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ESTABLISHMENT OF THE BACULOVIRUS EXPRESSION SYSTEM USING EGFP IN SF9 INSECT CELLS

BACHELOR THESIS

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List of Abbreviations

AcMNPV	*Autographa californica* nuclear polyhedrosis virus
BES	Baculovirus Expression System
BEVS	Baculovirus Expression Vector System
bp	Basepairs
BV	Budded virus
Cq	Quantitation cycle
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
fw	Forward
GFP	Green fluorescent protein
GV	Gransulosis virus
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobasepairs
LB	Lysogeny broth
MNPV	Multiple nuclear polyhedrosis virus
ng	Nanogram
nm	Nanometer
NPV	Nuclear polyhedrosis virus
OB	Occlusion bodies
P	Passage
PCR	Polymerase Chain Reaction
PEI	Polyethyleneimine
pFBD	pFastBac Dual vector
pfu	Plaque forming units
pHD	Polyhedrin promoter
qPCR	Quantitative real time PCR
rev	Reverse
RNase	Ribonuclease
rpm	Revolutions per minute

S.O.C.	Super Optimal Broth with Catabolite repression
SDS	Sodium dodecyl sulfate
SNPV	Single nuclear polyhedrosis virus
TAE	Tris-acetate-EDTA
TM	Melting temperature
Tris-HCl	tris(hydroxymethyl)aminomethane
U	Unit
VP	Virus passage

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1. Introduction

1.1. Overview of Baculovirology

The baculovirus expression vector system has gained in importance over the last decades, providing many benefits as high protein expression and rapidity. The baculovirus plays a major role in this system, as the name implies. Baculoviruses, although not pathogenic for humans, are known for hundreds of years. They do not infect humans, mammals, other vertebrates and plants [1]. Earlier baculovirus infections are described in ancient Chinese literature in form of silkworm disease. In 1940s these viruses, which infect invertebrates, were used as biopesticides. Nowadays baculoviruses are also used as vectors for the expression of heterologous genes. [2]

1.2. The Baculovirus

Baculoviruses are members of the *Baculoviridae* family of occluded viruses. They belong to the group of double-stranded DNA (dsDNA) viruses. This virus family is differentiated by the size of their occlusion bodies. The first type of them is named nuclear polyhedrosis viruses (NPVs), which produce *polyhedra*. *Polyhedra* is an envelope for many virions which is made from the protein polyhedrin and range from 1 to 15µm in size [1]. Polyhedrin will be specified in the next paragraph. Apart from that there are granulosis viruses (GVs). These viruses are principally one virion in a small occlusion body called granules which consisting of the protein granulin [3]. In the following only the more investigated NPV are described. NPVs are large dsDNA viruses, some of them have genomes in size of ca. 135 kb [4], which are located in nucleocapsids singly (SNPV) or in groups (MNPV). NPVs have several species of insects as hosts. Most common baculoviruses are derived from the Lepidoptera, but they also can infect marine organisms like shrimps, which leads to serious shrimp diseases in China since 1993 and is connected with big economic losses. [3, 5]

Baculoviruses have a notability *in vivo* life cycle. During a single infection cycle the virus particle exist in two forms (figure 1).

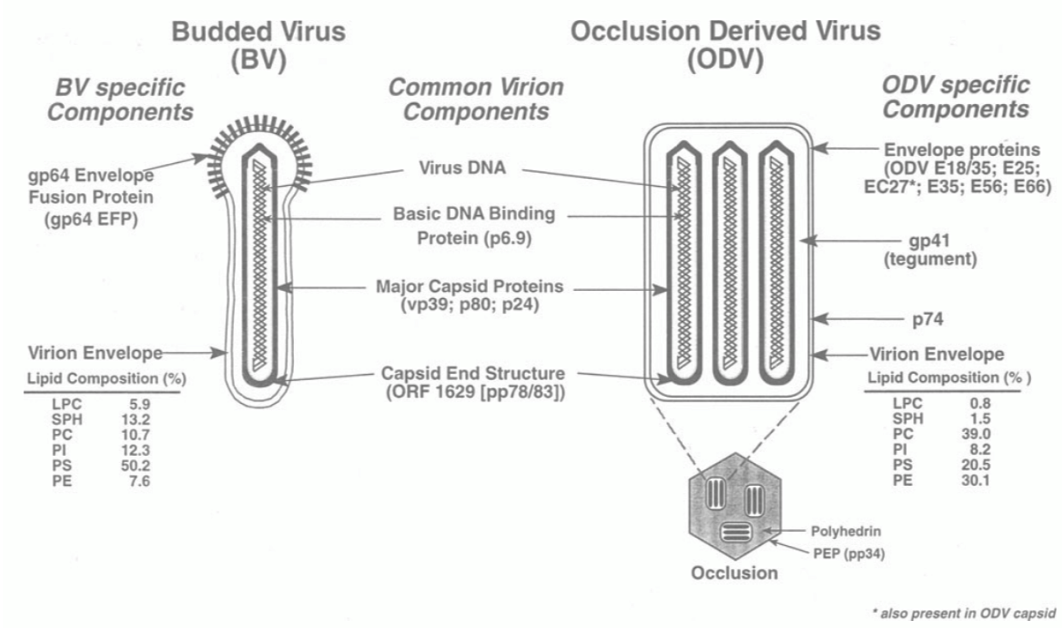

Figure 1: Budded and occlusion-derived virions. Locations of virally encoded proteins are also described. Modified from [3].

The first form is responsible for infecting insects is called occlusion body (OB). OBs are enclosed in a protein envelope which consist of the polyhedrin protein. When OBs are ingested by host insects by feeding contaminated diet, the alkali-soluble occlusion body dissolves due to high pH and proteases of the harsh environment of insect midgut and in conclusion occlusion derived virions (ODV) are released. ODVs infect epithelial cells and produce another virions. These virus particles relevant for second infection within insect tissue are non-occluded and called budded virus (BV). BVs are adapted for movement and infection of tissues and also have a lipid envelope consisting of the protein *gp64* for budding through the plasma membrane of tissue cells by endocytosis [1, 3]. After endocytosis, viral DNA is uncoated in the nucleus of the insect cell and after 6 hours post infection the DNA replication starts. After 10 hours post infection BVs are released from cells by budding and initiate a horizontal transmission within the host. OBs can be detected after 24 hours post infection [6]. During late stage of infection where BVs infected fat bodies, nerve cells and haemocytes insect cells produce both forms of virus particles including virus particles within polyhedra. In the terminal stages of infection the insect host has a swollen appearance because of accumulation of polyhedra within infected nuclei of cells, which are 5 to 10 times increased in size [7]. After host death the OBs are released into the environment [1, 3, 8]. Virus particles surrounded by the polyhedra envelope can persist for a long time in the environment if they are protected from

environmental influences such as UV radiation [8, 9]. For example, the NPV of tussock moth (*Orgyia pseudotsugara* NPV) can persist in forest soil for 41 years [9].

Baculovirus gene expression and replication *in vitro* can be divided into four temporal phases, namely immediate-early (α), delayed-early (β), late (γ) and very late (δ). In these four phases the gene expression increases per phase. During very late stage, ca. 15 h post infection, the virus assembles the occlusion body. Very late genes express the polyhedrin protein, which is the main component of the *polyhedra*, and also the p10 protein, which is believed to play a role in *polyhedra* assembly [1]. These two late promoters are decisive factors for the use of baculoviruses as expression vectors.

1.3. Baculovirus Expression System

A baculovirus expression vector (BEV) is a recombinant baculovirus with genetically modified circular double stranded DNA with an inserted gene of interest. Genetically modified baculoviruses efficiently transfer foreign genes into eukaryotic insect host cells as *Spodoptera frugiperda* (Sf9) cells. The majority of these modified baculoviruses are based on the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) [10]. As already mentioned the proteins polyhedrin and p10 play a crucial role in the baculovirus expression vector system (BEVS). Originally, the focus was on the polyhedrin protein. The particular characteristic about this protein is that, although it is obligatory for the infection of insect host via oral route, it is not essential for *in vitro* infection. As a result, the polyhedrin sequence could be replaced with a foreign gene sequence, thus expressed by the strong polyhedrin promoter. This has the advantage that the modified virus particles can be differentiated phenotypically under the microscope because of the absence of the polyhedra envelope. Because the p10 protein is also not necessarily needed for production of BVs, as it only plays a role in production of polyhedra matrix, it could also be replaced by a foreign gene [3]. The p10 promoter-based transfer vectors do not produce a recognizable phenotype of virus particles [1, 11]. The p10 promoter is used for example to produce biopesticides, because of its persistence in the environment and the ability to infect insects naturally as the polyhedra envelope is still present. Furthermore these two proteins have strong promoters, which express large amount of proteins for the polyhedra envelope. The other advantage of these very late genes is that the production of proteins occurs after the production of infectious budded viruses, so, inter alia, cytotoxic proteins can be produced. Besides that, the size of insert can be varied, because the genome of the baculovirus is large and the insert does not affect the normal replication of viral DNA. The other benefit of baculovirus induced expression

of genes is the safety, as they only have insects as hosts and the large scale production of proteins, as insect cells can be cultivated in large scale for example in bioreactors which is still cost effective and has high efficiency of expression [1, 12]. The disadvantages of the BES are manageable, but significant. The first downside is that BES provides only transient expression of proteins, because baculovirus-infection of insect cells result cell death, and so you have to re-infect fresh cell cultures for protein production. The other problem is the different glycosylation of proteins as in mammalian cells, which synthetize shorter and less complex N-linked glycans [1, 13]. Despite that, generally the posttranslational modifications such as fatty acid acylation, disulfide bond formation and phosphorylation are similar to processes in mammalian cells, which make baculovirus expression system in insect cells important for protein production [12, 14, 15].

With BEVS it is possible to produce multiple proteins simultaneously by using multiple promoters and transfer vectors were constructed with combinations of different viral promoters as pHD and p10 [16]. Nowadays several purchasable kits based on multiple-promoter vectors are available as the one, which was used in this thesis and will be described in the next paragraph.

1.4. Bac-to-Bac® Baculovirus Expression System from Invitrogen

Bac-to-Bac® is a method to generate genetically modified baculoviruses which produce recombinant proteins in insect cells. This method is based on an efficient site-specific transposition system. There are different donor plasmids available. In this thesis the pFastBac™ Dual vector will be used, which has two multiple cloning sites controlled by baculovirus promoters in which your genes of interest can be inserted. The baculovirus-specific promoters in pFastBac™ Dual vector are the pHD and the p10 promoter in immediate vicinity. The pHD promoter is located on the sense- and the p10 promoter is located on the antisense strand of DNA (see Appendix 9.1). The advantages of this method are the fast identification and purification of recombinant baculovirus in less than two weeks [17]. The figure below describes the procedure for producing recombinant baculovirus using the Bac-to-Bac® System.

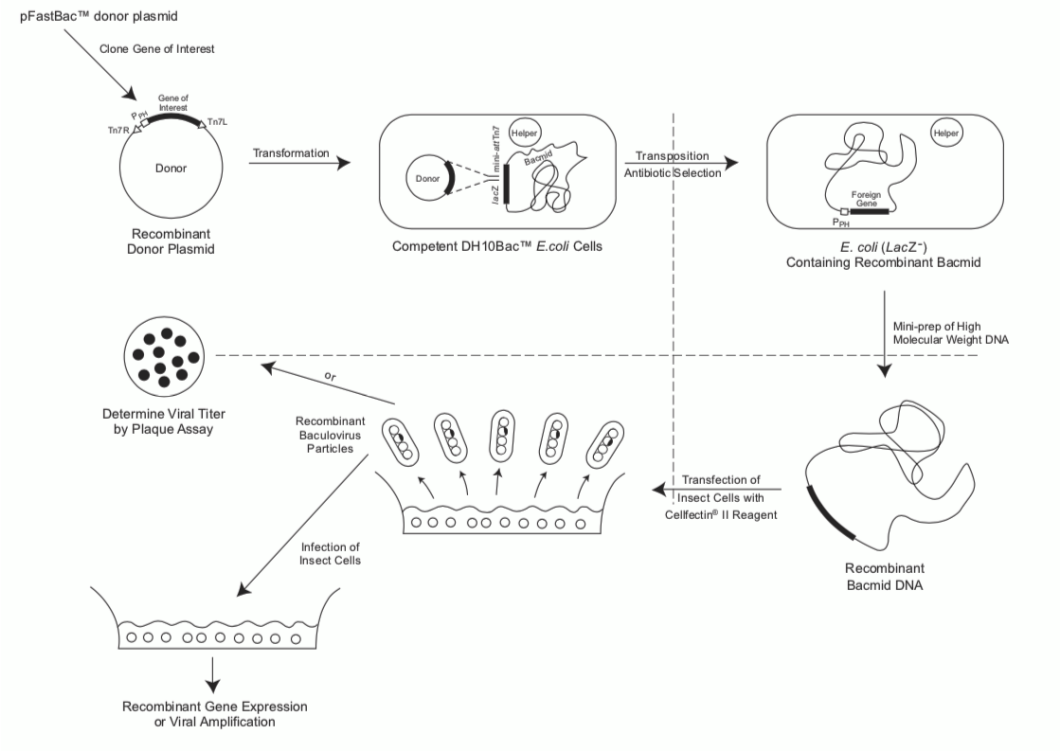

Figure 2: Generation of recombinant baculovirus using Bac-to-Bac® System. Modified from [17].

