Characterization of Alternative Promoters to Stagger and Control Multiple Gene Expression and Protein Production in the Baculovirus-Insect Cell System

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, 2014

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

The baculovirus-insect cell system is one of the most widely used systems for recombinant protein production. The ease of handling insect cells and their ability to produce mammalian-like post-translational modifications, as well as improved methods for the quantification of baculovirus, have led to substantial progress in understanding the dynamics of protein production through the baculovirus expression vector system (BEVS). Furthermore, single baculoviruses can be engineered for expression from multiple genes guaranteeing expression of the different proteins within a single cell (for example *Spodoptera frugiperda* – Sf-9 cells). The main benefit of using a co-expression system is for products such as virus-like particles (VLPs) that can only be formed by producing two or more proteins within a single cell. There are some problems, however, associated with co-expression systems, such as the manipulation of expression levels for different proteins. In baculoviruses, genes are under the control of different promoters and these promoters are classified by the different times during which they "promote" expression. These can be classified into early promoters, late promoters and very late promoters. Researchers have traditionally used the strong and very late "polyhedrin" promoter for gene expression. Very late phase expression has several problems, such as allowing expression during a period where there is breakdown of the host machinery. Several proteins require extensive post-translational modification, such as glycosylation, or require secretion into the extracellular environment, both of which require the host cell machinery to be fully functional. Problems with glycosylation and secretion can be exacerbated by protein over-production. Simultaneous production of two different proteins in insect cells can also result in "competition" for cellular resources, which can cause lower amounts of each protein being produced compared to if these proteins were produced individually. The use of alternative promoters (polh, p10, vcath, basic, ie1, and gp64), which are active at different times post-infection, provides flexibility to control expression level and timing of "genes of interest".

In our work, Green Fluorescent Protein (GFP) and Red Fluorescent Protein (RFP) are being used to detect the expression of two proteins under the control of different promoters. Placing genes under the control of different promoters, allows for the control of mRNA levels in the cell and this can then lead to control of protein production levels. In our studies, RFP is always under the control of the polyhedrin promoter, while GFP is either under the control of the p10, basic, vcath, ie1 or gp64 promoters constituting five different polycistronic virus vectors; note that p10 and polh are very late and strong promoters; basic, vcath and gp64 are late promoters; and ie1 is an early and weak promoter. These combinations can lead to temporal separation of RFP and GFP production. The effect of different promoters on protein production is investigated. Flow cytometry is used for detection of fluorescent protein production by the cells after infection at different multiplicities of infection (MOIs). Tracking of intracellular mRNA levels of GFP and RFP are conducted with reverse transcription real time polymerase chain reaction. The 28S rRNA has been tracked and used as a housekeeping gene to ensure uniformity between samples.

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Marc Gordon Aucoin for his continuous support and guidance during my entire Master's program. In addition to the valuable of research skills and technical knowledge I have acquired under his supervision, I have also learned how to communicate efficiently, how to conduct myself professionally, and how to approach any challenge with fortitude and determination. I would also like to thank Professor Eric Jervis for accepting me as a Master's student.

1 am especially thankful for the support and companionship provided by Steve George. Steve selflessly endured many hours for the progress of this thesis and was part of this research in each and every step.

I would also like to extend my thanks to the readers of my thesis, Prof. Maud Gorbet and Dr. Jason Grove. I am especially thankful to Prof. Gorbet who allowed us access to her lab and equipment.

I would like to thank past and present group members in the Aucoin lab: Steve George, Eric Blondeel, Stan Sokolenko, Bhavik Manocha, Jann Ang, Sandi Yen, Megan Logan, Valentin Malenkov, Biljana Todorovic, Sasha Kieβlich, and Simon Guillemette for their friendship, constant support and encouragement. My master's study in the Aucoin group is one of the most memorable experiences in my life.

I would like to express my deepest gratitude to my parents, and my brothers who have always been there for me. As I move on to the next chapter of my life, it is comforting to know that, regardless of wherever life takes me, I will always have a loving family to lean on.

And finally and by no means the least, my friends who have helped keep me balanced and sane. In particular, I would like to thank Aashish Gaurav, Mohamad Shoaib and Ibrat Husain for their constant support and encouragement.

Dedicated to my beloved Grandparent "Afzal Karim"

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List of Abbreviation

AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
BEVS	Baculovirus expression vector system
Ct	Cycle threshold number
DIP	Defective Interfering Particle
DNA	Deoxyribonucleic acid
EDPA	End Point Dilution Assay
eGFP	enhanced green fluorescent protein
ETL	Early-to-late promoter
FC	Flow cytometry
FSC	Forward Scatter
FL	Fluorescence
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCA	Growth cessation assay
GFP	Green fluorescent protein
GV	Granulovirus
HA	Hemagglutinin
hpi	hour post infection

hrs	Homologous regions sequence
mW	milliwatt
min	minutes
MOI	Multiplicity of Infection
NPV	Nucleopolyhedrovirus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Polh	Polyhedrin
qRT-PCR	Quantitative real time polymerase chain reaction
RFP	Red fluorescent protein
RNA	Ribonucleic acid
Sf-9	*Spodoptera frugiperda*
SSC	Side Scatter
TBP	TATA binding protein
TCID	Tissue Culture Infectious Dose
VLPs	Virus Like particles

Chapter 1
Introduction

This is a thesis based on the production of non-native proteins in cells, an endeavor that is highly exploited to manufacture proteins that can then be used, for example, as materials, vaccines or therapeutics. The production of a protein in a cell requires the cell to be able to "read" a gene and create an instruction sheet (transcript) from which a protein can be built (translation) – Figure 1.1. The ability of the gene to be "read" is partly based on the gene (sequence of codons) but also on nucleotide sequences surrounding the gene. This thesis focusses primarily on nucleotide sequences upstream of a gene, sequences known as promoters.

The Baculovirus Expression Vector System (BEVS) has been used for protein production for the past thirty years. The BEVS (genetically modified baculoviruses that carry a foreign gene to insect cells, where the cells then produce a protein of interest) is widely used to produce many protein products at the lab scale, and it is now being used increasingly for commercial scale protein production. The BEVS is primarily used for: recombinant protein synthesis ranging from cytosolic enzymes to membrane associated proteins; as a biopesticide; and as a model system (for studying apoptosis) (Vieira et al., 2010). It was first exploited for human use as an insecticide, however in the last decades the BEVS has been successfully used for biological medical products such as vaccines for humans and animals. GlaxoSmithKline's Cervarix™ (GSK, Rixensart, Belgium), a bivalent human papillomavirus (HPV) vaccine against cervical cancer, was the first approved human product using the BEVS. It was approved in 2009 for commercial use in the United States of America. Commercial uses of baculovirus and BEVS have been extensively reviewed elsewhere (Ikonomou et al., 2003).

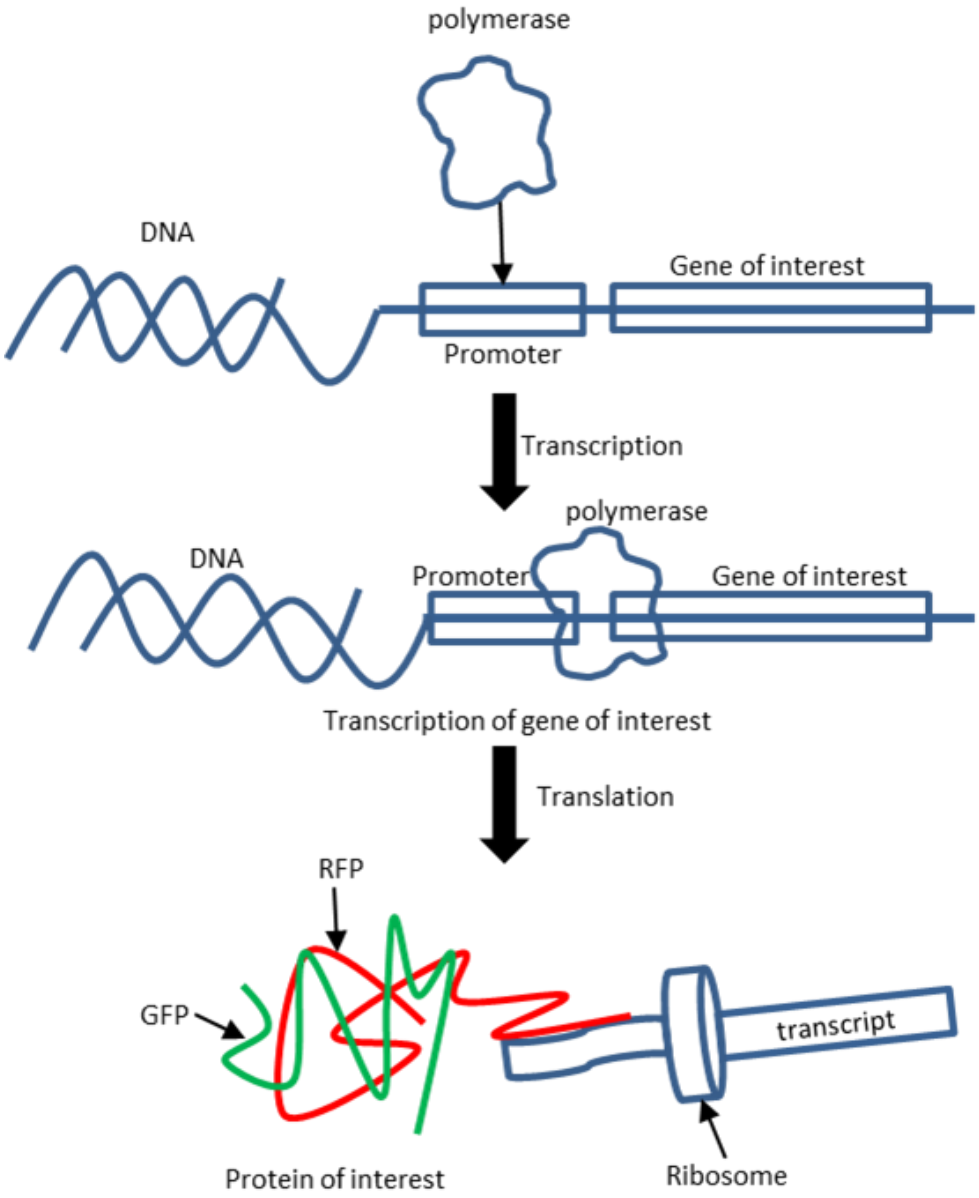


Figure 1-1: Protein synthesis: from transcription to translation.

The versatility of the BEVS is due to factors such as the robustness of insects cell; high growth rates of insect cells; the ability of insect cells to grow in a suspension culture and their ability to grow up to high cell density; the capabilities of insect cells to perform mammalian-like post-translation

modifications; the capacity of baculovirus vectors to accept large gene insertion; and most importantly, the non-infectivity of these baculovirus vectors towards humans, which makes it a safe biological tool for research use (as reviewed by Sokolenko et al., 2012). Moreover, combined with baculovirus vectors, insect cells can achieve high levels of protein production driven by the strongest animal cell promoter, the polh promoter. The utility of this system has been further extended by extensive research to improve factors such as the vectors used, and to improve insect cell lines to perform more mammalian like post-translation modifications (Kost, et al., 2005).

One particular application of the BEVS is its use for the production of multiple proteins within a single cell. This is useful for the production of multi-component proteins such as protein complexes and Virus-Like Particles (VLPs) (Aucoin et al., 2010; Hu, 2005; Kost et al., 2005). In addition, co-production of several proteins in a single cell can allow for the introduction of complex processing functions into cells, as foreign proteins such as chaperones can help in the folding of other proteins of interest (Ailor & Betenbaugh, 1998; Hartl, 1996; Kato et al., 2005; Tate et al., 1999). Key to the above is the need to have the chaperone and the protein of interest in the same cell. In a transient system, like the BEVS, where the genes for the additional proteins are being brought to the cells, there is a statistical probability that a cell does not receive/accept the gene(s) being delivered. This is in contrast to "stable cell lines" that express foreign genes. In the latter, all cells contain the foreign gene and continue to "keep" the foreign genes as the cells propagate. There are a number of reasons why a transient system may be chosen, one of which is the difficulty in obtaining a "stable" cell line expressing your protein(s) of interest. In the case where the BEVS is used and there is a desire to have multiple proteins produced, one can use multiple baculoviruses, each encoding one gene (co-infection), or use a single baculovirus, which can be engineered to express multiple foreign genes (polycistronic vectors). The advantages and disadvantages of co-infection and co-expression have been discussed in an earlier work from the Aucoin Lab (Sokolenko et al., 2012). Briefly, in the case of co-infection, gene expression can be

manipulated by varying the ratio of viruses to cells, also known as "multiplicity of infection" or "MOI". However, the probabilistic nature of infection means that all cells in the culture may not be infected; furthermore infected cells may be infected by different ratios of the different viruses. This may be sub-optimal for the production of VLPs and other protein complexes that require the production of different proteins within a single cell but not necessarily in equal proportions. While manipulation of gene expression is tedious in the case of co-expression, all infected cells will have all genes of interest, thus enabling all cells to produce a complete final product. Unfortunately, very few works to date have investigated ways to tailor expression levels in polycistronic vectors.

The expression of baculovirus genes in insect cells is controlled. Each gene is upstream of a different promoter, which serves as an "attractant" to the enzymes that are required for transcription to occur. Baculovirus promoters are classified into three groups according to their time of expression post-infection: early promoters, late promoters and very late promoters. Researchers have traditionally used the very late, and strong, polh and p10 promoters for driving expression of foreign genes as part of the BEVS. Although these promoters drive the gene expression at very high levels, these promoters are active at a time when infected cells are dying (Jarvis & Summers, 1989). Although, researchers have used promoters other than p10 or polh to drive foreign gene expression in insect cells (Bonning, et al, 1994a; Grabherr, et al, 1997), there has been no comprehensive study of how these promoters could be used in the context of multiple protein expression.

1.1 Hypothesis

The simultaneous production of multiple proteins in insect cells is thought to result in "competition" for cellular resources, which can reduce the amount of protein being produced. It is further believed that a reduction in such competition can be achieved by temporally separating expression of these co-

expressed genes using promoters active in different phases of the infection (early, late, very-late). The use of alternative promoters, which are active at different times is also hypothesized to yield controlled ratios of proteins produced.

1.2 Objectives

1. To generate five different baculovirus from plasmids prepared in our laboratory co-expressing GFP and RFP gene, where GFP is under control of different promoters (ie1, vcath, gp64, basic, or p10) while RFP always under control of the polh promoter, and characterize viral stocks through virus quantification assays.
2. To develop a protocol to use flow cytometry to track protein levels in cells, and track levels and timing of GFP and RFP production. To track GFP and RFP mRNA levels using reverse transcription and real time polymerase chain reaction (PCR), along with 28S rRNA as control.

An overall schematic of how the experiments were carried out to meet the objectives is given in Figure 1-2.

1.3 Thesis organization

While the first chapter of this thesis was a brief introduction, with the hypothesis and objectives of this research work, the second chapter of this thesis begins a detailed literature review of baculoviruses and BEVS, particularly of multiple protein production in co-infection and co-expression systems and use of alternative promoters for gene expression. Chapter 3 presents the materials and methods used throughout this work. Chapter 4 describes the work done to characterize the viral stocks to ensure that differences in virus stocks were not the cause of the differences observed in our experiments. In Chapter

5, protein production using the different viral vectors is studied using, primarily, flow cytometry, and the effect of using alternative promoters is examined. In Chapter 6, the effect of alternative promoter is investigated at the transcriptional level using reverse transcription real-time PCR.

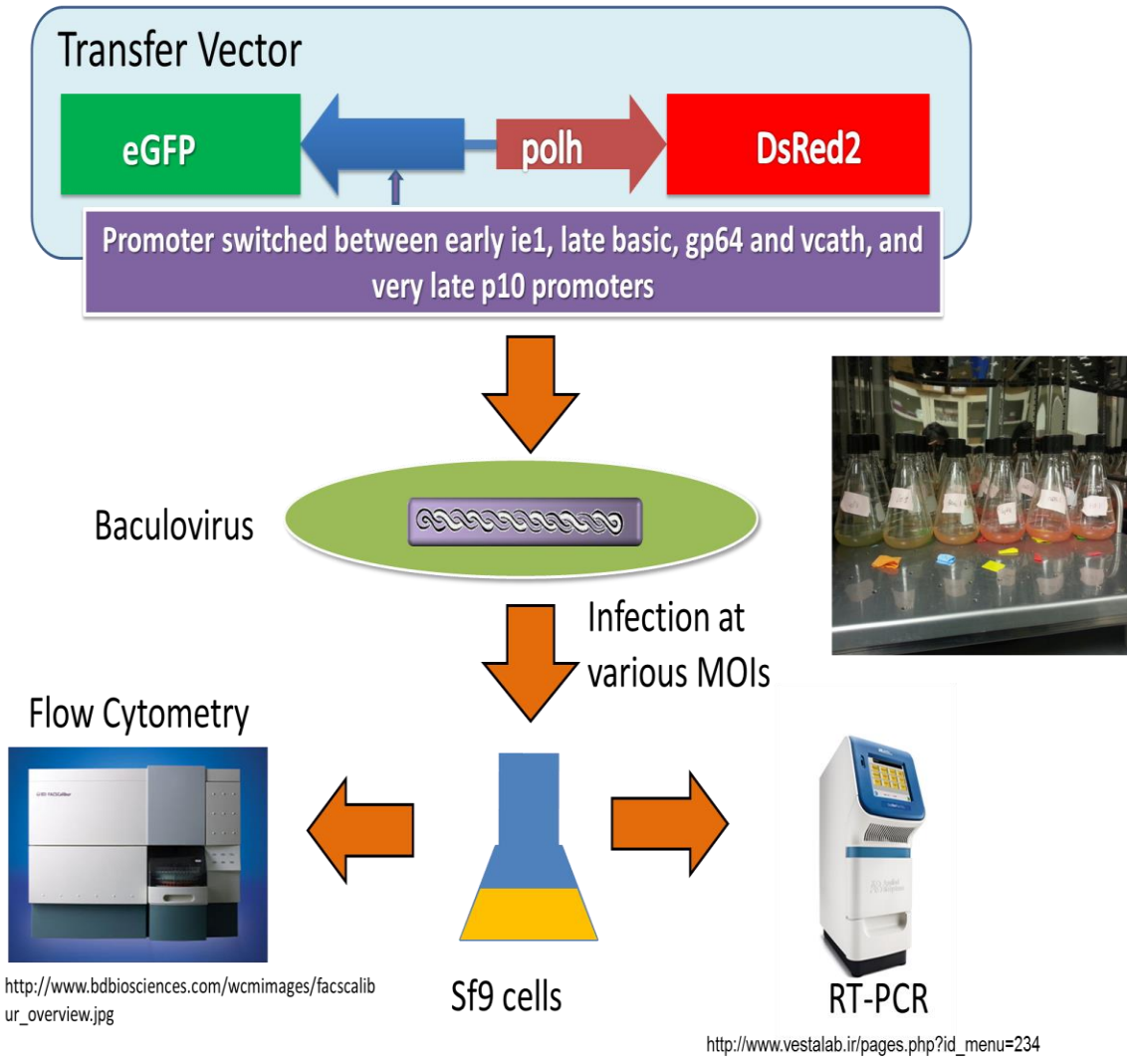


Figure 1-2: Schematic diagram of the experimental design and methodology for work presented in this thesis.

Chapter 2
Literature Review

2.1 Baculoviruses

Baculoviruses are a group of rod-shaped viruses with genomes made of double stranded, circular, supercoiled DNA. Baculovirus genomes are between 80-180 kb in size, encoding between 90-180 genes as reviewed elsewhere (Rohrmann & By, 2013). They are large viruses with sizes varying from 230-385 nm in length and 20-60 nm in diameter (Hill-Perkins & Possee, 1990). The infectivity of these viruses is restricted to the phylum Arthropoda, and most commonly to insect species of the order Lepidoptera (Lu & Miller, 1997a). Baculoviruses can also enter mammalian cells such as human, primate, rodent, fish and avian species but are incapable of replication and hence, are non-infectious to these species (Chen et al., 2011). There are two main types of baculoviruses: the nucleopolyhedroviruses (NPVs) have *polyhedra* consisting of single or multiple virions within a protein matrix, and the granuloviruses (GVs) consisting of one virion within smaller occlusion bodies called granules. NPV baculoviruses exist in two forms in their "life" cycle: the occluded form, in which the virus particles are embedded within a protein matrix composed mainly of a protein called Polyhedrin, and the budded form which derive their envelope when the virion buds through the host cell plasma membrane (as reviewed by Rohrmann & By, 2013). Occluded virions (OV) are efficient at infecting new hosts, but they are inefficient at spreading infection within insect tissue; budded virions (BV) are enveloped forms that are responsible for transmission within the cells and tissues of a host. Budded virions are produced late during the infection of a cell, while occluded virions are produced during the very late phase. The *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is the most extensively researched of the baculoviruses, and has been used for the present research work.

2.2 Baculovirus replication cycle

Baculovirus transmission within insect cell culture is mostly mediated by either absorptive endocytosis (Blissard et al., 1992; Volkman & Goldsmith, 1985), or by direct membrane fusion (Volkman & Goldsmith, 1985). Initially, baculoviruses release their nucleocapsid into the cell cytoplasm by fusing their membrane with the endosome membrane. The process is mediated by the baculovirus GP64 surface protein (Blissard & Wenz, 1992). Low pH and presence of endocytosis inhibitors can cause baculovirus entry by direct fusion (Dong et al., 2010). Nucleocapsids are transported into the nucleus through nuclear pores (Ohkawa, Volkman, & Welch, 2010). Transcription begins immediately after baculovirus DNA is released into the cell's nucleoplasm. The first genes to be expressed are immediate early (IE) or α class baculovirus genes. Baculovirus gene expression occurs in a temporal fashion, as successive gene expression depends on the products of each previous phase. NPV genes are categorized into α (immediate early) genes, β (delayed early) genes, ϒ (structural or late) genes, and temporally delayed and hyper-expressed late (very late) genes (Blissard & Rohrmann, 1990). Immediate early viral genes are transcribed utilizing host RNA Polymerase II. As infection progresses late and very late gene expression are mediated by a viral polymerase (Fuchs et al. 1983; Hoopes & Rohrmann, 1991; Huh & Weaver, 1990). Transcription, protein synthesis and initiation of viral DNA replication occurs within the first 6-8 hours post infection (hpi).

