of insect cell maintenance media consists of a mixture of carbohydrates, amino acids, vitamins, growth factors, trace elements, salts, metabolic precursors and hormones. Different cell lines require various concentrations and combinations of these constituents (Arunkarthick et al., 2017). The ideal temperature for maintenance is between 26 to 28 °C, with a lower temperature resulting in decreased growth rate and a temperature higher than 30 °C leading to lower viability (Arunkarthick et al., 2017).

In serum free and protein free medium cultures, insect cells have a fast specific growth rate. Running batch and fed-batch bioreactors with cells densities reaching high levels is quite straightforward and robust till the culture conditions are tightly monitored and controlled. Ability of insect cells to grow in serum free medium is highly beneficial as it eliminates the chances for occurrence of adventitious viruses thus reducing the need for more intensive purification processes (Vicente et al., 2011). The requirement for annual adjustments to influenza vaccine makes the insect cell-baculovirus expression vector system (IC-BEVS) suitable for the production of influenza VLPs (Cox and Hollister, 2009).

## 2.3 Protein production platform

Different platforms such as bacteria, yeast, plant cells, mammalian cells and insect cells are available for recombinant protein production. Egg-based technology has been used for a long time for the production of inactivated and live attenuated influenza vaccines (Milián and Kamen, 2015). In the biopharmaceutical industry, production of therapeutic proteins or vaccines using mammalian cell culture technology is well established (Le et al., 2010). Several mammalian cell lines such as MDCK (Madin Darby canine kidney cells) (Rimmelzwaan et al., 1998), PER.C6 (human embryonic retinal cells) (Pau et al., 2001), Vero (African green monkey derived kidney cells) (Barrett et al., 1998) and HEK293 (human embryo kidney cells) (Le et al., 2010) have been explored for the production of influenza particles. Optaflu/Flucelvax® (Novartis) vaccine approved by the FDA in 2012 is a trivalent vaccine produced in MDCK cells (Doroshenko and Halperin, 2009). Perflucel™ is another influenza vaccine produced in Vero cells, formulated with inactivated H1N1, H3N2 and influenza B virus (Agency, 2012).

Cervarix, a human recombinant papillomavirus VLP cancer vaccine, was successfully produced using the insect cells-baculovirus expression vector system. A well-accepted safety profile of insect cells has led to their consideration for commercial vaccine production (Vicente et al., 2011). A recombinant baculovirus carrying a gene of interest can be designed faster thus resulting in shorter turnaround periods when compared to other production methods

(Cox and Hashimoto, 2011).

## 2.4 Advantages of the BEVS

The baculovirus expression vector system has become a versatile and powerful eukaryotic vector system for recombinant protein production, since its introduction in 1983 (Smith et al., 1983a). The IC-BEVS works by inserting one or more gene(s) of interest into a baculovirus genome, using a transfer plasmid, under the control of a baculovirus or insect promoter sequence and at a site that does not interfere with the baculovirus replication (George, 2016). The ideal sites for insertion of the gene(s) of interest are situated downstream of the very strong very late baculoviral p10 or polyhedrin promoters, which leads to very high expression levels (Smith et al., 1983b).

BEVS offers a number of advantages over other expression vector systems. The restricted host range and non-pathogenicity to mammals and plants, makes baculovirus an ideal choice (Fuxa, 1991). The requirement of helper cell lines or helper viruses is eliminated as all the genetic information can be contained in the baculovirus genome. Recombinant products produced using BEVS can be easily scaled up for large scale production. High levels of recombinant gene expression can be achieved using this system (Sokolenko et al., 2012) along with the mammalian like post-translational modification capacity of insect cells.

The baculovirus has limited permissive cell lines for infection. The BEVS

has the advantage of lower risks of contamination with human adventitious agents over mammalian platforms and the ability to provide eukaryotic protein modifications including N-glycosylation (Toth et al., 2014). Expression systems such as bacteria and yeast are known to achieve higher yields, but they do not match the degree of complexity (expression of multiple/different gene(s) of interest) required for VLPs that can be achieved with IC-BEVS (Vicente et al., 2011). The product yields obtained are significantly higher using IC-BEVS when compared to egg-based or mammalian cell based VLP production and the production time is also reduced to 12 weeks (James, 2009).

## 2.5 Coinfection versus coexpression using the BEVS

Expression of one or more foreign proteins for the production of a protein complex can be achieved by one of the three most common infection strategies using IC-BEVS: coinfection, coexpression or a combination of both. On one hand, coinfection, using multiple virus constructs each carrying a gene of interest, provides an additional degree of flexibility that is beneficial for early exploratory work. On the other hand, coexpression, using a single virus construct carrying all the genes of interest, is preferred at the larger scale owing to the reduced number of different virus constructs required for infection (George, 2016).

The choice of infection strategy is critical for the production of a desired protein or protein complex. Some groups have focused on coexpression for the production of protein complexes (Road, 1995; George et al., 2015). This expression strategy not only reduces the total amount of baculoviruses, but also decreases the number of possible combinations of baculoviruses that can be found in one cell (Sokolenko et al., 2012). Coexpression using polycistronic baculovirus allows the desired production of all foreign proteins in every infected cell. The expression ratios in such cases can be controlled and adjusted by manipulating the promoters that control the levels of protein expression (Road, 1995). However, the use of only very strong very late p10 and polyhedrin promoters to drive expression does not provide much opportunity to tailor expression ratios within the cell. George et al., 2015 demonstrated that the use of alternative promoters to control the timing and expression of foreign proteins may prove to be a better alternative to modify coexpression.

Many other groups in the past have exploited coinfection due to the ability of insect cells to be infected by multiple baculovirus constructs, as the primary expression strategy for the production of complex products (Aucoin et al., 2006; Mena et al., 2010; Meghrous et al., 2005). For coinfection the crucial parameters of interest are multiplicity of infection (MOI) and time of infection (TOI) (Sokolenko et al., 2012). By varying the MOI, that is the ratio of infectious baculovirus to the number of viable insect cells at the time of virus addition, the infection conditions can be controlled. Coinfection offers the advantage of investigating the ratio of each gene required for the

optimal production of a desired product complex by altering the MOI of individual virus vectors carrying a foreign gene. The work by Aucoin et al., 2006 demonstrated how coinfection strategies with a combination of monocistronic (virus vector carrying one foreign gene) and polycistronic (virus vector carrying multiple foreign genes) baculoviruses led to differences in yield depending on baculovirus ratios selected, thus highlighting the necessity to understand the relationship between baculovirus constructs to optimize the coinfection strategy. The inherent instability of large baculovirus constructs containing multiple genes makes process scale-up unfavourable, projecting the importance of coinfection strategy (Aucoin, 2007).

## 2.6 Defective interfering particles and virus amplification

Defective interfering particles (DIPs) are spontaneous deletion mutants of viruses that can infect insect cells, but not complete the replication cycle, and replicate at the cost of the parent virus. It has been observed that the growth of viruses *in vitro*, as well as the establishment of a persistent infection is affected by DIPs (Kirkwood and Bangham, 2006). Multiple passage of baculovirus in cell culture medium, especially at high MOI results in the accumulation of DIPs.

These defective particles are known to interfere with the helper virus replication, lack considerable portions of the genome and cause the passage

effect during serial passaging in insect cell cultures. The presence of DIPs with a major genomic deletion upon serial passage of AcMNPV-E2 in Sf21 cells has been observed (Kool et al., 1991). Based on the above mentioned work by Kool and group (1991), another study demonstrated the presence of DIPs lacking 43% of the viral genome in low passage AcMNPV-E2 virus stocks and in polyhedra, but not in AcMNPV isolate obtained prior to cell culture passaging (Pijlman et al., 2001). It has been postulated that the rapid generation of DIPs is an intrinsic property of baculovirus infection in insect cell culture, involving several recombination steps (Pijlman et al., 2001).

Production of recombinant protein and baculovirus, after repeated virus amplification in insect cells at high MOI, is usually accompanied by the appearance and accumulation of DIPs (Wickham et al., 1991). The phenomenon of passage effect, that is, generation of defective particles upon recurrent passaging at high MOI in insect cells, complicates the scale-up of baculovirus and recombinant protein *in vitro* (Pijlman et al., 2001). On the other hand, during a low MOI infection all the cells in the culture are not infected and instead consists of multiple infection cycles. A low MOI infection results in some cells receiving replicative virus, some receiving DIPs and others not receiving any virus particles (Wickham et al., 1991). Several studies have shown the use of low MOI for virus amplification, hence reducing the passage effect. For the production of self-forming *Porcine parvovirus*-like particles using the IC-BEVS, a low MOI was used to avoid an additional virus amplification step and to minimize accumulation of DIPs (Maranga et al.,

2003). A study on the occurrence of DIPs within high passage AcMNPV, interference with recombinant protein and infectious virus production was presented using three insect cell lines. They observed significant reductions in the specific productivity of a recombinant protein with MOI over 0.01 pfu/cell (Wickham et al., 1991).

In addition to cell line and media used for production, factors such as pH, temperature, dissolved oxygen and, infection and harvest parameters (including MOI, TOI and time of harvest (TOH)) are critical for optimal baculovirus vector production process. Current production processes relies on cultures infected at low densities of 1 to 3 x $10^6$ cells/ml at low MOIs (Aucoin et al., 2010). High MOI ($>$ 3 pfu/cell) infections results in the entire cell population being simultaneously infected and is referred to as synchronous infection. In contrast low MOI ($<<$ 1 pfu/cell) infections leads to asynchronous infection where only a portion of the overall cell population is infected. Aucoin et al., (2010) described the dynamics of low MOI infection in four steps. The first step involves two cell populations being observed with one infected population and one uninfected population, following that the infected population produces progeny virus and the uninfected population keeps growing until additional viruses are produced after the primary infection. The final step involves infection of the non-infected population after a time period by the progeny viruses that bud from the primary infected cells. To minimize the production of DIPs, which interferes with process productivity and baculovirus quality, a low MOI is preferred for virus ampli-

fication (Pijlman et al., 2003). It has been reported that the accumulation of non-homologous repeated origin of replication regions (non-hr ori) causes defective particle formation, and the deletion of these regions from the viral DNA can help to prevent the accumulation of DIPs (Pijlman et al., 2003).

## 2.7 Influenza A virus and VLP

### 2.7.1 Influenza A virus

Influenza A viruses belonging to the Orthomyxoviridae family are enveloped RNA viruses with the genome divided into 8 negative sense single stranded RNA fragments (Krammer and Schinko, 2010). Five of the single stranded RNA fragments encode a single protein each and the other three encode two proteins each (Bouvier and Palese, 2008). The major glycoproteins and antigens of influenza A virus, HA and NA, are responsible for binding to cellular receptors of host cells and mediating the release of newly synthesized particles from the host cell surface, respectively (Nayak et al., 2009). The matrix protein, M1, of the influenza virus binds the viral RNA and along with the HA and NA forms the structural component of the virus. The neuraminidase (NA) surface protein releases newly formed virus from cell surfaces by cleaving the sialic acid residues that binds the cell to the HA protein. The HA glycoprotein, which is used in this project, is present as a trimer on the surface of the virus. A 17 amino acid residues is followed by a 511 amino acid extracellular domain, which is further followed by a helical 21

amino acid transmembrane domain and finally a 16 amino acid cytoplasmic tail domain (Winter et al., 1981). Other viral components and non-structural proteins of the virus are involved in virus infectivity and transmission of host cells.

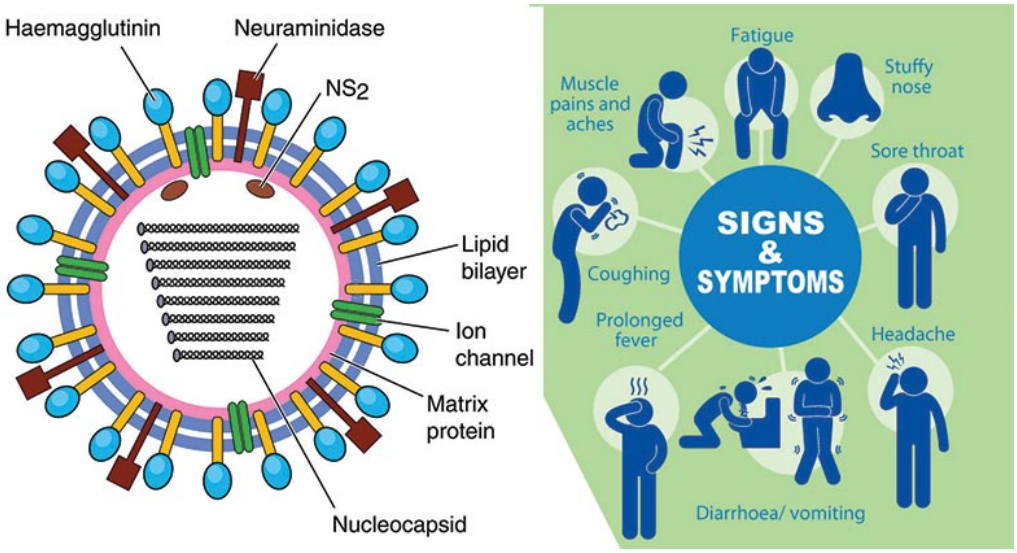


Figure 2.3: **Influenza virus morphology and symptoms of flu.**

### 2.7.2 Production of influenza VLPs

Influenza A VLPs have been produced in mammalian and insect cells previously. The baculovirus expression vector system is gaining popularity for the production of influenza VLPs mainly due to the lack of sialylation in insect cells, which prevents the binding of HA proteins present on the surface of influenza VLPs to the cells. This eliminates the need for viral NA to cleave the VLPs from the cell surface, unlike in mammalian cells (Mena et al., 1999).

The first multi-subunit influenza VLP was produced in BEVS using a bac-

ulovirus coexpressing HA, NA, M1 and M2 proteins (Latham and Galarza, 2001). The production of nontransducing influenza VLP in insect cell culture through the simultaneous expression of influenza HA, NA, M1 and M2 in a single baculovirus resulted in the release of particles in the supernatant that were morphologically similar to influenza virions (Latham and Galarza, 2001; Cox, 2008). Another work showed that coinfecting insect cells with monocistronic baculoviruses coding for HA or M1 proteins led to the production of VLPs comprising of HA and M1, and which provided protective immunity from influenza virus (Quan et al., 2007). Simultaneous expression of influenza HA and NA along with a viral core protein, such as M1, in insect or mammalian cells resulted in the production of enveloped VLP vaccines containing influenza HA and NA antigens. The hypothesis behind an influenza VLP vaccine is that the efficacy of these vaccines is linked to the particulate, multivalent composition along with correctly folded antigens with intact biological activities, and that these VLPs are highly immunogenic and protective to pathogenic influenza (Haynes, 2009). Infection with a single vector at a low MOI, comprising of the genes for HA, NA and M1 led to the successful production of influenza VLPs (Haynes, 2009).

