Real Time PCR Protocol Development for Rapid and Low Cost Quantification of Baculovirus and for Monitoring Progression of Infection

by

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AUTHOR'S DECLARATION

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

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ABSTRACT

The work presented in this thesis aims to further the understanding and implementation of the Baculovirus Expression Vector System (BEVS) for varied uses such as protein production and viral vector production. To this end, three projects have been presented, two of which deal with methods to quantify baculovirus titres and the last deals with tracking baculovirus transcripts in infected insect cells.

The first project examined assumption-free analysis as a method for data analysis of Real Time PCR data in order to enable direct comparison of baculovirus titres between samples, without the need for a traditional standard curve. It concluded that assumption-free analysis was well suited for this purpose and fold differences of baculovirus titres of different samples obtained using this method corresponded to real differences in sample titres.

The second project aimed to develop a cheap and reliable method for sample preparation for Real Time PCR which would remove the need for the use of commercially available extraction kits. Samples were subjected to various combinations of Triton X-100 at different concentrations and different numbers of freeze/thaw cycles in order to determine the combination which would provide the best baculovirus genome exposure. One of these combinations was found to be at least as good as commercially available kits in reliably extracting baculovirus DNA and providing baculovirus titres that are at least as accurate.

The third project was a preliminary study examining the effects of multiplicity of infection on the levels of baculovirus Gp-64 transcript in insect cell culture. The study concludes that at high multiplicities of infection, there seems to be no increase in baculovirus transcripts when the multiplicity of infection is further increased. This study served to allow for familiarization with tracking transcript levels, and the principles and techniques demonstrated here will form the basis for an exhaustive future study on the same subject.

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DEDICATION

To my friend David Suh

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CHAPTER 1

INTRODUCTION

Baculoviruses were first exploited for human benefit as insecticides, where their narrow host specificity and high infectivity was exploited to create highly safe insecticides. Over the past 20 years, however, their use has been expanded to applications such as recombinant protein production (T. A. Kost, Condreay, & Jarvis, 2005), and more recently as vectors for gene transfer to mammalian cells, and as scaffolds for the surface display of various proteins.

The most important current use of this system is for the production of recombinant proteins in insect cell culture and insect larvae. This is due to the use of suspension insect cell cultures which can be grown to high density, as well as the use of baculovirus promoters which can drive very high levels of expression of the gene (or genes) of interest, which in turn can be very large due to the large packaging capacity of the baculovirus genome. The utility of this system has resulted in its use in the production of a wide range of proteins, protein complexes and virus-like particles (M. G. Aucoin, Mena, & Kamen, 2010; Y. Hu, 2005; T. A. Kost et al., 2005), including several commercial products such as the human papillomavirus (HPV) vaccine Cervarix™ from GlaxoSmithKline and the influenza vaccine FluBlok® from Protein Sciences.

The increasing utility of this system has resulted in the requirement to be able to accurately quantify amounts of baculovirus in a sample, which is necessary for large scale protein and baculovirus production. Therefore, there have been substantial efforts to develop an accurate and reproducible method of baculovirus titration, with methods such as the plaque assay, end-point dilution assay and flow cytometry, among several others. Real Time PCR has also been

investigated as a rapid and accurate method for quantifying baculovirus in a sample.

The work presented in this thesis aims to further develop Real Time PCR as a method for quantifying virus and understanding the progression of infection. To do this, it looks at methods of analysing Real Time PCR data, as well as new methods for preparing baculovirus samples for analysis by Real Time PCR, and methods for tracking the transcription of viral genes.

The main hypotheses of this work are that non-traditional Real Time PCR analysis methods can be used to calculate differences in abundances of template in between samples, and that baculovirus sample generation can be simplified in order to reduce the cost and time associated with this process, and that the simplified method can be used at least as reliably as current established preparation methods.

The first chapter of this thesis is a detailed literature review which gives an overview of the baculovirus system and its various uses. It explains various factors involved in the usage of this system, particularly for protein production and concludes with a section on baculovirus quantification methods, with particular emphasis on Real Time PCR and Real Time PCR data analysis methods. The second chapter of this thesis is a common Materials and Methods section, with protocols common to subsequent chapters. Chapter 3 of this thesis describes the benefits of using assumption-free analysis as an alternative to comparative C_T analysis, especially for the evaluation of quantification protocol development. This was done to reduce the dependence of data analysis on the dilution of a set of standards and on the generation of a standard curve, as well as to address other deficiencies of the comparative C_T based method of data analysis. The fourth chapter of this thesis details efforts to validate a new method of baculovirus sample preparation for Real Time PCR, which is easier and cheaper than conventional kit based extraction methods. Various combinations of treatments using Triton X-100 and freeze/thaw

cycles were studied to find the treatment scheme which yielded sample as suited for Real Time PCR. The last chapter of this thesis presents work involving tracking levels of a baculovirus gene transcript in infected insect cells, over the course of an infection cycle, and forms the basis of future work in this area.

CHAPTER 2

Literature Review

2.1 *Baculoviruses*

Baculoviruses are a group of DNA viruses that infect only organisms of the phylum Arthropoda (Miller & Lu, 1997), with most viruses infecting species of the order Lepidoptera. Baculoviruses are present in two forms: the occluded form in which virus capsids are embedded within a protein matrix, and are responsible for virus transmission between hosts, while the budded form is an enveloped virus and mediated transmission within a host. Baculovirus capsids are usually 40 – 50 nm in diameter and 200 – 400 nm in length and enclose a genome that is between 80 – 200 kilo bases (kb) in length. Baculoviruses can be divided into two genera that differ by the size and composition of their occlusion bodies: the nuclear polyhedrosis viruses (NPVs), which encapsulate many virions in large structures called polyhedra and the granulosis viruses (GVs) which have only one or two virions encased in smaller occlusion bodies called granules (Funk, Braunagel, & Rohrmann, 1997). The best studied among these viruses is the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) which infects the alfalfa looper *Autographa californica*.

2.2 *Baculovirus replication cycle*

Much of what we know about baculovirus biology is from the study of AcMNPV infection of

insect cells in culture. Within insect cell culture, baculovirus transmission is mediated by budded virus which are taken up into insect cells mainly by receptor mediated endocytosis (Blissard & Wenz, 1992; Volkman & Goldsmith, 1985), but also by direct membrane fusion (Volkman & Goldsmith, 1985). Following entry, nucleocapsids travel to the nuclear pores, following which the genomic material is released into the nucleus, which is followed by synthesis of viral RNA and viral DNA. The baculovirus infection cycle is divided into three phases: the early, late and very late phases of infection. The early phase consists of the immediate-early and delayed-early phases where the immediate-early phase involves the expression of genes controlled by immediate-early promoters which can be transcribed by the host cell's RNA polymerase II (Grula, Buller, & Weaver, 1981), while expression of delayed-early genes rely on the presence of immediate-early gene products. Early gene products include transcriptional activators, anti-apoptotic factors, and components of virally encoded polymerases. The synthesis of these early genes starts immediately after cell infection (B. J. Kelly, King, & Posee, 2007) and extends to about 6 hours post infection and is followed by expression of the late proteins from about 6 hours post infection, which is mediated by a viral RNA polymerase (Fuchs, Woods, & Weaver, 1983), and continues till 24 hours post infection. This phase corresponds to replication of baculovirus DNA and also to the production of budded virus. Infection of insect cells by baculoviruses also corresponds to cell cycle arrest at the G2/M phase of replication (Braunagel, Parr, Belyavskyi, & Summers, 1998), the gradual shutdown of host protein production from about 6 hours post infection (Carstens, Tjia, & Doerfler, 1979) and the decrease in levels of several host mRNAs in the late phase of infection (Nobiron, O'Reilly, & Olszewski, 2003). The very late phase of the infectious cycle starts from at about 20 hours and involves the expression of high levels of proteins necessary for the production of occluded virus particles, such as the polyhedrin and p10 proteins. In insects the very late phase of infection would correspond to the embedding of virus capsids within a polyhedrin matrix, and the subsequent occlusion of these particles.

2.3 *Uses of the Baculovirus system*

Baculoviruses were first exploited for use as pesticide due to their narrow host range, high infectivity and high biosafety. Since then, baculovirus have been used for several different applications which make use of their versatility, with improvements being made continuously to expand their utility to new systems and applications. It must be noted that several works have been published which give much more comprehensive reviews of the uses and implementation of the baculovirus system (Y. Hu, 2005; T. A. Kost et al., 2005) and these may be referred to for further information. A few of these uses are given below.

2.3.1 *Protein production in insect cells:*

The most important use of the baculovirus system has been for the production of proteins, protein complexes and virus-like particles in insect cells and insect larvae, with the main advantage being the high levels of protein expressed and modified with eukaryotic processing mechanisms. The system also has the advantage of being able to accommodate large foreign genes, and is safe for use as it does not infect humans. Given that this remains the most common use of baculoviruses, a detailed review of protein production in insect cells using baculovirus is given in the section titled "The Baculovirus Expression Vector System".

2.3.2 *Baculovirus mediated gene delivery into mammalian cells*

Baculoviruses were first reported in 1983 to be able to be taken up into a variety of different non-target cells, without resulting in virus replication (Volkman & Goldsmith, 1983). This work was expanded upon subsequently by various research groups showing expression of genes of interest in mammalian cells, when placed under the control of a mammalian promoter such as the widely used cytomegalovirus (CMV) (C. Hofmann et al., 1995), or the Rous sarcoma virus (RSV)

promoter (Boyce & Bucher, 1996). Baculoviruses have been found to transduce a wide variety of cells with varying efficiencies, with the list of permissive cells including popular cell lines such as HeLa and CHO cells (Y. Hu, 2005), and primary cells such as human hepatic cells (C. Hofmann et al., 1995) and neural cells (Sarkis et al., 2000). These baculoviruses which were capable of gene expression in mammalian cells were termed BacMam viruses. The mechanism by which baculovirus can transduce this wide variety of mammalian cells is not known, but is thought to involve some form of endocytosis (Dee & Shuler, 1997).

The capability of baculovirus to introduce genes into a wide range of mammalian cells and cause transient expression of these genes, as well as the safety profile of baculoviruses, has been exploited for gene therapy, with studies being done on both *in-vivo* and *ex-vivo* gene transfer. However, *in-vivo* gene transfer suffers some limitations such as baculovirus inactivation by the complement system (C. Hofmann & Strauss, 1998) and the prevention of this phenomenon is an area of continuing research.

In addition to gene therapy, baculoviruses have been used as vectors for protein production in mammalian cells, and is especially advantageous if mammalian-like processing is required for the expressed protein products. The system has been used in the production of secreted proteins (Ramos et al., 2002), virus-like particles (Wang et al., 2005) and viral vectors like adeno-associated virus (AAV) vectors (Sollerbrant et al., 2001).

2.3.3 *Baculovirus surface display*

The ability of baculovirus to incorporate expressed membrane bound proteins into their outer membrane has led to surface modified baculoviruses being produced and used for various purposes. These modified viruses have been used in various applications such as improving transduction efficiencies of non-permissive cells using proteins such as avidin (Raty et al., 2004)

and antibody fragments (Mottershead, Alfthan, Ojala, Takkinen, & Oker-Blom, 2000). They have also been used for screening epitope libraries (Ernst et al., 1998), for the assembly of active protein complexes (Hayashi et al., 2004; Loisel et al., 1997; Masuda et al., 2003), and as vaccines by expressing antigens on their surface. To this end, baculovirus vaccines have been shown to induce immune responses in animals against viruses such as influenza (T. Abe et al., 2003), psuedorabies virus (Aoki et al., 1999) and Hepatitis C virus (Facciabene, Aurisicchio, & La Monica, 2004), among others.

2.4 *The Baculovirus Expression Vector System (BEVS)*

The use of baculoviruses coupled with insect cells has become increasingly popular due to the ability of the system to produce large quantities of protein product, which can sometimes approach the yield of prokaryotic systems (D. L. Jarvis, 1997). This is due to a combination of factors including the ability of insect cells to be grown in suspension culture, the high tolerance of these cells to osmolality and by-product concentrations (Ikonomou, Schneider, & Agathos, 2003), and the use of strong baculovirus promoters such as the *polyhedrin* and *p10* promoters to drive high levels of protein expression. This system has become particularly valuable for the expression of protein products which require eukaryotic post-translational modifications.

The BEVS system has been used to express many different types of proteins in insect cells, due to the properties listed above, as well as other factors like the large gene insert capacity of the baculovirus genome and their lack of pathogenicity. The production of proteins by the baculovirus expression system has been reviewed elsewhere (Y. Hu, 2005; T. A. Kost et al., 2005). In addition, the large packaging capacity of the baculovirus, and consequently the ability to package multiple foreign genes within a single baculovirus, as well as the ability to infect a cell

with multiple baculoviruses coding for one or more genes of interest, have made this system very valuable for the production of single and multi-subunit products such as virus-like particles (VLPs). The production of viral particles using the insect cell system has been reviewed elsewhere (Y. Hu, 2005; T. A. Kost et al., 2005), and a detailed list of VLPs produced using the system is given in other works (M. G. Aucoin, 2007). The system is also capable of producing enveloped virus particles such as influenza particles (Latham & Galarza, 2001; Pushko, Tumpey, Bu, Knell, & Smith, 2005), among others. In addition, infectious viral vectors such as AAV vectors have been produced in insect cells (Urabe, Ding, & Kotin, 2002).

A wide variety of insect cell lines are available for protein production, with the most commonly used being lines such as Sf9 and Sf21, isolated from *Spodoptera frugiperda*, and High Five cells isolated from *Trichoplusia ni*, due to their ease of culture. They also benefit from the ability to be grown in suspension culture. Detailed reviews of the cell lines used and comparisons between these have been given elsewhere (M. G. Aucoin et al., 2010; Ikonomou et al., 2003; Lynn, 2007). The main baculovirus vector which has been used to transfer genes to the insect cells is the AcMNPV virus which remains the best studied of the baculoviruses.

2.4.1 Recombinant Baculovirus Production

The production of recombinant baculoviruses containing the gene(s) of interest has been done using several techniques, which aim to produce purely recombined virus, with as little contaminating non-recombined virus as possible. All these techniques generally use a transfer vector, with one or more genes of interest under the control of various promoters, into a baculovirus genome. One of these techniques involves the use of transfer vectors and linearized baculovirus DNA with an essential gene deleted, which can be replaced when the linear DNA recombines with a transfer plasmid containing the deleted gene, along with the gene of interest,

thus producing recombined virus (Kitts & Possee, 1993). The transfer plasmid contains the gene of interest downstream of a baculovirus promoter sequence, and is flanked by sequences complementary to parts of the baculovirus genome which facilitates homologous recombination of the promoter-gene DNA fragment into a non-essential region of the baculovirus genome, usually the *polyhedrin* gene. The percentage of recombinant virus in the virus population can reach to nearly 100% using this method (Possee & King, 2007). Other methods include conducting the recombination with bacteria by inserting the gene of interest by site specific recombination into a bacmid containing the baculovirus genome, and selection using a marker gene (Airenne, Peltomaa, Hytonen, Laitinen, & Yla-Herttuala, 2003; Luckow, Lee, Barry, & Olins, 1993). A third method involves *in-vitro* site specific transposition for the insertion of a foreign gene, and incorporates a negative selection marker for the elimination of non-recombined baculovirus DNA (Zhao, Chapman, & Jones, 2003). Detailed reviews on the generation of recombined baculovirus are published in other works (T. A. Kost et al., 2005; Possee & King, 2007).

2.4.2 Limitations of the Baculovirus Expression Vector System

2.4.2.1 *Post-translational modifications in the baculovirus system:*

The BEVS system along with the insect cell has been used for the production of proteins which require eukaryotic post-translational modifications; however, the pathways in insect cell lines are not always similar to that of higher organisms. In addition, existing processing pathways may be affected due to the shutdown of host protein production and downregulation of host mRNA production mentioned earlier, and may also be overwhelmed by the large amounts of recombinant protein produced by most baculovirus vectors, both of which can lead to inefficient post translational modification of produced protein.

2.4.2.2 *Proteolytic cleavage:*

The baculovirus expression system has been used successfully to produce and process several proteins which require proteolytic cleavage of their prosequences for biological activity, such as interleukin-2 (Smith et al., 1985). However, there have been many reports of overexpressed proteins such as HIV gp160 (Wells & Compans, 1990), the influenza hemagglutinnin (Kuroda K, Hauser C, Rott R, Klenk HD, & Doerfler W, 1986) being inefficiently cleaved by insect protein processing mechanisms. However, this may be overcome by expressing processing enzymes in the insect cell system. It has been shown that expressing a mammalian furin convertase along with TGIFβ improved the amount of bioactive TGIFβ several fold (Laprise, Grondin, & Dubois, 1998).

2.4.2.3 *Glycosylation*

While insect glycosylation pathways are similar to those found in higher eukaryotes, they do show some differences (Altmann, Staudacher, Wilson, & Marz, 1999; D. L. Jarvis, 1997; T. A. Kost et al., 2005). These differences include incompletely elongated oligosaccharide chains with incomplete sialylation of N glycans in the N-glycosylation pathway, and lack of production of the sialylated galactose-O-N-acetylgalactosamine trisaccharide. These differences are due to the absence of various mammalian glycosyltransferase enzymes and so efforts have been made to introduce these transferases into insect cells, with one article reporting the generation of an insect cell line which incorporated five glycosyltransferases, along with two further enzymes for conversion of the sialyic acid precursor, and showed that this cell expressed a GST protein which was fully N-glycosylated, with no unglycosylated forms detected (Aumiller, Hollister, & Jarvis, 2003). In addition, cell lines such as Ea4 (Ogonah, Freedman, Jenkins, Patel, & Rooney, 1996) have been isolated which show higher glycosylation capabilities than Sf-9 cells. Detailed reviews have been published regarding glycosylation in insect cells and efforts to address its limitations, (Ikonomou et al., 2003; D. L. Jarvis, 1997; T. A. Kost et al., 2005)

In addition to the issues listed above, the baculovirus system has also been found to have other limitations such as inefficient phosphorylation and acylation, among others. Further information on these limitations can be found in the above mentioned reviews.

2.4.2.4 *Proteolysis in the BEVS system*

Proteases are produced in a baculovirus infected insect cell during the very late phase of infection, at a time when recombinant protein expression is the highest in conventional polyhedrin driven vector systems. These proteases are produced both by the cell, during cell lysis, or can be encoded by the baculovirus, probably to dissolve the cell and insect host to facilitate release of the polyhedra. These enzymes are mostly cysteine proteases (Gotoh, Miyazaki, Kikuchi, & Bentley, 2001), and can cause degradation of produced recombinant proteins. Deletion of protease genes such as *v-cath* and chitinase *chiA* have been shown to reduce degradation of recombinant proteins (Monsma & Scott, 1997) and can be used to prevent problems caused by the proteolytic nature of the BEVS system. In addition, the use of protease inhibitors has been explored (Gotoh et al., 2001). Another option to circumvent the problem of proteases during the very late phase of infection is to use promoters which are active at earlier phases of the baculovirus infection cycle (discussed later). A detailed review on this subject can be found in other published work (Ikonomou et al., 2003).

2.4.3 Promoters used for the expression of foreign genes

Many of the proteins produced till now have used baculovirus vectors in which recombinant gene expression is driven by the baculovirus very late polyhedrin (*polh*) promoter, which has been found to be one of the strongest known promoters and drives high level expression of the polyhedrin gene in wild-type baculovirus. In addition to the *polh* promoter, the very late *p10*

promoter has also been used for protein expression and is active slightly earlier than the *polh* promoter, but is weaker and drives lower levels of protein expression, and therefore is not as widely used (D. L. Jarvis, 1997). However, the use of very late baculovirus promoters means that the proteins are produced at a time when much of the host cell pathways such as the secretory pathway have been disrupted to some extent (D. L. Jarvis & Summers, 1989), and therefore cannot efficiently produce the active forms of these proteins efficiently (T. Tsao et al., 1990), especially due to the overwhelming amounts of protein produced by the strong very late promoters. In addition, the very late phase of infection corresponds to the presence of high levels of baculovirus and cell encoded proteases, as discussed earlier. Therefore, earlier baculovirus promoters have been used to drive gene expression, even though they are not as strong as the very late promoters. It has been found that earlier and weaker promoters like the early *ie-1* promoter (D. L. Jarvis, Weinkauf, & Guarino, 1996) and the late *p6.9* coding for the basic protein (Bonning, Roelvink, Vlak, Possee, & Hammock, 1994; Hill-Perkins & Possee, 1990; T. A. Kost et al., 1997; Lawrie, King, & Ogden, 1995), when used to drive protein expression, can be used to produce the same or higher levels of active protein than the polyhedrin promoter. The *p6.9* protein promoter has also been shown to drive higher levels of protein production than the two very late promoters (Bonning et al., 1994; T. A. Kost et al., 1997). The baculovirus *gp-64* promoter, which codes for the gp-64 transmembrane protein which is essential for infectivity of budded baculovirus and is expressed in the late phase of the infection cycle, can be used to drive expression of foreign genes, with the reasoning being that post-translational processing will be optimal during this phase in which the baculovirus proteins are being produced that have to undergo post-translational processing (Possee & King, 2007). Other promoters such as the *p39* capsid promoter have also been used in transfer vectors designed to produce recombinant baculoviruses (Possee & King, 2007).