Baculovirus infection blocks cell cycle progression and prevents cells from undergoing mitosis. AcMNPV infection of *Spodoptera frugiperda* cells leads to reduction of host mRNA levels by 24 hpi (Nobiron, 2003). The shutoff of most host gene expression increases availability of host transcription and translation machinery e.g.: transcription factors, secretory pathway and post-translation processing. The other factor responsible for high level gene expression in BEVs is that expression of most baculovirus genes is short-lived, and this shut-off of viral gene expression frees up viral RNA

polymerase for transcription of very late phase genes such as polh and p10 (as reviewed by Rohrmann & By, 2013).

The production of anti-apoptotic factors, which offsets the apoptosis response induced in insect cells after infection, is an important characteristic of baculovirus infection in insect cells. The product of the *p35* gene is formed during the early and late phases of baculovirus infection and blocks the apoptotic response (Hershberger, et al., 1994), and is also capable of preventing apoptosis in a wide range of cell types. The baculovirus *iap* (inhibition to apoptosis) genes are also capable of blocking the apoptotic response and are expressed by baculoviruses (as reviewed by Rohrmann & By, 2013). Many proteins are responsible for cell death. Most notably are the caspases (Clem & Duckett, 1997). It is assumed that *p35* and *iap* function by inhibiting caspase activity inside insect cells (Bump et al., 1995).

2.3 Baculovirus gene expression and control

The baculovirus gene expression is divided into immediate early, early, late and very late expression. The characterization of phases of infection is defined by the types of genes expressed at times post infection. Early gene expression in baculovirus infected cells starts from about 30 min post infection (Grula, et al., 1981) to 6 hour post infection (hpi). Early genes can be further divided into an immediate early phase and a delayed early phase, with the genes expressed in the delayed early phase requiring the expression of immediate early phase genes. The progression of baculovirus gene expression depends on gene products generated during the previous stage of gene expression, with evidence indicating that the timing and level of early and late baculovirus gene expression is regulated at the level of transcription (Lu & Miller, 1997b). During baculovirus infection, viral protein production inside infected cells is a major indicator of the progression of infection, along with the fading of host protein expression.

Early genes expression is dependent on host RNA polymerase II (Grula et al., 1981) and associated transcription factors. Three baculovirus transregulators (IE0, IE1 and IE2) are produced during the early phase of expression, which helps in the transcription of viral genes. The IE1 is the most important transregulator for transcription and DNA replication (Kool, et al., 1994; Stewart, et al., 2005). Furthermore, while IE1 and IE0 are essential genes for virus replication, both of them can work independently. Baculoviruses containing only the IE1 gene have higher replication levels than baculoviruses having only the IE0 (Stewart et al., 2005). The effect of IE1 on transcription greatly increases when the IE1 is linked to homologous regions (*hrs*) (Guarino & Summers, 1986). The IE1 protein actively binds to the *hrs* without any requirement of additional insect cell proteins (Choi & Guarino, 1995; Rodems, et al., 1997). Other than host cell RNA polymerase II, two other DNA elements are necessary for transcription: a TATA promoter motif and a CAGT transcriptional initiation consensus sequence. (Blissard & Wenz, 1992; Blissard & Rohrmann, 1990; Rohrmann & By, 2013). The CAGT motif helps in the initiation of transcription (Rohrmann & By, 2013), while the TATA element specifies the binding loci for the TATA binding protein (TBP), which helps RNA polymerase II to initiate transcription in eukaryotes (Lu & Miller, 1997b). Expression of baculovirus early genes, for the most part, shutoff during the late phase of infection, though some early genes are expressed in both the early and late phase of infection.

The early and late phases of infection are separated by DNA replication. Viral DNA replication and the presence of baculovirus RNA polymerase define the late phase of baculovirus infection (Yolanda et al., 1983b; Guarino, et al., 1998). Baculovirus late and very late gene expression occurs with DNA replication and requires the presence baculovirus RNA polymerase, whereas early viral gene transcription is mediated by host cellular RNA polymerase II. (Fuchs et al., 1983; Guarino et al., 1998). Baculovirus polymerase requires a TAAG sequence element for transcription initiation of late and very late gene expression (Morris & Miller, 1994). Late promoters start with (A/G/T)TAAG (Lu & Miller,

1997b). Furthermore, it has been discovered that eighteen baculovirus genes, various late expression factors (LEFs), the protein in DNA polymerase complex, IE1 and IE2 are necessary for optimal late and very late baculovirus gene expression and DNA replication. A regulator element known as a "burst sequence" is present in very late promoters between the TAAG sequence and the translation initiation for very high levels of expression (Ooi et al., 1989). This sequence activates transcription by binding to the very late factor 1 (VLF-1) protein, which activates very-late baculovirus genes (Mistretta & Guarino, 2005; Yang & Miller, 1999).

Genes coding structural proteins, e.g.VP39, p6.9, major capsid protein and basic core protein, are transcribed during the late phase of infection. The *Gp-64* and *pp31* are also late genes. Their expression takes place around 6 to 24 hpi. The *p35* is a late gene, which is anti-apoptic in nature and helps with host cell viability as well as promotes viral replication and late transcription.

Polyhedrin and granulin genes encoding polyhedrin and granule proteins, and the *p10* gene, which initiates nuclear disintegration in the final stage of baculovirus infection, are some of the genes transcribed during the very late phase of infection (Lu & Miller, 1997b). The *polh* and *p10* are highly expressed genes. Very late gene expression switches on at 24 hpi with high levels of expression till about 72 hpi. The A/T rich sequence downstream from the *polh* and *p10* genes are involved in the high level gene expression of these genes (Ooi, et al., 1989).

2.4 The Utility of baculoviruses and BEVS

The first commercial use of baculoviruses were as highly selective insecticides due to their limited host range and high infectivity (Bonning & Hammock, 1996). Baculoviruses have also been used as gene therapy vectors, and for screening applications using baculovirus for surface display of protein. Most importantly, in the last 30 years, its use has been extended to vectors for recombinant protein production and gene transfer in insect cells, mammalian cells and in insect larvae.

When the first commercial insecticide products came to market during the 1970s, the technology suffered various problems due its lack of development as a biological system, as well as being less effective in comparison with field chemicals. Furthermore, the system had high processing cost. The development of baculovirus molecular biology in the 1990s enabled baculoviruses, to once again be considered as insecticides (Lu & Miller, 1997b). Baculovirus insecticides have been produced successfully *in-vivo* and *in-vitro*, with each method having its own advantages and disadvantages (discussed in greater detail by Lu & Miller, 1997b).

As an expression vector for production of recombinant proteins, baculoviruses are genetically modified to carry a foreign gene, which is ultimately expressed in an insect host. The ability to produce protein at remarkably high levels is one of the trademark features of the baculovirus expression system, and this property has been used for last two decades (Kost et al., 2005). The foreign gene is put under the control of a viral promoter, which allows it to be efficiently expressed in the insect host cells. The BEVS is largely used for recombinant protein production at the lab scale, and as a research tool to study complex protein production. This system also has great potential for commercial production of products such as therapeutic agents, vaccines, biopesticides, complex proteins and pharmaceutical products.

The main advantages of BEVS include: its ability to produce large quantities of protein as compared to other eukaryotic cells; the ability of insect cells to grow easily in suspension culture; the capability of insect cells to perform mammalian-like post-translational modifications; the high tolerance of insect cells to osmolarity changes and by-product concentrations; the large genome size of baculoviruses which can hold large gene insertions; and the ability to express multiple genes in one viral genome (polycistronic vectors) (Ikonomou et al., 2003). The ease of maintenance of insect cells and lower cost compared to mammalian cell culture makes the BEVS a competitive alternative to traditional mammalian based expression systems.

A broad range of insect cell lines are used for protein production, with the most common being *Spodoptera frugiperda* (Sf-9 and Sf-21) and *Trichoplusia ni* (High Five™ Cells) cell lines. Detailed reviews of cell lines used and studies comparing them are presented elsewhere (Aucoin et al., 2010; Ikonomou et al., 2003).

The ability to carry multiple genes make BEVS a suitable system to produce multiple sub-unit products such as VLPs and multiprotein complexes, as well as being suitable for the production of proteins that require "helper" proteins such as chaperones. The extent to which this system has been used to produce virus particle is discussed thoroughly elsewhere (Aucoin et al., 2007; Y. Hu, 2005; Kost et al., 2005).

2.5 The Limitations of BEVS

There are several characteristics of the BEVS that limit its protein production capability. These include: a lack of complete mammalian-like post-translation modifications; significant proteolytic activity in infected cells; and the breakdown of the secretory pathways during the late phase of baculovirus infection, as cellular protein processing pathways are disturbed due to infection.

2.5.1 Post-translational modifications

Post-translational processing capabilities in insect cells are similar, but not identical to mammalian cells, which are critical when products are therapeutic proteins and other complex proteins. Insect cell post-translational modifications differ from higher eukaryotes for processes such as glycosylation and proteolytic cleavage, and the end products are not always similar to those of higher eukaryotes. Protein processing pathways in host insect cells are also strained due to the cessation of host gene transcription and translation mentioned earlier in this thesis. The situation is further exacerbated by the large amount

of foreign protein being produced in the infected cell. Post-translational modifications can be especially problematic after 2-3 days post infection when the host machinery is highly disrupted. This is also the time when very late promoters are most active and viral gene expression is at a maximum. Although large quantities of protein is produced, expression late in the infection can lead to non-functional or inefficient production of protein that require post-translational modification.

2.5.1.1 Proteolysis

The release of proteases can affect the quantity and quality of recombinant protein produced using the BEVS. Proteases are produced by infected insect cells as a stress response during cell lysis, and by baculovirus to facilitate baculovirus release from the cell. The two main proteases produced in the BEVS are carboxyl proteases and cysteine proteases (Gotoh, et al., 2001). This protease activity can be blocked with the addition of protease inhibitors such as pepstatin A (carboxyl protease inhibitor) and E64 (cysteine protease inhibitor) (Gotoh et al., 2001). Other significant cysteine protease inhibitors that have been used include cystatin, leupaptin and antipain (Gotoh, et al., 2001) . Other proteases which are produced in the baculovirus-insect cell system are v-cathepsin, from the *vcath* baculovirus gene (Slack, et al., 1995), and chitinase, from the *chiA* baculovirus gene (Hawtin et al., 1995). The v-cathepsin and chitinase proteases along with the cellular carboxyl and cysteine proteases are produced during the late phase of baculovirus infection. The deletion of vcath and *chiA* gene from baculovirus vectors has been used to increase protein production (Fitzgerald et al., 2006). Proteolysis in the baculovirus-insect cell system and approaches to reduce proteolysis has been reviewed elsewhere (Gotoh, et al, 2002; Ikonomou et al., 2003)

2.5.1.2 The secretory pathway

The secretory pathway includes folding, cleavage, assembly and transport of newly formed polypeptides in eukaryotes and some forms in prokaryotes as well. Each of these secretory processes is

facilitated by cellular proteins. During the late phase of baculovirus infection, the secretory pathway is significantly perturbed and stressed due to the deterioration of host cell machinery and the large amount of recombinant protein being produced. This results in overproduction of insoluble protein that cannot be trafficked to the membrane for secretion (Ailor & Betenbaugh, 1999). One of the approaches used for overcoming this challenge is the co-production of cytosolic chaperones hsp70 (heat shocking protein 70), calreticulin (Kato et al., 2005), and binding immunoglobulin protein (BiP) (Hsu & Betenbaugh, 1997), which assist in translocating polypeptide strands into the endoplasmic reticulum and can suppress aggregation of these polypeptides by inhibiting nonspecific intermolecular hydrophobic interactions (Hartl, 1996). The use of chaperones to increase the solubility and secretion of proteins of interest in insects cells has been reviewed elsewhere (Ailor & Betenbaugh, 1999; Sokolenko et al., 2012). Changing expression times of secreted and membrane bound protein using early and late promoters, has also been studied for improvement of protein production system (Grabherr et al., 1997; Higgins, et al., 2003; Lawrie, at al., 1995).

2.5.2 Glycosylation

The lack of mammalian-like glycosylation processing has somewhat restricted the use of the baculovirus-insect cell expression vector system. Glycosylation is an important factor for the biological activity of human therapeutics and prophylactic products. Generally, glycosylation present in human proteins is different from those produced in insect cells (Palomares, et al., 2003). Insect cells differ in their ability to produce trimmed *N*-glycan moieties with galactose or sialic acid residues at their ends (Jarvis & Finn, 1995; Kulakosky et al., 1998). Mammalian cells produce more complex N-glycans with terminal sialic acid, while insect cells produce mostly N-glycans with terminal mannoses (Harrison & Jarvis, 2006). Additionally, N-glycosylation in BEVS can be inefficient, particularly during the late

phase of infection (Lu & Miller, 1997b). Insect cell lines such as Ea4 (Ogonah, et al., 1996), *Pseudalitia unipuncta* (A7S) and *Danus plexippus* (DpNI) are capable of producing more complex N-glycans than traditionally used cell lines such as Sf-9 and Sf-21 (Palomares et al., 2003). Glycosylation pathways in insect cells can also be changed and improved by the introduction of mammalian glycosyltransferases such as galactosyltransferases and sialyltransferases. The generation of a cell line containing five mammalian glycosyltransferases and two enzymes involved in sialic acid synthesis has been shown to produce glutathione S-transferase with mammalian-like glycosylation and sialylation (Aumiller, et al., 2003). Another strategy to affect glycosylation has been the production of the protein of interest during the early phase of infection by placing the gene under control of an early promoter (Jarvis, Weinkauf, & Guarino, 1996a).

2.6 Multiplicity of Infection

Baculovirus infection varies within a cell culture, even if each cell has been infected within that culture. The differences in how individual cells behave can be explained in part by the number of viruses that infect a given cell. The multiplicity of infection (MOI) is one of the main parameters that describe the infection of a culture. The MOI is defined as the ratio of infectious virus particles to the number of cells in the culture. The average number infectious virus particles a cell receives is defined by the probabilistic nature of virus infection as given by a Poisson distribution (Murhammer, 2007) – Equation 2-1.

Equation 2-1: Probability (*P*) that a cell absorbs "*n*" infectious particles.

$$P(n, MOI) = \frac{MOI^n . e^{-\mathrm{MOI}}}{n!}$$

In a low MOI infection (MOI<<1), a small portion of cells get infected, after which the viruses start replicating and produce more virus which later infects the other uninfected cells, causing a high MOI "secondary infection" in the culture. The timing and method to define a secondary infection is not perfectly understood because non-infected cells continually divide. Prediction of low MOI systems is not trivial. Because not all cells are infected with small MOIs, the system is described as having an asynchronous infection. At high MOIs, synchronous infection can be achieved. A MOI of 3 can set up a synchronous infection where almost all cells in the population have been infected by at least one virus particle. Usually high MOI experiments are performed for efficient protein production. For large scale productions, high MOIs can be problematic due to the need for large volumes of virus inoculum.

The passaging and amplification of virus stock can result in the generation of defective interfering particle (DIPs) (Kool, et al., 1991). DIPs can cause decrease in recombinant protein production and as such, amplification and passaging should always be done with small MOIs << 1 (Wickham et al., 1991). Using small MOIs ensures that only productive viruses are taken up and replicated in cells.

Whereas better baculoviruses are produced at low MOIs, recombinant protein production can be compromised due to greater chance of proteolytic degradation (Radford et al., 1997). By carefully adjusting the MOI and the time of infection (TOI), high recombinant protein production can be achieved with low MOIs, capitalizing on higher cell final cell densities. Protein yield from insect cell cultures has been shown to vary with MOI; however it is also linked to the growth phase of the cells at the time of infection (Licari & Bailey, 1992; Schopf, et al., 1990). For recombinant protein production in BEVS, insect cells are generally infected when they are in their exponential phase, with a viability above 95 percent. As recombinant protein production is highly dependent on time and multiplicity of infection, therefore, there is a need for proper virus stock characterization.

2.7 Multiple Protein Production within a Single Cell

The expression of multiple proteins is performed by using either multiple monocistronic vectors (co-infection), a single polycistronic vector (co-expression) or a combination of the two (monocistronic and polycistronic vectors). Each method has its own advantages and disadvantages. Co-infection systems involve infection of single cells with more than one type of virus, each type of viruses coding different genes. The use of multiple viruses to deliver genes of interest inside insect cells allows control of expression levels through the use of different MOIs for each virus type (Hu & Bentley, 2001). Unfortunately, the probabilistic nature of infection means that not all cells will receive the same proportion of viruses in each cell and this could greatly affect the final product. In co-expression systems, single baculoviruses can be engineered for expression of multiple genes, guarantying expression of the different proteins within a single cell. The main benefit of using a co-expression system is for products such as VLPs that can only be formed by producing two or more proteins within single cell. There are some problems, however, associated with co-expression systems such as the manipulation of expression levels for gene of interest. The benefit of co-expression over co-infection have been shown repeatedly, with higher yields beyond achieved with use of polycistronic vectors (Pushko et al., 2005a; Shanks & Lomonossoff, 2000; Vieira et al., 2005).

The ability of BEVS to express multiple genes makes it a remarkable platform for complex protein production like transcriptional factor, RNA polymerase complexes, antibodies (Song et al., 2010) and virus-like particles (Betenbaugh et al., 1995), as reviewed elsewhere (Sokolenko et al., 2012). A co-expression system was used for expression of the light and heavy chains of an antibody under the control of oppositely oriented polh promoters over 25 years ago (zu Putlitz et al., 1990). Polycistronic vectors containing p10 and polh promoters have been used repeatedly to drive expression of light and

heavy chains (Bès et al., 2001; Liang et al., 2001; Poul et al., 1995; Song et al., 2010). A co-infection system has also been used for antibody production (Shen et al., 2009). There are several virus-like particle produced using co-infection and co-expression systems examples: parvovirus by using a co-expression system consisting of a bicistronic vector (Brown et al., 1991); and the blue tongue virus using co-expression (French & Roy, 1990). Other examples of virus-like particles produced by using BEVS include: rotavirus; poliovirus, enterovirus 71; human papilloma virus (HPV); herpes simplex virus (HSV); as well as enveloped VLPs such as influenza; simian immunodeficiency virus; human immunodeficiency virus and several others (Sokolenko et al., 2012).

Tailoring the expression levels of different component proteins produced in the BEVS system can be important for several reasons. Levels of individual component proteins may affect the composition of the final multiprotein product, such as in the case of parvovirus-like particles (Tsao, et al., 1996). It may also be important when a helper protein is needed for the production of a final product, but is not itself a part of the final product. Moreover, in the case of products where production levels of component proteins do not affect the composition of the final product, overproduction of some protein can be waste and an unnecessary cellular resource depletion (Sokolenko et al., 2012).

2.8 Competition effect among expressed gene

Controlling times and levels of foreign gene expression in BEVS could be important to avoid "competition" between expressed genes for cellular resources. This has been suggested to happen when two or more genes are expressed at high levels at the same time within a single cell (Hitchman et al., 2010). These expressed genes can inhibit the expression of the other (Chaabihi et al., 1993; Hitchman

et al., 2010), which can be possibly due to depletion of cellular resources. These cellular resources can be transcriptional factors necessary for mRNA production, cellular amino acid, protein synthesis machinery, post-translation modification machinery, essential factors for virus replication and various cellular metabolites.

There are several pieces of evidence in the literature that support this hypothesis but researchers have not looked into this phenomenon in detail. For example, the p10 and polh very late and strong promoters which drive high levels of gene expression, compete for limited viral or host transcriptional factors, and p10 is found to compete more successfully (Lu & Miller, 1997b).Deletion of the p10 promoter causes increase in protein and mRNA production from genes under the control of the polh promoter, but deletion of polh promoter does not cause an increase in gene expression driven by the p10 promoter (Chaabihi et al., 1993; Hitchman et al., 2010). In addition, when chaperones and proteins of interest were studied in as co-expression systems, these systems tended to produce more final product than their co-infection system counter-parts (Kato et al., 2005; Tate et al., 1999). Moreover, the expression of calnexin along with another chaperone such as calreticulin, or immunoglobulin heavy chain binding immunoglobulin protein (BiP), caused a decrease in expression of the protein of interest, as compared to when only one chaperone was expressed. This may have been be due to an overload on cellular protein synthesis machinery when three gene were expressed simultaneously under very strong promoters (Tate et al., 1999). That study also pointed to the fact that the ratio of expressed genes may be an important factor in maximizing the production of a protein of interest (Kato et al., 2005).

A competition effect was also thought to occur in an AAV producing co-infection system in which three baculoviruses (BacCap, BacRep and BacITRGFP) were used. In that case, an increase in MOI of the BacRep virus caused decrease in the number of non-genome containing virus particles composed of proteins VP1, VP2 and VP3, from their BacCap virus. It was speculated to be as a result

of competition between the production of replication and capsid proteins from BacRep and BacCap, respectively (Aucoin et al., 2006).

The idea of a limited pool of resources for which different processes are required to compete, is also consistent with the fact that beyond a certain MOI, there is little to no benefit on protein production. Reduction in protein production occurred at an MOI of 50, when compared with an infection at an MOI of 0.05, 0.5 and 5 (Bédard, et al., 1994a).