First the gene of interest is cloned into the multiple cloning sites under control of virus promoters. After successful cloning of desired gene into the donor plasmid, the donor plasmid was transformed into competent DH10Bac™ *E. coli* cells.. The DH10Bac™ cells contain helper plasmids and a shuttle vector between insect cells and *E. coli*, called bacmid. This shuttle vector can propagate in both host species [18]. The bacmid contains the genome for baculovirus generation. In the DH10Bac™ cells the transposition occurs. Transposition is a process, where genes can change their position between genomes or in the genome with the help of transposon elements as Tn7 which has the ability to insert into a specific site called “attachment” site or attTn7 [19]. The bacmid, bMON14272, contains a kanamycin resistance marker, a *lacZα* segment where the attTn7 element is inserted. For transposition is the enzyme transposase required, which is encoded in the helper plasmid pMON7124. After transformation into DH10Bac™ *E. coli* kanamycin-resistant colonies were selected and the isolated bacmid transfected into Sf9 cells, where baculoviruses are produced [17, 20]. The site-specific transposition to insert foreign genes has several advantages over producing baculoviruses by homologous recombination as the efficiency, which is higher [20].

1.5. Insect Cells for Production of Baculoviruses

Insect cells are baculovirus hosts *in vitro*. Commonly *Spodoptera frugiperda* cell cultures are used for baculovirus production and protein expression, like Sf21 cell line or its clonal isolate Sf9 [6, 21, 22]. These two cell lines are originally from the IPLBSF-21 cell line, derived from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda* [21]. The cell line Sf21 was developed by James E. Vaughn in 1977. He found out, that the cells are sensible for baculovirus infection [23]. In 1983 its clonal isolate Sf9 were developed. Sf9 cells were produced with several advantages compared to Sf21 cells; in fact they are more robust, more sensitive for baculovirus infection and produce high titers of budded viruses (BVs), cell growth is faster and they are adaptable to serum-free medium [14, 24]. Alternatively for protein expression a cell line from *Trichoplusia ni*, BTI 5B1-4 or commercially known as High Five cells, were used. The BTI 5B1-4 cell line was derived from the ovarian cells of the cabbage looper *Trichoplusia ni*. The High Five cells are more suitable for recombinant protein production as the protein expression level is 5-10 times higher than in the *Spodoptera frugiperda* cell cultures [21]. These three cell lines are the most used cell lines for baculovirus expression system. All of them do not require CO_2 and grow optimally at 27-28°C [14].

1.6. Green Fluorescent Protein as Expression Marker

The green fluorescent protein, short GFP, is a fluorescent protein originally isolated from the jellyfish *Aequorea victoria*. This protein is nowadays the most important reporter in cell biology, as you can visualize GFP under fluorescence microscope or flow cytometry. Because wild-type GFP has a low fluorescent signal, several mutants of the GFP were generated, such as the EGFP which will be used in this thesis [25, 26]. EGFP has an excitation wavelength of 488 nm and emission wavelength of 507 nm [27].

1.7. Aim of Thesis

Nowadays there are several protein expression systems. One of the most important is the baculovirus expression vector system. In this thesis the baculovirus expression system should be established in adherent Sf9 insect cells with the help of the Bac-to-Bac® system from Invitrogen. The protein to be expressed should be the EGFP, which serves as a protein expression marker. The produced EGFP shall be detected with fluorescence microscope. In addition, preliminary experiments for future baculovirus titer determination with qPCR will also be investigated. Different extraction methods

for virus DNA will be tested as kit-based and phenol/chloroform extraction. These results are significant for analysis of the virus DNA with qPCR. Furthermore the difference in Cq values between different virus titer will be analyzed.

2. Methods and Materials

2.1. Molecular biological Methods

2.1.1. Plasmid Isolation

2.1.1.1. Via Kit

The GenElute™ Plasmid Miniprep Kit (Sigma) was used to isolate the plasmid from the OneShot™ Top10 *E. coli* and from the MAX Efficiency™ DH10Bac™ cells. The procedure has been done without modification as provided in the manual. [17] The amount of centrifuged cell suspension was 2 ml. For the elution of the DNA from the column, nuclease free water was used. The diluted DNA was stored in the freezer at -20°C.

The PureLink™ HighPure Plasmid DNA Miniprep Kit was subsequently used to isolate the recombinant bacmid from the MaxEffeciency™ DH10Bac™ *E. coli*. The isolation was accomplished according to the instructions. The amount of centrifuged cells was 2 ml. Nuclease free water was used to elute the DNA from the column. The bacmid solution was aliquoted and stored at -20°C.

2.1.1.2. Via Alkaline Lysis

The isolation of DNA from the transformed *E. coli* (OneShot™ Top10 *E. coli* and DH10Bac™ E. coli) cells was performed with alkaline lysis. [28] The solutions will be described in table 1. First 1.5 ml of the overnight culture was centrifuged (12.000 x g, 2 min). Then the pellet was resuspended in 200 µl Solution I. Afterwards the cells were lysed with 200 µl of Solution II and mixed by inverting. Finally 350 µl of Solution III was added and inverted to neutralize the strong alkaline conditions. The mixture was centrifuged (12.000 x g, 15 min). The supernatant was subsequently transferred into new microtube with 800 µl isopropanol (100%) and mixed by inverting, whereby the DNA was precipitated and pelleted after centrifugation (12.000 x g, 15 min). After the centrifugation, the supernatant was discarded and the pellet was coated with 300 µl of ethanol (70%) and centrifuged again (12.000 x g, 3 min). After this wash step the ethanol was discarded and the pellet dried for 30 min at 37°C. Afterwards the

DNA was resuspended with 30 µl of nuclease free water and stored at -20°C in the freezer.

Table 1: Buffer for alkaline lysis

Buffer	
Solution I	50 mM Tris-HCl, pH 8
	10 mM EDTA
	RNase A (final concentration 100 µg/ml)
Solution II	200 mM sodium hydroxide
	1% SDS
Solution III	3 M potassium acetate
	pH 5.5 (adjusted with acetic acid)

2.1.2. Colony PCR

To verify positive clones of bacteria, so they successfully included the plasmid, single colonies were picked with a pipet tip from the agar plates and transferred into sterile 1.5 ml microtubes with 15 µl distilled water and incubated for 15 minutes at room temperature. After the incubation step, 5 µl of bacteria suspension were transferred into 0.5 ml microtubes and incubated for 5 min at 95°C, so the cell membrane lyse. Then the PCR-Mastermix was added, which is described in the table 2.

Table 2: Reaction setup for colony PCR with Taq Polymerase

Amount for each sample	**Components**
1.25 µl	10xPCR Buffer
0.5 µl	MgCl2 (50 mM)
0.5 µl	Forward Primer (10µM)
0.5 µl	Reverse Primer (10µM)
1.0 µl	dNTP Mix (10 mM)
0.04 µl	Taq Polymerase (5 U/µl)
3.75 µl	Nuclease free water
5.0 µl	Template-DNA

Table 3: Thermocycler conditions for colony PCR with Taq Polymerase

Step	Temperature	Time	Cycles
Initial	95°C	2 min	1
Denaturation	95°C	45 sec	35
Annealing	TM_{Primer}	40 sec	35
Extension	72°C	1 min	35
Final Extension	72°C	10 min	1

2.1.3. Bacmid PCR

A bacmid PCR was performed to verify the presence of the gene of interest in the recombinant bacmid. For this, primers were chosen which bind at the pUC/M13 region of the bacmid vector. The product size is predefined in the manufacturers user guide, which in this case supposed to be around 2560 bp plus the size of the insert (bacmid transposed with pFastBac™ Dual vector) [17]. In table 4 the mastermix and the program were described.

Table 4: Reaction setup for bacmid PCR with GoTaq® Polymerase

Amount for each sample	Components
4.0 µl	5x Green GoTaq® Reaction Buffer
1.0 µl	Forward Primer (10µM)
1.0 µl	Reverse Primer (10µM)
0.4 µl	dNTP Mix (10 mM)
0.1 µl	GoTaq® G2 DNA Polymerase (5 U/µl)
13 µl	Nuclease free water
0.5 µl	Template-DNA

Table 5: Thermocycler conditions for bacmid PCR with GoTaq® Polymerase

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	45 sec	30
Annealing	TM_{Primer}	45 sec	30
Extension	72°C	4:30 min	30
Final Extension	72°C	5 min	1

2.1.4. Standard PCR

A standard PCR was performed to verify the presence of the gene of interest in the pFastBac™ Dual vector. The chosen forward primer binds in a region before the multiple cloning site and the reverse behind the multiple cloning site, in which the gene was cloned. The sequences of the primer are listed in chapter 2.3.9. For the standard PCR the Phusion™ Polymerase, Taq Polymerase and the GoTaq® G2 Polymerase were used. The mastermix and the program which depend to various polymerases are described in tables 6-11.

Table 6: Reaction setup for standard PCR with Phusion™ DNA Polynerase

Amount for each sample	Components
4.0 µl	5x Phusion™ HF Buffer
1.0 µl	Forward Primer (10µM)
1.0 µl	Reverse Primer (10µM)
0.4 µl	dNTP Mix (10 mM)
0.2 µl	Phusion™ High-Fidelity DNA Polymerase (2 U/µl)
12.4 µl	Nuclease free water
1.0 µl	Template-DNA

Table 7: Thermocycler conditions for standard PCR with Phusion™DNA polymerase . Melting Temperature is individual for each primer pair.

Step	Temperature	Time	Cycles
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	TM_{Primer}	30 sec	30
Extension	72°C	30 sec	30
Final Extension	72°C	10 min	1

Table 8: Reaction setup for standard PCR with Taq Polymerase

Amount for each sample	Components
1.25 µl	10xPCR Buffer
0.5 µl	MgCl2 (50 mM)
0.5 µl	Forward Primer (10µM)
0.5 µl	Reverse Primer (10µM)
1.0 µl	dNTP Mix (10 mM)
0.04 µl	Taq Polymerase (5 U/µl)
8.2 µl	Nuclease free water
1.0 µl	Template-DNA

Table 9: Thermocycler conditions for standard PCR with Taq Polymerase . Extension time adjusted to EGFP. Melting Temperature is individual for each primer pair.

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	45 sec	35
Annealing	TM_{Primer}	40 sec	35
Extension	72°C	1 min	35
Final Extension	72°C	10 min	1

Table 10: Reaction setup for standard PCR with GoTaq® Polymerase

Amount for each sample	Components
4.0 µl	5x Green GoTaq® Reaction Buffer
1.0 µl	Forward Primer (10µM)
1.0 µl	Reverse Primer (10µM)
0.4 µl	dNTP Mix (10 mM)
0.1 µl	GoTaq® G2 DNA Polymerase (5 U/µl)
13 µl	Nuclease free water
0.5 µl	Template-DNA

Table 11: Thermocycler conditions for standard PCR with GoTaq® Polymerase . Extension time adjusted to EGFP. Melting Temperature is individual for each primer pair.

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	1 min	35
Annealing	TM_{Primer}	1 min	35
Extension	72°C	1 min	35
Final Extension	72°C	10 min	1

2.1.5. Gel Electrophoresis

The PCR products were separated in an agarose gel. To identify the length of the fragments, a DNA marker, such as GeneRuler™ 1 kb DNA Ladder Plus, was used. To prepare the samples for loading into wells, a loading dye was added. Loading buffer contains bromophenol blue, glycerin and other supplements. Bromophenol blue is a dye which helps to visualize DNA migration during gel electrophoresis. Glycerin causes the sinking of the sample into the well.

2.1.6. Preparing chemically competent DH10Bac™

The MaxEfficiency™ DH10Bac™ cells were incubated overnight in DH10Bac™ medium [LB medium with kanamycin (50µg/ml) and tetracyclin (10µg/ml)]. From the starter culture, 1.5 ml was picked and transferred into 60 ml of fresh DH10Bac medium and incubated at 37°C for few hours until it reached OD_{600} between 0.5 and 0.8. Then, 25 ml of the cell suspension was poured into pre-chilled 50 ml falcon tubes and incubated on ice for 15 min. The falcons were centrifuged (3.000 x g, 10 min, 4°C), the supernatant disrupted and the resulted pellet was resuspended with 15 ml of cold Tbf1 buffer and centrifuged (3.000 x g, 10 min, 4°C) again. After discarding supernatant the pellet was gently resuspended in 2.5 ml of Tbf2 suspension. The resuspended cells were incubated 5 min on ice. In this time liquid nitrogen was poured in a container and microtubes were placed in a rack into the liquid nitrogen. Then competent cells were aliquoted into the microtubes and stored at -80°C. The used solutions are described in table 12.

Table 12: Solutions for preparing chemically compentent DH10Bac™ *E. coli*

Solutions	
Tbf1	30 mM potassium acetate
	100 mM KCl
	10 mM $CaCl_2$
	50 mM $MnCl_2$
	15 % Glycerin
	pH = 5.8 (adjusted with acetic acid)
Tbf2	10 mM MOPS
	75 mM $CaCl_2$
	10 mM KCl
	15 % Glycerin
	pH = 6.8 (adjusted with potassium hydroxide)

2.1.7. Transformation

2.1.7.1. OneShot™ Top10 E. coli

To amplify the pFastBac™ Dual - EGFP vector, OneShot™ Top10 *E. coli* cells were used. To transform the plasmid into the bacteria first the *E. coli* was gently thawed on ice and 200 ng of the plasmid was added. Then the cells were incubated on ice for 30 minutes before the heat shock for 30 seconds at 42°C was performed. After the heat shock, the cells were incubated for another 2 minutes on ice. Then 250 µl of pre-warmed S.O.C. medium was added to each vial and incubated at 37°C for 1 hour at 225 rpm in a shaking incubator. Each incubated transformation mixture was spread on LB agar plates with appropriate antibiotic and incubated overnight at 37°C. In this case, we used Carbenicillin, so only transformed bacteria can grow because of the antibiotic resistance in the transformed vector.

