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COMPARISON OF BACULOVIRUS TRANSCRIPTIONAL REGULATORY ELEMENTS AND THEIR INFLUENCE ON THE EXPRESSION OF RECOMBINANT PROTEINS IN THREE DIFFERENT INSECT CELL LINES

Master Thesis

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ABSTRACT

The baculovirus expression vector system (BEV) is a versatile system with many advantages used in medicine and research. The aim of this work was to increase recombinant protein expression by modifying transcriptional regulatory elements. Specifically, the number of a transcription factor binding site, typically called burst sequence, was increased in the polyhedrin promoter. Promoters with one, two, three and four burst sequence were compared. Additionally, the transcription factor itself, very late factor 1, was overexpressed under the control of the immediate early viral protein 1 and the glycoprotein 64 promoters. The remaining polyhedrin protein sequence at the 3'end of the normally used polyhedrin promoter was removed as well. These modifications were compared by expression of yellow fluorescence protein, YFP, under the polyhedrin promoter influence, in three different insect cell lines. The insect cell lines used were *Spodoptera frugiperda* Sf9 cells, *Trichopulsia ni* BTI-TN5B1-4 "HighFive™ and the Tnms42 derived from the HighFive™ cell line. In BioLector® experiments two burst sequences yielded three times more protein than the original promoter. But more than two burst sequences resulted in a decrease of obtained YFP product. Also, simultaneous expression of the very late factor 1 and the YFP resulted in a decrease of YFP production, indicating a bottleneck later in the expression machinery. Unfortunately, because of virus susceptibility problems, only trends could be observed in the cell line comparison experiments. However, comparison of the most promising constructs showed potential for increased recombinant protein production in the new Tnms42 cell line as compared to the widely used Sf9 cell line.

Keywords: insect cells, baculovirus, recombinant protein expression, burst sequences, transcription factor

ZUSAMMENFASSUNG

Das Baculovirus-Expressions-Vektor-System (BEV) ist ein vielseitiges, vorteilhaftes System, welches in Medizin und Forschung verwendet wird. Das Ziel dieser Arbeit war es, die Expression eines rekombinanten Proteins zu erhöhen, indem die transkriptions regulierenden Elemente modifiziert wurden. Konkret wurde die Nummer an Transkriptionsfaktor-Bindungsstellen, Burst-Sequenzen genannt, im Polyhedrin-Promoter erhöht. Promotoren mit ein, zwei, drei oder vier Burst-Sequenzen wurden miteinander verglichen. Zusätzlich wurde der Transkriptionsfaktor, very late factor 1, selbst überexprimiert unter Kontrolle der ie1 und GP64 Promotoren. Die vorhandene Polyherin-Protein-Sequenz am 3‘ Ende des normalerweise verwendeten Polyhedrin-Promoters wurde ebenfalls entfernt. Diese Modifikation wurden mittels Expression des gelben, fluoreszierenden Proteins, YFP, beeinflusst durch den Polyhedrin-Promoter, in drei verschiedenen Insektenzelllinien, verglichen. Die verwendent Insektenzelllinien waren *Spodoptera frugiperda* Sf9 Zellen, *Trichopulsia ni* BTI-TN5B1-4 “HighFive™ und Tnms42 welche von der HighFive™ Zelllinie abgeleitet sind. In BioLector® Experimenten lieferten zwei Burst-Sequenzen dreimal so viel Protein als der originale Promotor. Aber mehr als zwei Burst-Sequenzen verringerten die Menge an produzierten YFP. Auch zeitgleiche Expression des very late factor 1 und YFP resultierte in verringerter YFP Produktion, was auf einen Engpass in den darauffolgenden Schritten der Expressionsmaschinerie hindeutet. Bedauerlicherweise konnten nur Tendenzen in den Zelllinien-Vergleichs-Experimenten beobachtet werden da es Probleme mit der Virusinfektiosität gab. Jedenfalls, der Vergleich der vielversprechenden Konstrukte zeigte Potential zur erhöhten rekombinanten Proteinproduktion in der neuen Tnms42 Zelllinie verglichen mit der oft verwendeten Sf9 Zelllinie.

ABBREVIATIONS

AcMNPV	*Autographa californica* multiple nucleopolyhedrosisvirus
APS	Alkaline phosphatase buffer
BCIP	5-bromo-4chloro-3'-indolyphosphate
BEV	Baculovirus expression vector
bp	Basepairs
BS	Burstsequence
BSA	Bovine serum albumin
BV	Budded virion
ddH_2O	Double destilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose containing nucleoside triphosphate
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylendiaminetetraacetic acid
FACS	Fluorescence activated cell sorting
GP64	Glycoprotein 64
GV	Granulosis virus
h.p.i.	hours post infection
ie1	immediate early viral protein 1
LB	Luria-Bertani
MES	2-(*N*-morpholino)ethanesulfonic acid
MNPV	*multiple nucleopolyhedrosisvirus*
MOI	Multiplicity of infection
ODV	occlution derived virion
Polh	polyhedrin
Rfu	relative fluorescence unit
RNA	ribonucleic acid
SDS	Sodium dodecyl sulphate

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNPV	single nucleopolyhedrosisvirus
$TCID_{50}$	50% Tissue Culture infective dose
TEMED	N,N,N',N'-Tetrametylethan-1,2diamin
TPBS	Phosphate buffered saline + Tween 20
TRIS	tris(hydroxymethyl)aminomethane
vlf-1	very late factor 1
WS	Working Stock
YFP	Yellow fluorescence protein

Inhalt

1. INTRODUCTION

1.1. THE BACULOVIRUS

Baculoviruses are a diverse group of enveloped viruses. Common to the whole group of *Baculoviridae* is the double-stranded, circular, supercoiled genome with the size of 80 to 180 kb. The genome is packaged in rod-shaped nucleocapsids of infectious virions (as reviewed by Rohrmann 2013, see figure 1-1) which are present in two genetically identical but morphologically and functionally distinct types: budded virions (BVs) and occlusion derived virions (ODVs) (Ackermann and Smirnoff 1983, see chapter 1.1.1).

Figure 1-1: rod-shaped virion. Taken from Rohrmann 2013

Baculovirus replication is restricted to invertebrates and they are highly pathogenic to multiple insect species. Phenotypical differences in their ODVs lead to their classification into two genera: nucleopolyhedroviruses (NPV) form polyhedron-shaped structure in the nucleus, whereas granulosis viruses (GVs) form granule-shaped virions in the cytoplasm of infected cells (Xeros 1952, Vago et al. 1974). Additionally, the nucleopolyhedrosis viruses are divided into two groups – single or multiple nucleopolyhedrosis viruses (SNPVs or MNPVs), depending on whether only a single (SNPV) or multiple (MNPV) nucleocapsids are packaged into one infectious virion (see figure 1-2).

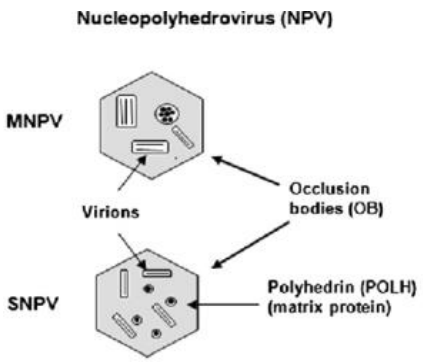


Figure 1-2: Either only one or multiple nucleocapsides are packed into a *virion. Of the nucleopolyhedrosis virus. Figure taken and modified from van Oers 2011.*

Another part of the NPV baculovirus nomenclatur indicates the insect species the virus was first isolated from. The most common hosts of the *Baculoviridae* are part of the Diptera (flies), Hymenoptera (sawflies, wasps, bees and ants) and Lepidoptera (butterflies and moths) insect orders. (Martignoni and Iwai 1986). The most commonly used baculovirus for recombinant protein expression is the *Autographa californica* multiple nucleopolyhedrosisvirus (*AcMNPV)* isolated from the alfalfa looper. This virus was also employed in the present work. It has a rather large genome, around 134kb which encodes for around 156 proteins (as reviewed in Rohrmann 2013).

1.1.1 Baculovirus life cycle

To establish an infection in the insect host, occlusion bodies (containing infectious ODVs) need to be eaten and digested. In the alkaline environment of the midgut the occlusion bodies dissolve and released ODVs infect the midgut epithelial cells (see chapter 1.1.2). After infection of the midgut, systemic infection of the insect is accomplished by budded virions, s which bud off from infected cells and initiate an infection cascade (Ghosh et al. 2002, see figure 1-3).

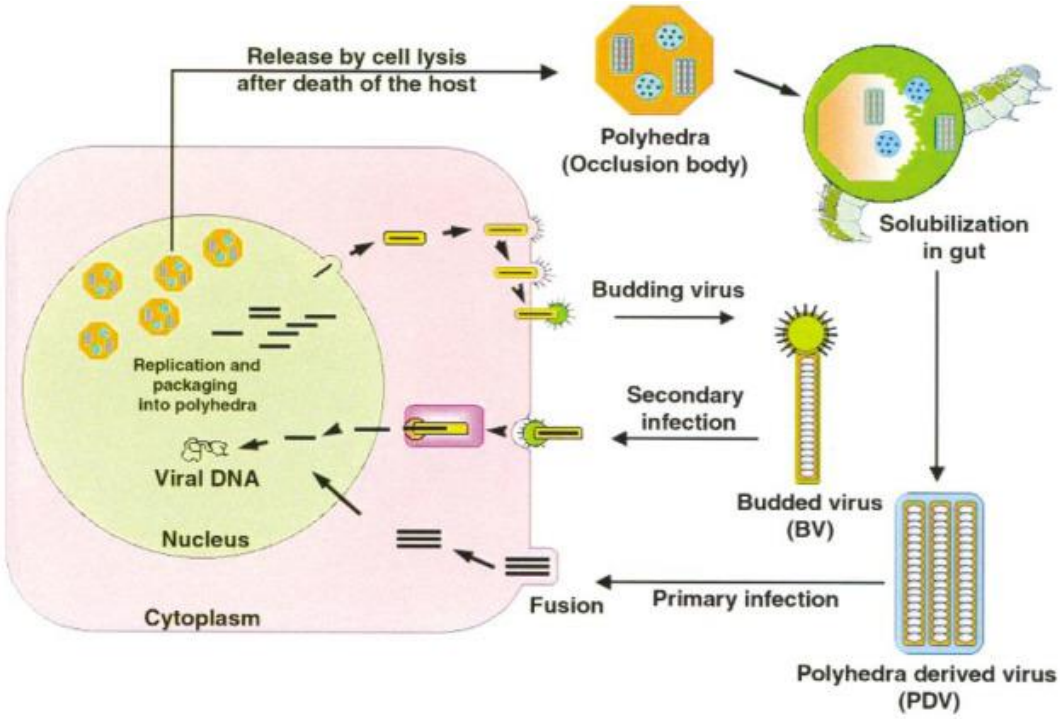


Figure 1-3 The polyhedral shaped occlusion bodies are solubilized in the gut and the virus can infect the first insect cells. In these cells the virus uses the host machinery to multiply the viral DNA and package it. Part of this buds of the cells and infects other cells as budded viruses. Another part is packaged into occlusion bodies and released at death of the host, ready to be eaten again. Figure taken from Ghosh et al. 2002.

The glycoprotein 64 (GP64) is a critical component of infectious *AcMNPVs* budded virions. The virion-membrane-anchored GP64 attaches to the host cell surface and triggers receptor-mediated endocytosis for viral entry. Upon virion take-up, GP64 undergoes a conformational change, enabling pH-triggered fusion with the host endosomal membrane and release of the nucleocapsid (as reviewed by Kataoka et al. 2012).

After their release into the cytosol, the nucleocapsids are transported into the nucleus, through nuclear pores. Viral transcription and DNA replication begins. A so called transcriptional cascade is initiated: The first genes synthesize transcription factors for the next set of genes and those synthesize factors for the next phase in virus replication, eventually resulting in four time-dependent phases of infection.

The first phase – the **immediate early phase** is a very short phase starting immediately after infection. It is characterized by the downregulation of the hosts mRNA levels and the exploitation of the host transcriptional apparatus (such as the host RNA-polymerase) for the synthesis of viral early proteins (Nobiron et al. 2003). Among these is the immediate-early protein 1 (ie1), which is the main transcriptional activator needed for initiation of the transcriptional cascade and controlling DNA replication of the virus (see chapter 1.6.2). Baculoviruses are the only nuclear-replicating DNA viruses that are known to use a combination of host and viral RNA-polymerases (which is produced later on).

The **delayed-early phase** (0 - 6 hours post infection) is characterised by replication of the viral genome and the generation of viral proteins, such as the viral RNA-Polymerase and the membrane glycoprotein 64 (see chapter 1.6.2).

In the **late phase** (6 - 24 hours post infection) of infection a variety of proteins, mostly the proteins needed for nucleocapsid assembly are expressed by the viral RNA-Polymerase (as reviewed by Rohrmann 2013, van Oers 2011, van Oers 2015). These include the very late transcriptional factor 1 (vlf-1), which has a function both in regulating the very late phase and as structural component of the nucleocapsid (Yang and Miller 1998). Additionally, GP64 expression peaks a second time, making the cell membrane ready for the virion budding (see chapter 1.6.2).

Assembled nucleocapsids are transported out of the nucleus and to the cell membrane, where they bud-off to generate infectious budded virions. Some nucleocapsids are retained inside the nucleus until the very late phase, where they get packaged in occlusion bodies, resulting in ODVs.

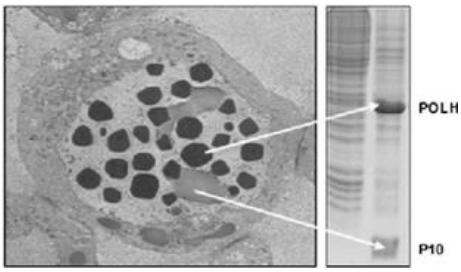


Figure 1-4: An occlusion body. The polyhedrin is the most abundant protein produced in the virus cycle. The second most abundant protein is p10. Figure taken and modified from van Oers 2011.

The **very late phase** (18 hours until up to 72 hours post infection) is characterized by the completion of ODVs. Two proteins are needed in relatively high abundance, the polyhedrin (polh) and p10 protein. The polyhedrin is the most abundant viral protein produced during the virus life cycle. It is the major component of the occlusion bodies, where viral nucleocapsids are embedded in (see figure 1-4). The second most abundant protein is p10, which is associated with the maturation of occlusion bodies (as reviewed by Rohrmann 2013, van Oers 2011, van Oers 2015). ODVs are enveloped in the nucleus and released upon cell lysis, resulting in the historically name “wilting disease” for a baculovirus infection. The disease is basically “spread” via plant leafs contaminated with occlusion bodies (Granados and Lawler 1981).

As the two most abundant proteins (the polh and p10 protein) are not needed for the generation of budded virions – the virus type employed for recombinant protein expression – the respective

promoters are being used for the high-level expression of recombinant proteins (as reviewed by Rohrmann 2013, van Oers 2011, van Oers 2015).

1.1.2 The infectious virus particles

The biphasic replication cycle of baculoviruses result in the generation of two infectious virus phenotypes:

Occlusion-derived virions are assembled in the nuclei and occluded in so called occlusion bodies. As the nomenclature of nucleopolyhedrosisviruses indicates, they are of polyhedral shape and approximately of a size of 0.6-2µm. Occlusion bodies consist of nucleocapsids embedded in a crystalline protein matrix and enclosed in an outer membrane, assembled in the nucleus. The protein matrix is simple and mostly consists of the protein polyhedrin (see chapter 1.6.2, as reviewed by Rohrmann 2013), which protects the virus from the environment and allows the virus to survive outside of the natural host (see figure 1-5, as reviewed by van Oers 2011). ODVs are resistant to heat and light-inactivation to retain their infectivity after being spread by liquefied insects on plant leafs. (Evans 1986). Insect-to-insect transmission occurs via these contaminated leafs that are ingested by another insect host (Granados and Lawler 1981).

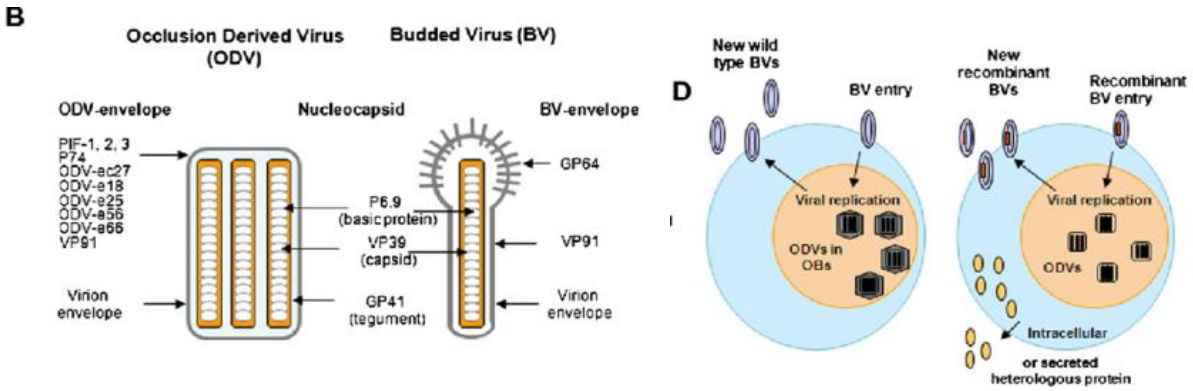


Figure 1-5: (B) Schematic representation of the two infectious particle types with the most important proteins. (D) Schematic representation or recombinant protein expression with the baculovirus. Figure taken and modified from van Oers 2011.

The budded virions, on the other hand, are more sensitive to the environment. These virions are responsible for cell-to-cell transmission and spread the infection by budding from the cell membrane into the insect hemocoel, thereby infecting neighbouring cells (Granados and Lawler 1981). During the budding process, virions get enveloped with the host plasma membrane, consisting of host and viral membrane proteins. The most important protein in the BV envelope is the protein needed cell-virus fusion GP64, for the virus to enter the cell (Granados and Lawler 1981, Oomens et al. 1995, see figure 1-5). BVs are approximately 40 - 60 nm in size and are smaller than occlusion bodies.

ODVs are only important for insect-to-insect transmission and are less efficient in virus cell-to-cell spread. This is why they are normally not utilized for recombinant protein expression in insect cell cultures and are not therefor not explained in further detail in this work.

1.2 THE BACMID SYSTEM

1.2.1 Mechanism

The polh and p10 proteins, produced in the very late stage of infection, are structural components of occlusion bodies, which are not utilized for recombinant protein expression in insect cell cultures. Therefore, the polh and p10 promoters can be used for recombinant protein expression. This discovery was the first step for the establishment of the baculovirus expression systems in insect cells. (as reviewed by Summers 2006). This system is also often called baculovirus expression vector system or BEV.

Originally homologous recombination, between the linearized virus genome (Baculogold™) and the plasmid with the gene of interest, was used to obtain recombinant viruses (Smith et al. 1983, Vlak et al. 1990). Owing to tedious purification steps needed to obtain recombinant baculovirus clones, other systems have been established.

Bac-to-Bac

The method used in this work depends on a bacterial artificial chromosome, also called bacmid, which contains the *AcMNPV* genome (Luckow et al. 1993). The *Escherichia coli* carrying the bacmid are commercially available (DH10Bac, Invitrogen; MultiBac, Geneva Biotech).

The Bacmid consists of multiple genetic elements that allow for its genetic manipulation in bacteria (see figure 1-6). The mini-F-replicon (7kb) is responsible for replication, maintenance and stable inheritance of the bacmid in the bacteria host (Lovett and Helsinki 1976, Tsutsui and Matsubara 1981, Shizuya et al. 1992). Additionally, a kanamycin resistance gene was incorporated into the bacmid for antibiotic selection. A T7 transposition site located within a lacZα reading-frame has been introduced into the bacmid for convenient introduction of foreign sequences and easy screening of recombinant clones. For this a plasmid with two Tn7 transposon sites, a bacmid with a Tn7 transposase

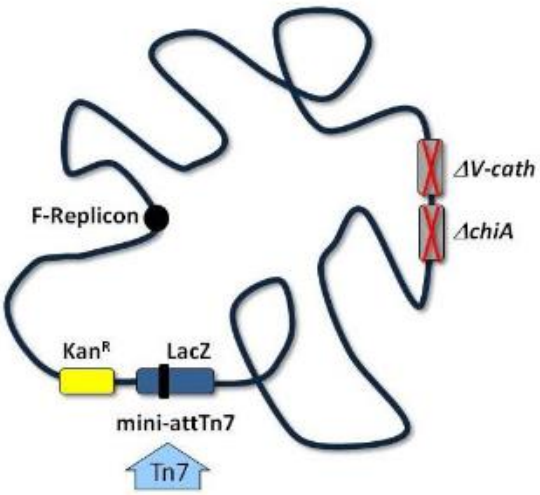


Figure 1-6: Bacmid of the Multibac System. The v-cath and chiA gene are disrupted. The Tn7 attachmentsite is located in the LacZ gene, making Blue/White Screening possible. There is also a Kanamycin Resistance Gene and the F-Replicon. Figure taken from the MultiBac Manual Version 3.0, 2011.

attachment site and the enzyme transposase is needed (Lichtenstein and Brenner 1981). Upon successful transposition, the lacZα sequence for the production of functional β-galactosidase is disrupted. Recombinant clones can be screened by using classical blue/white screening, as X-gal in the selection medium is cleaved only by functional β-galactosidase resulting in a blue coloured colonies. In contrast, colonies with no functional β-galactosidase stay white (Ullmann et al. 1967).

DH10Bac or MultiBac bacterial cells also contain a so called Helper Plasmid. This is ~13 kbp plasmid contains a transposase expression cassette and a tetracyclin selection marker for antibiotic selection.

