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THE EFFECT OF TIME AND OPTIMIZATION OF THE BACULOVIRUS EXPRESSION SYSTEM FOR MORE EFFICIENT RECOMBINANT PROTEIN PRODUCTION OF PORCINE EPIDEMIC DIARRHEA VIRUS IN INSECT CELLS

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ABSTRACT

The expression of recombinant protein for structure determination is one of the major challenges in pharmaceutical and academic research, since the number of potential drug targets has increased significantly in the last decade. Despite the fact that the baculovirus expression vector system is widely used for this purpose, the system is hampered by three very slow and tedious procedures, namely generation of high titer baculovirus stock, determination of the virus titer and discovery of the best conditions for protein expression. We herein describe the development of the Bac to Bac system to address and overcome these issues for protein expression in insect cells. We have established a new baculovirus expression technology for insect cells that is based on expression of PEDV with target gene, a new regime for cell culturing and a highly efficient purification and enrichment procedure for recombinant baculovirus particles. Expression of PEDV is used to monitor the infection of insect cells, to simplify titer determination and to optimize expression conditions. The new regime for cell culturing with increased viability of non-infected insect cells and its combination with the massive enrichment of virus particles via high-speed centrifugation enables the production of large amounts of recombinant virus in a very short period of time. By combining these techniques and by using the bicistronic vector pFastBacHTb, we have been able to cut the time-lines for protein expression in insect cells by half, approaching those for protein production in *Escherichia coli*. This new expression system is a significant step forward towards industrialized protein production in both, industry and academia.

Keywords: *Baculovirus, BEVS, Protein Expression, PEDV, Expression Conditions, Insect Cells*

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is the causative agent of PED, a highly contagious disease of pigs characterized by acute watery diarrhea, and vomiting. The disease has mortality as high as 100 % in new-born piglets, and infected pre-weaning pigs often succumb to severe dehydration (Song and Park, 2012; Jung and Saif, 2015). Since its first appearance in Europe, PEDV outbreaks have occurred persistently in Asia and recently in North America, resulting in enormous economic loss worldwide to the swine industry (Pasick *et al.*, 2014, Vlasova *et al.*, 2014, Wang *et al.*, 2014). PEDV is an enveloped RNA virus possessing a single-stranded, positive-sense genome with a 5' cap and a 3' polyadenylated tail and belonging to Alphacoronavirus genus in the family Coronaviridae (Masters, 2006; Park *et al.*, 2012). Based on the entire genome sequence of the well-characterized CV777 strain, PEDV genome is approximately 28 kb, bearing at least seven overlapping open reading frames (ORF) encoding non-structural proteins including replicase1a, 1b and ORF-3, and structural proteins including spike (S), envelope (E), matrix (M) and nucleocapsid (N) proteins (Kocherhans *et al.*, 2001).

The S protein of PEDV is a type I membrane glycoprotein composed of 1,383 to 1,386 amino acids (aa), depending on the strain. It contains a putative signal peptide (aa 1-24), a large extracellular region, a single transmembrane domain (aa 1,334-1,356), and a short cytoplasmic tail. Although PEDV has an

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uncleaved S protein because it lacks a furin cleavage site, the S protein can be divided into S1 (aa 1–735) and S2 (736–the last aa) domains based on homology with S proteins of other coronaviruses (Sturman and Holmes, 1984; Duarte and Laude, 1994; Jackwood *et al.*, 2001; Lee *et al.*, 2010). Like other coronavirus S proteins, the PEDV S protein is known to play a pivotal role, interacting with the cellular receptor to mediate viral entry and inducing neutralizing antibodies in the natural host (Chang *et al.*, 2002; Bosch *et al.*, 2003). More precisely, previous studies have shown that the S1 domain includes the main neutralizing epitopes and the receptor-binding region (Sun *et al.*, 2007; Lee *et al.*, 2011). Furthermore, along with the full-length S gene, the S1 portion is known to be a suitable region for determining genetic relatedness among the different PEDV isolates and for developing differential diagnostic assays (Lee *et al.*, 2010; Chen *et al.*, 2014). Considering these molecular and biological features of the S1 domain, it would be an appropriate target for developing effective vaccines against PEDV.

