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RESEARCH ARTICLE

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 signi cantly 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 e cient puri cation 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 signi cant step forward towards industrialized protein production in both, industry and academia.

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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 (Jung and Saif, 2015; Song and Park, 2012). 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

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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 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 (Duarte and Laude, 1994; Jackwood *et al.,* 2001; Lee *et al.,* 2010b; Sturman and Holmes, 1984). 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 (Bosch et al., 2003; Chang et al., 2002). More precisely, previous studies have shown that the S1domain includes the main neutralizing epitopes and the receptor-binding region (Lee *et al.,* 2011; Sun *et al.,* 2007). 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 (Chen *et al.,* 2014; Lee *et al.,* 2010a). 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 modi cations (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) identi cation 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 simpli es titer determinations (Cha *et al.,* 1997; Philipps *et al.,* 2004). GFP from the jelly sh 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 puri cation and protein/protein interaction studies (Katagiri and Ingham, 2002; Lippincott-Schwartz *et al.,* 2001; 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 ampli cation to go from a puri ed 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% con uence 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 rst 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 con uence 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 di erent sources (Giessauf *et al.,* 2002; O'Reilly *et al.,* 1994). 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 (Condreay *et al.,* 1999). This method, however, has not been applied previously to the enrichment of viruses for the infection of insect cells, nor have the yields been quanti ed. The resuspended pellet was either puri ed 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 puri cation of recombinant baculovirus particles, and a non-invasive uorescent readout procedure with PEDV for fast titer determination and e cient 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 e ciency to ful ll 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'-GGGGGAATTCATGATTT

CTTTTGTTACTCTGC-3'), and R_NotI (5'-TTTTGCGGC CGCCGCTGTAGAACATCCGTCT 3'). The PCR condition for the amplification by following: 5 minutes initial denaturation at 94ºC, followed by 30 cycles, each containing of 30 seconds at 94ºC, 30 seconds at 60ºC, and 1 minute 30 seconds at 72ºC, and a final extension at 72ºC 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 ddH_2O. 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 *Hin*dIII 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 moitored 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 42^0C 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 37^0C 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, 7µg mL^{-1} gentamicin, 10 µg mL^{-1} tetracycline, 100 µgmL^{-1} X-gal and 40 µgmL^{-1} IPTG. The plates were incubated for 48 h at 37^0C. 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 27^0C in TNMFH medium, supplemented with 10% FBS. For transfection, 9 x 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 27^0C. 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 27^0C for 144 h allowing baculovirus production. The recombinant virus was ampli ed twice to obtain virus stocks of the highest titer and harvested. Classical plaque assay was applied for virus titration (Summers and Smith, 1987).

Insect cell transfection and baculovirus concentration

Sf9 insect cells (Invitrogen) were seeded into a T75 tissue culture ask to around 50-60% con uence 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 ^{o}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 ask. After 3 more days (5 days post-transfection) the cells were harvested and centrifuged at 300g for 5 min. The sterile ltered supernatant was centrifuged for 1 h at 45,000g in an SS34 rotor (Kendro Laboratory Products AG, Zurich, Switzerland) at 20 ^{o}C. The resulting virus pellet was resuspended in 1 ml phosphate-bu ered saline (PBS) bu er, 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 uorescence 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 uorescent 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. Brie y, 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).

$$\text{Infectivity } [\frac{\text{pfu}}{\text{ml}}] = \frac{0.69}{10\log(\text{dil50}) - \left(\frac{\text{perab50}-50\%}{\text{perab 50}-\text{perbe50}}\right).\text{ml virus}\frac{\text{mlvirus}}{\text{well}}} \quad (*)$$

In uence of PEDV-S1 expression on the expression yield of target protein

Bacmid di ering only by the presence or absence of PEDV-S1 was constructed to analyze the e ect 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) (Fig. 3a). To compare the expression yield in the presence and absence of PEDV and in non-infected insect cells (Fig. 3b).

Analysis of di erent 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 uorescence intensity of the cells was determined on a Typhoon 9400 uorescence 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) (Fig. 4).