2.4.4 Multiplicity of Infection

One of the main parameters describing the infection of a cell culture with virus is the multiplicity of infection, or the MOI, which is the ratio of infectious virus particles to the number of cells in the culture. The MOI can be used to estimate the proportion of cells infected by viruses and the fraction (F) of cells infected by a specific number of virus particles (n) can be calculated using the following formula (Murhammer, 2007):

Equation 1:

$$F(n, MOI) = \frac{(MOI^n)\exp(-MOI)}{n!}$$

When the MOI is low, an asynchronous infection is set up where only a part of the cell population is infected, whereas a high MOI such as 3 will cause a synchronous infection, with nearly all the cells being infected by at least one virus particle. High MOIs are usually used for quick and predictable protein production in small scale experiments. However, during larger scale production, the establishment of high multiplicities of infection becomes problematic due to the need for large amounts of virus inoculum and therefore, becomes impractical. In addition, it has been found that defective interfering particles are formed during the passage of baculovirus in culture, due to the loss of parts of the genome (Kool, Voncken, Van Lier, & Tramper, 1991). These propagate in cell culture using helper functions from fully functional baculovirus and can cause reduction in protein production (Wickham, Davis, Granados, Hammer, & Wood, 1991). However, this can be avoided by producing baculoviruses at low multiplicities of infection (M. P. Zwart, Erro, van Oers, de Visser, & Vlak, 2008).

At low multiplicities of infection, baculovirus infect a small proportion of cells, which then release virus to infect other cells in the culture. In the meanwhile the non-infected cells continually divide. Therefore, the low MOI system is not easily predictable and this is

exacerbated by the sensitivity of low-MOI infections to initial conditions like virus titres and cell density, which can be inaccurate, and this reflects the importance of accurate quantification methods for baculovirus titration.

Additional factors to be considered when trying to maximize protein production include the need to reduce the time in which recombinant protein production takes place, in order to reduce proteolytic degradation of these proteins, which has been shown to be an issue in low MOI infections (Radford et al., 1997). This would indicate a need for synchronous infection as is found in high MOI infections. In addition, low MOI infections run the risk of having very high cell densities which would cause nutrient depletion and therefore, loss of productivity (Radford, Cavegn et al., 1997; K. T. K. Wong, Peter, Greenfield, & Nielsen, 1996). It has been shown that careful manipulation of the cell density and time of infection (TOI) can enable the use of very low multiplicities of infection (K. T. K. Wong et al., 1996). This approach requires the establishment of accurate baculovirus titres. Inaccurate MOI's could cause large cell death initially leading lowered productivities due to insufficient cell density, or alternatively an insufficient number of cells could be infected at the outset, leading to high cell densities and consequently, lowered protein productivities.

It has been previously reported that changes in protein yields from insect cell culture with respect to increase in MOI is significantly affected by the growth phase of cells in the cell culture (Licari & Bailey, 1991; Schopf, Howaldt, & Bailey, 1990) .In particular, it has been noted that when the cells are infected in the early exponential phase of cell culture, recombinant protein production shows no obvious correlation to the MOI. This is in contrast to what was observed when cells were infected in the late exponential phase of cell growth, where product yield showed a clear correlation to MOI, with higher MOI's resulting in higher product yields. By controlling the initial cell density, TOI and MOI carefully, low MOI strategies have been used to produce

proteins at comparable levels to high MOI strategies (K. T. K. Wong et al., 1996).

Further information on the effects of MOI in the BEVS system can be found in other works (M. G. Aucoin et al., 2010).

2.4.5 Co-infection

An extension to the infection of insect cells with more than one virus is the idea of co-infection, which involves infecting a single cell with more than one type of virus coding for different genes. This is especially important for the production of protein complexes such as virus-like particles (VLPs). It has also been used extensively for the provision of helper functions for protein processing by expressing enzymes necessary for post-translational modification of proteins, such as glycosyltransferases and enzymes for sialic acid synthesis, to provide mammalian-like modifications (Chang, Chen, Lin, Chen, & Chen, 2003; Hill, Aumiller, Shi, & Jarvis, 2006).

The use of multiple baculoviruses to deliver genes into insect cells allows for the control of expression of different genes by controlling the proportion of each virus added to cell culture (Y. Hu & Bentley, 2001; L. Palomares A., Lopez, & Ramirez, 2002). Since all cells in the cell culture will not be infected with all of the viruses used, protein complexes will have a range of compositions and particles being formed having a desired average composition. While this suggests that a high MOI is necessary to ensure that a large proportion of cells are infected with all recombinant virus, an alternative strategy involves setting up a low MOI infection, such that after the first round of baculovirus, a secondary synchronous high MOI infection will be set up for the rest of the culture (E. I. Tsao, Mason, Cacciuttolo, Bowen, & FolenaWasserman, 1996).

A further complicating factor involved in baculovirus co-infections is the fact that in some co-

infected populations of cells, the expression of genes delivered by co-infecting viruses differed significantly from what was predicted by analysing Poisson distributions (J. A. Mena, Ramirez, & Palomares, 2007). Therefore, production runs involving virus co-infection have to be optimized for every new system used.

2.5 *Baculovirus Quantification*

The accurate quantification of baculoviruses is critical for the establishment of multiplicities of infection, which is in turn critical for establishing a productive infection cycle, as discussed earlier. In brief, over or underestimation of baculovirus titres can cause the setting up of non-synchronous infections, prolonged protein production periods, thereby leading to the possibility of degraded proteins, and can cause lowered protein productivities due to sub-optimal cell densities at the time point when the bulk of protein production takes place.

Many methods have been described for the quantification of both infectious and total baculovirus particles. These include traditional techniques such as the plaque assay and the end-point dilution assay, as well as other methods based on cell size and viability, among others. Physical particle titres can be obtained by methods such as flow cytometry and Real Time PCR. Many of these methods, especially infectivity assays are usually cumbersome and lengthy (Roldão, Oliveira, Carrondo, & Alves, 2009) and sometimes have high variability between runs (L. Nielsen, 1992). In order to circumvent this, quantification methods have been developed involving the expression of reporter genes, as well as colorimetric assays. Some of the methods commonly used to obtain baculovirus titres are presented in the following sections:

2.5.1 Plaque Assay

The plaque assay involves using diluted virus samples to infect cells grown as a monolayer and counting the number of plaques formed. The number of plaques formed from several cultures infected with different dilutions of virus can be used to determine the titre of the virus stock.

The plaque assay is thought to be more accurate than the end-point dilution assay, but is more laborious (L. K. Nielsen, Smyth, & Greenfield, 1992). In addition, plaques formed by baculovirus are not always obvious and complicates the implementation of this method.

2.5.2 End Point Dilution Assay

The End Point Dilution Assay (EDPA) used for baculovirus titration is based on the Tissue Culture Infectious Dose 50 ($TCID_{50}$) method developed by Reed and Muench (Reed & Muench, 1938) and modified by others (Darling, Boose, & Spaltro, 1998; King & Possee, 1992) where different dilutions of virus are used to infect a number of cultures to statistically determine the point at which 50 per cent of the cultures infected with that dilution of virus show cytopathic effect. This is then used to extract the titre of the infectious agent, which in the case of baculoviruses infecting insect cell culture is reported as plaque forming units per ml or pfu/ml. A sample equation set (King & Possee, 1992) for the calculation of infectious titres is given below:

Equation 2a:

$$PD = \frac{[50\% - (\%\, rate\ of\ dilution\ next\ below\ 50\%)]}{[(\%\, rate\ of\ dilution\ next\ above\ 50\%) - (\%\, rate\ of\ dilution\ next\ below\ 50\%)]}$$

Equation 2b:

$$Log\ (TCID_{50}) = log(dilution\ next\ to\ and\ above\ 50\%\ positives) - PD$$

Equation 2c:

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

where PD is the proportional distance and V is the volume of added virus

The end-point dilution assay is especially useful in cases where plaques formed by virus are not readily distinguishable, and is easier to perform than the plaque assay. However, unless a large number of replicate cultures are infected with each virus dilution and analysed, the EDPA assay is not as accurate as the plaque assay (L. K. Nielsen et al., 1992). The use of reporter genes such as GFP (Cha, Gotoh, & Bentley, 1997) or β-galactosidase (Sussman, 1995) have been used to easily distinguish between infected and uninfected wells and therefore increase the accuracy of this method. In order to remove the problem of small plaques and non-obvious infection, a method has been developed whereby infected plates are incubated for 7 days and the supernatants are used to infect a plate with fresh cultures for an additional 4 days, which improved the ability to distinguish between infected and uninfected cultures (Roldão et al., 2009)

2.5.3 MTT assay

The MTT assay (J. A. Mena, Ramirez, & Palomares, 2002) is used to determine the presence of infected cells in a manner similar to the $TCID_{50}$ assay. In this assay, replicate cultures are infected with serial dilutions of virus and incubated. Following incubation, thiazolyl blue tetrazolium bromide (MTT) is added to the culture which forms insoluble MTT-formazan due to the action of dehydrogenases of living cells. The cultures can then be put through a spectrophotometer and the virus titre calculated by the following set of equations:

Equation 3a:

$$A = A_{bottom} + \frac{A_{top} - A_{bottom}}{1 + (\frac{dil}{EC_{50}})^{slope}}$$

Equation 3b:

$$TCID_{50} = \frac{1}{EC_{50} \times V}$$

Equation 3c

$$\log(pfu/ml) = 0.829 \times log(TCID_{50}) + 2.512$$

Where A is the absorbance, A_{top} is the maximum absorbance which is set to 100 after data normalization), A_{bottom} is the baseline absorbance which is set to 0 after data normalization, EC_{50} is the dilution that provokes a response halfway between maximum and baseline, and dil is the virus dilution. The MTT assay while accurate is also labour intensive and time consuming (Pouliquen, Kolbinger, Geisse, & Mahnke, 2006).

2.5.4 AlarmarBlue Assay

The AlarmarBlue assay (Pouliquen et al., 2006) is used to observe cell cycle arrest caused due to baculovirus infection. It is based on the principle that proliferating cells are capable of reducing the reagent AlamarBlue™ to a reduced fluorescent state which can then be measured using a fluorescence detector. The obtained data can be used to determine the infectious titre of the virus stock using the following equations:

Equation 4a:

$$GI = \frac{t_{24}virus - t_0control}{t_{24}control - t_0control} * 100$$

Equation 4b

$$GI = A_1 + \frac{A_2 - A_1}{1 + e^{-(D-D_0)*p}}$$

Equation 4c

$$TCID50/ml = \frac{1}{D_0 * V}$$

Equation 4d

$$Log(pfu/ml) = 0.643 * \log(TCID50/ml) + 5.867$$

Where GI is the growth inhibition, t_{24} and t_o values are the fluorescence values for control and infected cultures at 24 and 0 hours respectively A_1 is the minimum growth inhibition (undisturbed cell growth), A_2 is the maximum growth inhibition (100% infected cells), D is the dilution factor, D_0 is the dilution at which the growth inhibition was 50%, and p is a slope factor.

The AlamarBlue™ method is rapid as it can be done in about a day (Pouliquen et al., 2006; Roldão et al., 2009), and this prevents erroneous readings of fluorescence values due to secondary virus infections (Pouliquen et al., 2006).

2.5.5 Cell Size Assay

The cell size assay (Janakiraman, Forrest, Chow, & Seshagiri, 2006) is used to measure the titre of virus stock using the changes in cell size distribution after virus infection. Insect cells infected with virus generally exhibit size increases after infection and the increase in mean cell size of cultures infected with different dilutions of virus is used to determine the titre of virus stock. The cell size distribution can be determined using a cell counter. There are several methods which can be used to estimate the parameters required for titre determination (Janakiraman et al., 2006; Roldão et al., 2009).

The advantages of this method include the ability to titrate virus stocks in about a day. However, cell size readings have to be conducted less than 24 hours post infection to avoid the effects of secondary infection, which can seriously affect titre determination (Roldão et al., 2009). In addition, the method requires the use of a cell counter which may not be accessible to all

researchers.

2.5.6 Flow Cytometry Assay

The flow cytometry assay is used to determine the total number of viral particles in a sample. It involves labelling the virus with an agent such as SYBR green and then running the virus through a flow cytometer (Brussaard, Marie, & Bratbak, 2000) and has been adapted for use in for the quantification of baculovirus (Shen, Meghrous, & Kamen, 2002). This method is relatively rapid and has low run-to-run variability (Roldão et al., 2009; Shen et al., 2002).

2.5.7 Real Time PCR

Real Time quantitative PCR has been used extensively for the quantification of DNA (Kaltenboeck & Wang, 2005) and RNA (Bustin, 2000). It has been used for the quantification of several viruses (Mackay, Arden, & Nitsche, 2002) and has been proven to be a sensitive and accurate method for virus quantification and detection.

Real time PCR has been extended for use in the baculovirus system and has been used by several groups for baculovirus quantification. Its main advantages are its rapidity and the accuracy of measurement (Roldão et al., 2009). The use of Real Time PCR for baculovirus titration has been described by Lo and Chao (Lo & Chao, 2004) and Hitchman et al (Hitchman, Siaterli, Nixon, & King, 2007).

A wide variety of primers have been used to target various regions of the baculovirus genome including essential genes such as *gp-64* (Hitchman et al., 2007), *ie-1* (Kato, Manoha, Tanaka, & Park, 2009; C. Liu et al., 2008), the *gp-41* gene (C. Liu et al., 2008), the viral DNA polymerase

gene *dnapol* (Rosinski, Reid, & Nielsen, 2002), as well as the incorporated transgenes (Roldão et al., 2009; Vieira et al., 2005).

While several papers have stated that the titres obtained from Real Time PCR corresponds closely to titres obtained by other methods such as the EDPA (Lo & Chao, 2004) and the plaque assay (Hitchman et al., 2007), others have found that the titres obtained by Real Time PCR can be 1-2 logs higher than other methods, but with very high reproducibility between runs (Roldão et al., 2009). This discrepancy has been thought to be due to the presence of defective interfering particles (discussed earlier in the section on multiplicity of infection), but can also be caused by residual DNA in the samples used for quantification.

Real Time PCR involves monitoring the change in amounts of double stranded PCR product in the system, from which information can be gathered about the starting quantity of template material in the sample. This is done by using a fluorescent tag to monitor the levels of fluorescence in the system at each cycle of DNA amplification. The two most commonly used Real Time PCR detection chemistries are the SYBR Green and TaqMan® chemistries. SYBR green is a dye that can be excited and fluoresces when bound to double stranded DNA. Therefore this enables the monitoring of the accumulation of double stranded DNA in each cycle of a reaction. The TaqMan® chemistry, on the other hand, was first developed in 1991 (Holland, Abramson, Watson, & Gelfand, 1991) and has since been further developed by others (L. G. Lee, Connell, & Bloch, 1993). The technology as it stands currently involves the use of a probe which binds specifically to the region targeted for real time PCR. The probe contains a fluorophore attached to the 5’ end and a quencher on the 3’ end, which quenches the fluorescence from the fluorophore by Fluorescence Resonance Energy Transfer (FRET). When PCR occurs, the DNA polymerase in the reaction mixture displaces the probe which is then degraded by the polymerase exonuclease activity. The fluorophore is no longer quenched and can therefore fluoresce.

Successive synthesis cycles will cause the accumulation of fluorescence which can be monitored.

2.5.8 Real Time PCR data analysis

In a typical Real Time PCR, the fluorescence level of the reaction initially fluctuates randomly at a low background fluorescence level, with the signal, due to the amplified product, being too low to be differentiated from background noise. At a certain cycle during the reaction, the fluorescence signal increases in a linear fashion above the background noise and passes a threshold fluorescence level set by the RT-PCR analysis software or the user, and the cycle number at which this occurs is called the threshold cycle or the Ct value. As can be expected, a higher starting template concentration will translate to a lower Ct value. As the primers and nucleotides start becoming depleted, the reaction plateaus out until a constant fluorescence level is reached indicative of a constant amount of product DNA.

The most common method to analyse Real Time PCR data makes use of standard curves generated by running known standards through the reaction and recording Ct values for each of the standards, which is used to generate a standard curve. The Ct values of the samples can then be compared to this standard in order to obtain the titre of template in the sample. While it is relatively straightforward to implement and robust (Cikos, Bukovska, & Koppel, 2007), it is limited in that it makes assumptions such as the constant efficiency of the amplification reactions for the sample and standard, as well as assumptions regarding the same baseline fluorescence values for all reactions. In addition, the standard curve method requires the generation of a standard curve, even for studies where absolute quantification of DNA is not required. The standard curve method is the only one to be used for baculovirus quantification, as far as we know.

The basic equation for PCR product formation in a sample is given below:

Equation 5a:

$$C_n = C_o * (1 + E)^n$$

Where C_o is the initial copy number, C_n is the copy number at cycle n, n is the number of cycles and E is the efficiency of target amplification. Since the copy number is directly proportional to the intensity of fluorescence, the equation can be re-written as:

Equation 5b:

$$R_n = R_o * (1 + E)^n$$

Where R_o is the initial fluorescence level and R_n is the level at cycle number n.

To circumvent the limitations of the standard curve method, several groups have investigated alternative methods to analyse Real Time PCR data. Most of these methods were originally developed to aid comparison of fold differences of expression between genes of interest and an exogenous control (Cikos et al., 2007). Some of these are described below.

2.5.8.1 *Comparative C_t Method*

The comparative C_t method (Livak, 1997) attempts to remove the need for a standard curve by making the assumption of ideal efficiency. A modified method has been developed (Pfaffl, 2001) which allows for deviations from ideal efficiency. The equation can easily be adopted for absolute quantification of DNA by substituting the terms for the reference gene by those for a DNA standard of known quantity.

Equation 6:

$$R = \frac{E_{Target}^{\Delta C_{target}(control-sample)}}{E_{Ref}^{\Delta C_{Ref}(control-sample)}}$$

Where R is the ratio of target gene expressed in a sample versus a control in comparison to a target gene, E_{Target} is the PCR efficiency of a target gene transcript, E_{Ref} is the efficiency of the reference gene transcript, ΔC_{target} is the Ct difference between target control and sample reactions and ΔC_{ref} is the Ct difference between the reference control and sample.

2.5.8.2 *Liu and Saint method*

The Liu and Saint method (W. Liu & Saint, 2002a) determines the efficiency of each reaction from the florescence and cycle numbers at two threshold fluorescence levels, as shown below:

Equation 7:

$$E = (R_2/R_1)^{1/(C_{t2}-C_{t1})} - 1$$

Where E is the efficiency of a reaction, R_2 and R_1 are the fluorescence intensities and C_{t2} and C_{t1} are the cycle numbers of a reaction at these intensities. The initial fluorescence level (which correlates directly to initial template abundance) can then be calculated by the basic equation for a PCR amplification reaction (Equation 5b). However, this method depends on the subjective selection of two threshold points, which could confound efficiency estimation and initial fluorescence level determination.

2.5.8.3 *Sigmoidal Curve Fitting*

The Sigmoidal Curve Fitting (SCF) method (W. Liu & Saint, 2002b) uses the whole amplification data (not just the exponential phase, like other methods) to fit a sigmoidal equation to this curve. The equation used by the original authors to describe the curve is given below:

Equation 8:

$$R_n = R - R_b = \frac{R_{max}}{1 + \exp\left(-((n - n_{1/2})/k)\right)}$$

Where R is fluorescence dye strength in real time PCR, R_n is background- removed fluorescence dye strength in real time PCR at cycle n, R_b is the background fluorescence dye strength, R_{max} the maximal fluorescence dye strength, $n_{1/2}$ the cycle number when fluorescence dye strength is half of the R_{max}, k the slope factor of increase in fluorescence dye strength. The efficiency is given by the equation:

Equation 9:

$$E = \frac{R_n - R_{n-1}}{R_{n-1}}$$

Which, ignoring the R_b term from equation 4 becomes:

Equation 10:

$$E = \{1 + \exp\left(-\frac{n - 1 - n1/2}{k}\right)\} \Big/ \{1 + \exp\left(-\frac{n - n1/2}{k}\right) - 1\}$$

The initial fluorescence value R_o can be calculated using the equation:

Equation 11:

$$R_o = \frac{R_{max}}{1 + \exp\left((n_{1/2})/k\right)}$$

2.5.8.4 *DART-PCR*

The Data Analysis for Real-Time PCR (DART-PCR) method (Peirson, Butler, & Foster, 2003) is a combination method which uses linear regression to extract the efficiency of the reaction for each sample at the mid-point of the exponential phase, and a conventional C_t based method to determine initial fluorescence value R_o for the sample. The equations used are shown below:

Equation 12:

$$M = R_{noise} * \sqrt{R_{max}/R_{noise}}$$

Where M is the midpoint of the fluorescence signal range, R_{max} is the maximum signal intensity and R_{noise} is calculated is the background level of fluorescence.