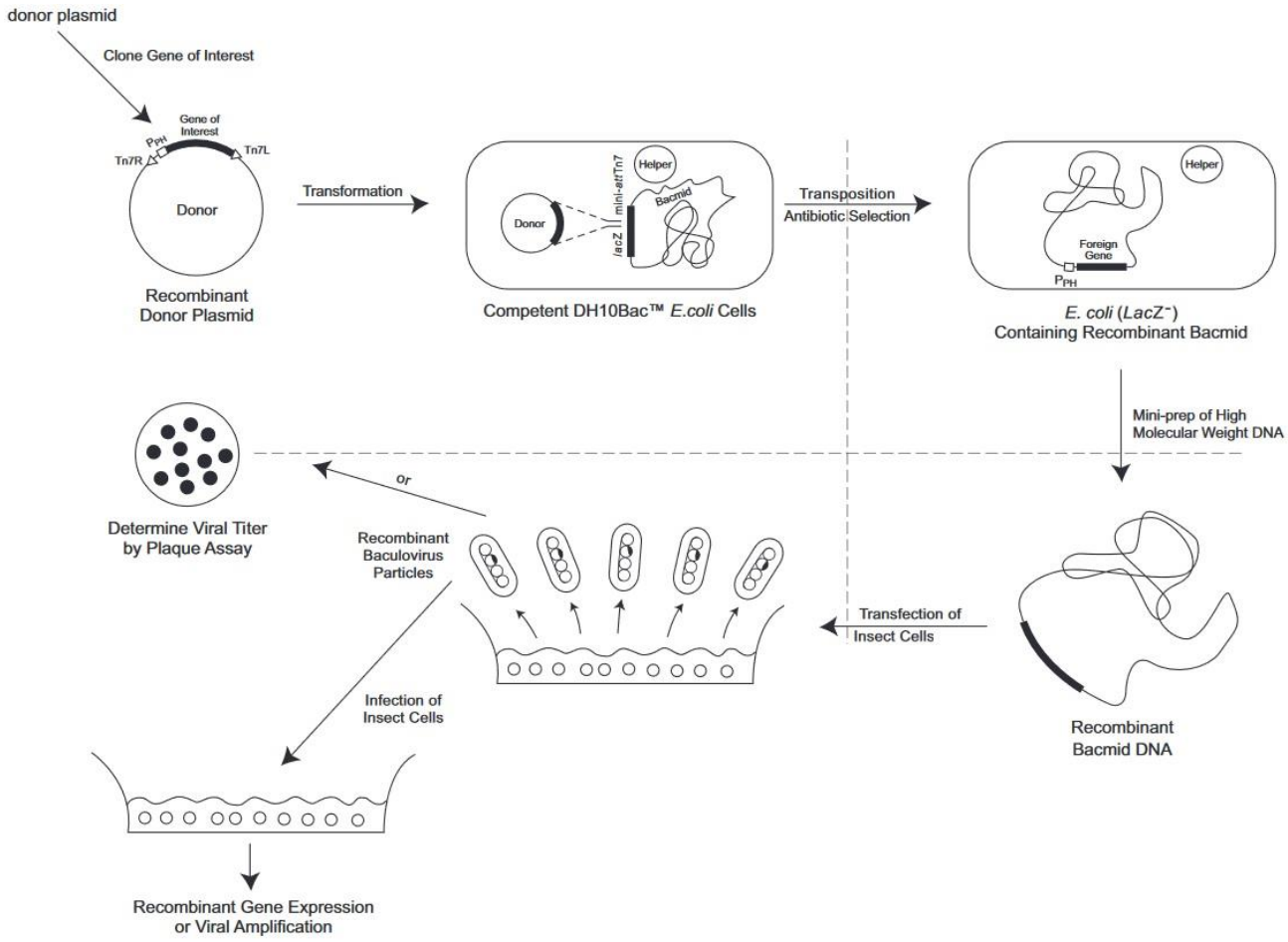


Figure 1-7: The Bacmid System. The gene located between the right and left arm of the Tn7 sites on the donor plasmid is inserted into the Tn7 attachment site on the bacmid. The bacmid is then multiplied in the E.coli cells and recombinant clones identified via blue/white screening. After this the bacmid is purified and transfected into insect cells. There infectious budded virions are produced, amplified and used for the experssion of the recombinant proteins. Figure taken from the MultiBac Manual Version 3.0, 2011.

Molecular cloning of the genes of interest is performed in transfer vectors/donor vectors, which contain Tn7L and Tn7R sites for recombination of the interjacent cloned DNA sequence into the bacmid. .

A schematic representation of the mechanism for the generation of the recombinant baculovirus genome can be seen in figure 1-7. When the plasmid with the Tn7L and Tn7R regions is transformed

into the DH10Bac or MultiBac bacterial cells, the transposase recognizes these regions and recombines the interjacent DNA sequence into the Tn7 attachment site on the bacmid. This results in the enlargement of the bacmid DNA and a disruption of the lacZα-reading frame. The transformed *E.coli* are then grown and spread out on Blue/White Screening plates. Then the bacmids are screened to re-confirm successful recombination. Then, exponentially growing insect cells are transfected with purified bacmid using a transfection reagents. In this system and the typically used host cells, both suspension and adherent culture is possible for protein expression. Once the bacmid is in the insect cells, the viral DNA is moved into the nucleus and the transciptional cascade is started. In the early transcription the viral DNA is replicated, in the late phase the virions bud out of the cell and infect other cells or stay in the supernatant. Budded are harvested from the supernatant and used for infection of a fresh batch of insect cells for the amplification of the virus. Additionally, the recombinant proteins are expressed. In case a fluorescence protein expression cassette (mCherry, YFP or others) is present on the bacmid, infected cells can be easily distinguished from uninfected cells to aid in the determination of the harvesting time point. Above that, cytopathic effects (cells become bigger and round-up, cell lysis) are monitored to determine the infection status (Bac-to-Bac ® Manual, MulitBac^{Turbo} Manual 2011).

Using the Bacmid system has advantages to the classical recombination system. First of all the adaption or mutation of the bacterial genome can be done with methods developed for bacterial systems therefore the system is easily applicable. Also the produced virus has a well-defined DNA compared to the DNA obtained by the recombination method (as reviewed van Oers et al 2015).

Disadvantages of the Bacmid system include, additionally to virus residues, the presence of bacterial DNA combined with the presence of antibiotic selection markers in the product. Additionally, the bacmid derived vectors appear to be relatively unstable. Therefore, for commercial use, the classical recombination into a linearized vector is preferably used, e.g. Baculogold ™ (as reviewed van Oers et al 2015).

1.2.2 Specialities MultiBac^{Turbo}

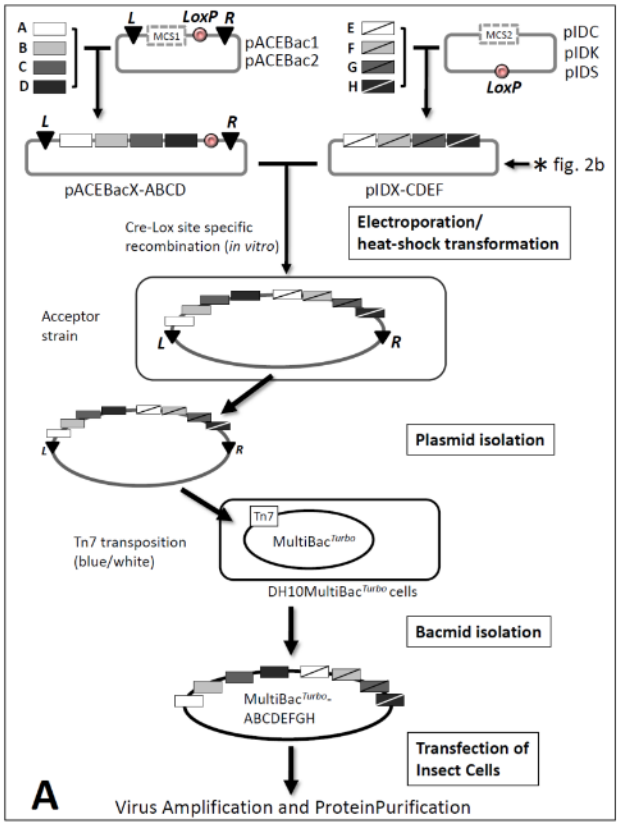


Figure 1-8: The MultiBac System. Two Plasmids are fused together with the Cre-Lox recombinase, transposed into the bacmid, multiplied, isolated and transfected into insect cells. Figure taken from the MultiBac Manual Version 3.0, 2011.

In this work the MultiBac^{Turbo} Kit (Geneva Biotech, Switzerland) was used. It addition to above-mentioned characteristics, donor vectors possess over lox-P fusion sites, which allow for the fusion of two plasmids to yield multi-expression cassettes using Cre recombinase. This allows for easy shuffeling of multiple expression plasmids for different applications. (see figure 1-8).

Additionally, two genes responsible for enhancing proteolytic breakdown (v-cath and chiA) of the infected host have been deleted from the bacmid. Allegedly this should result in improved maintanance of the cellular compartment during infection and improved quality of the proteins produced (MulitBac^{Turbo} Manual 2011, figure 1-6).

1.3 INSECT CELLS

Insect cell lines have been around for some time with the first continuously growing one being established in the 1960s. Their many advantages make them interesting for research and commercial use especially in combination with insect viruses like the *Baculoviridae*. (see chapter 1.4).

Insect cells of the order Lepidoptera (moths and butterflies) have been proven to be highly susceptible to *Baculoviridae* and cell lines derived from these species are frequently used for recombinant protein expression.

Sf9 cell line

Figure 1-9: fall armyworm pupae. Photograph taken from [1]

The SF9 cell line was originally obtained from the ovary tissue of *Spodoptera frugiperda,* also called fall armyworm (see figure 1-9). The cells are highly susceptible to the *AcMNPV* infection and are derived from the IPLB-SF-21 cell line (Vaighn et al. 1997). This cell line is routinely employed for the production of recombinant baculoviruses and for recombinant protein expression. Unfortunately a latent virus infection by the Sf-rhabdovirus was found (Ma et al. 2014). A virus contamination in the cell line used, could have unknown effects on behaviour studies and protein expression and could additionally lead to pathogenicity in vertebrates if the produced protein is applied as therapeutics or similar.

High Five cell line

This cell line is derived from ovarian cells of *Trichoplusia ni* - cabbage looper (see figure 1-10) - and is officially called BTI-TN-5B1-4. High Five cells have been proven to be superior in production yields for several recombinant intracellular and secreted proteins or virus like particles in comparison to the Sf9 cell line (Wickham and Nemerow 1993, Krammer et al. 2010). But were also found to contain a latent alphanodavirus infection (Li et al. 2007).

Figure 1-10: Adult cabbage looper. Photograph taken from [2]

Tnms42 cell line

With both major cell lines infected with a latent virus, the need for a virus free cell line arises. Tnms42 is a subclone from the High Five cells and have been established only recently. In contrast to the High Five cells, this cell line does not contain any detectable alphanodavirus, but is hardly characterised in terms of its performance in recombinant protein expression. (Chen et al. 2013, Chen et al. 2014).

1.4 ADVANTAGES AND DISADVANTAGES

Similar to every expression system, also the baculovirus expression vector system (BEVS) has advantages and disadvantages. To understand the different important factors one has to keep in mind that this system consists of modifying and multiplying the virus genome in bacterial cells, transfecting the DNA into insect cells and expressing the protein of interest in insect cells.

The advantages of using insect cells over mammalian cells is, they are fairly simple and cheap to grow. Insect cells do not require CO_2 and they can grow adherently or in suspension in serum-free media, making scale-up simple. Working with insect cells as expression system reduces the risk of contamination with vertebrate-specific pathogens as compared to the mammalian system. Additionally, the availability of strong viral promoters enables the expression of high amounts of recombinant protein (Boulaire et al. 2009). Above that, insect cells allow for proper protein folding and for post-translational modification, which is an advantage in comparison to bacterial and yeast systems (Wickham and Nemerow 1993).

Major advantages of using *Baculoviridae* includes their narrow host range, which makes them safe to work with. Additionally, the structure of the nucleocapsides allows for the insetion of large DNA fragments, which allows for the co-expression of several recombinant proteins (as reviewed van Oers et al. 2015).

There are unfortunately also major disadvantages. In table 1-1, it can be seen that cultivation of insect cells is more expensive and also slower in comparison to bacteria or yeast cells. Also the produced proteins might induce a stronger immune system reaction than bacterial or yeast cell products. This is due to the core fucosylation of N-glycans, called paucimannose, in insect cells being highly antigenic to humans. Above that, post translational modification do not always work, especially glycosylations as the side chains of the N-glycans are less complex than mammalian (Kuroda et al. 1990, Shi and Jarvis 2007, see table 1-1). But there are insect cell lines allowing for mammalian-type glycosylations and for modification of the sugar structure, for example SweetBac™ (Palmberger et al. 2012). Additionally, therapeutics are supposed to be free of any virus residues, which makes downstream processing more laborious than using non virus dependent expression systems. Especially for virus-like-particles there is no satisfactory method yet to separate viruses from the particles.

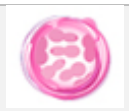
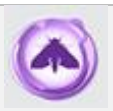
	E.coli	Yeast	Insect cells	Mammalian cells
				
Cell growth	rapid (30 min)	rapid (90 min)	slow (18-24h)	slow (24h)
Complexity culture conditions	minimum	minimum	moderately complex	complex
Cultivation expense	low	low	high	high
Expression speed	high	moderately high	low to moderately high[1]	low to moderate[2]
Expression properties	proteins rarely secreted cells well characterised	works well for secreted and intracellular proteins	works well for secreted and intracellular proteins	Works well for secreted and membrane proteins (low yields intracellular)
Volumeric production levels	high	high	medium	low
Process scale up	easy	moderately easy	moderately difficult	difficult
Protein folding	refolding usually required	refolding may be required	proper folding	proper folding
Other posttranslation modifications	none	high mannose, simple N-linked glycan structures different to mammalian	simple, no sialic acid N-linked glycan structures different to mammalian	complex most mammalian are possible
Contamination risk	endotoxins	low	low	Viruses, Oncogenes

Table 1-1: Comparison between most commonly used hosts cells for recombinant protein expression. Table modified from Fernandez and Hoeffler 1999, [4], [5], van Oers et al. 2015.

[1]low with stable expression, moderate with plasmid based transient expression and moderately high with virus based transient expression

[2]low with stable expression and moderate with transient expression.

In conclusion, for smaller, homogeneous, simple proteins higher levels of production can be achieved in insect cells than in mammalian cells. This makes insect cells highly interesting for vaccine production and structure/functional studies (Wickham and Nemerow 1993).

1.5 APPLICATIONS

Next to its increasing application in recombinant protein expression, baculoviruses have a long tradition in being used in agriculture and forestry for insect pest control. The first insect line for recombinant protein expression was established in 1959 (Gaw et al. 1959) and *AcMNPV* was first isolated in 1971 (Vail et al. 1971). In 1983 the BEV system was first used for the expression of a recombinant protein, human IFN-β (Smith et al. 1983). Ten years later, in 1993, the bacmid system was

developed (Luckow et al 1993). When the BEV system was ready to be established for industrial use, the yeast and bacterial systems, which were also fairly new at that time, were already successfully in use. There was not the capacity for a new system to be introduced, which might be the reason why the progress in BEV system research was slower than in the research of the already established systems at that time (as reviewed by van Oers et al. 2015).

Because high protein expression levels can be achieved by the viruses, the BEV system, especially with *AcMNPV*, is mostly used in research and medicine to produce therapeutics, subunit vaccines, virus like particles and so on (as reviewed by Mena and Kamen 2011, as reviewed by van Oers et al. 2015). Some of the products have already been commercialized, as summarized in table 1-2.

Product name	Company	Expressed product	Purpose	Use	Year of release	Cell line
Porcilis Pesti	MSD Animal Health	E2 glycoprotein	Subunit/marker vaccine against classical swine fever	Pigs	1998	Sf21
Bayovac CSF E2	Bayer Biologicals/Pfizer Animal Health Care	E2 glycoprotein	Subunit/marker vaccine against classical swine fever	Pigs	2001	Sf21
Circumvent PCV	MSD Animal Health	Porcine circovirus ORF2	VLP vaccine against porcine circovirus type 2	Pigs	2005	-
Cervarix	GlaxoSmithKline	Human papillomavirus L1 protein (serotype 16 and 18)	VLP-based vaccine against cervical cancer	Girls	2007	Hi5
CircoFLEX	Ingelvac	Porcine circovirus ORF2	VLP vaccine against porcine circovirus type 2	Pigs	2008	Sf9
Porcilis PCV	MSD Animal Health	Porcine circovirus ORF2	VLP vaccine against porcine circovirus type 2	Pigs	2009	Sf
Provenge (sipuleucel-T)	Dendreon	PAP-GM-CSF	Immunotherapy against prostate cancer	Men	2010	-
Glybera	UniQure	AAV vector with lipoprotein lipase transgene	Gene therapy against familial lipoprotein lipase deficiency	Humans	2012	Sf
Flublok	Protein Sciences	Influenza HA	Annual trivalent flu vaccine	Humans	2013	expressSf

Table 1-2: Approved vaccines and therapeutics, produced with the baculovirus expression vector system. Taken and modified from van Oers et al. 2015.

1.6 PROTEINS AND PROMOTERS OF INTEREST IN THIS WORK

1.6.1 Proteins

Yellow fluorescence protein

The green fluorescence protein was isolated first in 1961 from the jellyfish, *Aequorea victoria* and is commonly used in cell and molecular biology to study promoter activity, for bioimaging of intact organelles, cells or organisms or to study protein interactions (LIT). A T203Y mutation was introduced to enhance its stability, which resulted in a shift in its fluorescence spectrum and thus in the discovery of the yellow fluorescence protein, YFP. YFP has an excitation wavelength of 516 nm and an emission peak wavelength of approximately 525 nm. YFP is a cytosolic protein of 26.4 kDa in size and is present as a monomer or a dimer depending on concentration. [3]

Very late factor 1

Baculovirus late and very late promoters share a TAAG sequence as transcription initiation site, which is recognized by the viral RNA-Polymerase. In addition to that, the polh promoter contains a so called “burst sequence” at its 3’end. This sequence is bound by the viral transcription factor very late factor 1 (vlf-1), a protein that belongs to the lambda integrase protein family. Vlf-1-binding results in a burst in transcription from the polh promoter, allowing for high-level protein expression from this promoter (Ooi et al. 1989, McLachlin and Miller 1994). For proper activation of the very late polyhedrin promoter, a threshold level of vlf-1 is necessary. Also overexpression of this gene may drive earlier and stronger expression of the promotor but also results in too early cell disintegration (Yang and Miller 1998).

Additionally, vlf-1 is believed to be a structural component of the nucleocapsid. Deleting this gene would results in the generation of non-infectous virus particles (Yang and Miller 1998). Vlf-1 is approximately 44.4 kDa in size and present as a monomer (Rohrmann and Mikhailov 2013, McLachlin and Miller 1994).

1.6.2 Promoters

Promoters are required for transcription of the DNA into RNA. One particular promoter controls the transcription of one particular gene by recruiting the necessary transcription machinery and initiating the elongation. A promoter region is normally located upstream and close to its gene. The sequence includes different motifs to which transcription factors can bind and initiate gene transcription by aiding the RNA-Polymerase. Promoters can be differentiated into two groups, constitutive promoters and inducible promoters (Morse et al. 2016).

In the current work, baculovirus promoters are used for recombinant protein expression in insect cells. Apart from the ie1 promoter, which employs the host RNA-polymerase for transcription, all promoters active in the later phases of infection require the viral RNA-polymerase for being active.

Because recombinant gene expression is very dependent on the promoters used, information about the strength and kinetics of the promoter is a key information for any expression experiment. This is basically the information when gene expression starts, how long it lasts and how much protein is produced. In general can be said, the later the promoter is activated in the virus cycle the higher protein expression it supports.

"Recombinant baculoviruses were constructed to place the bacterial chloramphenicol acetyltransferase gene under the control of promoters strongly active in the early, late, or very late stages of virus replication. In fully permissive cells, expression from a very late promoter was 2- to 3-fold higher than expression from a late promoter and 10- to 20-fold higher than expression from an early promoter or from a virus-borne insect promoter." (Morris and Miller 1992)

Polyhedrin

The polyhedrin, approximately 33kDa in size, is the matrix protein of ODVs and thus only produced in the very late phase of infection (18 hours until up to 72 hours post infection), but there in enormous amounts. The protein forms a crystalline cubic lattice that embeds the virions in occlusion bodies (as reviewed by Rohrmann 2013). As the polyhedrin is not needed for budded virion production – the virus form employed in insect cell cultures - the polh promoter is highly interesting for recombinant protein expression (as reviewed by Summers 2006, see chapter 1.1.2).

Two regions in this promoter are of great interest in this work. The TAAG motif which is the transcription initiation site and the so called burst sequence, BS, at the 3' end of the promoter, to which the vlf-1 binds. Binding of the transcription factor to the burst sequence induces a "burst" in expression. This sequence is approximately 50 bp long and mutations in this stretch were found to reduce expression by 10 to 20 fold (Ooi et al. 1989). Higher levels of vlf-1 present in the cell as well as multiple burst sequences present within the promoter may lead to a higher level of gene expression (Manohar et al. 2010).

Glycoprotein 64

Glycoprotein 64 is the viral envelope fusion glycoprotein and is responsible for attachment and fusion of the virus particle with the host cell, resulting in secondary infection (see chapter 1.1.1).

Consequently deleting this gene results in the generation of non-infective budded virions (Oomens and Blissard 1999).

The protein was found to have two expression peaks, which is conform to the presence of an early and a late transcription initiation site (Zhou et al. 2003). First, in the early phase (0-6 hours post infection), to enable budding of non-replicated virions, already present in the virus and the second, in the late phase, to enable budding of the replicated virions. The second peak is significantly stronger than the first. GP64 is one of the three most abundant proteins in *AcMNPV* and thus the gp64 promoter is very interesting for recombinant protein expression (Wang et al. 2010).

Immediate early viral protein 1

The immediate early viral protein, ie1, is the activator of the transcriptional cascade in the replication cycle of many baculoviruses by transactivating early promoters. It is needed for the viral DNA replication and also has a task in blocking proapototic activity. Later in the cell cycle, however, the ie1 can trigger apoptosis of the cell (Schultz et al. 2009). The vlf-1 expression starts very early in the infection process until the late phase (6 - 24 hours post infection) at a rather contstant but weak level (Guarino and Summers 1986).

Because of the blocking of the antiviral response and the enabling of the multiplication, the ie1 is an important protein also in foreign gene expression and therefore cannot be deleted completely in experiments (Schultz et al. 2009).