Recombinant baculoviruses are widely used to express heterologous genes in insect cells (O'Reilly *et al.*, 1994). The baculovirus expression vector system (BEVS) has many strengths, such as the capacity for large inserts of DNA and a high yield of recombinant protein. Proteins produced in the BEVS are very similar to naturally occurring human proteins in terms of post-translational modifications (e.g., phosphorylation), biological activity, and protein stability. For this reason the BEVS is widely used in academia and industry. However, protein production with the BEVS is hampered by three slow and tedious procedures: (i) generation of high titer baculovirus stock; (ii) determination of the virus titer; and (iii) Identification of the best conditions for protein expression. The titer determination step is usually done either by an end-point dilution assay or by a plaque assay, which both take at least 7 days (O'Reilly *et al.*, 1994). Introduction of the Bac-PAK baculovirus rapid titer kit (BD Biosciences Clontech, Palo Alto, CA, USA) has reduced the time necessary to determine the baculovirus titer to only 2 days. This titer determination method is based on an immunological assay against viral antigens expressed on the surface of infected cells. Titer determination with this kit, however, is both, labor-intensive and expensive. The use of PEDV-S1 protein in a baculovirus expression system allows for the early and facile detection of recombinant viruses and simplifies titer determinations (Cha *et al.*, 1997; Philipps *et al.*, 2004). GFP from the jellyfish *Aequorea victoria* has become an excellent marker not only for studies of gene expression, protein localization, and dynamics in various biological systems, but also for protein purification and protein/protein interaction studies (Lippincott-Schwartz *et al.*, 2001; Katagiri and Ingham, 2002; Van Roessel and Brand, 2002). Using the protocol from the widely used Bac-to-Bac system (Invitrogen, Carlsbad, CA, USA), it takes three rounds of virus amplification to go from a purified bacmid to a high-titer baculovirus stock, a procedure that takes at least 10 days overall. In this process, both the transfection step and the two subsequent infection steps are performed with cells at 90% confluence or higher. Viruses are harvested 3–4 days later without refeeding of the cells (O'Reilly *et al.*, 1994). However, only about 10% of the cells are transfected with the bacmid or are infected by the virus during the first day. At least 90% of the insect cells are not infected and could in principle divide further with their characteristic half-life of the cell cycle. Due to the high confluence of the cells, however, cell division is no longer possible and the cells go into the resting state (G0) (Kioukia *et al.*, 1995). Infection during the stationary phase of growth leads to a lower amount of infectious particles compared to infection during the exponential phase. Compared to the classical Bac-to-Bac system, the number of infectious particles could be increased by improving the viability of the non-infected insect cells during the virus production phase. This would provide healthy, dividing insect cells, which could then be infected by a new baculovirus particle directly after its release into the medium from an already infected cell. Centrifugation has been widely used to purify and concentrate virus particles from different sources (O'Reilly *et al.*, 1994; Giessauf *et al.*, 2002). Ultracentrifugation with a sucrose gradient has been described for the concentration of baculovirus particles. This is a very time-consuming step, requiring both expensive equipment and specially trained personnel. In addition, only about 50% of the infectious material is generally recovered after the ultracentrifugation step. The use of a high speed centrifuge, which circumvents the disadvantages of the sucrose gradient in a ultracentrifugation step, has been reported for the concentration of baculovirus particles (Condeary *et al.*, 1999). This method, however, has

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not been applied previously to the enrichment of viruses for the infection of insect cells, nor have the yields been quantified. The resuspended pellet was either purified further by ultracentrifugation (Loisel *et al.*, 1997) or used for the infection of mammalian cells (Condreay *et al.*, 1999). Finding the conditions best suited for expression of large amounts of enzymatically active target proteins is still a very time-consuming step. During this optimization process the multiplicity of infection (MOI), time of infection (TOI), agitation rate, and temperature are altered and the expression yield of target protein is estimated by Western blotting.

With our novel expression system, termed pFastBacHTb, we now report a technique to address the three main bottlenecks for protein production with BEVS. Our approach is based on the simultaneous use of an optimized transfection protocol, a method for concentration and purification of recombinant baculovirus particles, and a non-invasive fluorescent readout procedure with PEDV for fast titer determination and efficient optimization of expression conditions. In summary, this technology enables us to produce recombinant proteins in insect cells in fairly short time, with high quality and high efficiency to fulfill the increasing demands in modern drug discovery and academic research.