Points in the amplification plot are selected which lie close to the M value, a line is fitted through these points, and efficiency E of the reaction is determined by the equation

Equation 13:

$$E = 10^{(1/slope)} - 1$$

Knowing the efficiency, the initial fluorescence value Ro can then be calculated from Equation 5b.

2.5.8.5 *Assumption-free analysis*

Assumption-free analysis (AFA) also uses linear regression, which is implemented slightly differently to DART-PCR (Ramakers, Ruijter, Deprez, & Moorman, 2003; Ruijter, Ramakers, Hoogaars, Karlen, Bakker, van den Hoff, & Moorman, 2009b). AFA is available for use as a program package called LineRegPCR and uses an algorithm to find the linear portion of the amplification plot, and fits a line to the points within the linear range. Using the slope and intercept of this line, the efficiency and initial fluorescence level of each individual reaction can be determined. The method is described in greater detail in Chapter 2 of this thesis.

Of these methods used, the methods using individual efficiencies to determine initial fluorescence are very sensitive to changes in individual reaction efficiencies due to small variations in initial conditions, thereby leading to issues of reproducibility. Therefore, using the average efficiency of

similar types of reactions has been shown to increase the robustness of these methods (Cikos et al., 2007).

All the data analysis methods listed (except for the standard curve method) give an arbitrary fluorescence value which can be compared with that of a standard to give an absolute value for quantification of template in a sample.

Detailed information on the relative benefits and disadvantages of each method and relative comparisons between methods have been published in other works (Cikos et al., 2007). Briefly, the assumption-free analysis method seems to be the best method to use due to its precision, and accuracy, which it shares with the DART-PCR method, but also due to its excellent reproducibility.

2.6 Summary

The use of baculoviruses in a wide range application illustrates the versatility of this virus and its potential for benefit to mankind. While much work has been done on improving its usage for various processes, many process related issues need to be further studied such as improvement of processing in cells and optimal production and infection conditions. In particular, the issue of baculovirus quantification needs to be urgently addressed due to the current lack of a universally accepted method of quantification as well as the lack of a standard reference material.

CHAPTER 3

Common Materials and Methods

The procedures given below are those which have been common to all of the work presented in this thesis. Variations to these methods in each separate chapter, as well as additional protocols and methods, are listed in the individual chapters.

3.1 Cell culture and Baculovirus Production

Spodoptera frugiperda clonal isolate 9 (Sf-9) cells (GIBCO, Carlsbad, California, USA) were maintained in capped glass Erlenmeyer flasks in Sf-900III media (GIBCO, Carlsbad, California, USA) at a temperature of 27ºC on an orbital shaker rotating at 130 rpm. Cultures were seeded at a density of $1x10^6$ cells/ml and infected with BacGFP which was kindly provided by Dr. Robert Kotin (National Institutes of Health, Bethesda, Maryland, U.S.A). The cultures were allowed to grow till the viability dropped to 70–80%, after which the culture medium was harvested and centrifuged at 1000g for 10 minutes in order to spin down cells and cell debris. The supernatant was removed and used as baculovirus stock for experiments.

3.2 Template Generation Using DNA Extraction Kits

Viral DNA was extracted and template for PCR was generated using three methods. The first two involved the use of two kits: the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Laval, Quebec, Canada) and the DNeasy Blood & Tissue Kit (QIAGEN, Mississauga, ON, Canada). Nucleic acid material from 200 µl of virus stock was purified using the two kits as described by

the manufacturer's protocols.

Briefly, for the High Pure Viral Nucleic Acid Kit, 200 µl of solution containing virus was lysed by incubating the sample at 72°C for 10 minutes with 50 uL of Proteinase K (20 g/L) and a 200 µl of a working solution made by mixing 4 µl poly(A) carrier RNA (supplied with the kit), with 250 µl Binding Buffer (6 M guanidine-HCl, 10 mM Tris-HCl, 20% Triton® X-100 (w/v), pH 4.4 (25°C)). An additional 100 µl of Binding Buffer was then added and the solution transferred to a High Filter Tube purification column. The solution was then centrifuged for 1 minute at 8,000 x g and the flow-through was discarded. This step was repeated with 500 µl of Inhibitor Removal Buffer (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6 (25°C), ethanol) and two 450 µl additions of Wash Buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5 (25°C), ethanol), with centrifugation steps being performed in between each addition. A final centrifugation step was done for 10s at 13,000 x g to remove residual Wash Buffer, after which DNA was eluted out of the column using 200 µl of Elution Buffer (nuclease-free, sterile, double distilled water) and centrifuged for 1 minute at 8,000 x g. DNA concentration was determined by NanoDrop® ND-1000 Spectrophotometer (NanoDrop products, Wilmington, U.S.A).

For the DNeasy Blood and Tissue Kit, the manufacturer's protocol was followed for cultured cells without initial centrifugation and re-suspension steps. Briefly, 200 µl of virus stock was lysed by incubating the sample at 56°C for 10 minutes with 20 µl Proteinase K solution (supplied with the kit) and 200 µl Buffer AL containing guanidine hydrochloride, after which 200 µl absolute ethanol was added to the sample and the solution placed into a DNeasy Mini spin column. The solution was then centrifuged for 1 minute at $\geq$ 6000 x g, and the flow-through was discarded. This step was repeated with additions of 500 µl of Buffer AW1 and 500 µl Buffer AW2, following which the column was centrifuged at 20,000 x g to dry the column membrane. The DNA was eluted out of the column using 200 µl of Elution Buffer and the DNA

concentration was determined by NanoDrop® ND-1000 Spectrophotometer (NanoDrop products, Wilmington, U.S.A).

3.3 Plasmid Standard Generation

The plasmid standards for RT-PCR analysis were generated by the following approach. Firstly, Gp-64F and Gp-64R primers were used to clone out a 72 bp region in the Gp-64 gene. This region was then cloned into the SacI site of the pAcSG2 plasmid (BD Pharmingen, San Diego, California, USA) using a combined restriction digestion/ligation reaction. The ligation of the Gp-64 fragment into the vector destroyed the SacI site preventing the recircularization without the desired fragment. The enzymes used were FastDigest SacI and T4 DNA Ligase (both from Fermentas Life Sciences, Burlington, ON, Canada). The new plasmid was then used to transform chemically competent DH-5α *Escherichia coli* bacteria, which were grown on LB plates with 100µg/mL ampicillin. Individual colonies were then isolated and grown in 5mL LB media supplemented with 150 µg/mL ampicillin. After 16 hours, the plasmid of interest was extracted from the cultures using the GeneJET™ Plasmid Miniprep Kit (Fermentas Life Sciences, Burlington, ON, Canada). The plasmid was tested for the presence of the inserted Gp-64 fragment by PCR using the Gp-64 F and R primers followed by running the PCR products on a 1.5% agarose gel to check for the presence of amplified product in the size range of the Gp-64 fragment.

3.4 Real time PCR

All RT-PCR reactions were conducted on a StepOne Plus Real-Time PCR system (Applied Biosystems, Burlington, ON, Canada) and were prepared in MicroAmp Fast Optical 96-well Reaction Plates (Applied Biosystems, Burlington, ON, Canada). Each reaction consisted of 2 µl

sample, 10 µl of 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Burlington, ON, Canada), forward and reverse primers at a concentration of 900 nM each, and nuclease free water, for a final volume of 20 µl. Successive sample dilutions were conducted by the addition of 2 µl concentrated sample into 18 µl of nuclease free water in 1.5 mL microcentrifuge tubes. 6 µl of these were then added along with 54 µl stock mix of 2X Power SYBR® Green PCR Master Mix, forward and reverse primers and nuclease free water into one well of an optical PCR plate. The solution was mixed and distributed between three wells of the PCR plate. The plate was then sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Burlington, ON, Canada) and centrifuged briefly at 1000g in a Eppendorf 5804R centrifuge (Eppendorf Canada, Mississauga, ON, Canada) centrifuge with an A2-DWP flat plate rotor (Eppendorf Canada, Mississauga, ON, Canada). Amplification cycles were conducted according to conditions which were described in literature for the primers used. Specific cycling conditions and primers used are described in each individual chapter.

Each sample was run in triplicate to provide statistical validity and confidence in the data obtained. Data obtained from each reaction was analysed by StepOne™ Software v2.0, which is the controller for the StepOne instrument, and assumption-free analysis was conducted on the data using the program LinRegPCR (11.5) (Ruijter, Velden, & Ilgun, 2009).

3.5 Titre Determination by Comparison with Standards

Prepared plasmid standard dilutions were subjected to RT-PCR along with samples, and a standard curve of threshold cycle (C_T) vs. standard concentration was generated. The threshold cycle is the cycle number at which fluorescence of a sample increases beyond a certain threshold, set automatically by the instrument control software StepOne™ Software v2.0 (Applied Biosystems, Burlington, ON, Canada) to be significantly higher than the background noise. The

C_T values of the samples were then compared with the standard series to determine the concentration of baculovirus DNA in samples.

3.6 Titre Determination by Assumption-free Analysis

The complete fluorescence profile over time for each individual well was used to determine the initial concentration fluorescence level of DNA in the well. In the portion of the profile where there is an exponential increase in the fluorescence, the fluorescence level is related to the amount of starting material by the following equation (Ramakers et al., 2003):

Equation 1:

$Log(N_C) = Log(N_0) + Log(Eff) \text{ X } C$

Where N_C is the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye; N_O is the starting concentration of material in arbitrary fluorescence units; Eff is the efficiency of the reaction; and C is the cycle number.

Briefly, the N_c values for each well, after each cycle were obtained and plotted against each other. The "window of linearity" was then determined and a linear equation was fitted to this line. The concentration (in arbitrary units) of starting material and efficiency of the PCR reaction for the well were determined from the intercept and slope, respectively. The arbitrary concentration was then compared to that of a standard well having a known concentration, and an absolute concentration of DNA was derived in terms of a ratio to the amount of standard DNA. All steps were performed using the program LinRegPCR (Ruijter, Velden et al., 2009) downloaded from http://LinRegPCR.nl on January 14, 2010. The assumption free analysis parameters uses different baselines and threshold fluorescence values for each group of samples, grouped as having different dilutions and treatments (See Chapter 3 for a description of dilutions and treatments).

CHAPTER 4

The Use of Assumption-free Analysis for Quantification of Baculovirus

4.1 Introduction

Real Time PCR has become an established method for general gene and transcript quantification (Kaltenboeck & Wang, 2005; Snider, Wechser, & Lossos, 2001), and for the quantification of both DNA and RNA viruses (A. Abe et al., 1999; Ngaosuwankul et al., 2010; Podgorska & Stadejek, 2010; Trujillo et al., 2006). The system has therefore been used for the quantification of baculovirus by various groups (Hitchman et al., 2007; Lo & Chao, 2004). Traditionally baculovirus quantification assays using Real Time PCR have required the generation of a standard curve of threshold cycle (Ct) vs. DNA copy number with various standard dilutions, to which the threshold cycles of samples are then compared and sample titres determined. This method assumes that the efficiency of sample and standard wells are the same, that efficiency of the PCR reaction remains constant over the course of a run (Ramakers et al., 2003). Slight differences in efficiencies between sample wells, however, can mean that samples with different levels of starting material can have similar Ct values which can cause a large variation in values obtained for the number of template copies.

Several groups have attempted to develop methods of analyzing Real Time PCR data to overcome the problems associated with the standard curve method. These include the Liu and Saint method, which account for variations in efficiency over the exponential phase of amplification (W. Liu & Saint, 2002a); the comparative Ct method (Pfaffl, 2001); the DART-

PCR method which calculates efficiency by trying to accurately estimate the exponentially increasing portion of an amplification plot (Peirson et al., 2003); sigmoidal curve fitting (Rutledge, 2004); and assumption-free analysis (Ramakers et al., 2003). Despite the emergence of these methods, the standard curve method remains one of the most widely used methods for nucleic acid quantification (Karlen, McNair, Perseguers, Mazza, & Mermod, 2007). A detailed review of the most well known methods in Real Time PCR data processing has been given in the Literature Review of this thesis.

Given the benefits of assumption-free analysis for the quantification of baculovirus, including its potential use for semi-quantitative determination of titres high level of precision (Cikos et al., 2007; Feng, Zeng, & Chen, 2008), we have decided to investigate its applicability to the quantification of baculovirus.

Assumption-free analysis of quantitative RT-PCR (Ramakers et al., 2003) is a method that, has seen widespread use lately for both DNA and RNA quantification (Buck et al., 2010; Wai et al., 2010), but as far as we know, has not been for establishing virus titres except for work done by Feng et al on the cucumber mosaic virus (Feng et al., 2008). Therefore, this method was tested to compare its accuracy, robustness and precision in baculovirus titre determination against standard curve based quantitative Real Time PCR. Assumption-free analysis was developed by Ramakers et al (Ramakers et al., 2003; Ruijter, Ramakers, Hoogaars, Karlen, Bakker, van den Hoff, & Moorman, 2009a) to address the limitations of traditional quantitative PCR using C_T comparisons with standards by determining the efficiency of each reaction from the exponential phase of each PCR reaction. This is done by first plotting the log of the fluorescence values against cycle number for each sample and using an algorithm to determine the region where the graph increases linearly (Ruijter, Ramakers, Hoogaars, Karlen, Bakker, van den Hoff, & Moorman, 2009a). This is done by an iterative algorithm which searches for a group of 4 to 6 points which have the

highest R^2 value and the maximum slope in the amplification curve. A line is then fitted to the linear portion of the graph, whose slope represents the efficiency of the reaction and from this, a starting concentration of sample in each well can be found in arbitrary fluorescence units, which is called an N_o value. The utilization of this method is demonstrated in Figure 4.1, with the bars on either side indicating the large changes in calculated No values which occur with small changes in efficiency. Therefore, small variations in reaction conditions can give large changes in the N_o value and so, an average efficiency value from replicate reactions has been shown to give more accurate and reproducible results as compared to when individual efficiencies are used (Cikos et al., 2007).

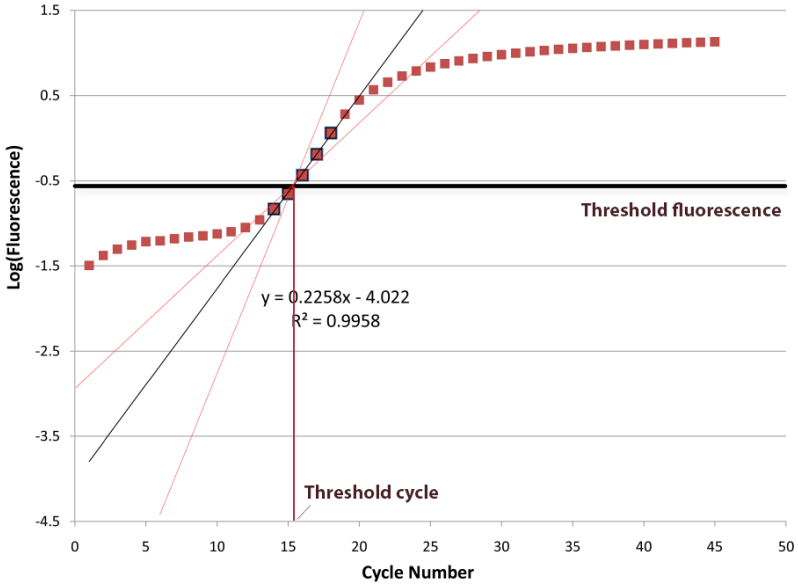

Figure 4.1: Assumption-free analysis: Log fluorescence values are plotted against cycle number and the window of linearity determined, where there is exponential amplification. A linear trendline is then fitted to the points within the window of linearity and the slope and intercept determined and used to calculate the efficiency and No values. Efficiency = 10^slope = 1.69, No = 10^intercept = 9.51E-5.

An absolute value for concentration of template can then be found, if desired, by comparing this arbitrary measurement with that of a defined standard. Because the fluorescence in a sample is directly proportional to the level of DNA inside a sample, the initial fluorescence level in a sample is indicative of the amount of template DNA present in the sample originally (Peirson et al., 2003). Therefore, an additional advantage to this method is that, in the absence of a standard,

N_o values can be directly compared between sample wells to determine the relative abundance of initial template in samples.

In this study we analyse two sets of preparations of 9 baculovirus stocks to determine whether there is an advantage to titres obtained using No analysis compared to those obtained by conventional standard curve comparisons. Furthermore, we investigated whether this method could be used to determine the fold difference of template material in these samples, without the use of standard curves.

4.2 Materials and Methods

The materials used for this study are described in chapter 3 with the modifications detailed below.

4.2.1 Cell culture and Baculovirus Production

Cell culture and baculovirus stock generation was conducted as described earlier. 9 unpurified samples of baculovirus (Stocks 1-9, culture supernatant) which were previously quantified by flow cytometry, were kindly provided by Dr. Amine Kamen (Biotechnology Research Institute, National Research Council Canada), and have been used elsewhere (Transfiguracion, Mena, Aucoin, & Kamen).

4.2.2 Template generation

Viral DNA was extracted and template for PCR was generated using two methods. The first involved the use of the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Laval, Quebec, Canada), as described in Chapter 3.

The second method involved diluting 2 µl virus stock in 198 µl PBS and then subjecting it to Triton X-100 at a concentration of 0.1% Triton X-100 volume/volume in the final reaction mixture, followed by 2 freeze/thaw cycles using dry ice and ethanol, and a 37^{O}C water bath

4.2.3 Real time PCR

Real Time PCR reactions were conducted as described earlier. The set of primer used in this reaction were targeted against a region in the Gp-64 gene (Hitchman et al., 2007). The sequences of the primers are given in Table 4.1. Amplification cycles were conducted according to conditions described previously (Hitchman et al., 2007). Briefly, initial denaturation was conducted at 95°C for 3 minutes, followed by 45 cycles of denaturation at 95°C for 30 seconds and annealing/extension at 60°C for 30 seconds. The results were then analysed by comparison with standards, or by assumption-free analysis.

Table 4.1: Sequences of primers used in this study.

Primer	Sequence (5' – 3')
Gp-64 F	CGGCGTGAGTATGATTCTCAAA
Gp-64 R	ATGAGCAGACACGCAGCTTTT

4.2.4 Titre Determination by Assumption-free analysis

Fluorescence data for individual wells at each cycle number were used to determine initial concentrations of material. The fluorescence level is related to the amount of starting material by this equation (Ramakers et al., 2003).

Equation 1

$$\text{Log}(N_C) = \text{Log}(N_0) + \text{Log}(\text{Eff}) \text{ X } C$$

Where N_C is the measured fluorescence data and is the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye, N_O is the starting concentration of material, in arbitrary fluorescence units, Eff is the efficiency of the reaction and is ideally 2.0, C is the cycle number

Briefly, the N_C values for each cell, at each cycle number were obtained and a fluorescence versus cycle number curve was plotted. The "window of linearity" was then determined and a linear equation was fitted to this line. The concentration (in arbitrary units) of starting material and efficiency of the PCR reaction for the well could then be determined from the intercept and slope, respectively. This measured arbitrary concentration was then compared to that of a standard well, and an absolute concentration of DNA in the sample well was derived in terms of a ratio to the amount of standard DNA. All steps were performed using the program LinRegPCR (11.6) (J. M. Ruijter, S. van der Velden, A. Ilgun, Heart Failure Research Center, Academic Medical Center, Amsterdam, the Netherlands) and downloaded from http://LinRegPCR.nl on January 14, 2010, which calculates threshold and Ct values and efficiency for each reaction (and then mean efficiency) from the window of linearity, which is then used to calculate the N_o value for each sample. The assumption free analysis parameters included using different baselines and threshold fluorescence values for each group of samples where each group contained different dilutions of DNA or treated baculovirus that had been prepared in a single consistent manner.

The average N_o values of samples were compared with the average N_o values of standards, computed by averaging N_o values of various dilutions of standards, to determine the absolute quantities of virus.

4.2.5 Statistical Data Analysis

Data analysis using the two tailed t-test as well as determination of confidence intervals of slopes of linear regressions was conducted on the data in Microsoft Excel 2007 (Redmond, Washington, U.S.A).

4.3 Results and Discussion

Assumption-free analysis has been used widely for titre determination of RNA and DNA, with the original paper by Ramakers et al (Ramakers et al., 2003) being referenced more than 600 times at the time this manuscript was being prepared. However, as far as we know, the use of this system for the quantification of viruses has only been reported once (Feng et al., 2008).