2.9 Alternative promoters in BEVS

Traditionally the polh and p10 promoters have been used to drive protein expression in BEVS, as these are very strong. The polh promoter is the strongest known natural promoter and drives high levels of gene expression (Lu & Miller, 1997b). The p10 promoter is marginally weaker and drives expression slightly earlier than the polh promoter (Lu & Miller, 1997b). Both the polh and p10 promoters drive expression of non-essential genes (polh and p10 gene respectively) in cell culture and can be replaced with a gene of interest. Using these promoters does not affect baculovirus replication. Both of these promoters are active at a time when there is extensive breakdown of host post-translational modifications and secretion mechanisms of the cell, and there is high level of cellular and baculovirus proteases present in the system. Moreover, due to the large amount of recombinant protein production, host protein processing machinery may be overwhelmed during the very late phase of infection when genes are under the control of the p10 and polh promoters. Several groups have investigated "alternative" promoters that are active earlier in the baculovirus infection cycle.

Secretory pathway protein are produced at much lower level and some of them are biologically inactive and insoluble when expressed under control of polh promoter (Arp et al., 1993; Tsao et al.,

1990). The p6.9 promoter (Bonning, et al., 1994b; Chazenbalk & Rapoport, 1995; Lawrie et al., 1995; Rankl, et al., 1994) and ie1 (Jarvis, et al., 1996a) promoter are active in the early phase and have been used successfully to get active and soluble protein. In some cases, the use of alternative promoters which are active earlier than the polh promoter and produce less mRNA can eventually produce large amount of active and soluble protein of interest. Several researchers have investigated the use of various alternative promoters, all with different gene expression strength. For example "protein kinase C- δ" which requires processing through the secretory pathway are often biologically inactive and insoluble when expression is mediated by the polh promoter, while functional when produced under control p6.9 promoter (Arp et al., 1993; Rankl et al., 1994; T. Tsao et al., 1990).

The early and weak ie1 promoter can also be used to express more biologically active secreted proteins (Jarvis, et al., 1996b). Other promoters such as gp64, which drives expression of the baculovirus transmembrane Gp64 glycoprotein (Grabherr et al., 1997), and the basic promoter which drives expression of baculovirus p6.9 basic DNA binding protein, have also produced greater amounts of protein requiring secretion (Bonning et al., 1994b; Chazenbalk & Rapoport, 1995) and complex proteins requiring secretion (Higgins et al., 2003). The p6.9 promoter has been used to drive higher levels of juvenile hormone esterase (JHE) and β-galactosidase proteins than the p10 and polh promoters (Bonning et al., 1994b). Some research groups have also looked beyond native baculovirus promoters to control timing and expression level of genes of interest, and have looked at synthetic and modified promoters such as the Pcappolh, a hybrid of vp39 and polh promoters exhibiting both late and very late regulation (Thiem & Miller, 1990), tandem ie1 promoters (Kojima, et al., 2001), and synthetic early promoters (Blissard et al., 1992). Other synthetic promoters used in BEVS have included truncated ie1 and p10 promoters (Urabe et al., 2006; Urabe, et al., 2002) and constitutive insect promoters like the Hsp70 promoter (Lu, et al., 1996; Prikhod'ko et al., 1998). In addition, mutation of the polh promoter has produced stronger promoters than the parent polh promoter (Rankin, et al., 1988).

Expression of VLP component proteins under the control of earlier promoters can increase the expression levels of some protein of interest and so increase VLPs levels. In the production of simian immune deficiency virus (SIV) VLPs, consists of Gag and Env protein, expressing the Env glycoprotein under the control of an earlier hybrid promoter resulted in higher expression level of Env protein on the cell surface which resulted in increased levels of assembled VLPs as compared to when Env was under control of very late promoters (Yamshchikov et al., 1995).

Chapter 3
Materials and Methods

3.1 Cell Culture

Spodoptera frugiperda clonal isolate 9 (Sf-9) cells (GIBCO, Carlsbad, CA, USA) stored at -140 °C or below, at a density of $1x10^7$ cells in 1.5 ml cryogenic vials were used as the source of cells for this work. Once thawed, the Sf-9 cells were maintained in SF-900™ III SFM (GIBCO, Carlsbad, California, USA) serum-free media. Cells were incubated at 27 °C on an orbital shaker (VBR, Champaign, Illinois, USA) rotating at 130 revolutions per minute (rpm). Sf-9 cell cultures were maintained between $0.5x10^6$ to $4x10^6$ cells/ml in 125 ml capped glass Erlenmeyer flasks having a working volume of 30 mL. The viability of the maintenance cell culture was above 95 percent. Cell culture stocks used for virus quantification, infection, and transfection had viabilities above 97 percent. Cell counts and viability were examined using a hemacytometer and the common trypan blue exclusion method.

3.2 Baculovirus construct generation

The baculovirus constructs used for this work were generated in the Aucoin lab by Steve George (PhD candidate). DsRed2 is a red fluorescent protein (RFP) and eGFP is an enhanced green fluorescent protein (GFP). In this work RFP is always upstream (under the control) of the insect virus polh promoter, while GFP is under control of other insect virus promoters, namely: the ie1, vcath, gp64, basic and p10 promoters. Altogether, 5 different bicistronic baculovirus vectors were used in this work (Table 3-1).

Table 3-1: Baculovirus constructs used in the experiments shown in this paper. All baculoviruses have been generated from pAcUW51.

Construct Name	Description			
	Promoter 1	Gene 1	Promoter 2	Gene 2
p10	p10	*GFP*	polh	*RFP*
ie1	ie1	*GFP*	polh	*RFP*
basic	basic	*GFP*	polh	*RFP*
gp64	gp64	*GFP*	polh	*RFP*
vcath	vcath	*GFP*	polh	*RFP*

3.3 Baculoviruses generation and amplification

Generation of the recombinant baculoviruses was done using the BD BaculoGold™ Transfection Kit (BD Biosciences, San Diego, CA, USA), following the protocol recommended by the manufacturer. After generating the baculoviruses, amplification of viruses to generate a first passage stock (P1) was achieved by infecting Sf-9 cells at a density $0.5x10^6$ cells per ml in a 30 mL culture with 3 mL transfection virus volume. Both cell density and viability were analyzed every 24 hours. The infected cell cultures were allowed to grow until the viability of the cells dropped to 70%. When the cell culture viability dropped to 70%, the culture was subjected to centrifugation at 1000 × g for 10 min. The resulting supernatants were collected and used as P1 virus stocks. To have enough virus for all experiments, P2 stocks needed to be generated. For generation of P2 stocks, Sf-9 cells were infected at a density of $0.5x10^6$ cells per ml in a 30 mL culture with 1.5 mL of P1 virus stocks (assuming high titre of P1 stocks). The second passage virus stocks (P2) were generated for all viruses and quantified.

3.4 Quantification of baculovirus in cell culture supernatant using real-time PCR

The real-time PCR quantification method used in this work was developed in the Aucoin Lab (George, et al., 2012). Briefly, the viral DNA to be quantified was obtained by treating cell culture supernatant containing virus with Triton-X 100 solution (final concentration of 0.1% v/v). More specifically, all viral stocks were diluted to 10^{-2} using PBS (1X) solution and 3 µL of Triton-X 100 was added to 297 µL of diluted virus solution making the final concentration 0.1% (v/v). Samples were subjected to two freeze-thaw cycles by storing samples in a -80 °C freezer (VWR, Alberta, Canada) for 30 min, then thawing them in a 37°C water bath. The real-time PCR reaction for each sample consisted of 2 µL of sample, 900 nM of a forward and a reverse primer for a segment of the *Gp-64* gene,, 10 µL of 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Burlington, ON, Canada), and UltraPure™ DNase/RNase-Free Distilled Water (Life Technologies Inc., Burlington, ON, Canada), making a final volume of 20 µL. The 20 µL solutions were put in wells of a MicroAmp Fast Optical 96 well Reaction Plate (Life Technologies, Burlington, ON, Canada). The reaction conditions for the plate include an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturing at 95°C of 30 seconds and annealing/extension at 60 °C for 30 seconds. Following DNA amplification, a melt curve analysis was performed by heating the final mixture for 15 seconds at 95°C, followed by an annealing stage of 15 seconds at 60°C. The data was analyzed by StepOne™ Software v2.0 and the results were analyzed by comparing Ct value with standards (see Section 3.4.1).

3.4.1 Preparation of standard for real-time PCR quantification

A plasmid containing the *Gp-64* gene was used as a standard. Plasmid standards were stored in a -20 °C freezer. Plasmids were thawed at room temperature and then incubated for 10 min at 60 °C on a heating plate. Plasmid samples (167.8 ng/mL) were serially diluted from 10^{-1} to 10^{-9} concentration, in duplicate for the creation of the standard curve. Primers were designed by using the Express 3.0 software (Applied Biosystems, Warrington, UK) to amplify a 72-bp region of the AcMNPV *Gp-64* gene from a baculovirus vector (Table 3.2)

Table 3-2: Primers used for baculovirus detection.

Primer	Sequence (5' -3')
Gp-64 Forward	CGGCGTGAGTATGATTCTCAAA
Gp-64 Reverse	ATGAGCAGACACGCAGCTTTT

3.5 Baculovirus quantification using flow cytometry

The virus titration protocol using a flow cytometer was adopted from Shen, et al.(2002a).

Briefly, 16% (w/v) methanol free paraformaldehyde (p-formaldehyde) solutions (Thermo Fisher Scientific Inc., Mississauga, ON, Canada) diluted to 2% (v/v) using PBS (1X) solution was used to fix the samples (diluted in PBS); final concentration was 0.04% (v/v). The final p-formaldehyde samples were stored at 4°C for an hour for fixation to take place. The fixed samples were subjected to three freeze-thaw cycles. Following the freeze-thaw cycles, 10% (v/v) Triton-X 100 (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) in PBS was added to each sample to reach a final concentration of 0.1% (v/v), and left to incubate for 5 min before starting the staining step. SYBR® Green I Nucleic

Acid Gel Stain, 10000X concentration in DMSO reagent (Life Technologies Inc., Burlington, ON, Canada) was diluted to 1/50X in PBS solution, before being added to the sample to reach a final concentration of 0.2% (v/v). Stained samples were transferred to a 20-well VWR® Digital Dry Block Heater (VWR International, Mississauga, ON, Canada) and incubated for 10 min at 80 °C. After incubation samples were cooled at on ice, and transferred to 5 ml polystyrene tube (Bioscience Technology, Mississauga, ON, Canada) for analysis with the flow cytometer. The calibration of flow cytometer was done using a 3 μm (nominal diameter) polystyrene fluorospheres Flow-Set™ (Beckman Coulter Canada, Mississauga, ON, Canada) having a bead concentration of 1×10^6 fluorospheres/mL (nominal concentration). The fluorospheres emit fluorescence between 515-800 nm when excited at 488 nm. Virus samples were analyzed for 30 seconds based on cumulative count. Samples were diluted to a concentration of 3×10^4 particles/ml, to avoid coincidence. The virus titre was determined by relating the number of particles detected to the number of fluorospheres (see appendix A.3 for additional information).

3.6 Quantification of baculovirus using a growth cessation assay

Sf-9 cells (GIBCO, Carlsbad, CA, USA), seeded at 1×10^6 cells/ml, were maintained in capped glass Erlenmeyer flasks in SF-900III media (GIBCO, Carlsbad, CA, USA) at a temperature of 27 ℃ on an orbital shaker rotating at 130 rpm

Sf-9 cells were infected with various volumes (μL) of virus stock and their growth profile was monitored. Five different cell cultures were infected with 0.1μL, 1μL, 10μL, 100μL, and 1000μL. Cell counts were performed for defined time intervals and a time delay (td) for growth to cease was recorded. The time delay is defined as the time post-infection when growth of cells ceases due to infection. Time

delays are plotted against volume of virus stock used, and the intersection of the x-axis (i.e. volume of virus stock that gives a time delay of 0) is taken to be at an infection at a MOI of 3.

3.7 Quantification of baculovirus using an end-point dilution assay

Virus titre was also determined using an end-point dilution assay. Sf-9 cells (GIBCO, Carlsbad, CA, USA) were diluted to 2×10^5 cells/ml in SF-900™ III SFM media (GIBCO, Carlsbad, CA, USA), added to MicroWell™ 96-Well Microplates and allowed to attach for an hour. The virus containing samples were serially diluted tenfold in SF-900™ III SFM media (GIBCO, Carlsbad, CA, USA) and added to the cells in the microplates. The plates were gently rocked and kept in a humidified sealed plastic container. After 7 to 10 days post-infection, wells were examined for green or red fluorescence. Titres were calculated as detailed in Appendix A.2.

3.8 Cell culture/infection experiments used to evaluate expression of novel bicistronic baculovirus vectors

Sf9 cells were seeded in a 1L glass Erlenmeyer flask (250 ml working volume) at a density of $0.5x10^6$ cells/mL. The cells were allowed to enter their exponential growth phase and grow to ~$3x10^6$ cells/ml before being passaged to $1x10^6$ cells/mL in 125 mL glass Erlenmeyer flask flasks. Within an hour, cultures were infected with P2 virus stocks. In a first set of experiments, each individual culture was infected with an MOI of 5 of a specific vector (except for the control which did not receive virus). The working volume in the 125mL flasks for this experiment was 35mL. In a second set of experiments all individual cultures were infected with an MOI of 25. The working volume in the 125mL flasks for this experiment was 35mL. The baculovirus vectors used in both of these experiments were those described that were described in Table 3.1. All cultures were performed in triplicate. Sampling was conducted at 0, 4, 8, 12, 16, 24, 36, 48, 72 and 96 hours post-infection. Sampling were done for 10 time

points between 0-96 hpi and sample at each time point consisted of 1mL, leaving 25ml of culture by the end of the MOI=5 and MOI=25 experiments, respectively. Samples were used to determine cell density, cell viability, mRNA levels, protein production, and baculovirus production. More specifically, sampling was done frequently between 0 to 36 hpi to examine onset of protein production, as gene expression driven by the promoters under study starts between 4 to 36 hpi. Later time points (48-96 hpi) were chosen to examine overall protein and baculovirus production levels.

Each 1mL sample was split into two micro-centrifuge tubes (500 µL each). Cells were separated from the supernatant by centrifuging at 300g for 7 min. One of the cell pellets was immediately fixed and analyzed using flow cytometry. The other was resuspended in PBS and frozen at -70 ºC for mRNA analysis. Both supernatants were immediately stored at -70 ºC for subsequent baculovirus and fluorescence analysis.

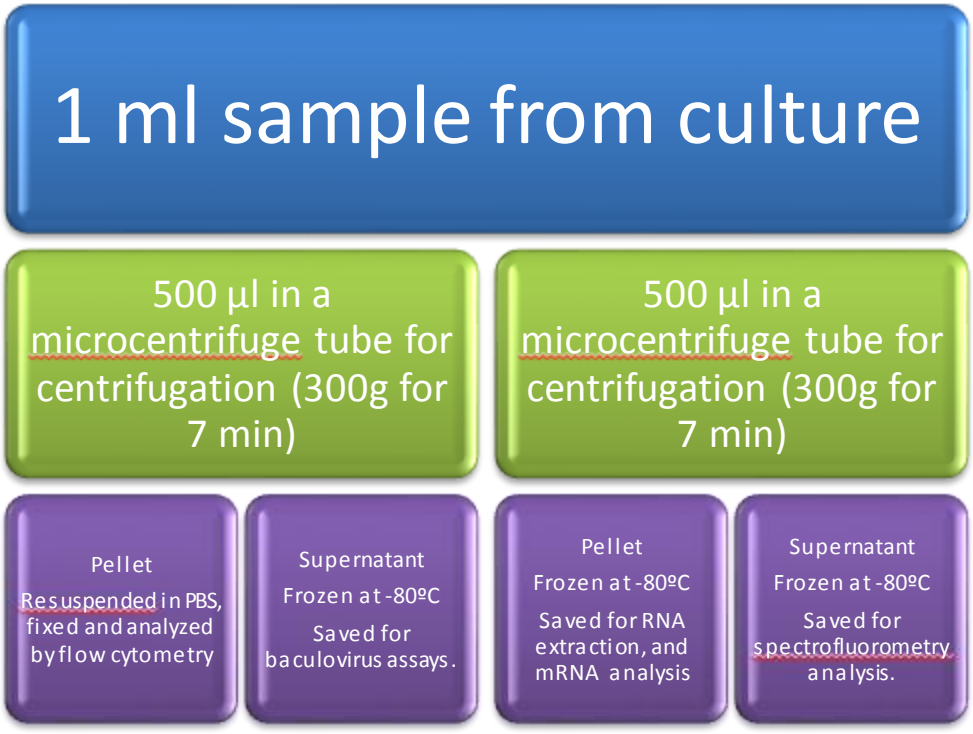


Figure 3-1: Schematic diagram of cell culture/ infection experiments used to evaluate expression of novel bicistronic baculovirus vectors.

3.9 Protein expression using flow cytometry

Infected Sf-9 cell cultures producing GFP and/or RFP were analyzed using a FACSCaliber Flow Cytometer (BD Biosciences, Mississauga, ON, Canada), equipped with a 15 milliwatt argon-ion laser. Red fluorescent carboxylate-modified microspheres 0.1 μm nominal diameter (2% Solid FluoSpheres®, Life Technologies, Burlington, ON, Canada) and Flow-Set™ Pro 3 μm nominal diameter Fluorospheres (Beckman Coulter Canada, Mississauga, ON, Canada) contains a dye which has a fluorescent emission range of 525 nm to 700 nm when excited at 488 nm were used to calibrate the detection of red and green fluorescence of the flow cytometer respectively.

Cell samples were treated with 2% formaldehyde in phosphate buffered saline (PBS) solutions and transferred to 5ml non-sterile disposable BD Falcon™ Polystyrene Round-Bottom Tubes (BD Biosciences, Mississauga, ON, Canada). All the samples were prepared in triplicate to reduce experimental error, and measured for 10000 events at a flow rate of 12 μL/min. The flow cytometry readings were collected using CellQuest Pro (BD Biosciences, Mississauga, ON, Canada) and analyzed using Flowjo (Treestar Inc., Asland, OR, United States). The system setting, instrument parameters, and significant variable used for the flow cytometry analysis are discussed further in Chapter 5.

3.9.1 Sample preparation for flow cytometer

Cell pellets collected at different time points during the culture were treated with 2% formaldehyde in phosphate buffered saline (PBS) solutions. Samples were stored at 4ºC for a minimum of 1 hour to ensure the fixation of the cells. Fixed samples were transferred to 5ml non-sterile disposable BD Falcon™ Polystyrene Round-Bottom Tubes (BD Biosciences, Mississauga, ON, Canada) and passed through the flow cytometer (BD Bioscience, Mississauga, ON, Canada).

3.9.2 Flow cytometry analysis

All samples were prepared in triplicate. 10000 cells were analyzed per sample. Samples were passed through the flow cytometer at a flow rate of 12 µL/min. The results were obtained using CellQuest Pro (BD Biosciences, Mississauga, ON, Canada) and subsequently analyzed using Flowjo (Treestar Inc., Ashland, OR, United States). Green fluorescence was detected by the FL1 detector (emission 530 nm, bandpass 30nm) and red fluorescence by the FL3 detector (emission 670 nm, longpass). A threshold based on forward-scattered light (FSC), which is proportional to cell-surface area or size, was used to capture events. FSC threshold was set at a value of 120, to eliminate unwanted noises from data acquisition. The side-scattered light (SSC), which is proportional to cell granularity or internal complexity, was also collected for all events. The SSC voltage was set at 200, FL1 at 310, FL2 at 350 and FL3 at 480 and remained constant throughout all experiments. Compensation was conducted to remove overflow of the GFP signal (FL1) into the RFP channel (FL3). Compensation was set at 11.5% of the FL2 (emission 564-606 nm).

3.10 Fluorescence spectroscopy

Fluorescence spectroscopy was conducted on samples of cell supernatant using a Synergy H4 Hybrid microplate reader (BioTek, Winooski, VT, USA). Briefly, 200 µl of supernatant were loaded into wells of a 96 well Nunc™ F96 microplate (Thermo Scientific, Rockford, IL, USA), and fluorescence was detected by the plate reader. Green fluorescence was measured with an excitation at 485 nm and emissions detected at 520 nm, while red fluorescence was measured with an excitation at 550 nm and emissions detected at 595 nm. A sensitivity of 75% was used for both readings.

3.11 Transcription tracking: quantifying mRNA levels

3.11.1 RNA extraction using TRIzol reagent

For quantitative analysis of gene transcripts, the first step involved RNA extraction from cell pellets. TRIzol (Life Technologies, Mississauga, ON, Canada) was used to homogenize cell pellets. The amount of TRIzol used depended on the cell density of the samples being processed (0.75 mL of TRIzol per $5x10^6$ cells). After cell lysing, 0.15 mL of chloroform was added for every 0.75 mL of TRIzol. Samples were shaken vigorously for 15 seconds by hand, incubated for 2-3 min, and then centrifuged at 12000g for 15 min at 4°C. The clear supernatant was transferred to a new centrifuge tube (while avoiding to draw any of the interface or organic layer) for RNA precipitation. 0.2 µL of RNase-free glycogen (Invitrogen, Life technologies, Burlington, ON, Canada), and 0.375 mL of 100% isopropanol were added for every 0.75 mL of TRIzol to the clear supernatant. Final solutions were incubated for 10 min at room temperature and then centrifuged at 12000g for 10 min. RNA was recovered by removing the supernatant without touching the pelleted RNA. In cases where the pellet was not visible, care was taken not to touch the walls of the centrifugation tubes. RNA was washed by adding 0.75 mL of 75% of ethanol for every 0.75 mL of TRIzol. The samples were briefly vortexed before being centrifuged at 7500g for 7 min at 4°C. The wash supernatant was discarded and the RNA pellets were air dried for 5-10 min without allowing the RNA to dry completely. The dried RNA pellets were resuspended in RNase-free water and incubated in a heating block at 55-60°C for 10 min. The samples were either used for analysis or stored at -70°C.