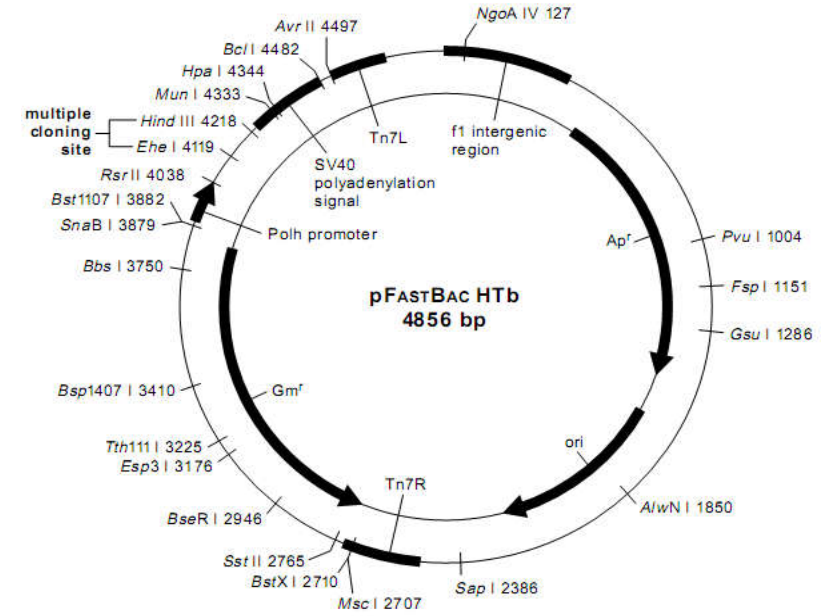
RESULTS AND DISCUSSION

We have developed a quick and easy protocol for titer determination, based on end-point dilution and PEDV-S1 uorescence 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})(Fig. 1a).

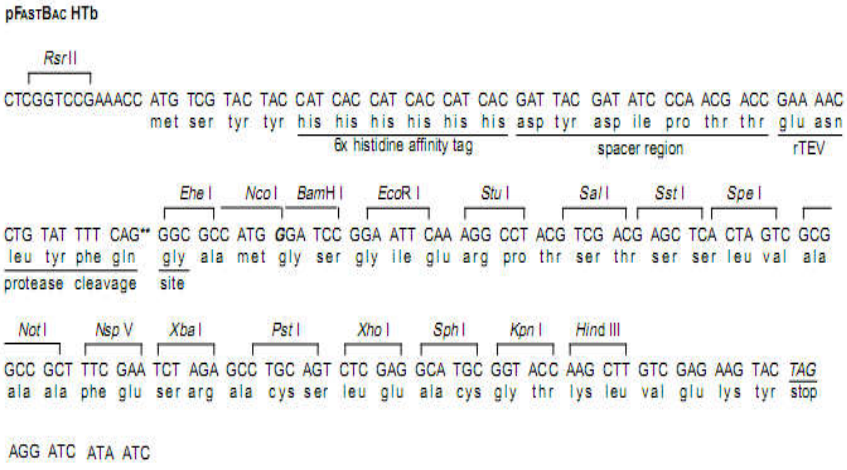
Thus, expression of PEDV-S1 starts already 6 h post-infection (O'Reilly *et al.,* 1994) and virus titer can easily be determined 2 days post-infection. The bicistronic vector pFastBacHTb described here in has an additional multiple cloning site (MCS) for expression of a target gene under control of the very late polyhedrin promoter (Ennis *et al*., 1999) (Fig. 1).

Multiple cloning site (MCS)

Titer determination with this PEDV-based end-point dilution assay and with the BacPAK rapid titer kit showed comparable virus titers (data not shown). To accelerate virus ampli cation, the transfection step was optimized to generate a high titer virus stock in 5 days starting from pure recombinant bacmid. The most likely reason for the high virus yield 5 days earlier than described in the Bac-to-Bac manual is an improvement in cell viability of the non-infected insect cells. To achieve this result, Sf9 cells were seeded in a T75 tissue culture ask at 60% con uence and transfected with the bacmid.