2.1.7.2. MaxEfficiency DH10Bac™ E. coli

To produce recombinant baculovirus for the expression of proteins, a shuttle vector for *E. coli* and insect cells, called bacmid, is needed. The DH10Bac™ cells contain a parent bacmid bMON14272 and a helper plasmid pMON7124 [17]. When the donor plasmid is transformed into the cells, a transposition occurs.

First the competent cells must be thawed on ice and then 100 µl of the competent cells was aliquoted into chilled round bottomed polypropylene tubes. After that 100 ng of

DNA, in this case pFastBac™ Dual EGFP vector, was added to the cell aliquot and gently mixed by tapping the tube. After the incubation of 30 min on ice the heat shock is following for 45 seconds at 42°C in a water bath. Then the vials are placed for another 2 minutes on ice and 0.9 ml of pre-warmed S.O.C. medium was added and incubated at 225 rpm for 5 hours at 37°C in a shaking incubator. When incubation time is over different dilutions (undiluted, 1:10, 1:100) with S.O.C. medium were made and spreaded on LB agar with gentamicin (7 µg/ml), kanamycin (50 µg/ml), tetracyclin (10 µg/ml), IPTG and Bluo-Gal.

2.1.8. Blue-White Screening

Blue-White Screening should be performed to prove the successful transposition of the donor plasmid into the parent bacmid. When the donor plasmid with the gene of interest is inserted into the parent bacmid with the help of the helper plasmid the lacZ-promoter is disrupted and cannot produce LacZα peptide. Colonies containing the recombinant bacmid appears white, in contrast of the blue colonies containing bacmids without the insert.

To perform Blue-White-Screening 5µl, 10µl and 20µl of IPTG (200 mg/ml) and 150 µl of Bluo-Gal (10 mg/ml) were spreaded on LB agar plates with appropriate antibiotics. The IPTG induce the expression of the LacZ gene and Bluo-Gal serves as a substrat for the β-Galactosidase and produces darker blue than the alternative X-Gal.

2.1.9. Viral Nucleic Acid Extraction

2.1.9.1. Via Kit

To isolate the baculovirus genome the innuPREP Virus DNA Kit from Analytik Jena was used. The procedure was done according to instructions. The amount of used virus stock was 200 µl. For the elution two steps were repeated with 30 µl of 70°C nuclease free water. The diluted DNA was stored for a few days at 4°C.

2.1.9.2. By Phenol/Chloroform Extraction

Another way to isolate the viral DNA is to perform a phenol/chloroform extraction. All solutions and buffers were described below. First 500 µl of virus stock was transferred into an ultracentrifuge microtube and centrifuged (100.000 x g, 4°C, 45 min). After that, the supernatant was discarded and resulted pellet was resuspended in 1 ml virus disruption buffer and incubated for 4 hours at 37°C in a shaking incubator. By the end of incubation, 800 µl of virus solution were transferred

into 2 ml microcentrifuge tube and 1 volume of phenol was added, mixed well and centrifuged (15.000 x g, 10 min). Thereafter the upper liquid phase, where the DNA is located, was transferred into a new microcentrifuge tube without touching the interphase, which consists of denaturated proteins, and 1 volume phenol/chloroform/isoamylalcohol solution was added and centrifuged again (15.000 x g, 10 min). The isoamylalcohol prevents foaming of the solution. The upper liquid phase was transferred into a new microcentrifuge tube. To the transferred nucleic acid solution 2,5 volumes of ethanol (100%) and 0,1 volume of potassium acid solution was added, mixed and stored at -20°C overnight. Next day the cold solution was centrifuged (maximum speed, 30 min, 4°C) and the supernatant was replaced with ethanol (70%) and centrifuged again (15.000 x g, 5 min). Then the pellet was placed at 37°C until the ethanol was evaporated and diluted with 60 µl nuclease free water.

Table 13: Virus disruption buffer for phenol/chloroform extraction. Modified from [29].

Buffer	
Virus disruption buffer	10 mM Tris-HCl
	10 mM EDTA
	0.25% SDS
	Proteinase K (final concentration 500 µg/ml)

2.1.10. Viral Quantitative Real Time PCR

To analyze the difference of gene expression in different titers of baculovirus stocks quantitative real time PCRs were performed. Gene specific primers with a product size of around 100bp were chosen. The master mix and the program were described in tables 14-15. A melt curve was also performed in the temperature range from 60°C to 95°C in steps of 0.1°C per second.

Table 14: Reaction setup for virus qPCR with SYBR® Green

Amount for each sample	**Components**
10.0 µl	SYBR® Green Master Mix
4.0 µl	Primer Mix (10µM)
0.5 µl	DMSO
x µl	Nuclease free water
>5 ng	Baculovirus-DNA

Table 15: Thermocycler conditions for virus qPCR with SYBR® Green

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	30s	45
Annealing	TM_{Primer}	10s	45
Extension	72°C	10s	45
Final Extension	72°C	10s	1

To analyze the quantification and melt curve data, CFX Manager™ Software from BioRad was used.

2.2. Cell Biology Methods

2.2.1. Subculture Sf9 Cells

Sf9 cells were grown adherent in a monoculture in T75 flasks. When density reached 90%, the cells were splitted. First, the old medium was aspirated and collected. To dislodge the monolayer, the flask was first tapped and then sloughed with 6 ml of new medium. In a new flask 3 ml of the cell suspension, 3 ml of sterile filtered conditioned medium and 9 ml of fresh Sf-900 II medium were poured in and incubated for approximately 1 week at 27°C, until the monolayer reached the density of 90% again.

2.2.2. Cell Counting

For cell counting the hemocytometer type Neubauer improved was used. To stain dead cells, trypan blue solution (0.4%) was added to the cell suspension. Trypan blue is a vital stain, which only integrates into dead cells trough defective membrane. As trypan blue is cytotoxic by binding to proteins, cell counting was carried out within 5 min after staining [30, 31].

2.2.3. Cryopreservation of Sf9 Cells

Per flask 1 ml of freezing medium was used to dislodge the monolayer by sloughing method or scrapping them. The resulted cell suspension was aliquoted in cryogenic vials and placed in a freezing container with isopropanol (100%), so the cells were cooled down slowly.

Table 16: Freezing medium for Sf9 cells

Freezing medium
80 % Grace's Insect Medium Supplemented
10% FBS heat-inactivated
10% DMSO

2.2.4. Thawing Sf9 Cells

To initiate cell culture from frozen stock, the vial was warmed in a water bath, so the cells are liquid. The cell suspension was put in a 15 ml falcon tube with 10 ml of fresh Sf-900 II medium, so that the DMSO in the freezing medium is diluted 1:100. Then the tube was centrifuged (100 x g, 5 min). After the old medium was discarded, new medium was poured to the cells and a cell count with hemocytometer was performed. After cell count 3.75E+06 cells, as GIBCO™ recommended, were seeded in each T75 flask with fresh Sf-900 II medium.

2.2.5. Transfection of Sf9 Cells with recombinant Bacmid

For the transfection 8E+05 cells in 2 ml of Sf-900™ II medium were seeded in each well of a culture plate with 6 wells. For each bacmid construct, 2 wells were used in columns. The plates were incubated for at least 2 hours at 27°C, so the cells are well attached to the bottom of the wells. After 1 h of incubation the transfection solutions were prepared, which are described in the table below.

Table 17: Transfection solutions

Solution A	Solution B
1-2 µg bacmid DNA	8 µl Cellfectin® II Reagent
100 µl serumfree medium	100 µl serumfree medium

In this case, Grace's Insect Medium (unsupplemented) was used for the transfection solutions. After preparing each solution, the solution B was added dropwise to solution A and gently mixed by tapping the bottom of the microcentrifuge tube. The merged solution was incubated for 45 min at room temperature. Following incubation, 0.8 ml of Grace's Insect Medium (unsupplemented) was added to the transfection solution and mixed gently again by tapping the bottom of the microcentrifuge tube. Then the incubated cells were placed under the sterile

workbench and the medium from the wells were removed and the transfection solution was added dropwise to each well. For the control cells in each culture plate 1 ml of Grace's Insect Medium (unsupplemented) was added instead of transfection solution. After addition of transfection solutions, the plates were incubated for 5 hours at 27°C. Following incubation the transfection solutions were removed and replaced with 2 ml of Sf-900™ II medium, supplemented with 50 µg/ml gentamicin, and left in the incubator at 27°C for approximately 5 days. The exact incubation length depends on the signs of viral infection, which are the increased cell size, the decrement of cell confluence, cessation of cell growth, granular appearance, detachment and cell lysis [17]. The cells were checked with an inverted phase microscope including fluorescence light mostly at 100-200X magnification.

2.2.6. Isolation of P1 Virus Stock

To collect the virus, which was released into the fluid after approximately 72h, the medium from each well was collected in a 15 ml tubes and centrifuged at 500 x g for 5 minutes. After the centrifugation step the clarified supernatant, which do not contain cells and large debris, was transferred into a fresh 15 ml tube, wrapped in aluminum foil to protect the virus from light, and stored at 4°C.

2.2.7. Amplification of P1 Virus Stock

To generate a high-titer P2 viral stock from a low-titer P1 viral stock, new Sf9 cells were infected with the P1 stock. Two different ways were described below.

2.2.7.1. Infecting Cells in a 6-Well Culture Plate

First 2E+06 cells in 2 ml of Sf-900™ II medium were seeded in each well of the culture plate and left in the incubator for 1-2 h at 27°C, so the cells are well attached to the bottom of the wells. Then 0.2 ml of P1 virus stock were added to each well and incubated at 27°C for 4-5 days, until infection sights are visible. Post infection time the medium from each well was collected in a 15 ml tube and centrifuged at 500 x g for 5 minutes. The clarified supernatant was transferred into a fresh 15 ml tube, which is wrapped in aluminum foil to protect the virus from light, and stored at 4°C.

2.2.7.2. Infecting Cells in a T75 Flask

First 1.2E+07 cells were seeded in a T75 flask with 15 ml of Sf-900™ II medium and let to be set in the incubator at 27°C for 1-2 h, so the cells are attached to the bottom of the flask. After that 1 ml of P1 baculovirus stock was added to the flask and

incubated at 27°C for 4-5 days, until infection sights are visible. Post infection, the medium from the flask was collected in a 15 ml tube and centrifuged at 500 x g for 5 minutes. The clarified supernatant was transferred into a fresh 15 ml tube, which is wrapped in aluminum foil to protect the virus from light, and stored at 4°C.

2.2.8. Viral Plaque Assay

To determine the titer of the baculovirus stocks, a viral plaque assay in 6-well plate was performed. To immobilize the infected cells Sf-900™ Plaquing Medium was used, which is a mixture of culture medium and low melting agarose. For this 30ml of Sf – 900™ Medium were mixed with 10ml of 4% Agarose gel. First, 30 ml cell suspension with a cell concentration of 5E+05 cells/ml was prepared and aliquoted into each well of two 6-well plates. Following incubation time of 1 hour, to allow the cells to settle to the bottom, a confluency of 50% should be obtained. After that, an 8 – log serial dilution of the clarified baculovirus stock in Sf-900™ II medium was prepared. The medium from the incubated culture plates was removed and replaced with each virus dilution, started from 10^{-4} to 10^{-8} dilution. As a negative control, 1 ml of appropriate medium without virus was added. The culture plate containing the baculovirus was incubated for 1h at room temperature. After the 1 h incubation, the medium containing virus was removed and replaced with 2 ml of plaquing medium, which was prepared and stored at 40°C in a water bath, and left for 1 hour at room temperature to harden the agarose before moving the cells in a 27°C incubator for 7 – 10 days. The plates were sealed with parafilm to keep the agarose moisturized and prevent desiccation.