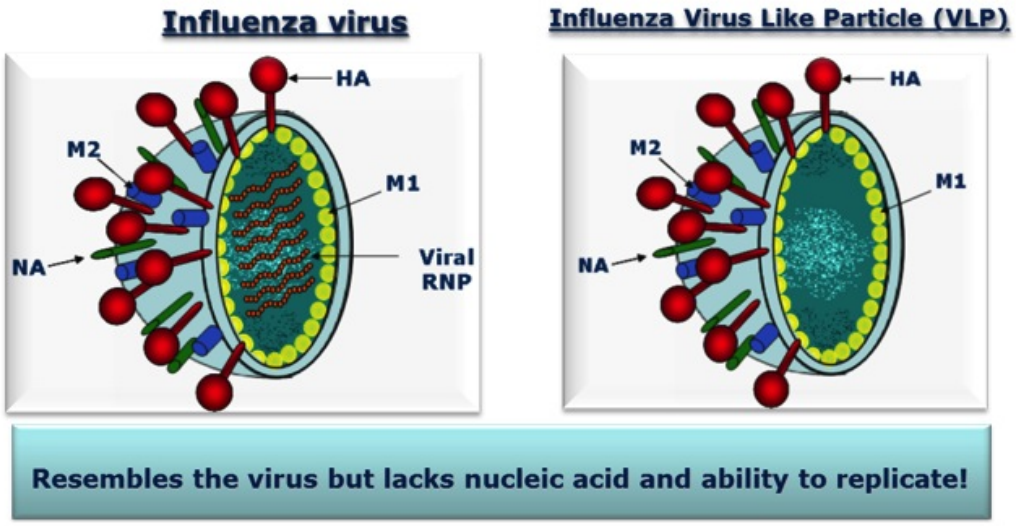


Figure 2.4: **Comparison between the structure of influenza virus and VLP.**

In addition, the GAG (group-specific antigen) protein from Human immunodeficiency virus (HIV) has been used for VLP production for a variety of applications. Retroviral GAG protein expression is an efficient VLP producing method (Chaves et al., 2019). GAG-based influenza VLPs have been generated that exhibit robust vaccine protection (Haynes et al., 2009; Cervera et al., 2013, 2017; Gutiérrez-granados et al., 2013; Haynes, 2009; Venereo-sanchez et al., 2017; Venereo-sánchez et al., 2019).

### 2.7.3 Influenza VLP as a vaccine candidate

Vaccines are effective in controlling and preventing disease severity, but high rate of mutations in the viral genome and genetic re-assortment makes the

production of new vaccines every flu season a necessity. Influenza vaccines containing live attenuated influenza virus and inactivated virions, both produced using embryonated chicken eggs, are two common types of influenza vaccines. The production of these vaccines is time consuming and labor intensive, and also unsuitable against H5N1 viruses which kills the chicken eggs (Quan et al., 2007). It takes around 3-6 months to produce vaccines using egg-based technology, thus increasing the cost associated with it. These limitations point to the production of influenza vaccines using cell culture based technology, which would accelerate the production rate and allow quick response to changes in circulating influenza subtypes. A recombinant trivalent hemagglutinin (rHA) vaccine called FluBlok produced in the IC-BEVS has emerged as a new alternative to egg-based vaccine (Cox and Hollister, 2009). The absence of egg-proteins (no cross-contamination) and preservatives, the presence of three times more HA in FluBlok than egg-based vaccines and the high purity of antigens makes the production and administration at a higher dose safe (Cox and Hollister, 2009).

Non-replicative VLPs are a potential vaccine candidate for many viruses (Pattenden et al., 2005). Antigen presenting cells (APCs) which activate the adaptive immune system, are responsible for professionally presenting VLPs (Buonaguro et al., 2006; Sailaja et al., 2007; Kang et al., 2009). Heat treatment of influenza VLPs, which causes denaturation of the surface HA glycoprotein, induces a lower titer of intact influenza specific antigens and provides negligible protection against influenza, and thus indicate the im-

portance of HA antigen in VLP based vaccines (Quan et al., 2007). There is evidence that influenza VLPs can induce a cytotoxic T cell response in mice, thus playing a vital role in protection from heterotypic subtypes of the influenza virus (Hemann et al., 2019).

Although commercial inactivated and live attenuated human influenza vaccines against H1N1 virus are immunogenic (Greenberg et al., 2009), there are certain limitations associated with manufacturing in embryonated eggs and adverse effect in particular populations (Horimoto and Kawaoka, 2006). The widespread 2009 pandemic H1N1 virus infection stressed the need for alternative vaccine strategies as opposed to the traditional egg-based method. A recombinant influenza VLP vaccine comprising of HA, NA and M1 proteins of the influenza A/California/04/2009 (H1N1) virus was developed using IC-BEVS. Infection of Sf9 insect cells with a baculovirus coexpressing HA, NA and M1 proteins resulted in the release of 120 nm influenza VLPs in the culture medium. When purified these VLPs were found to be morphologically similar to influenza virions and exhibited biological characteristics of influenza virus along with HA and NA activities. After being incorporated in a ferret challenge model, these VLPs produced high-titer serum HA inhibition antibodies for the H1N1 virus and repressed replication of the lower and upper respiratory tract tissues following H1N1 infection, in addition to complete clearance of the virus from the ferret lung using only a single 15 $\mu$g dose (Pushko et al., 2010). It was shown that intranasally administered VLPs derived from 1918 pandemic H1N1 virus protected animals against

lethal homologous and heterologous viruses (Perrone et al., 2009).

To combat avian influenza viruses a recombinant VLP vaccine comprising of HA, NA and M1 proteins of influenza A/Hong Kong/1073/99 (H9N2) virus was produced in Sf9 cells using a baculovirus coexpressing the above three mentioned structural proteins. The VLPs exhibited influenza virus characteristics including HA and NA activities. When BALB/c mice were infected with these VLPs, serum antibodies specific for the influenza A/Hong Kong/1073/99 (H9N2) virus was produced and influenza virus replication was inhibited. It was thus concluded that VLPs are a potential strategy for producing vaccines against H9N2 viruses (Pushko et al., 2005). The licensed trivalent vaccine produced using egg-based technology is efficient in healthy adults but are not very protective for high risk individuals such as the elderly and the immunocompromised. It was reported that Sf9 cells-derived influenza VLPs elicited high immune responses and effective protection in animal models against seasonal (Bright et al., 2007) and pre-pandemic strains (Bright et al., 2008). VLP comprising of HA, NA and M1 proteins of the influenza A/Fujian/411/2002 (H3N2) virus was produced in Sf9 cells using a baculovirus comprised of the three influenza structural proteins, namely HA, NA and M1. Intramuscular vaccination of mice or ferrets with these VLPs in a dose sparing experiment by varying the HA concentration and comparing the immune responses to those elicited in animals vaccinated with recombinant HA (rHA) or inactivated whole influenza virions (WIV) revealed broader immune response by the influenza VLPs (Bright et al., 2007).

# Chapter 3

# Materials and Methods

## 3.1 Cell Line and Maintenance

The clonal isolate 9 of *Spodoptera frugiperda*, Sf9 cells, were maintained in Sf-900 III serum free media (GIBCO, Carlsbad, CA, USA) for virus amplification and recombinant protein production. The cell cultures were maintained routinely between 0.6 x $10^6$ and 4.6 x $10^6$ cells/ml in 125 ml capped glass Erlenmeyer flask at 27°C on an incubator shaker (VWR International, Missisauga, ON, Canada) at 130 rpm (revolutions per minute). Cell density and viability were determined using a Countess II FL hemacytometer (Life Technologies, Carlsbad, CA, USA) and via trypan blue dye (HiMedia) exclusion method, respectively.

## 3.2 Amplification of Initial Baculovirus Stocks

Sf9 insect cell cultures seeded at 0.6 x $10^6$ cells/ml were allowed to grow to an exponential phase between 3.5 and 3.8 x $10^6$ cells/ml before being diluted back to 2 x $10^6$ cells/ml in fresh Sf-900 III media (GIBCO, Carlsbad, CA, USA) and infected with eGFP (a green fluorescent protein), mKate2 (a red fluorescent protein), GAG-GFP (retroviral group specific antigen tagged with a green fluorescent protein) or HA (influenza hemagglutinin) expressing baculovirus, herein referred to as p10GFP, p10RFP, p6.9GAG-GFP and p6.9HA respectively. The cell cultures infected at an MOI of 0.1 were allowed to propagate until the viability dropped to 70-80% at which point the cultures were harvested by centrifuging at 1000 x g for 15 minutes to spin down cell and cell debris. The supernatant from the cultures was collected and stored at 4°C to be used in the coinfection experiments.

## 3.3 Quantification of Infectious Baculovirus

Replication capable virus titers were determined by end point dilution assay as described by Reed and Muench (1938) and O'Reilly et al., (1994). Briefly each well of 96-well plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were seeded with 100 $\mu$L of exponentially growing Sf9 cells or GFP inducing Sf9 cells (GFP-Sf9, provided by a PhD student at the Aucoin Lab) diluted to 2 x $10^5$ cells/ml with Sf-900 III media and allowed to attach for 1 hour. Serial dilution of virus stocks were prepared, ranging from

$10^{-2}$ to $10^{-10}$, using Sf-900 III media. 10 $\mu$L of each virus dilution was added to each well of a row starting with $10^{-4}$ dilution, thus resulting in 12 replicates per dilution. The plates were incubated at 27°C for 5-7 days in a sealed box with a wet paper towel to prevent the plates from drying out. After the incubation period, the plates were observed under a fluorescence microscope and the wells were scored as infected or uninfected based on the fluorescence of the wells. Each plate was analyzed twice using two filters, green light for red fluorescence and blue light for green fluorescence, to determine the titer of both p10GFP and p10RFP. For the p6.9GAG-GFP/p6.9HA virus stocks, GFP inducing Sf9 cell line was used to detect the combined titer of the stocks. A detailed calculation of the titers can be found in **Appendix A**. The titers obtained from this assay were used to calculate the amount of virus stock to be added to cell culture to perform an infection at a particular MOI.

$$virus\ amount\ (ml) = \frac{MOI \times number\ of\ cells}{titer\ of\ virus}$$

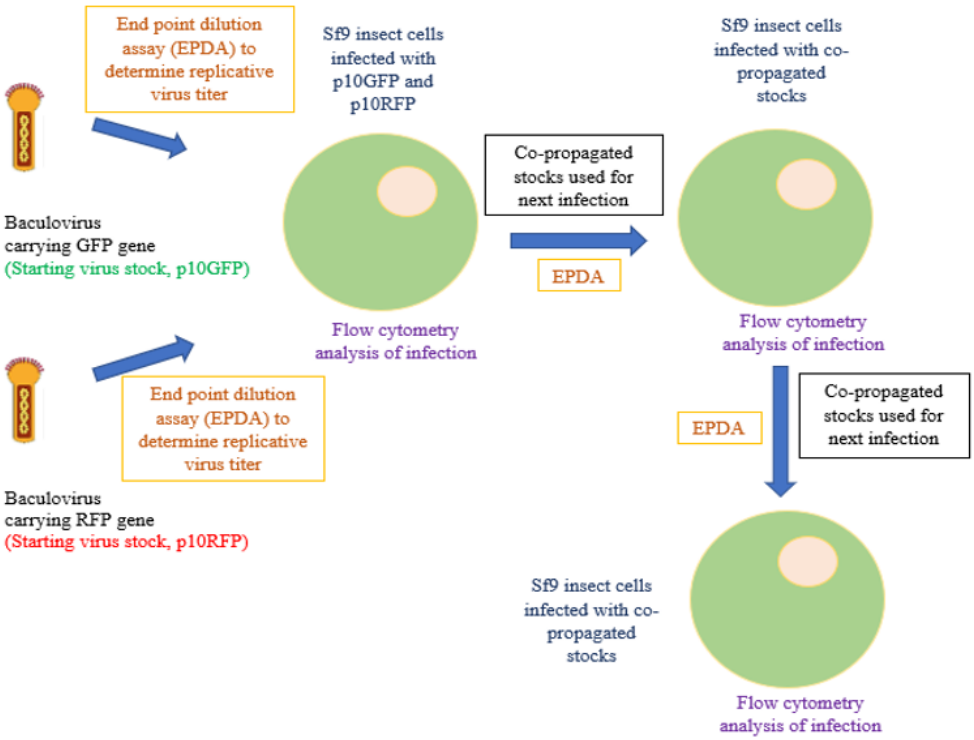


Figure 3.1: **An overview of the amplification process.**

## 3.4 Design of Dual Stock (Coinfection) Experiments

Sf9 cell cultures at a density of 2 x $10^6$ cells/ml in Sf-900 III media with a working volume of 35 ml were coinfected, in duplicates, with P3 stocks of p10GFP (recombinant baculovirus expressing eGFP under the very late p10 promoter) and p10RFP (recombinant baculovirus expressing mKate2 under

the very late p10 promoter) at a total MOI of 10 (high MOI) or 0.1 (low MOI). In addition, 2 x $10^6$ Sf9 cells/ml in Sf-900 III media were coinfected, in duplicates, with P3 stocks of p6.9GAG-GFP (recombinant baculovirus expressing GAG-GFP under the late promoter p6.9) and p6.9HA (recombinant baculovirus expressing HA under the late promoter p6.9) at a total MOI of 0.1. The different MOI combinations used are summarized in **Tables 3.1, 3.2 and 3.3**. Samples were taken at 0, 24, 36, 48, 72 and 84/96 hours post-infection (hpi).

| **MOI Ratio** | **MOI p10GFP** | **MOI p10RFP** | **Total MOI** |
|---|---|---|---|
| **10:0** | 10 | 0 | 10 |
| **7:3** | 7 | 3 | 10 |
| **5:5** | 5 | 5 | 10 |
| **3:7** | 3 | 7 | 10 |
| **0:10** | 0 | 10 | 10 |

Table 3.1: Different MOI combinations used in dual stock experiment at a total MOI of 10. MOI Ratio refers to the ratio of MOI of p10GFP to MOI of p10RFP.

Cell density and viability were determined at each of these time points using a Countess II FL Automated Cell Counter (Life Technologies, Carlsbad, CA, USA). Samples were centrifuged at 300 x g for 10 minutes, the supernatant was discarded and cell pellets were resuspended in 2% paraformaldehyde solution in phosphate buffered saline (PBS) and kept at 4°C for approximately 1 hour before dilution with 1x PBS and analysis by flow cytometry. The controls used were uninfected cultures seeded at the same density as

the infected cultures and infections with P3 stocks of p10GFP, p10RFP, p6.9GAG-GFP or p6.9HA. Supernatants containing co-propagated viruses (p10GFP and p10RFP) were collected from each culture condition (MOI Ratio) around 70-80% viability as described in **Section 3.2** and stored at 4°C to be used in the first set of co-propagated single infections. For infections with p6.9GAG-GFP and p6.9HA, after harvesting the cultures, the cell pellets were stored at -80°C, while the supernatants were stored at 4°C with half of it to be used as virus stocks in the first round of co-propagated single infections and the other half was filtered using a 0.2 $\mu$m membrane filter (VWR International, Mississauga, ON, Canada) and kept at 4°C for further analysis.

| **MOI Ratio** | **MOI p10GFP** | **MOI p10RFP** | **Total MOI** |
|---|---|---|---|
| **0.1:0** | 0.01 | 0 | 0.01 |
| **0.07:0.03** | 0.07 | 0.03 | 0.01 |
| **0.05:0.05** | 0.05 | 0.05 | 0.01 |
| **0.03:0.07** | 0.03 | 0.07 | 0.01 |
| **0:0.01** | 0 | 0.01 | 0.01 |

Table 3.2: Different MOI combinations used in dual stock experiment at a total MOI of 0.1. MOI Ratio refers to the ratio of MOI of p10GFP to MOI of p10RFP.

| MOI Ratio | MOI p6.9GAG-GFP | MOI p6.9HA | Total MOI |
|---|---|---|---|
| **0.1:0** | 0.01 | 0 | 0.01 |
| **0.07:0.03** | 0.07 | 0.03 | 0.01 |
| **0.05:0.05** | 0.05 | 0.05 | 0.01 |
| **0.03:0.07** | 0.03 | 0.07 | 0.01 |
| **0:0.01** | 0 | 0.01 | 0.01 |

Table 3.3: Different MOI combinations used in dual stock experiment at a total MOI of 0.1. MOI Ratio refers to the ratio of MOI of p6.9GAG-GFP to MOI of p6.9HA.

## 3.5 Design of Single Stock Experiments

Sf9 insect cells seeded at 2 x $10^6$ cells/ml in Sf-900 III media with 35 ml working volume were infected with P4 co-propagated stocks obtained from the previous dual stock (coinfection) experiment at a total MOI of 10 or 0.1. Sampling was done at 0, 24, 36, 48, 72 and 84/96 hpi.