3.11.2 Reverse transcription

Reverse transcription was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Burlington, ON, Canada). The protocol used was the one recommended by the

manufacturer. Briefly, the RNA samples were diluted 1 in 50 in RNase free water (GIBCO, Invitrogen, Grand Island, NY, USA) before running the reverse transcription reaction. 2µl of RNA sample was mixed with 2 µL of 10 × RT Buffer, 0.8 µL of 25 × dNTP mix, 2.0 µL of 28S-R primer at a concentration of 25 µM, 2.0 µL of eGFP-R primer having a concentration of 900 µM, 2.0 µL of DsRed2-R primer having a concentration of 900 µM, 1.0 µL Multiscribe™ Reverse Transcriptase and 8.2 µl nuclease-free water, for a total reaction volume of 20 µl. The conversion of all three transcripts of interest (GFP, RFP and 28S rRNA) were done in the same tube to avoid run-to-run experimental error and maintain uniformity among the reactions. The samples were then placed in a VeritiTM 96 Well Thermal Cycler (Applied Biosystems, Burlington, ON, Canada) and run at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. These samples were then stored at -20°C until further analysis with real-time PCR.

28S rRNA was used as a house keeping element to control for sample to sample variability. Reverse primers have been described by others (Xue, et al., 2010a). GFP RNA, RFP RNA and 28S rRNA were converted to cDNA using specific reverse primers (Bustin, 2000a). The reverse primers used to convert 28S rRNA, GFP RNA and RFP RNA into cDNA are given in Table 3-3.

Table 3-3: Primers (Integrated DNA Technologies, Coralville, IA, USA) used in reverse transcription study.

Description	Primer sequence	Primer name
Primer for 28SrRNA	5′-GCAACGACAAGCCATCAGTA-3′	28S rRNA-R

Primer for eGFP	5' - ACTGGGTGCTCAGGTAGTGG - 3'	GFP-R
Primer for DsRed2	5' - CAGCCCATGGTCTTCTTCTG - 3'	RFP-R

3.11.3 Real time PCR for transcript quantification

Quantitative real time PCR was used to amplify cDNA samples from the reverse transcription reaction. The PCR reactions were conducted using a StepOne Plus Real-Time system (Applied Biosystems, Burlington, ON, Canada) and were prepared in MicroAmp Fast Optical 96 well reaction plates (Applied Biosystems, Burlington, ON, Canada). Each transcript was analyzed through separate reactions with separate master mixes. Each reaction consisted of 2 µl of cDNA sample mixed with 900 µM final concentration of reverse and forward primer, 10 µl of 2X Power SYBER® Green PCR Master Mix (Applied Biosystems, Burlington, ON, Canada), and 6.56 µl of nuclease free water (GIBCO, Invitrogen, Grand Island, NY, USA) making a final volume of 20 µl. Table 3-4 lists the GFP, RFP and 28S rRNA forward and reverse primers used in this study. The master mix to amplify GFP, RFP and 28S rRNA gene transcripts was prepared separately and then mixed with cDNA samples in a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, Burlington, ON, Canada). The reaction plate was sealed using MicroAmp Optical Adhesive Film (Applied Biosystems, Burlington, ON, Canada), and then centrifuged briefly for 3-4 seconds in an Eppendorf 5804R centrifuge (Eppendorf Canada, Mississauga, ON, Canada) with an A2-DWP flat plate rotor (Eppendorf Canada, Mississauga, ON, Canada). The amplification of individual cDNA was conducted using specific reverse and forward primers, and specific cycling conditions. Each sample was run in triplicate to ensure statistical validity and confidence in the data obtained.

The initial denaturation was run at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 5 seconds, annealing at 58°C for 20s and extension at 72°C for 20s. Melt curve analysis, for distinguishing the different species of DNA amplified during the reaction, was conducted by holding samples at 55°C for 10 seconds and then increasing the temperature in 0.3°C increments in 15s to 95°C, with fluorescence being measured during the ramping stage. The reaction conditions were previously described in the literature (Xue et al., 2010b) with one minor alteration. The annealing temperature was changed from 55°C as described in the literature to 58°C to improve tracking of the transcripts at early times post-infection.

The amplification curve obtained by the real-time PCR was analyzed using the StepOne™ Software v2.0. Further analysis was performed using LinReg PCR (Ruijter et al., 2009) to obtain the efficiency of individual reactions. GFP and RFP transcript levels were normalized to the 28S rRNA levels to control for experimental error associated with RNA extraction.

Table 3-4: Primers (Integrated DNA Technologies, Coralville, IA, USA) used in real-time PCR work.

Description	Primer sequence	Primer name

Primers for amplifying the 28SrRNA gene	5′-GCAACGACAAGCCATCAGTA-3′ 5′-CGACGTTGCTTTTTGATCCT-3′	28SrRNA-R 28SrRNA-F
Primers for amplifying the eGFP gene	5' - ACTGGGTGCTCAGGTAGTGG - 3' 5' - ACAAGCAGAAGAACGGCATC - 3'	eGFP-R eGFP-F
Primer for amplifying the DsRed2 gene	5' - CAGCCCATGGTCTTCTTCTG - 3' 5' - CGGCTGCTTCATCTACAAGG - 3'	DsRed2-R DsRed2-F

Chapter 4
Virus Stock Characterization

4.1 Introduction

Multiplicity of infection (MOI) is an important parameter that plays a critical role in recombinant protein production. The quantification of baculoviruses is very important to establish reproducible MOIs. Quantification can be established as the number of infectious virus particles or the total number of virus particle. Infectious virus particles are those that can carry out a productive infection cycle, and can be determined using plaque assay, end-point dilution assay and or growth cessation assay. These infectivity assays are labor intensive and time consuming (Roldão, et al., 2009). Total virus particle assays do not account for functional virus. Assays for total particles rely on detecting the physical attributes of the virus and can include: genome quantification by PCR, and/or flow cytometry; viral structural protein detection by western blot or ELISA; and full virion imaging by electron microscopy.

The use of native promoters to drive the expression of the genes of interest in the novel bicistronic vectors presented in this work were speculated to possibly cause a perturbation in transcription of native *ie1*, *gp64*, *vcath* or *basic* genes because these genetic elements were now repeated in the genome. As the genes, that these promoters natively control are essential for baculovirus replication, hindering the expression of these genes could reduce baculovirus replication, and consequently lead to a decrease in baculovirus particles, baculovirus genomes, or viable virus particles that are produced.

Baculovirus replication was assessed by genome counts using RT-PCR and flow cytometry, while infectious virus particles were assessed using an end-point dilution assay and a growth cessation assay. The virus stocks were quantified with a number of different methods, reflecting the importance

of the virus stock characterization to this work. To compare the effect of the promoters, there was a need to make sure the virus vectors were as similar as possible, in terms of their quality (infectiousness, potency, etc). Before starting this work, it was unclear whether using native baculovirus promoters to drive the expression of the gene of interest (GFP), would interfere with the expression of the native gene (given that a second copy of the promoter was now present).

4.2 Results and Discussions

Second passage (P2) virus stock were generated, quantified and used for protein production in insect cells. Passaging of virus stock involves the progressive amplification of virus stocks through a cell culture. P2 stocks were generated to have enough virus to conduct all of the experiments described in this work.

4.2.1 Comparison of virus titre with different methods

The titres obtained from real time PCR and flow cytometry (FC) reflect the concentration of virus genomes, which are much higher than the values obtained from the end point dilution and growth cessation assays, both of which count infectious virus particles. Both real-time PCR and flow cytometry detect "stained" genomes. In the flow cytometry protocol, the SYBR green reagent binds to double stranded DNA but does not account for any specific genome sequence. In real-time PCR, a specific DNA sequence is amplified using specific DNA primers. The DNA is continuously being stained with SYBR green reagent throughout the PCR amplification. Titres obtained using real-time PCR are of genomes containing a specific gene sequence. The protocol to prepare samples for quantification using real- time PCR is derived from a previously published method for sample preparation for quantification using flow cytometry (George et al., 2012; Shen et al., 2002a). As a result, it is expected that the values

obtained for either method should be comparable. As seen in Figure 4-1, this is indeed the case. On the other hand, the growth cessation assay and end point dilution assay look at infectious virus titres. The growth cessation assay looks at when all cells cease to divide, whereas the end-point dilution assay looks at the cytopathic effect on the cells i.e. the damage to host cells caused by virus infection. Again, because both of these assays examined the infectious nature of the vectors, it was expected that the values obtained from each of these assays would be comparable. The values obtained for the infectious assays were all similar and approximately one order of magnitude less than the assays used to determine the viral genome concentrations.

The differences in titres between these methods are hypothesized to be because only a portion of the total number of virus genomes produced in a system is infectious, and many of the budded viruses are non-infectious.

A more in-depth look (Figure 4.2), reveals that certain constructs might have larger differences between the infectious particle count and total particle count (genome count). The vcath and gp64 constructs both have infectious virus titres almost 30 times lower than the total number of particles. This is about 3 times larger than most other constructs. Given the variability of the assays, this difference may not be significant (significance could not be assessed because of the number of replicates run).

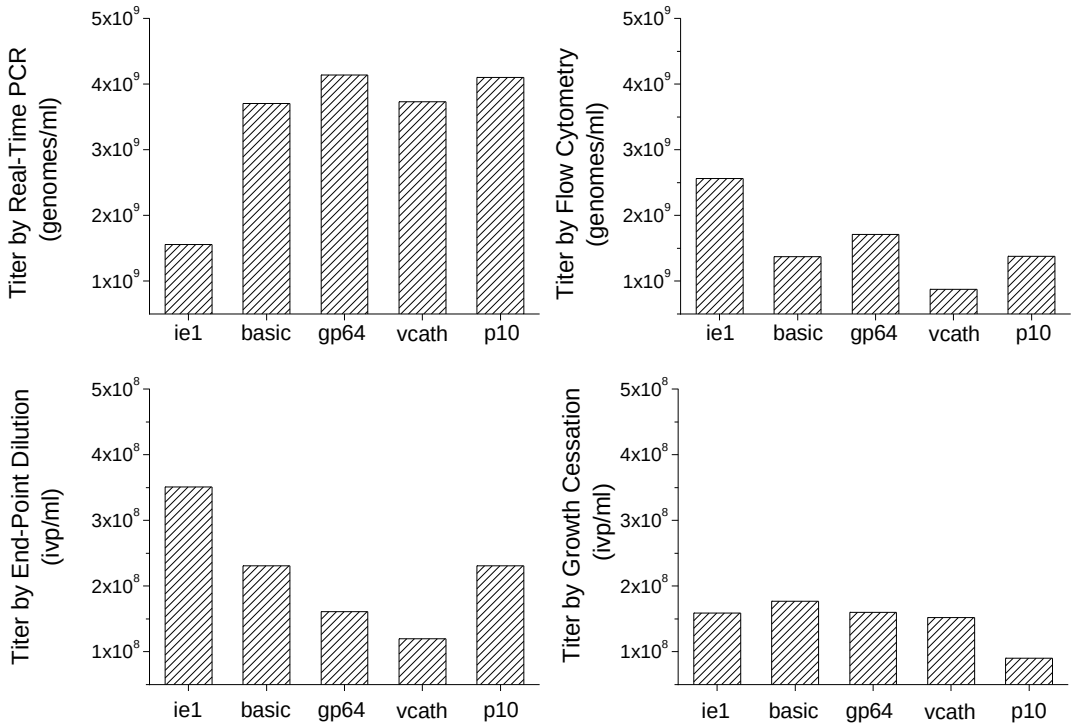


Figure 4-1: Titres obtained using four different methods to quantify the five baculovirus vectors used in this study. The constructs were: p10; basic; vcath; gp64 and ie1. Real-time PCR titres and flow cytometry titres are in virus genomes/mL; end-point dilution assay titres and growth cessation assay titres are expressed in infectious virus particles/mL, often used interchangeably with plaque forming units (pfu/mL).

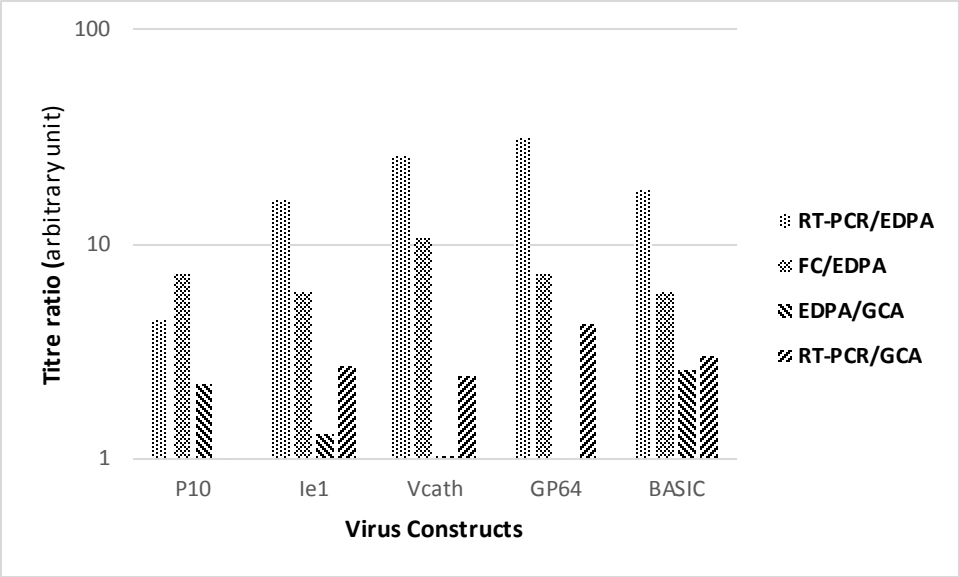


Figure 4-2: Plot comparing titre ratios between different baculovirus constructs.

Recombinant protein production significantly depends on MOI, which depends on the infectious virus particles in the culture. Given that the vectors used in this work enable the production of fluorescent protein, infection is easily detectable with a fluorescence microscope. Because the EDPA readily uses this protein fluorescence as a marker, and because the assay inherently involves replication of results, the values obtained using the EPDA were used as a basis to determine amount of virus solution added to the insect cell cultures for all subsequent experiments.

4.2.2 Baculovirus genome tracking in supernatant at different hour post infection

Given that there was no significant differences observed in the viral stocks generated, a comparative study was done on how these viruses behaved when they were added to cultures in equal amount. To assess if the use of alternative promoters affected the infectious nature of the baculovirus or baculovirus replication, the number of virus genomes in the culture supernatant was monitored over time after infection using real-time PCR (Section 3.4). Using this methodology, and sampling scheme,

both the uptake and release of baculovirus vectors could be clearly observed (Figure 4.3). Genome counts increase during the later time points due to replication inside cells and virus particles are progressively released in the supernatant (Figure 4.3). A similar pattern was observed for all the different virus constructs for experiments at both MOIs. The p10 construct showed a comparatively higher genome concentration at 72hpi for the MOI of 5 experiment, while the same pattern was not seen for the MOI of 25 experiment (Figure 4.3). Further experimentation (data not shown) revealed that this was most likely an experimental artifact.

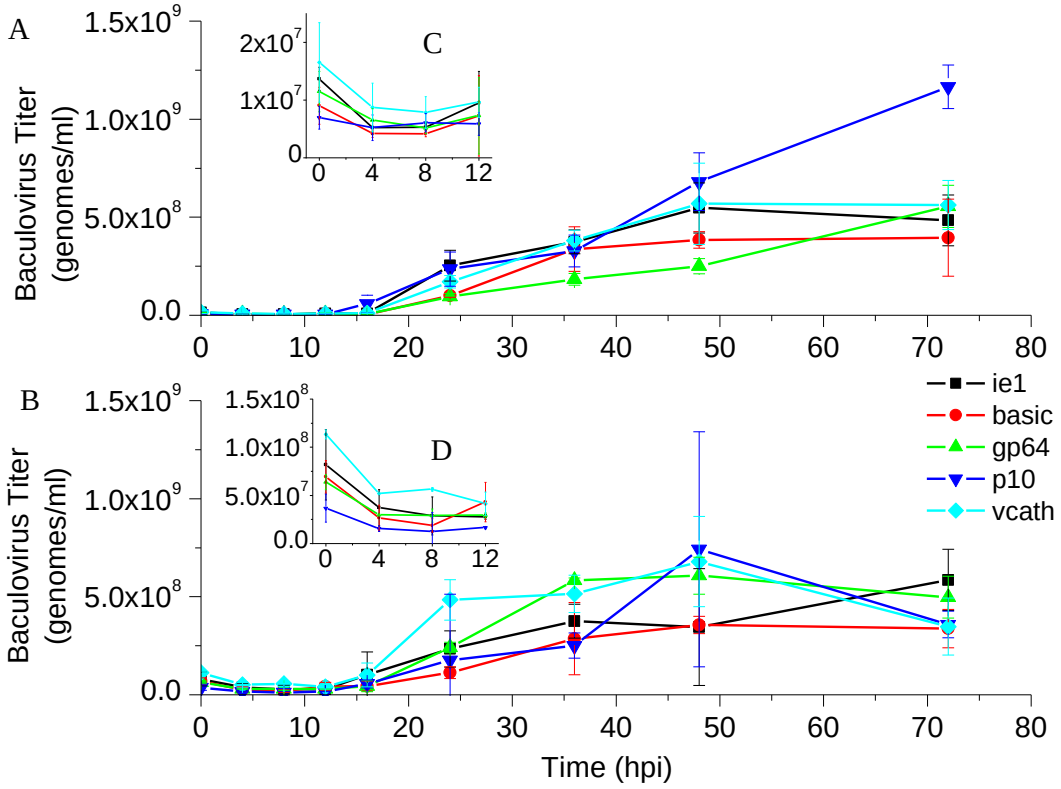


Figure 4-3: Genome counts in baculovirus infected culture supernatants over time, as determined by using real-time PCR. The various constructs used were: p10; basic; vcath; gp64 and ie1. Figures (a) and (b) represent baculovirus genome concentrations in culture supernatant from 0 to 72 hours post infection during experiments where cultures were infected at an MOI of 5 and 25 respectively. Figures (c) and (d) represent genome concentrations at 0 – 12 hours of cultures infected at MOIs of 5 and 25 respectively

Virus uptake of different constructs in infected cells was calculated using Equation 4-1. For all MOIs, the average uptake varied between 50 and 70% (Table 4-1). For the MOI of 5 experiments, the number of vectors taken up by the cells was approximately 10^6 particles. When the MOI was increased from 5 to 25, the number of virus taken up by the cells increased by a factor of 10 on average. It is interesting to note that the number of viruses taken up (based on genome quantification) is similar to

the actual MOI that was administered to the cells (based on infectious counts), indicating that indeed the extra genomes counted are of non-infectious particles.

Equation 4-1: Virus uptake rate of infected cells

$$\%\ \text{vector particle taken up by cells} = \frac{Initial\ genome\ count - Genome\ count\ at\ 4\ hpi}{Initial\ genome\ count}$$

Table 4-1: Average percent of vector taken up in co-expression experiments at MOIs of 5 and 25.

Vector	% Vector/Number of Vector Particles Taken Up By Cells			
	MOI = 5	MOI = 5	MOI = 25	MOI = 25
p10	25%[1]	$1.76x10^6$	66%	$2.13x10^7$
ie1	62%	$8.43x10^6$	73%	$4.47x10^7$
Basic	67%	$4.86x10^6$	54%	$4.28x10^7$
gp64	55%	$4.95x10^6$	66%	$3.39x10^7$
Vcath	52%	$7.77x10^6$	64%	$6.15x10^7$

In order to characterize the infectivity of the virus produced, when infected with equivalent amounts of virus stock (MOI=5), supernatant collected at 48 hpi was compared using EDPA. There was no significant difference observed in viable viruses produced in the supernatant of cultures infected with different constructs (Figure 4.4).

[1] Note: upon repeated experiments (data not shown), this value was never observed again.

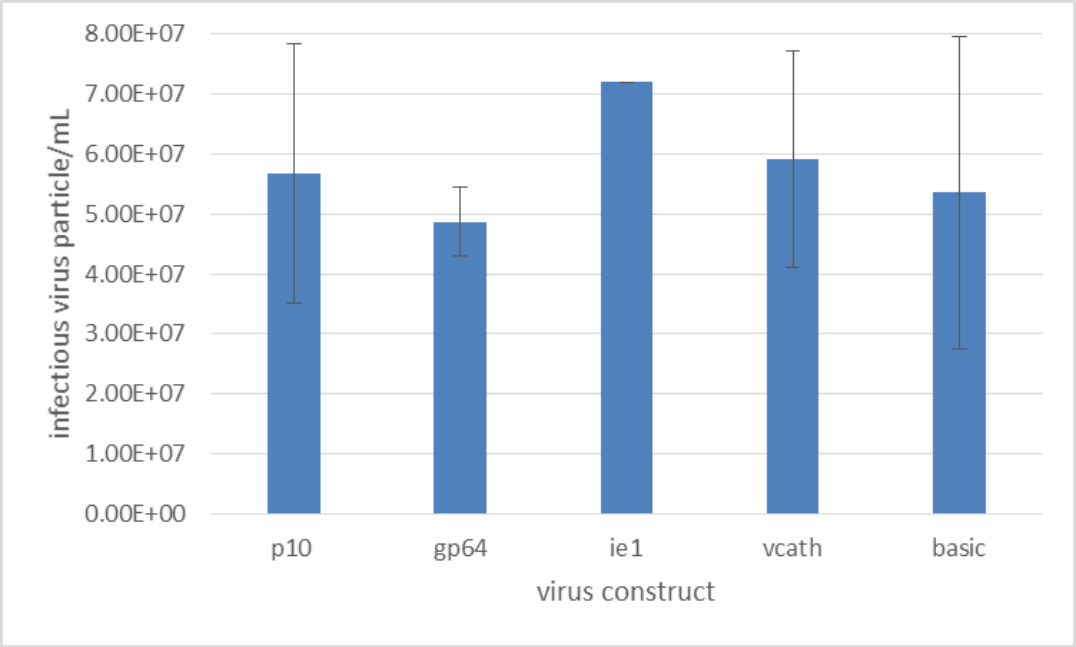


Figure 4-4: pfu/ml counts for baculovirus in infected culture supernatants at MOI=5 for 48 hpi. The various constructs used are: : p10; basic; vcath; gp64 and ie1, with the bar graphs representing the average of counts for two replicate flasks, and the error bars representing one standard deviation between replicate flask.