MATERIALS AND METHODS

Plasmid Constructions and Bacmid Preparation

The pFastBacHTb plasmid was constructed by cloning the nucleotide sequence of the gene encoding for the S1 protein. The sequence was PCR-amplified from a previous vector, using the primers: F_EcoRI (5'-GGGGGAATTCATGATTTCTTTTGTACTCTGC-3'), and R_NotI (5'-TTTGTGCGGCCGCGCTGTAGAACATCCGTCT 3'). The PCR condition for the amplification by following: 5 minutes initial denaturation at 94°C, followed by 30 cycles, each containing of 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute 30 seconds at 72°C, and a final extension at 72°C for 10 seconds. PCR was carried out in 10 µl volume containing 1 µl of DNA template, 5 µl of 2x Master mix, 0.5 µl of each primer, 3 µl of ddH₂O. The PCR products were loaded to agarose gel (1.2 %, w/v) for confirmation. The PCR products containing the PEDV-S1, were digested with *Bam*HI and *Hind*III restriction enzymes in order to insert the DNA, in frame, into the pFastBacHTb transfer vector (Gibco BRL, USA) of the Baculovirus Expression System (Invitrogen, USA). The recombinant plasmids were used to transform competent *E.coli* DH10Bac (containing the wild-type baculovirus genome in bacmid form) to generate the corresponding recombinant bacmids upon transposition. Cloning steps were monitored by using sequence analysis and controlled by Genetyx-Win Program.

One vial of DH10Bac competent cells (100 µl) were thawed on ice for each transformation. 3 µl pFastBacHTb plasmid DNA were added to the cells and mixed gently. The cells were incubated on ice for 30 min. Heat shock was applied to the cells for 45 s at 42°C without shaking and immediately transferred the tubes to ice and chilled for 2 min. LB (Luria Broth) medium was added 900 µl at room temperature and shake at 37°C at 225 rpm for 4 h. Serial dilutions of the cells (10^{-1} , 10^{-2} , 10^{-3}) was prepared with LB (Luria Broth) medium. Each dilution was plated on LB (Luria Broth) agar that contained 50 µg mL⁻¹ kanamycin, 7 µg mL⁻¹ gentamicin, 10 µg mL⁻¹ tetracycline, 100 µg mL⁻¹ X-gal and 40 µg mL⁻¹ IPTG. The plates were incubated for 48 h at 37°C. White colonies, which include recombinant bacmid *E.coli* clones, were picked by blue/white-screening (Invitrogen).

After transformation of *E. coli* DH10Bac cells, *Spodoptera frugiperda* (Sf9) cells were cultured at 27°C in TNMFH medium, supplemented with 10% FBS. For transfection, 9×10^5 cells were plated in 6 well tissue culture dishes and incubated for 1 h in 2 mL TNMFH medium containing 10%FBS (without antibiotics) to allow adhesion of the cells to surface. Recombinant bacmid DNA had been preincubated for 45 min at room temperature with Cellfectin II (8 µl). Cells were incubated with the liposome-DNA complex for 5 h at 27°C. The transfection medium was removed and 2 mL of TNMFH medium, containing antibiotics was added. The DNA was transfected into Sf9 cells. Transfected cells were incubated at 27°C for 144 h allowing baculovirus production. The recombinant virus was amplified twice to obtain virus stocks of the highest titer and harvested. Classical plaque assay was applied for virus titration (Summers and Smith, 1987).

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Insect Cell Transfection and Baculovirus Concentration

Sf9 insect cells (Invitrogen) were seeded into a T75 tissue culture flask to around 50-60% confluence in 20 ml TMN-FH's medium containing 10% fetal calf serum (FCS). A transfection solution of 2 ml TMN-FH's medium with 15 lg bacmid DNA and 70 µl CELLfectin (Invitrogen) was prepared and incubated for 30 min at room temperature. The medium of the cells was aspirated and the transfection solution was diluted with 8 ml TMN-FH's medium containing 10% FCS and added to the cells. After 5 h of incubation at 27 °C the transfection medium was aspirated and the cells were overlaid with 20 ml fresh FMN-FH's medium containing 10% FCS. The cells were harvested 2 days post-transfection and seeded with 50 ml fresh medium into a T225 tissue culture flask. After 3 more days (5 days post-transfection) the cells were harvested and centrifuged at 300g for 5 min. The sterile filtered supernatant was centrifuged for 1 h at 45,000g in an SS34 rotor (Kendro Laboratory Products AG, Zurich, Switzerland) at 20 °C. The resulting virus pellet was resuspended in 1 ml phosphate-buffered saline (PBS) buffer, pH 7.4, per 50 ml original cell culture volume.