2. OBJECTIVES

The baculovirus expression vector system uses insect cells as hosts and is an important system used in research and medicine. For this work transcriptional regulatory elements were modified with the aim to optimize yield and kinetics of recombinant protein expression. Based on two papers the aim was to incorporate multiple burst sequences into the polyhedrin promoter (Manohar *et al*. 2010) and overexpress the very late factor 1 (McLachlin and Miller 1994) under control of the GP64 and ie1 promoter.

- Modification of the polyhedrin promoter
- Modification of the transcription factor binding site in the polyhedrin promoter
- Simultaneous overexpression of the transcription factor and cytoplasmic recombinant protein regulated by different promoters
- Comparison of different constructs in three different cell lines

The comparison in the different cell lines especially aimed to observe and establish the fairly new Tn42 cell line for laboratory use. Mainly because different cell lines might experience different limitations.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Cells

E.coli

- JM109, New England BioLabs, USA
- NEB 5-alpha competent E.coli (High Efficiency) for chemical transformation, C2987H(New England BioLabs, USA)
- pirHC cells (EMBL-Grenoble, France)
- DH10MultiBacY cells (EMBL-Grenoble, France)
- DH10MultiBac (-Y) cells (EMBL-Grenoble, France)

Insect Cell lines

- *Spodoptera frugiperda* Sf9 cells (ATCC CRL-1711)
- *Trichopulsia ni* BTI-TN5B1-4 "High Five" cells (ATCC CRL-10859), abbr. Hi5
- Tnms42 subcloned from BTI-TN5B1-4 cells (Chen et al. 2013), abbr. Tn42

3.1.2 Nucleic acids

(see chapter 6 Appendix for sequences)

Plasmids

- pACEBac1 (EMBL-Grenoble, France)
- pIDK (EMBL-Grenoble, France)

Promotores

- Glycoprotein64,
- Immediate early viral protein 1, ie1
- Burst sequence
- Polyhedrin promoter

Proteins

- Very late factor 1, vlf-1
- YFP

3.1.3 Antibodies for Western Blots

Hm (human Ab C2G12 (anti-GP 120HIV) Lot: T590304-A Conc: 13.1 mg/ml Polymon Scientific GmbH	1:5000
AntiHuman-IgG (γ-Chain specific) Alkaline Phosphatase, AB produced in goat Sigma Aldrich (A3187-.5ML)	1:2000
AntiMouse-IgG (γ-Chain specific) Alkaline Phosphatase Sigma Aldrich (A1047-1ML)	1:2000
Anti Mouse-IgG (whole molecule) Alkaline Phosphatase A5153-1ML	1:2000
Tetra-His Antibody Anti-Mouse-Antibody	1:2000
Pierce® Anti 6 x His Epitope Tag Monoclonal Mouse Antibody Prod # MA1-81023	1:1000
AntiGFP antibody Mouse Monoclonal (6AT316)	1:2000
Anti-Rabbit IgG (whole molecule) Alkaline Phosphatase antibody produced in goat A9919-25ML	1:72000

3.1.4 Media

Lysogeny broth –media (LB) pH 7.5 adjusted with NaOH

The components listed in table 3-1 were dissolved in ddH_2O and autoclaved at 120°C for 20 minutes. After autoclaving the medium was stored at 4° C. If required, antibiotics and additives were added just before usage. For LB Agar the agar was added before autoclaving and the medium heated in the microwave before the plates were poured.

Table 3-1: Lysogeny Broth – Media

10 g/l	Peptone casein

5 g/l	yeast extract
10 g/l	NaCl
15 g/l	Agar Agar (if desired)

Super optimal broth with (SOC)

All ingredients in table 3-2 were dissolved and autoclaved. The sterile SOC medium was stored at 4°C.

Table 3-2: Super optimal broth with catabolite repression (SOC)

20 g/l	BactoTryptone/Peptone Caseine
5 g/l	yeast extract
10mM	NaCl
3mM	KCl
10mM	$MgCl_2*6H_2O$
10mM	$MgSO_4*7H_2O$
20mM	glucose

Cell culture media

HyCloneTM SFM4 Insect media with glutamine (GE Healthare, GB) supplemented with 0.1% pluronic was used as cultivation medium for insect cells. The pluronic is a non-ionic detergent which reduces hydrodynamic damage.

3.1.5 Antibiotics and media additives for selective growth media or plates

Antibiotics and media additives were added to media and agar to ensure selective *E.coli* growth. Table 3-3 shows the used working concentrations.

Table 3-3: Antibiotics and additives

Working concentration	**Additive**
15 µg/ml	Gentamycin
40 µg/ml	IPTG
50 µg/ml	Kanamycin
10 µg/ml	Tetracyclin
100 µg/ml	X-Gal

3.2 MOLECULAR CLONING EMPLOYED IN THIS WORK

This is a short description of the steps done to receive the desired virus constructs. All promoters and protein sequences were cloned into the multiple cloning site of the plasmids. Sequences of inserts and primers can be found in the appendix. The description for each method can be found in chapter 3.3.

3.2.1 Generation of promoter constructs

The **GP64 and the ie1 promoters** were already present on plasmids and therefore amplified by bacterial growth, purified and subsequently digested for ligation. Plasmids (pACEBac1 and pIDK) and promoters were digested with the same enzymes. By separation with agarose gels and gel purification, the sequences were purified and used for sticky end ligation. Subsequently cloning was continued with the instructions found chapter 3.2.3.

The plasmid with the **polh promoter** was first also purified with a silica membrane but then was amplified with PCR to discard the piece of polyhedrin protein sequence which still is attached to the normally used polh promoter. The components and conditions of the mastermix can be found in table 3-4.

Table 3-4: PCR Components and Conditions for 2 x 50 µl reaction mixtures

20 µl	5xQ5 reaction buffer	**Conditions:**	
2 µl	10mM dNTPS	98°C	2 min
5 µl	Primer 16.120	98°C	10 s
5 µl	Primer 16.121	55°C	30 s
-	Template: 10-20 ng/µl	72°C	10 s
1 µl	High Fidelity Q5 DNA Polymerase	72°C	2 min
-	ddH_2O	x34	

The obtained sequences were subsequently purified by separation with an agarose gel and gel purification. Then the sequence was digested, purified with the Gel and PCR purification kit and sticky-end ligated with the also digested pACEBac1 vector. Subsequently cloning was continued with the instructions in chapter 3.2.3.

The next step was to replicate the **burst sequences, BS,** in the polh promoter. A forward and a reversed primer were designed to have the same sequence as the burst sequence. The only difference was that one restriction site, right after the BS, was crippled when a BS was ligated to the wild-type. This resulted in only one restriction site on the 5' end of the original BS and no restriction sites in between the BS. To ensure no BS are cut out in further ligation steps, this needed to be done. These two primers were annealed to each other in a Thermo Cycler. The dimers were then diluted and observed on a 3% agarose gel. Subsequently the dimer were digested overnight with a blunt end restriction enzyme and purified by agarose gel electrophoresis and Gel and PCR purification kit. Meanwhile the vector, so the already modified, cloned and sequence verified polh promoter on the pACEBac1 plasmid, was digested

with the same blunt end enzyme, dephosphorylated to reduce self-ligation and purified with Gel and PCR clean up kit (see chapter 3.3.13). The insert and vector were then ligated followed by steps of electroporation, colony screening and sequence verification (see chapter 3.2.3). These steps, beginning with the blunt end digestion, were repeated until polh promoter constructs with one, two, three and four burst sequences were obtained. Subsequently cloning was continued with the instructions in chapter 3.2.3.

3.2.2 Generation of inserts

Vlf-1 is present on the bacmid DNA which is too big for normal digestion. As a result the sequence was amplified with PCR, with a primer designed to give it a His-Tag as well. The components and conditions can be found in table 3-5.

Table 3-5: PCR Components and Conditions for 2 x 50 µl reaction mixtures

20 µl	5 x Q5 reaction buffer	**Conditions:**	
2 µl	10mM dNTPS	98°C	2 min
5 µl	Primer 16.118	98°C	10 s
5 µl	Primer 16.124	59°C	30 s
-	Template: 200 ng	72°C	45 s
1 µl	High Fidelity Q5 DNA Polymerase	72°C	2 min
3 µl	DMSO		x34
-	ddH_2O		

The amplified sequences were then purified by agarose gel electrophoresis and gel purification. Subsequently the sequence was digested, again purified with the PCR and Gel purification kit and ligated by sticky end ligation with the vector plasmid, pIDK. Afterwards cloning was continued with the instructions in chapter 3.2.3.

The **YFP** was obtained similarly to the GP64 and the ie1 promoters. It was amplified on a plasmid in bacteria. Then the plasmid was purified and digested. Afterwards the vector plasmid, pACEBac1, modified with the polh promoter constructs or the other two promoters, was also digested. All sequences were then purified and ligated together. Subsequently cloning was continued with the instructions in chapter 3.2.3.

3.2.3 Electroporation, colony screening and sequence verification

After ligation, electroporation was done. The overnight incubated agar plates were then used for screening of the colonies, to find colonies with successful ligation. For this a PCR was done but instead of a certain amount of template DNA, the colony in question was used directly. A pipette tip was used to take up the targeted colony, stir it inside the reaction tube with the reaction mixture and inoculate a master plate. 8 colonies were screened normally. The primers were chosen to anneal on both sides

on the vector next to the restriction sites of the digest. In table 3-6 an example for the used PCR components and conditions can be found. This example was used for screening of the BS in the polh promoter on the pACEBac1 plasmid.

Table 3-6: PCR Components and Conditions for one 25 µl reaction mixture

5 µl	5 x One Taq reaction buffer	**Conditions:**	
0.5 µl	10mM dNTPS	94°C	2 min
0.5 µl	Primer 16.10	94°C	20 s
0.5 µl	Primer 16.9	46°C	20 s
-	One colony	68°C	30 s
0.2 µl	One Taq DNA Polymerase	68°C	5 min
18.3 µl	ddH_2O		x30

Positive colonies were then fractionated streaked out on agar plates. With one colony of these plates, an overnight culture was inoculated, which was then used for plasmid purification with a silica membrane. The purified plasmids were control digested with one or multiple restriction enzymes to ensure no mixed population of vectors with and without inserts was obtained. Positive plasmids were sent for sequencing to control for point mutations. In the example in table 3-6, sequencing was done with the primer 16.68.

After verification the plasmids were used for further ligation steps, for cryo stocks, Cre-loxP recombination or insertion into bacmids.

3.2.4 Generation of fused plasmids

The purified and sequence confirmed pIDK and pACEBac1 plasmids were fused by Cre-loxP recombination. After reaction incubation the fused plasmids were electroporated and incubated in SOC media overnight. The cell suspension was then spread on an agar plate, with gentamycin and kanamycin added for selection, and incubated overnight. The obtained colonies were fractionated streaked out and used for inoculation of an overnight culture. The plasmids in this culture were then purified with a silica membrane, control digested and run on an agarose gel to confirm the fusion. The obtained fused plasmids were then used for cryo stocks and for insertion into bacmids.

3.2.5 Generation of cloned bacmids

The plasmids were electroporated into bacmid containing cells and incubated overnight in SOC media. The Tn7 transpositions happened in this incubation time. After incubation the cell suspension was then spread on blue/white screening plates and incubated for two days. A masterplate with seven white colonies and one blue colony was made and again incubated. Then, of three chosen clones, a fractionated streak was made and incubated. These steps ensured bacmids without an insert were lost

in case of a mixed population. Subsequently one colony per clone was used for inoculation of an overnight culture and the bacmid was purified. Then a similar screening to the one mentioned in chapter 3.2.3 was done to ensure no mixed population was obtained and the sequence was inserted fully. PCR components and conditions for this screening can be found in table 3-7.

Table 3-7: PCR Components and Conditions for one 25 µl reaction mixture

5 µl	5 x One Taq reaction buffer GC rich	**Conditions:**	
0.5 µl	10mM dNTPS	94°C	4 min
0.5 µl	Primer 16.78 (M13)	94°C	20 s
0.5 µl	Primer 16.79 (M13)	53°C	20 s
-	100 ng bacmid	68°C	4.5 min
0.3 µl	One Taq DNA Polymerase	68°C	3 min
	ddH_2O		x30

The purified bacmids were then transfected into insect cells and the steps found in chapter 3.4 were followed.

3.2.6 Obtained constructs

With this cloning methods the following constructs were obtained.

pACEBac1 polh 1BS YFP + pIDK GP64 vlf1-His (denoted YG1)

pACEBac1 polh 1BS YFP + pIDK ie1 vlf1-His (denoted Yi1)

pACEBac1 polh 2B YFP + pIDK GP64 vlf1-His (denoted YG2)

pACEBac1 polh 2B YFP pIDK ie1 vlf1-His (denoted Yi2)

pACEBac1 polh 3B YFP + pIDK GP64 vlf1-His (denoted YG3)

pACEBac1 polh 3B YFP + pIDK ie1 vlf1-His (denoted Yi3)

pACEBac1 polh 4B YFP + pIDK GP64 vlf1-His (denoted YG4)

pACEBac1 polh 4B YFP + pIDK ie1 vlf1-His (Yi4)

pACEBac1 polh YFP (denoted Y1)

pACEBac1 polh 2B YFP (denoted Y2)

pACEBac1 polh 3B YFP (denoted Y3)

pACEBac1 polh 4B YFP (denoted Y4)

pACEBac1 GP64 YFP (denoted G)

pACEBac1 ie1 YFP (denoted i)

pACEBac1 GP64 YFP + pIDK GP64 vlf1-His (denoted GG)

pACEBac1 GP64 YFP + pIDK ie1 vlf1-His (denoted Gi)

pACEBac1 ie1 YFP + pIDK GP64 vlf1-His (denoted iG)

pACEBac1 YFP + pIDK ie1 vlf1-His (denoted ii)

3.3. MOLECULAR BIOLOGICAL AND BIOCHEMICAL METHODS

3.3.1 Plasmid purification:

Here the more traditional isopropanol precipitation was used to obtain DNA as well as extraction with a silica spin column and purification with an anion exchange chromatography was carried out.

Plasmid purification with a silica spin column

The NucleoSpin® Plasmid Quick Pure Kit from Macherey-Nagel, Germany, was used for plasmid purification. This kit depends on the principle of alkaline lysis. The protocol included in the kit was followed except for the elution step. Here 50 µl of sterile, 70°C preheated, ddH_2O was spread on the column and incubated for 5 min at room temperature instead of the provided buffer.

The concentration of obtained nucleic acid was determined as described in chapter 3.3.2.

Plasmid purification with anion exchange chromatography

If higher volumes of plasmid DNA than obtainable with plasmid purification with a silica spin column were desired, the Midi Prep Kit, NucleoBond® Xtra Midi from Macherey-Nagel, Germany, was used. This kit depends on DNA binding to an Anion-exchange chromatography column. For this method the protocol included in the kit was followed. The only exceptions were a repeat of the pellet washing step with 70% ethanol. Pellet dissolving was done with 400 µl ddH_2O (endotoxin-free) instead of the provided buffer.

The concentration of obtained nucleic acid was determined as described in chapter 3.3.2.

Nucleic acid purification with alcohol precipitation (bacmid purification)

For the bacmid purification a special protocol has to be used as they are too big (>135 kb) for purification in the commercial columns. The isopropanol used here is needed in smaller volumes and can precipitate lower concentrations than the normally used ethanol, but has to be exchanged later on with the more volatile solution. For isopropanol precipitation buffers from the Midi Prep Kit, NucleoBond® Xtra Midi from Macherey-Nagel, Germany, were used. Because the DNA is so large and shear forces might break it apart, mixing was kept to a minimum and genomic tips were used as frequently as possible.

A 4 ml overnight culture (up to 18 h) with the transfected DH10 E.coli cells was prepared. After incubation the 4 ml were centrifuged on top of each other in 2 ml miccrocentrifugation tubes at 14000 x g for 1 min. The supernatant was discarded and the pellet carefully resuspended in 0.5 ml Solution I (15 mM Tris-HCL pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). Then, to start cell lysis, 0.5 ml of Solution II (0.2 N NaOH, 1 % SDS) were added and the tube inverted a few times to gently mix. This reaction mixture was incubated for 5 min at room temperature. Afterwards 0.5 ml of Solution III (3 M potassium

acetat pH 5.5), were added to neutralize and stop the reaction. This was inverted 10 times to ensure proper mixing and incubated again for 5 - 10 min on ice. To obtain the bacmid DNA, the mixture was then centrifuged at 14000 x g for 10 min.

For every sample two tubes were prepared with 600 µl isopropanol in each. 700 µl supernatant of the centrifuged mixture were then transferred into each of the two tubes. Subsequently the tubes were placed on ice for 5 - 10 min to ensure complete isopropanol precipitation. Then the tubes were centrifuged at 11000 x g for 15 min and the supernatant removed. To wash the pellet, 0.5 ml 70 % ethanol were transferred on the pellets, the tubes were inverted a few times and centrifuged at 11000 x g for 6 min. The washing step was repeated another time.

Afterwards as much ethanol as possible was removed and the pellet was air dried at room temperature. Then the pellet was dissolved in 50 µl ddH2O (endotoxin free) by gentle pipetting or tapping. This bacmid solution was stored at 4 °C.

The concentration of obtained bacmid was determined as described in chapter 3.3.2.

3.3.2 Quantification of nucleic acid concentration

For determination of nucleic acid concentration, ultraviolet spectroscopy based on the Lambert Beer law were used. The concentration of the obtained nucleic acid solution was determined with the photometer Nanodrop 1000 Spectrometer from peQLab Biotechnologie GmbH, Germany.

With only 1.5 µl of sample, the whole spectrum between 220 nm and 340 nm was measured. The absorbance at 260 nm was used for calculation of the concentration, absorbances at other wavelengths were used to determine the purity of the sample.

3.3.3 Digestion with restriction enzymes.

Enzymes that produce overhangs, "sticky ends" and enzymes that produce a straight cut, blunt ends, were used. All the enzymes were bought from New England Biolabs Inc., USA, and the provided buffers were used.

Digestion for ligation

Two times 50 µl of reaction mix were used to ensure enough cleaved DNA will be available. 2-3 µg DNA were mixed with 5 µl of the specific enzyme buffer and approximately 20 Units of the enzyme were added. Then ddH_2O was added to obtain 50 µl. The mixture was incubated at the optimal enzyme activity temperature for 3 h to overnight, depending on the enzyme stability. Special treatments e.g. inactivation of the enzyme after digest are enzyme dependent and were loperformed seperately for each enzyme.

Digestion for control digest

Because here only a qualitative answer was needed and the product was not further used, only 1 µg DNA in 20 µl reaction mix was incubated with only 10 Units of enzyme. Incubation time was also shortened to 1 h.

3.3.4 Ligation

For successful sticky end ligation the two DNA pieces have to have complementary overhangs. To ensure directional insertions, the overhangs at each side should be different. Additionally, this ensures self-ligation is unlikely. In blunt end ligation this is not possible, making wrong insertions or self-ligation more common and therefore control digests and sequence confirmation are more important.

The T4 DNA Ligase from New England BioLabs Inc. (USA) with the provided buffer was used.

For one reaction mixture maximal 10 ng/µl DNA was used. To minimize the probability of self-ligation and to ensure insertion, 5 times more moles of the insert than moles of the vector were added to the mixture.

$$\text{required mass of insert (g)} = \frac{\text{insert}}{\text{vector}}\ \text{molar ratio x mass of vector (g)x}\ \frac{\text{insert}}{\text{vector}}\ \text{length ratio}$$

$$\text{moles dsDNA (mol)} = \frac{\text{mass of dsDNA (g)}}{\text{length of dsDNA (bp) x 617.96 g/mol} + 36.04\ \text{g/mol}}$$

2 µl ligase, 2 µl of the T4 DNA ligase reaction buffer and the calculated amounts of vector and insert were mixed with ddH_2O for 20 µl of reaction mix. In this work the bacteriophage T4 DNA Ligase was used because of its high efficiency. Because of the combination of the enzymes optimal working temperature (37°C) and the melting temperature of the termini (~0°C) the chosen incubation temperature was 4°C for overnight ligation and 25°C for 30 min ligation. For blunt end ligation low temperatures were always used because of the bad productivity.

After incubation the mixture was heated to 65°C for 5 min to inactivate the ligase.

3.3.5 Dephosphorylation of the vector

This was done to increase the outcome of the blunt-end-ligation.

The here used phosphatase was calf intestinal (CIP) alkaline phosphatase by New England BioLabs Inc. (USA). 50 µl of the vector were mixed with 6 µl CutSmart Buffer by New England BioLabs Inc. (USA), 1 µl CIP alkine phosphatase and 3 µl ddH_2O. This reaction mixture was incubated for 30 min at 37°C. Afterwards, because heat inactivation is not possible for this enzyme, the nucleic acid was purified with the NucleoSpin® Gel PCR Clean up Kit by Macherey Nagel (Germany).

3.3.6 Preparation of electrocompetent cells

Cells used for electroporation have to be made competent first. Excessive washing steps are employed to clean away culture media traces and therefore to avoid arcing.

Table 3-8: 10 x $KHPO_4$ Buffer

0.17 M	KH_2PO_4
0.72 M	K_2HPO_4

Table 3-9: Terrific Broth-Medium (TB)

12 g/l	Tryptone
24 g/l	yeast extract
0.4 % (v/v)	Glycerol
10 % (v/v)	10 x $KHPO_4$ buffer

Here, DH10MultiBacY and DH10MultiBac (-Y) cells (see chapter 3.1) were made competent.

2 x 250 ml TB-Medium (see table 3-9) with kanamycin and tetracyclin were each inoculated with 2 ml of an overnight culture. This culture was incubated at 37 °C until an OD600 of 0.4 - 0.6 meaning the cells were in their exponential growth phase. This optimal OD600 is a compromise between the fact that the smaller the cell density the better the competence but also the smaller the yield. As soon as the cell density was in the optimum region the flask was cooled on 4 °C for 15 - 20 min, to ensure a stop in cell growth. Then the cell suspension was filled into cooled centrifugation beakers and centrifuged at 3000 x g at 4 °C for 10 min. Afterwards the supernatant was removed, the pellet dissolved in 150 mL, chilled, 1mM HEPES Buffer from Sigma Aldrich (USA) and centrifuged again at 3000 x g at 4 °C for 10 min. This washing step was repeated another two times. The pellet was then dissolved in 1 mM HEPES and transferred into a 50 ml tube. Then the washing steps with 1 mM HEPES were repeated as above another time with a smaller volume of 1mM HEPES. In the end the supernatant was removed and the pellet dissolved in 2 ml of a 30 % glycerine solution. The cells were then aliquoted into 50µl aliquotes and shock frozen in liquid N_2. They were stored at -80°C.