Assumption-free analysis was originally investigated in this lab as part of an effort to find methods to compare the amounts of template DNA in different samples without the use of a standard curve. This method was used due to its ability to account for changes in amplification efficiency between samples (Ramakers et al., 2003), its accuracy over other methods of data processing (Cikos et al., 2007; Feng et al., 2008) and because of its relative ease of implementation (Feng et al., 2008). Alternative methods such as comparing Ct's of various samples cannot be used for determining fold differences in template material without the use of standards, as this would require the use of an efficiency factor, which would involve either using a standard curve to determine efficiency (which would again introduce the assumption of uniform efficiency in all wells of the reaction plate) or would require the assumption of perfect efficiency.
Due to the nature of assumption-free analysis, it is a very sensitive method; small changes in the efficiency of a sample can cause large dispersion of N_o values (Cikos et al., 2007; Karlen et al., 2007), even between replicates of the same sample. This is a limitation of the method as it can confound determination of the "real" N_o value of the sample. This variation in efficiency is most likely caused due to random error and may not be indicative of a real difference between reactions (Nordgård, Kvaløy, Farmen, & Heikkilä, 2006). The accuracy and reproducibility of this method is greatly improved when the mean efficiency of a group of reactions is averaged and used as the basis for determining N_o values (Cikos et al., 2007). A group consists of similar samples, which were treated in a similar manner. An example would be that all standard dilutions

were considered as one group and their mean efficiency was used as the basis of N_o calculations for standards.

4.3.1 Determination of efficiencies

The efficiencies of the different reactions were measured by assumption-free analysis and compared to the average efficiency and the efficiency of the overall reaction as determined by the slope of the standard curve. The results are given in Table 3.2.

Run 1			
Number of copies	Efficiency of sample	Average efficiency	Efficiency from standard curve
2.00E+07	89.3 (0.84)	93.1	100.8
2.00E+06	93.3 (1.93)		
2.00E+05	94.2 (0.95)		
2.00E+04	94.2 (0.27)		
Run 2			
Number of copies	Efficiency of sample	Average efficiency	Efficiency from standard curve
2.00E+08	94.2 (0.95)	92.1	96.3
2.00E+07	83.5 (3.74)		
2.00E+06	87.5 (1.98)		
2.00E+05	100.2 (0.20)		

Table 4.2: Efficiencies (expressed in % form) for two sets of plasmid standards analysed in two Real Time PCR runs on two different days. Individual and average efficiencies have been determined by assumption-free analysis, while the efficiency from standard curves by plotting the $\log_{10}$ (fluorescence) values against the copy number of the standard. Standard deviations obtained from triplicate runs are given in parentheses.

It can be seen that concentration does not appear to play a role in determining the efficiency of a reaction, as there seems to be no clear trend. However, efficiencies obtained between different wells and samples do change and the mean efficiency obtained using assumption-free analysis differs substantially from the mean obtained from a standard curve. This is a result which has been observed earlier and can be attributed to various factors including the assumption that the reaction at the threshold cycle (C_t) is at optimal efficiency in the exponential amplification stage (Rutledge & Stewart, 2008), which is made to be common for all reactions when subjected to

analysis using standard curves. It can be seen from Table 4.2 that there is not much variation in efficiency between triplicate samples, which could be due to the methodology used, where each triplicate reaction is prepared together and then dispensed into the different wells

In addition, it can be seen that the efficiency obtained from standard curves is higher than that obtained from assumption-free analysis. This is in accordance to previous reports which state that the standard curve method seems to over-estimate reaction efficiencies (Tichopad, Dilger, Schwarz, & Pfaffl, 2003; M. L. Wong & Medrano, 2005).

4.3.2 Correlations between titres obtained using standard curves and using N_o analysis

Data obtained from two separate runs were analysed by comparison with standard curves and by assumption free analysis and the titre obtained from each method was determined. The results of this analysis are shown in Figure 4.2. It can be seen that the titres obtained are correlated almost exactly, even if they do not have exactly the same values. Therefore, it is likely that any fold differences seen in N_o values of different samples can be regarded as fold differences in between template concentrations of these samples. This is based on the assumption that titres obtained using standard curves are correlated with the real titres of virus. The slopes of both graphs were found to be significantly different from 1 at the 95% confidence interval (with 95% confidence limits for the slopes being 0.759 ± 0.0167 and 0.9271 ± 0.011 respectively). This could be indicative of a bias between the two methods of treatment, where the standard curve method reports higher titres when compared to assumption-free analysis.

This was further confirmed when data obtained for dilutions of standards used to generate standard curves were also analysed by N_o analysis. When the N_o values of different dilutions of standards were used to calculate to the N_o value of the lowest dilution of standard (i.e. calculating back to the N_o value of the most concentrated sample by multiplying the N_o value of diluted

standard with the dilution factor), it was seen that the values obtained from various standard dilutions appeared to be almost equal (Figure 4.3). This gives added evidence that fold differences in N_o values correspond to real fold differences in template concentrations. Variations in N_o values such as those observed in Figure 4.3 can probably be attributed due to a combination of factors such as the possible presence of inhibitors (Wilson, 1997)and variations in reaction components and variations in early amplification (Bustin, 2000; M. L. Wong & Medrano, 2005).

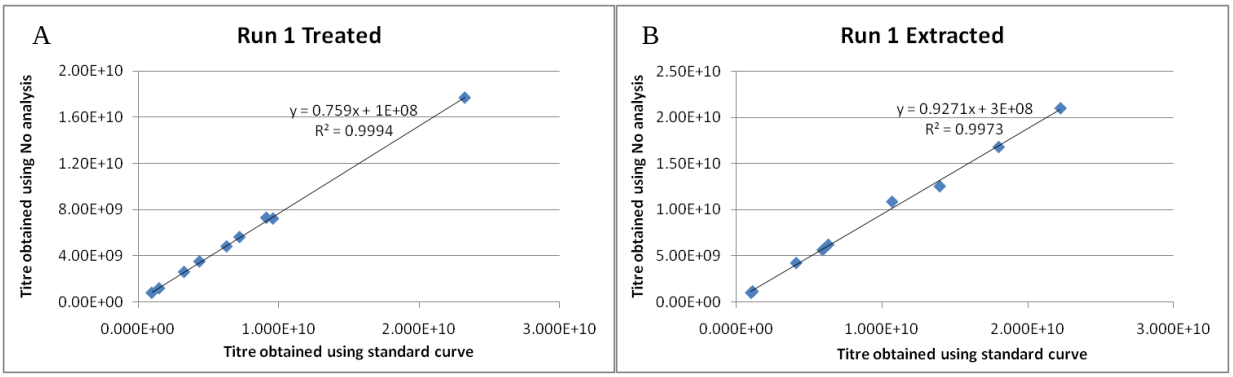

Figure 4.2: Titres of various stocks of virus, treated by two methods to expose DNA, was obtained by analysing RT-PCR data by comparison with standard curves as well as by assumption-free analysis. The two titres were plotted against each other to obtain the graphs shown above. 4.2 (a): Treated samples, 4.2 (b): Extracted samples.

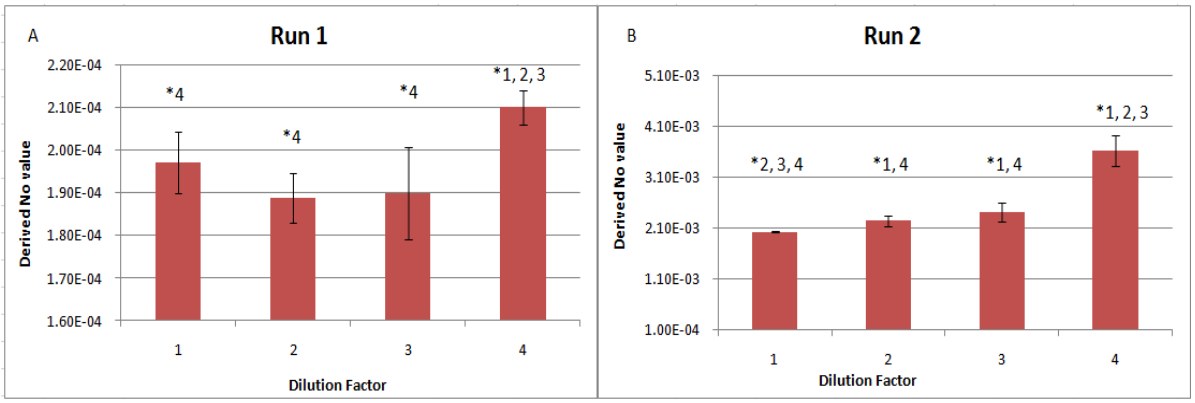

Figure 4.3: N_o values of dilutions of standards, which have been used to calculate the N_o value of the lowest dilution. Two sets of dilutions were run on two separate days on separate plates. A and B are two representative runs. The different dilutions were compared by 2 tailed student t-tests, with the results being presented by * values above each bar on the two graphs, with *n meaning that the data in the dilution factor is significantly different from the data in dilution factor n at the 95% confidence level.

It was decided to look at inter-day variability in baculovirus titres obtained using both standard curve analysis and assumption-free analysis, for baculovirus stocks subjected to kit extractions and for those subjected to freeze/thaw and Triton X-100 treatments (Figure 4.4). These samples

were separately extracted and treated from the same stock for each of the two runs. Therefore, discrepancies between values obtained between the two days can be attributed to inconsistency in treatment or extraction between the two days, as well as due to error due to running the Real Time PCR reaction on two different days, as described earlier.

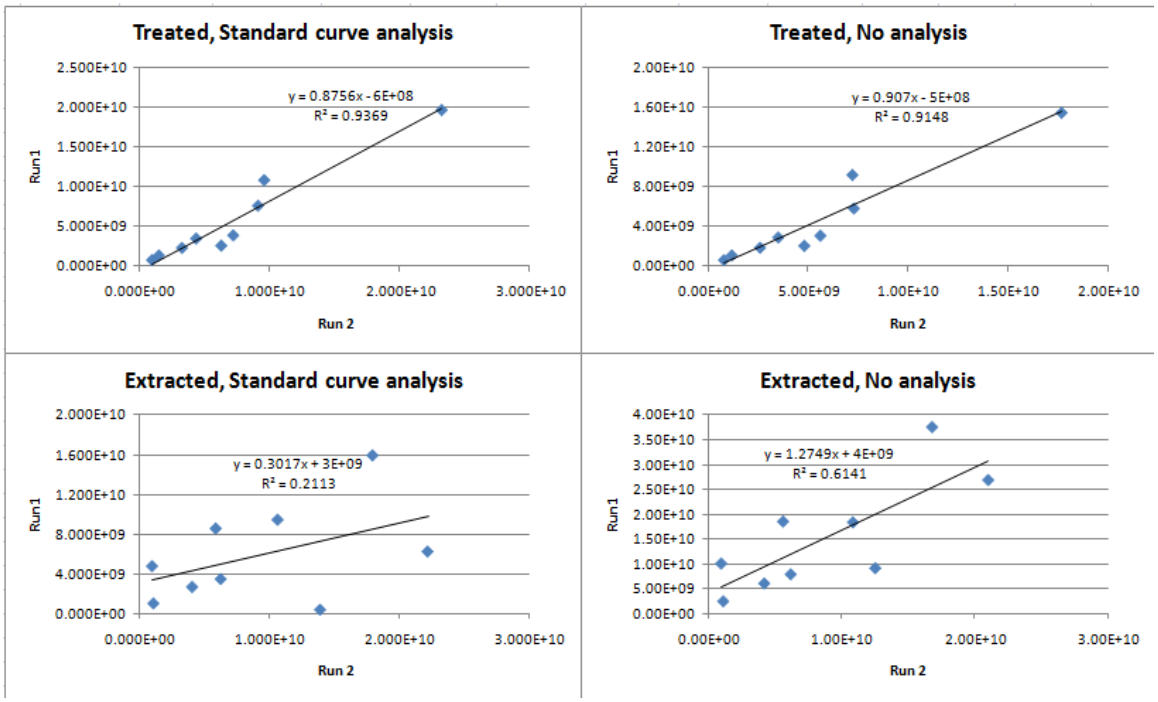

Figure 4.4: Titres of various stocks of virus, treated by two methods to expose DNA, was obtained by analysing RT-PCR data by comparison with standard curves as well as by assumption-free analysis. The two titres were plotted against each other to obtain the graphs shown above. Run 1 samples were run on Day 1 on the same plate, whereas run 2 samples were run on Day 2 on a different plate.

It can be seen that for the treated samples, was good correlation between values obtained from the two runs (R^2 values between 0.91 and 0.93), when results were analysed by the two analysis methods. In contrast, there was relatively poor correlation between samples isolated by extraction kits. However, this correlation seems to be improved slightly (R^2 increases from 0.21 to 0.61) when the RT-PCR data is analysed by assumption free analysis, as can be seen in Figure 4.4 and it can be seen that using N_o analysis to determine titres for the two runs helped reduce some of the variability in between the runs. Therefore, it is possible that using N_o analysis can reduce errors associated with determining titres using standard curves, due to the assumption of uniform efficiency, and therefore, help better elucidate the effects of other underlying factors. In this case,

it may allow better visualization of error in titre determination introduced due to sample extraction efficiency.

4.4 Conclusions

It can be seen that assumption-free analysis seems to correlate well to titre determination using standard curves and can be used to compare the template copy numbers between samples without the use of standards. It was seen that for the our reactions, there was no great effect due to efficiency differences, but it was desired that in the future Real Time PCR analysis could be conducted without having to worry about the effects of variation in efficiencies between reactions. Therefore, this method was implemented in further research for this purpose.

CHAPTER 5

Alternative Sample Preparation involving freeze/thaws and Triton X-100 for Baculovirus Titre Determination by Real Time PCR.

The following chapter details studies on a simplified method to expose baculovirus DNA, for use as a template preparation method for Real Time PCR.

Alternative Sample Preparation involving freeze/thaws and Triton X-100 for Baculovirus Titre Determination by Real Time PCR.

5.1 Overview

The increasing use of the Baculovirus Expression Vector System (BEVS) has generated significant interest into techniques for quantifying baculovirus stocks of which one method involves the use of quantitative Real Time Polymerase Chain Reaction. This work aims to further develop this method for the quantification of baculovirus by examining a simple method for template generation from baculovirus stock, involving freeze/thaw and Triton X-100 treatments, with the final goal of reducing the cost associated with sample preparation for baculovirus quantification by Real Time PCR. From our experiments we concluded that combinations of these treatments could, in fact, provide a valid sample preparation method for Real Time PCR.

5.2 Background

The capability of baculovirus to infect insect cells has been harnessed in the Baculovirus Expression Vector System in which baculovirus are used to insert a gene of interest into insect cells, where they are expressed. Briefly, this process involves the production of recombinant baculoviruses, which have a gene of interest under the control of a baculovirus promoter. These baculoviruses are then inserted into insect cells where the gene of interest is expressed. The *Autographa californica* nuclear polyhedrosis virus (AcMNPV) has become the most widely used baculovirus for this purpose due to its stability and easy propagation in cell culture (Miller, 1997). In recent years, the Baculovirus Expression Vector System (BEVS) has become one of the most

widely used systems for the production of recombinant proteins (T. A. Kost et al., 2005). This stems from the ability of this system to produce high yields of recombinant proteins and the ability of the system to produce proteins with post-translational modifications such as glycosylation, disulfide bond formation, phosphorylation and fatty acid acylation which are similar to mammalian cell produced proteins (B. J. Kelly, King, & Possee, 2007). In addition, this system is relatively rapid, and cheap to implement, when compared to mammalian cell based systems (Cox, 2004).

Baculoviruses have also been studied as biosafe insecticides (B. J. Kelly et al., 2007), and as vectors for gene delivery into mammalian cells (Cox, 2004; T. A. Kost et al., 2005). Recent research has focused on improving this system by retargeting these vectors to different types of cells, and using these cells in applications such as gene therapy or as scaffolds for surface display, among other applications (T. A. Kost et al., 2005).

The increasing interest in baculovirus biotechnology, particularly the BEVS system has stimulated research into eliminating bottlenecks within the production process, of which one of the most significant has been the quantification of baculovirus stocks (Lo & Chao, 2004). Quantification is important because accurate titres are a prerequisite for establishing accurate multiplicities of infection (MOI) which have been shown to be critical in baculovirus stock amplification, where high MOIs cause formation of defective particles (Kool et al., 1991). Also, accurate MOIs are required for optimal recombinant protein production (M. G. Aucoin, Perrier, & Kamen, 2006a; Roldão et al., 2009) and for batch to batch process consistency (Roldão et al., 2009). Traditionally, methods such as plaque assays, end-point dilution assays and antibody based assays have been used to quantify baculovirus titres (Ramakers et al., 2003); however, these assays are time consuming and labour intensive and so, alternative assays have been developed using techniques such as flow cytometry (Shen et al., 2002), magnetic cell sorting

technology (Philipps, Forstner, & Mayr, 2004), microfluidics based bioanalysers (Malde & Hunt, 2004), measurement of changes in cell size distribution (Janakiraman et al., 2006), cell viabilility assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (J. A. Mena, Ramirez, & Palomares, 2003), quantitative real time polymerase chain reaction (Lo & Chao, 2004) and assays based on reporter genes such as β-galactosidase (Sussman, 1995) and green fluorescent protein (Cha et al., 1997). Among these, real time PCR (RT-PCR) appears to be the most rapid technique, only requiring about two to three hours to complete (Roldão et al., 2009), as well as being highly amenable to high throughput titre determination (Hitchman et al., 2007). These advantages could possibly allow the use of RT-PCR for routine baculovirus titre determination, in spite of its higher cost as compared to other techniques (Roldão et al., 2009).

RT-PCR has been demonstrated to be a valid and accurate method for determining baculovirus titre as compared to plaque assays (Hitchman et al., 2007) and end-point dilution assays (Lo & Chao, 2004) and has been widely used for baculovirus titre determination (Durand & Lightner, 2002; Garnier, Gaudin, Bensadoun, Rebillat, & Morel, 2009; Kato et al., 2009; Ke, Wang, Deng, & Wang, 2008; C. Liu et al., 2008; Rosinski et al., 2002; Vanarsdall, Okano, & Rohrmann, 2005; M. P. Zwart et al., 2008; M. P. Zwart et al., 2008). Sample preparation for Real Time PCR requires the use of nucleic extraction kits, which is necessary because traditional methods such as phenol\chloroform extraction do not allow for consistent nucleic acid recovery from baculovirus samples (Lo & Chao, 2004). In our study, we compare the yield and quality of sample baculovirus DNA isolated by two different kits, the Roche High Pure Viral Nucleic Acid Kit and the QIAGEN DNeasy Blood & Tissue Kit.

In addition, we explore the use of two previously described sets of primers for conducting quantitative real time PCR. These include a set of primers targeting a region in the *Gp-64* gene (Hitchman et al., 2007) and a set of primers amplifying a region in the *Ie-1* gene (Lo & Chao,

2004). Although others have chosen to amplify regions in foreign genes in baculovirus (Vieira et al., 2005), the use of sequences such as *Ie-1* and *Gp-64* allow for the quantification of all types of baculovirus and are not dependent on a single transgene.

One of the factors contributing to the expense associated with baculovirus using Real Time PCR is the high cost of sample preparation for those who rely on commercial kits. Since routine implementation Real Time PCR as a baculovirus quantification method requires that the cost associated with its implementation be reduced, we decided to look at alternative methods of sample preparation. In addition to the cost, these kits could suffer from problems such as loss of nucleic acid material during the purification process, during the binding of nucleic acids to membranes and the subsequent washing and elution steps. Therefore, we decided to study a method of sample treatment which involved subjecting the baculovirus to multiple freeze/thaw cycles followed by treatment with Triton X-100. This treatment strategy was based on methods previously used to prepare baculovirus samples for quantification by flow cytometry (Shen et al., 2002). This method would be attractive because of the much lowered sample preparation cost and lack of steps in which nucleic acid material could be lost, due to the absence of nucleic acid purification by binding to a membrane. Our final goal was to determine if a combination of freeze/thaw and Triton X-100 treatments would provide samples suitable for use as Real Time PCR template, as kit extracted DNA.

5.3 Materials and Methods

The materials used for this study are described in the chapter Common Materials and Methods, with the modifications detailed below.

5.3.1 Cell culture and Baculovirus Production

Cell culture and baculovirus production was conducted as described previously. In addition, 9 unpurified samples of baculovirus (Stocks 1-9, culture supernatant) which were previously analysed by flow cytometry and high-performance liquid chromatography (HPLC) were kindly provided by Dr. Amine Kamen (Biotechnology Research Institute, National Research Council Canada) (Transfiguracion et al.,).

5.3.2 Template generation using DNA extraction kits

Viral DNA was extracted and template for PCR was generated using two methods. The first two involved the use of two kits: the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Laval, Quebec, Canada) and the DNeasy Blood & Tissue Kit (QIAGEN, Mississauga, Ontario, Canada), as described earlier.