Titer Determination

The titer of the generated high titer virus stock was determined with end-point dilution and PEDV fluorescence detection. Ten microliter aliquots of serial dilutions of the high titer stock virus (10^{-2} to 10^{-9} dilution) were mixed with 50 µl aliquots of Sf9 cell suspension (10^5 cells/ml) and 12 aliquots of each mixture were seeded into a 96-well half area plate (Cha *et al.*, 1997). After 2 days, the number of wells with at least one PEDV fluorescent cell was counted for every dilution. The basic principle of the method is the assumption that all cultures infected at a particular dilution would have been infected at all lower dilutions, and conversely, that all cultures not infected at that dilution would not have been infected at all higher dilutions (Reed and Muench, 1938). The virus titer was calculated according to the 50% tissue culture infectious dose (TCID₅₀) method. Briefly, the proportions of infected cells per dilution are determined. The infectivity in plaque forming units (pfu) is then calculated according to Eq. (*), with dil₅₀ being the dilution above 50% infection, perab₅₀ being the percentage of infection above 50% infection and perbe₅₀ being the percentage of infection below 50% infection (Hughes and Wood, 1986, O'Reilly *et al.*, 1994).

$$\text{Infectivity} \left[\frac{\text{pfu}}{\text{ml}} \right] = \frac{0.69}{10 \log(\text{dil}_{50}) - \left(\frac{\text{perab}_{50} - 50\%}{\text{perab}_{50} - \text{perbe}_{50}} \right) \cdot \text{ml virus} \frac{\text{ml virus}}{\text{well}}} \quad (*)$$

Influence of PEDV-S1 Expression on the Expression Yield of Target Protein

Bacmid differing only by the presence or absence of PEDV-S1 was constructed to analyze the effect of PEDV-S1 expression under the control of the P_{Basic} promoter on the expression of a target gene. In the construct, the PEDV-S1 gene was cloned into the MCS of pFastHTb downstream of the P_{PH} promoter. Sf9 cells were infected with the recombinant baculoviruses at a multiplicity of infection (MOI) of one and cells were harvested 3 days post-infection. Expression of all samples was analyzed by Western blotting with either an anti- antibody (Rockland, Gilbertsville, PA, USA) or an anti-His6 antibody (Sigma, Buchs, Switzerland) (Figure 3a). To compare the expression yield in the presence and absence of PEDV and in non-infected insect cells (Figure 3b).

Analysis of Different Conditions for Protein Expression

In a 96-well plate, insect cells were infected with baculovirus for a target protein and at ten different MOIs for each series. Total fluorescence intensity of the cells was determined on a Typhoon 9400 fluorescence plate imager (Amersham Biosciences, Freiburg, Germany). The expression yield of PEDV-S1 and target gene was analyzed by Western blotting using an anti-His antibody (Sigma) and an anti-PEDV antibody (Santa Cruz Biotechnology, Heidelberg, Germany) (Figure 4).

RESULTS AND DISCUSSION

We have developed a quick and easy protocol for titer determination, based on end-point dilution and PEDV-S1 fluorescence as readout. To pursue this, we changed the promoter controlling EGFP expression from the very late p10 promoter to the late basic protein promoter (P_{Basic}) (Figure 1a). Thus, expression of PEDV-S1 starts already 6 h post-infection (O'Reilly *et al.*, 1994) and virus titer can easily be determined

[illegible]

PEDV-S1, Enhanced Green Fluorescent Protein; P_{Basic}, Basic Promoter; P_{PH}, Polyhedrin Promoter; HSV tk Poly (A), Herpes Simplex Virus Thymidine Kinase Polyadenylation Signal; SV40 Poly(A), Simian Vir us 40 Late Polyadenylation Signal. The Construction of the Vector pFastBacHTb is Described Under Materials and Methods. The Lengths of the Vector Features in this Figure are Relative to their Respective Length in Nucleotides in the Plasmid

Centrifugation of the high titer virus stock is a quick and easy method for concentration and purification of the baculovirus particles. We developed a centrifugation step without the need for a sucrose density gradient in a high speed laboratory centrifuge. Only about 1% of the virus particles from the starting material was found to remain in the supernatant after the centrifugation, whereas the vast majority of the infectivity of the sample was present in the resuspended pellet (Figure 2). A combination of the techniques developed herein was applied to a series of bacmids and titer determination was performed at different stages of virus production (Figure 2).

To analyze the influence of PEDV-S1 expression on the expression yield of target genes, we compared the target gene expression in the absence and in the presence of PEDV-S1. The expression of PEDV-S1 showed no visible effect on the expression yield as analyzed by Western blot analysis and by PEDV-S1 activity assay (Figure 3).