Cell density and viability were determined using hemacytometer and trypan blue dye exclusion method. Samples were prepared as described in **Section 3.4** to be assessed by flow cytometry. The controls used were uninfected cultures at the same density and infections with only P4 p10GFP, p10RFP, p6.9GAG-GFP or p6.9HA virus stocks as obtained from the previous single infections. While, the supernatants obtained from this round of p10GFP/p10RFP co-propagated single infections when the viability dropped to around 70-80% were collected and stored at 4°C for future experiments, the supernatants from p6.9GAG-GFP/p6.9HA co-propagated single infec-

tions were stored at 4°C with half of it to be used as the next co-propagated stocks in the second round of co-propagated single infections and the other half was filtered using a 0.2 $\mu$m membrane filter and kept at 4°C for further analysis. Moreover, the cell pellets were stored at -80°C for determining the HA activity.

## 3.6 Flow Cytometry Analysis

A BD FACSCalibur (BD Biosciences, San Jose, CA, USA) was used for flow cytometry analysis at each of the time points. Green fluorescence was detected using the FL1 detector, whereas, the red fluorescence was detected by the FL3 detector. Samples were run using a medium flow rate of 35 $\mu$L/min and 10,000 events (each cell is considered an event) were acquired for each sample. The amplification mode was set to linear for FSC and SSC, and to logarithmic for FL1 and FL3. The results were acquired using CellQuest Pro (BD Biosciences, Missisauga, ON, Canada) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA) and the R programming language.

## 3.7 Sucrose Cushion Ultracentrifugation

The filtered supernatants obtained from each p6.9GAG-GFP/p6.9HA culture condition when the viability dropped between 70-80% were concentrated by ultracentrifugation using a 25% sucrose cushion to remove low density par-

ticles and semi-purify the VLPs. 25% sucrose solution was prepared by dissolving sucrose in chilled 1x NTC buffer (1 M NaCl, 0.2 M Tris-HCl pH 7.4, 50 mM CaCl2). Samples were layered over 1.5 ml of the prepared sucrose cushion and centrifuged using a Beckman Coulter Optima XPN-100 ultracentrifuge (Beckman Coulter, Mississauga, ON, Canada) with SW41 Ti rotor (Beckman Coulter, Mississauga, ON, Canada) at 26,000 rpm for 1.5 hours at 4°C. Following the ultracentrifugation, the supernatant was discarded carefully and the pelleted virions were resuspended in 2 ml of chilled 1x NTC buffer. The samples were then shaken at 200 rpm on ice for 20 minutes before being further purified by a density gradient.

## 3.8 Iodixanol Density Gradient Ultracentrifugation

Iodixanol gradients were prepared in 14 x 89 mm polyallomer centrifuge tubes (Beckman Coulter, Mississauga, ON, Canada). Each gradient had six steps comprising of 60%, 50%, 40%, 30%, 20% and 10% iodixanol in 1x NTC buffer. Using a pipette, 1.5 ml of each step was layered slowly taking precautions not to mix the layers. 2 ml of the resuspened virion obtained by sucrose cushioning was then layered slowly on top of the final 10% step, followed by a layer of 1ml 1x NTC buffer. Centrifugation was done using Beckman Coulter Optima XPN-100 ultracentrifuge (Beckman Coulter, Mississauga, ON, Canada) with SW41 Ti rotor (Beckman Coulter, Mississauga,

ON, Canada) at 35,000 rpm for 2.5 hours at 4°C. Following iodixanol density gradient ultracentrifugation, 1 ml fractions (the fraction obtained from the top was considered Fraction 1) were collected by puncturing the side of each centrifuge tube. The weight of the fractions were determined and the densities were calculated before storing them at 4°C for further analysis.

## 3.9 Hemagglutination Assay

Influenza VLPs have an envelope protein called hemagglutinin (HA) that is capable of binding to sialic acid receptors on cells as well as to erythrocytes (red blood cells) thus resulting in the formation of a lattice. This phenomenon, called hemagglutination, gives an indirect indication of the presence of influenza virus. The bulk supernatants and cell lysates obtained at 72, 84 or 96 hpi (depending on the time point at which the culture reached 70-80% viability), in addition to the purified fractions from density gradient ultracentrifugation, were checked for HA activity by Hemagglutination assay (HA assay). In particular, 50 $\mu$L of 1x PBS was added to each well (except the first well of each row) of 96-well round-bottom plates. The last row of each plate was used as a negative control with only 1x PBS in all the wells and 100 $\mu$L of a virus sample was added to the first well of a row. The virus sample was serially diluted 1:2 (two-fold dilution) across the row with each row comprising of a different virus having the same dilution pattern. 50 $\mu$L of 0.5% chicken red blood cells (RBCs) (Rockland Immunochemicals Inc.

Limerick, PA, USA) in 1x PBS was added to all the wells of the plates. After addition of RBCs, the plates were incubated at room temperature overnight. Following the incubation period, the wells were examined for signs of hemagglutination. Formation of lattice or coating of the well by RBCs attached to virus particles marked the presence of hemagglutination, while the lack of it was an evidence that there were no virus particles containing HA to which RBCs could attach and the blood settled to the bottom of the well as a button or pellet. The HA titer of a virus stock was indicated as the hemagglutination units (HAU)/50 $\mu$L and was equivalent to the reciprocal of the highest dilution upto which complete hemagglutination was observed. The HAU/ml of a sample was obtained by multiplying the HAU/50 $\mu$L by 20.

## 3.10 Baculovirus Quantification by Flow Cytometry (SYBR Green Staining)

The baculovirus quantification method was adapted from the paper "Quantification of Baculovirus Particles by Flow Cytometry" (Shen et al., 2002). Briefly, virus stocks were serially diluted to $10^{-2}$ and $10^{-3}$ in sterile PBS. The virus samples were then fixed in 2% paraformaldehyde at 4°C for 1 hour to get a final concentration of 0.02%. This was followed by a freeze-thaw cycle comprising of freezing the samples at -80°C for 30 minutes and thawing them for around 5 to 10 minutes in a water bath at 27°C. The fixed and

frozen-thawed samples were subjected to permeabilization with 10% Triton X-100 for 5 minutes to obtain a final concentration of 0.01%. The permeabilization step was succeeded by staining with SYBR Green I Nucleic Acid Gel Stain 10000x (Thermo Fisher, Mississauga, ON, Canada) diluted to 5 x $10^{-3}$. The SYBR Green I stained samples were eventually transferred to a 20 well VWR Digital Dry Block Heater (VWR International, Mississauga, ON, Canada) and incubated in the dark for around 6 minutes at 80°C. This was followed by a cooling step in ice before transferring the samples to 5 ml tubes (VWR International, Radnor, PA, USA) for flow cytometry analysis.

A BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with 15 mW, 488 nm argon-ion laser was used for analysis. Green fluoroscence of the baculovirus particles was detected using a 530 nm band-pass filter. The discriminator was set on the green fluorescence (FL1) and the volatge of photomultiplier was regulated to distinguish between the green fluorescence from the viral particles and the background. All samples were run three times for 30 seconds at a medium flow rate of 35 $\mu$L/min. The data obtained was processed using FlowJo software (Tree Star, Ashland, OR, USA) and the R programming language. For calibration purposes 3 $\mu$m FlowSet fluorospheres (Beckman Coulter, Mississauga, ON, Canada) with a concentration of 1 x $10^6$ fluorospheres/ml were used. A detailed flow settings and calculations used for quantification can be found in **Appendix C**.

# Chapter 4

# Repeated propagation of two baculovirus vectors expressing GFP or RFP simultaneously in cell culture at high or low MOI

## 4.1 Introduction

The use of the insect cell-baculovirus expression vector system (IC-BEVS) as a production platform has increased in the past few decades. The BEVS is widely used for the production of proteins and protein complexes both industrially and at laboratory scale. One of the crucial questions that form the foundation of production is the infection strategy to be used. The pros and cons of the different infection strategies have been debated by many researchers. While few groups have brought forward the importance of co-expression, others have proved the requirement for coinfection. From an industrial point of view, coexpression is always preferred as it reduces the virus burden and the different possible product combinations upon infection. One of the main drawbacks associated with coexpression though is the lack of control on the ratios of different proteins being expressed by a virus to make a particular product. Infection of insect cells using multiple baculoviruses (coinfection) has been exploited for production of higher order protein and protein complexes (Aucoin et al., 2006; Pushko et al., 2005). Although the choice of infection strategy may seem arbitrary, it has a notable impact on product development (Aucoin et al., 2006). When the formation of a product requires multiple genes to be expressed in different proportions, using one (or more) polycistronic baculovirus carrying the genes for all proteins of interest may not be fruitful. Also, the level of expression of each protein of interest may vary and not all the genes needs to be expressed at the same level

(George, 2016). In addition, large baculovirus constructs containing multiple genes of interest maybe unstable (Zhai et al., 2019), thus not helpful for scale-up. The benefit of using coinfection at a small scale is the additional degree of freedom to tailor virus ratios by altering the MOI (multiplicity of infection) of each virus construct. A lot of work has been done on the propagation of viruses at high and low MOI, and the accumulation of defective interfering particles in culture medium (Kool et al., 1991; Gotoh et al., 2002; Aucoin et al., 2006), but not much preference has been given to the possibility of maintaining the ratios during the simultaneous amplification process of multiple viruses.

The objective of this work was to take advantage of coinfection to explore different ratios of two virus constructs and track the virus populations over repeated passaging. For the purpose of this project two baculovirus constructs expressing an easily traceable reporter protein, in particular eGFP (green fluorescent protein, GFP) or mKate2 (red fluorescent protein, RFP), were amplified together to follow the population distribution of the resulting co-propagated stocks upon subsequent amplifications. The resultant virus stocks, hereby known as single or co-propagated stock(s), were used for successive infections. Following the coinfection experiment (P3, passage number of starting stocks) two sets of co-propagated single infection experiments were run. Two different total MOIs were selected for infection: high MOI of 10 and low MOI of 0.1, and the entire set of one coinfection and two co-propagated single infections were run for each chosen total MOI. The aim

was to see if the virus population/ratio changes or if it is maintained over repeated amplification so that coinfection can be avoided at industrial scale.

## 4.2 Results and Discussion

From literature we know that baculovirus infection in cell culture follows a Poisson distribution. According to Poisson distribution, upon baculovirus infection each cell can receive one, multiple or no virus vector. In order for each cell to be infected with at least one virus, cell cultures must be infected at a MOI $> 3$, thus resulting in synchronous infection. Infections at a MOI $<< 1$ leads to asynchronous infection, where all the cells are not infected simultaneously. In this work we wanted to see the population distribution upon repeated propagation at high ($> 3$) or low ($<< 1$) MOI.

A schematic of coinfection of Sf9 cells with the two different virus vectors, p10GFP and p10RFP, and the techniques used to track the population distribution upon infection is shown in **Figure 4.1**. Following this, the budded virus comprising of both p10GFP and p10RFP was used for subsequent amplification, as can be seen in **Figure 3.1**, and the same techniques were used for the analysis.

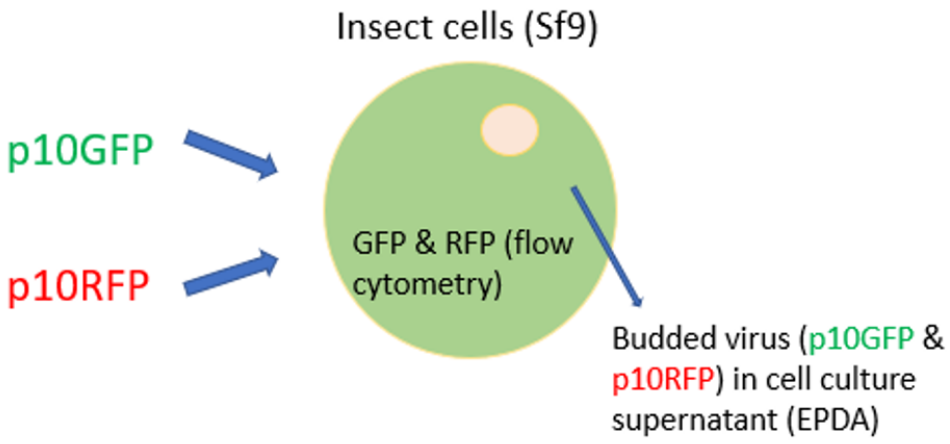


Figure 4.1: **Schematic of the p10GFP and p10RFP infection analysis techniques.**
GFP and RFP produced inside the cell were tracked by flow cytometry. The budded virus (p10GFP and p10RFP) in the cell culture supernatant, at the end of the infection cycle was determined by EPDA.

### 4.2.1 High MOI

It is known from literature that although high MOI is beneficial for protein production and synchronous infection, for virus amplification it is not deemed ideal. Despite the fact that high MOIs are known to cause accumulation of defective interfering particles in cell cultures, we sought to investigate how the population of two different viruses change via the expression of reporter proteins (GFP and RFP) when (a) cells are coinfected; and (b) cells are infected with virus resulting from a co-propagated culture. After investigating different high MOIs (5, 10 and 20), we saw that although there is an im-

provement from MOI 5 to 10, there is not much difference between MOIs 10 and 20. Thus, we can say that after increasing the MOI to a certain level, it reaches a saturation point and only increases the virus load in the cultures. This led us to use a high MOI of 10 for the synchronous infections. The different MOI combinations of the two virus vectors in the coinfection experiments were selected keeping in mind that we achieve synchronous infection even at the individual infection level.

#### 4.2.1.1 Flow Cytometry Analysis of Infection

The two easily traceable reporter proteins eGFP (GFP) and mKate2 (RFP) used in this work were tracked by flow cytometry. As the infection progressed three distinct populations of cells corresponding to two singly infected populations with either p10GFP or p10RFP and a coinfected population were observed **(Figure 4.2)**. These three populations were gated manually using the FlowJo software, based on the single infections with only p10GFP or only p10RFP.

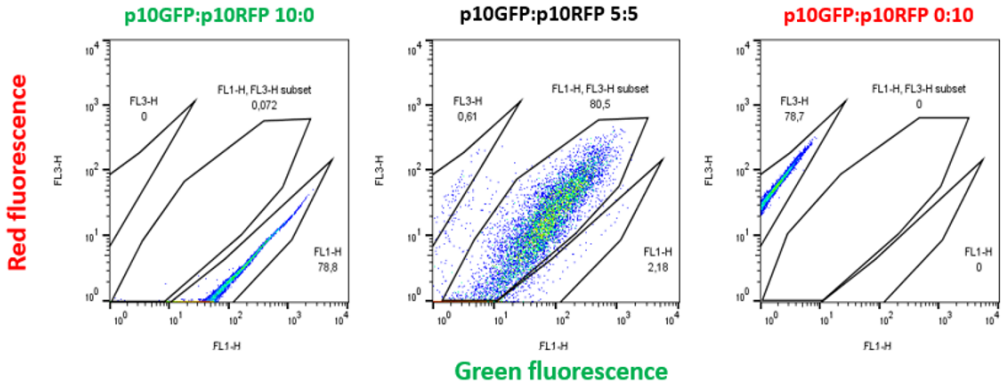


Figure 4.2: **A representation of flow cytometer scatter plots showing distinct populations of p10GFP+p10RFP coinfected Sf9 cells as compared to single infected (with either p10GFP or p10RFP) Sf9 cells at 48 hpi at a total MOI of 10.**
FL3 corresponds to red and FL1 to green fluorescence. p10GFP:p10RFP is the ratio of the two viruses used in the coinfection experiment. A certain percentage of low fluorescent events are at the edge of the chart, which are not considered for analysis, thus resulting in the total infected populations to be less than 100%.

If we look at the distribution of populations with repeated passaging, there is a drop in the percentage of the coinfected population for all the different culture conditions. The maximum variation in population distribution from P3 to P4 was observed for the cell cultures infected with equal concentrations of both the viruses (MOI p10GFP:p10RFP 5:5), while the minimum variation was seen in the cultures infected with higher concentration of p10GFP (MOI p10GFP:p10RFP 7:3). However, the population distribution between the two co-propagated single infections (P4 and P5) varied the most for the higher p10GFP infections. Also, it is to be noted

that the coinfected population shifted towards the lower fluorescence levels for all the cultures with repeated passaging (**Figure 4.3**).