Post 7-10 days of incubation, the plates were stained with 1 mg/ml Neutral Red solution to visualize the plaques. For this, 0.5 ml of Neutral Red solution was added to each well and incubated for 1-2 hours at room temperature. After that the stain was removed and the plaques were visible to count. The titer was calculated with the formula below:

$$titer\ (pfu/ml) = number\ of\ plaques \times dilution\ factor \times \frac{1}{ml\ of\ inoculum/well}$$

2.3. Utilized materials

2.3.1. Instruments

Table 18: List of used instruments

Name	Manufacturer
Balance BP2100S	Sartorius (Göttingen)
Balance PB3002-S DeltaRange	Mettler Toledo (Greifensee)
Balance XS205DU	Mettler Toledo (Greifensee)
BioDocAnalyze	Biometra (Göttingen)
BioPhotometer	Eppendorf (Hamburg)
Centrifuge 3-8K	Sigma Aldrich (St. Louis, US)
Centrifuge 5417 R	Eppendorf (Hamburg)
Centrifuge 5424 R	Eppendorf (Hamburg)
GeneAmp® PCR System 2700	Applied Biosystems™ (Darmstadt)
Heraeus FunctionLine Incubator	Thermo Scientific™ (Darmstadt)
HeraSafe™ Workbench	Heraeus (Hanau)
Mastercycler® gradient 5331	Eppendorf (Hamburg)
Mastercycler® pro	Eppendorf (Hamburg)
Micro-Centrifuge DW41	Qualitron Inc (Bloomfield Hills, US)
NanoDrop™ One	Thermo Scientific™ (Darmstadt)
Rotilabo®-mini-centrifuge "Uni-fuge"	Carl Roth (Karlsruhe)
Shaking Incubator CERTOMAT® BS-1	B.Braun Biotech International (Melsungen)
Stirrer RCT basic	IKA WERKE (Staufen im Breisgau)
Thermomix Thermostat	B.Braun Biotech International (Melsungen)
Thermomixer® comfort	Eppendorf (Hamburg)
Ultracentrifuge	Beckman Coulter (Brea, US)

2.3.2. Kits

Table 19: List of used kits

Name	Manufacturer
GenElute™ DNA Miniprep Kit	Sigma Aldrich (St. Louis, US)
PureLink™ HiPure Plasmid Miniprep Kit	Invitrogen™ (Darmstadt)

2.3.3. Chemicals

Table 20: List of used chemicals

Name	Manufacturer
2-Propanol	Merck (Darmstadt)
3-Morpholinopropanesulfonic acid (MOPS)	Fluka (Seelze)
Acetic acid	Carl Roth (Karlsruhe)
Bluo-Gal	Invitrogen™ (Darmstadt)
Calcium chloride dihydrate	Carl Roth (Karlsruhe)
DMSO	Carl Roth (Karlsruhe)
Ethanol	Merck (Darmstadt)
Ethanol	Carl Roth (Karlsruhe)
Ethylenediamine tetraacetic acid	Carl Roth (Karlsruhe)
Glycerol	Carl Roth (Karlsruhe)
IPTG	Carl Roth (Karlsruhe)
LB-Broth (Lennox)	Carl Roth (Karlsruhe)
Manganese(II) chloride 4-hydrate	Carl Roth (Karlsruhe)
MgCl2 (50 mM)	Invitrogen™ (Darmstadt)
MidoriGreen Advance DNA Stain	NIPPON Genetics EUROPE (Düren)
NEEO Ultra Quality Rotgarose	Carl Roth (Karlsruhe)
PIPES	Sigma Aldrich (St. Louis, US)
Potassium acetate	Carl Roth (Karlsruhe)
Potassium chloride	Carl Roth (Karlsruhe)
Rotiphorese® 50x TAE Buffer	Carl Roth (Karlsruhe)
SDS	Carl Roth (Karlsruhe)
Sodium hydroxide	Carl Roth (Karlsruhe)
Tris hydrochloride	Sigma Aldrich (St. Louis, US)
Trypanblue 0.4% (v/v)	GIBCO™ (Darmstadt)

2.3.4. Enzymes

Table 21: List of used enzymes

Name	Manufacturer
GoTaq® G2 DNA Polymerase	Promega (Madison, US)
Phusion™ HighFidelity DNA Polymerase	Thermo Scientific™ (Darmstadt)
RNase A	Thermo Scientific™ (Darmstadt)
Taq Polymerase	Invitrogen™ (Darmstadt)

2.3.5. Antibiotics

Table 22: List of used antibiotics

Name	Manufacturer
Carbenicillin	Carl Roth (Karlsruhe)
Gentamicin	GIBCO™ (Darmstadt)
Kanamycin	Carl Roth (Karlsruhe)
Tetracyclin	Sigma Aldrich (St. Louis, US)

2.3.6. Cell Culture

Table 23: List of tools, reagents, media, cells that been used in cell culture lab

Name	Manufacturer
Cellfectin® II Reagent	GIBCO™ (Darmstadt)
CELLSTAR® multiwell culture plates (6 wells)	Greiner Bio-One (Kremsmünster, AU)
Falcon® Cell Scraper	Becton Dickinson (New Jersey, US)
Mr. Frosty™ Cold Storage	Thermo Scientific™ (Darmstadt)
S.O.C. medium	Invitrogen™ (Darmstadt)
Sf-900™ II SFM	GIBCO™ (Darmstadt)
T75 Flasks	Sarstedt (Nürnbrecht)
Grace's Insect Medium, unsupplemented	GIBCO™ (Darmstadt)
4% Agarose Gel	GIBCO™ (Darmstadt)
Neutral Red	Sigma Aldrich (St. Louis, US)

2.3.7. Plasmid

Table 24: List of used expression vectors

Name	Manufacturer
pFastBac™ Dual Expression Vector	GIBCO™ (Darmstadt)

2.3.8. Cell lines

Table 25: List of used cell lines

Name	Manufacturer
MAX Efficiency™ DH10Bac™ Competent Cells	GIBCO™ (Darmstadt)
OneShot™ Top10 Chemically Competent *E. coli*	Invitrogen™ (Darmstadt)
XL10-Gold® Ultracompetent Cells	Agilent Technologies (Santa Clara, US)
Sf9 Cells	GIBCO™ (Darmstadt)

2.3.9. Primer sequences

Table 26: List of used primer sequences in 5`-3`direction

Name	Sequence
Bacmid_fw	CCCAGTCACGACGTTGTAAAAACG
Bacmid_rev	AGCGGATAACAATTTCACACACAGG
pFastBacDual_fw	CATACCGTCCCACCATCG
pFastBacDual_rev	TTAGCTGCAATAAACAAGTTAAC
PHD_pFastBac_fw	TTCATACCGTCCCACCAT
qPCR_EGFP_fw	AGGACGACGGCAACTACAAG
qPCR_EGFP_rev	TTGCCGTCCTCCTTGAAGTC

2.3.10. Standards and Additives

Table 27: List of used standards and additives for several methods

Name	Manufacturer
5x Green GoTaq® Reaction Buffer	Ambion® (Darmstadt)
5x Phusion™ HF Buffer	Thermo Scientific™ (Darmstadt)
DNA Gel Loading Dye (6x)	Thermo Scientific™ (Darmstadt)
dNTPs 10 mM	Thermo Scientific™ (Darmstadt)
FastRuler High Range DNA Ladder	Thermo Scientific™ (Darmstadt)
GeneRuler 1 kb Plus DNA Ladder	Thermo Scientific™ (Darmstadt)
Orange DNA Loading Dye (6x)	Thermo Scientific™ (Darmstadt)

3. Results

3.1. Amplification of pFastBac™ Dual_EGFP

The plasmid pFastBac™ Dual_EGFP (pFBD_EGFP), which was kindly provided by University of Hamburg, was transformed into chemically competent OneShot™ Top10 *E. coli* in order to acquire higher amount of the plasmid (see chapter 2.1.7.1.). The initial DNA amount was 200 ng. After 24 hours there were many colonies on the agar plate, which was supplemented with carbenicillin. To verify the positive colonies, which contain the pFBD_EGFP vector, six of single colonies were taken and cultured in 3 ml LB-Lennox, which was also enriched with carbenicillin.

A Mini-Preparation with the GenElute™ Kit was performed on the cultures to extract the plasmid (see chapter 2.1.1.1.). The isolated plasmid was diluted in 30 µl nuclease free water. Afterwards the DNA amount was measured with the NanoDrop™. In table 28 the measurements are listed.

Table 28: DNA concentrations and ratios of extracted pFastBacDual_EGFP plasmids. The measurements were performed with NanoDrop™ One. For pure DNA ratios should be A260/A280 = 1.8-2.0 and A260/A230 ≥ 2.0.

Samples	ng/µl	A260/A280	A260/A230
1	411.2	1.84	1.81
2	612.8	1.66	1.21
3	459.5	1.89	2.23
4	392.1	1.87	2.30
5	314.6	1.87	2.04
6	408.1	1.88	2.30

3.2. Verifying the presence of EGFP gene in pFastBac™ Dual

Standard PCRs with Phusion™ Polymerase and GoTaq® Polymerase were performed (see chapter 2.1.4.) to verify the presence of the inserted EGFP gene that is located under the pHD promoter. The plasmid DNA amount in each master mix was approximately 200ng. For forward primer “pFastBacDual_fw” and for reverse primer “pFastBacDual_rev” were used (see chapter 2.3.9.). Agarose gel electrophoresis was performed to visualize the amplified fragments as seen in figure 3.

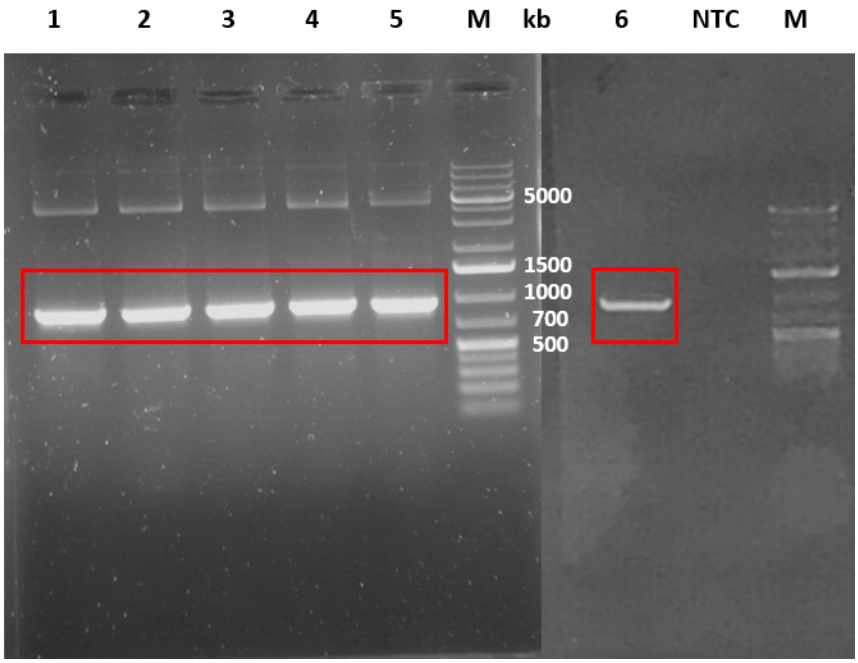

Figure 3: Gel electrophoresis of amplified pFBD_EGFP plasmid. Gel electrophoresis of amplified pFBD_EGFP plasmid with GoTaq® Polymerase using 'pFastBacDual_fw' and 'pFastBacDual_rev' primers. The PCR product bands, shown in red boxes, are around 1000bp. M = GeneRuler™ 1kb Plus DNA Ladder; NTC = no template control; 1-6 = pFBD_EGFP

3.3. Production of of bacmids in chemically competent DH10Bac *E. coli*

In order to produce bacmids, purchased DH10Bac™ *E. coli* were made chemically competent and were transformed with the pFBD_EGFP plasmid, subsequently cultured on agar plates (see chapter 2.1.7.2.). A blue-white selection was performed to identify positive colonies (see chapter 2.1.8.). Agar plates were supplemented with three different antibiotics (Gentamicin, Kanamycin, Tetracycline), Bluo-Gal and IPTG. Because the exact amount of IPTG was not known, three IPTG amounts (5µl, 10µl, 20µl) were tested. After 48 hours there are ideally two phenotypes of colonies on the agar plates as it is shown in Figure 4. The blue colonies are negative and the white colonies show successful transposition of the insert into the bacmid DNA.

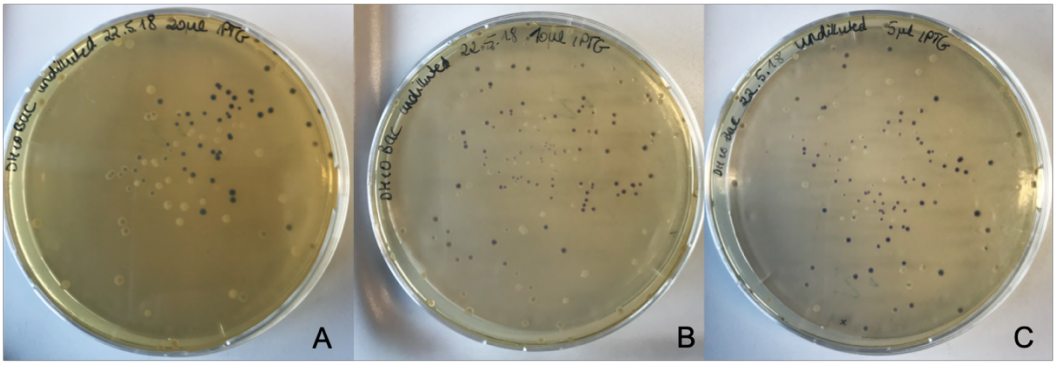

Figure 4: Blue-White-Selection of DH10Bac™ *E. coli*. Different IPTG amounts were used. A= 20µl IPTG on agar plate; B=10µl IPTG on agar plate; C=5µl IPTG on agar plate.

3.4. Verifying positive bacmid with EGFP insert

Bacmid PCR was performed to be certain, that the required insert with the EGFP gene is transpositioned into the bacmid. To analyze the bacmid, the plasmid of DH10Bac™ *E. coli* was isolated. Different plasmid isolation methods were performed, e.g. PureLink™ HiPure Plasmid DNA Miniprep Kit from Invitrogen™, GenElute™ Plasmid Miniprep Kit from Sigma Aldrich, and alkaline lysis (see chapter 2.1.1.1 – 2.1.1.2.). Afterwards, all DNA samples were measured with NanoDrop™ One. The measurements are listed in table 29.

Table 29: DNA concentrations and ratios of different mini preparation methods. The measurements were performed with NanoDrop™. For pure DNA ratios should be A260/A280 = 1.8-2.0 and A260/A230 ≥ 2.0.