3.3.7 Electroporation

To avoid arcing from high salt buffers in the mixture from past steps, the nucleic acid was diluted 1:100 before electroporation.

The used cuvetts (Electroporation cuvettes 2mm gap, Fisherbrand ®, Austria) were put on ice before electroporation. Additionally, the SOC media was prewarmed to 37 °C for optimal recovery of the bacteria. Then 50 µl of electrocompetent cells made in chapter 3.3.6 were transferred into the cuvette and 5 µl of the 1:100 diluted nucleic acid were added without generating air bubbles. The used conditions for electroporation of *E.coli* were: 2.5 kV, 200 Ω, 25 µF. After the pulse 500 to 900 µl of the

prewarmed SOC media were added as fast as possible. The cells were incubated at 37°C for 1 to 16 h depending on the selection antibiotics used. After incubation the cells were spread on LB-agar plates with the necessary antibiotics and incubated at 37°C overnight.

The electroporation device used here, ECM630 Electro Cell Manipulator® with Safety stand 630B from BTX Harvard Apparatus (USA)

3.3.8 Chemical transformation/ heat shock transformation

For this protocol NEB ® 5-alpha competent *E.coli* for chemical transformation (C2987H) from New England BioLabs Inc. (USA) were used. The vial of *E.coli* was thawned for 10min on ice. Afterwards the cells were pipetted into a transformation tube on ice. Then 1-5 µl of 1 pg to 100 ng plasmid DNA solution was added to the cell vial. Subsequently the tube was inverted 4 times to mix without disrupting the cells and placed on ice for 30 min. Next, the tube was heated in a waterbath at 42°C for exactly 30 seconds. This step enhances the membrane permeability. The cells were then rapidly cooled again by being placed on ice for 5 min. To help the cells recover, 950 µl SOC medium were pipetted into the tube and put on a shaker with 37°C and 250 rpm for at least 1 h. Selection LB-agar plates were pre-warmed to 37°C and the cells were spread in several dilutions. The plates were incubated at 37°C overnight or at 30°C for 24-36 h.

3.3.9 Polymerase chain reaction (PCR)

The polymerase chain reaction was used to amplify DNA sequences which were needed for further cloning steps and to screen colonies.

Primer design

For a polymerase chain reaction small oligonucleotides are needed. They are complementary to the two 3' ends of the wanted sequence and therefore determine the start and the end of the PCR product sequence. The guideline "qPCR Assay Design and Optimization" from Bio-Rad (USA) for primer design was used, but in general secondary structures were avoided and both primers had similar annealing temperature.

Estimation annealing temperature (T_m)

The temperature for the annealing of primers is specific for the pair used and has to be estimated. In general the temperature can be calculated by hand by looking at the amount of A/T and G/C bases. Unfortunately sometimes this temperature does not work. In this case a gradient PCR was done by using different temperatures on different tubes and looking at the amount of produced sequences therefore getting a gradient.

PCR Conditions

Table 3-10: Master Mix for Polymerase Chain Reaction

-	5 x One Taq Buffer / Q5 Buffer
10 mM	dNTPs
10 µM	forward primer
10 µM	reverse primer
0.03-0.04 units/ µl **0.02 units / µl**	PCR Polymerase (One Taq) PCR Polymerase Q5
-	ddH_2O

Enzymes and provided buffer were used from New England BioLabs Inc. (USA).

Polymerases used in this work:

- One Taq was used for colony screening. It is inexpensive, slow (1000 bp/min) and has no proofreading activity.
- Q5 was used for sequence amplification. It is expensive, faster (1000bp /20-30 s) and has proofreading activity.

The PCR mastermix (see table 3-10) was mixed and aliquoted into PCR-Tubes. Then 10 ng of the DNA with the desired sequence or a colony directly from an LB-agar plate was added to the mix. The tubes were then put into the Thermo Cycler and run, for the conditions see table 3-11. Used Thermocycler Devices here were PIKO 24, Thermo Scientific (USA) and C1000™ Thermal Cycler, Bio-Rad (USA)

Table 3-11: Thermocycler conditions

Step	**One Taq**		**Q5**	
1	94 °C	2 - 5 min	98 °C	2 min
2	94 °C	20 s	98 °C	10 s
3	T_m	20 s	T_m	30 s
4	68 °C	60 s / 1000 bp	72 °C	20 - 30 s / 1000 bp
5	68 °C	5 min	72 °C	2 min

After the run the samples were either stored at 4°C, -20°C or used immediately to e.g. run on an agarose gel.

3.3.10 Annealing of primers/ generation of short, individual DNA strands

For the burst sequence generation, two primer were designed and annealed to each other.

50 µM of the primers were mixed with 10 µl of 5 mM Tris/HCL Buffer with pH 8.5, in an overall volume of 20 µl. This was put into one of the Thermo Cycler mentioned in chapter 3.3.9 for 5 min at 95°C and

left to cool for 1 h. To control if the procedure worked a gel was run with the supposedly double stranded DNA and the single stranded primers. The double stranded DNA should appeared bigger on the gel because more ethidium bromide can bind on a double strand than on a single strand.

3.3.11 Gel electrophoresis

Gel electrophoresis is used to separate differently sized, charged macromolecules in an electric field through movement through a porous gel. The resulting band pattern can be observed for information. Additionally, single bands can be cut out with razor blades to purify certain DNA fragments. Two different gel electrophoresis types were used here.

Agarose gels

These gels are used for separation of nucleic acids. To make DNA visible ethidium bromide was used here.

Table 3-12: 50 x TAE Buffer

242 g/L	Tris Base
5.71 % (v/v)	Glacial acetic acid
18.61 g/L	EDTA
	ddH_2O

In this case 1-2.5% agarose gels were used as no big molecules needed to be separated.

For casting of a gel, agarose, 50 x TAE Buffer (see table 3-12) and ddH_2O are mixed and heated until the agarose is melted. This mixture is poured into a casting mould and cooled off at room temperature.

To run a gel the sample was mixed with 6 x loading buffer from New England BioLabs Inc. (USA) to make it heavier and dye it for estimation of the end of the run. The gel was put into a running chamber and covered with 1 x TAE buffer (diluted 50 x TAE supplemented with 0.03 % (v/v) EtBr). Then the samples and the DNA ladder, for example 2-Log DNA ladder by New England BioLabs Inc. (USA) were pipetted into the pockets. For a 1% agarose gel the conditions for the run were 110 to 130 V, 400 mA and 20 to 60 min. The bands were then observed in the Molecular Imager® Gel Doc™ XR+ Imagine System from Bio-Rad (USA). If the run was done for purification, the target band was then cut out with a razor blade and the DNA extracted as explained in chapter 3.3.13.

Polyacrylamid gels

The here used sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE, is used to separate proteins soley on their size, independent of secondary structures. The mobility of the molecules depends therefore on their length.

In this work all of the SDS-PAGEs where followed up by western blotting to make the band pattern visible. (see chapter 3.3.12) The here used methods for polyacrylamide gels were the Laemmli method and the Bis-Tris buffer system.

Table 3-13: Composition of a SDS-PAGE gel with 1 mm thickness for the laemmli methode

	Separation Gel (T:C=12.6:1)
1406 µl	Separation Buffer(1.5M Tris/HCL, pH 8.8)
1752 µl	40 % acrylamide
369 µl	2 % bisacrylamid
56.25 µl	10 % SDS
33.27 µl	10 % APS
3.327 µl	TEMED
2026 µl	ddH_2O
	Stacking Gel (T:C=5.7:2.2)
312.5 µl	Stacking Buffer(0.5M Tris/HCL, pH 6.8)
178 µl	40 % acrylamide
81.25 µl	2 % bisacrylamid
12.5 µl	10 % SDS
10 µl	10 % APS
1 µl	TEMED
675 µl	ddH_2O

Table 3-14: Composition the 20 x MES SDS Running Buffer

50 mM	MES
50 mM	Tris Base
0.1 % (w/v)	SDS
1 mM	EDTA

Table 3-15: Composition the 10 x Laemmli Running Buffer

144 g/L	Glycine
30 g/L	Tris Base
10 g/L	SDS

Casting gels

For the Bis-Tris buffer system, NuPAGE™ 4-12% Bis-Tris Protein Gels by Thermo Fisher Scientific (USA) were bought.

For the Laemmli method, gels were cast by hand. The components are listed in table 3-13. APS and TEMED are the polymerisation ingredients were added immediately before gel casting. First the ingredients for the separation gel were mixed and pipetted into the casting chamber (SE245 Dual Gel Caster from Hoefer, USA). To have a smooth finish a thin layer of isopropanol was pipetted on top while the gel was still in a liquid stage. After 30-45 min the isopropanol was taken off with filter paper

and the stacking gel was added. The comb was added immediately. This was incubated for another 30-45 min. For storage the chamber was taken out of the frame, wrapped in moist clothes and stored at 4°C.

Sample preparation

For 60 µl of running sample 15 µl NuPAGE™ SDS Buffer (4x), 6 µl NuPAGE™ Sample Reducing Agent (10x), both by Thermo Fisher Scientific (USA), and 39 µl of the protein sample were mixed. Cell pellets were first solved in 200 to 250 µl PBS (see chapter 3.3.12, table 3-16) before being used. This mixture was then boiled at 95 °C for 5 min to ensure complete denaturation of the proteins.

Running the gel

The electrophoresis chamber was prepared by filling it with Running Buffer. 1 x Laemmli running buffer for the Laemmli method (see table 3-14), 1 x MES running buffer for the Bis-Tris system (see table 3-15). Then 0.5 ml NuPAGE™ Antioxidant by Thermo Fisher Scientific (USA) was added to prevent the proteins from reoxidation while moving through the gel. Subsequently the samples, controls and protein ladder were loaded in the pockets. For the laemmli method the SE260 Mini vertical gel electrophoresis unit from Hoefer (USA) was used. For the Bis-Tris system the XCell Secure Lock™ Novex® Mini cell Electrophoresis system from Invitrogen (USA) was used.

Conditions for running the gels were the following:

For the Laemmli method: 125V, 90 mA, 1.5 h

For the Bis-Tri System: 200 V, 120 mA, 40 min

3.3.12 Semi dry western blot

Western blotting is used to identify proteins. The proteins in the SDS-PAGE gel were transferred onto a membrane with the help of an electric field. Subsequently they were detected here with two antibodies. First an antibody which was specific to the target protein was incubated with the membrane. After a washing step the second antibody which was specific to the first antibody and conjugated to alkaline phosphatase was added. By adding the alkaline phosphatase substrates a colourizing reaction occurred and the target protein bands were made visible.

Table 3-16: Composition of the Phosphate buffered saline + Tween 20 (PBS-T)

8 g/L	**NaCl**
0.2 g/L	KCl
14.4 g/L	$Na_2HPO_4{*}7H_2O$
20.48 g/L	KH_2PO_4
0.1% (v/v)	Tween-20

Table 3-17: Composition of the TOW-BIN Buffer

30.3 g/L	**Tris Base**
145 g/L	Glycine
20 % (v/v)	Methanol

Table 3-18: Composition of the alkaline phosphatase buffer (APS) pH 9.5

5 mM	**$MgCl_2*6H_2O$**
100 mM	NaCl
100 mM	Tris
0.1% (v/v)	Tween 20

For each gel 6 filter paper pieces were used as well as one polyvinylidene difluoride membrane (Amersham™ Hybond™ Low Fluorescence 0.2 µm PVDF Blotting Membrane) in the size of the gel. The membrane was activated with pure methanol for 10 s and then immersed in TOW-BIN Buffer for composition see table 3-17) with methanol. The filter papers were also soaked in TOW-BIN Buffer with methanol. The percentage of methanol used in this buffer depended on target protein size, here 20% methanol was used in general. The SDS-PAGE gel after the run was sandwiched into the semi dry western blotting device in the following order: lower part of device - 3x filterpaper - activated membrane - gel - 3x filterpaper - upper part of device. After making sure there are no bubbles in the sandwich and the stack was wetted with buffer, the run was started. The conditions for the run depend on the amount of membranes in the device. E.g. two membranes: 50 min, 170 mA, 60W

At the end of the run, most of the ladder should have been transferred onto the membrane. To lessen background signals, the membrane was blocked overnight with a 3% bovine serum albumin, BSA, in PBS-T (see table 3-16) or TBS-T (Tris buffered saline from PAA Laboratories, USA, supplemented with 0.1% (v/v) Tween 20) solution at 4°C.

The next day the membrane was washed with PBS-T or TBS-T. Then 12 ml of a dilution of the first antibody (see 3.1.3) mixed with 0.5 % BSA, was added. The BSA should prevent unspecific interactions.

The membrane was then incubated on the shaker for 1 h. Afterwards it was washed three times with PBS-T or TBS-T for 5 min each to make sure no antibodies are remaining. The second antibody was then added, incubated and washed off the same way as the first. For the substrate reaction the membrane was pre-equilibrated with APS-Buffer (for composition see table 3-18) and the dye mixture was prepared. It consisted of 5 ml APS-Buffer, 33 µl NBT and 16.5 µl BCIP, both from Promega (USA) per membrane. This mixture was poured on the blot and incubated at room temperature until the bands were visible. The reaction was stopped by washing the blot with water. Subsequently the blot was dried and scanned.

3.3.13 Purification of DNA after PCR or agarose-gel-electrophoresis

After PCR or cutting out a band after agarose-gel-electrophoresis the obtained DNA needed to be purified to clean it of unwanted substances e.g. enzymes and/or the agarose. For this the Nucleo Spin®Gel and PCR Clean-up kit from Macherey-Nagel, Germany, was used. This technique depends on a silica-membrane similar to the plasmid preparation kit (see chapter 3.3.1). The provided protocol was followed except that elution was done with 50 µl ddH_2O instead of the provided buffer.

The concentration of obtained nucleic acid was determined as described in chapter 3.3.2.

3.3.14 Sequencing

For verification of cloned constructs and sequence control small DNA segments of up to 1000 bp were sequenced by Microsynth AG (Switzerland). Both PCR proucts and plasmids were sent for sequencing.

Samples were supplemented with 3 µl of a primer as sequencing starting point, before being sent. Approximately 100 ng/µl in 12µl plasmid were sent. For sequencing PCR products 18 ng per 100 bp in 12 µl were sent. The used primer sequences can be found in the appendix.

3.3.15 Cryo stocks

To avoid cell rapture from ice crystals and osmotic shock, the cryoprotectant glycerol was used for storage of bacterial cell.

700 µl of a 30 % glycerol solution was mixed with 700 µl *E.coli* in exponential growth phase in appropriate tubes. They were stored immediately at -80°C after mixture.

3.3.16 Cre-loxP recombination:

Cre-loxP recombination makes use of the Cre recombinase to fuse two plasmids with both one loxP site. The commercially bought pIDK and pACEBac1 plasmids used in this work are designed to have inverted Cre-LoxP recombination sites (see chapter 1.2.2)

The Cre Recombinase from New England BioLabs Inc. (USA) and the provided buffer were used.

For the reaction 1-2 µg of each of the selected plasmids, in equimolar amounts, were combined. This was mixed with 2 µl Cre Buffer (10x), 1 µl Cre Recombinase and filled up with ddH_2O to a final volume of 20 µl. Then the mixture was incubated for 1 h at 37°C.

3.3.17 Purification viral nucleic acid

The principle of this purification is the binding of nucleic acid to a glass fiber fleece with the help of a chaotropic salt similar to the silica membrane method in 3.3.1. The purified viral DNA was used for PCR to verify the amount of burst sequences. For this PCR the Primers 16.120 and 16.96 were used to obtain the polh sequence with the added burst sequences.

This was done with the High Pure Viral Nucleic Acid Kit from Roche (Switzerland). The provided protocoll was followed except for the elution step. Here 50 µl ddH_2O was used instead of elution buffer.

3.4 INSECT CELLS/CELL CULTURE METHODS

The used insect cells were always cultivated in HyClone Media as described in chapter 3.1.4. The cell lines used for the different methods are Sf9, Hi5 and Tn42 as described in chapter 3.1.1.

3.4.1 Cell counting

To estimate cell concentration and viability cell counting is necessary. For this 10 µl of cell suspension and 10µl of trypanblue were mixed. Then the cells were counted on in the Luna II™ Automated Cell Counter from Logos Biosystems (USA) or the TC20™ Automated Cell Counter from Bio-Rad (USA).

3.4.2 Passaging

For optimal cell growth, the cells were kept in their exponential growth phase. For this passaging was done every 3 to 4 days. For suspension growth the old cells were seeded with a concentration of 0.6 x 10^6 to 0.8 x 10^6 cells/ml. The cells should not exceed a concentration of 3.5x10^6 cells/ml. Hi5 and the Tn42 cells are prone to clumping so heparin was added to prevent that.

3.4.3 Transfection and production of virus particles in Sf9

Transfection of the bacmids

For transfection the FuGENE®HD Transfection Reagent by Promega (USA) was used. The here used reagent consists of a highly branched organic compound to induce bacmid entry into the cells.

For transfection the chosen cell concentration was 1 x 10^5 cells / cm^2 surface. Here 6 well plates with 9.6 cm^2 per well were used. The cells were seeded into the wells in at least 1 ml suspension and incubated for 30 min at 27°C.

The bacmid was mixed with the used medium to obtain a concentration of 0.05 µg/ µl. At the same time 8 µl of, well mixed and hand warm, transfection reagent were mixed with 92 µl of medium. 100 µl of the bacmid dilution and 100 µl of the diluted transfection reagent were then mixed and incubated at room temperature for 15 min.

After the cells in the wells were attached to the well surface the old medium was removed and 1 ml new medium was distributed atop of the cells. Then the 200 µl bacmid-transfection reagent mixture was distributed in the well. The cells were incubated overnight at 27°C. Subsequently 1 ml new medium was added to the well and the cells were incubated again for 3-4 days at 27°C.

In the fluorescence microscope (Leica, Germany) the amount of infected cells was observed. When enough cells were infected the supernatant was harvested and centrifuged at 800 x g for 5 min. The supernatant of this should contain the infectious virus particles and was called virus stock, v0. If the cell pellet was also desired for further experiments, the pellet was solved again in 250 µl DPBS.

Intermediate stock

To generate more virus particles the virus stock, v0, was used to infect more cells. In a T80 flask 1 x 10^5 cells/cm^2 were seeded and medium was added to an overall volume of 7 ml. Then the cells were incubated until they were growing adherently, approximately 30 min, at 27°C. If cell concentration was relatively small and not much new medium was added the old medium was exchanged with new. Then 150 µl of virus stock, v0, was added and the cells were incubated overnight at 27°C. Then 7 ml medium were added on top and the incubation continued for 3 days at 27°C.

Harvest was conducted as described in the chapter Transfection of the bacmids. This virus stock was called intermediate stock, v1.

Working stock

To get an amount of virus particle solution working is possible with, a working stock, ws, needed to be generated. This was done similarly to the intermediate stock with the only difference of bigger volumes. T175 flasks were used and 2 times 12 ml of medium. For infection only 150 µl of the intermediate stock, v1, was used.

3.4.4 50% tissue culture infective dose ($TCID_{50}$)

For estimation of infectious virus particle concentration in the working stocks the $TCID_{50}$ method was used, because all the constructs produce YFP. IT is possible to calculate the plaque forming unites per ml from the $TCID_{50}$ value. This information was determined to be able to compare the constructs with each other by infecting cells with the same multiplicity of infection, MOI.

For easy handling 96 well plates were used. Per virus sample, one 96 well plate was used. To ensure statistical accuracy each virus was titrated on at least two different plates. 4 x 10^4 cells in 100 µl medium were pipetted in every well. Cells appeared 25 - 40% confluent after attaching themselves. To ensure the cells were adherent, the $TCID_{50}$ plates were incubated at 27°C for at 1 h. Meanwhile the virus dilutions were prepared. The dilutions were chosen depending on the estimated virus concentration. In the last row no virus dilution was added to obtain a negative control. For higher accuracy the dilutions were made from the rows A to G, for more dilution steps the dilutions were made from the columns 1 to 11. Another 96 well plate was used as a dilution plate.

Example for preparation of the virus dilutions: After prediluting the virus stock, the stock was diluted on the whole 96well in 1:5 steps from rows A to G. For this in one column of the dilution plate 240 µl of medium was pipetted into each well. Then 60 µl of the virus stock were added to the first well, so the well 1A. The mixture was pipetted up and down multiple times and 60 µl of the dilution in 1A was pipetted into the well 1B. This was mixed again and pipetted into the next well until well 1G. As always

one row in the $TCID_{50}$ plate received the same virus dilution the result was 7 dilution steps with each 12 replicates.

After incubation and control of the cells in the microscope, 15 µl of virus dilution were pipetted in each well of the $TCID_{50}$ plate. The plates were then wrapped in a plastic bag with moist towels to ensure they do not dry out. Incubation time was 7 days at 27°C.

After 7 days the plates were evaluated with the fluorescence microscope (Leica, Germany). For each well information on it being infected or not was collected. Table 3-19 and the formulas below show the calculation of the pfu/ml. The dilution factor (h) was 5 in this case (1:5 dilutions on the dilution plate). The volume of virus inoculation was 15 µl so 0.015 ml.

Table 3-19: Calculation of pfu/ml with the $TCID_{50}$ method.