(A)



(B)

Fig. 1. Map (a) and unique restriction endonuclease sites of the multiple cloning site (MCS) (b) of the vector pFastBacHTb. PEDV-S1, enhanced green uorescent protein; P_{Basic}, basic promoter; P_{PH}, polyhedrin promoter; HSV tk poly(A), herpes simplex virus thymidine kinase polyadenylation signal; SV40 poly(A), simian virus 40 late polyadenylation signal. The construction of the vector pFastBacHTb is described under materials and methods. The lengths of the vector features in this gure are relative to their respective length in nucleotides in the plasmid

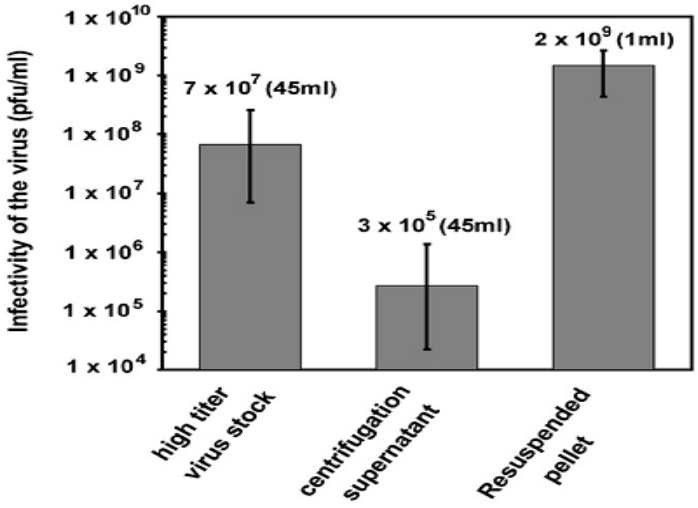


Fig. 2. Comparison of the infectivity of the baculovirus at the di erent preparation stages. The plaque forming units per milliliter sample (pfu/ml) of 10 di erent preparations of various baculoviruses have been determined after the harvesting of the high titer stock, the supernatant of the centrifugation step, and the pellet of the centrifugation step resuspended in PBS bu er. Titer determination was performed by the end-point dilution method with PEDV-S1 uorescence 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

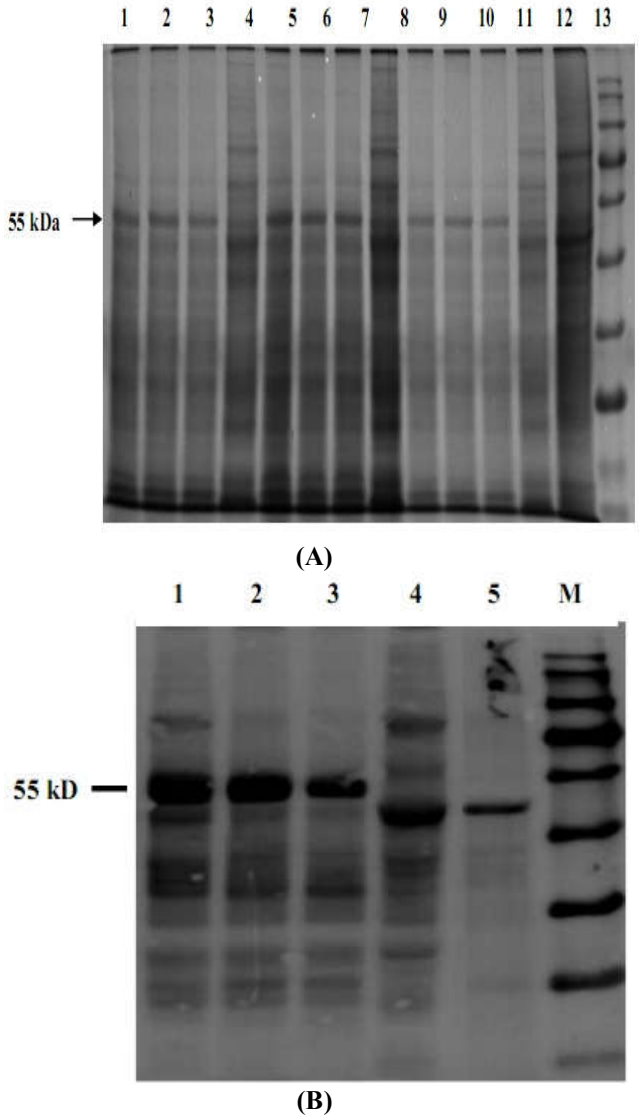


Fig. 3. The expression yield of a target protein under the control of the very late polyhedrin promoter with and without expression of PEDV-S1 under the control of the late basic promoter. The expression yield of target protein and PEDV was estimated by SDS-PAGE and Western blot using an anti-His antibody for target protein detection (middle panel) and an anti-PEDV antibody for detection of PEDV expression (lower panel)