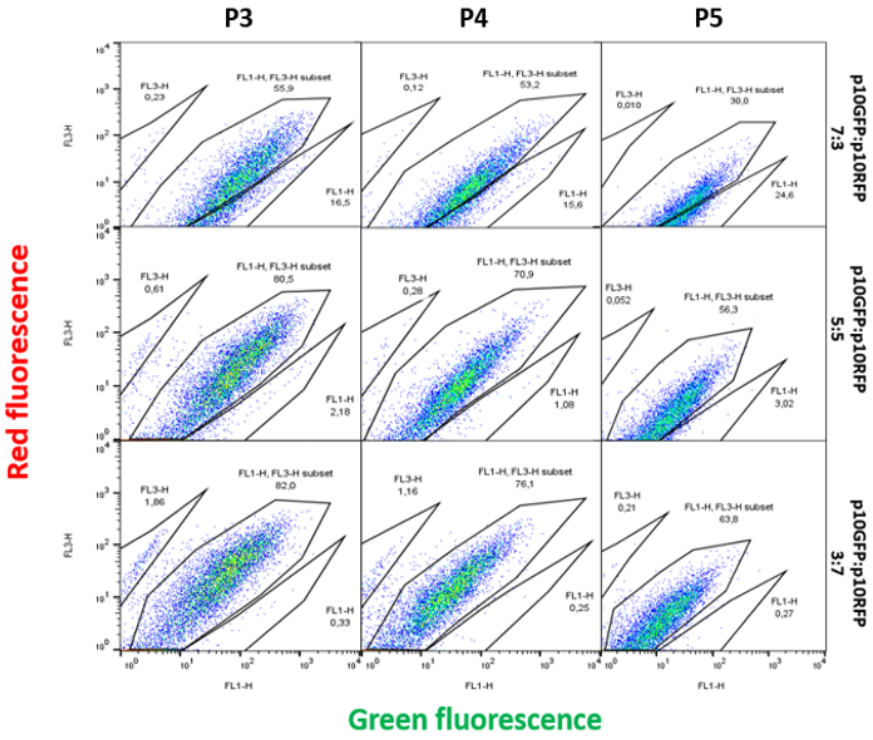


Figure 4.3: **A representation of flow cytometer scatter plots showing distinct populations of p10GFP+p10RFP coinfected Sf9 cells at 48 hpi at a total MOI of 10.**
FL3 corresponds to red and FL1 to green fluorescence. P3, P4 and P5 refer to the passage number of virus stocks used in the coinfection, and the first and second co-propagated single infection experiments, respectively. p10GFP:p10RFP is the MOI ratio of the two viruses used in the coinfection experiment. The first, second and third panel (top to bottom) represents the population distribution of only p10RFP, p10RFP+p10GFP and only p10GFP infected population for the MOI ratios of p10GFP:p10RFP 7:3, 5:5 and 3:7, respectively. A certain percentage of low fluorescent events are at the edge of the chart, which are not considered for analysis, thus resulting in the total infected populations to be less than 100%.

The different cell populations became more prominent around 48 hpi and were used for further analysis. The percentage of GFP+RFP, GFP and RFP positive cells upon infection and the intensity of fluorescence expressed by these cells are based on the three selected populations.

The single infected red population (Percent.FL3, p10GFP:p10RFP 0:10) decreased by 50%, whereas the single infected green population (Percent.FL1, p10GFP:p10RFP 10:0) decreased by 22% from P3 to P5. For the coinfected populations (Percent.co) the percentage of cells expressing fluorescence was higher for the conditions having higher (p10GFP:p10RFP 3:7) or equal (p10GFP:p10RFP 5:5) p10RFP. There was a 12-30% decrease in all the coinfected population from P3 to P5 (**Figure 4.4**). Due to the sensitivity of flow cytometer (overlapping) we did not see similar percentages for p10GFP:p10RFP 7:3 and 3:7 although they were normalized to have similar outputs.

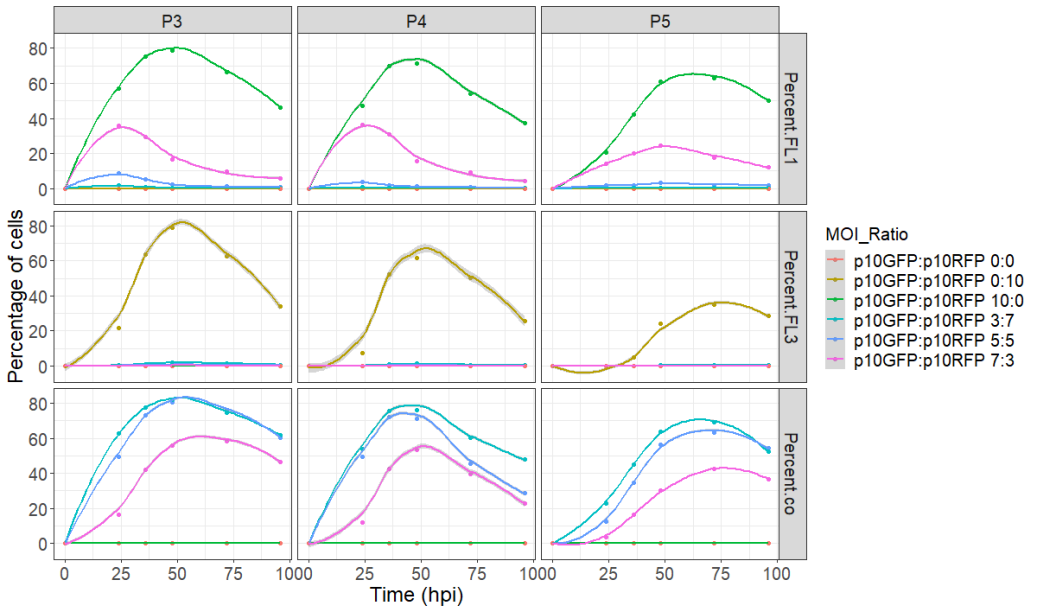


Figure 4.4: **Percentage of cells expressing fluorescence at different time points when infected at a high MOI.**
P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p10GFP and p10RFP used in the coinfection experiment, while P4 and P5 are the virus stocks of p10GFP+p10RFP used in the subsequent co-propagated single infection experiments. Percent.FL1 corresponds to the percentage of cells expressing green fluorescence, Percent.FL3 is the percentage of cells expressing red fluorescence and Percent.co is the population of cells expressing both green and red fluorescence. The data points shown here are an average of values for duplicate flasks.

The decrease in green fluorescence is more prominent for the single infection (Mean.FL1, p10GFP:p10RFP 10:0), while for the red fluorescence (Mean.FL3), the drop is more for the higher p10GFP (p10GFP:p10RFP 7:3) and only p10RFP (p10GFP:p10RFP 0:10) infections. The intensity of coinfected population decreases 1.3-1.6 times from P3 to P4 and 1.1 to 1.5 times from P4 and P5 (**Figure 4.5**).

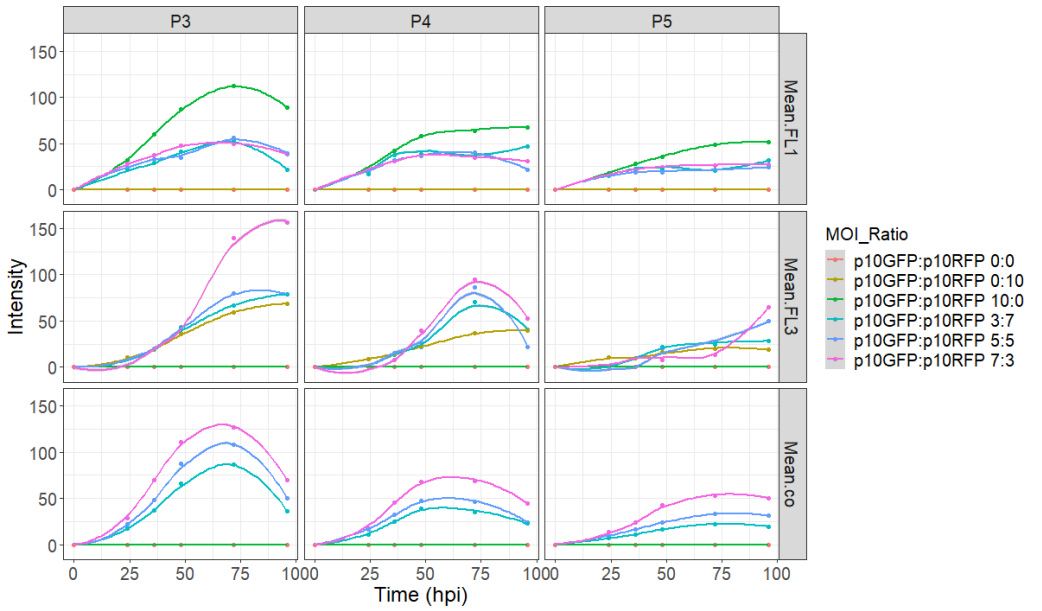


Figure 4.5: **Intensity of fluorescence at different time points post infection at high MOI.**
P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p10GFP and p10RFP used in the coinfection experiment, while P4 and P5 are the virus stocks of p10GFP+p10RFP used in the subsequent co-propagated single infection experiments. Mean.FL1 corresponds to the green fluorescence, Mean.FL3 is the red fluorescence and Mean.co is the combined fluorescence expressed by the coinfected population. The data points shown here are an average of values for duplicate flasks.

#### 4.2.1.2 Titer of Virus Stocks

P3 stocks of p10GFP and p10RFP amplified from P2 stocks in Sf9 cell cultures at an MOI of 0.1 had high titers in the order of $10^8$. With repeated passage at a high MOI of 10 the titers dropped from approximately 6-7 x $10^8$ for P3 to 1-3 x $10^7$ for P6 (**Figure 4.6**). This is in line with literature, which represents that the use of high MOI for virus amplification is not ideal.

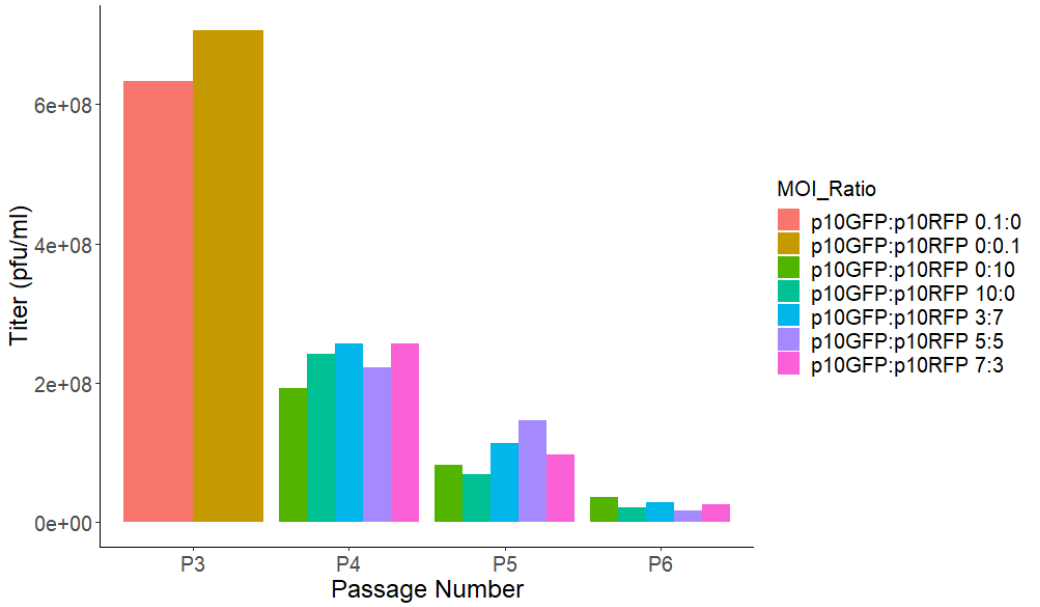


Figure 4.6: **Titer of baculovirus in cell culture supernatant at 70-80% viability as obtained by end point dilution assay (amplification at high MOI).**
P3, P4, P5 and P6 refer to the passage number of virus stocks. P3 being the virus stocks of p10GFP and p10RFP used in the coinfection experiment, while P4, P5 and P6 are the subsequent co-propagated stocks of p10GFP+p10RFP. The titers plotted are an average of duplicates.

### 4.2.2 Low MOI

From literature it is known that a low MOI is preferred for virus amplification. Infecting cell cultures with a low MOI results in asynchronous infection where cell growth persists post infection. It is also known that a low MOI can reduce the passage effect, which is highly undesirable during repeated amplification. Thus, we wanted to inspect how two virus populations change by expressing easily traceable reporter proteins, when (a) cells are coinfected;

and (b) cells are infected with the resultant co-propagated viruses, at a low MOI. In order to achieve asynchronous infection (MOI << 1), total MOI of 0.1 is selected, which is a 100 fold drop from the high MOI of 10. The different MOI combinations of p10GFP and p10RFP in the coinfection experiments, also dropped by 100 fold from the high MOI ones to maintain consistency.

#### 4.2.2.1 Flow Cytometry Analysis of Infection

Three cell populations corresponding to two single populations infected with either p10GFP or p10RFP, and a coinfected population were observed with the advancement of infection **(Figure 4.7)**. Similar to the high MOI infections, the three distinct populations observed here, were gated manually using the FlowJo software, based on the single infections with only p10GFP or only p10RFP.

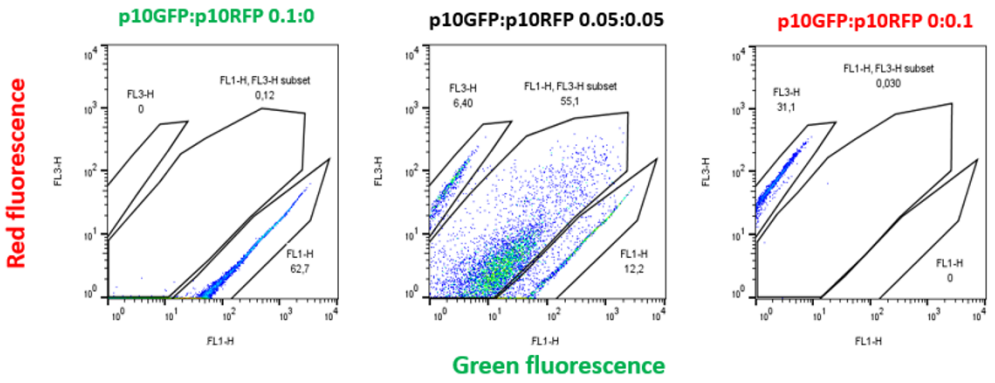


Figure 4.7: **A representation of flow cytometer scatter plots showing distinct populations of Sf9 cells coinfected with p10GFP and p10RFP in equal ratios, as compared to single infected (with p10GFP or p10RFP) Sf9 cells at 48 hpi at a total MOI of 0.1.** FL3 corresponds to red and FL1 to green fluorescence. p10GFP:p10RFP is the ratio of the two viruses used in the coinfection experiment. A certain percentage of low fluorescent events are at the edge of the chart, which are not considered for analysis, thus resulting in the total infected populations to be less than 100%.

As compared to the distinct populations observed for high MOI infections, the percentage of a single population expressing cells has increased and the percentage of the coinfected population has dropped for low MOI infections (**Figure 4.9**). These populations became more prominent around 48 hpi and used for further analysis. The maximum coinfected population for each passage is observed for the higher p10RFP infections (p10GFP:p10RFP 0.03:0.07) at 48 hpi. The distribution of the coinfected population did not vary much between the coinfection and the subsequent co-propagated single infection for all the coinfected conditions as can be seen in **Figure 4.8**. With

progress in the infection cycle, the coinfected population shifted more towards the red or green fluorescence depending on the condition of the coinfected cultures.