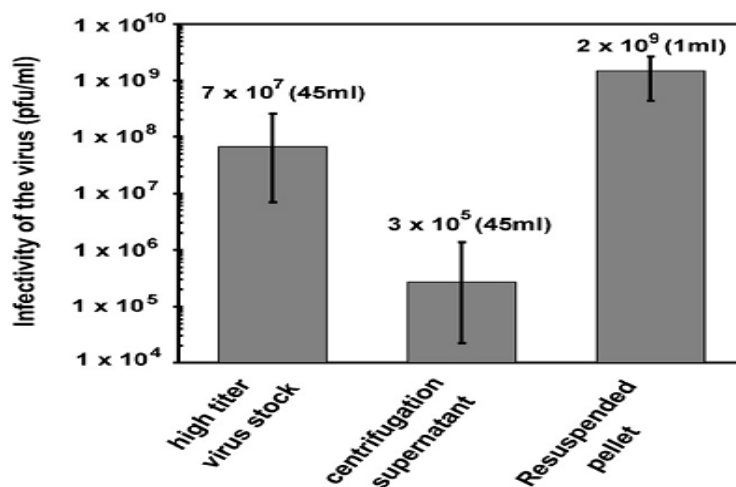


Figure 2: Comparison of the Infectivity of the Baculovirus at the Different Preparation Stages. The Plaque Forming Units per Milliliter Sample (pfu/ml) of 10 Different Preparations of Various Baculoviruses have been Determined after the Harvesting of the High Titer Stock, the Supernatant of the Centrifugation Step, and the Pellet of the Centrifugation Step Resuspended in PBS Buffer. Titer Determination was Performed by the End-Point Dilution Method with PEDV-S1 Fluorescence Readout. The Results were Comparable to the Determination Using the BacPAK Baculovirus Rapid Titer Kit (BD Biosciences). After the Centrifugation Step only about One Percent of the Infectivity Remained in the Supernatant, whereas the Majority of the Infectivity was Detected in the Pellet

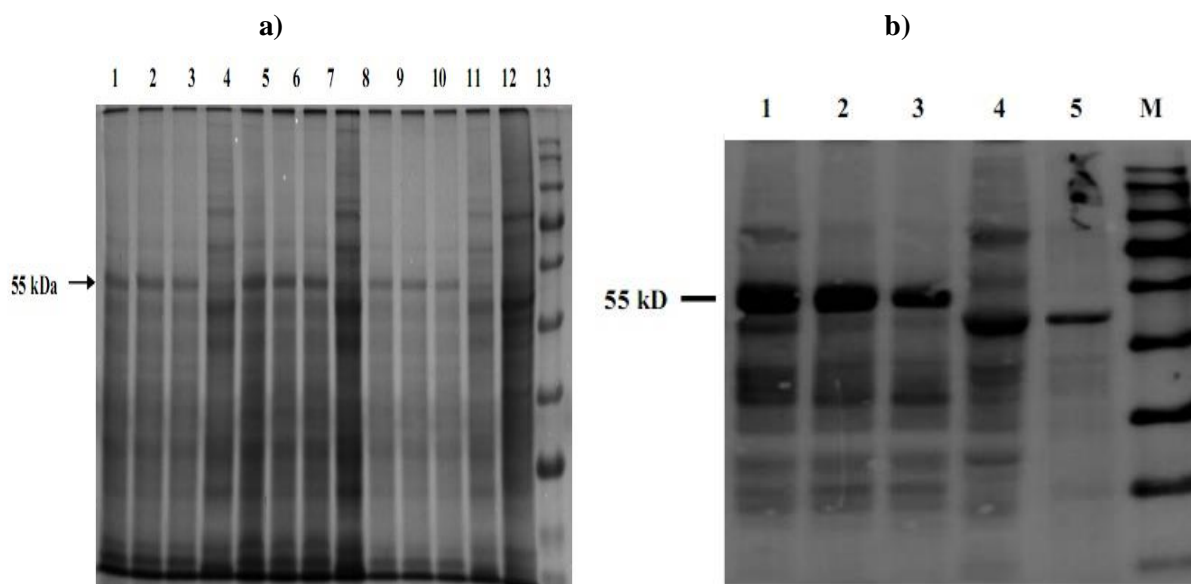


Figure 3: The Expression Yield of a Target Protein under the Control of the Very Late Polyhedrin Promoter with and without Expression of PEDV-S1 under the Control of the Late Basic Promoter. The Expression Yield of Target Protein and PEDV was Estimated by SDS-PAGE and Western Blot