5.3.3 Experimental strategy for determining optimal combination of freeze/thaw cycles and Triton X-100

A third method for template generation, not relying on kits, involved subjecting baculovirus stocks to various combinations of freeze/thaw and Triton X-100 treatments. Freeze/thaw cycles were conducted by alternating between freezing the samples in dry ice and ethanol and thawing them in either a 37°C water bath or at room temperature (22 – 23^{O}C). The five factors which were combined in all possible combinations were:

1. Concentrations of sample material
2. Concentrations of Triton X-100
3. Number of freeze/thaw cycles
4. Temperature of thaw
5. Order of treatments

Different concentrations of sample were evaluated by diluting samples in 1X phosphate buffered

saline (PBS) by factors of 10^1, 10^3 and 10^5. Triton X-100 concentrations were varied between 0, 0.01 and 0.1% in final solution and the number of freeze/thaw cycles varied between 0 or 2 freeze/thaw cycles. Thawing was conducted at either 37^OC or room temperature (22^OC). Samples were subjected to three treatment schemes: the "Forward" treatment, where the sample was subjected to freeze/thaw cycles and then treated with Triton X-100, the "Reverse" treatment where Triton X-100 was added before subjecting samples to freeze/thaw cycles and the "Room Temperature" treatment which is a variation of the Forward treatment with the thawing occurring at room temperature.

5.3.4 Real time PCR

The two sets of primers used for the reactions have been described earlier and were targeted against a region in the Gp-64 gene (Hitchman et al., 2007) or against a region in the *ie*-1 gene (Lo & Chao, 2004). These are given in Table 5.1. Some experiments involving the *ie*-1 primers contained additions of 25 mM $MgCl_2$, for reaction optimization, with nuclease free water being used to adjust the final reaction volume to 20 µl. Amplification cycles were conducted according to conditions described in literature previously (Hitchman et al., 2007; Lo & Chao, 2004). Briefly, for the Gp-64 primers, initial denaturation was conducted at 95°C for 3 minutes, followed by 45 cycles of denaturation at 95°C for 30 seconds and annealing/extension at 60°C for 30 seconds. For the Ie-1 primers, initial denaturation was conducted at 95°C for 30 s for one cycle, followed by 45 cycles of denaturation at 95°C for 30 s, 5 s of annealing at 63°C, and 10 s of primer extension at 72 °C. Following PCR, a melt curve analysis was performed by heating the final mixture to 95°C for 15 seconds followed by annealing at 60°C for 1 minute. The temperature was then ramped up in 0.3 degree increments to 95°C for 15 seconds, with fluorescence being measured during the ramping stage.

Table 5.1: Sequences of primers used in this study.

Primer	Sequence (5' – 3')
Gp-64 F	CGGCGTGAGTATGATTCTCAAA
Gp-64 R	ATGAGCAGACACGCAGCTTTT
Ie-1 F	CCCGTAACGGACCTCGTACTT
Ie-1 R	TTATCGAGATTTATTTGCATACAACAAG

5.3.5 Statistical Data Analysis

Statistical data analysis to determine confidence intervals of linear regressions was conducted using MicroExcel 2007 (Redmond, Washington, U.S.A).

Two factor ANOVA analysis was used in data analysis in section 5.4.3 and was implemented using the Analysis TookPak in Microsoft Excel 2007 (Redmond, Washington, U.S.A). The data used was normalized to untreated samples for each dilution. All ANOVA tests were conducted at the 95% confidence level.

In addition, two tailed t-tests were used in section 5.4.4, and the paired comparison test in section 5.4.3, both of which were implemented in Microsoft Excel (Redmond, Washington, U.S.A).

5.4 Results

5.4.1 Comparison of the primers targeting regions in *Ie-1* and *Gp-64*

Two previously described sets of primers were compared to see the choice of primer could affect titre determination. The primers targeted regions in the AcMNPV *Gp-64* gene (primers Gp-64 F and Gp-64 R) and in the *Ie-1* gene (Ie-1 F and Ie-1 R). A plot of threshold cycles vs. sample dilutions using both sets of primers is shown in Figure 5.1.

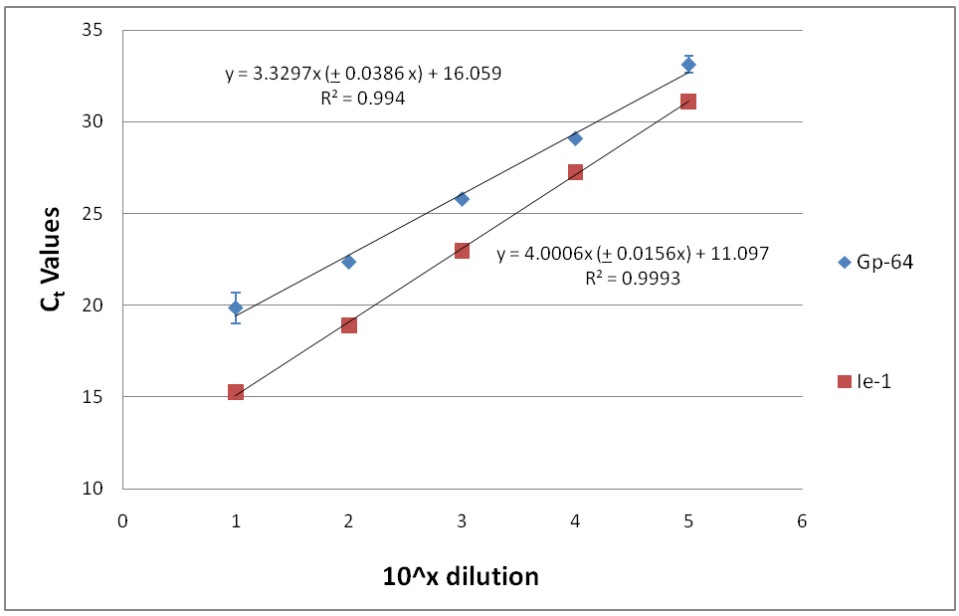

Figure 5.1: Mean Ct values of triplicates of different dilutions from two different PCR reactions. Two sets of primers Gp-64 F, Gp-64 R and Ie-1 F, Ie-1 R were used in reactions using serial dilutions of QIAGEN purified DNA as template. The Ct values were plotted against dilutions and a linear equation was fitted to the linear portions of the plot. Here the Ct values obtained from both kits were plotted against the dilution factors of the samples with respect to the original sample. Error bars signify 1 standard deviation above and below the mean of replicates. The confidence intervals on the slopes denote the error of the slopes at the 95% confidence interval.

It was seen that the amplification efficiencies of the reaction were about 73% with the Ie-1F and Ie-1R primers, while the reactions with Gp-64 primers show an efficiency of 103%, which correspond to slopes of 3.2396 and 4.186, respectively (Figure 5.1) and these were distinctly different at the 95% confidence level. Therefore, it was attempted to optimize the Ie-1 reaction in accordance with what was stated in the paper from which these primers were obtained (Lo & Chao, 2004). Accordingly, reactions were supplemented with 25mM $MgCl_2$ in order to increase Mg^{2+} ion concentrations in the reactions by +1, +2 and +2.5 mM. The results of these reactions are given in Figure 5.2A.It can be seen that increasing the Mg^{2+} concentration by +2.5 mM did not increase the efficiency of the reaction to acceptable levels. Further increasing Mg^{2+} concentrations by +3 and +4 mM had very little effect on efficiencies (Figure 5.2B).

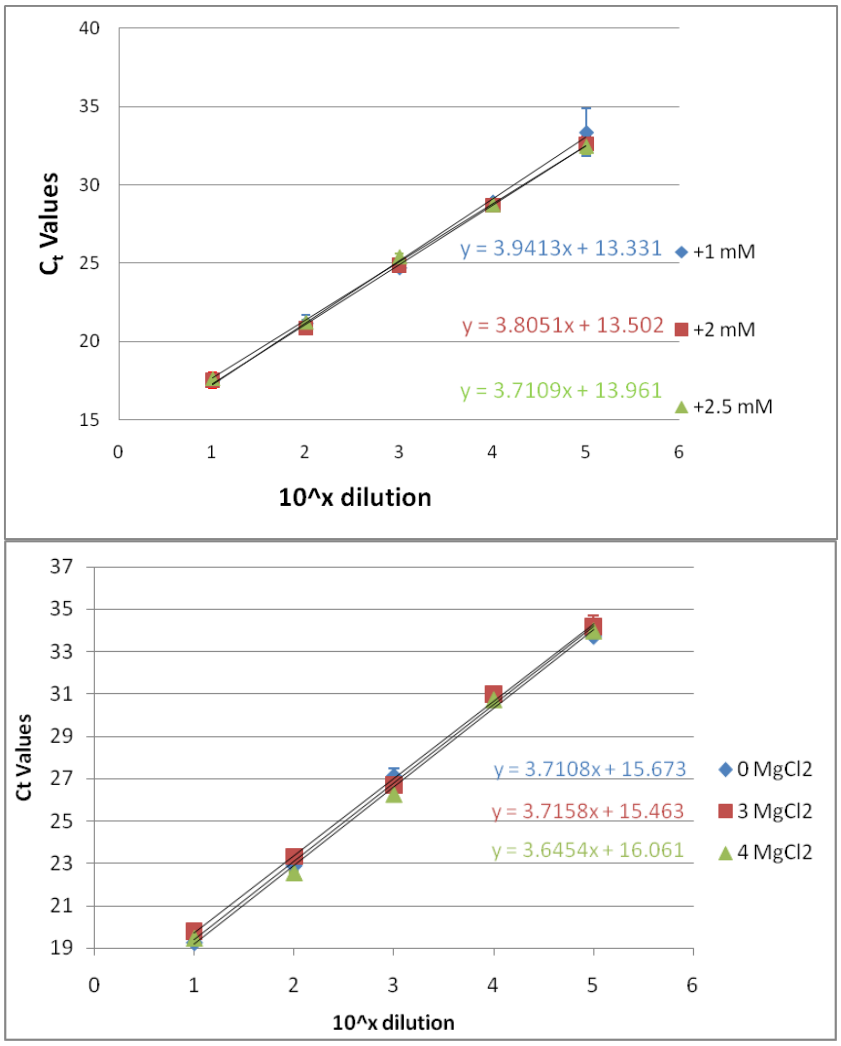

Figure 5.2: Mean Ct values of triplicates of different dilutions, obtained from a single RT-PCR experiment. The primer set Ie-1 F, Ie-1 R was used in reactions using serial dilutions of QIAGEN purified DNA as template, along with different amounts of additional 25 $MgCl_2$, to increase Mg^{2+} ion concentrations in the reactions by (a) +1, +2, and +2.5 mM and (b) +3 and +4 mM. The Ct values were plotted against dilutions and a linear equation was fitted to the linear portions of the plot. Here the Ct values obtained from both kits were plotted against the dilution factors of the samples with respect to the original sample. Error bars signify 1 standard deviation above and below the mean of 3 replicates.

To further investigate the suitability of these primers for Real Time PCR, the presence of primer dimerization was checked for by melt curve analysis. A comparison of the negative reactions (no template) using either sets of primers showed the reduced presence of primer-dimers in reactions using the Ie-1F and Ie-1R set of primers, as can be seen in Figure 5.3. In addition, it can be seen in Figure 5.4 that the peaks observed in the negative (no template) samples for both sets of primers are distinct from those seen in samples with template. Therefore, the peaks in the negative samples are not caused due to amplification of template and must result from primer-

dimerization. It should also be noted that the fluorescence due to primer dimerization was much greater in negative samples with Gp-64 primers than for Ie-1 primers, indicating that there is more final Gp-64 primer-dimerization product.

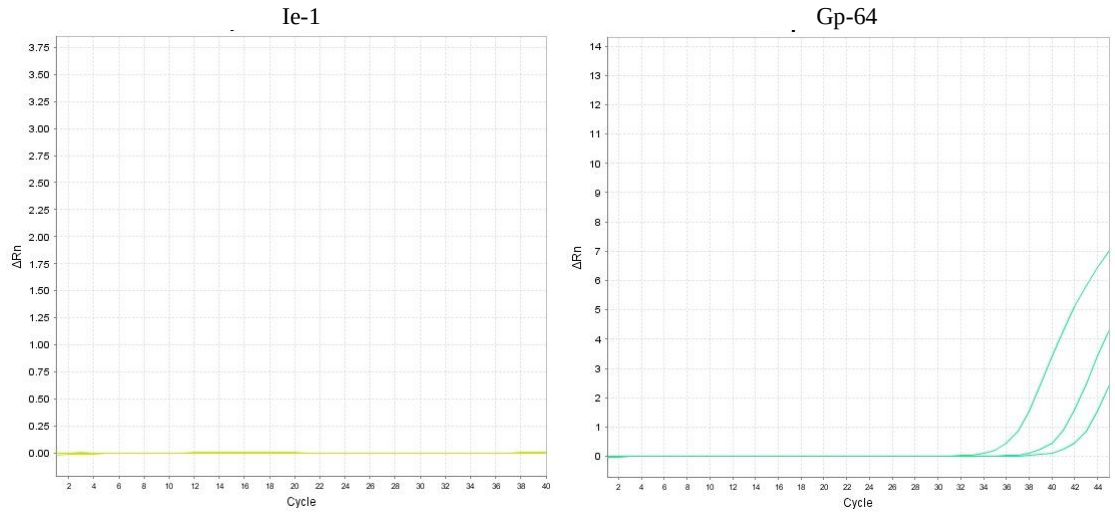

Figure 5.3: Amplification plots of Real Time PCR reactions with no template with two different sets of primers- Gp-64F and R and Ie-1F and R

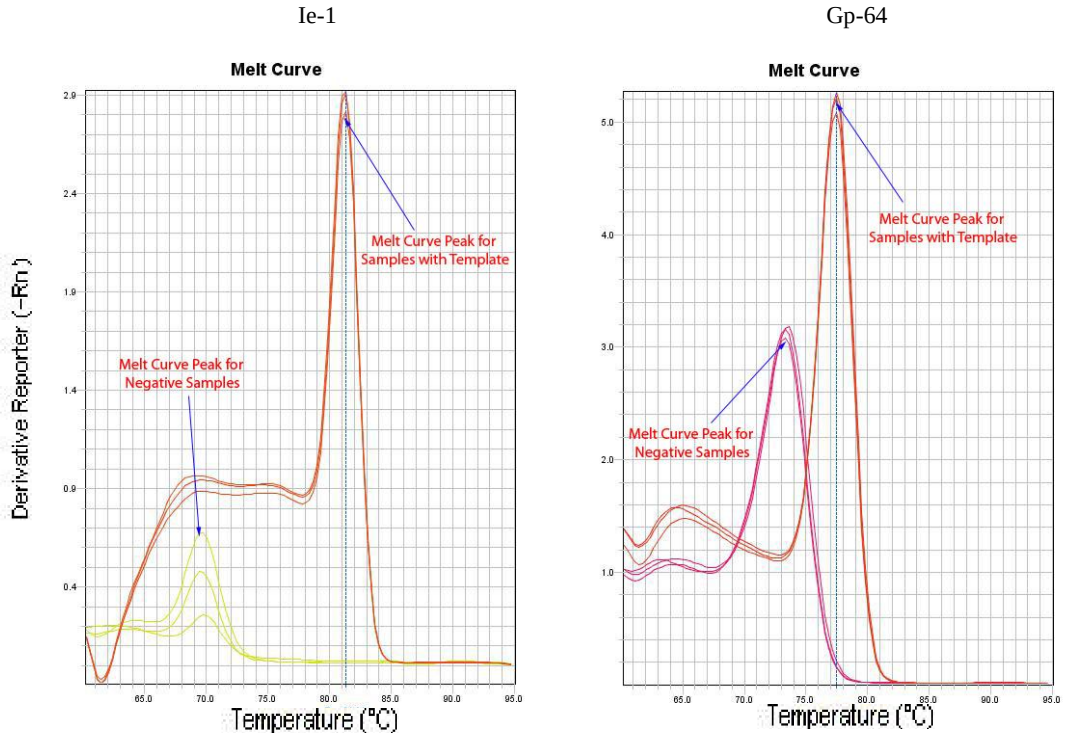

Figure 5.4: Melt curves of Negative samples (no template) and samples with template for reactions with two different primer sets- Gp-64F and R and Ie-1F and R

Based on the above results, it was decided to use the Gp-64 based primers .These primers were suited to our needs because our sample abundances were sufficiently high so as to overwhelm the signal from primer-dimerization. In addition, these primers were used in a later study to track baculovirus expression levels (Chapter 6) and so they were used here in order to have commonality between projects and to become more familiar with the system.

5.4.2 Real Time PCR template generation using DNA extraction kits and comparison of recovered DNA

Baculovirus stocks were used to generate template material for RT-PCR using two nucleic acid extraction kits: the High Pure Viral DNA Extraction kit from Roche Diagnostics and the DNeasy Blood & Tissue Kit from QIAGEN. The yields of the two extracted samples could not be confirmed directly by UV spectrophotometry as the presence of poly(A) RNA masks the signal generated by viral DNA. Therefore, we chose to compare the nucleic acids extracted by the 2 kits by conducting RT-PCR on dilutions of kit extracted DNA and comparing the N_o values of each of these dilutions. The results of this are given in Figure 5.5. The N_o value was used to compare the original template concentrations for each of the extracted samples as this enabled a direct comparison of the concentrations, unlike Ct values

It can be seen that in general, the DNeasy kit seems to have extracted roughly half the amount of DNA as the High Pure kit. While the data below indicates that the DNeasy Blood and Tissue kit extracts nucleic acid more consistently than the High Pure Viral Nucleic acid kit, we desired to recover as much of the DNA present in the sample as possible. Therefore, it was decided to use the High Pure extraction kit for all future studies.

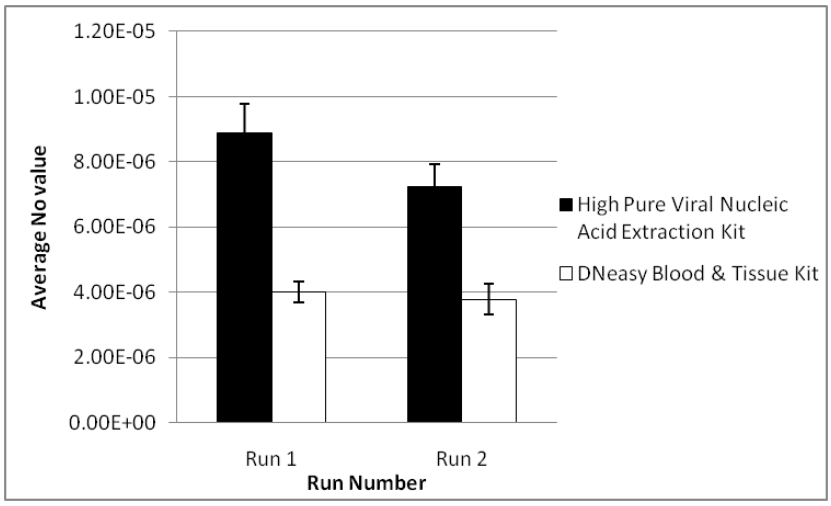

Figure 5.5: Figure representing the No value of the undiluted samples obtained by extracting DNA from two nucleic acid extraction kits: the High Pure Viral DNA Extraction kit from Roche Diagnostics and the DNeasy Blood & Tissue Kit from QIAGEN and putting samples through Real Time PCR using the primers Gp-64F and Gp-64R. Each value was obtained by averaging the N_o values of triplicates of successive dilutions of each sample. Standard deviations were obtained by averaging the standard deviations between triplicates of each dilution of each sample.

5.4.3 Protocol Development for Alternative Real Time PCR template generation

To determine the combination of freeze/thaw and Triton X-100 treatments which would give the best result in terms of viral genome exposure, it was decided to perform a systematic series of experiments as described in the materials and methods section. Briefly, samples were subjected to 0 and 2 freeze/thaw cycles and the addition of Triton X-100 concentrations of 0, 0.01 and 0.1% in the final reaction mixture. In addition, the order of treatments and thawing temperature was varied. The generated samples were run through Real Time PCR and the results analysed by assumption free analysis (LineRegPCR), to generate N_o values for each sample, with a higher N_o value corresponding to a higher level of genome exposure. The N_o values of different samples were then compared to give fold differences between samples. The error bars for each figure represent the standard deviation of triplicate N_o values divided by the mean N_o value of the untreated sample. The error bars for the untreated samples represent the standard deviations between N_o values of triplicate samples.

It was observed that in general, increasing the concentration of Triton X-100 in the reaction

beyond 0.01% seemed to have very little effect in influencing the N_o values obtained for the different dilutions of baculovirus sample (Figure 5.6). When ANOVA analysis was conducted, it was determined that both Triton X-100 concentrations and dilution factors seemed to have an effect on genome exposure, with an interaction effect also being observed at the 95% confidence level. It is possible that this interaction effect is caused due to the increase in the abundance of Triton X-100 relative to the number of baculovirus particles, at higher dilutions.