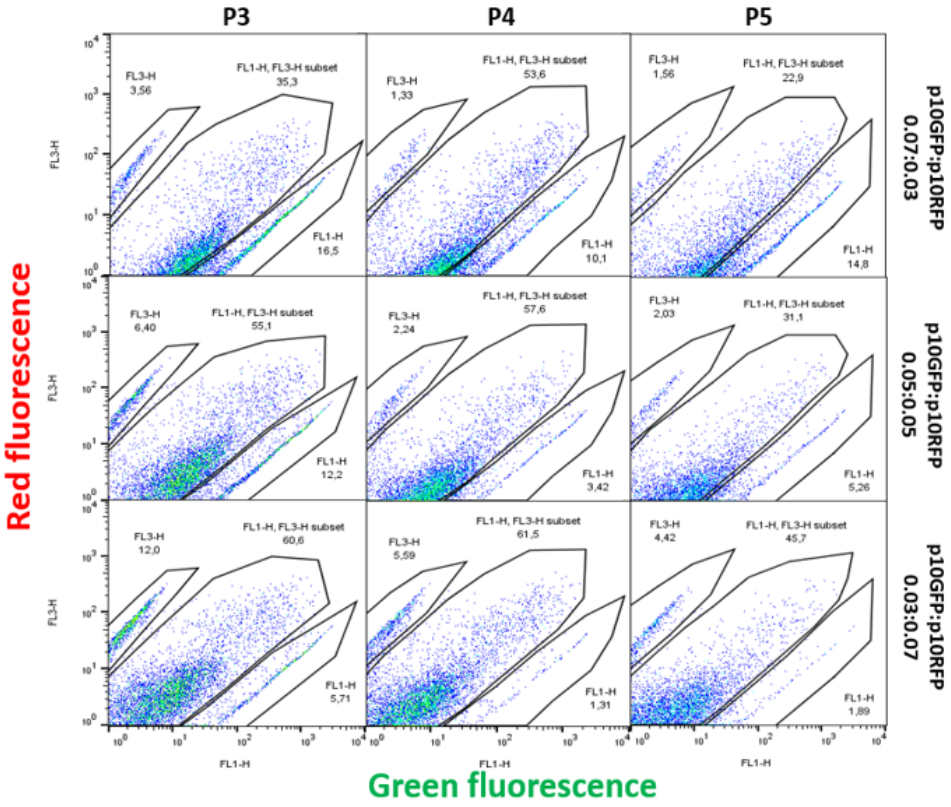


Figure 4.8: **A representation of flow cytometer scatter plots showing distinct populations of coinfected Sf9 cells at 48 hpi at a total MOI of 0.1.**
FL3 corresponds to red and FL1 to green fluorescence. P3, P4 and P5 refer to the passage number of virus stocks used in the coinfection, and the first and second co-propagated single infection experiments, respectively. p10GFP:p10RFP is the MOI ratio of the two viruses used in the coinfection experiment. p10GFP:p10RFP refer to the MOI ratio of the two viruses used in the coinfection experiment. The first, second and third panel (top to bottom) represents the population distribution of only p10RFP, p10RFP+p10GFP and only p10GFP infected population for the MOI ratios of p10GFP:p10RFP 0.07:0.03, 0.05:0.05 and 0.03:0.07, respectively. A certain percentage of low fluorescent events are at the edge of the chart, which are not considered for analysis, thus resulting in the total infected populations to be less than 100%.

The single infected red population (Percent.FL3, p10GFP:p10RFP 0:0.1) exhibited a decrease of around 2.44 times from the coinfection experiment (P3) to the first round of co-propagated single infections (P4) and an increase of 1.04 times from P4 to P5 (second round of co-propagated single infections), whereas the single infected green population (Percent.FL1, p10GFP:p10RFP 0.1:0) showed an almost 1.85 times decrease from P3 to P4 and a 1.72 times increase from P4 to P5. For the coinfected populations (Percent.co) the percentage of cells expressing fluorescence is higher for the conditions having higher (p10GFP:p10RFP 0.03:0.07) or equal (p10GFP:p10RFP 0.05:0.05) p10RFP. There is a 1.02 times increase in the coinfected population from P3 to P4 and a 1.09-1.48 times drop from P4 to P5. Due to the sensitivity of the flow cytometer and overlapping nature of the green and red fluorescence we don't see similar percentages for p10GFP:p10RFP 0.07:0.03 and 0.03:0.07 even after normalizing the conditions.

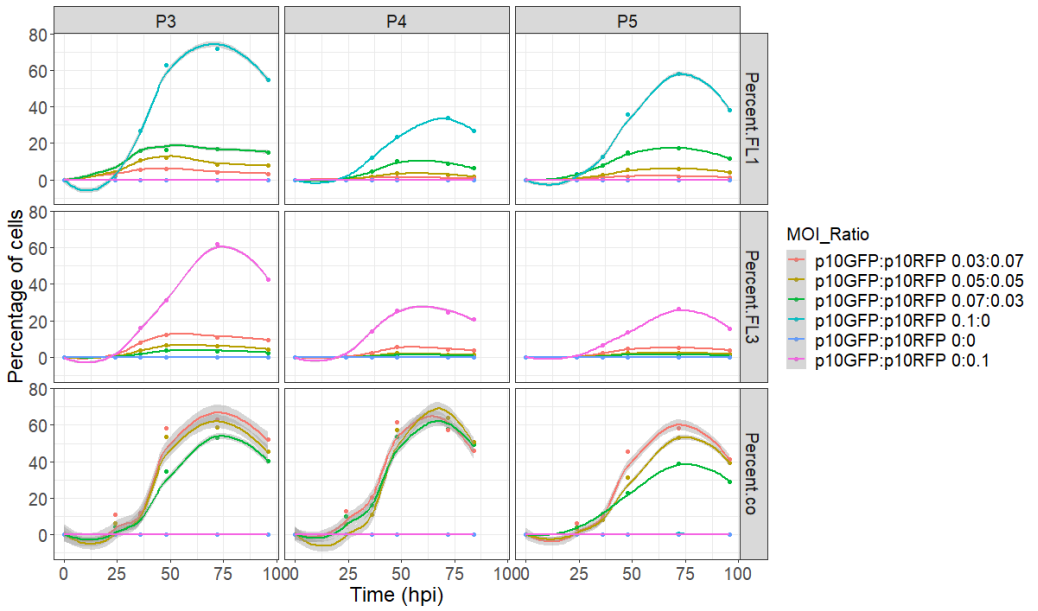


Figure 4.9: **Percentage of cells expressing fluorescence at different time points when infected at low MOI.**
P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p10GFP and p10RFP used in the coinfection experiment, while P4 and P5 are the virus stocks of p10GFP+p10RFP used in the first and second round of co-propagated single infection experiments respectively. Percent.FL1 corresponds to the percentage of cells expressing green fluorescence, Percent.FL3 is the percentage of cells expressing red fluorescence and Percent.co is the population of cells expressing both green and red fluorescence. The values plotted here are an average of values for duplicate flasks.

The decrease in green fluorescence (Mean.FL1) is more prominent from the first round of co-propagated single infections (P4) to the second round of co-propagated single infections (P5) with the highest drop of 2.05 times for the condition p10GFP:p10RFP 0.03:0.07, while for the red fluorescence (Mean.FL3) there is an increase of 1.31 times from P3 (coinfection experi-

ment) to P4 and a drop of 1.61 times from P4 to P5 (**Figure 4.10**). The intensity of coinfected population shows a significant decrease of around 3.82 times from P3 to P4 and 1.17 times from P4 to P5.

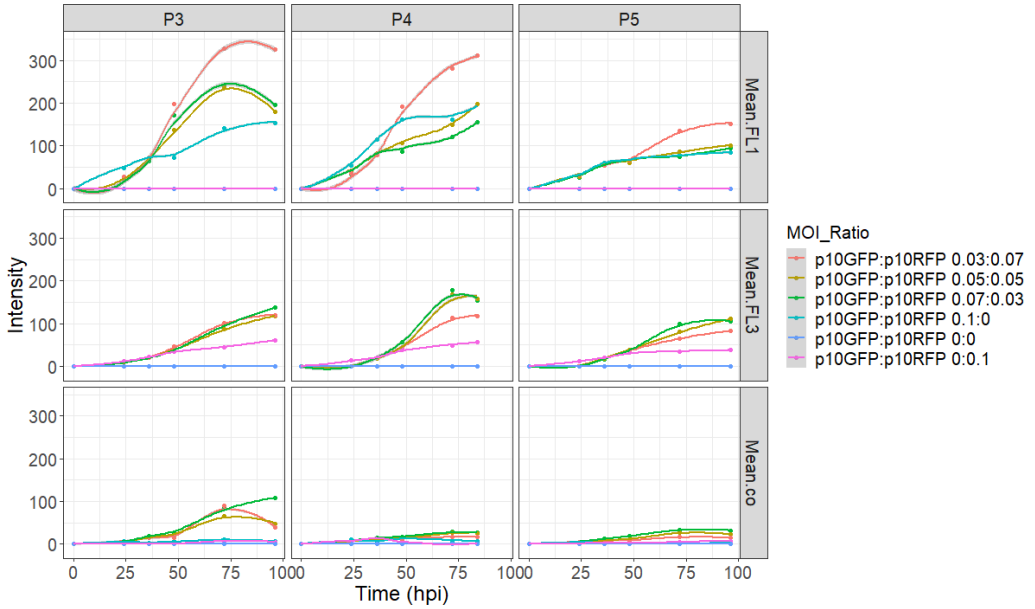


Figure 4.10: **Intensity of fluorescence at different time points post infection at low MOI.**
P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p10GFP and p10RFP used in initial dual stock experiment, while P4 and P5 are the virus stocks of p10GFP+p10RFP used in co-propagated single infection experiments. Mean.FL1 corresponds to the green fluorescence, Mean.FL3 is the red fluorescence and Mean.co is the combined fluorescence expressed by the coinfected population. The values plotted here are an average of values for duplicate flasks.

#### 4.2.2.2 Titer of Virus Stocks

P3 stocks of p10GFP and p10RFP amplified from P2 stocks in Sf9 cell cultures at an MOI of 0.1 had titers in the order of $10^8$. With repeated passage

at a low MOI of 0.1, the titers did not drop drastically from P3 to P5 (**Figure 4.11**). Additionally, there is an increase in the titer of the P4 co-propagated stocks. The minimal change in titers over subsequent passaging is indicative of the benefit of using low MOI for virus amplification, as known from literature.

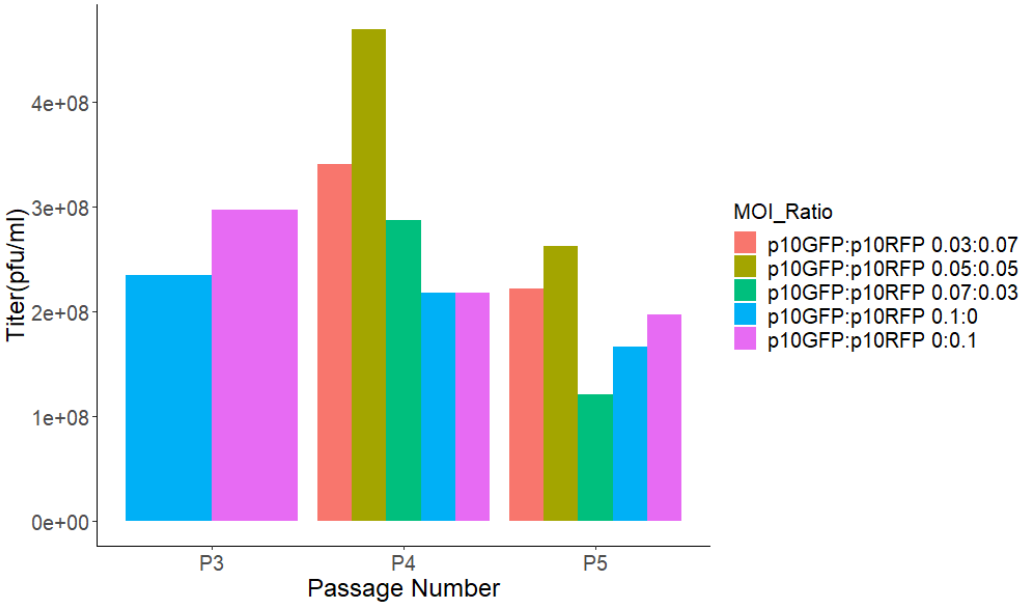


Figure 4.11: **Titer of baculovirus in cell culture supernatant at 70-80% viability as obtained by end point dilution assay (amplification at low MOI).**
P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p10GFP and p10RFP used in the coinfection experiment, while P4 and P5 are the subsequent single stocks of p10GFP+p10RFP. The titers plotted are an average of duplicates.

## 4.3 Conclusions

Coinfection is a necessary strategy as large constructs with multiple genes maybe unstable and thus not favourable for process scale-up. In addition, coinfection also comes with the advantage of controlling the ratios of different viruses to be used for product formation. The main aim of this work was to track the distribution of two virus populations over repeated passaging by co-amplifying them at high and low MOIs, and then using the virus resulting from the coinfected cultures for succeeding infections, such that the use of coinfection can be eliminated at larger scale.

Although high MOI is known to increase the accumulation of defective interfering particles (DIPs), known as the passage effect, the aim was to see if the virus distribution or ratio changes, or is maintained with repeated passages. The titer of virus stocks kept decreasing over passages when a high MOI of 10 was used. The titer dropped from higher $10^8$ order to lower $10^7$ order for P3 stocks (used for the coinfection experiment) and P6 stocks (single stocks obtained from the second round of co-propagated single infections). Along with the huge reduction in titer over passage number the drop in viability of cells post infection got delayed by 24 hours for the second round of co-propagated single infections when MOI of 10 was used **(Appendix D, Figure D:1)**. In addition, there were changes in the population distribution, as well as, reduction in the percentage of cells expressing fluorescence and the intensity of fluorescence from the three distinct populations over subsequent

amplifications.

From literature, we know that the use of low MOI is preferred for amplification of viruses as it can minimize the accumulation of DIPs. The titers of the virus stocks obtained after repeated passaging improved instantly when a low MOI of 0.1 was used. There were some variations in the replicative virus titer with passage number but not as drastic as those observed for the high MOI infections. Also, the coinfected population did not vary much between the coinfection experiment and the succeeding co-propagated single infection experiment. However, the intensity of fluorescence showed some decrease with passage even for low MOI infections. Due to the overlapping nature of the two fluorescent proteins and the sensitivity of the flow cytometer, a different combination of viruses expressing industrially relevant proteins could be used to predict the production capacity of the system over passages.

# Chapter 5

# Repeated propagation of two viruses expressing GAG-GFP or HA proteins simultaneously in insect cell cultures

## 5.1 Introduction

Influenza is an enveloped RNA virus belonging to the Orthomyxoviridae family. The HA (hemagglutinin) glycoprotein found on the surface of influenza viruses is partly responsible for the infectivity of the virus. HA is a class I fusion protein acting as both an attachment factor and a membrane fusion protein thus forming a bond between the virus and sialic acid on the surface of taget cells (Russell et al., 2008). Influenza poses significant risk of morbidity and mortality connected with seasonal exposure to continually drifting influenza strains. It is believed that the next influenza pandemic is on the outlook and it is associated with a new influenza A subtype (antigenic drift) (Haynes et al., 2009). Thus, there is a constant need to improve the flu vaccine to protect against pathogenic influenza strains. The GAG protein derived from the retrovirus has the ability to self-assemble and bud at the plasma membrane resulting in the formation of enveloped non-infectious VLPs (Chaves et al., 2019; Cervera et al., 2013). GAG-based influenza VLPs containing influenza antigens have been reported to exhibit robust vaccine protection and suggested applicability towards other respiratory viruses (Haynes et al., 2009; Cervera et al., 2013).