4.2.3 Conclusions

Virus stock characterization protein production depends, in part, on the ability of the virus vector to replicate inside cells. Furthermore, from an overall process standpoint, where virus amplification would be necessary for large productions, there is an inherent need to have fully replication-competent virus vectors. It was not clear at the onset of this work if doubling the number of instances of a native baculovirus promoter in the vector genome could cause a hindrance with the expression of the native gene or with the replication capacity of the vectors. In order to examine these effects, extensive characterization of the virus stocks was undertaken. No significant differences in the virus stocks were observed. This result enabled us to confidently probe the effect of promoter use on the production of protein.

Chapter 5
Determining the effect of non-conventional promoters by tracking Protein expression level using Flow cytometer

5.1 Introduction

Co-expression systems generally rely on the very late p10 and polyhedron (polh) promoters to drive expression of foreign genes. Because these promoters trigger expression at almost the same time and with almost equal strength, they do not provide much opportunity for tailoring gene expression ratios within cells. Many researchers have already looked into the use of alternative promoters in co-expression systems, with two patents considering use of weak baculovirus promoters for production non-structural protein for virus-like particles production (Oker-Blom & Summers, 1992; Hu and Lin, 2013). However, there is not much characterization of alternative promoter that has been done to carefully modulate protein expression ratios, which would be the next step in implementing a "designed" protein expression system for the production of complex proteins. This chapter aims to demonstrate the use of different promoters to control the timing and expression of two easily traceable fluorescent proteins, green fluorescent protein (GFP) and red fluorescent protein (RFP), in a co-expression system using BEVS. In this work, the RFP gene was placed under the control of the very-late polh promoter, while the GFP gene was placed under the control of either an early (ie1), a late (basic, gp64 or vcath) or a very-late (p10) promoter, and the expression levels of these two proteins in cells was tracked using flow cytometry.

5.2 Results

5.2.1 Progression of infection: cell density and viability tracking

Figure 5-1 tracks cell density and viability over time at MOIs of 5 and 25. Both sets of experiment resulted in synchronous infections as seen by the cessation of growth (Figure 5-1); i.e., there was no increase in cell density after virus was added. Regardless of the vectors, viabilities of the cells remained high (above 90%) until 36 hpi, after which there was a significant drop in viabilities observed. Depending on the vector used, there was a significant difference in the viabilities of the cultures at 72 hpi (Figure 5.1). When an MOI of 5 was used, the ie1 and basic vectors showed the highest viability, while vcath and gp64 vectors showed viabilities between basic and p10 vectors, and the p10 vector showed the lowest viabilities (Figure 5-2). The same trend was observed when an MOI of 25 was used (Figure 5-2), with slightly earlier drops in viabilities (48 hpi). While, comparing viabilities between experiments conducted with MOIs of 5 and 25, those conducted at an MOI of 25 showed bigger differences in viabilities between the cultures infected with the different vectors.

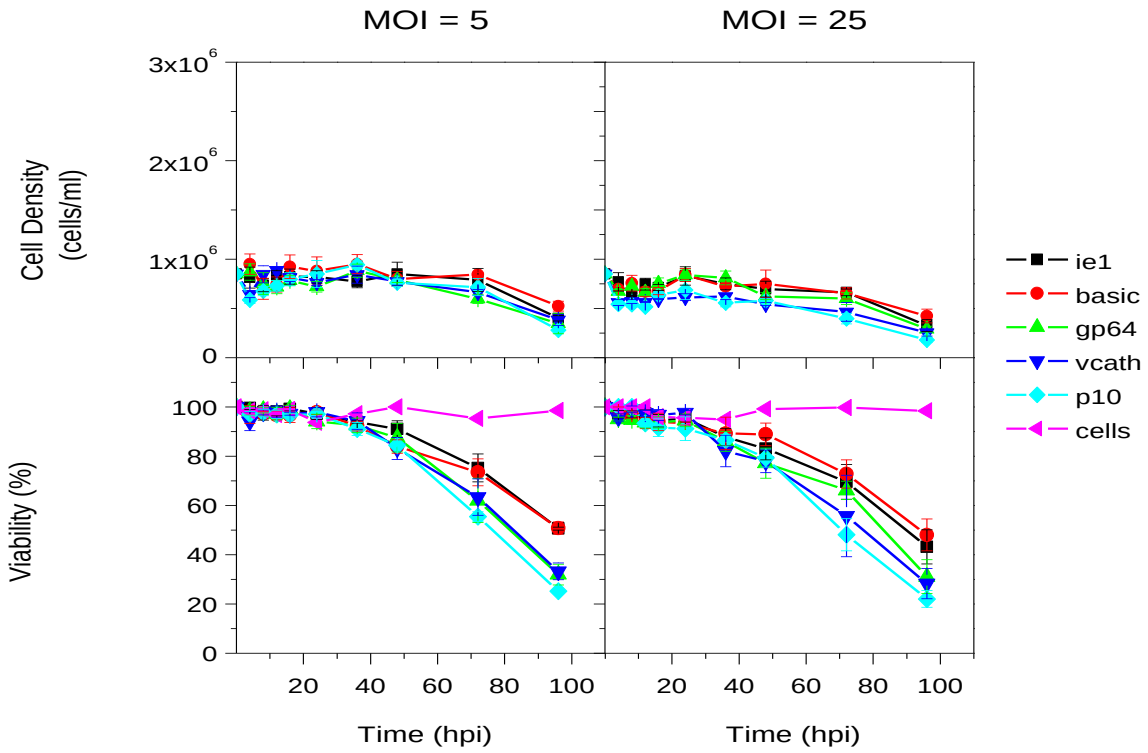


Figure 5-1: Tracking cell densities and cell viabilities of infected cell culture during course of time post infection. The 35mL Cell cultures were infected in triplicate at a MOI of 5 or 25 with various baculovirus vectors (p10; basic; vcath; gp64 and ie1). Error bars represent the standard deviation of three replicate cultures (n=3).

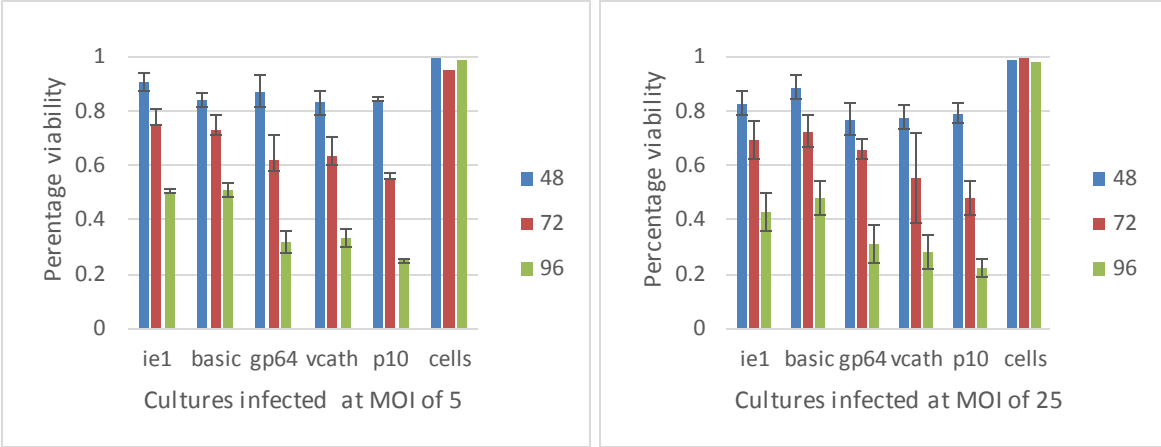


Figure 5-2: Tracking cell viabilities of infected cell cultures at 48, 72 and 96 hpi. The 35mL cell cultures were infected in triplicates at a MOIs of 5 and 25 with various baculovirus vectors (p10; basic; vcath; gp64 and ie1). Error bars represent the standard deviation of three replicate cultures (n=3).

5.2.2 Flow cytometric analysis of GFP and RFP fluorescence in cells: methods and observations

5.2.2.1 Use of a monocistronic baculovirus vector expressing GFP to evaluate signal bleed over

While analyzing both GFP and RFP fluorescence levels in insect cells with the flow cytometer using the FL1 and FL3 channels, the long emission spectrum tail of the green fluorescence detected in the FL1 channel bleeds over into the FL3 channel used to detect the RFP signal. Therefore, the signal obtained the FL3 is not an absolute signal from the RFP produced, but a combined signal from GFP and RFP. Figure 5-3 represents bleeding of FL1 into FL2 channel (denoted by A) and bleeding of FL2 into FL1 channel (denoted by B). If one can estimate the level of bleed over i.e. area A, then a "compensation" factor can be applied to obtain a more accurate signal in the channel of interest i.e. FL2 (in the example given in 5-3) or FL3 in the work presented here.

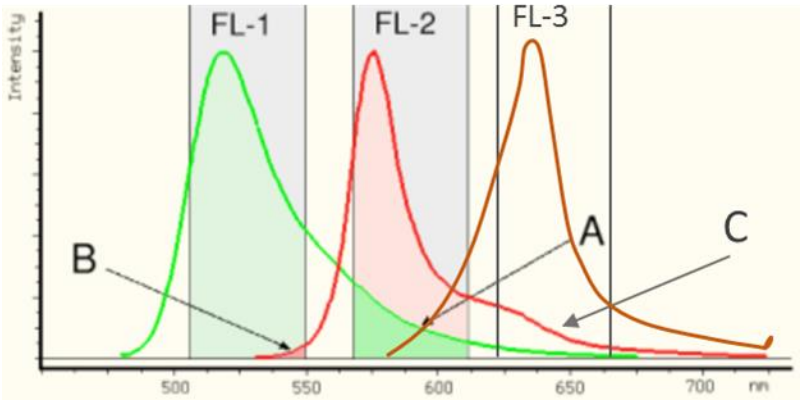


Figure 5-3: Diagrammatic representation of bleeding of FL1 and FL2 into each other.

(*COMPENSATION MIT Flow Cytometry Core Facility*, n.d.).

In this work, a monocistronic baculovirus vector that carries the GFP gene was used to establish the degree of compensation needed for the FL3 signal. Evidence of bleed over was evident with signal being captured in the FL3 channel without the expression of RFP (Figure 5.4). This meant that signal produced by the fluorescence of GFP bled through the FL2 channel and into the FL3 channel. Compensation for FL3 signal was therefore done as a percentage of the signal captured in the FL2 (there is no direct method to compensate FL3 as a percentage of FL1). Using a compensation factor of 11.5% (of the signal in the FL2 channel) eliminated the bleed over (Figure 5.4).

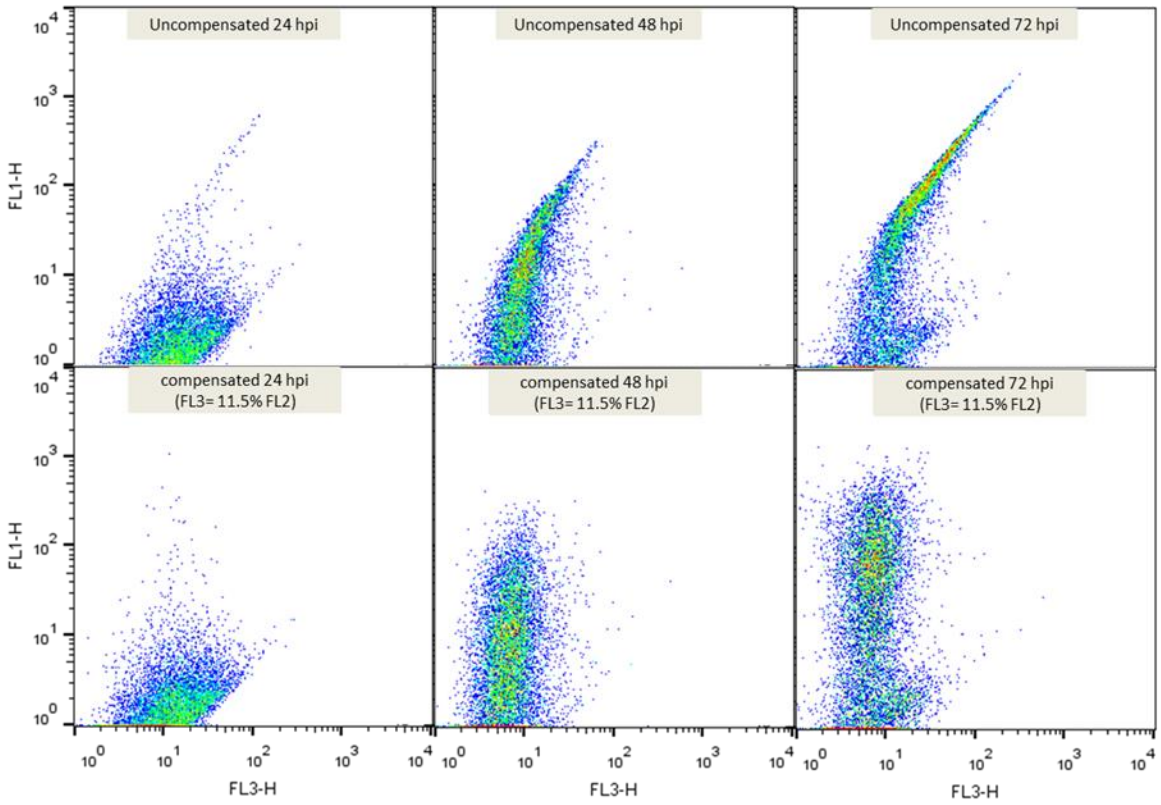


Figure 5-4: Flow cytometry scatterplots of green fluorescence (FL1) vs red fluorescence (FL3) for cell cultures infected with a monocistronic baculovirus vector carrying the GFP gene at an MOI of 5. The plots are of samples taken at 24, 48, and 72 hpi.

5.2.2.2 Distinct populations of cells infected with bicistronic vectors

Intracellular GFP and RFP were tracked by flow cytometry. Figure 5.5 is an example of how GFP and RFP levels changed over time when infected with the basic and gp64 baculovirus vectors (Table 3.1). It can be seen that fluorescence increased during the course of infection and attained a peak value within the time frame observed. Green fluorescence (FL1) started increasing earlier than the red fluorescent protein (as seen in Figure 5.4).

Up until 48hpi, the population moves from a low fluorescent state to a high fluorescent state but between 72 and 96 hpi there is backward shift in the population from a high fluorescent level to a low fluorescent state. This "low fluorescent population" is thought to be dying/leaky fluorescent cells. These populations were not artifacts of the fixation process, as the populations were also seen in untreated (unfixed) cell samples (data not shown).

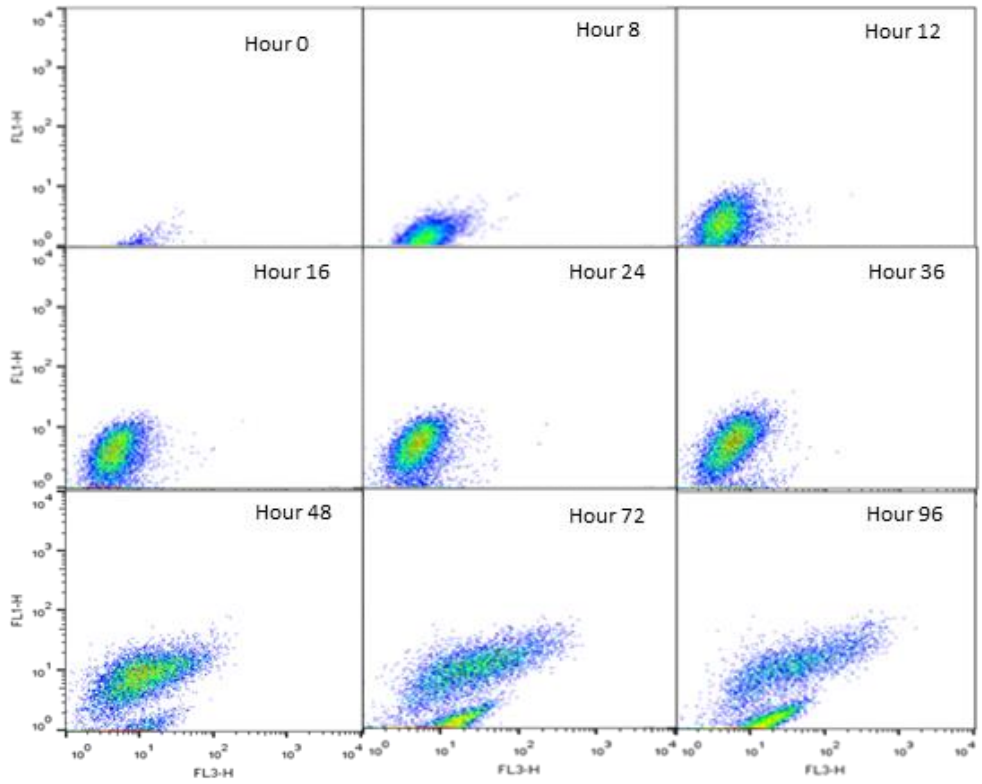


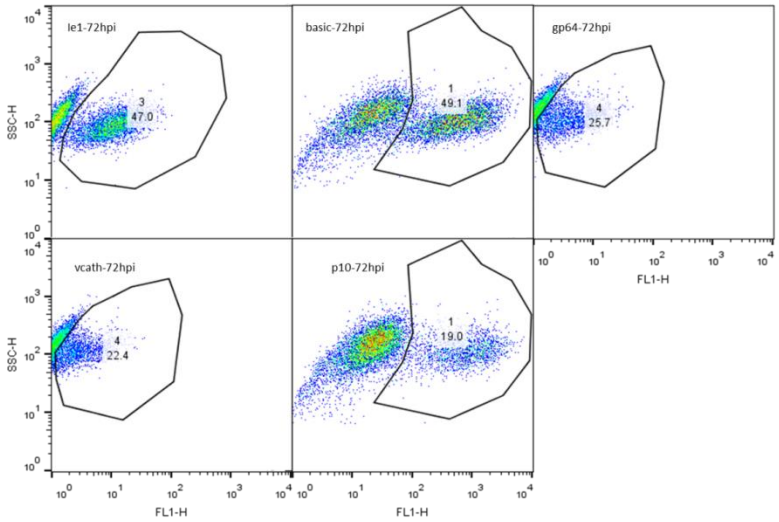
Figure 5-5: Flow cytometry scatterplots of green fluorescence (FL1[2]) vs red fluorescence (FL3) for cell cultures infected with the basic, gp64 and ie1 vectors at an MOI of 5. The plots are of samples collected at 0, 8, 12, 16, 24, 36, 48, 72 and 96 hpi.

The "high" and "low" fluorescence populations were further explored to understand the significance of the separation. First, the high fluorescence population was gated out from the low fluorescence population. The best discrimination of the populations occurred when looking at the SSC vs FL1 plots (Figure 5-6). One the gating was applied the geometric mean of the green and red fluorescence signals could be calculated. A consistent gating was applied to each sample infected by

[2] The "H" attached to FL1 and FL3 refers to the height of voltage signal when particle passes through laser beam.

the same vector at the same MOI; however, each infection condition had unique population signatures and a common gating for all conditions was not possible (Figure 5-6).

A



B

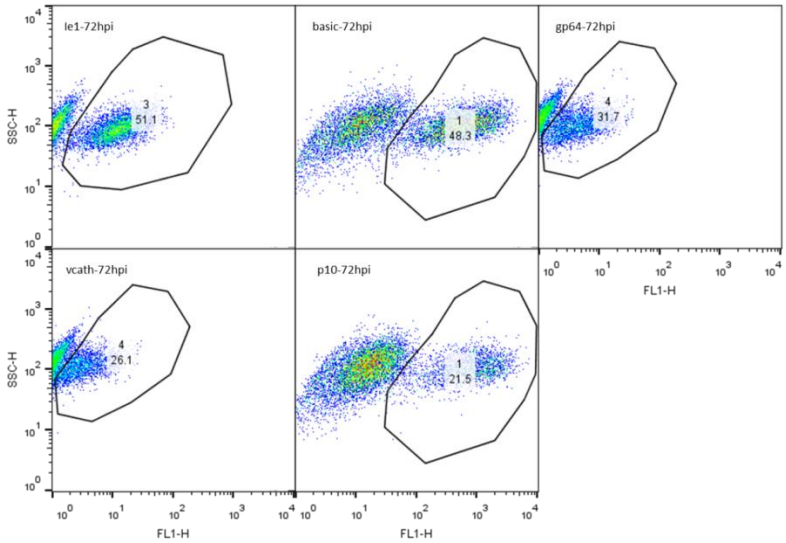


Figure 5-6: SSC vs FL1 flow cytometry scatter plots plot of cultures infected with various constructs (ie1, basic, gp64, vcath and p10), at A) an MOI of 5 at 72 hpi, B) an MOI of 25 at 72 hpi, showing the applied high fluorescence population gating.