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using an Anti-His Antibody for Target Protein Detection (Middle Panel) and an Anti-PEDV Antibody for Detection of PEDV Expression (Lower Panel)

To find the conditions best suited for expression of a target protein, PEDV-S1 fluorescence of infected insect cells was measured. For this purpose, insect cells were infected with virus at different multiplicities of infection in a 96-well plate. The total fluorescence of the different wells was measured on a Typhoon 9400 fluorescence plate imager (Figure 4). To compare the expression level of the proteins with the expression of PEDV, the amount of expressed proteins was estimated by Western blotting using anti-His and anti-PEDV antibodies (Figure 4). The expression levels of the target proteins show strong correlation with both, fluorescence intensities and PEDV-S1 expression levels (Figure 4).

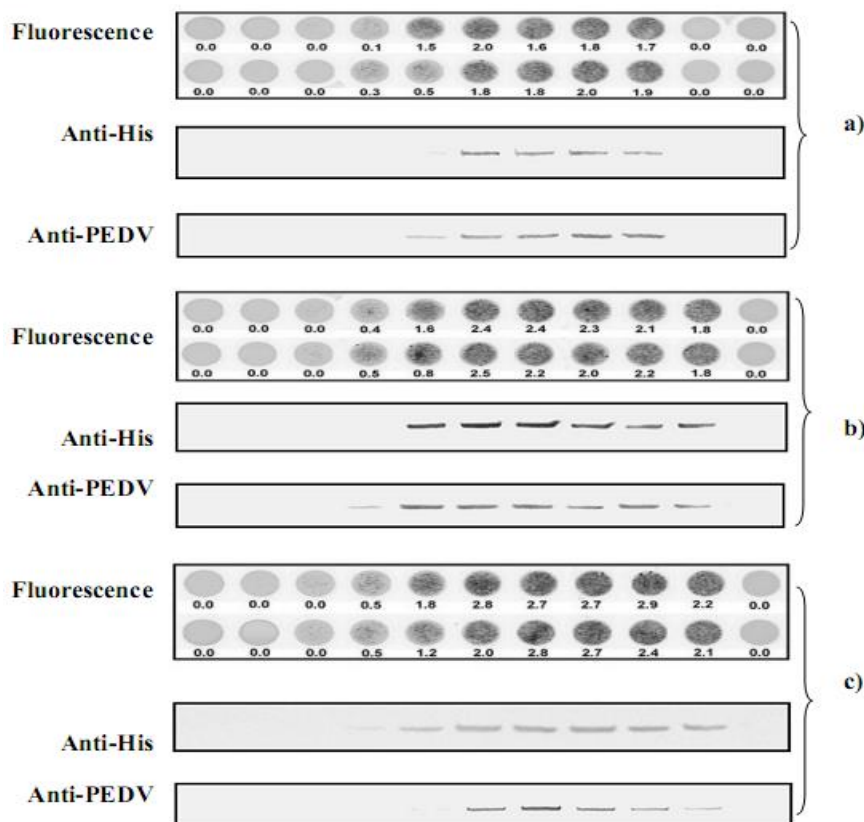


Figure 4: Correlation of the Expression Yield of a Target Gene with the Expression of PEDV-S1. Insect Cells were Infected at Different MOIs with Baculovirus Expressing Human Interleukin-1 Receptor-Associated Kinase 4 (IRAK4)

(a). Human Spingosine Kinase 1 (SK)

(b). and Human Interleukin 6 Signal Transducer (gp130)

(c). The Total fluorescence of the Wells was Determined in Duplicates with a Typhoon 9400 Fluorescence Plate Imager (Shown in the Upper Panel) and the Fluorescence Values are Shown (Arbitrary Units/106)

The generation of a high titer baculovirus stock from a recombinant bacmid by standard methods requires three amplification rounds and takes 10 days at least, as described in the Bac-to-Bac manual (Invitrogen). In the study presented here, we have been able to cut the time required for virus generation by half, going from a pure recombinant bacmid to high titer baculovirus stock with only one amplification round in 5 days. The most likely reason to achieve this acceleration is an increase of the viability of the non-infected

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insect cells during virus production. In the classical transfection step protocol, the cells are seeded to 90% confluence and transfected with the bacmid. As only 10% of the cells are transfected with the bacmid at best, the 90% non-in-fected insect cells cannot divide further due to the high confluence of the cells. Therefore, the cells go into the resting state (G0) (Kioukia *et al.*, 1995). However, baculovirus infection during the G0 phase is difficult and leads to a significantly lower amount of new infectious virus particles produced, compared to infection during the exponential growth phase. For dividing insect cells, the infectivity of the baculovirus is significantly dependent on the cell cycle stage of the insect cells. Baculovirus infection yields of insect cells in the G1 or S phase are about 1.5- to 1.8-fold higher than infection yields of cells in the G/M phase (Saito *et al.*, 2002).