Influenza vaccine generation using the influenza antigen(s) presented as VLPs has attracted a lot of attention (Venereo-sanchez et al., 2017). Well-assembled VLPs can mimic the original virus from which it is derived, without the presence of virus genome and induce similar immune response in

the host cells, along with keeping the conformation intact (Zeltins, 2013; Haynes et al., 2009; Roldão et al., 2010). Egg-based manufacturing technology comes with a bundle of limitations including susceptibility to potential avian influenza pandemic thus threatening global egg supply, being labour intensive and long production time which would be an issue in case of a pandemic. Hence, production of such vaccines using a reliable platform is the need of the hour.

The need to replace traditional egg-based technology for influenza vaccine production has led researchers to investigate different production platforms. The VLPs produced in cell cultures have been reported to elicit broader immune response as compared to recombinant HA vaccine and whole influenza inactivated virus (Bright et al., 2007), thus proving to be a candidate of interest to combat seasonal and pandemic influenza. The IC-BEVS is a promising alternative for vaccine production. The ease of use, capability to carry out post-translational modifications and high protein yield via infection has made this system a favorable platform for biopharmaceutical production (Peixoto et al., 2007). This system has been used for the production of influenza VLPs leading to the development of influenza vaccines such as NanoFlu (Novavax) which is expected to initiate Phase III clinical trials in Fall 2019 (Novavax, 2019). The occurrence of baculovirus budded particles (BVs), and baculovirus encoded GP64 envelop protein, in VLP preparations is undesirable for vaccine manufacturing (Chaves et al., 2019). To combat the issue the GAG gene was expressed in the absence of baculovirus gp64

gene, which resulted in an improved VLP production method in insect cells (Chaves et al., 2019).

The work shown in **Chapter 4** with the simple virus system of p10GFP and p10RFP was extended to a more complex industrially relevant system. The objective of this project was to track the population of two viruses, amplified simultaneously, over repeated passage. Two baculovirus constructs, expressing the retroviral GAG protein fused to eGFP (GAG-GFP) or the HA protein, were co-amplified to monitor the population distribution of the resultant co-propagated stocks upon successive passaging. The virus stocks from the coinfected cultures, hereby known as single or co-propagated stock(s), were used for subsequent infections. Following the coinfection experiment (P3, passage number of starting stocks), two sets of co-propagated single infection experiments were run; at a low MOI of 0.1. The aim was to see if the virus population/ratio changes or if it is maintained over repeated amplification so that coinfection can be avoided at large scale.

## 5.2 Results and Discussion

As established in literature, a low MOI was used for repeated virus amplification in this work. The MOI ratios used here was kept same as those used in the p10GFP and p10RFP coinfection experiments at low MOI to maintain uniformity. A schematic of Sf9 cells coinfected with the two different virus vectors, p6.9GAG-GFP and p6.9HA, and the techniques used to track the population distribution upon infection and VLP analysis is shown in **Figure**

**5.1**. Following this, the budded virus comprising of both p6.9GAG-GFP and p6.9HA was used for subsequent amplification, as can be seen in **Figure 3.1**, and the same techniques were used for the analysis. The virus populations were tracked after each infection. In addition, after harvesting the cultures at 70-80% viability, the supernatants were purified by sucrose cushioning and iodixanol density gradient ultracentrifugation; and the purified fractions were further analyzed for the presence of VLPs.

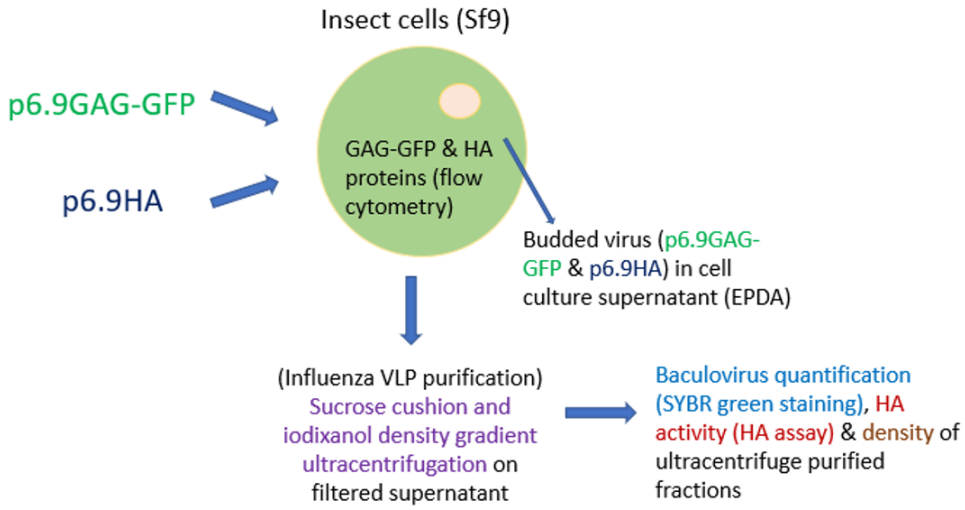


Figure 5.1: **Schematic of the p6.9GAG-GFP and p6.9HA infection and VLP analysis techniques.**
GAG-GFP and HA proteins produced inside the cell were tracked by flow cytometry. The budded virus (p6.9GAG-GFP and p6.9HA) in the cell culture supernatant, at the end of the infection cycle was determined by EPDA. The filtered supernatant obtained at the same time point were purified by two ultracentrifugation steps. Fractions obtained from the second ultracentrifugation were analyzed by HA assay and SYBR green staining.

### 5.2.1 Flow Cytometry Analysis of Infection

The progression of infection was tracked by flow cytometry. The **Figure 5.2** shows a shift in the population distribution at 48 hpi, when the culture condition is changed. The gated events are based on the infections with only p6.9GAG-GFP or p6.9HA, and an uninfected culture. For the higher (p6.9GAG-GFP:p6.9HA 0.07:0.03) and equal (p6.9GAG-GFP:p6.9HA 0.05:0.05) p6.9GAG-GFP infections, the population of GAG-GFP positive cells did not change much between the coinfection experiment (P3) and the first co-propagated single infection experiment (P4). With increasing concentration of p6.9HA in the cell culture, the number of GAG-GFP positive events moved towards the lower fluorescence level, for all the passages. Although, the population distribution is somewhat similar across the passages for each culture condition, there is a change in the percentage of GAG-GFP positive cells over subsequent amplifications.

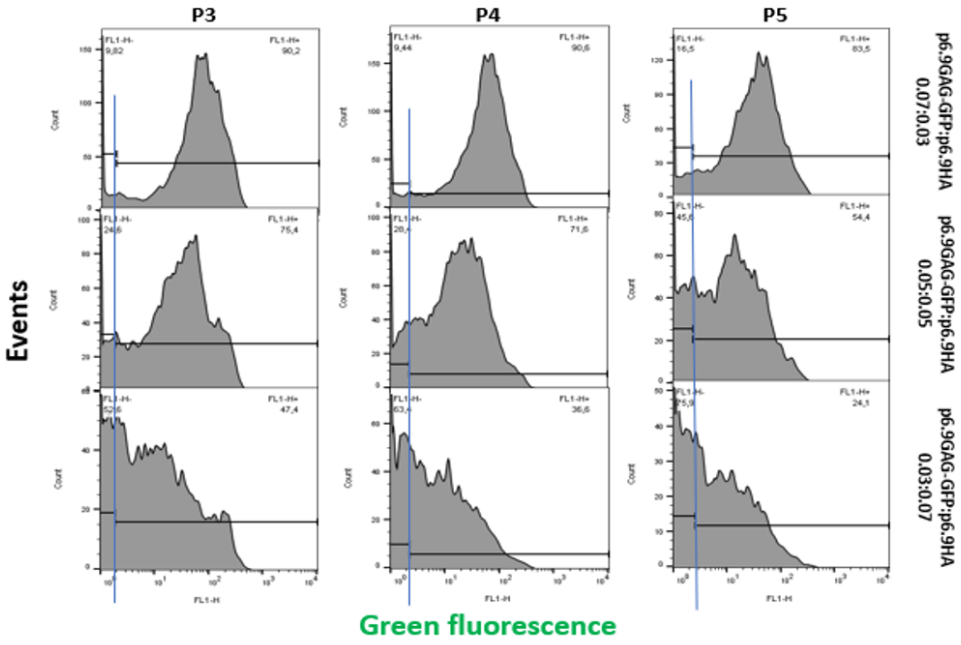


Figure 5.2: **Population of cells expressing green fluorescence at 48 hpi.**
P3, P4 and P5 refer to the passage number of virus stocks used in the coinfection, and the first and second co-propagated single infection experiments, respectively. p6.9GAG-GFP:p6.9HA is the MOI ratio of the two viruses used in the coinfection experiment. The first, second and third panel (top to bottom) represents the population distribution for the MOI ratios of p6.9GAG-GFP:p6.9HA 0.07:0.03, 0.05:0.05 and 0.03:0.07, respectively. The vertical line separates the population distribution on the left and right hand side of each chart. The population on the left hand side of each chart corresponds to an uninfected or only p6.9HA infected population, whereas that on the right hand side represents a p6.9GAG-GFP infected or a coinfected population. A small percentage of low fluorescent events (cells) are at the edge of the chart, which are not considered for analysis, thus resulting in the total infected populations to be less than 100%.

The single p6.9GAG-GFP infection (Percent.FL1, p6.9GAG-GFP:p6.9HA 0.1:0) showed negligible change with passage number. For the coinfected con-

ditions the percentage of cells expressing green fluorescence (Percent.FL1) dropped from P3 (coinfection experiment) to P4 (first co-propagated single infection experiment) especially for the higher (p6.9GAG-GFP:p6.9HA 0.03:0.07) and equal (p6.9GAG-GFP:p6.9HA 0.05:0.05) p6.9HA culture conditions and increased slightly from P4 to P5 (second co-propagated single infection experiment) (**Figure 5.3**).

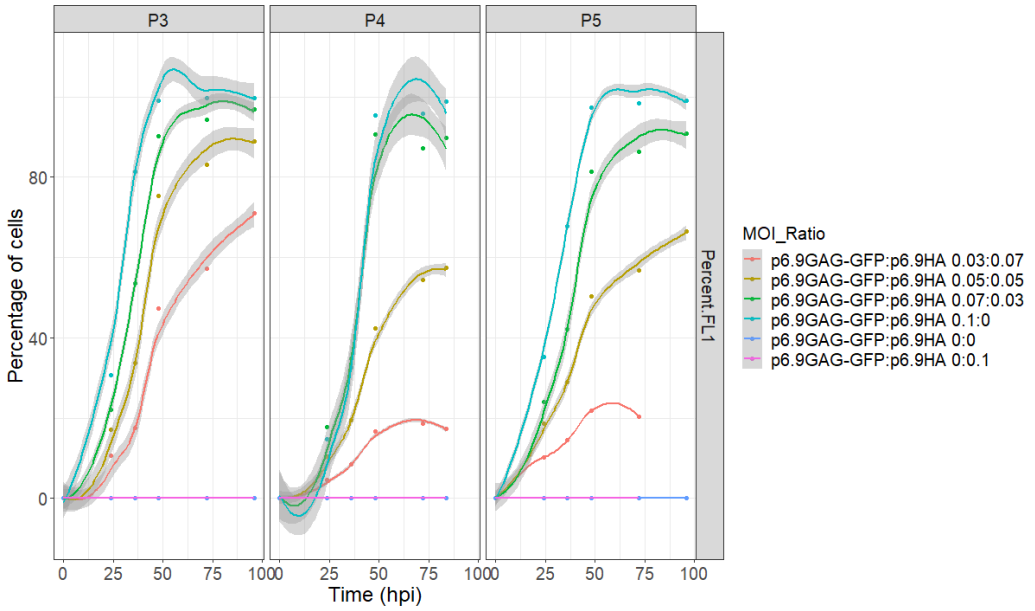


Figure 5.3: **Percentage of cells expressing green fluorescence at different time points post infection with p6.9GAG-GFP and/or p6.9HA baculovirus.**
P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p6.9GAG-GFP and p6.9HA used in the coinfection experiment, while P4 and P5 are the virus stocks of p6.9GAG-GFP + p6.9HA used in first and second rounds of co-propagated single infection experiments respectively. Percent.FL1 corresponds to the percentage of cells expressing green fluorescence. The plotted data points are an average of values for duplicate flasks.

For the single p6.9GAG-GFP infection (p6.9GAG-GFP:p6.9HA 0.1:0), the green fluorescence (Mean.FL1) portrayed an increase of 1.05 times from P3 to P4 and a drop of 1.12 times from P4 to P5. However, the intensity of green fluorescence of the coinfected conditions decreased from P3 and P5 (**Figure 5.4**).

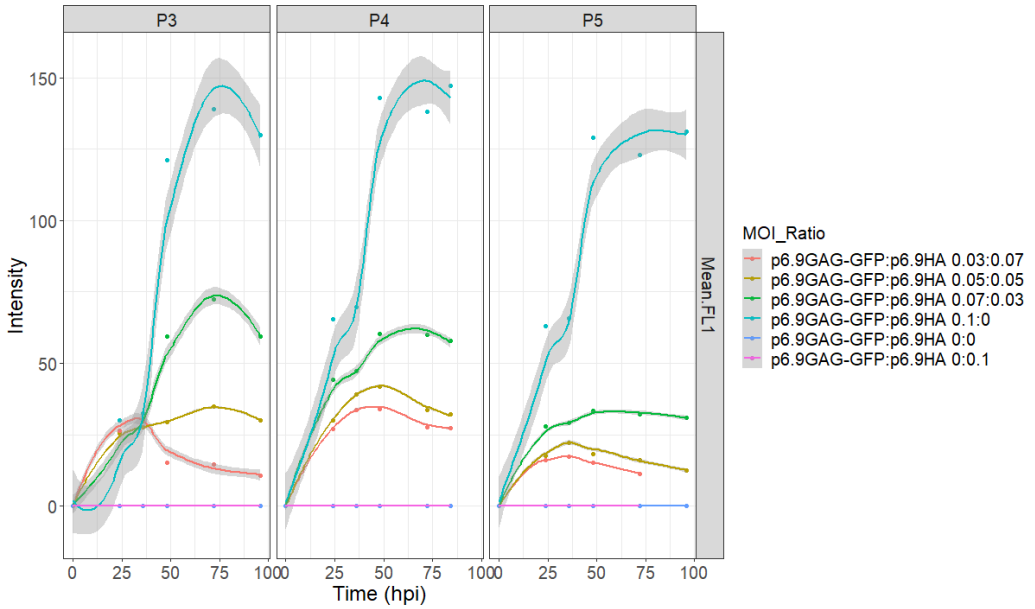


Figure 5.4: **Intensity of green fluorescence at different time points post infection with p6.9GAG-GFP and/or p6.9HA baculovirus.** P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p6.9GAG-GFP and p6.9HA used in the coinfection experiment, while P4 and P5 are the virus stocks of p6.9GAG-GFP + p6.9HA used in the co-propagated single infection experiments. Mean.FL1 corresponds to the green fluorescence of the GAG-GFP positive cells. The plotted data points are an average of values for duplicate flasks.

### 5.2.2 Titer of Virus Stocks

P3 stocks of p6.9GAG-GFP and p6.9HA were amplified at a low MOI of 0.1 from P2 stocks in Sf9 cell cultures and were used as the starting virus stocks for the coinfection experiment. Since the HA did not have a fluorescent tag, the replicative titer of the virus stock by end point dilution assay (EPDA) could not be determined using Sf9 cells. Thus, the total replicative virus titer (by EPDA) after each infection was determined by infecting GFP inducing Sf9 cell line (GFP-Sf9), which was kindly provided and validated by Mark Bruder (PhD student at the Aucoin Lab). Serial passaging of the resultant co-propagated stocks did not portray much variation among different passages (**Figure 5.5**). There is a minimal fluctuation in titers of the virus stocks obtained from the Sf9 cell cultures coinfected with p6.9GAG-GFP and p6.9HA. This variation could be due to the variability associated with the end point assays.