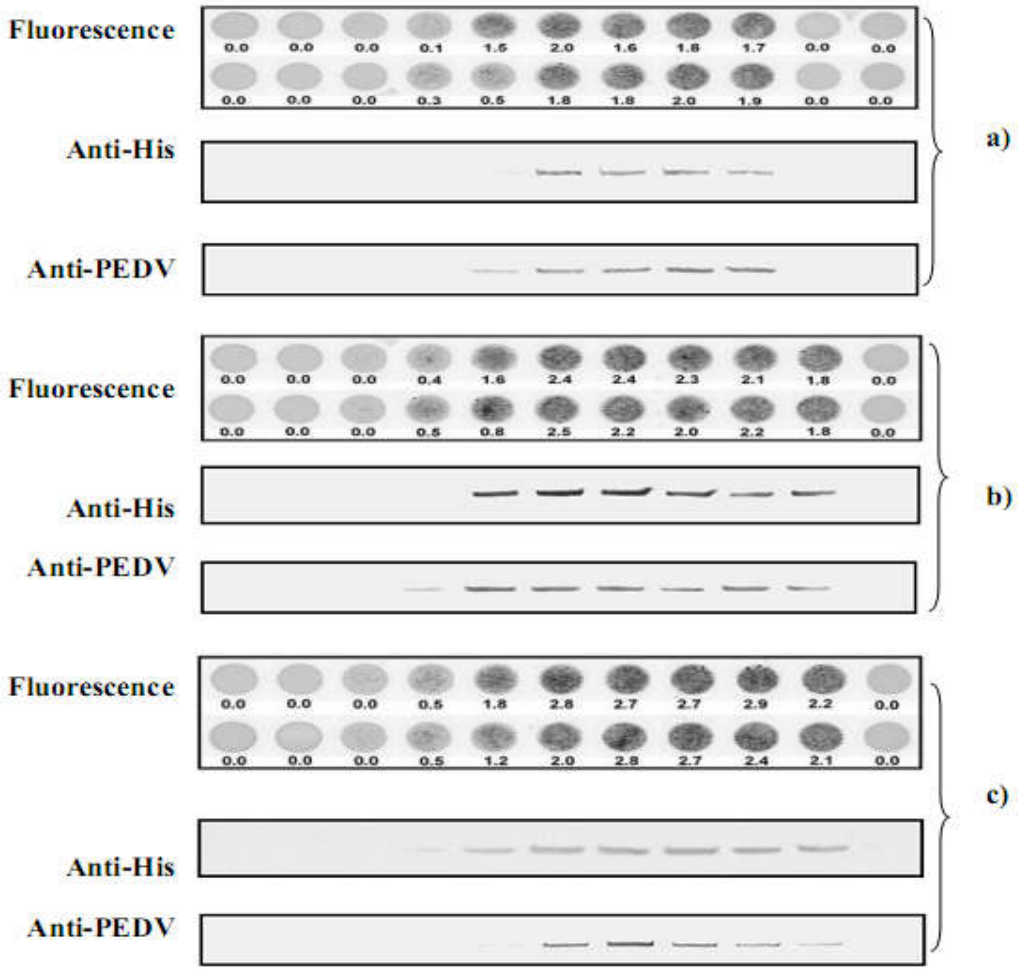


Fig. 4. Correlation of the expression yield of a target gene with the expression of PEDV-S1. Insect cells were infected at di erent MOIs with baculovirus expressing human interleukin-1 receptor-associated kinase 4 (IRAK4) (a), human sphingosine kinase 1 (SK) (b), and human interleukin 6 signal transducer (gp130) (c). The total uorescence of the wells was determined in duplicates with a Typhoon 9400 uorescence plate imager (shown in the upper panel) and the uorescence values are shown (arbitrary units/106)