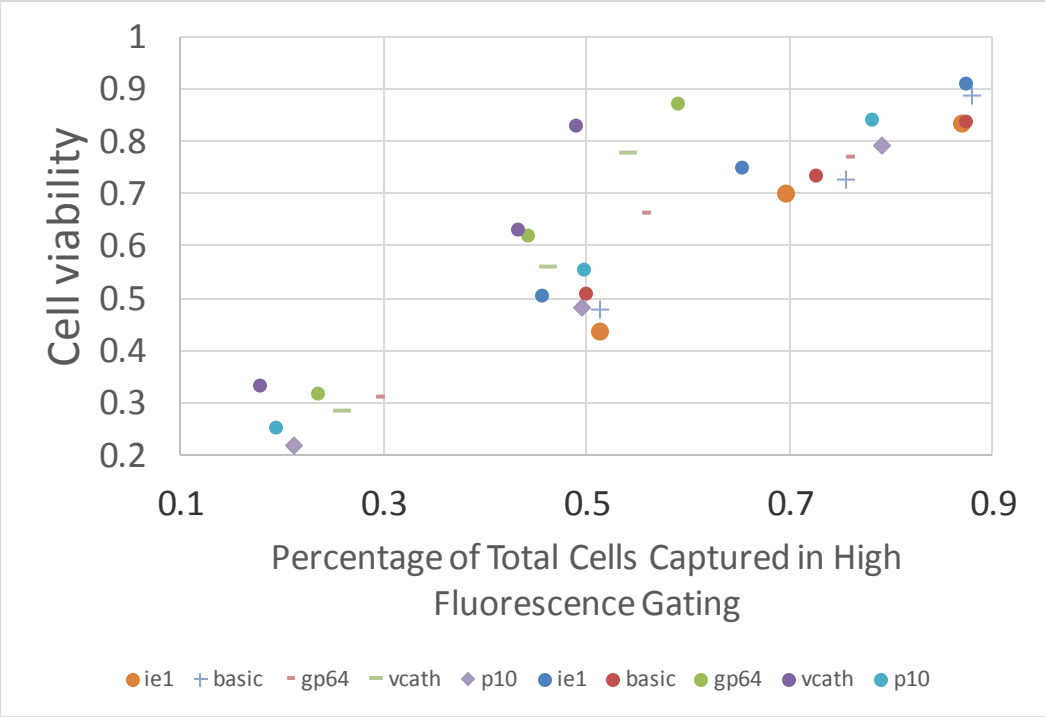
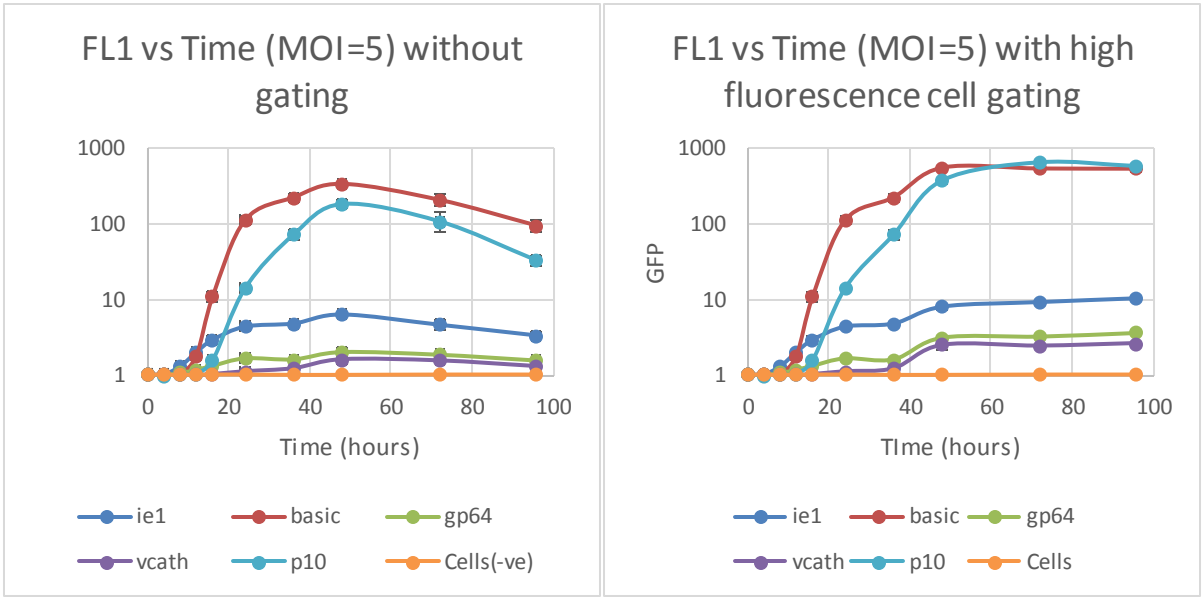


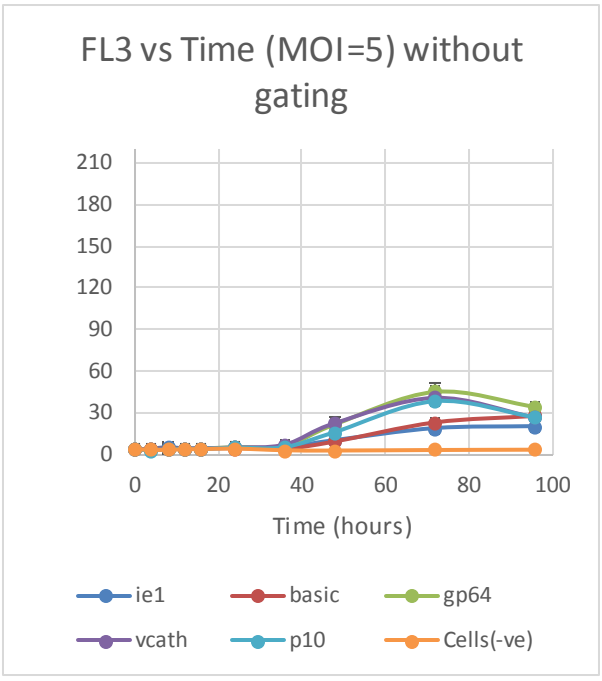
Figure 5-7**:** Correlation between cells gated as a "high fluorescence" population and cell viability for cultures infected with various constructs (ie1, basic, gp64, vcath and p10) and sampled at 48, 72 and 96 hpi (when viability starts dropping due to infection).

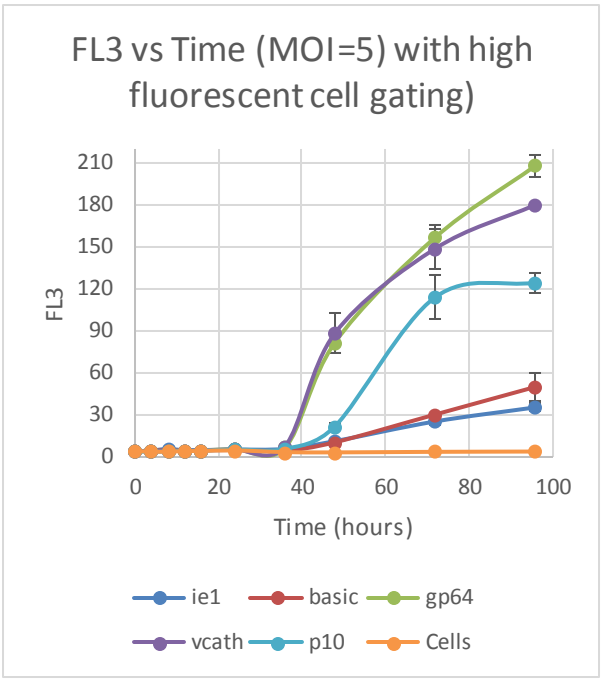
It was found that the high fluorescence population, which was gated out from the low fluorescence population correlated with viability of cell samples (Figure 5-7). Furthermore, it was observed that the removal of low fluorescence population drastically increased the red fluorescence (FL3) level, while it did not affect the green fluorescence (FL1) level much (based on the geometric means of the signal intensities of the populations, Figure 5-8). This result was consistent for both the MOI of 5 and 25 experiments. The large increase red fluorescence may be explained by the fact that the viable cells are still producing RFP late in the culture because the expression of gene is driven by the very late polh promoter. Production of GFP, on the other hand, may be more constant at the later time points because of the "earlier" promoters used to drive the expression of the GFP gene.

The high fluorescence population was gated out to account for the protein production in viable cells, as the total foreign protein production is important. While applying geometric mean analysis on total population reduce overall productivity of the system from 48 hpi, due to split of cell population between viable cells emitting high fluorescence signal and dead or cell debris emitting low fluorescence signal. Therefore, the protein expression in alive cells was higher at later time points, "overall" protein production was much lower. This is a very important factor to consider when producing a protein of interest in cell culture.

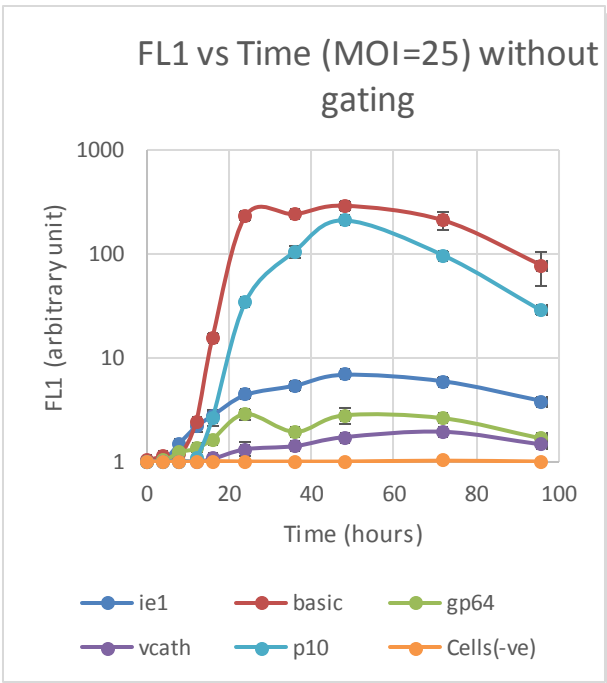
A

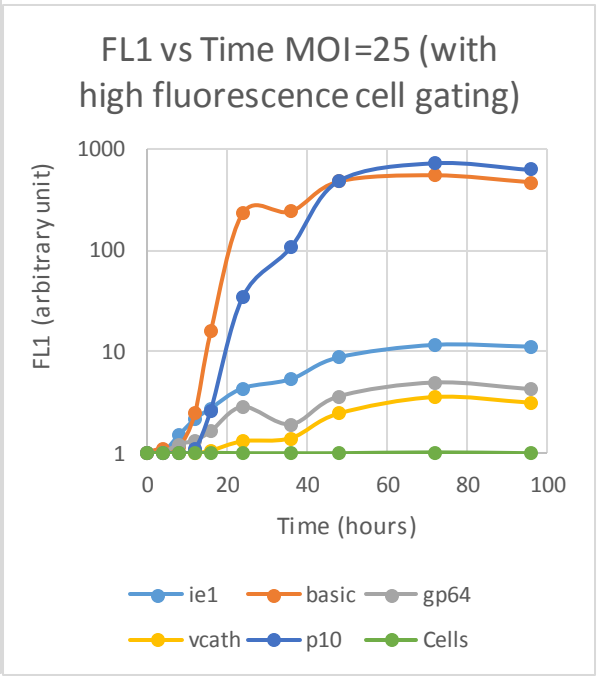



FL3 vs Time (MOI=5) without gating
210
180
150
120
90
60
30
0
0
20
40
60
80
100
Time (hours)
ie1
basic
gp64
vcath
p10
Cells(-ve)


FL3 vs Time (MOI=5) with high fluorescent cell gating)
FL3
210
180
150
120
90
60
30
0
0
20
40
60
80
100
Time (hours)
ie1
basic
gp64
vcath
p10
Cells

B


FL1 vs Time (MOI=25) without gating
FL1 (arbitrary unit)
1000
100
10
1
0
20
40
60
80
100
Time (hours)
ie1
basic
gp64
vcath
p10
Cells(-ve)


FL1 vs Time MOI=25 (with high fluorescence cell gating)
FL1 (arbitrary unit)
1000
100
10
1
0
20
40
60
80
100
Time (hours)
ie1
basic
gp64
vcath
p10
Cells

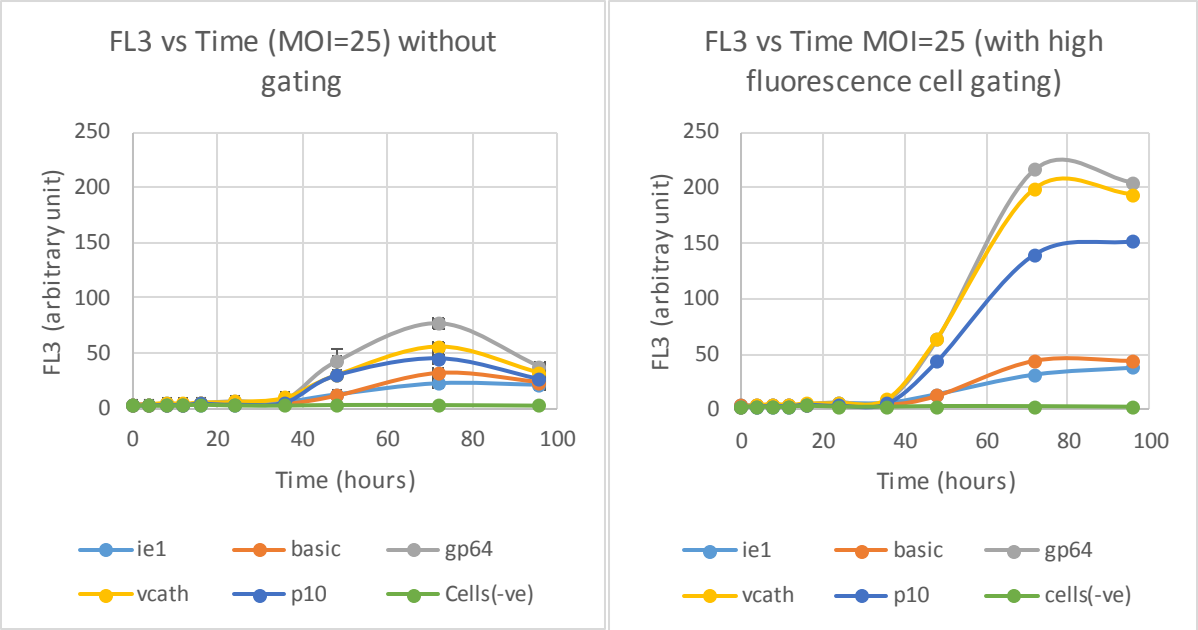


Figure 5-8: Comparison of GFP and RFP level when high fluorescence cells population are removed from the low fluorescence cells population. Geometric mean statistic are applied to obtained FL1 and FL3 level for high fluorescence and total population for culture infected with A) a MOI of 5; and B) a MOI of 25.

In subsequent analysis, to describe the average levels of protein produced, only the "viable" (high cell fluorescence population was considered). When examining the total protein produced, both low and high fluorescence populations were considered.

5.2.3 GFP and RFP Production

The purpose of this work was to observe gene expression patterns in cultures infected with bicistronic baculovirus vectors having at least one foreign gene under control of a non-conventional promoter. Figure 5.9 shows the relative overall level of green and red fluorescence observed over time in insect cells when infected at MOIs of 5 and 25.

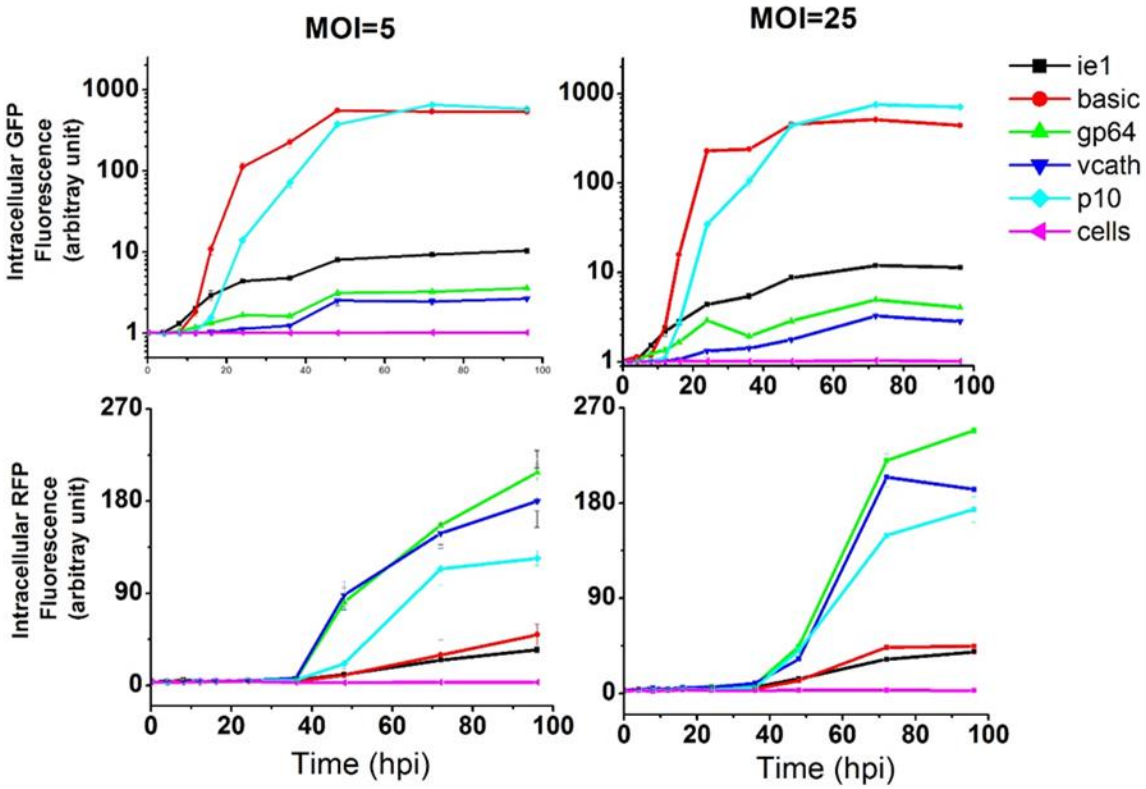


Figure 5-9: Relative fluorescence levels of GFP fluorescence and RFP fluorescence in cell cultures infected at an MOI of 5 and 25 with various constructs (p10; basic; vcath; gp64 and ie1). Increases in fluorescence beyond 48 hpi are correlated with a decrease in cell viability. Error bar represent the standard deviation observed from three replicate cultures.

The start of GFP production depended on the promoter controlling the gene, with the earliest signs of GFP being observed 4-8 hpi when under control of ie1, basic and gp64, 12-16 hpi when under control p10 and 16-24 hpi when under control vcath for MOI of 5 (Table 5-1). While, an increase in MOI from 5 to 25 does not seem to change the pattern of the emergence of green fluorescence except in case basic

and p10, there does seem to be a difference that can be observed for the red fluorescence (Table 5-1). The effect of increasing the MOI to 25 appears is only 'somewhat' noticeable for the experiments done with the "basic" vector where the peak level of GFP is reached earlier (24 hpi, Figure 5-9). In either case (MOI of 5 or 25), the basic vector yielded the highest levels of GFP (Figure 5-9).

Table 5-1: Average time protein was observed.

Vector	Time at which protein production is detected (hpi)			
	GFP		RFP (polh)	
	MOI = 5	MOI = 25	MOI = 5	MOI = 25
p10	12-16	8-12	24-36	24-36
ie1	4-8	4-8	24-36	16-24
basic	4-8	0-4	36-48	24-36
gp64	4-8	4-8	24-36	12-16
vcath	16-24	16-24	24-36	12-16

Table 5-2: Average time peak fluorescence levels was observed.

Vector	Time at which peak fluorescence is detected (hpi)			
	GFP		RFP (polh)	
	MOI = 5	MOI = 25	MOI = 5	MOI = 25
p10	48	48	72	72
ie1	48	48	96	72
basic	24	48	96	72
gp64	48	48	72	72
vcath	48	72	72	72

Table 5-3: Peak fluorescence level observed with different construct.

Vector	Peak fluorescence level is detected (hpi)			
	GFP		RFP (polh)	
	MOI = 5	MOI = 25	MOI = 5	MOI = 25
p10	183	209	38	46
	(649)	(756)	(124)	(174)
ie1	6.34	6.9	20	23
	(10.4)	(11.9)	(35)	(39.2)
basic	337	289	28	32
	(552)	(516)	(50)	(44.8)
gp64	2.02	2.8	41	77
	(3.61)	(4.97)	(208)	(248)
vcath	1.63	1.95	45	56
	(2.66)	(3.27)	(180)	(204)

Note: The value in brackets "()" represents high fluorescence level when low fluorescence cells were gated out.

RFP production differed (Figure 5-9) depending on the vector used even though the RFP gene was always under the control of the polh promoter. Changes in production levels (based on fluorescence intensities) were under 1.5 fold. The increase in MOI from 5 to 25 caused more significant increases in red fluorescence compared to the levels of green fluorescence (Figure 5-9). The maximum increase was observed when the vcath and gp64 vectors were used to infect the cell cultures. The p10 and basic vectors had lower increases in red fluorescence and there was no increase in red fluorescence observed in the case of the ie1 vector.

RFP expression was first observed between 12-16 hpi for vcath and gp64, 16-24 for ie1 construct, 24-36 for basic and p10 constructs, and difference in level was significantly observed from 36 hpi for MOI of 25. For MOI of 5, RFP expression was observed between 24-36 hpi for all constructs except basic where it was delayed to 36-48 hpi (Table 5-1). The earliest appearance of RFP occurred when the GFP gene was under the control of either the gp64 or vcath promoters. Both the vcath and

gp64 vectors yielded very low levels of green fluorescence but produced very high levels of red fluorescence.

The highest level of GFP fluorescence occurred at 48 hpi for all vectors for both high and low MOI experiments, with the exception of infection with the gp64 vector at an MOI of 25, where the maximum level was reached at 24 hpi. Furthermore, for infection with the gp64, ie1 and basic vectors, it was observed that at 36 hpi there was a decrease in GFP fluorescence (a dip in green fluorescence level or FL1 signal). This phenomenon may be due to dual regulation of protein production or may be related to the onset of RFP production. In the case of RFP production, the peak level was observed at 72 hpi in all cases (Figure 5-9) except for the culture which were infected by the ie1 and basic vectors at an MOI of 5, where the maximum fluorescence level was observed at 96 hpi.