	Dilution	Infected wells	Uninfected wells	Cumulative Infected	Cumulative Uninfected	% of wells Infected
1	**$1*10^{-4}$**	12	0	47	0	100
2	**$2*10^{-5}$**	12	0	35	0	100
3	**$4*10^{-6}$**	12	0	23	0	100
4	**$8*10^{-7}$**	7	5	11	5	68.8
5	**$1.6*10^{-7}$**	1	11	4	16	20.0
6	**$3.2*10^{-8}$**	3	9	3	25	10.7
7	**$6*10^{-9}$**	0	12	0	37	0.0

Proportionate distance (PD)

$$= \frac{\%\text{ of wells infected at dilution rate above } 50\% - 50\%}{\%\text{ of wells infected at dilution above } 50\% - \%\text{ of wells infected at dilution below } 50\%}$$

$$= \frac{68.8 - 50}{68.8 - 20} = 3.85 \text{ x } 10^{-1}$$

$$\mathbf{Log_{10}(TCID_{50})} = \log \text{total dilution above } 50\% - (\text{PD x } \log \text{h})$$

$$= \log 8 * 10^{-7} - (3.85 \text{ x } 10^{-1} * \log 5) = -6.37$$

$$\mathbf{TCID_{50}} = 10^{\text{Log}_{10}(\text{TCID}_{50})} = 10^{-6.37} = 4.31 \text{ x } 10^{-7}$$

$$\frac{\mathbf{1}}{\mathbf{TCID_{50}}} = \frac{1}{4.31 \text{ x } 10^{-7}} = 2.32 \text{ x } 10^{6}$$

$$\mathbf{TCID_{50/ml}} = \frac{\frac{1}{\text{TCID}_{50}}}{\text{inoculation volume}} = \frac{2.32 \text{ x } 10^{6}}{0.015} = 1.55 \text{ x } 10^{8}$$

$$\underline{\mathbf{Pfu/ml} = 0.69 \text{ x TCID}_{50}\text{/ml} = 0.69 \text{ x } 1.55\text{x}10^{8} = 1.07 \text{ x } 10^{8} \text{ pfu/ml}}$$

3.4.5 BioLector® experiments

The BioLector® from m2p-labs (Germany) is a microbioreactor for fermentation of multiple samples at once. It keeps the incubation conditions stable and at adjusted time intervals measures biomass, pH, dissolved oxygene and fluorescence, in this case every 15 min. The MTP-R48-BOH plates were used here. Here the different produced virus constructs in the titrated working stocks were measured and compared with each other. Additionally, constructs in all three cells lines were also compared.

In general the total volume per well was 1.5 ml, consisting of cell suspension and the virus working stock. In each well 1.5 x 10^6 cells were pipetted and a MOI of 10. This means that for every cell there were 10 infectious particles in the mixture. The MOI was calculated based on the $TCID_{50}$ titrations. Also 1 ml Turbidity Verification Standard, 10 NTU from Hach (Austria) was pipetted on every plate as well as a negative control to confirm sterility. The growth conditions were 27°C, 0% CO_2, 20.95% O_2, 85% humidity and 700 rpm.

3.4.6 Flask fermentation experiment

To control the results of the BioLector® experiments two selected viruses were fermented in flask with all three cell lines. Samples were taken at the post infection hours 0, 16, 20, 24, 48, 72 and 93. The virus consturcts Y2 and YG2 were used (see chater 3.2.6). The negative control in this experiment was a construct without YFP production, sweetBac GP120 native.

50 ml with 1 x 10^6 cells/ml in exponential growth phase were infected with a MOI of 5. The flasks were then incubated on the shaker at 27°C for 93 hours. At every sampling time 2 x 10^6 cells were taken, counted, their viability measured, the pH controlled and the cell size measured. Then the cell suspensions were centrifuged at 3000 rpm for 5 min. 2 x 100 µl of supernatant were collected for fluorescence measurement. The pellet was washed with 1 ml DPBS, divided into two microcentrifugation tubes and centrifuged again at 3000 rpm for 5 min. 100 µl of the wash supernatant were collected for fluorescence measurement, the leftover was discarded. One of the two pellets was solved in 1 ml DPBS and the percentage of infected cells was measured by FACS measurement with the Gallios™ Flow Cytometer from Backman Coulter (USA). The other pellet was either frozen for later experiments or the cells were lysed with the method described in chapter 3.4.7. 2 x 100 µl of the obtained lysate were collected again for fluorescence measurement, the rest was prepared for SDS-PAGE and western blotting as described in chapter 3.3.11. The fluorescence measurement were then done in the Tecan Infinite® M1000 (Switzerland) with appropriate plates.

3.4.7 Insect cell lysis for protein extraction

Pellets were lysed with I-PER® Reagent (Insect Cell Protein Extraction Reagent) from Thermo Fischer Scientific (USA), which is specifically used for baculovirus infected cells grown in suspension or monolayer.

First an aliquote of the I-PER® reagent was mixed with protease inhibitor. The pellet with 1 x 10^6 cells (see chapter 3.4.6) was resuspended in 0.25 ml I-PER® reagent and vortexed for 5 s. Then it was incubated for 10 min on ice and centrifuged at 15000 x g for 15 min at 4°C. The obtained supernatant contained the expected proteins. These were either used for measurements, western blotting or storage at 4°C.

4. RESULTS AND DISCUSSION

4.1 CONFIRMATION OF BURST SEQUENCES

To obtain the desired constructs, the burst sequence (BS), of the polyhedrin promoter was amplified and inserted, resulting in constructs with one, two, three or four BS in the polyherdin promoter. (For promoter sequences see chapter 6 appendix)

In figure 4-1 agarose gels of PCR products a) in the first step of burst sequence addition b) in the second step of burst sequence addition can be seen. This means in a) one burst sequence, additionally to the original, should have been ligated into the construct, resulting in two burst sequences and, with the chosen primers, in two bands. In b) one burst sequence, additionally to the original and the one added in the step before, should have been ligated into the construct, resulting in three burst sequences and three bands.

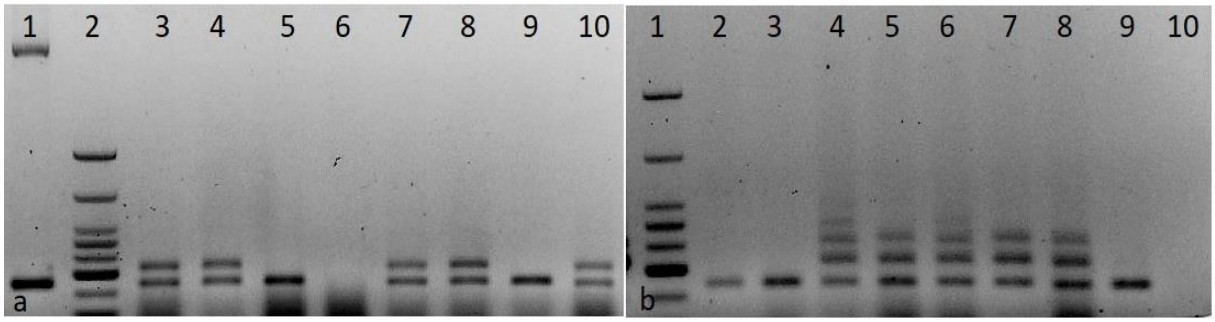


Figure 4-1: Agarose Gels of the burst sequence ligation steps. The Low Molecular Weight Ladder was used. The primers chosen bind inside of the burst sequence resulting in multiple bands with 40 bp size difference if multiple burst sequences are present. a) This is an agarose gel after the first step of ligating. Two burst sequences should be now in the promoter. b) This is an agarose gel after the second step of ligating. Three burst sequences should be now in the promoter. The dark smears below the bands are so called primer smears.

In figure 4-1a lane 1 the positive control with one band at around 220 bp, which represents the PCR product with one burst sequence, can be seen. The second, much higher band is unamplified plasmid as the used concentration was rather high. Lane 2 shows the Low Molecular Weight Ladder. Lanes 3,4,7,8 and 10 show constructs with two burst sequences indicating that the ligation worked. In contrast, lanes 5 and 9 show good examples of self-ligation, where after digest no new burst sequence was ligated into the construct.

In figure 4-1b lane 1 shows the Low Molecular Weight Ladder. Lanes 5,6,7 and 8 show three bands with 40 bp difference between each of them, indicating that here the ligation worked as expected and three burst sequences can be found in the construct. In lane 4 even four bands can be seen, meaning two burst sequences were inserted in this ligation step.

Lane 6 in 4-1a and lane 10 in 4-1b show no burst sequence at all. Probably the additional burst sequence was inserted inverted. This would result in formation of a loop which has a much higher

melting temperature than the temperatures reached in the PCR reactions. This results in the polymerase not having access to that region so no amplified product is obtained.

As the sequence, used for the PCR shown on the gel in 4-1, already consisted of two burst sequences, lanes 2, 3 and 9 show that one burst sequence was lost. The primers for this PCRs were designed to cripple restriction sites that would be generated between inserted burst sequences, only leaving one at the 5' end of the wild type. So no burst sequence can be lost in the digestion process. But it is known that tandem repeats in *E.coli* might be deleted or rearranged (Bzymek and Lovett 2001). This probably also happened here, leaving only the original burst sequence in the construct. With the suspicion of construct instability, the amount of burst sequences was confirmed after multiple spread outs and after every major cloning step. No other loss of burst sequences was observed.

For final confirmation, viral DNA from the produced working stocks was purified, the sequence with the burst sequences amplified and sent for sequencing. Figure 4-2 shows the amplified sequence from the virus DNA. Because different primers than in figure 4-1 were chosen, the obtained bands have

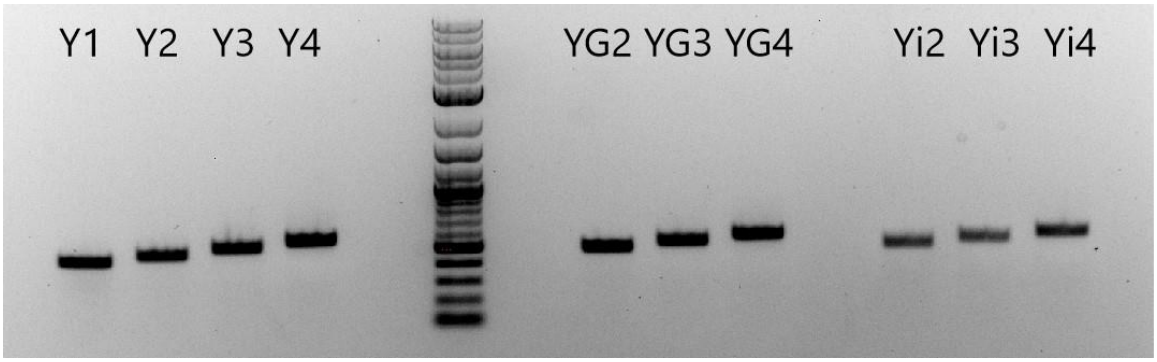


Figure 4-2: Agarose Gel of PCR product with the polh promoters and different amounts of burst sequences. The DNA ladder is a 2-Log ladder.

approximately the size of 500 bp. The 40 bp difference between the amount burst sequences can be seen well in figure 4-2. Sequencing then also confirmed this observation. No burst sequences were recombined.

4.2 TEMPERATURE-GRADIENT PCR FOR SREENING OF BACMIDS

The M13 Primers were frequently used for screening of the bacmids to confirm successful sequence integration. When calculating the annealing temperature based on the sequence, 42°C were recommended. But when applied, multiple bands instead of one, for this construct expected, at around 7500 bp, could be observed. With a temperature gradient PCR with the Cre-fusioned-plasmid and the bacmid made from it, optimal annealing temperature was estimated. In figure 4-3 the resulting band

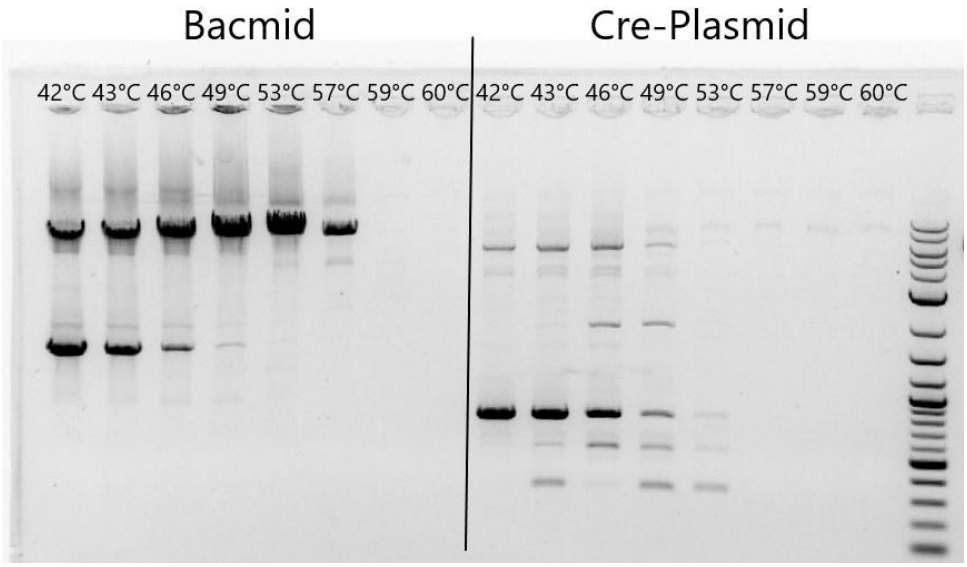


Figure 4-3: Agarose Gel of Temperature Gradient PCR product with the two M13 Primer. On the left side with a bacmid, on the right side with the cre-fusioned-plasmid from which the bacmid was made. The 2-Log ladder was used here. Construct: pACEBac1 GP120 + pIDK ie1 vlf-1

pattern can be seen. The 2-log DNA ladder was used here. The M13 primer should not bind to the Cre-Plasmid in general, as there is only low sequence similarity, but it does at low annealing temperatures. In combination with sequencing, the band pattern was solved. Apparently, even with multiple mismatches, the primers are able to anneal in the Cre-Plasmid at low temperature on multiple places. Additionally, the reverse primer anneals strongly - with a few bases - in the Cre-Plasmid, resulting in the relatively thick band at around 2000bp if the forward primer anneals to its estimated place in the bacmid. This phenomena only occurs in pACEBac1 plasmids fusioned with a pIDK plasmid. For later screening a annealing temperature of 53°C was chosen as the targeted band is strong at this temperature but no background bands can be observed anymore.

4.3 COMPARISON OF YFP EXPRESSING CONSTRUCTS

The BioLector® was used for screening of the virus construct yielding to the highest YFP signal in Sf9 cells. The following figures show the yield, calculated with the relative fluorescence unit and the biomass measured. All the virus constructs were measured in multiple experiments and similar trends were observed. For simplification only one experiment is shown in this work.

In all experiments the fluorescence curves decreased after a peak, possibly because the, for the cell stressful and useless, YFP is degraded again.

The used polh promoter in all the construct is the modified one without the leftover polh protein sequence as seen in chapter 6 appendix. The exact construct description can be found in chapter 3.2.6.

4.3.1 Promoter strength and kinetics

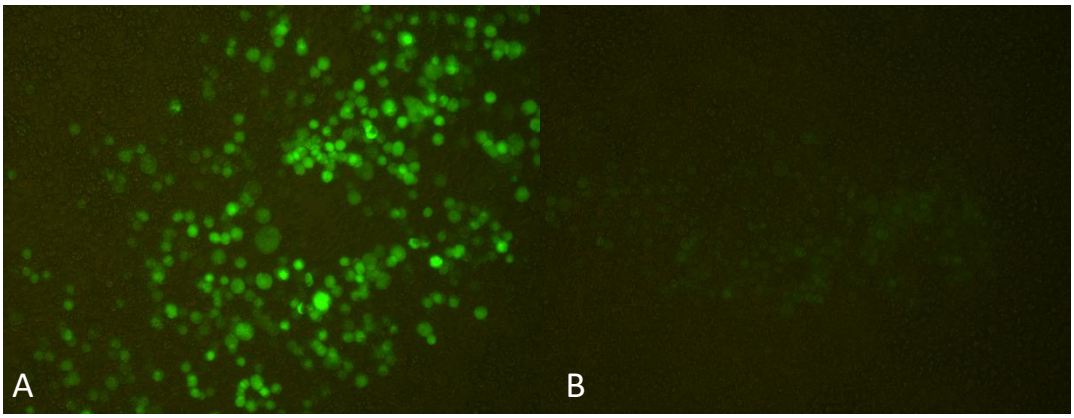


Figure 4-4: Difference of promoter strength in infected Sf9 cells with constructs GP64 YFP and ie1 YFP seen in the $TCID_{50}$ determination.

In figure 4-4, photographs taken in the $TCID_{50}$ determination can be seen. The difference in promoter strength between ie1 and GP64 is apparent. The GP64 YFP construct in A yielded to much more YFP production and therefore to a stronger fluorescence signal than the ie1 YFP construct in B. Both infections were done with the same cells and the photos taken at the same time post infection.

The, from the BioLector® experiments obtained, figure 4-5 shows the same trend. The ie1 promoter reached its expression peak very early and continued to steadily produce a low amount of recombinant protein. The GP64 promoter expression peaked 40 hours earlier than the polh and appears to also have yielded more YFP. There is the possibility that the cells were already too stressed from producing other virus components, at the late hour post infection the polh promoter had its peak, to be able to properly produce YFP with correct folding. It is also possible that this late into the infection the cell already missed essential building blocks for YFP production.

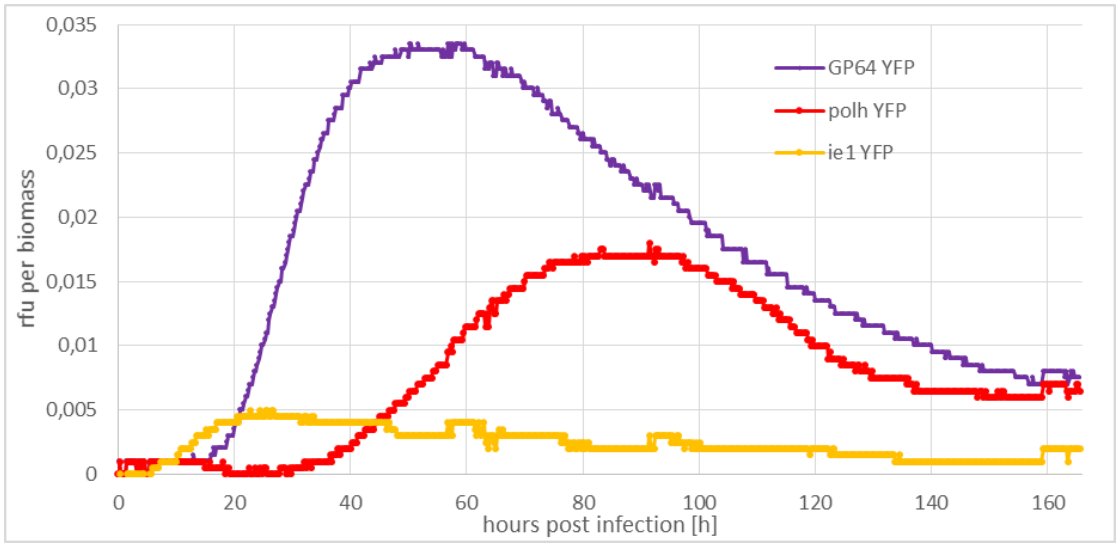


Figure 4-5: Comparison of strength and kinetics of the different promoters used in this work if they control the YFP transcription.

4.3.2 Impact of the burst sequence multiplication

In figure 4-6 a comparison of the yields of the polh promoter constructs without the additional vlf-1 His is shown. The promoter with only the original burst sequence appears to have yielded the least amount of YFP. Interestingly, the promoter with only one additional burst sequence yielded the highest protein production. If the curve is compared to the construct with the original burst sequence, three times as much YFP was produced by the 2B construct. If the curve is compared to figure 4-5, nearly double of the GP64 YFP construct was produced by the 2B construct. With a higher amount of burst sequences, on the other hand, the amount of protein production decreased again with each additional burst sequence. This result is in agreement with the results of Manohar et al. 2010 who concluded that

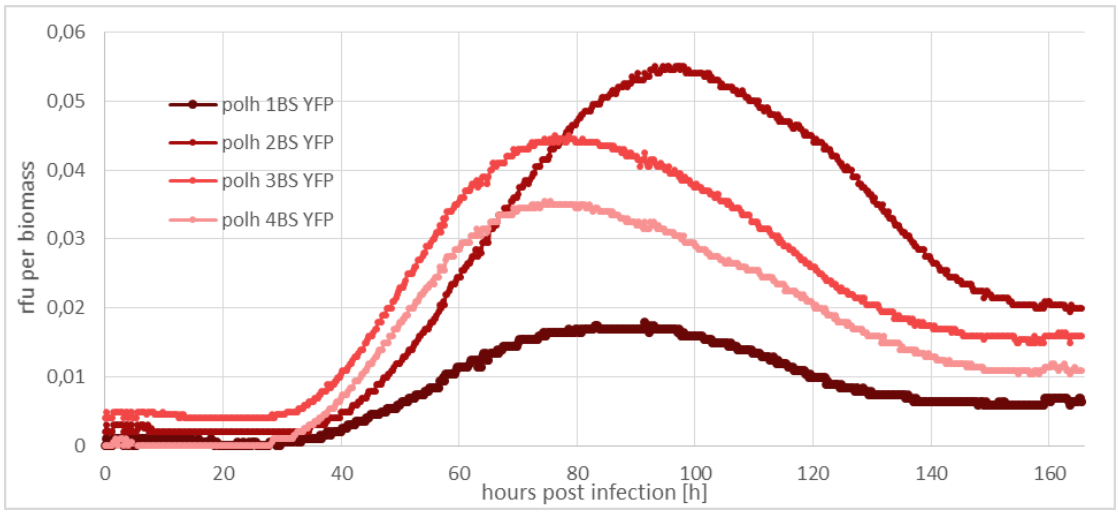


Figure 4-6: Comparison of the influence in YFP production with different amounts of burst sequences in the polh promoter

two burst sequences are the optimum for insect cells. An explanation for this phenomena could be based on the 5'-UTR length. The longer the 5'-UTR the higher the chance for translation to already start in this region rather than at the actual start codon (Peabody 1988). Because the transcription initiation site is situated upstream of the burst sequences the mRNA contained these repeated sequences. If the translated protein got this additional "tail" the probability it was not folded correctly is higher and the so obtained protein did not give a fluorescence signal, therefore was not measured here.

Also worth noting appears to be the hour post infection, h.p.i., the protein expression peak was reached by the constructs with three and four burst sequences. Both reached their peak approximately 10 -15 h.p.i. earlier and with a much steeper increase in the beginning than the other two constructs. This might indicate that more protein expression is possible and even perhaps happening but also as if the possible 5`UTR dependent mistranslation happened unproportionally more often the more protein was produced.