Method	Samples	ng/µl	A260/A280	A260/A230
PureLink™ HiPure	**1**	31.1	1.67	1.47
Plasmid MiniPrep	**2**	15.6	1.85	1.01
Kit	**3**	5.5	2.06	0.37
	4	7.3	2.21	0.46
	5	3.2	3.57	0.22
	6	5.0	2.30	0.48
GenElute™	**7**	112.7	1.92	1.98
Plasmid MiniPrep	**8**	87.8	1.90	2.03
Kit	**9**	101.2	1.86	1.72
	10	90.9	1.94	2.06
Alkaline Lysis	**11**	1308.3	1.90	2.27
	12	2629.3	1.90	2.34
	13	2084.0	1.91	2.31
	14	2626.1	1.93	2.38
	15	3344.0	1.79	1.80
	16	2669.5	1.92	2.37
	17	2714.1	1.91	2.42
	18	2689.7	1.91	2.42
	19	1445.7	1.88	2.34
	20	546.3	1.79	1.75
	21	3412.5	1.89	2.59
	22	3301.8	1.89	2.76
	23	2677.0	1.88	2.38
	24	2624.2	1.85	2.43
	25	2526.5	1.84	2.51
	26	2852.0	1.90	2.50
	27	2915.5	1.87	2.42
	28	2551.9	1.86	2.57
	29	2817.0	1.84	2.42
	30	2030.3	1.86	2.44

After the isolation, bacmid PCR was performed to verify the insert. The primers 'Bacmid_fw' and 'Bacmid_rev' were used (see chapter 2.3.9.). The samples were isolated with PureLink™ HiPure Plasmid DNA Miniprep Kit and GenElute™ Plasmid MiniPrep Kit showing no bands at the desired fragment size, which is expected to be around 3200bp (figure 5). On the other hand, nearly all samples isolated with alkaline lysis show bands at around 3200bp (figure 6), which is an indicator for successful bacmid production and isolation.

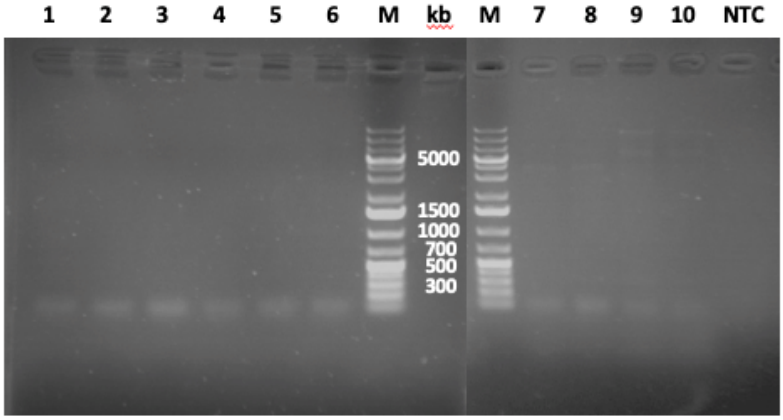

Figure 5: Gel electrophoresis of isolated bacmids. Samples 1-6 are bacmids isolated with PureLink™ Kit. Samples 1-10 are bacmids isolated with GenElute™ Kit. No significant bands are visible. M = GeneRuler™ 1 kb Plus DNA Ladder; NTC = no template control

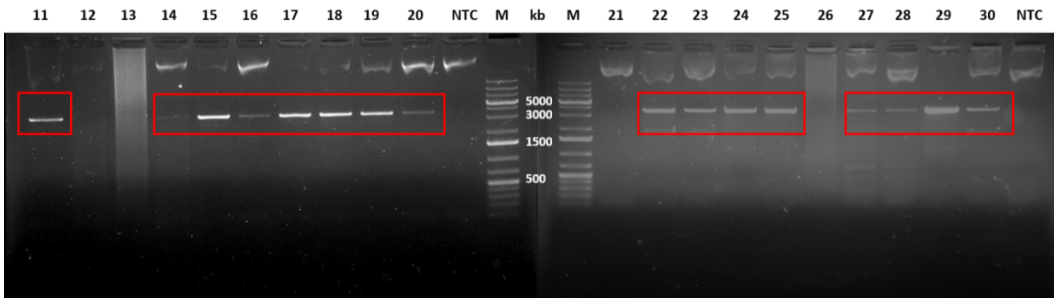

Figure 6: Gel electrophoresis of isolated bacmids. Samples 11-30 are bacmids isolated with alkalic lysis. Significant bands, framed in red boxes, of insert are visible at around 3000bp. M = GeneRuler™ 1 kb Plus DNA Ladder; NTC = no template control

3.5. Producing of recombinant Baculoviruses

The positive bacmids were processed to produce recombinant baculoviruses. Several indications for successful transfection were observed. The expression of EGFP was monitored by using fluorescence microscopy. Additionally, positive transfected cells display characteristic phenotypes, such as increased cell diameter, granular appearance, decrease of confluence and cell detachment. These phenotypes can be monitored with an inverted phase microscope. In figure 7, the progress of EGFP

expression of one transfection experiments were shown, whereas the characteristic phenotype of transfected cells is displayed in figure 8.

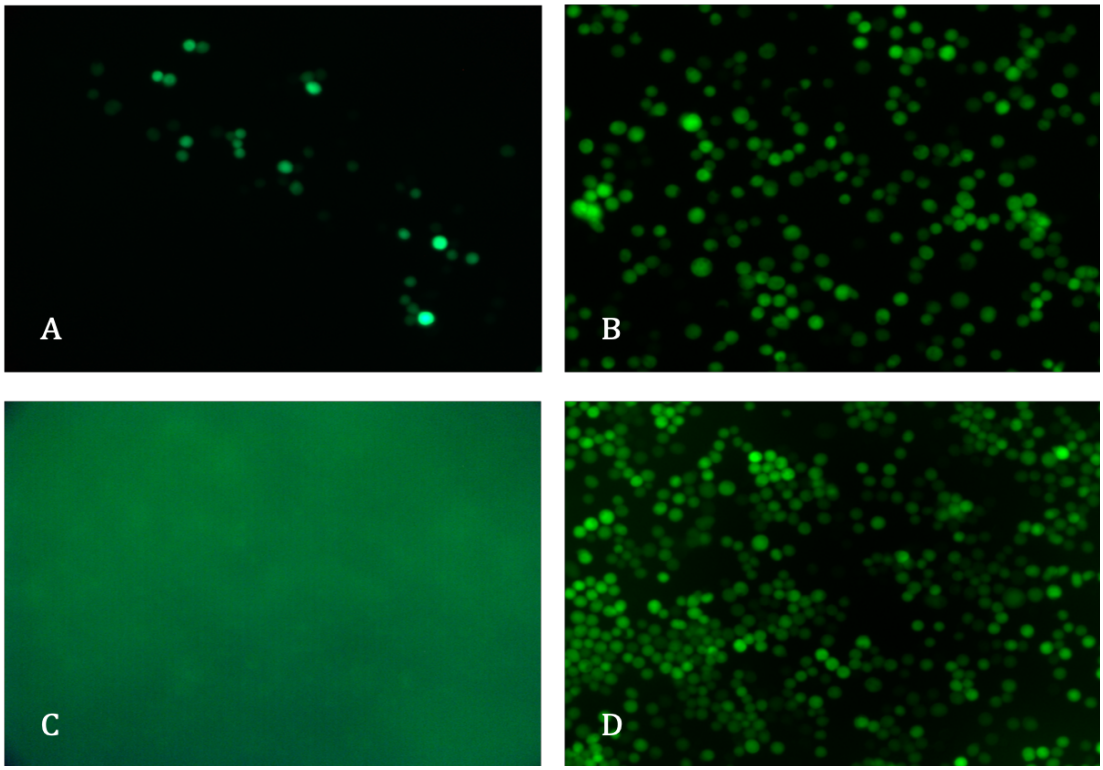

Figure 7: Fluorescence microscopy of transfected cells. Transfection experiment started on 06.06.18. A = EGFP expression of sample 1 after two days. B = EGFP expression of sample 1 after seven days. C = EGFP expression of sample 3 after two days. D = EGFP expression of sample 3 after seven days.

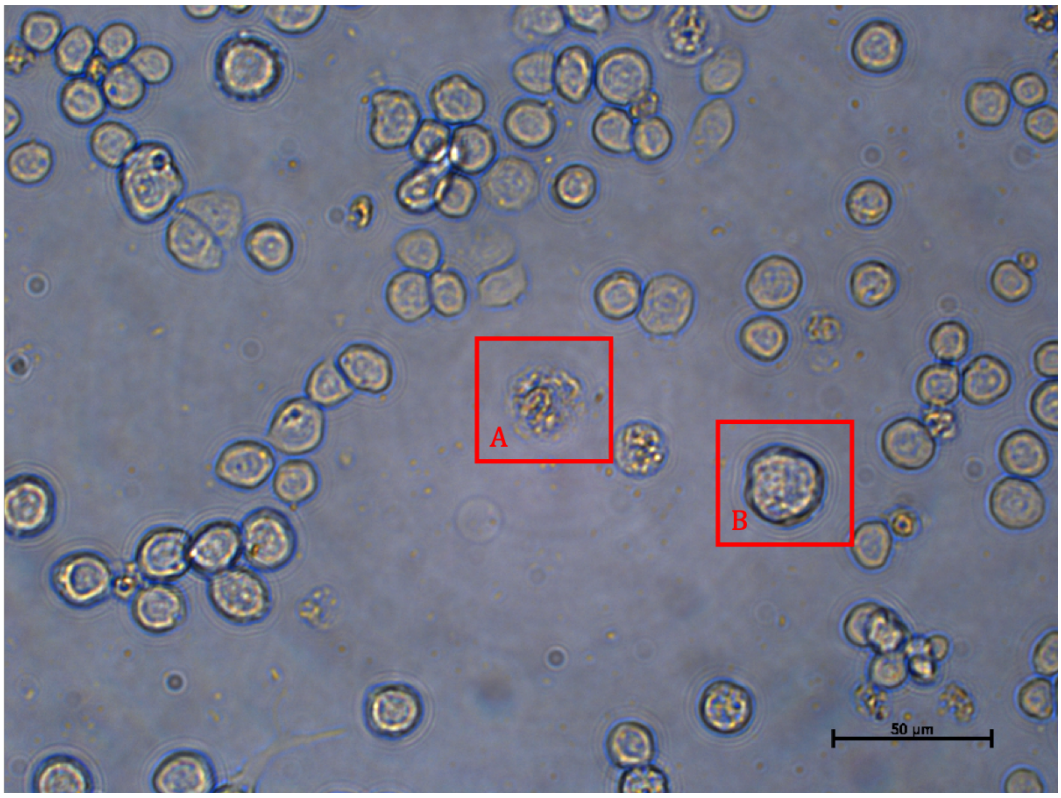

Figure 8: Characteristic phenotype of transfected insect cells. Transfection experiment performed on 06.08.18. Microscope picture were taken 3 days post transfection. A = typical granularity of cells; B = increased size of cell (ca. 25µm).

Post transfection, the baculovirus is located in the supernatant and can be harvested (see chapter 2.2.6.). The harvested baculovirus stock is the P1 viral stock. Because the amount of P1 viral stock is limited, cells were infected with the VP1 stock to obtain a P2 viral stock with higher titer. (see chapter 2.2.7.)

3.6. Determining Titer of Baculovirus Stock

In order to prove how concentrated the virus is, a plaque assay was performed. (see chapter 2.2.8.) Overall four virus stocks were analyzed. The results are shown in figure 9.

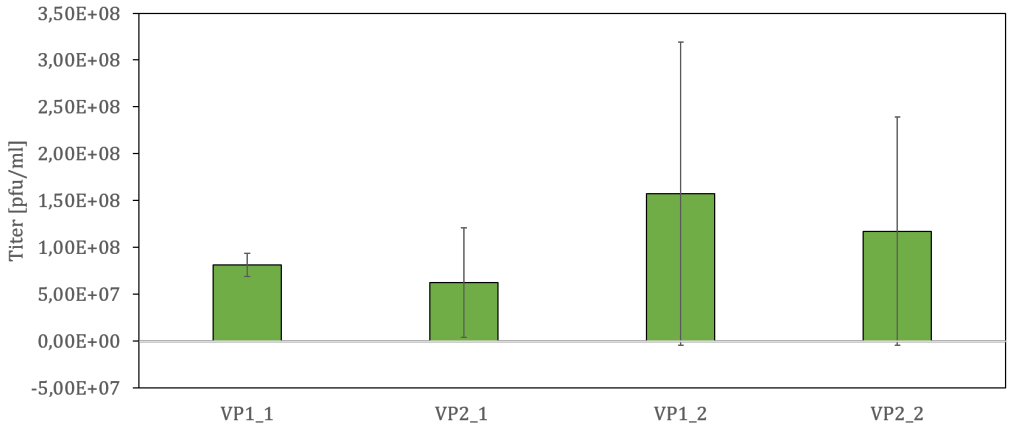

Figure 9: Plaque Assay analysis of four virus stocks. Standard error of mean is shown as bars. T-Test was performed, no significant differences existing (p>0.3).

1. Virus quantitative real time PCR

A virus qPCR was performed to determine differences between VP1 and VP2. For this, the Baculovirus DNA was isolated with the InnuPrep™ Viral DNA Kit. Additionally, the viral DNA was also isolated with phenol/chloroform extraction, to analyze differences between Kit-based and chemical extraction. The collection of VP1 samples have been done on 13.06.18 (VP1_1) and 07.07.18 (VP1_2), whereas VP2 samples were produced on 18.06.18 (VP2_1) and 13.07.18 (VP2_2). The virus stock sample which was isolated with Phenol/Chloroform extraction was the sample VP2_1. In table 30 - 31 the DNA amounts and ratios of each methods and stocks were summarized.