We have therefore decided to seed insect cells at 60% confluence and transfect them with the bacmid at a similar DNA-to-cell ratio as described in the classical protocol. To increase the viability of the non-infected cells further, the medium was removed after 2 days and the cells were harvested and reseeded to 30% confluence. Aspiration of the used medium may enhance the viability of the cells by removing toxic byproducts of cell metabolism. More importantly, reseeding of the cells at low confluence allows the non-infected cells to divide further until they in turn will be infected by virus particles generated during the amplification step. Virus stock obtained with this protocol 5 days post-transfection showed a titer comparable to the titer achieved after 10 days and three amplification rounds with the Bac-to-Bac protocol (data not shown).

In addition to the virus particles, high titer baculovirus stock also contains fetal calf serum (FCS) from the growth medium and catabolic side products of cell metabolism, which may show toxic effects on the insect cells. The serum proteins in the medium can cause difficulties in subsequent protein purification, especially if the target gene has been expressed as a secreted protein. Even the addition of 50 ml virus stock containing about 10% FCS to 1 L of serum-free insect cell culture is sufficient to cause such purification difficulties. The simplest method to separate the virus particles from the FCS and the metabolic byproducts is a centrifugation step, using the difference in density of virus particles on the one hand and serum proteins and toxic catabolites on the other hand. Purification of baculovirus particles by sucrose gradient based ultracentrifugation has been reported (O'Reilly *et al.*, 1994). This, however, is a very time and work intensive step, requiring costly equipment and resulting in only low virus recovery. We therefore used a standard high-speed centrifuge to separate the virus particles from all lighter particles of the virus production step. The serum proteins and the catabolic byproducts remain in the supernatant, whereas the virus particles are found in the pellet. The pellet can then be easily resuspended in a small volume of serum-free buffer, allowing for the production of a virus stock depleted of FCS and other contaminants. To determine the yield of virus recovered after the high speed centrifugation, we determined the titers of a non-centrifuged virus sample, the supernatant of the centrifugation, and the resuspended pellet (Figure 2). The majority of the initial infectivity could be found in the pellet after centrifugation and only about 1% of the infectivity is detected in the supernatant and lost for subsequent work (Figure 2).

Titer determination is an additional, very time consuming step in protein production with BEVS and is a matter of intense investigation. We have recently described an optimized end-point dilution method with PEDV fluorescence readout (Cha *et al.*, 1997; Philipps *et al.*, 2004). This method was initially developed utilizing PEDV expression under the control of the very late polyhedrin promoter (Ennis *et al.*, 1999), allowing titer determination 4 days post-infection (Cha *et al.*, 1997).

Replacement of the P_{PH} by the earlier expressing late basic protein promoter (P_{Basic}) controlling the expression of a membrane-bound fusion protein consisting of CD4 and PEDV-S1 led to titer determination already 2 days post-infection (Philipps *et al.*, 2004). We now modified this system by exchanging the gene under control of P_{Basic} from the CD4-PEDV-S1 fusion protein to soluble PEDV-S1. The expression level of soluble PEDV-S1 is significantly higher and fluorescence appears brighter due to the cytoplasmic localization of PEDV-S1, compared to the membrane bound CD4-PEDV-S1 fusion protein (data not shown). This improvement allows for an easier detection of the infected insect cells, leading to a simplification of the titer determination step.

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All methods described here are based on bicistronic vectors, giving the opportunity to clone target genes in a second multiple cloning site and express the corresponding protein under the control of the very late polyhedrin promoter (Figure 1). As shown in Figure 3a, expression of PEDV-S1 under the late basic promoter has no detectable effect on the expression yield of different target genes, as determined by Western blot analysis. Three target proteins – IRAK4, a tyrosine kinase; SK, a lipid kinase; and gp130, a signal transducer protein showed similar expression levels independent of the presence or absence of co-expressed EGFP. Comparable expression levels of b-galactosidase for these constructs were detected by a Western blot analysis (Figure 3a) and a b-galactosidase activity test of the cell lysates (Figure 3b). Similar results have been observed for b-galactosidase expression with and without simultaneous expression of a fusion gene consisting of CD4-PEDV-S1 under control of P_{Basic} (Philipps *et al.*, 2004). Moreover, we can also monitor the stability of a baculovirus by determination of GFP fluorescence. In several cases, we were able to detect loss of infectivity during storage over time (data not shown).