In the cell culture infected with different virus vectors, low levels of green fluorescence are produced when the GFP gene is under control of either the vcath or gp64 promoters. These same infections (those with the GFP gene under the control of either the vcath or gp64 promoters) produced greater levels of red fluorescence than the culture infected with the vectors that had GFP under the control of either the p10 or basic promoters. The ie1 vector was the only exception, where the levels of GFP could not be used to predict in the levels of RFP (Figure 5-10).

5.2.3.1 Spectrofluorometric analysis of GFP and RFP fluorescence in cell culture supernatant

Fluorescence spectroscopy was conducted on cell supernatant from cultures infected with different vectors to analyze extracellular fluorescence levels. Low levels of fluorescence were observed during early times of infection, while it increased progressively with time. An increase in fluorescence level was significantly observed beyond 36 hpi for both MOI of 5 and MOI of 25 experiments (Figure 5-10). Progressive increases in fluorescence levels in the supernatant time are correlated with decreases in cell

viability (data not shown). The decrease in cell viability is believed to cause the cells to "bleed" GFP and RFP in the supernatant. The highest extracellular fluorescence levels were seen for the p10 vector, which also caused the greatest decrease in cell viability. The ie1 vector showed the lowest extracellular fluorescence level, and also caused the smallest decrease in viability (Figures 5-1, 5-10).

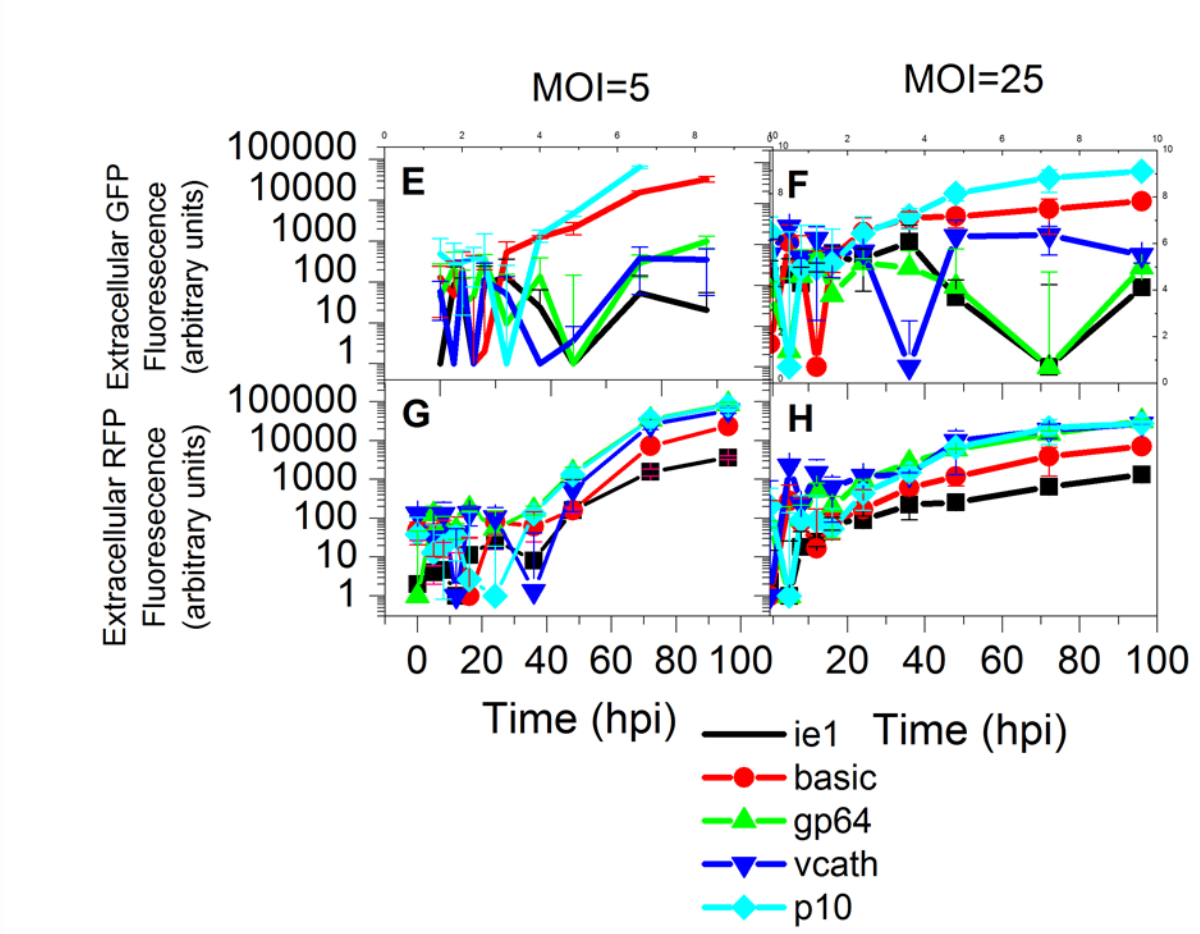


Figure 5-10: Shows an extracellular GFP and RFP fluorescence level for cultures infected with the various vectors at a MOI of 5 and 25. To obtain the extracellular fluorescence measures, fluorescence spectroscopy was performed on the supernatant of cell culture. The plotted values are mean value of triplicate flasks, and represents the geometric means of fluorescent population after gating to remove dead cells. Error bar represents the standard deviation around three replicate cultures (n=3).

5.3 Discussions

Over the last twenty years, plenty of work has been done improving the productivity of the insect cell system by manipulating cell culture conditions (Ikonomou et al., 2003), modifying protein production pathways (Kost et al., 2005), and improving vectors (Airenne, 2003). One aspect that researchers have not focused on much is the use of non-conventional promoters to control expression levels, and nobody had gone ahead and looked into the benefits of offsetting expression levels through promoter's control. The main objective of this work was to carefully characterize the use of promoters in bicistronic baculovirus vectors with the aim of controlling the timing and level of gene expression – ultimately to be used as a model for the production of more complex final products. A prime example of where it is believed that this knowledge will be useful is in the optimization of influenza vaccine particle production, which can be synthesized through the production of the influenza HA, NA and M genes (Bright et al., 2007; Pushko et al., 2005b; Pushko et al., 2007). Given that the HA and NA proteins are glycosylated and are embedded in the membrane (Daniels et al., 1987), it may be beneficial to produce these proteins earlier in the infection. The M protein, on the other hand, needs to interact with the cytoplasmic tail domain of the HA protein (Quan, Huang, Compans, & Kang, 2007; Veit & Thaa, 2011), and may benefit from a later production. A secondary example, may be in the production of chaperones or helper elements to help the formation of a complex product. The chaperones and helper elements may not necessitate high expression levels. Being able to control expression levels is thought to be beneficial. In this work, we have established vectors that allow different expression levels of two different genes (Figure 5-11).

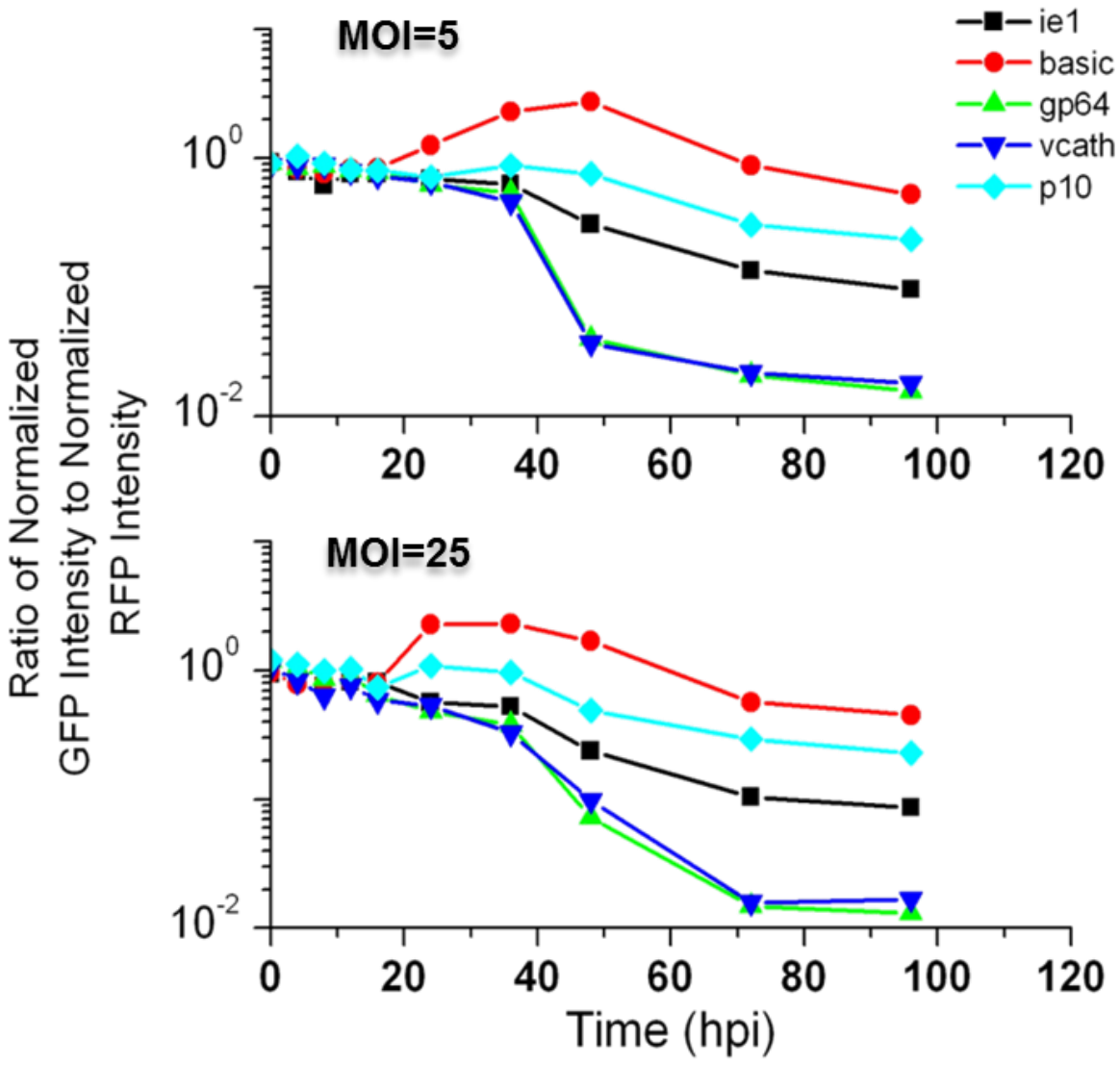


Figure 5-11: Ratio[3] of normalized GFP intensity to normalized RFP intensity over time plot for all construct at an MOI of 5 and 25 respectively. This represents the profile plot of p10, basic, vcath, gp64 and ie1 vectors at an MOI of 5 and 25.

[3] $Ratio\ of\ normalized\ value = \frac{fl1/\max(fl1)}{fl3/\max(fl3)}$

Another aspect of this work was aimed at determining if the synthesis of one protein affected the synthesis of the other in a negative manner, a "competition effect" of sorts. This could be due to genes competing for cellular resources that are required for the production of proteins i.e. competing for transcription factors or ribosomes for example. While this effect has been speculated to be present in both co-expression and co-infection systems, there has been no systematic studies showing this effect within the baculovirus-insect cell system.

GFP and RFP were selected because of their obvious ability to produce green and red fluorescence, which are relatively easy to detect. Five different virus vectors were generated for this experiment. The promoters controlling the expression of the GFP gene were varied in the five constructs: the p10 promoter, which has been reported to be active during the very late phase of baculovirus infection, starting at 19 hpi (Bonning et al., 1994a); the basic promoter, which has been reported to be active starting at about 13 hpi (Bonning et al., 1994a; Hill-Perkins & Possee, 1990); the gp64 promoter, which has been reported to be active in both the early phase (starting from 6 hpi) at low levels and during late phase (starting around 24 hpi) at relatively higher level (Garrity, Chang, & Blissard, 1997); and the vcath promoter, which has been reported to be active during the late phase starting at around 22 hpi (Hodgson, Arif, & Krell, 2007; Hom, Ohkawa, Trudeau, & Volkman, 2002; Slack et al., 1995). The results obtained from the work presented in this thesis corresponds with these finding for most of the part, however detection of GFP when under control of the p10 and vcath promoter are observed earlier, which may be due to the sensitivity of detection used in study.

This work demonstrates the use of alternative promoters to control the timing and expression of proteins within BEVS, which we believe is the next step in improving the system for the production of complex proteins. In addition to the large number of baculovirus promoters available within the baculovirus genome, foreign protein expression can be further modulated by the use of other

baculovirus regulatory elements such as homologous regions (HRs) (Guarino & Summers, 1986; Ishiyama & Ikeda, 2010), as well as non-baculoviral, and truncated (Urabe et al., 2006, 2002), hybrid (Thiem & Miller, 1990) or tandem (Kojima et al., 2001) promoters that extend the duration of protein expression, or change expression levels of native promoters. Artificial promoters (Gary W Blissard et al., 1992; Rankin et al., 1988) can also be used to regulate gene expression level.

The expression level of proteins in BEVS system can be dependent on the MOI used for infection. In this work, a cell density of approximately $1x10^6$ cells/ml was used to ensure that nutritional limitations would not be a limiting factor. Furthermore, two MOIs were investigated, 5 and 25, both ensuring synchronous infection. In all cases, the volume of virus stock used was always 5 times larger for the MOI of 25 experiments when compared t the MOI of 5. MOIs were also based on an assumption that the seeding density was exactly $1x10^6$ cells/ml even though the cell density in some cases was below this target value (effectively increasing the MOIs used).

The results presented in this document do point to a "competition effect" within the system when producing multiple proteins within the insect cells. The effect is seen mostly in the levels of red fluorescence achieved. Competition effects are not expected to arise only when vectors use combinations of promoters to drive gene expression, but can also arise due to the number of vectors introduced in the system. This phenomenon was observed by Bédard et al. as early as 1994 when they showed that there was no longer any increase of protein production as the MOI approached 50 (Bédard et al., 1994b). In this work, only moderate increases in protein production were observed when increasing the MOI from 5 to 25, but there did not seem to be any negative effect resulting from the increase in vectors. Prior to completing this work, we speculated that increasing the number of vectors would increase the GFP production, which would then lead to a decrease in RFP production. This did not occur.

As mentioned in the results section of this chapter, the levels of GFP were somewhat correlated to the levels of RFP achieved i.e. the higher the GFP levels, the lower the RFP levels; however not for the ie1 vector. The ie1 vector, which produced low levels of GFP, did not produce higher levels of RFP protein (Figure 5-10). Currently this behavior is not well understood but it could due to interference of the ie1 promoter with the polh promoter driving RFP gene. It should also be noted that transcription of genes downstream from the ie1 promoter is mediated by a cellular polymerase (Grula et al., 1981), while all of the other promoters examined in this work rely on a viral polymerase (Fuchs et al., 1983b; Guarino et al., 1998).

5.4 Conclusion

In this work, it was shown that placing one of the genes (GFP) under the control of earlier, and weaker promoters (gp64 and vcath) allowed increases in RFP production compared to when high levels of GFP were produced (when the gene was under the control of the basic promoter, for example). Furthermore, we have characterized five novel vectors able to produce different ratios of GFP and RFP. With the knowledge of the expression patterns, it may be possible now, to appropriately choose a vector design for the production of molecules that require the expression of multiple genes.

Chapter 6
Investigating the effect of non-conventional promoters at transcription level by tracking mRNA using real time PCR

6.1 Objective

Given that the main objective of this work was to observe protein production in insect cells infected with a polycistronic baculovirus, having one foreign gene under the control of a non-conventional promoter and a second under the control of the traditional polh promoter, it was important to also look at gene expression patterns. In the previous chapter, the effect of non-conventional promoters for use in co-expression systems on protein production levels was studied; the results indicated that there was an effect on the levels of proteins produced when producing more than one foreign protein. By looking at gene expression patterns, it was hoped that competition effects could be attributed to either competition at the transcriptional or the translational level.

6.2 Results

This set of experiments aimed to compare RNA transcript levels in cell cultures infected with various vectors at MOIs of 5 and 25. The approach consisted of extracting RNA from cell pellets using TRIZOL, reverse transcribing the RNA to cDNA and amplifying the resulting cDNA via real-time PCR. 28S rRNA was chosen as a control to account for the variability introduced in the experiment due to different efficiencies in the RNA isolation process and also to account for potential difference in starting cell concentrations. 28S rRNA has been used effectively with the BEV system by others (Xue et al., 2010b).

Individual PCR reaction efficiencies were determined using an assumption-free analysis with the LinRegPCR 11.6 program (Heart Failure Research Center, Academic Medical Center, Amsterdam, Netherlands). The efficiency used for different construct at high and low MOI experiments are provided in Table 6.1. These efficiencies were used in the determination of GFP gene and RFP gene transcripts as well as for 28s rRNA.

Table 6-1: Reaction efficiencies used for different RNA transcripts.

	MOI=5			MOI=25		
Constructs	GFP RNA	RFP RNA	28SrRNA	GFP RNA	RFP RNA	28SrRNA
p10-polh	1.828	2.084	2.17	1.94	2.277	2.25
basic-polh	1.821	2.112	2.088	1.865	2.229	2.317
gp64-polh	1.908	2.143	2.117	1.942	2.123	2.249
vcath-polh	1.89	2.27	2.120	2.1	2.284	2.44
ie1-polh	1.903	2.181	2.302	1.969	2.212	2.279
Cells(uninfected)	1.882	2.154	2.249	1.976	2.284	2.13

The cycle threshold (Ct) values obtained from the amplification of the GFP and RFP cDNAs were normalized against the Ct obtained from the amplification of 28S rRNA cDNA. 28S rRNA remains constant in a cell, and is one of the least affected RNA pools after infection (Xue et al., 2010b). In the uninfected cultures that served as controls, the quantity of 28S rRNA increased (Figure 6-1), matching the increase in cell density (data not shown). The 28S rRNA transcript levels in infected cultures showed only a small increase in transcript level, before plateauing as growth ceased due to infection. 28S rRNA transcripts started decreasing from 48 hpi for MOI of 5, while transcript levels started decreasing from 24-36 hpi for MOI of 25 (Figure 6-1), which also corresponded to the viability profile of the cultures (Figure 5-1).

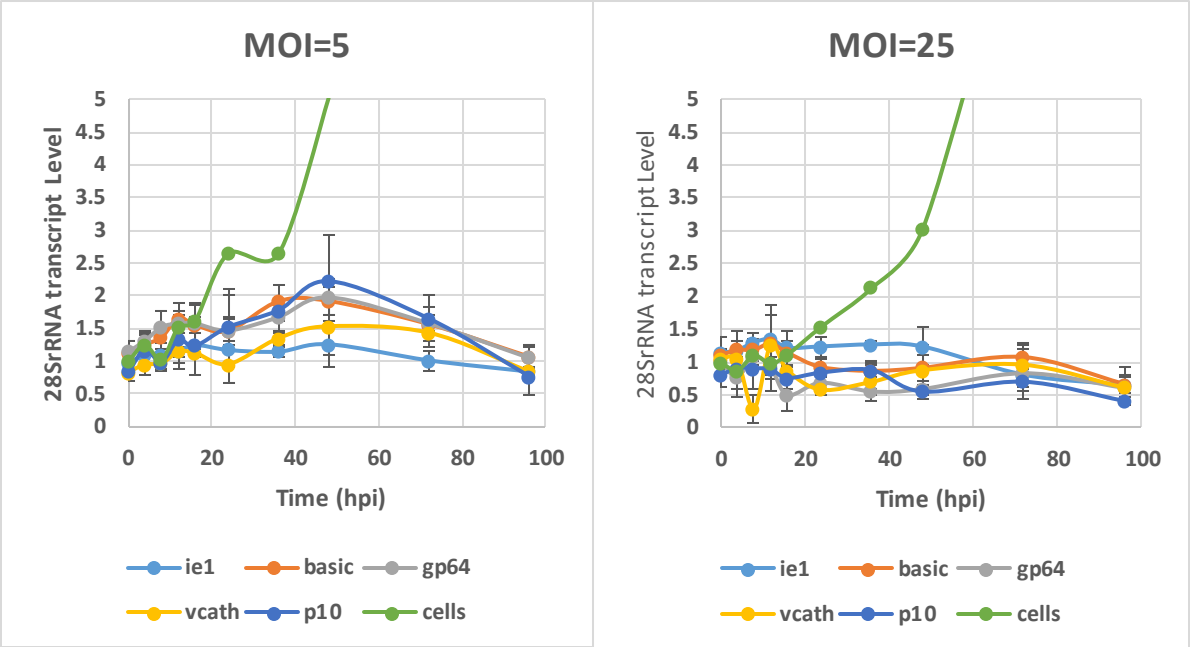


Figure 6-1: Tracking 28SrRNA transcript level of infected cell culture during course of time post infection. The 30mL Cell cultures were infected in triplicate at MOIs of 5 and 25 with various baculovirus vectors (p10, basic, vcath, gp64 and ie1). Error bars represent the standard deviation around three replicate cultures (n=3).

Figure 6-2 shows the overall level of GFP RNA transcript observed over time for cultures infected with MOIs of 5 and 25. The trend in RNA transcript levels was similar in the high and low MOI experiments, although lower levels of transcripts were detected for cultures infected at an MOI of 25, and the average transcript levels changed with respect to each vector when going from an MOI of 5 to 25. Increasing the MOI from 5 to 25 decreased the expression of GFP RNA transcript levels for the GFP gene under the control of the ie1 or vcath promoter. A smaller decrease was seen for the gp64 vector. The p10 and basic promoter drive almost same amount of GFP RNA level in both MOI of 5 and 25 infection (Figure 6-2). In both the MOI 5 and 25 experiments, the highest GFP transcript levels were observed when the gene was under the control of the p10 promoter.