Table 30: Viral DNA Isolation with InnuPrep™ Viral DNA Kit . VP1_1 (three biological replicates), VP2_1 (five biological replicates), VP1_2 (five biological replicates), VP2_2 (five biological replicates). For pure DNA ratios should be A260/A280 = 1.8-2.0 and A260/A230 ≥ 2.0.

Virus Stock	Samples	ng/µl	A260/A280	A260/A230
VP1_1	**1**	3.70	1.42	0.2
	2	3.20	1.99	0.17
	3	2.40	1.28	0.12
VP2_1	**1**	2.20	1.67	0.08
	2	1.90	1.65	0.1
	3	1.90	1.79	0.06
	4	2.60	1.58	0.07
	5	1.80	1.82	0.16
VP1_2	**1**	2.23	0.13	0.14
	2	2.50	2.26	0.14
	3	2.30	2.35	0.09
	4	2.10	2.62	0.12
	5	3.60	1.58	0.15
VP2_2	**1**	1.70	2.13	0.11
	2	2.50	1.44	0.10
	3	2.00	1.37	0.05
	4	1.80	1.14	0.18
	5	2.00	1.46	0.05

Table 31: Phenol/Chloroform Extraction of two biological replicates of VP2_1 stock . For pure DNA ratios should be A260/A280 = 1.8-2.0 and A260/A230 ≥ 2.0

Virus Stock	Samples	ng/µl	A260/A280	A260/A230
VP2_1	**1**	7.00	1.43	0.69
	2	14.30	1.34	1.47

Viral DNA, which was isolated with the Kit, was used to perform a qPCR. The primer are gene-specific, in this case 'qPCR_EGFP_fw' and 'qPCR_EGFP_rev' primers (see chapter 2.3.9.). The amplification and melt curve data are shown in figures 10 - 11. Overall, there are two biological replicates for VP1 and VP2. Furthermore two technical replicates of each biological replicate were analyzed. Housekeeping genes were not used and also a standard curve was not generated.

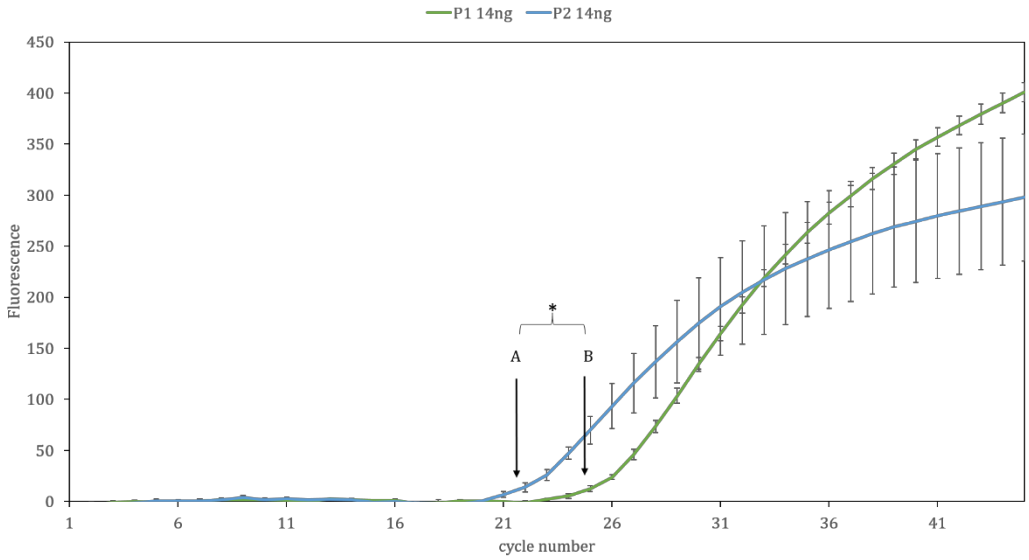

Figure 10: Amplification plot of VP1 and VP2 stocks extracted with kit. P1 and P2 virus stocks were isolated with InnuPrep Viral DNA Kit. DNA amount for master mix was 14 ng. Standard error of mean is shown as bars. Cq = quantitation cycle. Cq value of P1 virus stock is 21.76 and cq value of P2 virus stock is 24.85. T-Test was performed; difference of cq value is significant (* $p<0.05$).

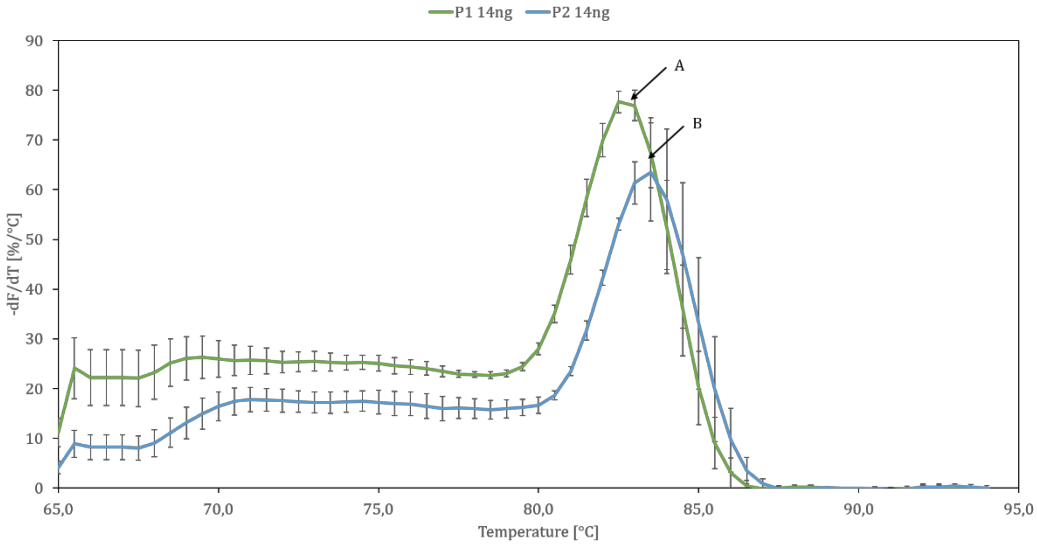

Figure 11: Melt curve of VP1 and VP2 stock extracted with kit. P1 and P2 virus stocks were isolated with InnuPrep Viral DNA Kit. DNA amount for master mix was 14ng. Standard error of mean is shown as bars. MT = melting temperature. MT of P1 virus stock is 82.75°C and MT of P2 virus stock is 83.25°C. T-Test was performed; MT difference is not significant ($p>0.09$).

Furthermore, the virus DNA was analyzed with virus qPCR to observe how phenol and chloroform residues affect amplification and melt curve plots of same virus DNA. Three technical replicates of each dilution were analyzed. Amplification plot and melt curve data of virus DNA which was extracted with phenol/chloroform are shown in figures 12 – 13. Housekeeping genes were not used and also a standard curve was not generated.

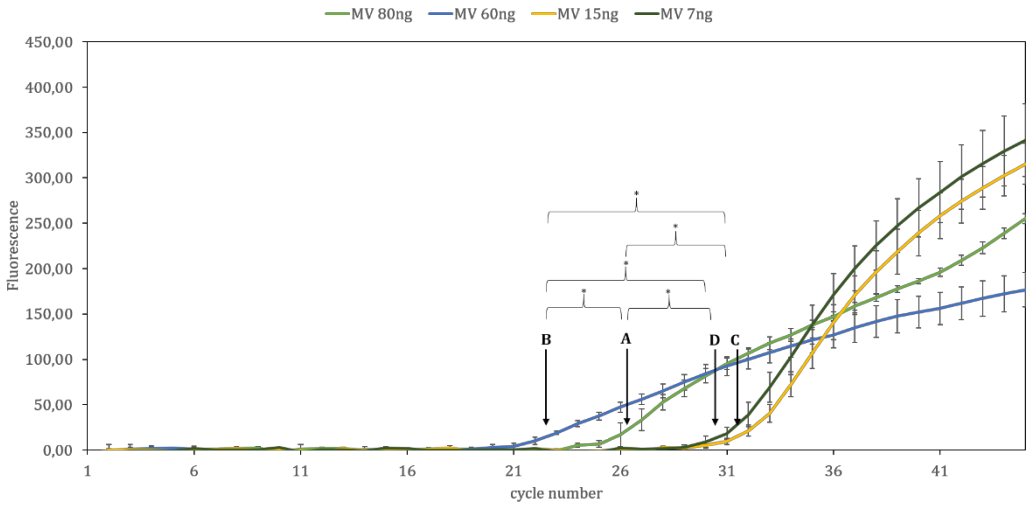

Figure 12: Amplification plot of VP2 stock extracted with phenol/chloroform. P2 virus stock was isolated with phenol/chloroform extraction. DNA dilution series were made. Standard error of mean is shown as bars. A = Quantitation cycle (cq) value of sample with virus DNA amount of 80 ng is 26.24; B = Cq value of sample with virus DNA amount of 60ng is 22.26; C = Cq value of sample with virus DNA amount of 15ng is 31.34; D = Cq value of sample with virus DNA amount of 7ng is 30.46. T-Test was performed: * p<0.05.

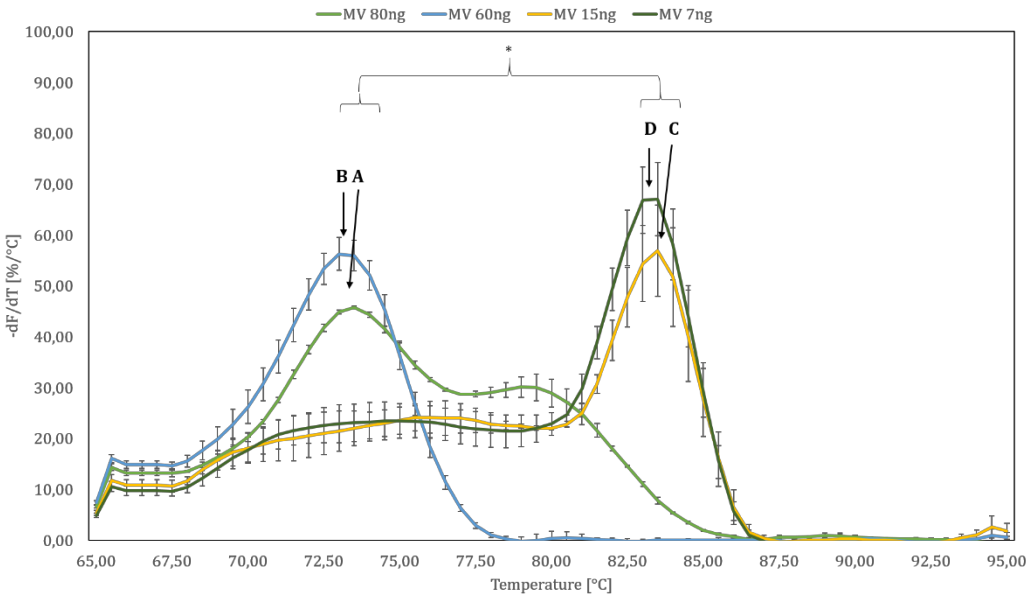

figure 13: Melt curve of VP2 stock extracted with phenol/chloroform extraction. P2 virus stock was isolated with phenol/chloroform extraction. DNA dilution series were made. Standard error of mean is shown as bars. A = Melt peak of sample with virus DNA amount of 80ng is 73.50°C; B = Melt peak of sample with virus DNA amount of 60ng is 73.17°C; C = Melt peak of sample with virus DNA amount of 15ng is 83.50°C; Melt peak of sample with virus DNA amount of 7ng is 83.33°C. T-Test was performed, melt peaks A and B are significantly different than melt peaks D and C (* p<0.05).

4. Discussion

4.1. Amplification and Analysis of Vector pFastBacDual_EGFP

There are many polymerases to choose like Taq-based and Pfu-based enzymes. *Taq* polymerase is an enzyme extracted from *Thermus aquaticus*, which is a thermophilic bacteria with a temperature optimum of 72°C. The specific feature of Pfu Polymerase obtained from *Pyrococcus furiosus* is that it can tolerate 95°C for over 4 hours and possess the ability for low error rate [32]. In this case two different polymerases were tested: GoTaq® G2 DNA Polymerase and Phusion™ HighFidelity DNA Polymerase. GoTaq® is a Taq-based polymerase, which is optimal for GC rich templates and produce high yield of PCR product but slow in comparison to Phusion™ Polymerase. This pfu based polymerase stands out through high accuracy and speed, high fidelity and producing blunt ends, which permits the fast TOPO cloning. Otherwise Phusion™ polymerase is not optimal for GC rich templates [33-35].

In our case the EGFP insert has a high GC content of 62 percent. In figure 3 the PCR was performed with the Taq-based Polymerase which produced bright and thick bands at around 1000bp, which shows the EGFP insert which should also have this fragment size because EGFP gene is around 720bp long plus around 200bp of vector backbone, where the primers are located [17, 36]. The bands in the upper part of gel at around 5000bp show the pFBD vector without insert, which is an indicator for colonies with mix of positive and negative plasmids. Sample 6 shows a thinner band, which could possibly be because this sample were located on another gel. The left gel picture shows not good photo quality, because DNA marker is also not bright. Figure 17 (see appendix 9.3) shows unsuccessful PCR amplification with Phusion™ Polymerase where no bands after gel electrophoresis are visible at desired fragment size. Figure 18 (see appendix 9.4) shows gel electrophoresis picture of an empty bacmid without an insert with fragment size of 300 bp.

