scientific reports

Check for updates

OPEN

A beginners guide to Sf9 and Sf21 insect cell line culture and troubleshooting

Joanna Kwiatkowska[1,2,5]✉, Ewa Stein[1,2,5], Anastasiia Romanenko[1,2], Martyna Plens-Gałąska[1,2], Małgorzata Podsiadła-Białoskórska[3], Ewa Szołajska[3], Uwe Kühn[4] & Kinga Kamieniarz-Gdula[1,2]

Recombinant proteins are not only a crucial research tool but are also widely implemented in biomedicine. There are a number of expression systems used for recombinant protein production. Among them *Spodoptera frugiperda* (Sf) insect cell system is a powerful tool for multiprotein expression. Most commonly used are Sf9 and Sf21 cell lines due to their cost-effectiveness and availability. While a collection of protocols describing the Sf cell lines culturing is available, we have found them incomplete and their adaptation to real laboratory conditions challenging. Here we created a user-friendly hands-on protocol suitable for beginners. Our work combines the efforts of three independent laboratories which culture Sf cells, two labs with long experience, and one which recently successfully set up this system from scratch. We propose novel tricks and tips that allow for culturing of healthy Sf cells, and high protein yield production. Besides catering for beginners, our protocol can serve as a troubleshooting guide for more experienced researchers. We believe that this work is useful for biochemistry all the way to biomedical laboratories. Starting with an exhaustive description of Sf cell lines, through baculovirus expression vector system characteristic, this publication is a protocol, troubleshooting guide and compendium in one.

Recombinant proteins are widely used in research as well as in biomedicine and pharmaceutics[1,2]. With the growing market size of recombinant therapeutic proteins, there is a need to design expression systems that will allow for obtaining the product of desired functionality cost-effectively. Expression in insect cells with the baculovirus expression vector system (BEVS) gained much interest in recent decades.

The lifecycle of baculoviruses as a basis for BEVS

The background of BEVS lies in the interactions between the baculoviruses and their insect hosts. The *Baculoviridae* family is represented by four genera of either nucleopolyhedroviruses or granuloviruses infecting arthropods with a high host specificity. Baculoviruses have a large, circular dsDNA genome (80–150 kbp), encoding up to 150 genes, protected by a rod-shaped nucleocapsid[3]. The virions alone or in groups can be surrounded by a protein matrix, forming occlusion bodies that protect the virus in the environment. The main difference between nucleopolyhedroviruses and granuloviruses is that for the first group large (0.5–10 µm) polyhedral occlusion bodies are formed by the polyhedrin protein and contain many virions; and for the second group smaller (approximately 0.4 µm) occlusion bodies are formed around just one virion and the protein responsible for it is granulin. The lifecycle of baculoviruses can be described with the example of AcMNPV (*Autographa californica* nuclear polyhedrosis virus) which is also the one most commonly used in laboratories.

The infection starts when occlusion bodies are ingested by the host. Then, in the midgut, virions are released from their coating and can enter the midgut epithelial cells. Later, via the tracheal system and hemolymph, the newly formed virions that have obtained an envelope with gp64 and F fusion proteins, important for subsequent cell entry, can infect the other types of cells in the body of the host during secondary infection. At this step two types of viruses can be produced: extracellular virus particles (Budded virions, BVs) and occlusion-derived virus particles (ODVs)[3–5]. Eventually, the ODVs accumulate in the nuclei, and, upon the host's death, they can be released into the environment in the form of occlusion bodies. At the cellular level, during the first hours

[1]Center for Advanced Technologies, Adam Mickiewicz University, 61-614 Poznań, Poland. [2]Department of Molecular and Cellular Biology, Faculty of Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, 61-614 Poznań, Poland. [3]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. [4]Institute of Biochemistry and Biotechnology, Charles Tanford Protein Center, Martin Luther University, Halle, Germany. [5]Joanna Kwiatkowska and Ewa Stein contributed equally to this work. ✉email: joanna.kwiatkowska@amu.edu.pl

after infection, the viral genome is released into the nucleus and early genes, encoding the transcription factors, as well as those promoting replication and preventing apoptosis, are transcribed by the host RNAP II. The late genes are mainly transcribed at 6–12 h post-infection. Among them there are viral DNA and RNA polymerases and structural proteins. New nucleocapsids start to assemble around 6–24 h after the infection and the formation of ODVs corresponds to 18–72 h post-infection[5].