4.3.3 Very late factor-1 His impact

Figure 4-7 shows the different vlf-1 His constructs. The impact on protein production when one additional burst sequence was incorporated into the polh promoter can also be seen. If the figure with the original burst sequence in the polh promoter is observed, the construct with additional vlf-1 His produced under the GP64 promoter led to the highest protein yield and also led to an earlier

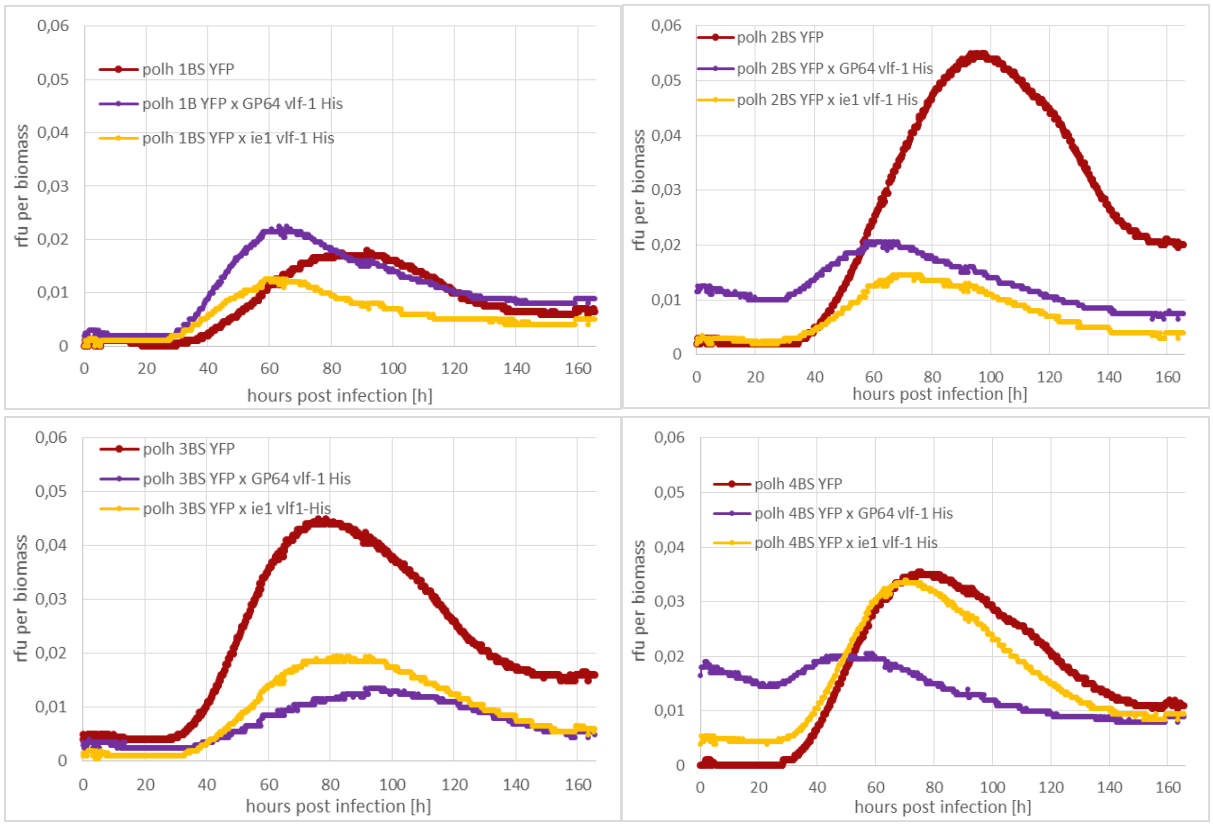


Figure 4-7: Comparison of poh constructs with different amounts of burst sequences fused with the different vlf-1 His constructs.

production start. The vlf-1 His produced under the ie1 promoter appeared to have a negative impact on the YFP production. Both constructs with vlf-1 His showed a similar sharp decrease of YFP signal after their peak in contrast to the construct without the transcription factor overexpression. This led to the conclusion that the produced proteins were degraded immediately after production, perhaps as a reaction to cell stress. In explanation, both proteins stayed in the cytoplasm and accumulated there, which comprises additional stress to the actual virus infection.

With more burst sequences the additional vlf-1 His appeared to have a negative impact on the protein production in general. This indicated that at some point the protein expression machinery is overloaded and is not capable of producing two proteins at such high concentrations. Especially with the three and four burst sequences it could be seen that the ie1 vlf-1 His construct led to more YFP production than the GP64 vlf-1 His construct. Because the GP64 promoter starts earlier than the polh and leads to strong expression, this might have overloaded the machinery faster and therefore caused a decrease in YFP production. The polh promoter with two burst sequences and the GP64 vlf-1 His constructs showed an earlier peak of YFP expression than the other constructs. This was expected and actually a goal of this work. However, the metabolic overload caused an overall decrease in expression of these constructs.

It appears that only in case of the ie1 vlf-1 His constructs higher protein production rates were initiated, directly correlating with higher number of more burst sequences. However this conclusion could be misleading. Perhaps the very early expression of vlf-1 His helped to reduce the overload on the protein expression machinery, resulting in the same amount of YFP being produced with or without vlf-1 His.

In addition to Sf9 cells, also Hi5 and Tn42 cell lines were infected with the different constructs and incubated in the BioLector®. However, in both cases infections were severely hampered and inefficient. We suspect, that the BioLector® might not be suitable for the Hi5 and Tn42 cell lines, therefore, a flask fermentation experiment was started with the two most promising virus constructs.

4.4 COMPARISON OF CONSTRUCTS IN DIFFERENT CELL LINES

In the flask fermentation experiment all three cell lines were infected and fermented with either Y2, YG2 or a SweetBac pACEBac1 GP120 construct which was used as the negative control and is denoted as neg. At post infection hours 0, 16, 20, 24, 48, 72 and 93 samples were taken. Cell count, viability, pH, relative fluorescence in the supernatant, relative fluorescence in the cell pellet and percentage of cells infected were measured at each sampling time. The two constructs used for this experiment appeared to be the most promising in terms of YFP production after the BioLector® experiments. Even though the YG2 construct appeared to yield less protein than other constructs, an early start of protein production could be observed. This made it interesting to observe in other cell lines.

On one hand this experiment is less accurate than the BioLector® experiment, because the machine measured the parameters every 15 min and also no handling steps were involved. Additionally, for handling reasons, only two constructs could be measured. On the other hand a broader variety of information could be obtained and the problem, that the BioLector® was in our hands not applicable to Hi5 and Tn42 cell lines was circumvented.

4.4.1 Experimental data obtained

In figures 4-8, 4-9 and 4-10 the cell count in comparison to the viability at the representative h.p.i. is portrayed. Every figure shows the results for one cell line. When the cells were infected, they were in the exponential growth phase and therefore had a doubling time of approximately 24h under normal conditions. In figure 4-8 the trends of the Sf9 cell line can be observed. Here, after infection, the growth decreased and only one doubling was achieved in the 93 hours of observation. Additionally, the viability decreased, especially in the last hours of observation. As the cells do not propagate after virus infect and are lysed at a certain point of the infection cycle, the trends in figure 4-8 are expected. The viability of cells infected with the negative control decreases particularly rapid. While figure 4-8 looks as expected figure 4-9 and figure 4-10 do not. In both, the Hi5 and the Tn42 cell lines, close to no decrease in viability could be observed. In correlation with this, major cell growth could be observed until the point of a too dense cell concentration. This indicated already that Hi5 and Tn42 are not as susceptible to the baculovirus as are the Sf9. Apparently this did not depend on the virus construct as the negative control also appeared to be less infective.

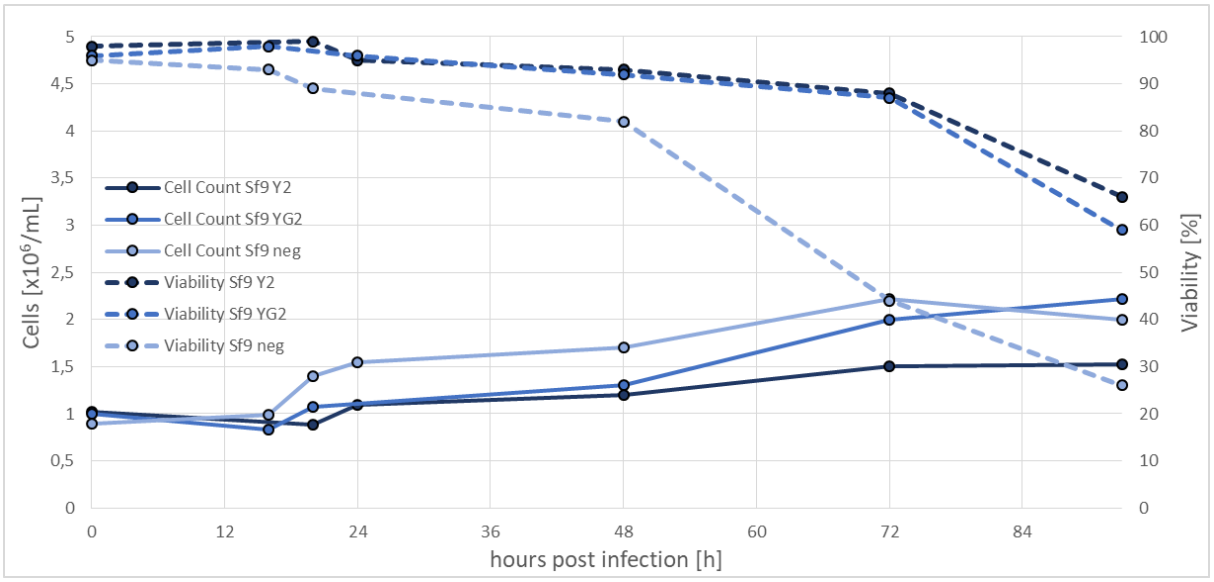


Figure 4-8: Comparison of cell count and viability at the h.p.i. of the Sf9 cell line and all measured constructs.

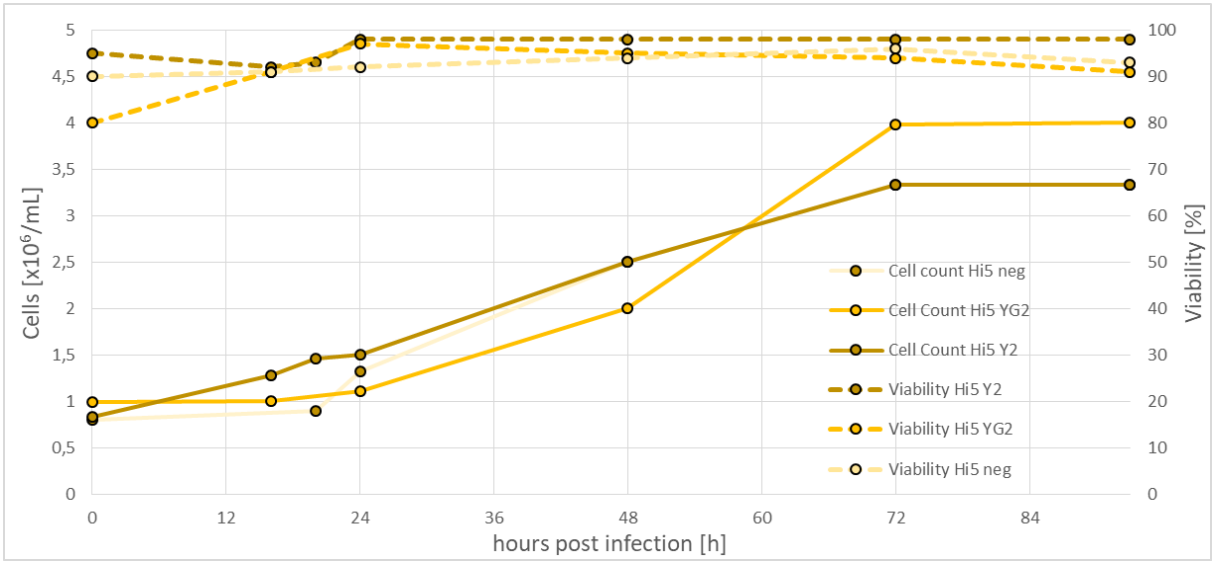


Figure 4-9: Comparison of cell count and viability at the h.p.i. of the Hi5 cell line and all measured constructs.

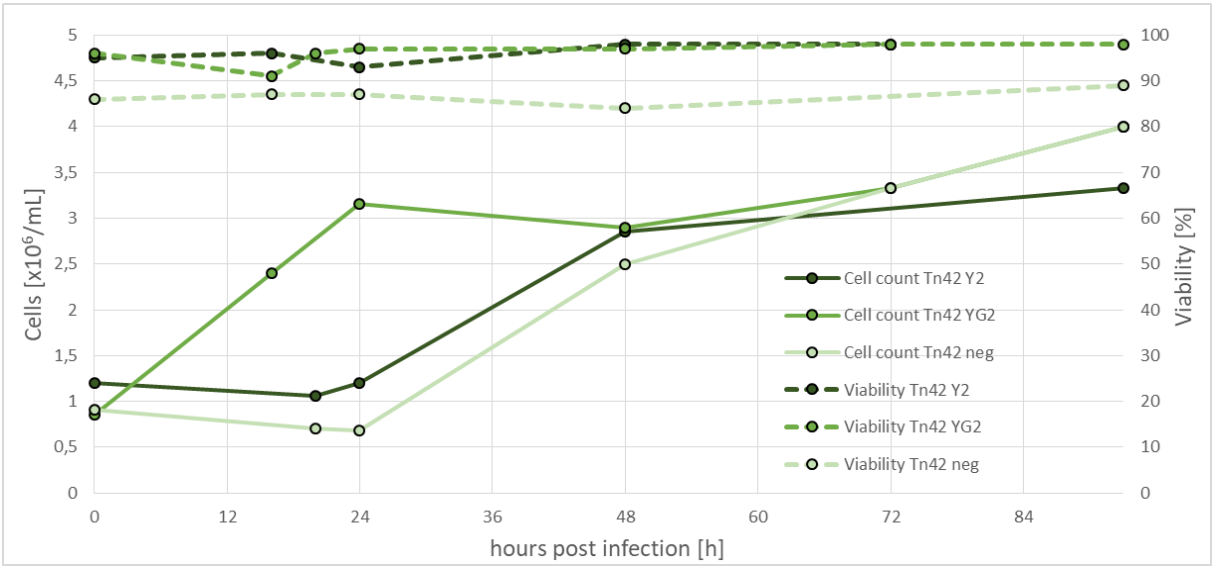


Figure 4-10: Comparison of cell count and viability at the h.p.i. of the Tn42 cell line and all measured constructs.

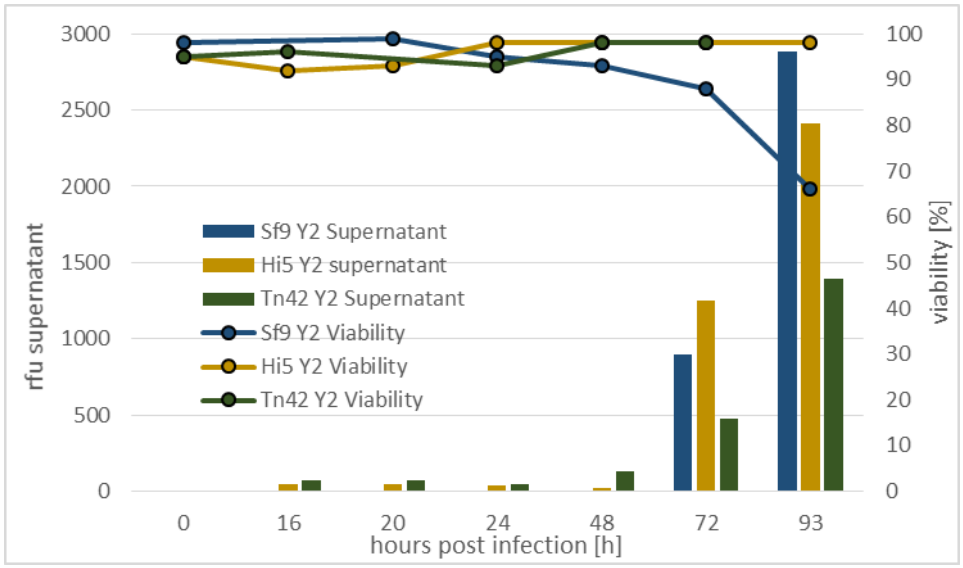


Figure 4-11: Comparison of relative fluorescence in the supernatant and viability at the respective h.p.i. of the Y2 construct in each cell line.

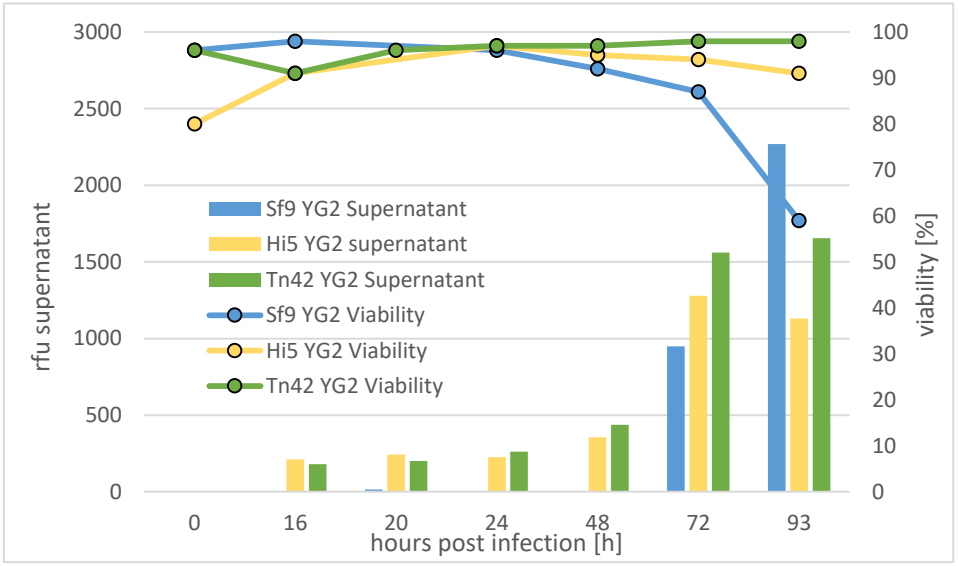


Figure 4-12: Comparison of relative fluorescence in the supernatant and viability at the respective h.p.i. of the YG2 construct in each cell line.

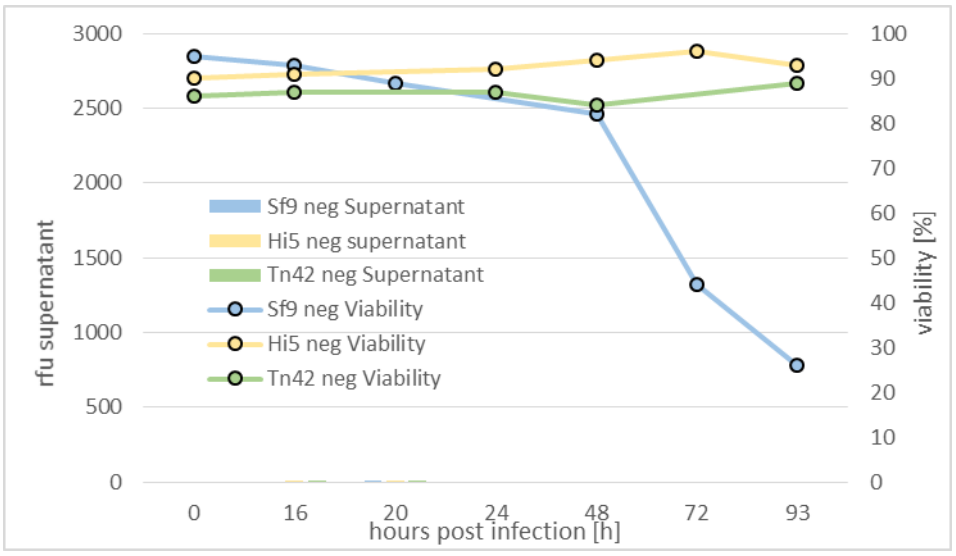


Figure 4-13: Comparison of relative fluorescence in the supernatant and viability at the respective h.p.i. of the negative control construct in each cell line.

The next three figures, figure 4-11, 4-12, 4-13, show the viability in comparison to the relative fluorescence of the YFP in the supernatant of the cell suspension. As YFP is a cytosolic protein and also did not receive a signal peptide for outward transfer, all YFP in the supernatant originates from already lysed cells. This explains why major amounts of YFP were only found in the supernatant from h.p.i. 72 onwards, in contrast to the YFP found in the cell pellet seen in the next three figures. The figures 4-11, 4-12 and 4-13 show each one construct in the different cell lines. Because there was no YFP gene incorporated in the construct of the negative control, no fluorescence was measured even though a decrease of viability, which is a sign for virus infection, is observable in figure 4-13. The Sf9 cells in figure 4-11 and 4-12 show a decrease in viability, correlating with an increase in YFP found in the supernatant, which correlates with the expected cell lysis. The Hi5 and Tn42 cell lines, on the other hand, reacted unexpectedly. Even though the relative fluorescence signal in the supernatant was not as strong as the one produced by the Sf9, there was clearly YFP produced, but the viability did not appear to decrease. This leads to three possibilities. Either the YFP is transferred out of the cells by not lysing them, but this is rather unlikely because of the missing signal peptide. The second possibility would be that the few Hi5 and Tn42 cells that were lysed produced a much higher amount of YFP per cell than the Sf9. Figure 4-17 shows this could at least be the case partly. The third possibility - and the most probable one - is that because only a small percentage of cells was infected (see figures 4-14, 4-15, and 4-16) the propagation of the cells was so strong, the small amount of lysed cells only had a small impact on the obtained viability value. There is of course also the possibility the answer is a combination of the one or more explanations.