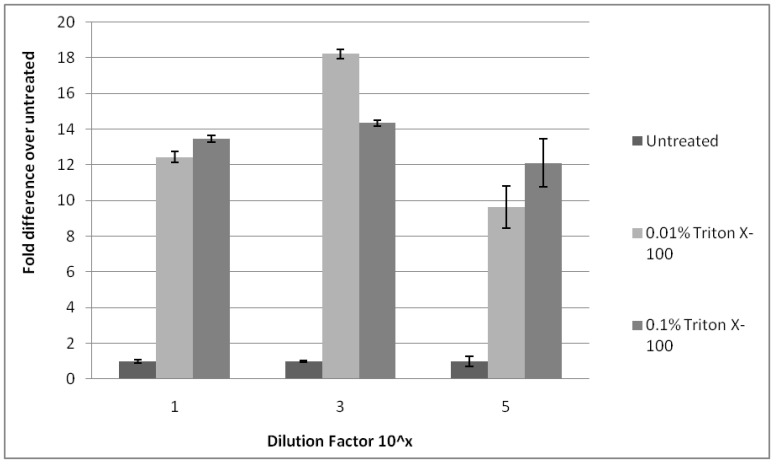

Figure 5.6: Effect of Triton X-100: Samples were treated with 0, 0.01 and 0.1% Triton X-100 and run in triplicate through Real Time PCR. The results were analysed by LineRegPCR. The figure shows the fold increase of N_o value of the different treated samples at different dilution factors, as compared to untreated sample at that dilution factor.

It was observed that freeze/thaw cycles seemed to have some effect on genome exposure (Figures 5.7 and 5.8). However, as can be seen, the temperature at which the thawing is performed affects the level of genome exposure, with genome exposure being increased when the samples were thawed at room temperature (22^OC). ANOVA analysis determined that the number of freeze/thaw cycles at 37^OC was significant in genome exposure at the 95% confidence level. However, when thawing was conducted at room temperature, the dilution factor seemed to play a role in genome exposure as well, as determined by ANOVA at the 95% confidence level, the reason for which is not known.

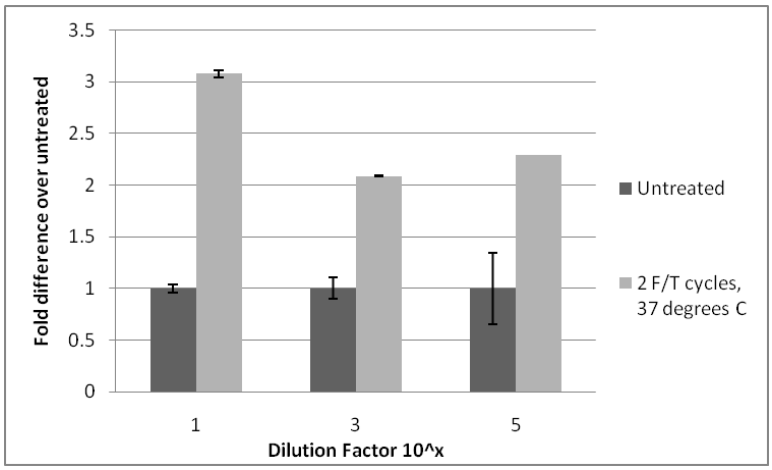

Figure 5.7: Effect of freeze/thaw cycles: Samples were treated with 0 and 2 freeze/thaw cycles with thawing at 37^{O}C and run in triplicate through Real Time PCR. The results were analysed by LineRegPCR. The figure shows the fold increase of N_o value of the different treated samples at different dilution factors, as compared to untreated sample at that dilution factor.

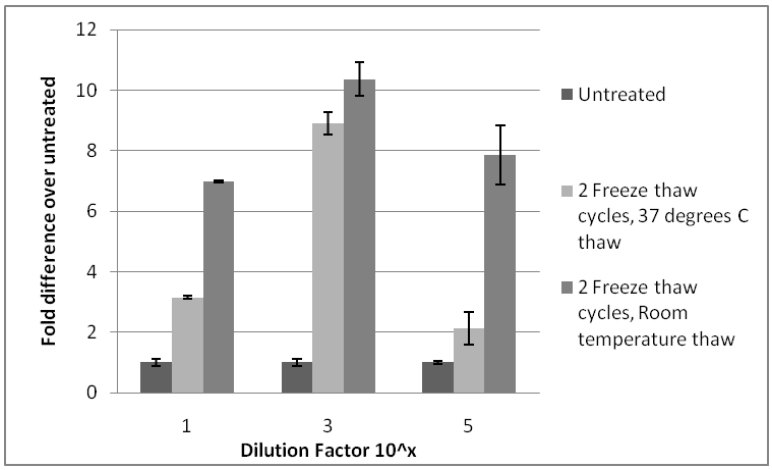

Figure 5.8: Effect of thaw temperature in freeze/thaw cycles: Samples were treated with 0 and 2 freeze/thaw cycles with thawing at 37^{O}C and 22^{O}C, and run in triplicate through Real Time PCR. The results were analysed by LineRegPCR. The figure shows the fold increase of N_o value of the different treated samples at different dilution factors, as compared to untreated sample at that dilution factor.

Combinations of the two treatments (freeze/thaw and Triton X-100 treatments) were then conducted (Figure 5.9). When the two treatments were combined in the Forward treatment scheme, it could be seen that freeze/thaw cycles did not increase genome exposure when coupled with Triton X-100 treatments. Results obtained from two factor ANOVA experiments conducted on this data which indicated that this conclusion was valid at the 95% confidence level.

In contrast to this result, freeze/thaws seemed to have an effect in the Reverse treatment scheme

(Figure 5.10) and to a lesser extent in the Room Temperature scheme (Figure 5.11). In both these schemes, increasing freeze/thaw cycles and Triton X-100 concentrations seemed both have an effect on genome exposure, as determined by two factor ANOVA. In addition, ANOVA analysis on all three samples determined that the dilution factor seemed to play a role in level of genome exposure in the various samples. It is possible that this is because of experimental inaccuracies which have been amplified during assumption-free analysis, especially for samples with lower abundance of DNA such as those diluted by a factor of 10^5.

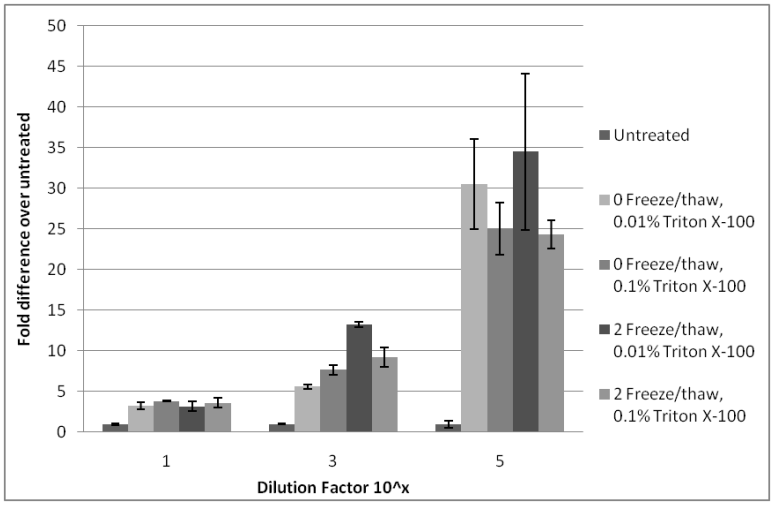

Figure 5.9: Samples treated by the Forward treatment scheme: freeze/thaw cycles followed Triton X-100 treatments. Samples were treated with 0 and 2 freeze/thaw cycles with thawing at 37^{O}C and run in triplicate through Real Time PCR. The results were analysed by LineRegPCR. The figure shows the fold increase of N_o value of the different treated samples at different dilution factors, as compared to untreated sample at that dilution factor.

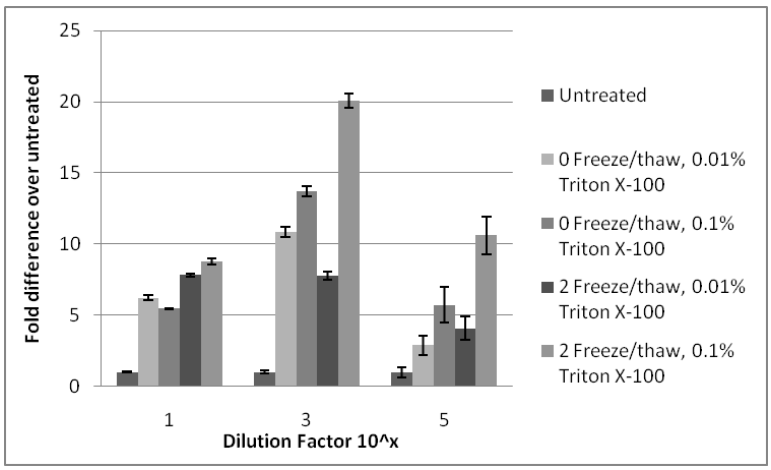

Figure 5.10: Samples treated by the Reverse treatment scheme: Triton X-100 treatments followed by freeze/thaw cycles. Samples were treated with 0 and 2 freeze/thaw cycles with thawing at 37^{O}C and run in triplicate through Real Time PCR. The results were analysed by LineRegPCR shows the fold increase of N_o value of the different treated samples at different dilution factors, as compared to untreated sample at that dilution factor.

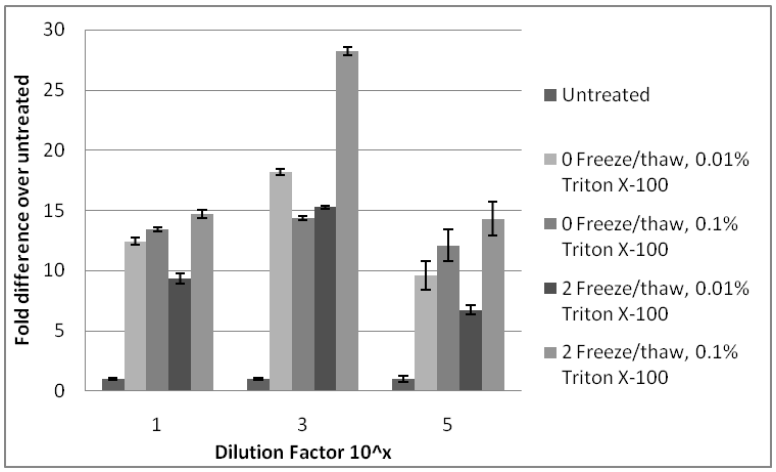

Figure 5.11: Samples treated by the Room Temperature treatment scheme: freeze/thaw cycles followed Triton X-100 treatments. Samples were treated with 0 and 2 freeze/thaw cycles with thawing at 22^{O}C and run in triplicate through Real Time PCR. The results were analysed by LineRegPCR. The figure shows the fold increase of N_o value of the different treated samples at different dilution factors, as compared to untreated sample at that dilution factor.

5.4.4 Comparison of Freeze-thaw and Triton X-100 samples with kit extracted DNA for use as RT-PCR template

It was decided to compare the efficiency of the various treatment strategies at various concentrations of baculovirus sample, with genome exposure by extraction using the High Pure extraction kit. The results of one such experiment are given in Figure 5.12. It can be seen that at higher concentrations of baculovirus sample, the High Pure Viral Nucleic Acid Extraction Kit seems to result in a higher N_o value than that obtained for the sample treated using the "best" treatments for each of the three treatment strategies (Forward, Reverse and Room Temperature). However, it can be seen that at lower concentrations of sample, the Reverse treatment strategy with 0.1% Triton X-100 in the final reaction, seemed to result in higher N_o values when compared with the kit extracted samples as can be seen in Figure 5.12(b), which was confirmed by t-tests at the 95% confidence level. The trends for the Forward and Reverse treatment schemes were observed in multiple experiments. The room temperature experiments were conducted only once and so, the reproducibility of its trend is not known. The error bars for each figure represent the standard deviation of triplicate N_o values divided by the mean N_o value of the untreated sample.

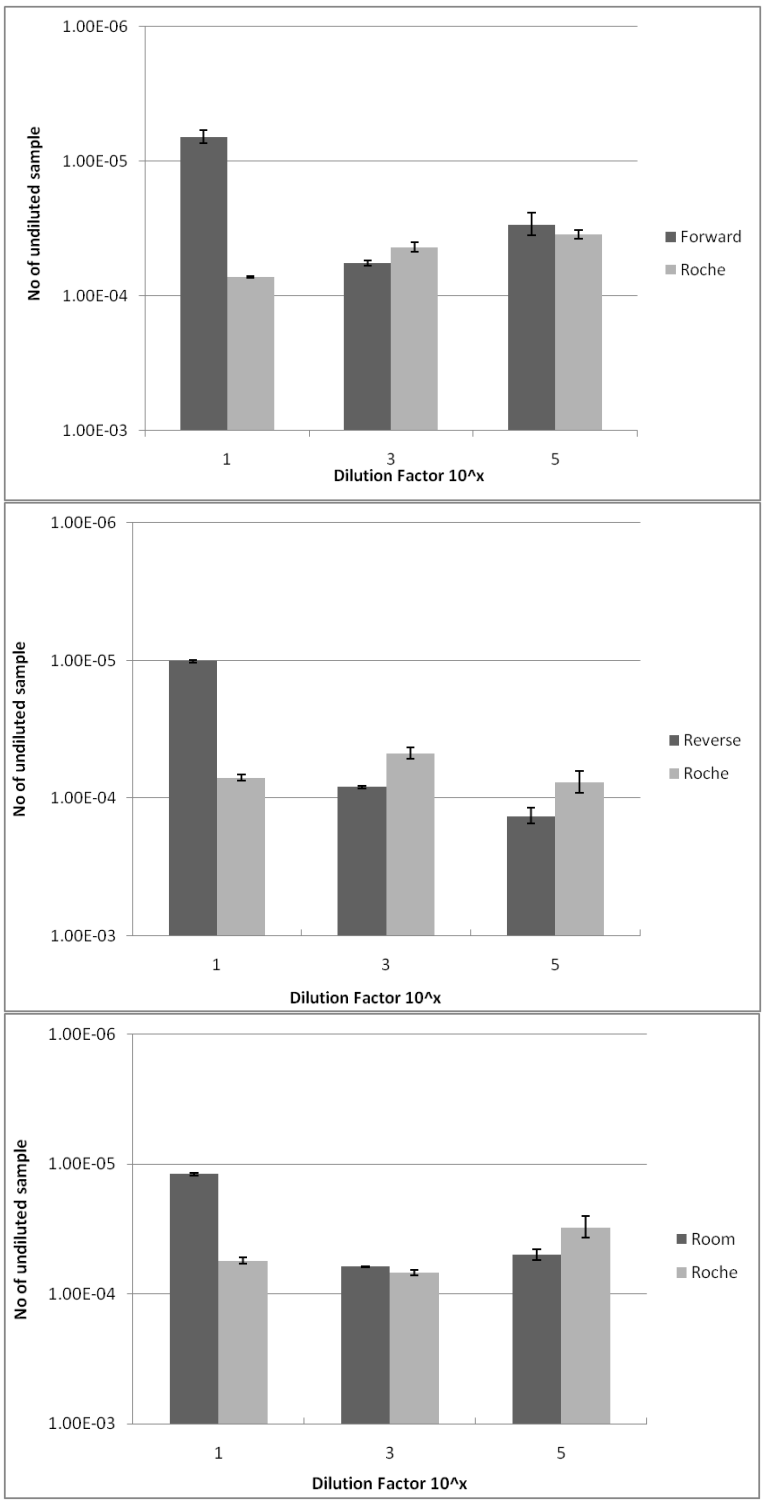

Figure 5.12: Comparison of effectiveness of treatment schemes and kit extraction: The Forward, Reverse and Room Temperature treatment schemes involve sample treatment using 2 freeze/thaw cycles and treatment with 0.1% Triton X-100. Samples for kit extraction were diluted by various dilution factors in PBS, extracted and run in triplicate through Real Time PCR along with treated samples. The results were analysed by LineRegPCR and are shown as fold increases of N_o value of the different treated samples at different dilution factors, as compared to untreated sample at that dilution factor. Figure 5.12 (a) Forward Treatment scheme compared with kit extraction Figure 5.12 (b) Reverse treatment scheme compared with kit extraction. Figure 5.12(c) Room Temperature treatment compared with kit extraction. The error bars denote 1 standard deviation above and below the mean of triplicate samples.

5.4.5 Quantification of viral stocks by Real Time PCR: comparing titres obtained using extraction kits and treated samples

The treatment method which seemed to give the best genome exposure (Reverse treatment with 0.1% Triton X-100 followed by 2 freeze/thaw cycles with thawing at $37^{O}C$) was compared with the DNA extracted with the Roche High Pure Viral Nucleic Acid Extraction kit to see which method would allow for accurate titre determination of 9 different stocks of viruses using Real Time PCR. Two sets of the same 9 samples were subjected to the two methods as follows: one set of samples was diluted 100 fold and subjected to the Reverse treatment and the other set of 9 samples was put through the Roche kit according to the recommended protocol. Both samples were then subjected to Real Time PCR and titres determined by comparing C_T values with a standard curve produced using plasmid DNA standards. Validity of results were examined by comparing the titres with baculovirus counts using FACS and HPLC data, which directly measure the number of virus particles in the samples. The results are given in Figure 5.13 and Table 5.2.

As can be seen from Figure 5.13, the titres for the various baculovirus stocks obtained using Real Time PCR seem to correlate far better to titres obtained by FACS when the stocks have been subjected to treatment using Triton X-100 and freeze/thaw cycles, than when they have been put through the DNA extraction kit. This indicates that recovery of DNA may not always reflect the abundance of DNA in the sample being put through the kit.

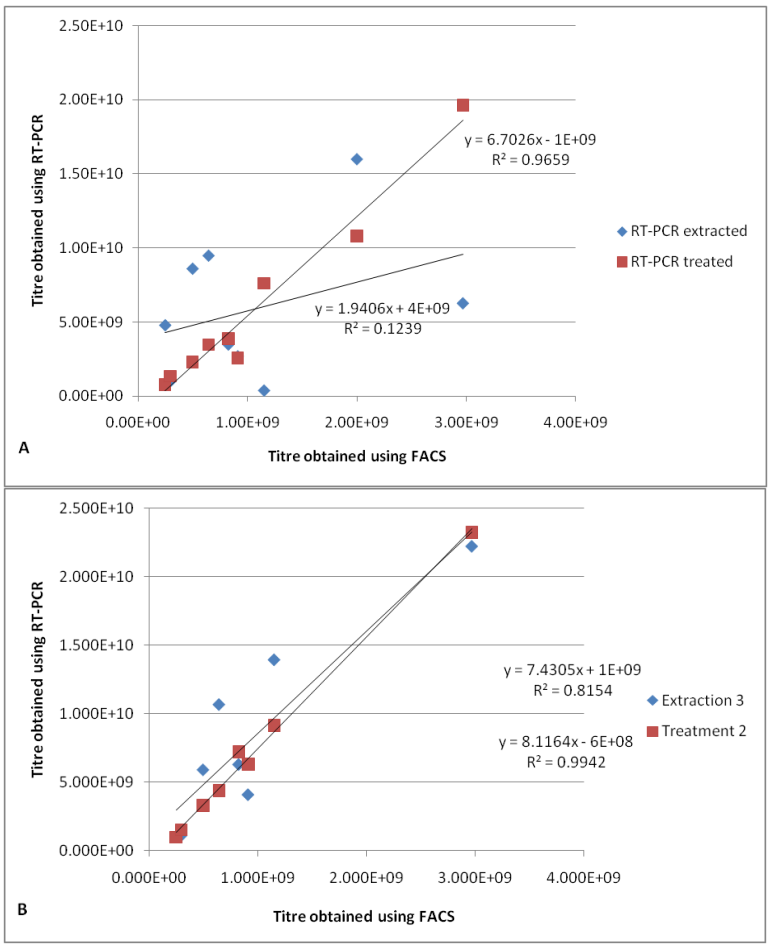

Figure 5.13: Comparison of Reverse treatment and kit extraction: Each reaction was run in triplicate using treated baculovirus (Stocks 1-9 diluted 100 fold and treated with 0.1% Triton X-100 and 2 freeze/thaw cycles) and Roche kit isolated baculovirus DNA as template. The Ct's were compared to a standard curve generated by running dilutions of plasmid standard in the same reaction, and the average titre of 3 triplicates was determined. The values obtained from this analysis were plotted against values for the same stocks obtained from FACS and a linear trend line was fitted through the resulting points. 5.13 (a): Run 1: Baculovirus titres obtained from Real Time PCR analysis for various stocks of baculovirus, using DNA extracted using the Roche kit, and samples subjected to the Reverse treatment, plotted against titres obtained by FACS, for these samples. 5.13 (b) Run 2 with samples treated separately from Run 1 samples.

In general, the titres obtained through Real Time PCR using extracted and treated samples seemed to be greater than titre values obtained from FACS analysis or HPLC (Table 5.2). A paired comparison test conducted on the data given in Table 2 revealed that while the titres obtained from Real Time PCR using the two different genome extraction methods seemed to belong to the same population at a confidence level of 95%, they were significantly different from titres obtained by the flow cytometry and HPLC methods, which in turn were significantly different from each other at the 95% confidence interval. Therefore, it can be concluded that the

use of the treatment scheme involving Triton X-100 and freeze/thaw cycles is at least as effective as kit based extraction for baculovirus genome exposure.