Development of BEVS for recombinant protein expression

The establishment of insect cell lines as well as the studies on the baculoviral genome and lifecycle revealing the dispensability of the polyhedrin (*polh*) gene for viral infectivity are the factors that stimulated the implementation of BEVS in research. As mentioned above, baculoviruses are produced in two forms: occlusion-derived virus particles (ODVs) and budded virions (BVs). The first form is needed for horizontal transfer of virus between hosts and requires polyhedrin and p10 proteins to be expressed in order to create occlusion bodies; the second form is able to cause systemic infection in host organism or insect cell culture, and the polyhedrin, as well as p10 proteins, are dispensable in this case. The strong promoter of *polh* gene allows for high productivity of gene expression. The first case of usage of this promoter for expression of a foreign gene was in 1983 when Smith and colleagues reported the expression of human IFNβ with the recombinant AcMNPV virus[6]. As many insect cells are susceptible to AcMNPV infection, this virus is used the most often as BEVS. Sometimes *Bombyx mori* nucleopolyhedrovirus (BmNPV) and *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV) can also be used[7].

Several strategies were employed to create baculovirus-based vectors for expression in insect cells. A gene of interest (GOI) insertion into a baculoviral genome can occur either already in insect cells via homologous recombination or in a specialized bacterial strain via site-specific transposition after which the insect cells will be transfected with a prepared vector. The most commonly used system, Bac-to-Bac, employs the second strategy. Here, the *Escherichia coli* DH10Bac strain, carrying a bacmid, which is a baculoviral genome with lacZ-mini-*att*Tn7 site, is used to create the recombinant bacmid with GOI insertion by site-specific recombination that then can be readily transfected into insect cells. Alternatively, an approach with Cre-LoxP recombination is also possible[3].

Additionally, to enable multiprotein expression, the MultiBac system was developed. Here, as the first step, a multigene transfer vector has to be created. The sequences of MultiBac donor (usually containing dual expression cassette, multiplication module, and loxP site) and acceptor (containing additionally Tn7 transposition sequences) vectors are assembled by in vitro Cre-loxP fusion or via the multiplication module located between the expression cassettes in vectors[8]. Then the multigene transfer vector is incorporated into the baculoviral genome in *E. coli* DH10MultiBac cells according to the Bac-to-Bac system described above.

Insect cell lines in the production of recombinant proteins with BEVS

Up to now, more than 400 cell lines from over 100 insect species have been used to produce baculoviruses and recombinant proteins. Most of them are derived from *Lepidoptera* species; undifferentiated ovarian and embryonic tissues are mainly used for establishing stable diploid cell lines. Essentially, three insect cell lines are now most frequently used for recombinant protein expression with BEVS. Two lines, Sf21 and its subclone Sf9, come from the pupal ovarian tissue of fall armyworm *Spodoptera frugiperda*. The third cell line, HighFive, is isolated from the adult ovarian tissue of the cabbage looper *Trichoplusia ni.* Some less commonly used lines are Bm5 from *Bombyx mori*, Tn368 from *Trichoplusia ni,* and Ea88 isolated from *Estigmene acrea*[7].

All these cell lines have specific features making them suitable for different applications. Both Sf21 and Sf9 cells are highly susceptible to viral infection, but Sf9 seems to be better for maintenance as it has a higher growth rate, and is more tolerant to high densities, shear stress, and changes in culturing conditions. HighFive may offer a higher level of expression of secreted proteins, but these cells also produce more proteases which may result in degradation of the target protein[3]. It seems that Sf21 cells give the best results for plaque assays and assessing virus cytopathic effects, while Sf9 cells are very good for virus amplification and protein synthesis by being able to grow well in suspensions of large volumes. Below there is Table 1 summarizing the main features of Sf21 and Sf9 insect cell lines according to the data from the Oxford Expression Technologies Insect cell culture manual as well as our experience[9].