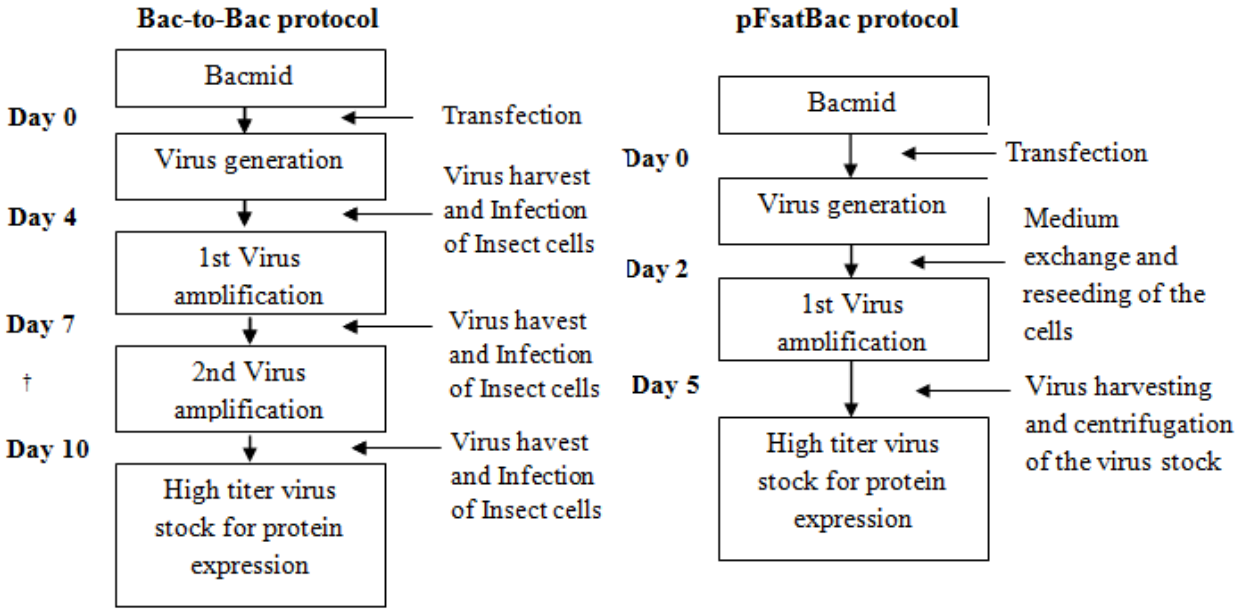


Fig. 5. Work ow for virus generation from bacmid to high titer virus stock. The newly developed pFsatBac protocol was compared to the classical Bac-to-Bac protocol (Invitrogen). By the use of the ultraBac protocol, the same amount of virus particles can be produced in half the time, and due to the centrifugation step, the virus is more concentrated and puri ed

After 2 days, the medium was removed and the cells were harvested. Reseeding of the cells into a T225 tissue culture ask resulted in cell con uence of about 30%. This low con uence during the virus production allows the non-infected cells to divide further until they in turn will be infected. The baculovirus stock was harvested 5 days post-transfection and yielded on average $2x10^9$ plaque forming units (pfu) per 10^8 cells in 50 ml cell culture medium (Fig. 2). Centrifugation of the high titer virus stock is a quick and easy method for concentration and puri cation 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 (Fig. 2). A combination of the techniques developed herein was applied to a series of bacmids and titer determination was performed at di erent stages of virus production (Fig. 2). To analyze the in uence 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 e ect on the expression yield as analyzed by Western blot analysis and by PEDV-S1 activity assay (Fig. 3).