Stock\treatment	**RT-PCR, kit extracted samples**	**RT-PCR, treated samples**	**Flow cytometry**	**HPLC**
Stock 1	3.411E+09 (2.296E+08)	3.770E+09 (2.124E+08)	8.223E+08 (5.08E+07)	2.27E+09
Stock 2	1.600E+10 (1.706E+09)	1.053E+10 (1.644E+08)	1.999E+09 (8.31E+07)	2.96E+09
Stock 3	4.679E+09 (1.366E+08)	7.377E+08 (2.437E+07)	2.441E+08 (2.09E+07)	2.65E+08
Stock 4	1.224E+10 (4.344E+08)	1.913E+10 (8.473E+08)	2.970E+09 (1.57E+08)	5.01E+09
Stock 5	3.657E+09 (1.857E+09)	7.411E+09 (3.325E+08)	1.150E+09 (1.70E+07)	2.58E+09
Stock 6	8.398E+09 (4.695E+08)	2.238E+09 (5.785E+07)	4.946E+08 (3.43E+07)	7.23E+08
Stock 7	1.010E+09 (4.074E+07)	1.321E+09 (2.346E+07)	2.937E+08 (1.70E+07)	2.90E+08
Stock 8	9.258E+09 (4.059E+08)	3.385E+09 (2.222E+07)	6.409E+08 (8.91E+07)	9.43E+08
Stock 9	2.636E+09 (6.627E+08)	2.512E+09 (2.084E+07)	9.088E+08 (8.04E+06)	3.66E+09
Semi-Purified Stock 10	1.205E+12 (7.868E+10)	1.845E+11 (6.303E+09)	1.660E+10	

Table 5.2: Table showing titres of baculovirus genomes in various samples (From Stocks 1-9 and Purified Stock 10) obtained from a single RT-PCR experiment, and compared to values obtained from flow cytometry and HPLC. The values in brackets indicate standard deviations between triplicate samples. Each reaction was run in triplicate using treated baculovirus (sample diluted 100 fold and treated with 0.1% Triton X-100 and 2 freeze/thaw cycles) and Roche kit isolated baculovirus DNA as template. The semi-purified stock was diluted 10,000 fold and then treated with 0.1% Triton X-100 and 2 freeze/thaw cycles. The Ct's were compared to a standard curve generated by running dilutions of plasmid standard in the same reaction and the average values of 3 triplicates determined and reported as baculovirus titres. All values, other than that for BacITRGFP were obtained from a single Real Time PCR run.

5.5 Discussion

Quantitative Real Time Polymerase Chain Reaction has been examined in the past by several groups as a rapid and accurate method to quantify baculovirus (Hitchman et al., 2007; Lo & Chao, 2004). As has been described earlier these groups use a comparative C_T method whereby C_T values of samples are compared to those of standards to generate an absolute value of virus titre, and they report satisfactory quantification results using this method. The advantages of this method are its speed as well as the capability to quantify multiple baculovirus samples in the same reaction. Factors such as sample cost, however, prevent the method from being widely used

in baculovirus quantification. In addition, factors like suboptimal primer design and template preparation could confound baculovirus quantification by Real Time PCR. Therefore, we decided to examine these factors to better understand the quantification process and ultimately to develop strategies to simplify and reduce the costs associated with Real Time PCR for quantifying baculovirus.

Two previously described sets of primers were studied in our experiments and were found to have varying efficiencies of amplification and extents of primer-dimer formation. An ideal primer would allow for a ~100 % amplification efficiency and cause little to no interference from primer-dimer formation. The primers targeting the Ie-1 region of the baculovirus genome were found to result in poor amplification efficiencies, with little primer-dimerization. It was decided against using this set of primers as lowered reaction efficiency would correspond to later Ct values for each reaction, due to which low abundance samples may not be detected. The primers targeting the Gp-64 region, while providing good efficiencies of amplification, were found to create primer-dimers. However, the signal generated by primer-dimers was considered to be low enough to not affect analysis for the template concentrations being used in our experiments and melt curve analysis was used to screen for samples containing primer-dimers. Primer-dimerization, however, will hinder the interpretation of signals from samples with low abundance of template DNA and therefore serves to highlight the importance of primer design in RT-PCR. However, the Gp-64 based primers were well suited for our work, as explained earlier and so, these were used in subsequent experiments.

To further advance RT-PCR as a viable method for baculovirus quantification we examined the process of template generation from baculovirus samples. Generally, this has involved extraction of DNA from samples using either column kits or methods such as lysis and ethanol purification of DNA. It has been found that column purification is a superior method for DNA isolation, as it

has a recovered DNA with greater consistency than lysis and phenol/chloroform extraction (Lo & Chao, 2004). Accordingly, it was decided to evaluate two kits in terms of their ability to generate template to be used in quantitative RT-PCR. Both kits non-specifically isolate all nucleic from samples, in spite of one, the kit from Roche, being labelled specifically as a viral nucleic acid kit[1]. It was seen that the Roche High Pure Viral Nucleic Acid kit seemed to isolate more DNA than the QIAGEN DNeasy kit. Therefore, we decided to use the High Pure extraction kit for all subsequent DNA extractions, as it would give a better estimate of the total amount of viral nucleic acid in each sample, due to its higher recovery.

In light of the high cost of these kits, an alternate method was pursued for template generation which involved subjecting the baculovirus samples to multiple freeze/thaw cycles and treatment with Triton X-100. This approach was adapted from previous works which involved permeabilizing baculovirus with freeze/thaw and Triton X-100 treatments to fluorescently label baculovirus DNA with SYBR Green I for detection by flow cytometry (Shen et al., 2002). Based on this method, combinations of freeze/thaw and Triton X-100 treatments were conducted with the number of freeze/thaws, concentrations of Triton X-100 in the final reaction and the order of treatments being varied to determine which treatment would cause the maximum level of genome exposure and facilitate quantification by Real Time PCR. Additional evidence for the validity of this approach comes from the finding that baculovirus seem to be degraded when exposed to concentrations of Triton X-100 as low as 0.01% volume/volume (Transfiguracion et al.,).

It was found that the Reverse treatment involving 0.1% Triton X-100 followed by freeze/thaw cycles seemed to be the most effective method for exposing baculovirus genomes, and at low concentrations of template material the treatment seemed to perform at least as well as sample preparation by kit extraction. The reason for the improved performance of the Reverse treatment over the forward treatment, where freeze/thaw cycles seemed to have no effect when coupled

with Triton X-100 treatments, is thought to be due to the fact that the Triton X-100 treats the outside membrane first after which the capsid proteins are denatured by freeze/thaw cycles, whereas in the Forward treatment scheme, the membrane may be intact during the initial freeze/thaw cycles, which could influence the degree to which capsids are broken down. Therefore, it seems possible that a kit free sample preparation method could involve diluting samples followed by treatments by the Reverse treatment, which would provide a cheap, easy, rapid and easily scalable method for sample preparation. The only source of error in this sample could be at the time when the sample is diluted, whereby small differences in measurement of the sample to be diluted could translate into large errors in baculovirus numbers in the diluted sample, and therefore, in the amplified DNA obtained by PCR.

When comparing treatment strategies, we analysed Real Time PCR results using assumption-free analysis, to compare fold differences in template abundance between samples without relying on plasmid standards. In addition, assumption-free analysis of Real Time PCR experiments addresses the shortcomings of traditional comparative quantitative Real Time PCR experiments, particularly the assumptions about uniform efficiency in all wells of the reaction. While assumption-free analysis does not remove the need for pure standard for absolute titre determination, variations in efficiency between sample and standard is no longer an issue. In addition, this method removes the need for a standard curve and so, uses less material than traditional comparative methods, which should decrease both the cost and time associated with quantitative RT-PCR.

It was expected that the lack of binding to a membrane and subsequent purification and elution steps in our treatment methods would ensure that there was no loss of sample material and therefore, the amount of DNA detected from treated samples would be a “true” reflection of the amount of DNA in the sample. To determine if this was true, nine stocks that were previously

quantified using flow cytometry and by a recently developed HPLC method (Transfiguracion et al.), were subjected to both extraction using the Roche High Pure viral nucleic acid kit, and our Reverse treatment method. It was found that the titres obtained from samples treated by the Reverse treatment method consistently correlated closely with titres from FACS analysis, while titres obtained from extracted DNA did not correlate as much, and were inconsistent between extraction runs. Therefore, it is possible that variability in extraction procedure causes the poor correlation between titres obtained from these samples and from flow cytometry or that possibly there is a higher specificity for packaged viral nucleic acid. It was also seen that in most cases the baculovirus titres obtained from Real Time PCR were almost the same when the sample was treated or extracted, which gives support to our argument that the Reverse treatment method can expose baculovirus genomes to at least the same extent as kit extraction, for diluted samples.

The similarity of the treatment methods for our Real Time PCR and FACS analysis, which also involves freeze/thaw and Triton X-100 treatments, raises another possible explanation for the discrepancy between titres obtained from Real Time PCR with extracted samples and FACS analysis. This is the possibility that the treatment method degrades or reduces the signal from baculovirus DNA in some way. However, the variability seen between baculovirus titres obtained from DNA extracted during separate extraction runs indicates that the extraction procedure causes the poor correlations.

It was seen that the titres of all baculovirus samples (Stocks 1-10) obtained using Real Time PCR were usually much higher than titres obtained using FACS (Table 4.2). One possible explanation for this phenomenon is that defective interfering particles (DIPs), which are particles lacking capsid or enveloped proteins which contain genetic material (Kool et al., 1991), may act as template during the real Time PCR reaction and so, confound titre determination by Real Time PCR (Roldão et al., 2009). In addition, genetic material baculovirus DNA which was present

outside of the capsids may also be the reason for the higher baculovirus titre determined by Real Time PCR. Another possible explanation for this phenomenon could be that there is some inherent limitation to the flow cytometry method, whereby not all baculovirus DNA is labelled and therefore detected.

In conclusion, a low cost method for preparing virus samples is presented in this work, which aims to address the issue of the high cost of baculovirus titration by Real Time PCR. It is hoped that this work would be instrumental in increasing the usage of Real Time PCR as a routine quantification method for baculovirus.

[1.] Private communication with Roche Technical Services

CHAPTER 6

This project was conducted in collaboration with Janelle Tam, a student at the Waterloo Collegiate Institute, and YeongHo (David) Suh, my fellow graduate student. The project was undertaken as part of the Sanofi-Aventis Biotalent Challenge in which Janelle Tam was a participant. I would like to thank David Suh for his work which included training Janelle and preparing all cell cultures and monitoring and sampling from these cultures at different time points. Janelle was involved in the processing of these extracted samples and analysing them.

I was involved in designing the experiment and was responsible for the analysis of the Real Time PCR generated, as well as for presenting the results in the form of this chapter.

Tracking baculovirus infections in cell culture by monitoring levels of Gp-64 transcript.

6.1 Introduction:

Recent years have seen a large increase in the use of the baculovirus-insect cell system for the production of a wide range of products such as proteins and virus particles (T. A. Kost et al., 2005), including well known products such as the vaccine Cervarix™ produced by GlaxoSmithKline, as reviewed by Aucoin et al (M. G. Aucoin et al., 2010). The optimal production of these products involves careful monitoring of factors such as the time of infection, the multiplicity of infection (MOI) and external factors such as pH, temperature and oxygen (Olejnik, Czaczyk, Marecik, Grajek, & Jankowski, 2004). As a result of this need, there has been substantial effort dedicated to tracking the progression of baculovirus infections in cultures and

the health of the cultures, including tracking the oxygen uptake rate and carbon dioxide evolution rate (Kamen, Bedard, Tom, Perret, & Jardin, 1996; L. A. Palomares & Ramirez, 1996), optical density (Bédard, Perret, & Kamen, 1997), intracellular ATP measurements (Olejnik et al., 2004), NADH dependent fluorescence (Hensler & Agathos, 1994) and relative permittivity of cell cultures (Zeiser, Bedard, Voyer, Jardin, & Kamen, 1999). These methods rely on physically quantifying some aspect of the cell culture and give an idea of the level of infection in a cell culture.

Reverse Transcription Polymerase Chain Reaction (RT-PCR), which involves using a reverse transcriptase enzyme to convert RNA to cDNA, coupled with PCR or Real Time PCR has been used widely to detect and to quantify RNA transcripts in a sample (Bustin, 2000). To this end it has been used in applications like detecting the presence of viral pathogens (Morris, Robertson, & Gallagher, 1996), for monitoring levels of various transcripts in cancers (Bieche et al., 1999) and for tracking the course of viral infections (Moody, Sellers, & Bumstead, 2000).

The objective of this work was to examine how the multiplicity of infection (MOI) of baculovirus changes the transcription of the viral gene *Gp-64* in insect cells, over the course of an infection. Gp-64 is a transmembrane protein which is necessary for virus entry into the cell and is expressed during the intermediate and late stages of baculovirus infections (Blissard & Rohrmann, 1991). The Gp-64 protein is essential for infectivity of budded baculovirus by endocytosis (Volkman & Goldsmith, 1985), and therefore the levels of baculovirus Gp-64 transcript should reflect the levels of infectious virus present in the culture. The Gp-64 gene has been previously used for baculovirus quantification with good results (Hitchman et al., 2007) and therefore, we chose to track transcription levels of this gene to monitor levels of infectious virus in culture. In addition, there is some interest in using its promoter to express proteins, especially glycosylated proteins in the BEVS system, which require post translational modifications (D. L. Jarvis, 1997), and

therefore, studying the kinetics of Gp-64 transcript production would be of use in this aspect.

In this work, cultures were infected with high MOI's of viruses and the levels of baculovirus transcript were tracked over the course of the culture. To strengthen our analysis, we normalized baculovirus transcript levels to the level of a housekeeping gene transcript. The housekeeping gene which was selected was the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene which has been used previously to study various aspects of baculovirus infection of insect cells (J. C. Lee, Chen, & Chao, 1998; Lu & Miller, 1995). The GAPDH gene catalyses the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate during glycolysis and has been shown to be a good control for RNA quantification. Therefore, comparison of the Gp-64 transcript levels with GAPDH transcript levels gives the levels of baculovirus infection in culture almost on a "per cell" basis.

This work was meant to serve as a preliminary study to allow familiarization with the system for a more exhaustive study using a wider range of conditions, which will be performed in the future.

6.2 Materials and Methods

6.2.1 Cell culture and baculovirus infections, sample harvesting

Spodoptera frugiperda clonal isolate 9 (Sf-9) cells were seeded into 9 capped 125mL Erlenmeyer flasks at a density of 1 X 10^6 cells/mL with 30 mL of Sf-900III media, counted, infected at a multiplicity of infection of 12, 24 and 36 and kept in an incubator at 27^{O}C and an orbital shaker shaking at a speed of 130 rpm. The 9 flasks consisted of three different experimental conditions (different MOIs) performed in triplicate. A semi-purified and quantified baculovirus stock obtained Dr. Amine Kamen (Biotechnology Research Institute, National Research Council Canada) having a titre of 1.66E10 particles/mL and an infectious particle count of 1.15E10

pfu/mL was used for infection. Each flask was counted at 0, 12, 18 and 24 hours. At each time point a 500uL sample was harvested and frozen at -20^{O}C until RNA extractions could be conducted. Cells were counted using a Fuchs Rosenthal Counting Chamber (Hausser Scientific, Pennsylvania, U.S.A).

6.2.2 RNA Extraction

The High Pure RNA isolation Kit (Roche Diagnostics, Laval, Quebec, Canada) was used to isolate total RNA from all samples. RNA was isolated from 200 µl of material according to the manufacturer's protocol. Briefly, 200 µl of sample was lysed by adding in 400 µl Lysis Buffer (4.5 M guanidine-HCl, 50 mM Tris-HCl, 30% Triton ® X-100 (w/v) pH 6.6 (25°C) and vortexing for 15s. The solution was then transferred to a High Pure Filter Tube purification column, centrifuged for 15s at 8,000 x g and flow-through was discarded. The flow through was discarded and DNase I was added to the filter tube along with 90 µl of DNase I incubation buffer and the tube was incubated at room temperature (22^{O}C) for 15 minutes. 500 µl of Wash Buffer I ((5 mM NaCl, 2 mM Tris-HCl, pH 6.6 (25°C), ethanol) was then added to the tube and this was centrifuged at 8000 x g for 15s. This step was repeated with 500 µl of Wash Buffer II (20 mM NaCl, 2 mM Tris-HCl, pH 7.5 (25°C), ethanol). A third repetition was done with 200 µl Wash Buffer II and the tube centrifuged at 13,000 x g for 2 minutes to remove residual Wash Buffer, after which DNA was eluted out of the column using 100 µl of Elution Buffer (Nuclease-free, sterile, double distilled water) and centrifugation for 1 minute at 8,000 x g. The extracted RNA was stored at -80^{O}C.

6.2.3 cDNA Generation

The conversion of total RNA to cDNA was conducted using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Burlington, Ontario, Canada) using the protocol recommended by the manufacturer. Briefly, 10 µl of extracted RNA was mixed with 10 µl of a

master mix consisting of 2.0 µl of 10X RT Buffer, 0.8 µl of 25X dNTP mix, 2.0 µl 10X RT Random Primers, 1.0 µl MultiScribe™ Reverse Transcriptase and 4.2 µl Nuclease-free water, for a total reaction volume of 20 µl. The samples were then placed in a Veriti™ 96 Well Thermal Cycler (Applied Biosystems, Burlington, Ontario, Canada) and cycled at the following conditions: 25^{O}C for 10 minutes, 120 minutes at 27^{O}C followed by 85^{O}C for 5 minutes. The converted cDNA was stored at -20^{O}C until analysis by Real Time PCR.

6.2.4 Real time PCR

All RT-PCR reactions were conducted on a StepOne Plus Real-Time PCR system (Applied Biosystems, Burlington, Ontario, Canada) and were prepared in MicroAmp Fast Optical 96-well Reaction Plate (Applied Biosystems, Burlington, Ontario, Canada). The two sets of primers used for the reactions have been described earlier and were targeted against a region in the baculovirus Gp-64 gene (Hitchman et al., 2007) and against a region in the GAPDH gene of the insect cell (Lu & Miller, 1995). Each reaction consisted of 2 µl sample, 10 µl of 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Burlington, Ontario, Canada), forward and reverse primers at a concentration of 900 nM each, and nuclease free water, for a final volume of 20 µl. 6 µl of sample were then added along with 54 µl stock mix of 2X Power SYBR® Green PCR Master Mix, forward and reverse primers and nuclease free water into one well of an optical PCR plate and then, the solution was mixed and distributed between three wells of the PCR plate. The plate was then sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Burlington, Ontario, Canada) and centrifuged briefly. Each plate had two sets of sample triplicates, with one set using primers targeted against the Gp-64 reporter gene (Gp-64 F and R), and the other set using primers for the control gene GADPH (GADPH F and R). The sequences of the primers used are shown in Table 6.1. Amplification cycles were conducted according to conditions which were described in literature for the Gp-64 primers (Hitchman et al., 2007; Lo & Chao, 2004). Briefly, initial denaturation was conducted at 95°C for 3 minutes, followed by 45 cycles of

denaturation at 95°C for 30 seconds and annealing/extension at 60°C for 30 seconds. Following PCR, a melt curve analysis was performed which involved initial denaturation at 95°C for 15 seconds followed by annealing at 60°C for 1 minute. The temperature was then ramped up in 0.3 degree increments to 95°C for 15 seconds, with fluorescence being measured during ramping stage. The results were then analysed by assumption-free analysis.

Each sample was run in triplicate to provide statistical validity and confidence in the data obtained. Data obtained from each reaction was analysed by StepOne™ Software v2.0, which is the controller for the StepOne and assumption-free analysis conducted on the data using the program LinRegPCR (11.6) (J. M. Ruijter, S. van der Velden, A. Ilgun, Heart Failure Research Center, Academic Medical Center, Amsterdam, the Netherlands)

Table 6.1: Sequences of primers used in this work.

Primer	Sequence (5' – 3')
Gp-64 F	CGGCGTGAGTATGATTCTCAAA
Gp-64 R	ATGAGCAGACACGCAGCTTTT
GADPH F	GCC ATC GCA GCG CCA TT
GADPH R	ATC GTT GAC GGC CAC CA

6.2.5 Titre Determination by Assumption-free analysis

Data analysis was conducted by conducting assumption-free analysis on all samples and comparing N_o values of samples run with Gp-64 primers and samples run with GAPDH primers.

6.2.6 Statistical Data Analysis

Two factor ANOVA analysis was implemented using the Analysis TookPak in Microsoft Excel 2007 (Redmond, Washington, U.S.A). The data used was normalized to untreated samples for each dilution.

6.3 Results

6.3.1 Cell counts over 24 hours

The cell counts in each of the flasks over the period of 24 hours are given below in Figure 6.1. It can be seen that the cell counts in each flask seemed to vary over the course of the experiment, which is most likely due to the inherent large variability associated with cell counting using a hemacytometer.