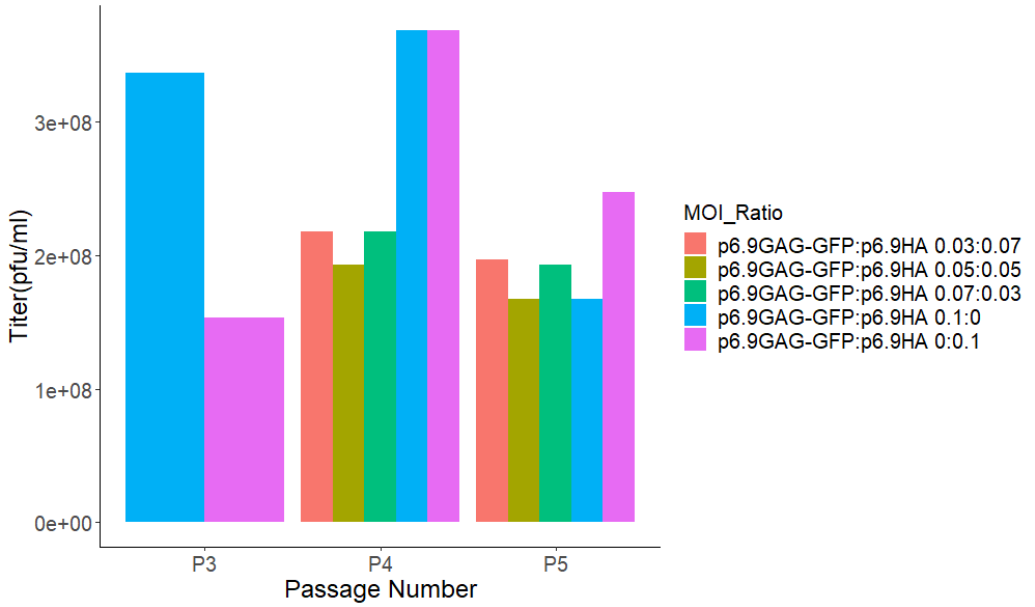


Figure 5.5: **Titer of infectious GAG-GFP/HA expressing baculovirus in cell culture supernatant harvested at 70-80% viability as obtained by end point dilution assay.**
P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p6.9GAG-GFP and p6.9HA used in the coinfection experiment, while P4 and P5 are the subsequent co-propagated stocks of p6.9GAG-GFP + p6.9HA. The titers plotted are an average of duplicates.

### 5.2.3 Analysis of fractions

The filtered supernatants obtained from each infection at 70-80% culture viability were concentrated by ultracentrifugation using a 25% sucrose cushion, followed by iodixanol density gradient ultracentrifugation to purify the virus-like particles. The density, HA activity and baculovirus particle concentration of each fraction from the ultracentrifuge purified density gradient of each culture condition after every infection were examined. The different

steps followed to purify the influenza VLPs are shown in **Figure 5.6**.

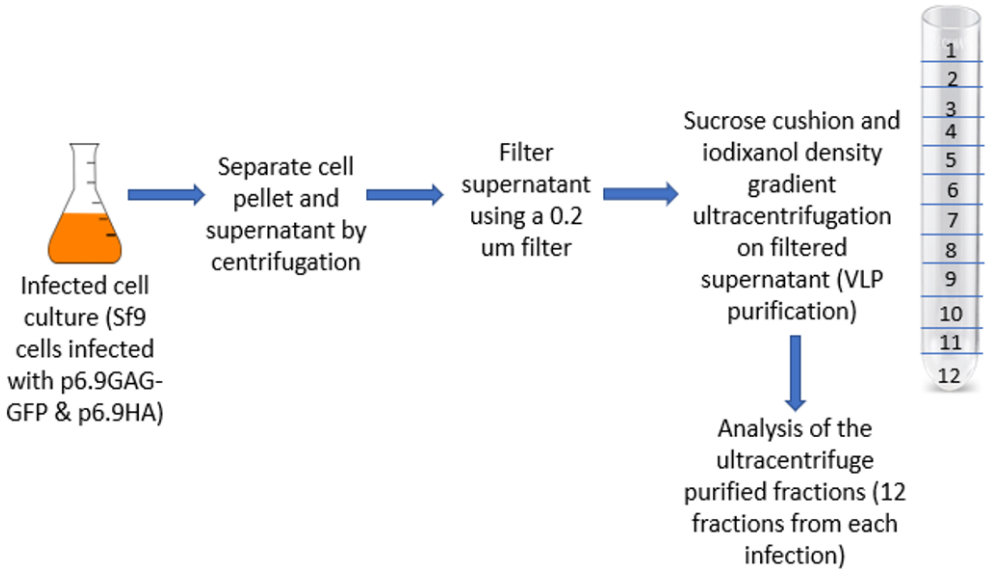


Figure 5.6: **A schematic of the steps followed to obtain ultracentrifuge purified fractions.**

HA assay is used to determine the presence of influenza virus/VLPs containing the HA protein. The highest HA activity was observed for the ultracentrifuge purified fractions 5 or 6 for each culture condition and remained consistent with repeated passaging (**Figure 5.7**). That being said, the HA activity of the fractions obtained from the coinfected cultures dropped by a factor of 2 from P3 to P5, and for the single p6.9HA infection it dropped by a factor of 2 from P3 to P4 but did not change from P4 to P5. HA activity was completely absent for all the fractions obtained from single p6.9GAG-GFP infections (p6.9GAG-GFP:p6.9HA 0.1:0) and the two least dense fractions (1 and 2) for all the other MOI ratios used. This was constant over the coinfec-

tion and both the co-propagated single infection experiments. Although, the HA activity of the fractions reduced with passage number, it is still considered high. Even though it can't be deduced with confidence, the variability maybe due to the accumulation of defective particles which begins the replication cycle but cannot complete it, thus producing lesser VLPs containg the HA. Also, as mentioned by George (2016) some of the HA activity gets lost in the ultracentrifugation step. The densities of the highest HA activity fractions (5 or 6) for the different conditions (MOI Ratios) varied between 1.09 to 1.19 g/ml for P3 (coinfection experiment), 1.10 to 1.22 g/ml for P4 (first round of co-propagated single infections) and 1.10 to 1.24 g/ml for P5 (second round of co-propagated single infections).

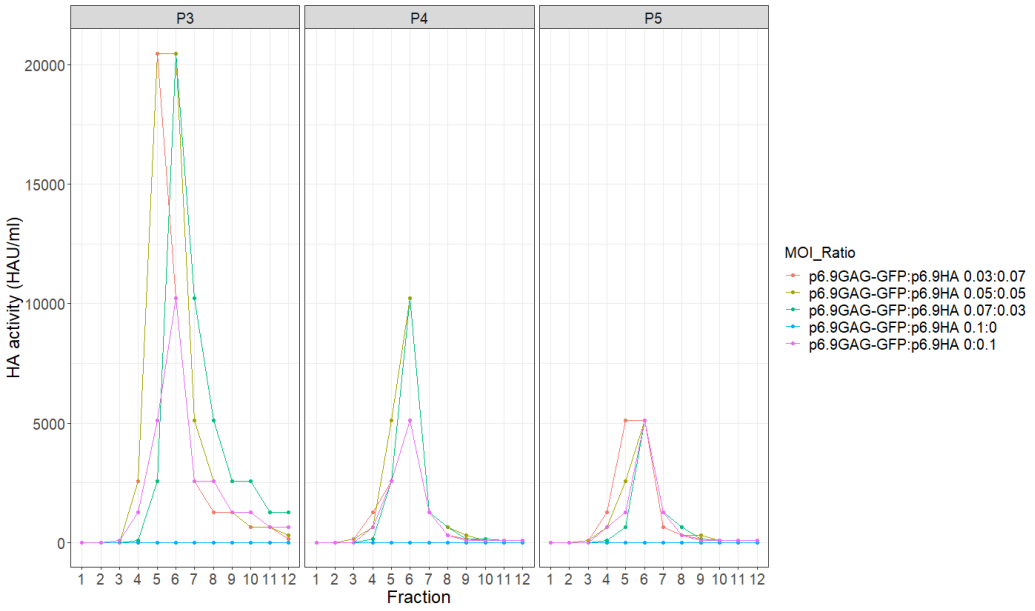


Figure 5.7: **HA activity of ultracentrifuge purified fractions.** P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p6.9GAG-GFP and p6.9HA used in the coinfection experiment, while P4 and P5 are the virus stocks of p6.9GAG-GFP + p6.9HA used in the co-propagated single infection experiments. The plotted data points are an average of duplicate values.

The use of a nucleic acid dye to stain the viral particles and then taking advantage of the fluorescence to determine the baculovirus particle concentration by flow cytometry provided to be a fast and easy technique (Shen et al., 2002). Particle counts (baculovirus particles/ml) revealed the maximum baculovirus particle concentration was observed from fractions 5 to 8 with the peak at fraction 6 for most of the conditions (**Figure 5.8**). The only three exceptions were for the P3 higher p6.9GAG-GFP (p6.9GAG-GFP:p6.9HA 0.07:0.03), P4 only p6.9GAG-GFP (p6.9GAG-GFP:p6.9HA 0.1:0) and P5

only p6.9HA (p6.9GAG-GFP:p6.9HA 0:0.1) where the peak was seen in fractions 7, 5 and 5 respectively. However, it is to be noted that the density of P4 only p6.9GAG-GFP fraction 5 is same as its corresponding P5 fraction 6; and the densities of P3 higher p6.9GAG-GFP fraction 7 and P5 only p6.9HA fraction 5 are quite similar to those of their corresponding fraction 6. Hence, we can say that the highest baculovirus particle concentration was observed for the fractions with the same densities, or the highest concentration of baculovirus particles was always observed at a particular density. Although, the peak baculovirus particle concentration did not show a prominent variation between passage numbers for the higher p6.9GAG-GFP, visible changes were observed for the equal and higher p6.9HA conditions. Also, the peak concentration for the coinfected conditions varied between 3.87E + 07 (p6.9GAG-GFP:p6.9HA 0.07:0.03, Fraction 7, P4) to 6.32E + 07 (p6.9GAG-GFP:p6.9HA 0.05:0.05, Fraction 6, P5) with passage number. Since the other fractions (1-4 and 9-12) did not have a high baculovirus particle concentration they were dropped. Nevertheless, baculovirus was detected in all the fractions in varying concentrations.

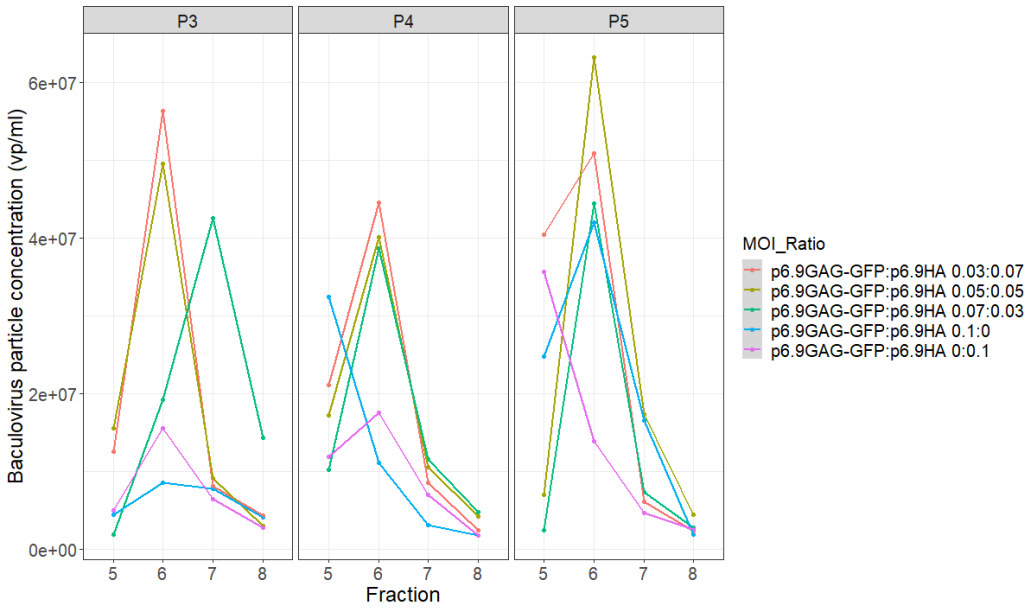


Figure 5.8: **Baculovirus particle concentration of ultracentrifuge purified fractions.**
P3, P4 and P5 refer to the passage number of virus stocks. P3 being the virus stocks of p6.9GAG-GFP and p6.9HA used in the coinfection experiment, while P4 and P5 are the virus stocks of p6.9GAG-GFP + p6.9HA used in co-propagated single infection experiments. The plotted data points are an average of duplicate values.

A detailed information on the density gradient ultracentrifuge purified fractions from each infection over subsequent passages can be seen in **Table 5.1**. The maximum baculovirus particle concentration of the highest peak were quite comparable among the coinfected conditions for each passage. The densities do not vary much over passages and are also similar for the different conditions. In addition, the fraction expressing the highest HA activity exhibited the highest baculovirus particle concentration with a few exceptions. These results give away that although density gradient ultracen-

trifugation can be a useful step in the VLP purification process, it is not the most effective method to purify VLPs from baculovirus.

Table 5.1: Comparison of density, HA activity and baculovirus particle concentration of ultracentrifuge purified fractions between passage numbers.

| MOI ratio (p6.9GAG-GFP:p6.9HA) | Passage number | Density range (g/ml) | Highest baculovirus particle concentration (vp/ml) | Density of peak baculovirus particle concentration fraction (g/ml) | Fraction with highest baculovirus particle concentration | HA activity (HAU/ml) | Fraction with highest HA activity |
|---|---|---|---|---|---|---|---|
| **0.1:0** | **P3** | 1.02 to 1.41 | 8.55E+06 | 1.14 | 6 | N/A | N/A |
| **0.1:0** | **P4** | 0.95 to 1.37 | 3.24E+07 | 1.10 | 5 | N/A | N/A |
| **0.1:0** | **P5** | 1.04 to 1.39 | 4.20E+07 | 1.10 | 6 | N/A | N/A |
| **0.07:0.03** | **P3** | 1.03 to 1.37 | 4.25E+07 | 1.11 | 7 | 20480 | 6 |
| **0.07:0.03** | **P4** | 0.99 to 1.39 | 3.87E+07 | 1.22 | 6 | 10240 | 6 |
| **0.07:0.03** | **P5** | 0.94 to 1.37 | 4.44E+06 | 1.15 | 6 | 5120 | 6 |
| **0.05:0.05** | **P3** | 1.03 to 1.39 | 4.95E+07 | 1.11 | 6 | 20480 | 6 |
| **0.05:0.05** | **P4** | 1.06 to 1.28 | 4.01E+07 | 1.16 | 6 | 10240 | 6 |
| **0.05:0.05** | **P5** | 1.07 to 1.41 | 6.32E+07 | 1.10 | 6 | 5120 | 6 |
| **0.03:0.07** | **P3** | 0.96 to 1.33 | 5.63E+07 | 1.19 | 6 | 20480 | 5 |
| **0.03:0.07** | **P4** | 1.00 to 1.39 | 4.45E+07 | 1.16 | 6 | 5120 | 6 |
| **0.03:0.07** | **P5** | 1.07 to 1.35 | 5.09E+07 | 1.24 | 6 | 5120 | 6 |
| **0:0.1** | **P3** | 1.09 to 1.36 | 1.55E+07 | 1.09 | 6 | 10240 | 6 |
| **0:0.1** | **P4** | 1.01 to 1.43 | 1.76E+07 | 1.16 | 6 | 5120 | 6 |
| **0:0.1** | **P5** | 0.94 to 1.31 | 3.57E+07 | 1.04 | 5 | 5120 | 6 |

## 5.3 Conclusions

The importance of selecting an infection strategy has been debated time and again by different researchers. In this work, not only did we take advantage of the flexibility provided by coinfection at small scale, but also tried to explore an amplification strategy that could be used with some modifications to avoid the application of coinfection at the large scale. Amplifying different viruses together at lab scale helps to control the ratio of each protein required for product formation, and the resulting stocks from the coinfected cultures can

be used for successive infections. Based on the results from **Chapter 4**, it was concluded that using a high MOI for repeated passaging would lead to significant drops in replicative virus titer. Thus, a low MOI of 0.1 was selected for the repeated amplification of industrially relevant proteins: GAG-GFP and HA.