To nd the conditions best suited for expression of a target protein, PEDV-S1 uorescence of infected insect cells was measured. For this purpose, insect cells were infected with virus at di erent multiplicities of infection in a 96-well plate. The total uorescence of the di erent wells was measured on a Typhoon 9400 uorescence plate imager (Fig. 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 (Fig. 4). The expression levels of the target proteins show strong correlation with both, uorescence intensities and PEDV-S1 expression levels (Fig. 4). The generation of a high titer baculovirus stock from a recombinant bacmid by standard methods requires three ampli cation 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 ampli cation round in 5 days. The most likely reason to achieve this acceleration is an increase of the viability of the non-infected insect cells during virus production. In the classical transfection step protocol, the cells are seeded to 90% con uence 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 con uence of the cells. Therefore, the cells go into the resting state (G0) (Kioukia *et al.,* 1995). However, baculovirus infection during the G0 phase is di cult and leads to a signi cantly 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 signi cantly 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% con uence 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% con uence. 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 con uence allows the non-infected cells to divide further until they in turn will be infected by virus particles generated during the ampli cation step. Virus stock obtained with this protocol 5 days post-transfection showed a titer comparable to the titer achieved after 10 days and three ampli cation 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 e ects on the insect cells. The serum proteins in the medium can cause di culties in subsequent protein puri cation, 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 su cient to cause such puri cation di culties. The simplest method to separate the virus particles from the FCS and the metabolic byproducts is a centrifugation step, using the di erence in density of virus particles on the one hand and serum proteins and toxic catabolites on the other hand. Puri cation 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 bu er, 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 (Fig. 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 (Fig. 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 uorescence 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 modi ed 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 signi cantly higher and uorescence 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 simpli cation of the titer determination step. 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 (Fig. 1). As shown in Fig. 3a, expression of PEDV-S1 under the late basic promoter has no detectable e ect on the expression yield of di erent 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 (Fig. 3a) and a b-galactosidase activity test of the cell lysates (Fig. 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 uorescence. 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 quanti cation 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 uorescence of the growing insect cells without interfering with the cell culture. For this purpose, we investigated the expression of three target proteins at di erent MOIs. Strong PEDV uorescence of the cells correlated signi cantly with the highest expression levels of both, FP and target protein, as determined by Western blotting (Fig. 4).

Thus, PEDV uorescence 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 puri cation or SDS-PAGE are necessary to nd 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 signi cant savings of time and resources. 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 (Fig. 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 (Fig. 5).

The amount of recombinant protein produced with this method in 10 days is usually su cient to provide enough protein for a complete protein structure determination process. The signi cant acceleration of protein production in BEVS now allows us, for the rst time, to approach timelines for recombinant protein expression in insect cells comparable to those for protein production in E. coli.

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REFERENCES

Bosch, B.J., van der Zee, R., de Haan, C.A.M. and Rottier, P.J.M. 2003. The coronavirus spike protein is a class I virus fusion protein: Structural and functional characterization of the fusion core complex. *Journal of Virology*, 77, 8801-8811.

Cha, H.J., Gotoh, T., Bentley, W.E., 1997. Simplification of titer determination for recombinant baculovirus by green fluorescent protein marker. *Biotechniques.,* 23, 782-786.

Chang, S.H., Bae, J.L., Kang, T.J., Kim, J., Chung, G.H., Lim, C.W., Laude, H., Yang, M.S., Jang, Y.S., 2002. Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. Molecules and cells 14, 295-299.

Chen, Y., Shi, Y., Deng, H., Gu, T., Xu, J., Ou, J., Jiang, Z., Jiao, Y., Zou, T. and Wang, C. 2014. Characterization of the porcine epidemic diarrhea virus codon usage bias. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 28, 95-100.

Condreay, J.P., Witherspoon, S.M., Clay, W.C. and Kost, T.A. 1999. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proceedings of the National Academy of Sciences*, 96, 127-132.

Duarte, M. and Laude, H. 1994. Sequence of the spike protein of the porcine epidemic diarrhoea virus. *The Journal of General Virology*, 75 (Pt 5), 1195-1200.

Ennis, F.A., Cruz, J., Jameson, J., Klein, M., Burt, D. and Thipphawong, J. 1999. Augmentation of human influenza A virus-specific cytotoxic T lymphocyte memory by influenza vaccine and adjuvanted carriers (ISCOMS). Virology 259, 256-261.

Giessauf, A., Flaim, M., Dierich, M.P. and Würzner, R. 2002. A technique for isolation of rubella virus-like particles by sucrose gradient ultracentrifugation using Coomassie brilliant blue G crystals. *Anal Biochem*., 308, 232-238.

Hughes, P., Wood, H., 1986. In vivo and in vitro bioassay methods for baculoviruses.

Jackwood, M.W., Hilt, D.A., Callison, S.A., Lee, C.W., Plaza, H. and Wade, E. 2001. Spike glycoprotein cleavage recognition site analysis of infectious bronchitis virus. *Avian Dis*., 45, 366-372.

Jung, K. and Saif, L.J. 2015. Porcine epidemic diarrhea virus infection: Etiology, epidemiology, pathogenesis and immunoprophylaxis. Vet J.