4.2. Production and Extraction of Bacmids

Genes encoding components for baculovirus production are located on a vector, which can propagate in *E. coli* and in insect cells. This shuttle vector is called bacmid. The procedure for producing bacmids is explained in chapter 2.1.7.2. Nevertheless through blue white selection you can determine positive transposed bacmids. Because the exactly required IPTG amount was not known, several quantities were tested with different dilutions of bacteria suspension. All colonies on each agar plates

were counted afterwards. Noticeable is – contrary to expectations – that the 1:100 dilution show just few colonies. So it is recommendable to use undiluted or low diluted DH10Bac™ suspension. Furthermore positive white colonies on agar plate with 20µl IPTG appeared bigger than on the other plates with distinctive IPTG amounts. Overall our recommendation for IPTG is 10 or 20µl for distinguishable positive colonies. Also it is important to check more carefully putative positive colonies because if white colonies appears grey in the center, they are also negative.

To observe, if alleged positive bacmids actually have the desired insert, performing bacmid PCR is recommended. The size of the bacmid is around 135kb long and very sensitive to mechanical exposure. To extract it out of the transformed DH10Bac™ *E. coli* they are several methods. In this thesis three of them were compared. In table 2 it can be seen, that the nucleic acid samples isolated with the PureLink™ HiPure Plasmid MiniPrep Kit from Invitrogen are extremely low concentrated as 3.2 ng/µl to 31.1 ng/µl and the ratios not in the desired range for pure DNA. The GenElute™ Plasmid MiniPrep Kit from Sigma result also low DNA concentrations between 87.8ng/µl and 112.7 ng/µl, but contrary to the other Kit the ratios are much closer to the suggested range. Otherwise the alkaline lysis worked very well for isolating bacmids. The DNA concentrations ranges between 0.5 kb and 3.4 kb. Also the ratios of A260/A230 and A260/A280 are in suggested range, which means the DNA is theoretically free of inhibitors like phenol etc. and proteins. The utilized primer are modified pUC/M13 primer, which sequences were kindly provided by University of Hamburg. According to manufacturer the size of the PCR product should be 2560 bp plus size of insert, which is 720 bp in our case. So the PCR product is supposed to be around 3300 bp long. In figure 5 the samples extracted with both kits were analyzed with bacmid PCR. There are no bands in the desired size visible and at around 100 bp you can see primer dimer bands. Also negative bacmids, without transposed insert, have PCR products at the size of approximately 300 bp. The assumption here is because of Kit-based extraction bacmids got damaged because of mechanical DNA precipitation with spin columns. Otherwise in figure 6 you can see bright bands at the required fragment size of around 3500 bp, which is an indicator for existing insert. Alkaline lysis works better for extracting bacmids out of the DH10Bac™ *E. coli*, because there is no need for mechanical DNA preparation with spin columns etc. and the sensitive bacmid will not be impaired. Also mostly isolation kits are based on alkaline lysis but with different DNA precipitation methods like silica-based filter and anion exchanger columns.

4.3. Production of recombinant Baculoviruses

EGFP were cloned behind the pHD promoter. While production of virus components, the EGFP were also expressed and released into the environment and if cell is not lysed, you can see the accumulated EGFP therein. Though located in supernatant, the protein alone is too small to detect through microscope. This fluorescent protein is a good marker for transfection or virus infection because of detecting with fluorescence microscope or flow cytometer. In this thesis the detecting method is fluorescence microscopy. EGFP is a modified mutant of the wild type GFP with a extinction wavelength of 488 nm and emission wavelength of 507 nm [26, 27]. For that fluorescence filter were used to expose the sample with excitation wavelength of the fluorophore [37].

In figure 5 you can see the expressed EGFP, which is accumulated in the cells at that time. The partial pictures A and B in the upper part of the collage shows the process of transfection experiment, performed on 06.06.2018, of the first sample. You can monitor the early EGFP expression after two days, which is an indicator for optimal transfection conditions. In the lower part of the collage seen in figure 7 you can see the transfection process of second sample of the same experiment. In C there is still no EGFP expressed after two days but otherwise after seven days there are lot of transfected cells visible. That shows, that you have to do the transfection with technical and biological replicates, to be sure to have positive viruses with inserted gene. If you do not have a fluorescent protein like EGFP or mCherry as a marker, you have to evaluate the characteristic phenotype of transfected cells which is not secure because necrotic or rather apoptotic cells also show related phenotypes as shown in figure 8 [37].

4.4. Determining Virus Titer

For baculoviruses the titer is defined as plaque forming units per milliliter (pfu/ml), which basically means the infectious virus particles per milliliter of virus stock. To determine titer different methods are existing. In this thesis only plaque assay was performed. Plaque assay is a method were adherent cells were infected with 10–fold diluted virus solutions. The virus can infect cells for one hour and subsequently will be removed. After that the cells were covered with a mix of 4% agarose and cell culture medium, so that virus particles cannot spread over the whole cell culture but focuses on contiguous cells. Infected cells in immediate environment lyse and form cell free areas in the monolayer known as plaques (figure 14) [38].

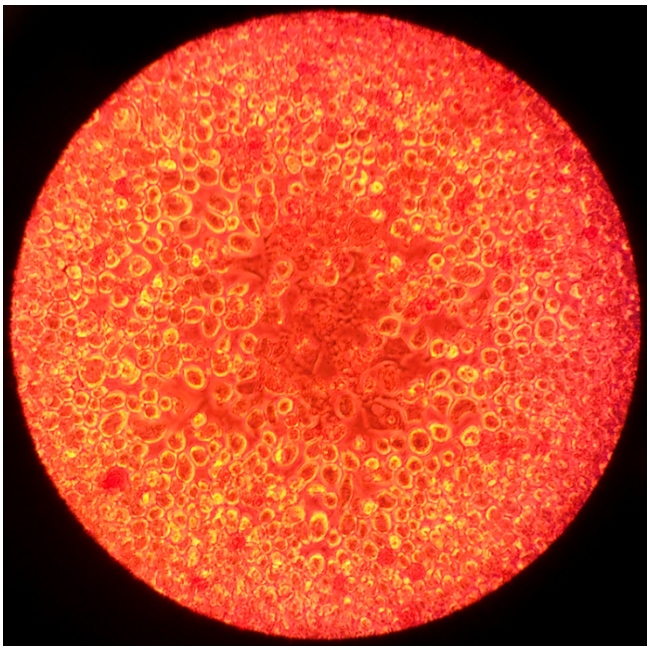

Figure 14: Plaque in Sf9 cell monolayer. Cell monolayer stained with Neutral Red. Transmitted light microscope with 10x magnification.

In this thesis all four virus stocks were analyzed by plaque assay shown in figure 9. Our assumption is that plaque assay is not a reliable method for determining titer because you always have to repeat same conditions for every virus stock like cell viability, temperature, virus dilution etc. Most of dilutions cannot be evaluated, because whether some plaques merge together or one big plaque over half well appeared. The calculated titer in pfu/ml can deviate between virus dilutions which are shown in the error bars in figure 9.

Another method to analyze baculovirus particles and to determine titer is to perform a qPCR with extracted virus DNA. In this thesis pilot experiments with virus qPCR were performed to analyze differences in amplification data and melt curves of different virus titers and to compare different methods to isolate virus DNA which should be suitable for qPCR. First virus DNA was isolated with a Kit purchased from Analytik Jena. The measurements of nucleic acid concentrations, shown in table 30, are very low as 1.7 – 3.7 ng/µl and the ratios are also not in the recommended range, which means that the DNA was not pure. Nevertheless the extracted DNA was used to perform a virus qPCR. Conspicuous is, that the Cq value of P2 virus stock sample is around 3 cycles earlier than the Cq value of P1 virus stock sample. In literature standard curves for determining titer of baculoviruses are already defined: the higher the titer, the earlier the Cq value [29]. In this thesis this statement was verified. Our assumption is, that high virus titer means that there are more infectious virus particles per milliliter, and that means that more of virus DNA with active promoter

were isolated from virus stock with higher titer. Correspondingly the melt peak of the P2 virus is shifted by plus 0.5°C than the P1 virus, the cause for that could probably be point mutations of insert but the correct reason for that have to be reviewed in other experiments. Virus DNA was also extracted with phenol/chloroform. This method is also not recommended because DNA concentrations are as low as with the Kit. Besides that, this method is very complex and also toxic for the user since phenol is highly corrosive [32] Nucleic acid isolated with phenol/chloroform is commonly full of PCR inhibitors as phenol and remaining proteins for example [39, 40]. Nevertheless virus DNA extracted with this method also were analyzed by qPCR. As result they are atypical amplification graphs of samples with 80 ng and 60 ng virus DNA. The Cq value of samples with 15 ng and 7 ng virus DNA are very late. In figure 13 you can see large shifts in melt peaks of 10 degrees although the initial nucleic acid is identical. The assumption here is, that the melt peaks at 73 degrees show phenol residues, because melt curves and amplification plots looks abnormal. Phenol inhibits Taq-based polymerases which are present in the SYBR Green II MasterMix [39].

5. Outlook

The baculovirus protein expression with Bac-to-Bac procedure from Invitrogen can be varied and optimized for individual aspirations. In the following an outlook of variations of single methods and reagents are subscribed.

Nowadays numerous different transfection reagents based on several methods are available. Cellfectin® II from GIBCO™, which were used in this thesis, is only one of many liposomal – based transfection substances. Furthermore you can also purchase non – liposomal transfection reagents. Some of existing liposomal and non – liposomal reagents with their prices, distributors and characteristics are listed in table 31. Non – liposomal transfection has the benefit of non – toxicity for cells, so you do not have to perform a medium change [41].

Table 32: Comparison of transfection reagents. Modified from [41].

Method	Name	Price per ml	Distributor
Liposomal	Cellfectin™ II	832€	GIBCO
	BaculoPORTER™	437€	Amsbio
	INSECTOGENE	150€	Biontex
	FlyFectin™	252€	Biozol
	Roti® Insectofect	185€	Carl Roth
	Insect GeneJuice®	299€	Merck Millipore
Non-liposomal	TransIT® Insect	281€	MirusBio
	baculoFECTIN II	460€	Cell Concepts

Furthermore we used adherent Sf9 cell culture for production of baculoviruses instead of suspension cell culture in shake flasks or bioreactor. The advantage of suspension culture is higher amount of P1 virus stock after transfection. Besides that for protein expression High Five cells are more suitable as protein expression is more increased compared to Sf9 cells [21].

For titer determination the virus qPCR must be advanced. First the virus DNA must be higher concentrated for efficient dilution series. For that other kits should be tested as HighPure Viral Nucleic Acid Kit from Roche as seen in other papers [29, 42] or usage of baculoQUANT All-in-One Virus Extraction and Titration Kit from Oxford Expression Technologies, which contain reagents to extract viral DNA and determine titer with qPCR. [43] Further housekeeping genes should be used for qPCR to compare expression of desired protein. In this case genes of AcMNPV, for example the IE-1 gene or the p80 can be utilized [44, 45]. Standard curve is essential for titer determining. For this known titer virus stock were diluted and afterwards cq values and titer in pfu/ml were plotted [29, 42].

In literature there are also other transfection reagent alternatives described such as polyethyleneimine (PEI), which is commonly used for mammalian transfection which is cost effective as you can use 50 ml of PEI stock for 25000 transfections [46]. In this thesis the qPCR-label SYBR Green II was used, which intercalate unspecific into synthesized double-stranded DNA. However, this fluorescent label can bind into other double-stranded DNA such as primer-dimers or contamination. To avoid this, specific fluorescent DNA labels like TaqMan can be used. These probes are custom for each target DNA, because they bind on primer with specific sequence and have a high

accuracy [42]. There are also different baculovirus titration methods described in literature. Some of them use the viable cell growth as parameter [47]. The measuring of cell diameter is also an indicator for baculovirus infection and with this method a rapid baculovirus titer estimation can be performed. Automated trypan blue exclusion assay can be used to measure cell parameters like viability, diameter and cell density. Because infected insect cells increase three times in cell diameter, virus titer can be obtained with this method [48]. Furthermore immunological assays for baculovirus titer determination were described in literature. For this viral antigens, which were expressed in infected insect cells, could be detected where virus infections can be spotted earlier than traditional visual methods [49]. Immunological assays were improved and optimized over the time as nowadays you can determine the titer within 48 hours with the help of 96-well plates [49].

6. Abstract

The baculovirus expression vector system is one of most used expression system both on large and laboratory scale protein production. The system used for this thesis, the Bac-to-Bac® Kit from Invitrogen, convinces by easy handling, fast results within four weeks and high amounts of produced recombinant baculoviruses, which is can be used for further experiments. In this thesis, the expression of the fluorescent protein EGFP, which can be used in future experiments as a transfection marker, was first investigated. This protein is helpful in determining successful transfection, whereby phenotypic characteristics are not the only factors that need to be taken into consideration. It was also confirmed that bacmids are isolated most effectively with alkaline lysis, which provides the most DNA yield. Also, the titer determination by plaque assay has not produced reliable results and should be further investigated. On the other hand, in the preliminary experiments for titer determination via qPCR, it was observed that lower cq value comes with higher virus titer. This information is usefull when creating a standard curve to determine virus titer via virus qPCR. Isolation of viral DNA by phenol/chloroform extraction was not suitable for further investigation via qPCR as the Taq-polymerase was inhibited by the remaining phenol. This could be observed by the variable melt peaks and cq values of identical DNA samples in the qPCR diagrams. Subsequently, it can be said that in this thesis the EGFP was successfully expressed as a transfection marker and the Bac-to-Bac by Invitrogen system was established and partially validated. Preliminary experiments and preliminary considerations for titer determination using virus qPCR were also approved.