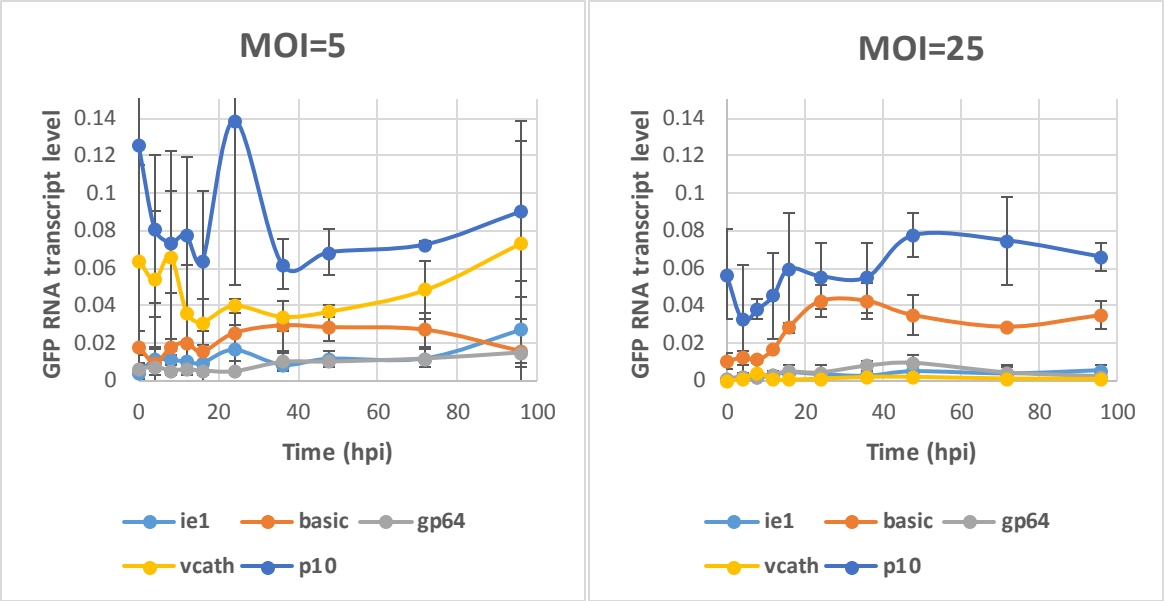


Figure 6-2: Relative GFP RNA transcript levels post-infection (relative to 28S rRNA). 30 mL cell cultures were infected in triplicate at MOIs of 5 and 25 with various baculovirus vectors (p10, basic, vcath, gp64 and ie1). Error bars represent the standard deviation of three replicate cultures (n=3).

The RFP RNA levels increased to different extents during the late phase of infection for the different vectors (Figure 6-4) albeit with no clear trends except for the gp64 vector.

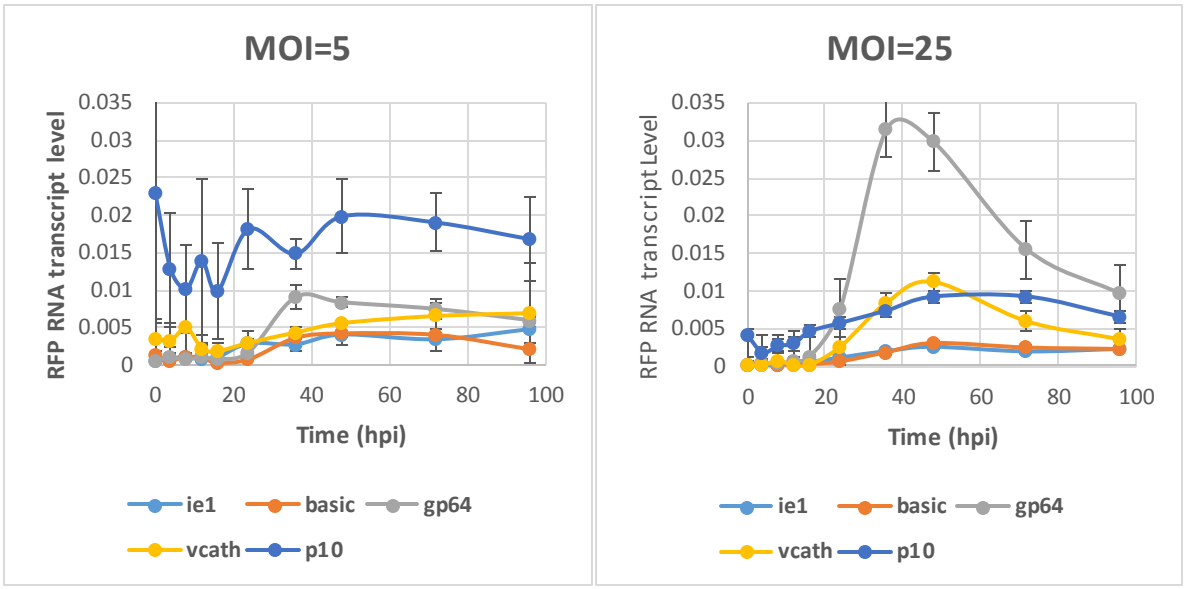


Figure 6-3: Tracking RFP RNA transcript level of infected cell culture during course of time post infection. Cell cultures were infected in triplicate at MOIs of 5 and 25 with various baculovirus vectors (p10, basic, vcath, gp64 and ie1). Error bars represent the standard deviation around three replicate cultures (n=3).

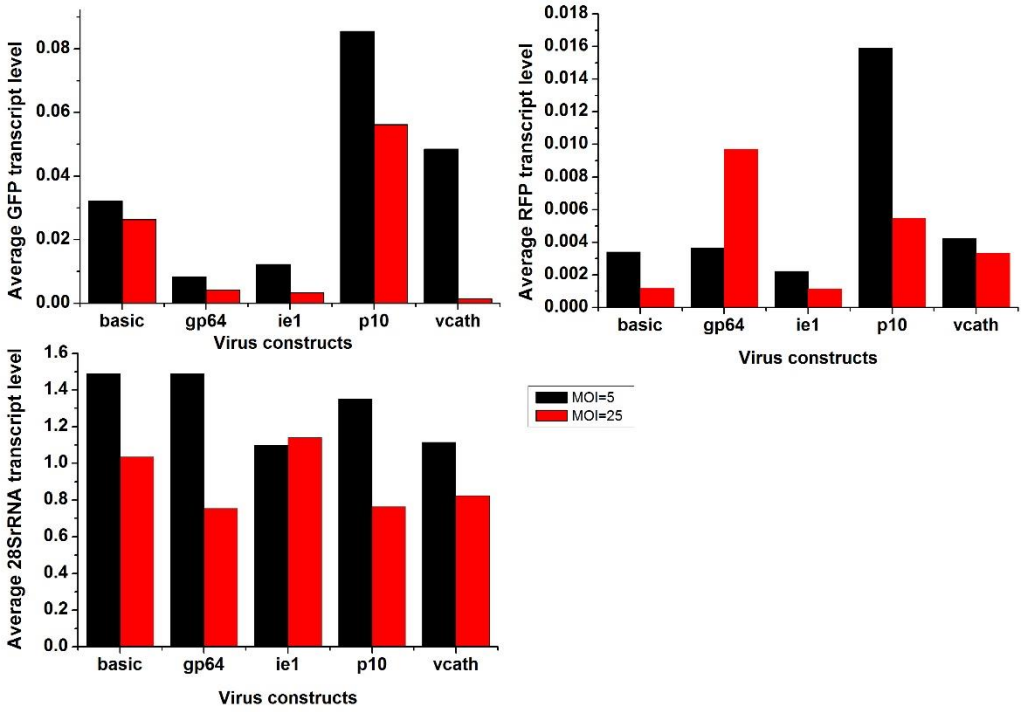


Figure 6-4**:** Comparison of average transcript level for GFP RNA, RFP RNA and 28SrRNA over entire course of time between MOI of 5 and 25 experiments.

6.3 Discussions

In all, the results are at best inconclusive. Protein production is a chain of events, starting with gene transcription followed by protein synthesis. In the BEVS, gene transcription is preceded by the delivery of the gene to the cell by the baculovirus vector. In this work, the expression levels of GFP and RFP mRNA under the control of different promoters was investigated. The overall 28SrRNA transcript level remained conserved for cultures infected at MOI of 5 and 25. The 28S rRNA level was lower for MOI of 25 infected cultures (Figure 6.1), which may be due to a higher death rate of MOI of 25 infected culture. Given that GFP mRNA and RFP mRNA were normalized to 28S rRNA, there may be some

unintended consequences of using 28S rRNA as a control. It is unclear why the results that were observed are so inconsistent. Many of our expectations were not realized. It is unclear why the RFP mRNA levels seem to be lower than the GFP mRNA levels or why increases in baculovirus vector do not result in higher overall mRNA or rRNA levels. At best, these results are inconclusive and require additional study.

6.4 Conclusions

Transcription represents a first step in the protein production process; therefore, the combined study of effect of non-conventional promoters at both the transcription and translation levels were thought to give a better understanding of co-expression system using alternative promoters. The correlation between RNA level and protein production level for genes of interest with different promoters was another aspect we aimed to establish. Unfortunately, the work completed thus far on gene transcription is inconclusive and will require study beyond this thesis.

Chapter 7
Conclusions and Recommendations

7.1 Conclusions

Following the creation of five novel baculovirus vectors, an in-depth characterization of vector stocks was undertaken. The baculoviruses were quantified using an end-point dilution assay, a growth cessation assay, a flow cytometer and a real time PCR. The characterization of the vectors showed that the stocks that were created were very similar and that there was no early signs that the use of baculovirus promoters chosen was problematic in terms of baculovirus generation. It was then possible to focus on the effect of protein production

Through flow cytometry, production of multiple proteins in insect cells infected with the novel bicistronic baculovirus vectors was examined. The promoter combinations chosen did indeed modulate protein production. Clearly, we demonstrated that the production of a second protein affects protein production when its gene expression is under the control of the polh promoter. This competition effect was observed at translation level, though it was not observed at the transcription level.

7.2 Recommendations

The goal of this study was the characterization of baculovirus promoters by analyzing gene expression at transcription and translation levels in a co-expression system. To expand on this work, new promoter combinations can be examined. These could make use of other baculovirus promoters such as vp39 and ETL (early to late promoter) promoters in various combinations. Studying more promoter combinations would give more freedom to attain a greater variety of ratios of different proteins of interest. In addition,

this work focused on a simple two reporter-protein system. This work can be extended into the creation of vectors with three or four co-expressed genes, with further research into the manipulation of expression levels. The use of RFP and GFP, two very simple proteins that need very little post-translational modifications and that are not secreted, may be an oversimplification of the system. Therefore, a system producing VLPs or complex proteins which need multiple subunit proteins produced at different levels, and with varying requirements for post- translational processing should be studied. In addition, there is need to look deeper into the system at the RNA level, as data shown in this work was inconclusive.

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Appendix A
Baculovirus quantification methods

A.1 Plaque assay

Plaque assay can be performed by using ten-fold dilutions of virus stocks prepared to infect cell samples grown as monolayer on plates. The monolayer usually formed up of nutrient medium called agar, which cause the formation of gel. After incubating cell samples, viruses are allow to be attached to the cells, and movement of newly formed viruses are restricted to neighboring cells by gel. Therefore, infectious particle formed a circular zone around infected cells called plaque, and by using dye better visibility is obtained between live and dead cell. The titre value of virus stocks can be determined by the number of plaques formed from several cultures infected with different dilutions. The use of Plaque assay is limited that it only account for the viruses with cause visible damage to the cells. The accuracy of Plaque assay is considered to be more than End-point dilution assay, but it is also more laborious (Nielsen, Smyth, & Greenfield, 1992).

A.2 End-point dilution assay

The end-point dilution assay (EPDA) was developed for virus quantification before plaque assay was developed and still used for this purpose where viruses do not form plaques. The EDPA used for infectious baculovirus quantification based on the Tissue Culture Infectious Dose 50 ($TCID_{50}$) assay developed by Reed and Muench in 1938 and further modified by many others (Darling, Boose, & Spaltro, 1998). Serial dilutions of virus stocks are used to infect cell culture in 96 well plate to statistically determine the point at which 50 percent of cell culture got infected and shows cytopathic effect. After incubation period, low diluted virus shows more infection; however at low dilution there will be no infection due unavailability of infectious particles. The titration of infectious particles in the

case of baculoviruses is determined in term of plaque forming unit per unit (pfu/ml). The equations used to determine infectious titres of virus stock are given below.

Equation A-7-1: Proportional response calculation

$$PD = \frac{[Percent\ rate\ dilutions\ next\ greater\ than\ 50\% - 50\%]}{[(\%\ rate\ dilutions\ next\ greater\ than\ 50\%) - (\%\ rate\ dilutions\ next\ less\ than\ 50\%)]}$$

Equation A-7-2: $TCID_{50}$ dose calculation

$Log_{10}(TCID_{50})$ = *(Log of the dilutions giving response greater than 50% - PD)*

Equation A-7-3: Titre calculation by EDPA

$$pfu/ml = \frac{TCID50}{V} \text{x } 0.69$$

Where PD is the proportional response and V is the volume of added virus

The end-point dilution assay is easier to perform than the plaque assay; it is particularly useful where plaques formed by virus that are not as easily distinguishable. The accuracy of EPDA is low compared to plaque assay, hence, large number of replicate cultures are needed to analyze titre value for virus stock (Nielsen et al., 1992). The use of reporter genes have been significantly used to differentiate between infected and non-infected wells such as β-galactosidase (Sussman, 1995) and Green fluorescent protein (Cha, Gotoh, & Bentley, 1997), and resulting an increase in the accuracy of the assay.

A.3 Flow Cytometry Assay

Flow Cytometery is used to count the virus particle in solution which passes through its capillary and hence, determine the total number of virus particle in a samples. The mechanism involves labelling the virus with a dye SYBR green and then running them through flow cytometer (Brussaard, Marie,

& Bratbak, 2000). The addition Triton-X 100 or NP-40 sometimes shows improved signal (Marie, Brussaard, Thyrhaug, Bratbak, & Vaulot, 1999). This method is relatively less labor intensive and time consuming, and shows high reproducibility on a run to run basis. The methods of the flow cytometer assay was modified for baculovirus quantification (C. F. Shen, Meghrous, & Kamen, 2002b).

A.4 Real Time PCR

Real Time PCR technique has been used progressively for nucleic acid sequence detection and quantification (Kaltenboeck & Wang, 2005), mRNA (Bustin, 2000b) and for quantification of several viruses (Mackay, Arden, & Nitsche, 2002). It has been used for quantitating gene expression and have also been compared with different kinetic PCR system. The RT PCR has been proven to be a versatile tool for quantification and detection due to its improved reproducibility, sensitivity. However, there are problems associated with its true sensitivity, specificity reproducibility; it also suffers from problems associated with PCR as well.

Real Time has been used for quantification of baculovirus particles for several groups of baculovirus, considering its main advantage in rapidity and accuracy of measurement (Roldão et al., 2009). The RT PCR has been exploited for the use of baculovirus quantification by Lo and Chao (Lo & Chao, 2004) and Hitchman et al (Hitchman, Siaterli, Nixon, & King, 2007).

RT PCR has been successfully used for baculovirus gene sequence detection and quantification; for this purpose wide variety of primers are used. Essential genes include *Gp-64* (Hitchman et al., 2007), *ie1* gene (Kato, Manoha, Tanaka, & Park, 2009; Liu et al., 2008), the viral DNA polymerase gene *dnapol* (Rosinski, Reid, & Nielsen, 2002), the *gp-41* gene (Liu et al., 2008), and the incorporated transgenes (Roldão et al., 2009; Vieira et al., 2005).

Several groups have compared titres value, obtained from RT PCR value with other quantification methods. The titre obtained by RT PCR are very close to titre obtained by EPDA assay (Lo & Chao, 2004), and plaque assay (Hitchman et al., 2007). The other groups found out that titres obtained by RT-PCR can be ten to twenty time higher than being obtained by EDPA and Plaque assay, and with consisting high reproducibility between runs (Roldão et al., 2009). The differences among different methods are due Defective Interfering Particle (DIP) and also due to the nature of quantification methods, as the plaque assay and EDPA assay describe infective particle titre assay while RT-PCR consider the total number of particles.

A.5 Calculation for Standard Plasmid used in RT PCR

Dilutions of ie1/Gp64 plasmids and Calculations of Concentrations

Plasmid Stocks Used: 167.8 ng/μL, Molecular weight of Plasmid = 3.4869931×1015 ng/mole

Number of moles per microliter=(mass/μl)/(Molecular weight)

=4.80811798×10-11 mole/μL

Number of particles per microliter of sample = 4.80811798×1011 mole/μl × 6.023 × 1023 particle/mole (NA)

NA = Avogadro number

= 2.89592946×1010 particles/μL

A.6 Calculation of virus titre by flow cytometer

Equation A-4: Titre calculation by flow cytometry

$$\textbf{Virus titre by flow cytometer}\left(\frac{\textbf{particles}}{\textbf{mL}}\right) = \frac{(\boldsymbol{Np - Ng})}{\boldsymbol{Ns}} \times \boldsymbol{Df} \times \boldsymbol{Cs}$$

Where

Np = Count of virus particle detected in the virus particle region

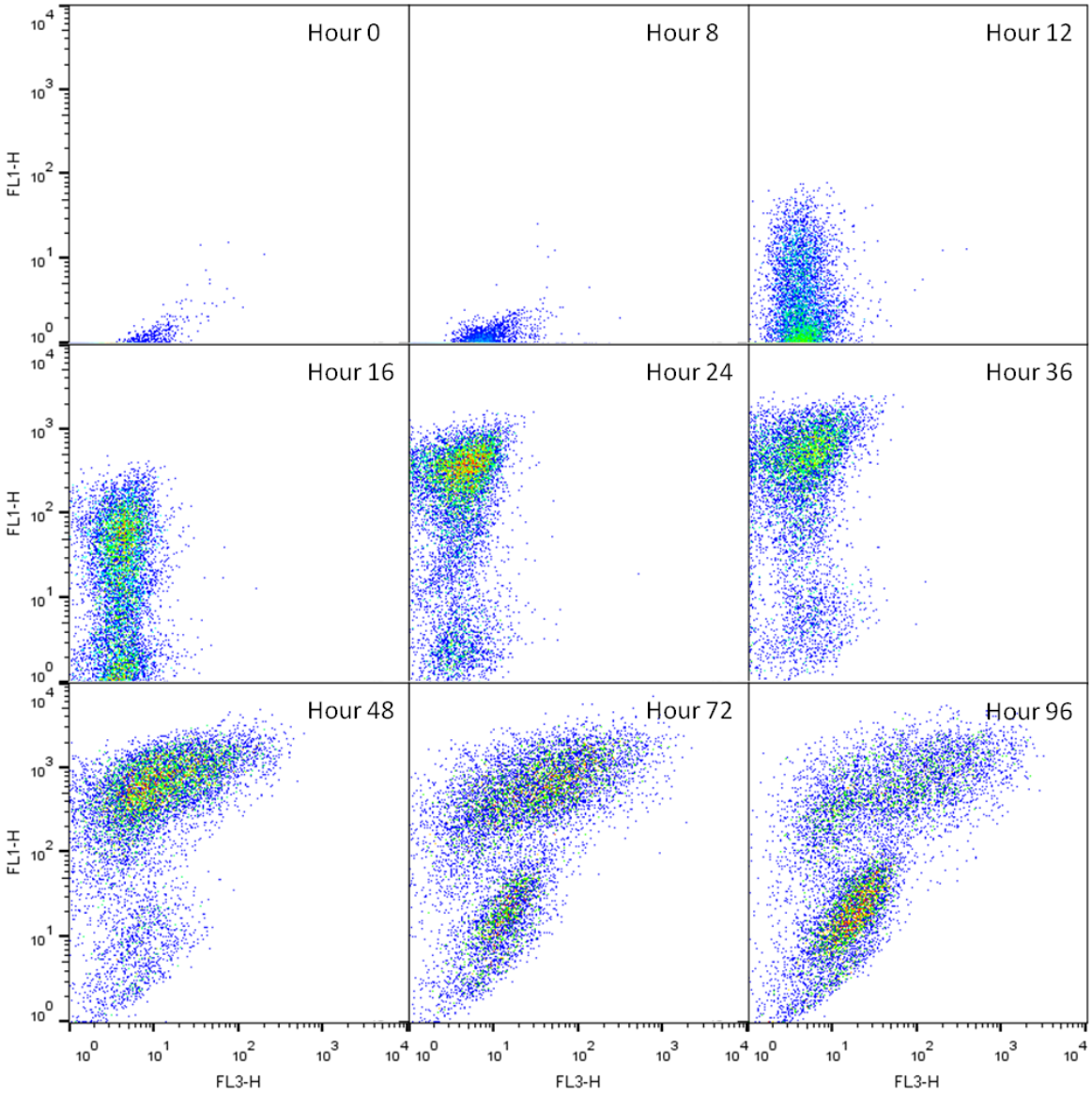
Ng = Count of virus particle detected from negative control in the virus particle region

Ns = Count of particle detected using standard fluorescence beads

DF = dilution factor of virus samples = (Volume of loading solutions/volume of initial virus supernatant)

Cs = Dilutions factor corrected concentration of fluorescence bead standards (particles/mL)

A.6 Scatter plot culture infected with basic vector



Flow cytometry scatterplot of green fluorescent (FL1-H) vs red fluorescent (FL3-H) for a cell cultures infected with the basic vector (basic) at an MOI of 5. The plots are of the samples 0, 8, 12, 16, 24, 36, 48, 72 and 96 hpi. The ‘H” in FL-H and FL3-H represents height.