Katagiri, Y. and Ingham, K.C. 2002. Enhanced production of green fluorescent fusion proteins in a baculovirus expression system by addition of secretion signal. Biotechniques 33, 24-26.

Kioukia, N., Nienow, A., Emery, A. and Al-Rubeai, M. 1995. Physiological and environmental factors affecting the growth of insect cells and infection with baculovirus. *J Biotechnol*., 38, 243-251.

Kocherhans, R., Bridgen, A., Ackermann, M. and Tobler, K. 2001. Completion of the porcine epidemic diarrhoea coronavirus (PEDV) genome sequence. *Virus Genes*, 23, 137-144.

Lee, D. K., Cha, S. Y. and Lee, C. 2011. The N-terminal region of the porcine epidemic diarrhea virus spike protein is important for the receptor binding. *Korean Journal of Microbiology and Biotechnology,* 39, 40-50.

Lee, D.K., Park, C.K., Kim, S.H. and Lee, C. 2010. Heterogeneity in spike protein genes of porcine epidemic diarrhea viruses isolated in Korea. *Virus Research*, 149, 175-182.

Lippincott-Schwartz, J., Snapp, E. and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nature Reviews Molecular Cell Biology*, 2, 444-456.

Loisel, T.P., Ansanay, H., St-Onge, S., Gay, B., Boulanger, P., Strosberg, A.D., Marullo, S. and Bouvier, M. 1997. Recovery of homogeneous and functional b2-adrenergic receptors from extracellular baculovirus particles. *Nat Biotechnol., 15*, 1300-1304.

Masters, P.S. 2006. The molecular biology of coronaviruses. *Adv Virus Res,* 66, 193-292.

O'Reilly, D.R., Miller, L.K. and Luckow, V.A. 1994. Baculovirus expression vectors: a laboratory manual. Oxford University Press on Demand.

Park, S.J., Kim, H.K., Song, D.S., An, D.J. and Park, B.K. 2012. Complete genome sequences of a Korean virulent porcine epidemic diarrhea virus and its attenuated counterpart. *Journal of Virology,* 86, 5964.

Pasick, J., Berhane, Y., Ojkic, D., Maxie, G., Embury-Hyatt, C., Swekla, K., Handel, K., Fairles, J. and Alexandersen, S. 2014. Investigation into the role of potentially contaminated feed as a source of the first-detected outbreaks of porcine epidemic diarrhea in Canada. Transboundary and emerging diseases 61, 397-410.

Philipps, B., Forstner, M. and Mayr, L.M. 2004. Baculovirus expression system for magnetic sorting of infected cells and enhanced titer determination. Biotechniques 36, 80-83.

Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol.,* 27, 493-497.

Saito, T., Dojima, T., Toriyama, M. and Park, E.Y. 2002. The effect of cell cycle on GFPuv gene expression in the baculovirus expression system. *J Biotechnol*., 93, 121-129.

Song, D. and Park, B. 2012. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. *Virus Genes*, 44, 167-175.

Sturman, L.S. and Holmes, K.V. 1984. Proteolytic cleavage of peplomeric glycoprotein E2 of MHV yields two 90K subunits and activates cell fusion, In: Molecular Biology and Pathogenesis of Coronaviruses. *Springer*, 25-35.

Summers, M.D. and Smith, G.E. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures.

Sun, D.B., Feng, L., Shi, H.Y., Chen, J.F., Liu, S.W., Chen, H.Y. and Wang, Y.F. 2007. Spike protein region (aa 636789) of porcine epidemic diarrhea virus is essential for induction of neutralizing antibodies. *Acta Virologica* 51, 149-156.

Van Roessel, P. and Brand, A.H. 2002. Imaging into the future: visualizing gene expression and protein interactions with fluorescent proteins. *Nature Cell Biology*, 4, E15-E20.

Vlasova, A.N., Marthaler, D., Wang, Q., Culhane, M.R., Rossow, K.D., Rovira, A., Collins, J. and Saif, L.J. 2014. Distinct characteristics and complex evolution of PEDV strains, North America, May 2013-February 2014. Emerging infectious diseases 20, 1620-1628.

Wang, L., Byrum, B. and Zhang, Y. 2014. New variant of porcine epidemic diarrhea virus, United States, 2014. *Emerging Infectious Diseases,* 20, 917-919.

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