7. Zusammenfassung

Das Baculovirus-Expressionssystem ist heutzutage eines der wichtigsten Expressionssysteme für die Produktion von rekombinanten Proteinen sowohl in dem Groß- und Labormaßstab. Das dafür verwendete Kit in dem Rahmen dieser Thesis, nämlich das Bac-to-Bac® Kit von Invitrogen, überzeugt durch einfache Handhabung, schnelle Ergebnisse innerhalb vier Wochen und hohe Menge produzierter rekombinanter Baculoviren, die man für weitere Experimente gut nutzen kann. In dieser Thesis wurde als Erstes die Expression von dem fluoreszierenden Protein EGFP untersucht, welcher in zukünftigen Experimenten als Transfektionsmarker genutzt werden kann. Dieses Protein ist hilfreich, um eine erfolgreiche Transfektion zu erkennen, wobei man sich nicht nur noch auf phänotypische Merkmale stützen muss. Ebenfalls wurde bestätigt, dass Bacmide am besten mit Alkalischer Lyse zu isolieren sind, da dies die meiste DNA-Ausbeute mit sich bringt. Ebenfalls hat die Titer-Bestimmung mittels Plaque Assay keine verlässlichen Ergebnisse hervorgebracht und sollte weiter untersucht werden. Andererseits wurden in den Vorexperimenten zu der Titer-Bestimmung via qPCR beobachtet, dass mit höherem Virustiter niedrigerer Cq-Wert einhergeht. Darauf aufbauend ist diese Information hilfreich bei der Erstellung einer Standartkurve zur Bestimmung des Virus-Titers mittels viraler qPCR. Die Isolation viraler DNA mittels Phenol/Chloroform Extraktion stellte sich als nicht geeignet für die weitere Untersuchung via qPCR dar, da die Taq-Polymerase durch das verbleibende Phenol inhibiert wurde. Dies konnte man durch die variierenden Schmelzpunkte und Cq-Werte der qPCR-Produkte selber DNA Proben in den Diagrammen beobachten. Anschließend kann man sagen, dass in dieser Thesis EGFP als Tranfektionsmarker erfolgreich expremiert wurde und das Bac-to-Bac System von Invitrogen etabliert und partiell validiert wurde. Ebenfalls wurden Vorexperimente und Vorüberlegungen zu der Titer-Bestimmung mittels viraler qPCR durchgeführt.

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9. Appendix

9.1. pFastBac™ Dual Vector Map

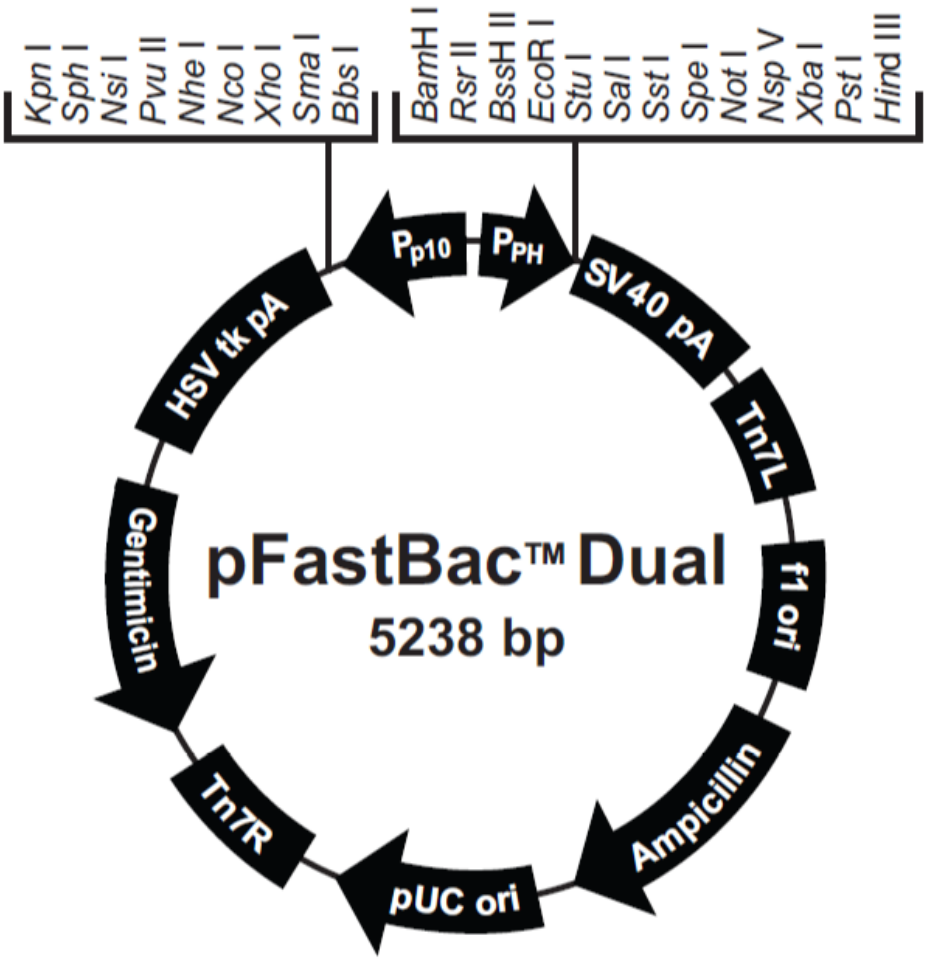

Figure 15: pFastBac™ Dual vector map. Modified from [17].

9.2. Microscope Picture of Transfection Experiment

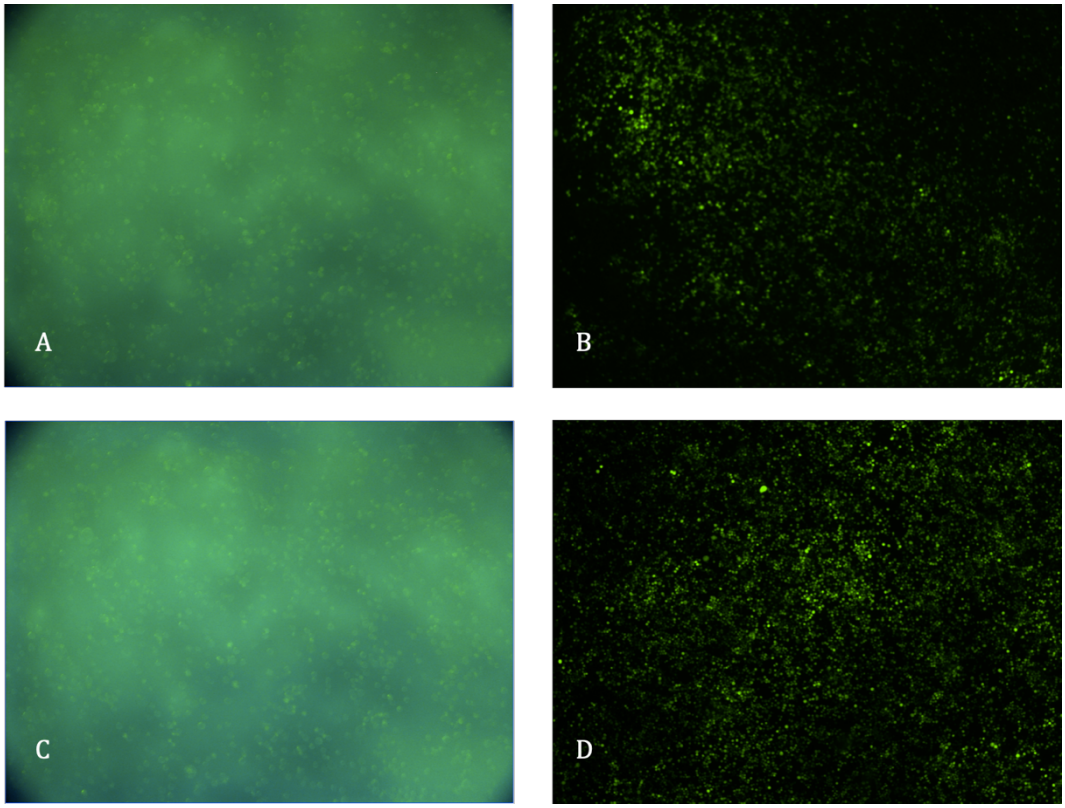

Figure 16: Fluorescence microscopy of transfected cells. Transfection experiment started on 02.07.18. A = EGFP expression of sample 7 after two days. B = EGFP expression of sample 7 after seven days. C = EGFP expression of sample 8 after two days. D = EGFP expression of sample 8 after seven days.

9.3. Standard PCR of pFBD_EGFP with Phusion™ Polymerase

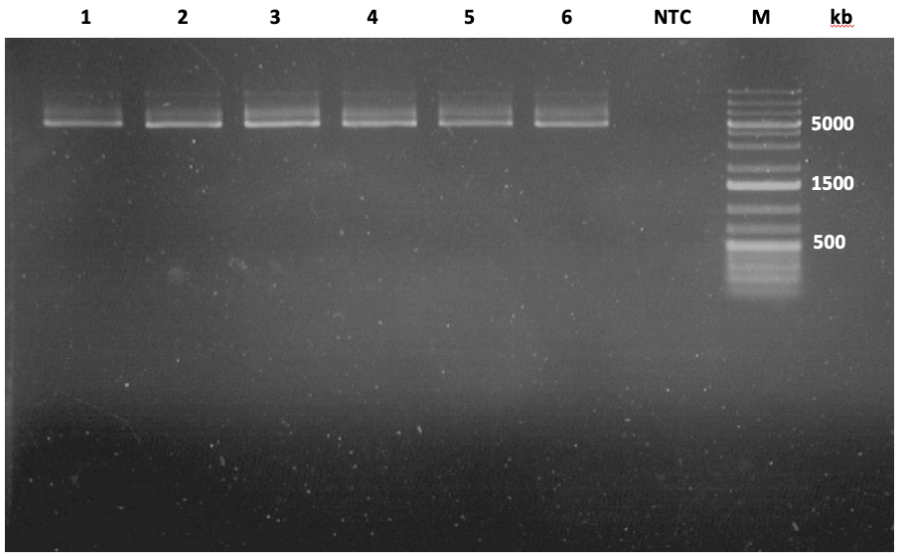

Figure 17: Gel electrophoresis of pFBD_EGFP with Phusion™ Polymerase. Standard PCR with Phusion™ polymerase was performed using 'pFastBacDual_fw' and 'pFastBacDual_rev' primers. No bands are visible. M = GeneRuler™ 1kb Plus DNA Ladder; NTC = no template control; 1-6 = pFBD_EGFP

9.4. Empty Bacmid

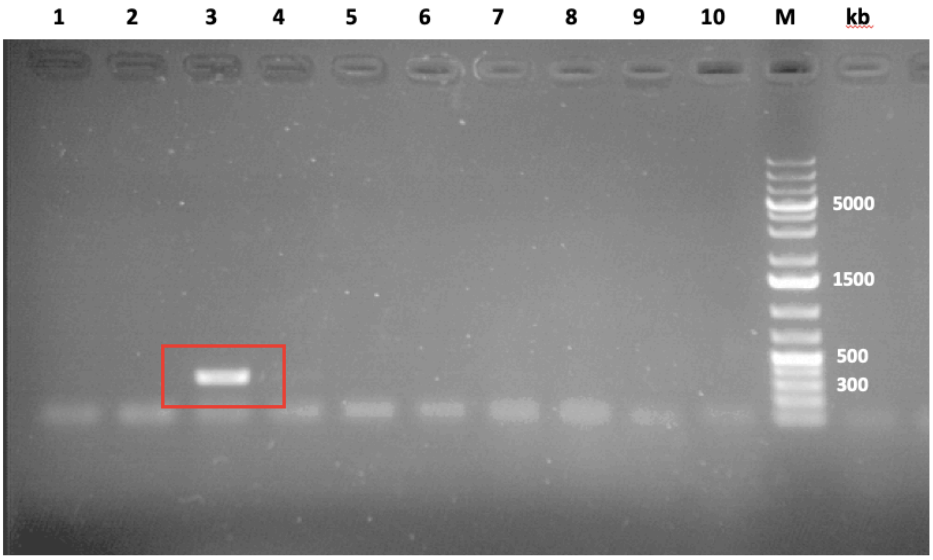

Figure 18: Gel electrophoresis of empty bacmid. Bacmid PCR with GoTaq® was performed with primers 'Bacmid_fw' and 'Bacmid_rev' (see chapter 2.3.9.). In line 3 an empty bacmid is visible which has a product size of approximately 300bp. M = GeneRuler 1kb Plus DNA Ladder; 1-10 = bacmid samples isolated with alkaline lysis.

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11. Eigenständigkeitserklärung

Ich versichere, dass die vorliegende Arbeit selbstständig und ohne unerlaubte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Alle Stellen, die inhaltlich oder wörtlich aus Veröffentlichungen stammen, sind kenntlich gemacht. Diese Arbeit lag in der gleichen oder ähnlichen Weise noch keiner Prüfungsbehörde vor und wurde bisher noch nicht veröffentlicht.

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Ort, Datum Unterschrift