Interesting is that already from h.p.i. 16 onwards YFP was found in the supernatant of the Hi5 and the Tn42 cells, especially observable in figure 4-12 but also in figure 4-11. Perhaps this indicates that the cells lysed earlier in the infection cycle than the Sf9, as a reaction to stress. This would partly be in agreement with Yang and Miller 1998 who concluded that early overexpression of vlf-1 causes premature cell lysis, even though their experiments were conducted in Sf21 cells. Another possibility would be they really carried the YFP outside of the cell. But this actually does not explain why there was already YFP found at h.p.i. 16 as the very late phase is not reached at this h.p.i. yet. It is possible that the construct, which was decided to be the best in Sf9, has a completely different impact on the *Trichoplusia ni* cell lines. Maybe an additional burst sequence or more vlf-1 induced the polh promoter earlier in the virus cycle of these cells. The relative fluorescence value of the supernatant, measured at h.p.i. 0, was taken as a blank value for calculations. At h.p.i. 72 it appears as if Hi5 and Tn42 produced more protein than Sf9 with both constructs. This observation is reversed at h.p.i. 93, indicating that Hi5 and Tn42 cells either started producing YFP faster than Sf9 cells or just lysed earlier after infection, resulting in faster accumulation, especially with the YG2 virus construct. In regards to the construct, a higher fluorescence signal was observed with Y2 than with YG2 at h.p.i. 93. On the other hand, at h.p.i.

72, the YG2 construct appears to have produced more YFP. This leads to the assumption that YFP production in the YG2 construct starts earlier than in the Y2 construct.

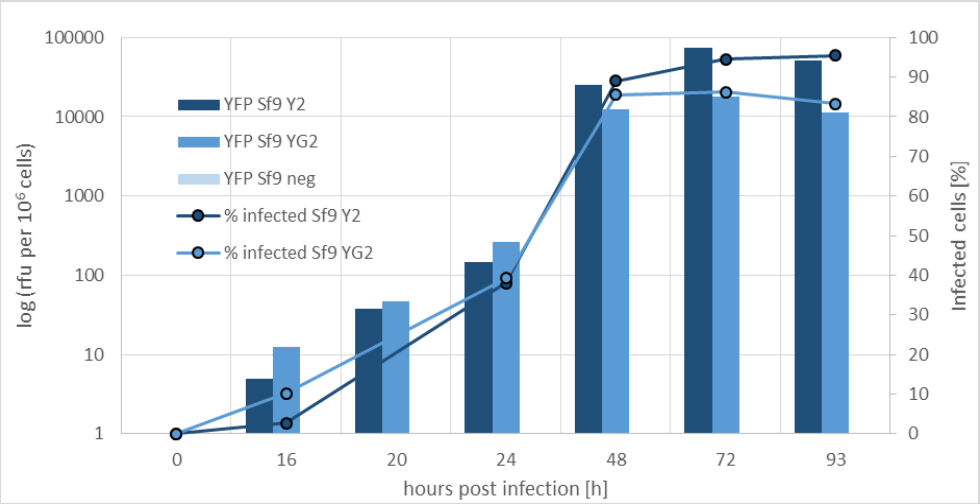


Figure 4-14: Comparison of relative fluorescence in the pellet of 106 cells and percentage of infected cells at the respective h.p.i. of the Sf9 cell line infected with each construct.

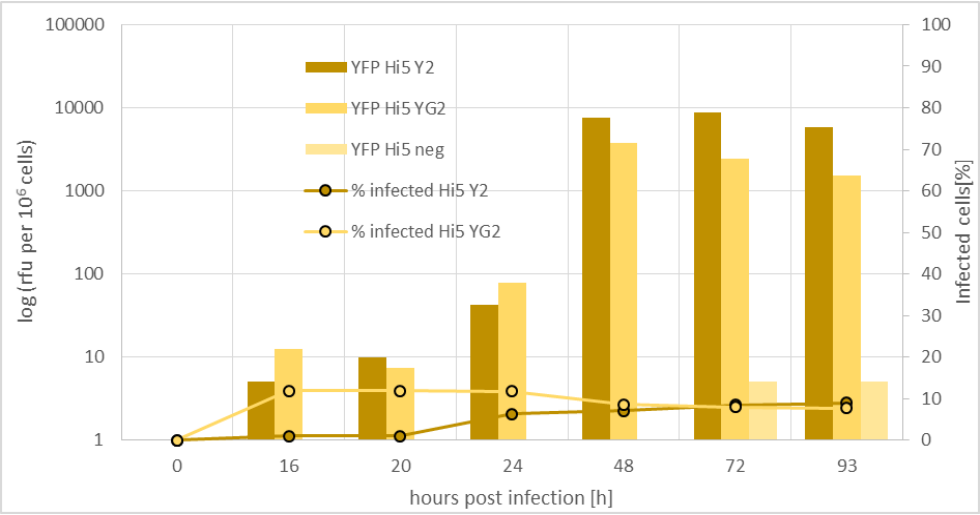


Figure 4-15: Comparison of relative fluorescence in the pellet of 10^6 cells and percentage of infected cells at the respective h.p.i. of the Hi5 cell line infected with each construct.

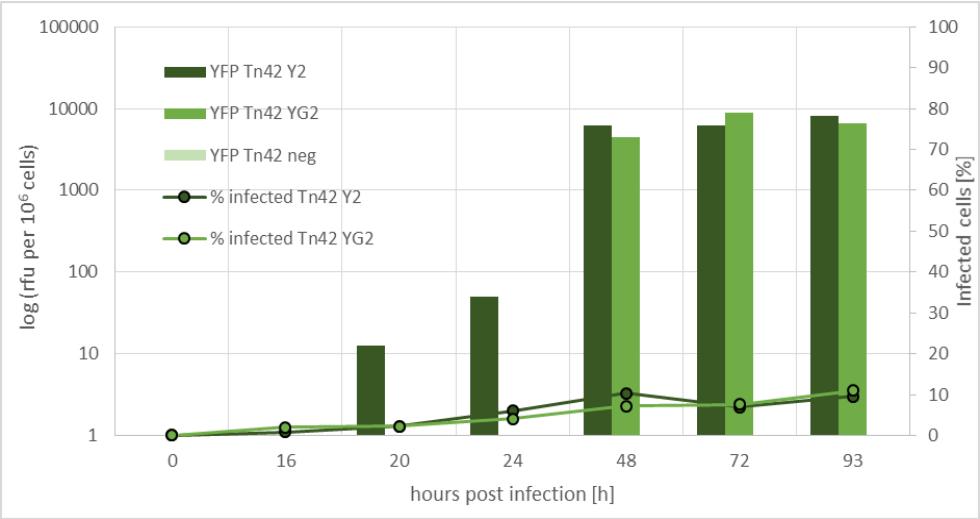


Figure 4-16: Comparison of relative fluorescence in the pellet of 10^6 cells and percentage of infected cells at the respective h.p.i. of the Tn42 cell line infected with each construct.

Figures 4-14, 4-15, 4-16 show the percentage of infected cells, measured in FACS, compared to the $\log_{10}$ of the relative fluorescence measured in a lysate of a pellet with 10^6 cells. Correlating with the viability observations made before, Tn42 and Hi5 cells appeared to not be as susceptible as the Sf9 to the virus infection. Figures 4-15 and 4-16 show a steady but low percentage of infection, around 8 %, throughout the whole observation time. The Sf9 cells in figure 4-14, on the other hand, show a steep increase in infected cells, to nearly 100%, between h.p.i. 24 and 48. The steep increase can be explained by looking at the virus cycle. In the late phase, between h.p.i. 6 to 24, the virions exit the cell by budding and infect the next cell. To be detected by FACS, YFP production has to have already started in the infected cell, therefore, the very late phase has to be already reached resulting in an observable spread in infection at around h.p.i. 25-30. In the Hi5 and Tn42 cells the percentage stayed approximately the same possibly because the cells died in the same speed as new cells were infected and not many cells were infected in general. In figure 4-15 can be seen that the YG2 construct in Hi5 cells appears to have induced spreading of the infection earlier than the Y2 construct. But to put forward an accurate statement on that, more experiments need to be done on this matter.

In contrast to figure 4-11 and 4-12, only very small amounts of YFP were found in the cell pellets at the first few hours of infection. This indicated that cells producing YFP, were lysed very early during infection and therefore, the YFP was not detected in the cell pellet. The small fluorescence values at the early h.p.i. appear bigger because of the $\log_{10}$ scale in figures 4-14, 4-15 and 4-16.

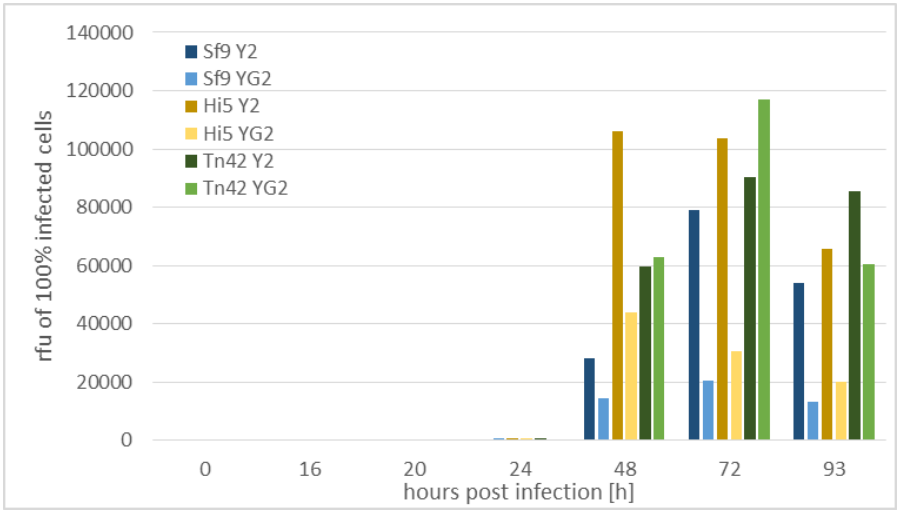


Figure 4-17: Relative fluorescence signal in the cell pellet of 10^6 cells if 100% of cells would have been infected by the respective virus construct.

Figure 4-14 shows more fluorescence signal than figures 4-15 and 4-16 but this depends on the amount of infected cells. In figure 4-17 the expected relative fluorescence values, if all the 10^6 cells in the pellet were infected by the virus, is portrayed at its respective h.p.i. Comparing figures 4-11 and 4-12 with each other it can be observed that more fluorescence was found in the supernatant of YG2 infected Tn42 than in the Y2 infected. Figure 4-16 on the other hand shows close to the same signal in both constructs. But in figure 4-17 the same trend as in figure 4-11 and 4-12 can be seen. It seemed that, if

more cells had been infected, the YG2 construct in Tn42 would have led to increased YFP production, in general and earlier in the infection cycle. For the Hi5 and the Sf9 cells the amount of signal in the supernatant and in the pellet correlated better. In these two cell lines the Y2 construct appeared to have yielded to the most YFP as indicated in the BioLector® experiments. All in all it appeared in figure 4-17 as if the *Trichoplusia ni* cell lines would have produced more YFP than the Sf9, if the same amount of cells were infected. The Hi5 cell line might even have started production earlier than the other two. Additionally, figure 4-17 shows the actual YFP production started between the h.p.i. 24 and 48.

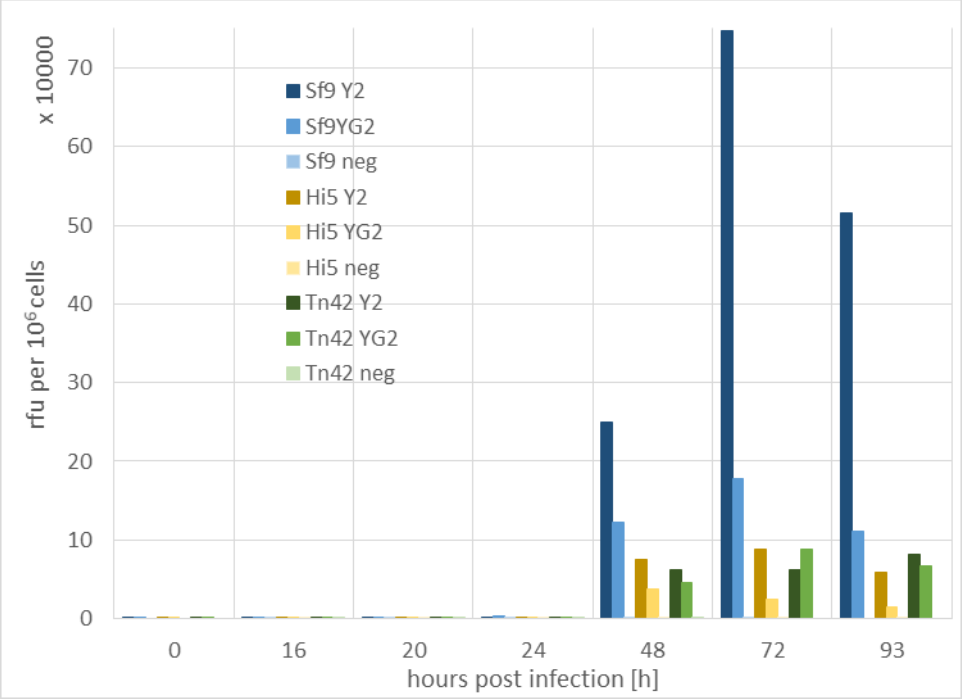


Figure 4-18: Relative fluorescence signal in the cell pellet of 10^6 cells. Comparison between all constructs and cell lines.

Figure 4-18 shows the relative fluorescence of the pellet with 10^6 cells at the respective h.p.i. This is important for an overall impression of the amount of protein produced in the different cell lines and with the different constructs. Here the produced signal showed that Sf9 infected with the Y2 construct yielded the highest fluorescence values by far. The YG2 construct yielded a lot less but the values produced by the other cell lines are even smaller. As figure 20 shows, this was probably due to the small amount of infected cells. As also already observed, YG2 appeared to induce a relatively strong production of YFP in Tn42 in contrast to the other two cell lines.

Because both, the Hi5 and the Sf9 cell lines are infected with a latent virus (Ma et al. 2014, Li et al. 2007) and the Tn42 cell line is virus free, the conclusion that poor YFP production with YG2 depended on the latent virus, is possible. Especially as the Tn42 cell line is a subclone of the Hi5 cell line and therefore, should react in a similar way. With the YG2 construct YFP production is supposed to start a bit earlier and amount to higher yields. Perhaps the latent viruses could react to too fast YFP of vlf-1 production in too high amounts, inducing a bottleneck further down the protein expression pathway.

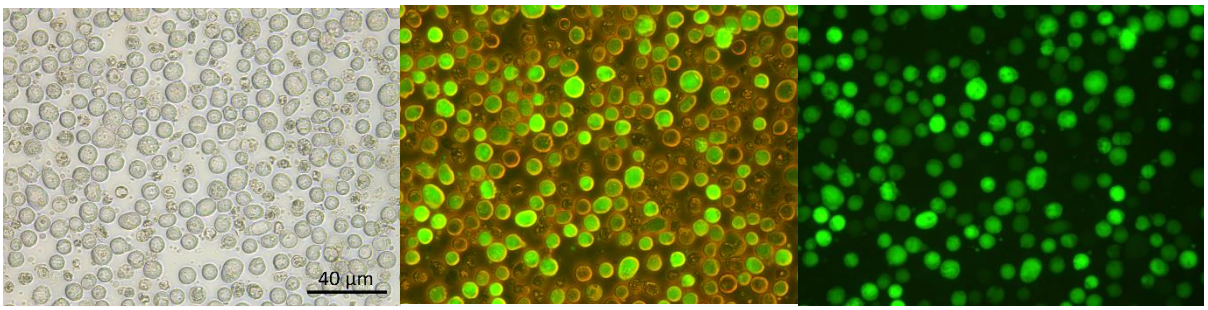
Also a possibility is that the strain of the additional vlf-1 production lyses or exhausts the Sf9 and Hi5 cell lines earlier after infection resulting in less YFP being produced.

All in all for this experiment can be said that the low percentage of infection in Hi5 and Tn42 cells made accurate comparison difficult and might have led to wrong conclusions. A repeat of the experiment might lead to more conclusions. Also some problems with the used cell counter were witnessed so accurate cell numbers might not have been obtained. This is the reason for the experiment in chapter 4.4.3.

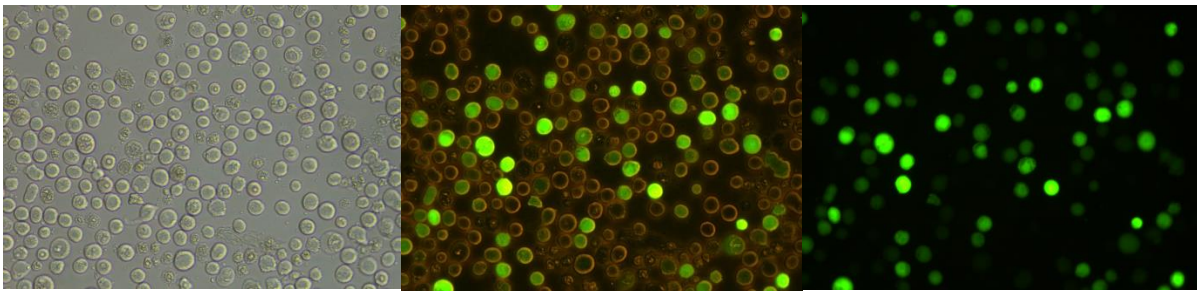
4.4.2 Fluoerscence microscope observations

After observing an unexpectedly poor infection of the Hi5 and Tn42 cell lines with FACS, the cells were observed under the fluorescence microscope at 93 h.p.i for confirmation. The results can be seen in figures 4-19 and 4-20.

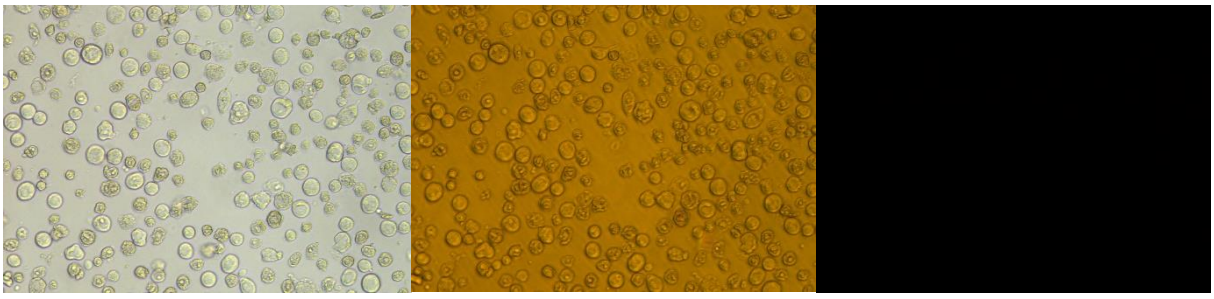
Sf9 at 93 h.p.i infected with construct Y2



Sf9 at 93 h.p.i infected with construct YG2



Sf9 at 93 h.p.i infected with negative control



Hi5 at 93 h.p.i infected with construct Y2

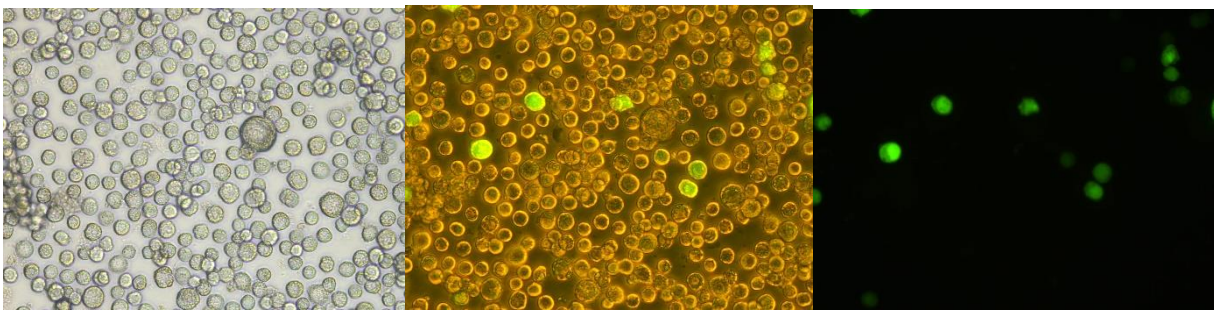
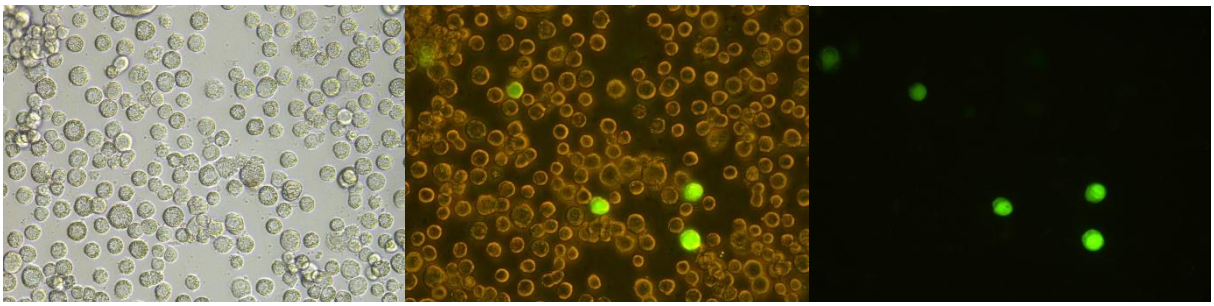
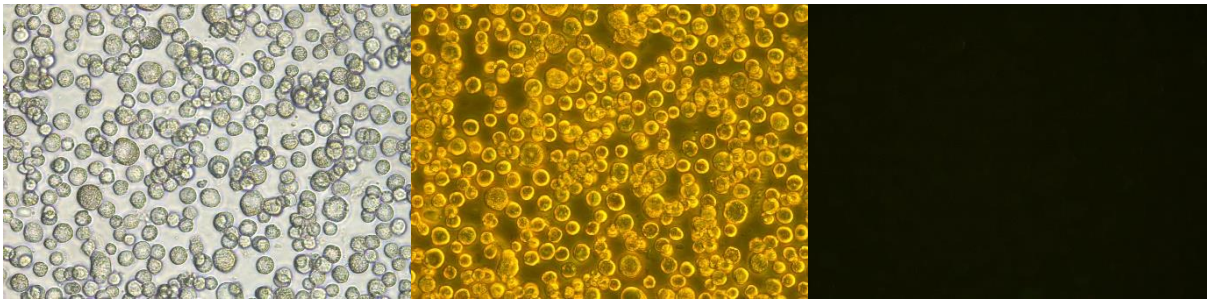


Figure 4-19: Different cell lines infected with different constructs at the end of the flask fermentation experiment (93 h.p.i.)