Optimization of the conditions (e.g., MOI, TOI, temperature, and stirring rate) for target gene expression in the baculovirus system takes up time and resources. The quantification of the expressed target protein for the different conditions is usually done by Western blot analysis after disruption of the cells, which is a very time- and labor-intensive procedure. We developed a method to estimate the best conditions for expression of the target genes by monitoring the PEDV fluorescence of the growing insect cells without interfering with the cell culture. For this purpose, we investigated the expression of three target proteins at different MOIs. Strong PEDV fluorescence of the cells correlated significantly with the highest expression levels of both, FP and target protein, as determined by Western blotting (Figure 4). Thus, PEDV fluorescence can be used as an estimate of target gene expression without interfering with cell growth. With this method, neither harvesting and disruption of the cells, nor protein purification or SDS-PAGE are necessary to find the best conditions for protein expression. This optimization is now used routinely to optimize temperature, MOI, TOI, and stirring rate for the expression of recombinant target proteins in our group. It is obvious that this opens a possibility for significant savings of time and resources.

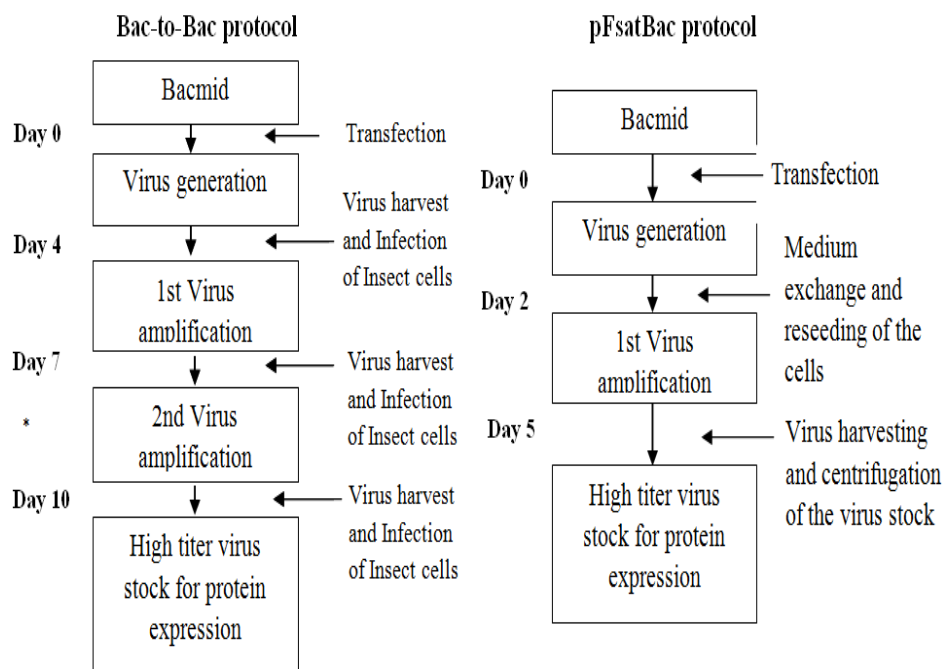


Figure 5: Workflow for Virus Generation from Bacmid to High Titer Virus Stock. The Newly Developed pFsatBac Protocol was Compared to the Classical Bac-to-Bac Protocol (Invitrogen). By

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the Use of the UltraBac Protocol, the Same Amount of Virus Particles can be Produced in Half the Time, and Due to the Centrifugation Step, the Virus is More Concentrated and Purified

Since its introduction in our laboratory, the herein reported protocol has replaced the standard Bac-to-Bac protocol and is now used on a routine basis within Novartis (Figure 5).

We have productively used this advanced system for the preparation of more than 20 new recombinant target proteins. The average time for the process from bacmid to pure protein was reduced from 3 to 2 weeks (Figure 5).

The amount of recombinant protein produced with this method in 10 days is usually sufficient to provide enough protein for a complete protein structure determination process.

The significant acceleration of protein production in BEVS now allows us, for the first time, to approach timelines for recombinant protein expression in insect cells comparable to those for protein production in *E. coli*.

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