The infectious virus titer, as determined by end point dilution assay, did not vary much over repeated passaging for the co-propagated stocks. Since, the GAG was fused to GFP, its fluorescence was tracked by flow cytometry. The drop in GAG-GFP positive cells and intensity of green fluorescence over passage for the coinfected conditions could be due to more population being coinfected with p6.9GAG-GFP and p6.9HA, but that can't be said with certainty as the distribution of the coinfected only population is not known separately. Following the influenza VLP purification steps, the ultracentrifuge purified fractions showed high HA activity with a two factor drop from P3 to P5, which maybe due to loss over repeated amplification; and baculovirus was detected in varying concentrations in all the fractions. Overall from the results, it was observed that the use of low MOI could help to sustain the virus distribution over subsequent amplifications. Nevertheless, ultracentrifugation alone does not have the resolution to separate baculovirus from VLPs. Thus, in order to purify the VLPs for any biopharmaceutical application different purification techniques need to be used in conjugation with ultracentrifugation.

# Chapter 6

# Conclusions and Recommendations

The main aim of this work was to track the population distribution over repeated propagation of multiple viruses simultaneously in insect cell culture at high or low MOI, so that coinfection can be avoided at industrial scale. Two virus vectors, p10GFP and p10RFP, expressing GFP and RFP respectively, were used to establish a baseline for repeated virus amplification at high and low MOI. It was found that using a high MOI changes the virus population, as well as results in drastic drop in virus titers over subsequent passages. When a low MOI was used, the population distribution and the virus titers did not change drastically with repeated amplification.

The use of low MOI for virus amplification has been well established in literature. For repeated simultaneous propagation of multiple viruses in

insect cell culture a low MOI should be used, which is also known to minimize the passage effect.

Using only a low MOI alone won't be sufficient to combat the issue of passage effect and other techniques need to be implemented to reduce the accumulation of these interfering particles. One such way can be the use of genetic engineering to eliminate/modify the genes responsible for the occurrence of DIPs.

Also, different reporter proteins that do not overlap can be used to track the virus populations over repeated passaging.

Based on these results, low MOI was used for the repeated propagation of a complex system of virus vectors, p6.9GAG-GFP and p6.9HA, expressing the proteins GAG-GFP and HA, respectively. As expected, the titers showed minimal variation with passages, in addition to sustained population distribution observed over at least one passage. However, following the influenza VLP purification procedure, it was found that baculovirus was present in varying concentrations in all the ultracentrifuge purified fractions.

In order to produce VLPs for therapeutic or biopharmaceutical applications a more robust purification system needs to be implemented. It has been established that sucrose cushioning and/or density gradient ultracentrifugation does not have the resolution to completely purify or separate VLPs from baculovirus. Additional purification techniques such as ion exchange chromatography and cellulose membrane-based steric exclusion chromatography (Lothert et al., 2019) can reduce baculovirus contamination in VLP

preparations.

Moreover, modification of the baculovirus genomes by deleting genes that are known to cause/increase baculovirus presence in VLP samples can improve VLP production in insect cells (Chaves et al., 2019).

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# Appendix A

# End Point Dilution Assay

The end point dilution assay (EPDA) technique is based on the Tissue Culture Infectious Dose 50 (TCID50) assay (Reed and Muench, 1938). TCID50 is the virus dose that causes 50% of the culture to be infected by a virus sample. Titers are calculated by using equations outlined by King and Possee, 1992.

**Equation A-1.** Calculation of proportional distance (PD):-

$$\mathbf{PD} = \frac{A - 50}{A - B}$$

where A is the percent response greater than 50% and B is the percent response less than 50%.

**Equation A-2.** Calculation of TCID50 dose:-

The dose that would give a 50% response is calculated as:

$\log_{10}(TCID_{50})$ =*(log of the dilution giving a response greater than 50%) - (PD)*

**Equation A-3.** Virus titer is the reciprocal of the TCID50:-

$$\textbf{Titer (pfu/ml)} = \frac{1/TCID50}{0.01\,ml} \times 0.68$$

| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Name | Madhuja Chakraborty | | | | | | | | | |
| 2 | Date | February 26, 2019 | | | | | | | | | |
| 3 | Virus stock (Passage) | EGFP (b)- February P3 Stock | | | | | | | | | |
| 4 | Cell line | Sf9 | | | | | | | | | |
| 5 | | | | | | | | | | | |
| 6 | | | | Cumulative | | | | | | | |
| 7 | Dilutions | Number of infected wells | Number of uninfected wells | Total Number of Infected | Total Number of Uninfected | Percent infected | Above 0.5 | % Greater than 50% | % less than 50% | Log dilution greater than 50 % | |
| 8 | | | | | | | | | | | |
| 9 | 1.00E-04 | 12 | 0 | 42 | 0 | 100 | TRUE | 0 | 0 | 0 | |
| 10 | 1.00E-05 | 12 | 0 | 30 | 0 | 100 | TRUE | 0 | 0 | 0 | |
| 11 | 1.00E-06 | 10 | 2 | 18 | 2 | 90 | TRUE | 90 | 0 | 0.000001 | |
| 12 | 1.00E-07 | 6 | 6 | 8 | 8 | 50 | FALSE | 0 | 50 | 0 | |
| 13 | 1.00E-08 | 2 | 10 | 2 | 18 | 10 | FALSE | 0 | 0 | 0 | |
| 14 | 1.00E-09 | 0 | 12 | 0 | 30 | 0 | FALSE | 0 | 0 | 0 | |
| 15 | 1.00E-10 | 0 | 12 | 0 | 42 | 0 | FALSE | 0 | 0 | 0 | |
| 16 | | | | | | | | 90 | 50 | 1.00E-06 | |
| 17 | | | | | | | | | | | |
| 18 | Prop Dist | 1 | | | | | | | | | |
| 19 | Log10(TCID50) | -7.00E+00 | | | | | | | | | |
| 20 | TCID50 | 1.00E-07 | | | | | | | | | |
| 21 | Virus titre | 1.0000E+09 | | | | | | | | | |
| 22 | Titer (pfu/mL or IU/mL) | 6.90E+08 | | | | | | | | | |
| 23 | | | | | | | | | | | |

Figure A.1: **An excel spreadsheet example for caltulating titers.**

# Appendix B

# Hemagglutination Assay

The hemagglutination assay or HA assay is based on the agglutination of red blood cells in the presence of the influenza virus envelope protein HA (hemagglutinin). The reciprocal of the last virus dilution in a row without the occurrence of a pellet or button at the bottom of the well represents the hemagglutination units (HAU) per 50 $\mu$L. In order to achieve HAU/ml, the HAU/50 $\mu$L is multiplied by 20.

If the last agglutination was observed for the dilution 1:256, then the HA activity is, (256 HAU/50 $\mu$L) x (1000 $\mu$L/1 ml) = 5120 HAU/ml.

# Appendix C

# Baculovirus Quantification by Flow Cytometry

The following settings were used to analyze samples in the BD FACSCalibur flow cytometer.

Table C.1: Settings on flow cytometer.

| Channel | Scale | Voltage |
|---|---|---|
| **FSC** | **log** | **E01** |
| **SSC** | **log** | **490** |
| **FL1** | **log** | **480** |

Samples were run at a medium flow rate of 35 $\mu$L/min for 30 seconds.

The following equation was used to determine the concentration of viral particles using the gated events.

**Equation C-1.** Viral particle concentration:-

$$viral\ particle\ concentration = Cv \times D \times 1000 \times \frac{50000}{Cf \times Vol}$$

where

Cv, Cf are the particle counts for viral particles and FlowSets respectively.

D is the dilution rate of viral solution.

Vol is the volume of diluted viral solution used for sample preparation.

1000 is the final volume of each sample.

50000 is the particle concentration of diluted FlowSet.

# Appendix D

# Viability of Sf9 Cells Post Infection

Cells were monitored at different time points post infection to assess their progression. When the cultures were infected at an MOI of 10 with p10GFP and p10RFP, it resulted in synchronous infection. The viability of the cell cultures kept on decreasing as the infection progressed with 70-80% viability being achieved at 72 hpi for P3 and P4, and at 96 hpi for P5.

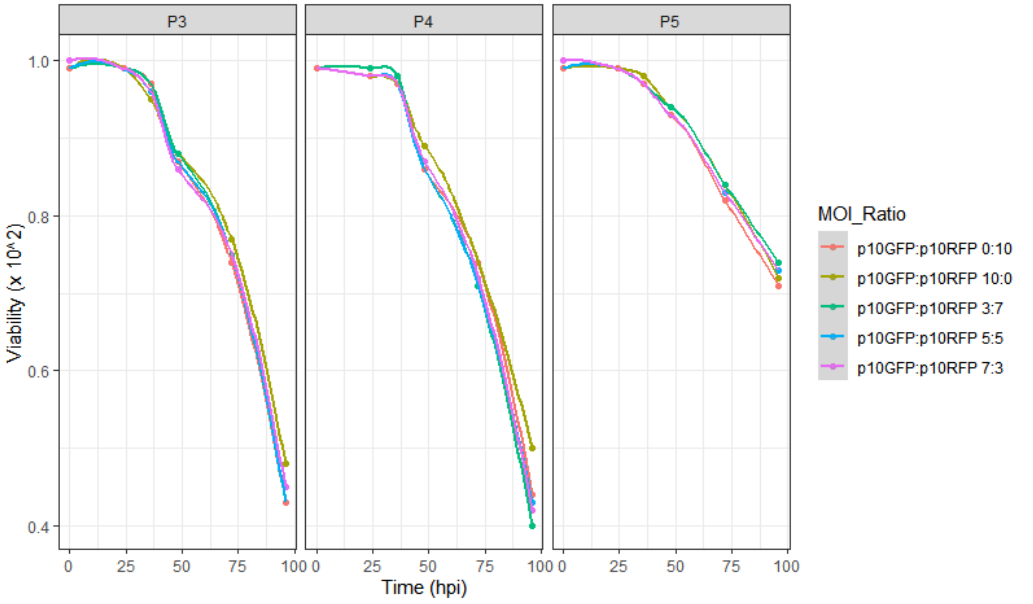


Figure D.1: **Sf9 cells infected at a total MOI of 10:**
The viability of the infected cell cultures are shown over the duration of an infection at MOI 10. P3, P4 and P5 refer to the passage number of virus stocks used in successive experiments.

Infection at a low MOI of 0.1 with p10GFP and p10RFP, resulted in asynchronous infection. The cultures followed a similar trend for viability with the exception of viability drop between 70-80% at 84 hpi when infected with P4 co-propagated stocks.

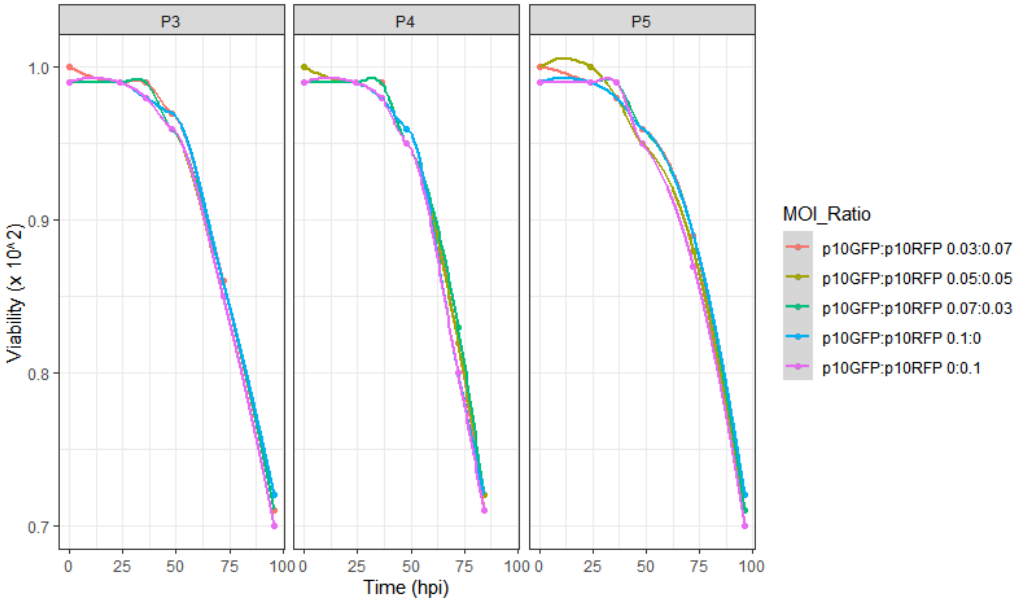


Figure D.2: **Sf9 cells infected at a total MOI of 0.1:** The viability of the infected cell cultures are shown over the duration of an infection at MOI 0.1.

As expected, a low MOI infection with p6.9GAG-GFP and p6.9HA resulted in asynchronous infection. The cultures followed a similar trend for viability with the exceptions of viability drop between 70-80% at 84 hpi when infected with P4 co-propagated stocks, and at 72 hpi for the single (p6.9GAG-GFP:p6.9HA 0:0.1) and higher (GAG-GFP:HA 0.03:0.07) p6.9HA conditions when infected with P5 single stocks.

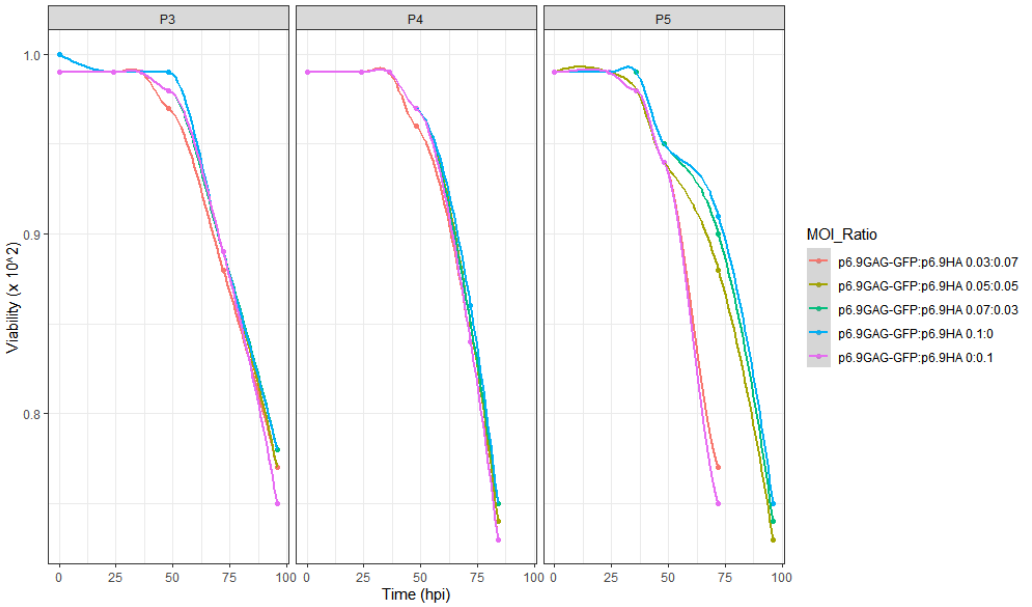


Figure D.3: **Sf9 cells infected with p6.9GAG-GFP and p6.9HA baculoviruses at a total MOI of 0.1:**
The viability of the infected cell cultures are shown over the duration of an infection at MOI 0.1. P3, P4 and P5 refer to the passage number of virus stocks used in subsequent experiments.