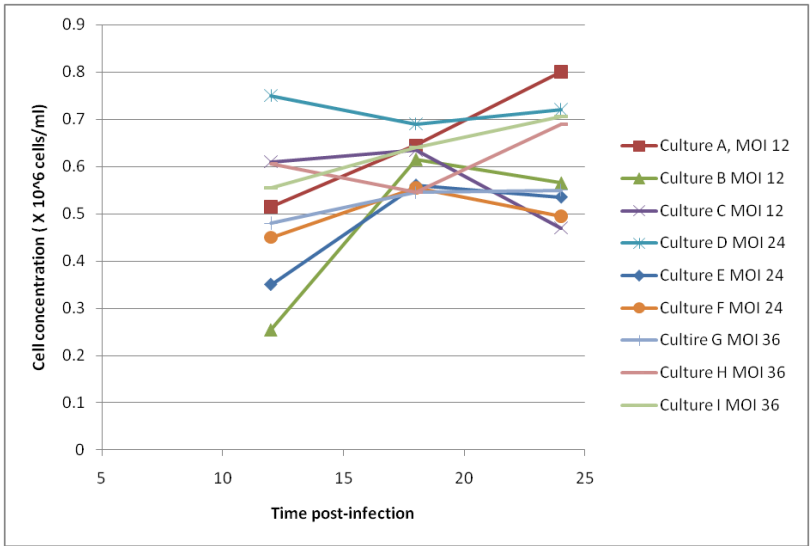

Figure 6.1: Total cell counts obtained at various time points of cultures infected with various multiplicities of infection of a pre-quantified stock of virus.

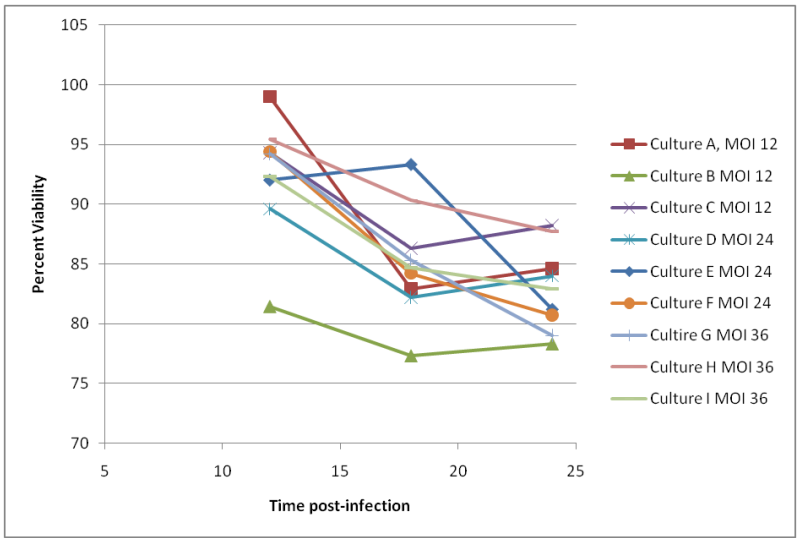

Figure 6.2: Percent viabilities of cell cultures at various time points, infected with various multiplicities of infection of a pre-quantified stock of virus.

6.3.2 Tracking GAPDH levels

All samples were subjected to Real Time PCR using two sets of primers targeting regions in the *Gp-64* gene and the *GAPDH* gene. Each sample was run twice on the same plate, once with Gp-64 primers and once with the GAPDH primers, with each reaction being conducted in triplicate to provide statistical significance to the values obtained from Real Time PCR. All reactions were run using conditions optimized for the Gp-64 primer pair as it was desired to have a GAPDH control on the same reaction plate to compare Gp-64 and GAPDH transcript levels without the confounding effect of variability between runs. The PCR data obtained was then subjected to N_o analysis, which allowed for comparisons between the initial template concentrations of *GAPDH* and *Gp-64* cDNA irrespective of differences in efficiencies of amplification. The N_o values obtained for the samples containing GAPDH cDNA are shown in Figure 6.3.

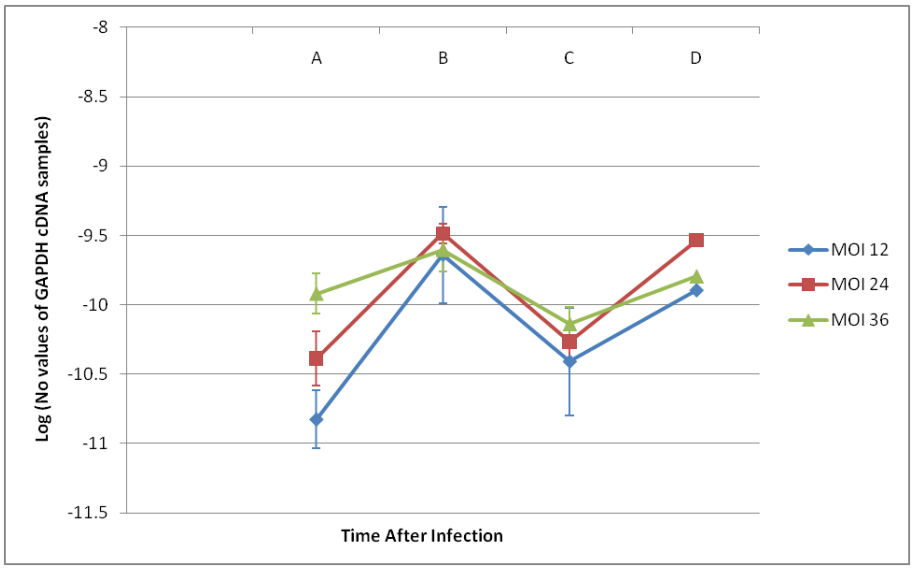

Figure 6.3: GAPDH transcript levels: Samples were extracted using the High Pure RNA Isolation kit, followed by reverse transcription and subjected to Real Time PCR using primers targeting the insect cell GAPDH gene. The results were subjected to assumption-free analysis and the No values obtained were observed at the four time points A: 0 hours post infection (p.i), B: 12 hours post infection (p.i), C: 18 hours post infection (p.i) and D: 24 hours post infection (p.i). The error bars represent one standard deviation above or below the mean of triplicate runs of three cultures run at the same conditions and were generated using the standard deviations of the log of No values.

As can be seen, there was variability in the GAPDH levels over the course of the infection, however, no clear increase or decrease in values was apparent.

6.3.3 Gp-64 transcript levels

It was decided to normalize the N_o values for the samples subjected to Real Time PCR with Gp-64 targeting primers, with the N_o values for the samples run with GAPDH primers, thereby giving a normalized and dimensionless fold difference between Gp-64 and GAPDH levels for each sample. As can be seen in Figure 6.4 the Gp-64 and GAPDH treated samples showed a similar pattern for N_o values for the different time points examined.

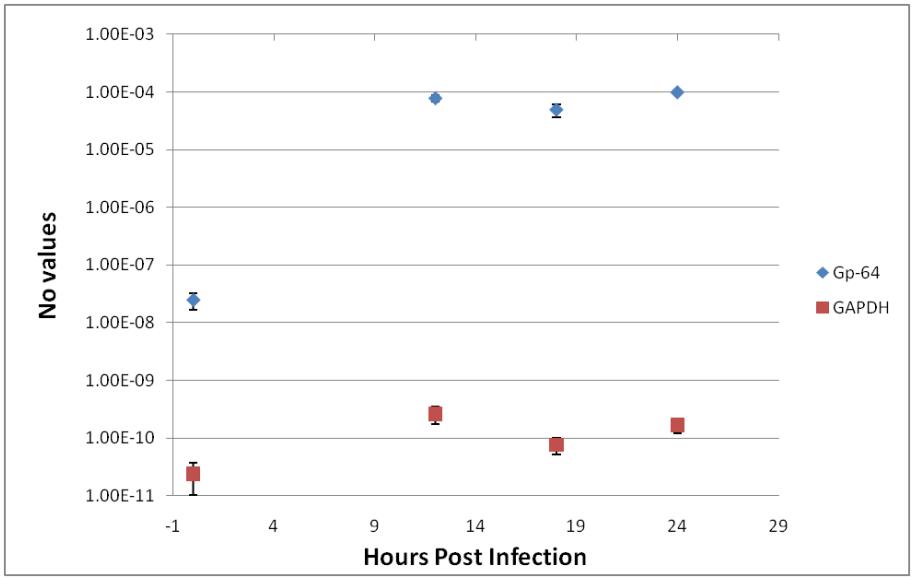

Figure 6.4: GAPDH and Gp-64 transcript levels: Samples were extracted using the High Pure RNA Isolation kit, followed by reverse transcription and subjected to Real Time PCR using primers targeting the insect cell GAPDH gene. The results were subjected to assumption-free analysis and the No values obtained were observed at the four time points 0 hours post infection (p.i), 12 hours post infection (p.i), 18 hours post infection (p.i) and 24 hours post infection (p.i). The error bars represent one standard deviation above or below the mean of triplicate runs of three cultures run at the same conditions and were generated using the standard deviations of the log of No values.

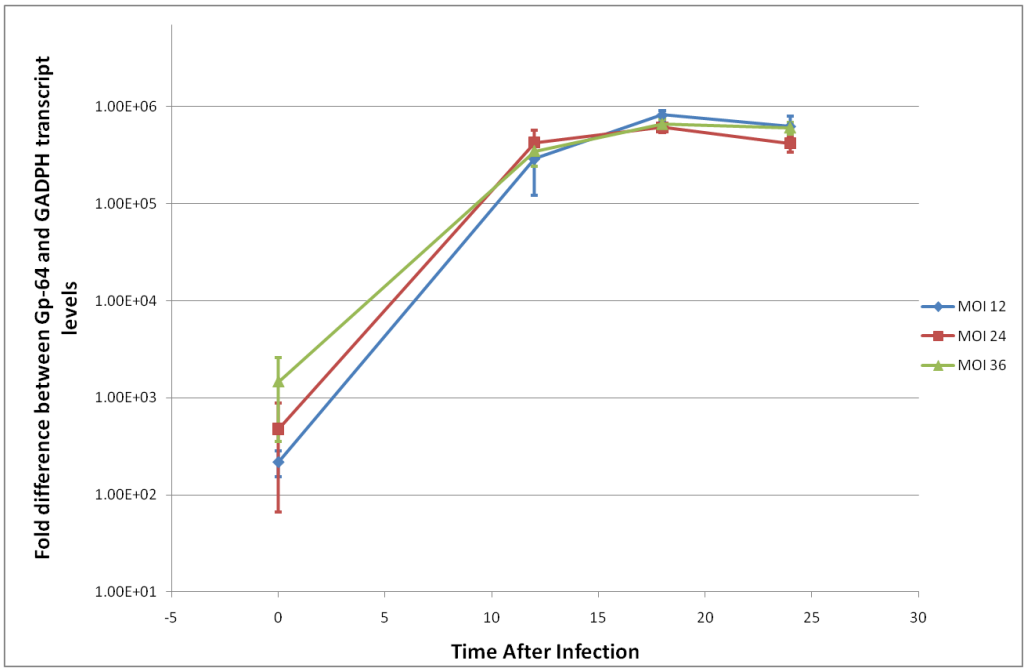

Figure 6.5: Normalized Gp-64 transcript levels: Samples were extracted using the High Pure RNA Isolation kit, followed by reverse transcription and subjected to Real Time PCR using primers targeting the insect cell GAPDH gene and the viral Gp-64 gene. The results were subjected to assumption-free analysis and the No values obtained were observed at the four time points 0, 12, 18 and 24 hours post infection (p.i) and normalized with respect to GAPDH transcript levels. The error bars represent one standard deviation above or below the mean of triplicate runs of three cultures run at the same conditions.

The graph showing the normalized Gp-64 transcript levels is given in Figure 6.5. It can be seen that in general, the Gp-64 transcript levels seem to increase by a large amount during the first 12 hours, with comparatively little change seen at later time points. However, when analysed by a two factor ANOVA test, it was found that Gp-64 values were significantly different at time points 12, 18 and 24 (95% confidence level), and that there was no significant difference in Gp-64 transcripts in samples infected with different MOI's of baculovirus. In addition, it can be seen that at Time 0 in Figure 6.5, where baculovirus transcript levels are expected to be non-existent, there seems to be some baculovirus DNA present.

6.4 Discussion

The objective of this work was to determine if changing the multiplicities of infection caused a change in the levels of one baculovirus transcript in infected insect cells. This was done by tracking baculovirus transcript levels in a culture and normalizing these levels with respect to the levels of a housekeeping gene, the GAPDH gene. To do this, reverse transcription followed by Real Time PCR was conducted on extracted DNA samples and the results analysed by assumption-free analysis. The levels of Gp-64 transcripts were then compared to the levels of GAPDH transcripts. This was done by running each sample twice on the same plate, once with primers targeting the Gp-64 gene and the other targeting the GAPDH gene. Another approach to achieve the same ends would have been to conduct a multiplex reaction with both primer sets in the same well. Multiplex PCR(Ballabio, Ranier, Chamberlain, Zollo, & Caskey, 1990) involves the use of multiple primers in the same reaction, with different amplification products being produced and detected in the same reaction, therefore enabling direct comparison between several targeted genes, (as reviewed by Elnifro et al (Elnifro, Ashshi, Cooper, & Klapper, 2000)), and has been widely used for this purpose. It was decided to use the conventional SYBR green based chemistry due to its lower cost of implementation, compared with chemistries required for multiplex reactions.

The GAPDH gene codes for glyceraldehyde 3-phosphate dehydrogenase and has been used as controls in several different types of tissues (Glare, Divjak, Bailey, & Walters, 2002) as well as for baculovirus (Lu & Miller, 1995). The advantage of using

housekeeping gene levels over cell counts as a normalization factor lies in the inherent variability in cell counts obtained from the cell counting method. This was clearly illustrated in the results in Table 1, where some cultures seemed to show drastically different cell counts when counted at different times, with no clear increase or decrease over time.

It was desired to see how the effects of multiple baculovirus infecting a single cell would change levels of baculovirus transcripts in the cell. While the monitoring of transcripts in a single cell has been done previously, (as reviewed by Dixon et al (Dixon, Richardson, Pinnock, & Lee, 2000)) and implemented by various groups, this is by no means a trivial undertaking. Therefore, it was decided to use high multiplicities of infection of baculovirus to ensure that all cells were synchronously infected by at least one baculovirus. Further increasing the multiplicities of infection would then cause multiple infections of one cell and therefore, through examining the culture, the effects of infection by multiple viruses could be observed. It has been estimated that almost all cells in a culture are infected at an MOI of above 10 (Y. Hu & E. Bentley, 2000), and therefore, the lowest MOI was set at 12.

Some variability in the transcript levels of GAPDH primers at different time points was observed. While this might be due to some artefact in the Real Time PCR reaction and amplification using the GAPDH primers, and from the assumption-free analysis, it is also possible that this error is due to differences in extraction efficiencies when the RNA was being extracted, as RNA from each of the time points was extracted separately at

different times. Both explanations are valid since RNA from samples at each time point was extracted and the Real Time PCR reactions run separately.

While GAPDH has been shown to be a valid control for normalizing transcript levels (Lu & Miller, 1995), some groups report that the GAPDH gene is not suitable for use as a control due to its variability between tissue types (Glare et al., 2002; Radonic et al., 2005; Xue, Salem, Turney, & Cheng, 2010). To our knowledge this has not been shown to be an issue in the baculovirus-insect cell system. One of the unsatisfactory features of using GAPDH as a control gene has been the high threshold cycle number associated with amplifying the gene, indicative of low abundance of the gene. Other housekeeping genes such as for 28S rRNA (Xue et al., 2010) have been proposed to correct these deficiencies. In addition, because of the need for the control reactions being present on the same plate as the sample reactions amplifying the Gp-64 fragment, the GADPH reactions were run at sub-optimal cycling conditions, and this may have contributed to the high threshold cycle number of the wells amplifying GADPH. In addition, the cell densities used may have been too low, thereby explaining the low N_o values observed.

In Figure 6.3, Gp-64 levels increased drastically in the first 12 hours, which corresponds to the transcription of where late genes are seen (6-15 hours post infection) (A. Roldao et al., 2007). After 12 hours post-infection, the levels of gp-64 transcript do not seem to change by a large amount, which could indicate that beyond 12 hours the rate of transcription is drastically reduced. However, the fact that these levels do not fall, indicates that some Gp-64 transcription was still maintained. If the infections were

followed for a longer period of time, it is possible that we would have seen a decrease in Gp-64 transcript levels, similar to what has been observed for late proteins past 24 hours post infection (B. J. Kelly et al., 2007).

It was also seen that there seemed to be amplification present in the samples which were taken from cultures at 0 hours post infection, where no Gp-64 RNA was expected to be seen. While the extraction kit was supposed to isolate only the RNA from each sample, it is possible that there was some baculovirus DNA contaminant, either due to some DNA being isolated by the kit, or due to contamination from outside sources. Unfortunately, no samples were taken prior to the addition of virus as a pure negative control.

It should be noted that there was almost no difference seen in the Gp-64 transcript levels between cultures infected at MOI 12, 24 and 36. This could be due to the cell transcription machinery being used up when one or more viruses infect the cell at a MOI of 12. The study concluded that increasing the MOI did not seem to significantly change the total level of Gp-64 transcript, during initial stages of baculovirus infection. This has important implications in the study of "competition" between several viruses infecting a single cell. Previous groups have shown that more than one baculovirus can be used to infect a cell and produce various levels of different recombinant proteins based on the multiplicity of infection of each virus type (M. G. Aucoin, Perrier, & Kamen, 2006b; Y. Hu & Bentley, 2000; J. A. Mena et al., 2007; A. Roldao, Vieira, Alves, & and, R.Oliveira and Manuel J T Carrondo, 2006). However, it has also been shown that recombinant protein production remains constant when cells in the early exponential phase are

infected with baculovirus at increasing MOI (Licari & Bailey, 1991; Schopf et al., 1990). Most of this work has been done with proteins expressed during the very late phase of infection. This work adds to this research by showing that during the early phase of the infection cycle in cells, the overall total level of transcription seems to remain constant, when the cell is infected by increasing numbers of virus. This limitation of transcription could be because there is an upper limit of the number of viruses infecting a cell beyond which additional infections would not cause the production of extra transcript, but would compete for the limited transcription machinery and metabolite pools. The alternative would be that there is some block which prevents multiple (beyond some number) infections of cells in their early exponential phase.

CHAPTER 7

Recommendations and Conclusions

The first study presented in Chapter 4 was done to validate the use of assumption-free analysis as a method to determine relative and absolute concentrations of samples of baculovirus. The study concluded that this was in fact, a viable method which could be used to do fold difference comparisons between samples without relying on an external standard having the same reaction profiles. This was based on experiments comparing the titres of viruses obtained by assumption-free analysis, with an established analysis method, which provided proof that fold differences in template concentrations seen using the assumption-free analysis method were observed in the established standard curve method.

The second study, presented in Chapter 5, investigated an alternative method for preparing unpurified samples of baculovirus for baculovirus titre determination by Real Time PCR, using freeze/thaw and Triton X-100 treatments. It was concluded from this study that combinations of these treatment methods, could yield samples that could be used for Real Time PCR analysis for baculovirus titre determination. It was also found that baculovirus titres determined by this method showed better correlation to values obtained by flow cytometry, than when kit extraction was used to purify baculovirus DNA for Real Time PCR. This sample preparation method suffers in that it requires that the sample be diluted before treatment, as undiluted sample seem to be better exposed by DNA extraction techniques using kits. A future direction for research would be to study the effects of increased Triton X-100 concentrations and further freeze/thaw cycles on the exposure of baculovirus genomes for use in Real Time PCR in concentrated baculovirus samples.

The final study presented in Chapter 6 investigated the effects of increasing baculovirus titres on the levels of baculovirus transcript during the early to late phase of the infection cycle. The study concludes that cells infected by increasing amounts of baculovirus in the early phase of the baculovirus infection cycle, do not seem to show a corresponding increase in the level of baculovirus mediated transcripts. This could be important in process considerations where increasing the MOI of infection beyond a point would not result in greater product yields. While this is already known, this study forms the basis for a more extensive future study on baculovirus transcript levels. Recommendations for future studies include elements such as a wider range of MOIs in order to determine if there is a certain MOI below which protein production can be affected by changing the MOI, following the infection for more time, perhaps several days to observe the effect of MOI on levels of transcripts beyond 24 hours post infection and the use of a wider range of cell densities to determine the effect of infecting cultures at different growth phases, with respect to changing transcript levels at different MOIs. Future studies could also look at infected cell cultures at several time points between 0 and 12 hours post infection, to track transcript levels during this phase of the infection cycle, and could use the GAPDH primers with optimized cycling conditions to track levels of the housekeeping *GAPDH* gene, or use housekeeping genes which have higher transcript levels, such as the 28S rRNA gene. In addition, further studies should include an uninfected culture which can be tracked over the infection period, as a further negative control.

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