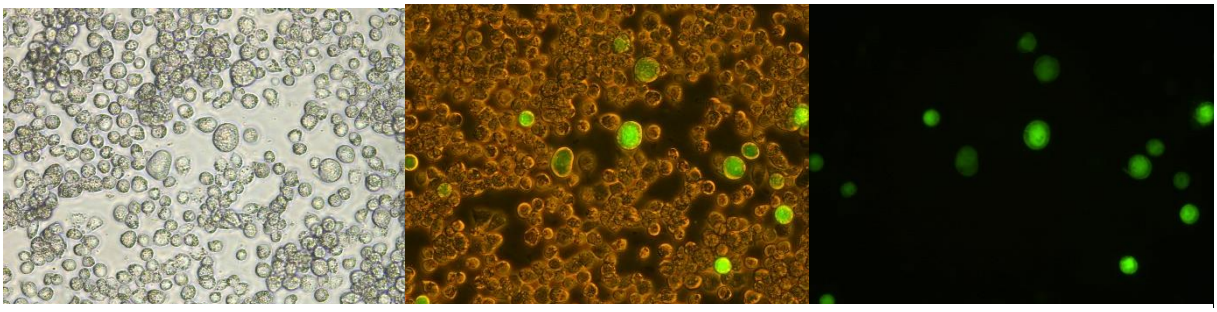
Hi5 at 93 h.p.i infected with construct YG2



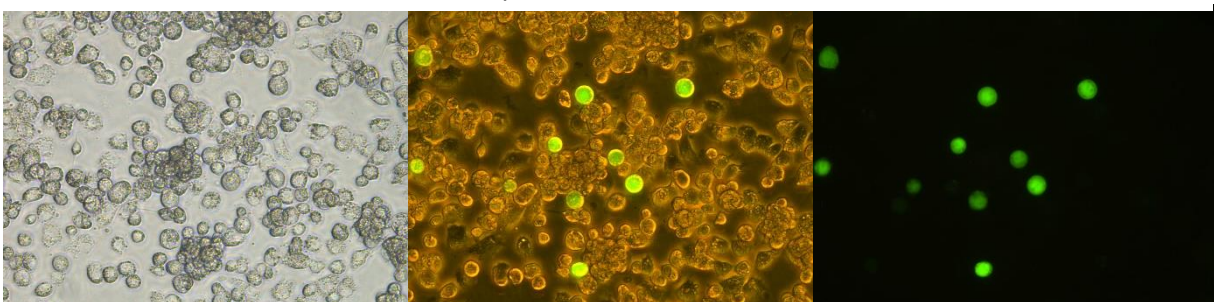
Hi5 at 93 h.p.i infected with negative control



Tn42 at 93 h.p.i infected with construct Y2



Tn42 at 93 h.p.i infected with construct YG2



Tn42 at 93 h.p.i infected with negative control

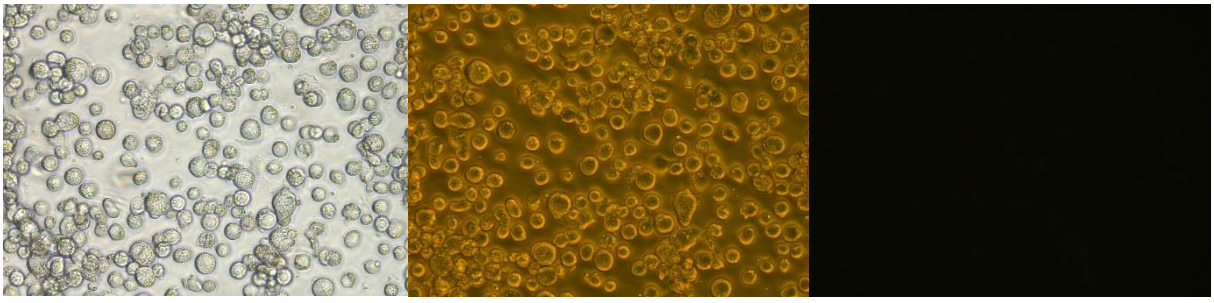


Figure 4-20: Different cell lines infected with different constructs at the end of the flask fermentation experiment (93 h.p.i.)

Similar to the figures in chapter 4.4.1 the poor infection of the Hi5 and Tn42 cells can be seen in figures 4-19 and 4-20. Except for YFP production, infected cells can also be distinguished from uninfected by their rounder and slightly bigger morphology. Nearly all Sf9 cells infected with Y2 either produced YFP or were already lysed but the other two cell lines showed only slight infection. Most of the Sf9 cells infected with YG2 showed YFP production and virus infected morphology even though less strongly compared to the Y2 infection. In the other two cell lines too few infected cells can be seen to allow for a conclusion like that. Correlating with the cell count and the amount of cells infected in chapter 4.4.1, the Hi5 and Tn42 cell lines appear to be in much higher cell density than the Sf9, even though they were seeded with the same concentration. As already mentioned the negative control virus did not produce YFP but infected the cells nonetheless. Especially in the Sf9 the cell debris of lysed cells and rounded morphology of the negative control cells can be seen well. Even with the addition of heparin to Hi5 and Tn42 cell culture flasks, the Tn42 appeared to have clumped a lot until the 93 h.p.i. compared to the Hi5. This might have influenced cell count and viability results as well as cell growth and virus susceptibility.

A possible reason for the low susceptibility of the Hi5 and Tn42 cells to the virus might be the standard heparin addition, which should avoid clumping. It appears as if heparin can inhibit virus infections in some case (Nahmias and Kibrick 1964, Lin et al. 2002), because some viruses use heparin or a very similar protein for virus entry. On the other hand, heparin appears to not have any effect on virus entry into Sf9 cells (Wu and Wang 2012). Sf9 cells were not prone to clumping and thus heparin was only added to Hi5 and Tn42 cells. For these two cell lines no data, in terms of heparin influence on virus entry, could be found.

4.4.4 Detection of YFP with equalized protein concentrations

An inconsistency of amount of pelleted cells was suspected because of problems with the cell counter. Therefore, to get comparable results, a bicinchonic acid assay was conducted with the lysed Y2 cell pellets after the flask fermentation experiment, see table 4-1.

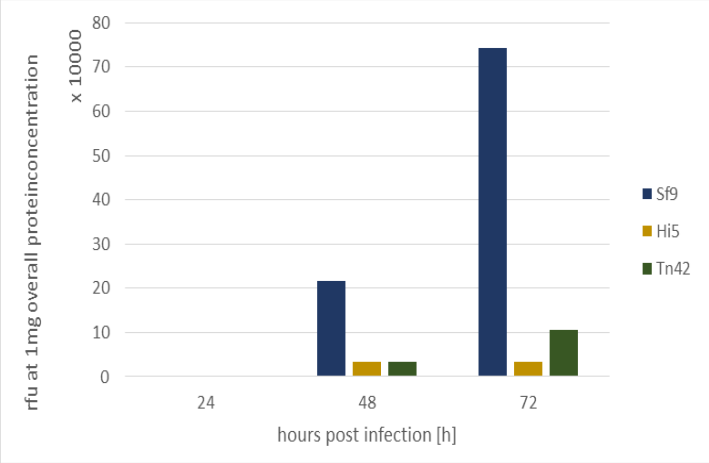


Figure 4-21: Relative fluorescence signal received if 1 mg overall protein amount would be in every pellet lysate.

	Protein concentration [µg/ml]	Rfu at 1 mg protein
Sf9 24 h.p.i.	269	2193
Sf9 48 h.p.i.	463	216307
Sf9 72 h.p.i.	402	742886
Hi5 24 h.p.i.	652	261
Hi5 48 h.p.i.	886	33928
Hi5 72 h.p.i.	1033	34153
Tn42 24 h.p.i.	1185	169
Tn42 48 h.p.i.	726	33967
Tn42 72 h.p.i.	236	106186

Table 4-1: Obtained protein concentration and calculated relative fluorescence unit.

Figure 4-21 shows, as figures before in chapter 4.4 flask fermentation,that recombinant protein production started between the h.p.i. 24 and 48. As also seen in figure 4-18 Sf9 produced by far more protein than the other two cell lines, but Tn42 produced also a little more than the Hi5 cells. In figure 4-18 the Tn42 cells only appear to have produced more YFP than the Hi5 cells in h.p.i. 93 and not already in h.p.i. 72 which might indicate that fewer Tn42 or more Hi5 were observed than 10^6 cells, at h.p.i. 72 in figure 4-18. In any way the trend appeared to be the same and also making a statement with figure 4-21 is difficult as the produced YFP should have a relatively high impact on the overall protein concentration and therefore underestimation is possible.

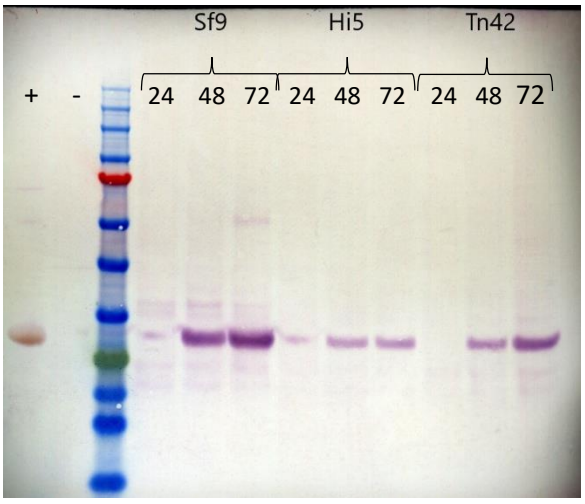


Figure 25: Westernblot with Pelltes obtained in the Flask Fermentation. The numbers indicate the h.p.i. the pellet was taken. Ladder: coloured prestained protein standard, broad range (11 - 245 kDa)Blot detected with YFP antibody. YFP has approximately a size of 26 kDa.

With the values for protein concentration in table 4-1 taken into account, a westernblot analysis, figure 4-22, was carried out to detect YFP concentrations qualitatively in the lysed pellets of the Y2 construct at the respective h.p.i. The obtained YFP bands at approximately 26 kDa confirmed what could be seen in figure 4-21 in terms of strength.

4.4.3 Detecting vlf-1 His

To prove vlf-1 His was really produced in the YG2 construct, a western blot analysis with an antibody to detect the Hexa-His-Tag on the protein was conducted, see figure 4-24. To also have an indication if overall protein concentration in all samples was similar, a SDS-PAGE was conducted and the proteins were dyed, see figure 4-23.

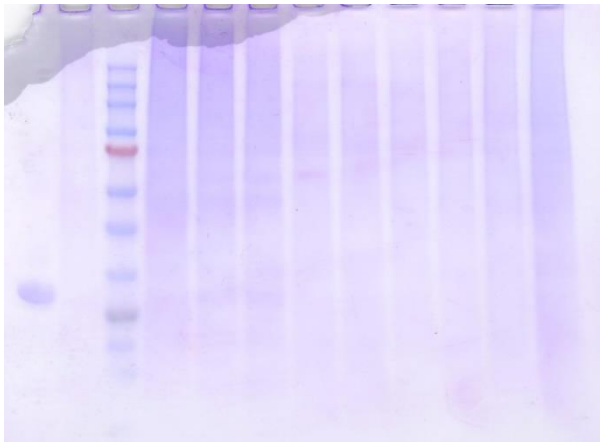


Figure 4-23: SDS-PAGE with dyed protein

The overall protein concentration in the samples used appears to be fairly similar, making figure 4-24 representative.

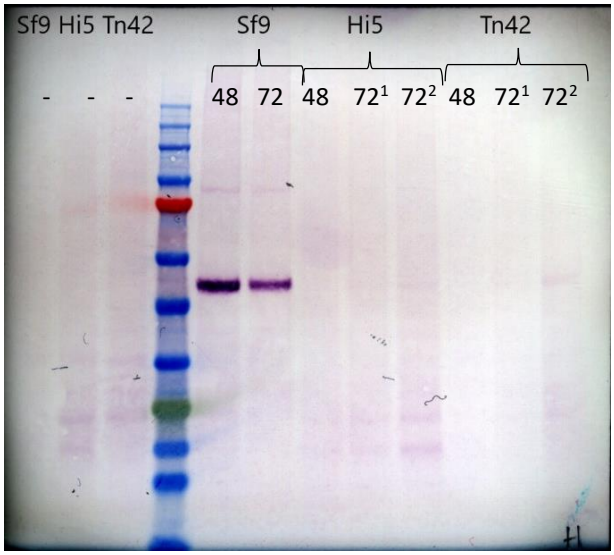


Figure 4-24: Westernblot with Pelltes obtained in the Flask Fermentation. The numbers indicate the h.p.i. the pellet was taken at. Ladder: coloured prestained protein standard, broad range (11 - 245 kDa).Blot detected with Hexa-His-Antibody. The negative control is lysed pellet of the flask fermentation negative control. Vlf-1 His has approximately a size of 50 kDa.

[1] volume of lysed pellet taken according to estimated protein concentration.

[2] maximal volume possible of lysed pellet taken.

In figure 4-24 strong vlf-1 His production can be seen in the Sf9 cells. The vlf-1 His production of Hi5 and Tn42 was probably too weak because of the bad infection resulting in only very light bands. But both 72^2 h.p.i. lanes showed light bands at the right size, indicating that vlf-1 His was also produced in Hi5 and Tn42. In h.p.i. 72 of the Sf9 cells, it appeared as if less vlf-1 His was present in the cells. Perhaps it was already starting to be degraded by the cells because the promoter regulating its expression was GP64. Therefore the vlf-1 His had been produced earlier than YFP.

5. CONCLUSION

The aim of this work was to increase recombinant protein expression in different insect cells lines when using the baculovirus expression vector system. To achieve this, the number of burst sequences in the polyhedrin promoter as well as the amount of the vlf-1 transcription factor production, which binds to this sequence, was increased.

Prior to the protein expression experiments, the amount of burst sequences in the virus constructs was confirmed to ensure reliable results. Then BioLector® experiments were carried out. Surprisingly, the GP64 promoter yielded more YFP in this case than the polyhedrin. Two burst sequences in the polyhedrin promoter resulted in the highest YFP production compared with the other burst sequence constructs. The problem that a long 5' UTR region might lead to early translation initiation and therefore misfolded proteins might play a role here. The experiments with combinations of different vlf-1 His constructs and different burst sequence numbers in the polyhedrin promoter showed that simultaneous YFP and vlf-1 His production led to a bottleneck in the expression machinery resulting in less YFP production.

In comparison with the *Spodoptera frugiperda* cell line, the *Trichoplusia ni* cell lines showed a lack of infection. With calculations however, some information of trends could be determined. It appeared that more recombinant protein could be obtained by Hi5 and Tn42 than using Sf9 if the problem with infection can be fixed. Especially Tn42 with the GP64 promoter controlling vlf-1 His production looked promising in the conducted experiments. Another finding was the YFP present in the supernatant very soon after infection in both *Trichoplusia ni* cell lines. To ensure the transcription factor was really produced, westernblot analyses were carried out and the vlf-1 His presence was confirmed.

Overall it can be said that the aims were accomplished. A novel promoter construct was generated which yielded, in Sf9 cells, higher YFP amounts compared to normally used viral vectors. Three different insect cell lines were compared in terms of YFP expression using the optimised construct. While different infection rates hampered our experiments, the overall trend showed that Tn42 cells and Hi5 cells have potential. Especially Tn42 showed different behaviour and stronger capacity for the construct overexpressing vlf-1 compared to the other two cell lines. This means in Tn42 higher protein expression than in Sf9 cells, could be achieved. Testing further constructs in all cell lines would perhaps reveal new findings, because the chosen ones were only proven to be the best in Sf9.

Unfortunately the protein expression was not as high as desired and a number of different problems need attention and further investigation. An interesting experiment would be to compare mRNA levels of YFP between the different polyhedrin promoter variations to answer the question if more protein

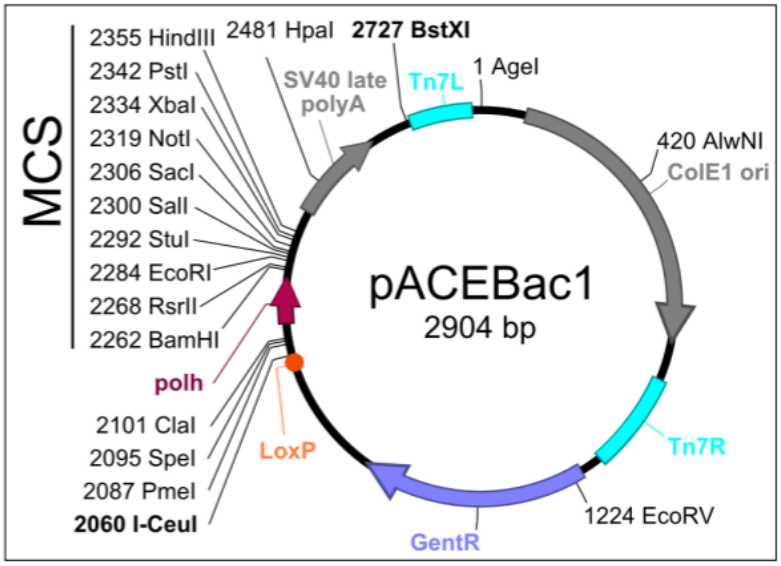
is transcribed with more burst sequences present. Another interesting experiment would be to exchange the YFP with a secreted protein to investigate the limitations of the secretory pathway. If the promoter appears to initiate higher transcription rates with other proteins after experimentation, further use for recombinant protein production is possible.

6. APPENDIX

PLASMIDS

pACEBac1

E.4.1.1 pACEBac1: 2904 bp



Multiple Cloning Site

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  NotI       BstBI  XbaI      PstI           HindIII
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5'-

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pIDK

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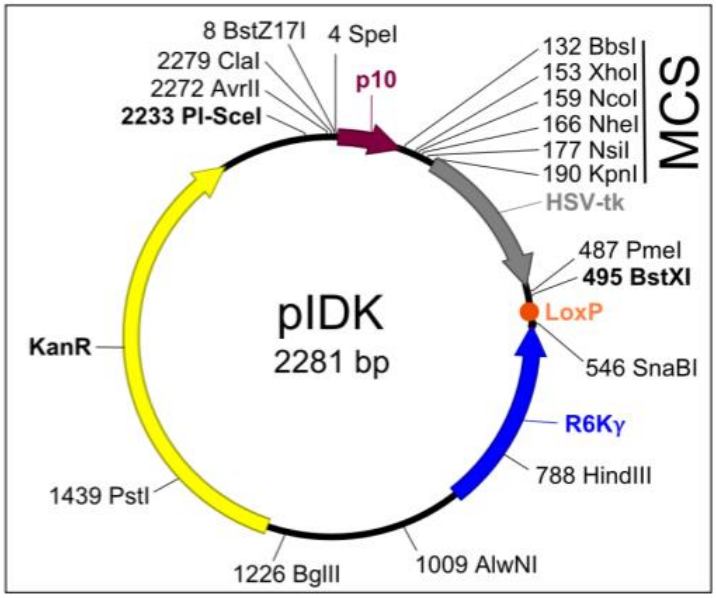

Multiple Cloning Site

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                Acc65I
NsiI            KpnI
ATGCATAGCATGCGGTACCGGG
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5’-
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PROTEINS

Yellow Fluorescence Protein

5‘-
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very late factor 1

5’-
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g-3‘

PROMOTERS

Polyhedrin Promoter

The normally used polyhedrin promoter has the following sequence.

5’-
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ttattcataccgtcccaccatcgggcgcgg-3’

Original Polyhedrin Promoter

The modified and in this work used polyhedrin promoters have the following sequences.

5’-atggagataattaaaatgataaccatctcgcaaataaataagtatttactgttttcgtaacagttttgtaataaaaaaacctataaat-3’
(denoted 1BS polh)

5’-atggagataattaaaatgataaccatctcgcaaataaataagtatttactgttttcgtaacagttttgtaataaaaaaacctataaat
ctgttttcgtaacagttttgtaataaaaaaacctataaat-3’
(denoted 2BS polh)

5’-
atggagataattaaaatgataaccatctcgcaaataaataagtatttactgttttcgtaacagttttgtaataaaaaaacctataaatctgttttcg
taacagttttgtaataaaaaaacctataaatctgttttcgtaacagttttgtaataaaaaaacctataaat-3’
(denoted 3BS polh)

5’-
atggagataattaaaatgataaccatctcgcaaataaataagtatttactgttttcgtaacagttttgtaataaaaaaacctataaatctgttttcg
taacagttttgtaataaaaaaacctataaatctgttttcgtaacagttttgtaataaaaaaacctataaatctgttttcgtaacagttttgtaataaa
aaaacctataaat-3’
(denoted 4BS polh)

Initiation Codon

Burst sequence

parts of polyherin protein gene

PRIMERS

16.9 SV40 polyA for

5’-cct cta gta ctt ctc gac aag-‘3

16.10 -44 back

5’-ttt act gtt ttc gta aca gtt ttg-‘3

16.68 pACEBac1 prom screen back

5’-ttc cac ggt gtg cgt c-‘3

16.78 M13 fw

5’-gtt ttc cca gtc acg ac-‘3

16.79 M13 rv

5’-cag gaa aca gct atg ac-‘3

16.96 YFP 316 for

5’-tcg tcc ttg aag aag atg g-‘3

16.118

5’-atg aac ggt ttt aat gtt cg-‘3

16.120 polh promoter-ClaI fw

5’-atg atg atg atg atc gat atg gag ata att aaa atg ata acc-‘3

16.121 polh promoter-PvuII-NheI-Ba

5’-atg atg atg atg gga tcc gct agc cag ctg att tat agg ttt ttt tat tac aaa act g-‘3

16.124 Ac-vlf-1-6 His KpnI rv

5’-atg atg atg atg ggt acc cta tta cta atg gtg atg gtg gtg atg aga acc acc ttc gtt gcg ata gta caa c-‘3

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