Feature	Sf21	Sf9
Origin	Pupal ovarian tissue isolated from primary explants of *S. frugiperda*[10]	Derived from the Sf21 cell line by Summers and Smith, 1987[11]
Morphology	Spherical	Spherical, more regular size
Suitability for monitoring cell viability	Worse	Better
Culturing conditions	Suspension and monolayer cultures, serum-containing and serum-free medium	Suspension and monolayer cultures, serum-containing and serum-free medium
Virus amplification and protein expression	Suitable for virus amplification and protein expression	Large-scale culturing for virus amplification and protein expression
Use in plaque assay	Easily detectable plaques after 3–4 days	Smaller plaques after 4 days
Tolerance to variation in experimental conditions and high cell densities during suspension culture	Low	High
Susceptibility to baculovirus infection	High	Moderate

Table 1. Comparison between Sf21 and Sf9 insect cell lines.

As it was mentioned above, the important problem of insect cell expression is improper *N*-glycosylation of mammalian proteins. Therefore, the main direction in the engineering of insect cells is altering the glycosylation pathways. For example, the SfSWT-1 cell line is derived from the Sf9 line and has the insertion of genes encoding for five mammalian glycosyltransferases; this allows for obtaining the mammalian patterns of terminally sialylated *N*-glycans. In order to prevent metabolic imbalance, the expression of these enzymes is inducible; it is activated by adding doxycycline to the culture medium.

The second direction of insect cell engineering is connected to prolonging their survival and resisting apoptosis upon baculoviral infection which may help in increasing the yield of target protein expressed. For example, constitutive expression of the anti-apoptotic vankyrin gene (P-vank-1 from *Campoletis sonorensis* ichnovirus) in Sf9 cells delayed their death after baculoviral infection[3].

The insect cells-baculovirus protein expression system was established in our laboratory to utilize it for the reconstitution of human cleavage and polyadenylation complex, responsible for the 3'-end processing of pre-mRNA molecules, including recognition of a polyadenylation signal, cleavage, and polyadenylation. While optimizing conditions for culturing insect cell lines and protein expression using BEVS, we took into account the experience and publications of groups working on similar tasks involving in vitro reconstitution of the cleavage and polyadenylation complex[12–14] as well as available protocols for protein expression in insect cells[9,10]. All of them used slightly different approaches which we adjusted for our needs. For example, Schmidt and colleagues[14] used suspension culture of Sf21 cells for protein expression, while Boreikaite and collaborators[13] as well as Zhang et al.[12] chose Sf9 cells for this purpose. In our experience, Sf9 cells are more tolerant to condition variation and higher cell densities during suspension cultivation than Sf21 cells. Together with the fact that they also have more uniform morphology facilitating the monitoring of cell viability and concentration, this made the Sf9 cell line our choice for most procedures, including virus amplification and protein expression. Sf21 cells were more susceptible to baculovirus infection and formed clear plaques afterward, this made them suitable for virus titer determination in plaque assays. In addition to the choice of the insect cell line, we also concentrated on the optimization of culturing conditions with the main attention given to the medium composition, the use of antibiotics, and the time between cell passaging. Importantly, our publication combines the efforts of three independent laboratories which culture Sf cells. Two of them have been working with baculovirus expression system for more than 20 years (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland and Institute of Biochemistry and Biotechnology, Charles Tanford Protein Center, Martin Luther University, Halle, Germany), and one which recently successfully set up this system from scratch (Center for Advanced Technology and Faculty of Biology, Adam Mickiewicz University, Poznan, Poland).

Experimental design

We take advantage of the MultiBac System, and electroporate DH10 MultiBac *E. coli* cells with pUCDM/pSPL donor vectors, and pFBDM/pKL/pFL acceptor vectors, subsequently[10]. As an example: we used pUCDM vector containing *mCherry* gene encoding fluorescent protein that can be fused via Cre-lox recombination to the artificial chromosome of DH10 MultiBac (DH10MB) *E. coli*. All steps are presented in the Fig. 1. Firstly, we transformed cells with Cre recombinase expressing pBAD-HisCre vector to generate electrocompetent DH10 MultiBac^{Cre} cells. Then these cells were electroporated with pUCDM-mCherry vector to obtain DH10 MultiBac^{Cre}-mCherry^{lox} strain. In further step pFBDM-CSTF vector was electroporated into these cells. *cstf* DNA sequence was incorporated within bacterial chromosome by Tn7 transposition. If the sequence of interest is incorporated by Tn7 transposition—white colonies are targeted. When employing Cre recombination—blue

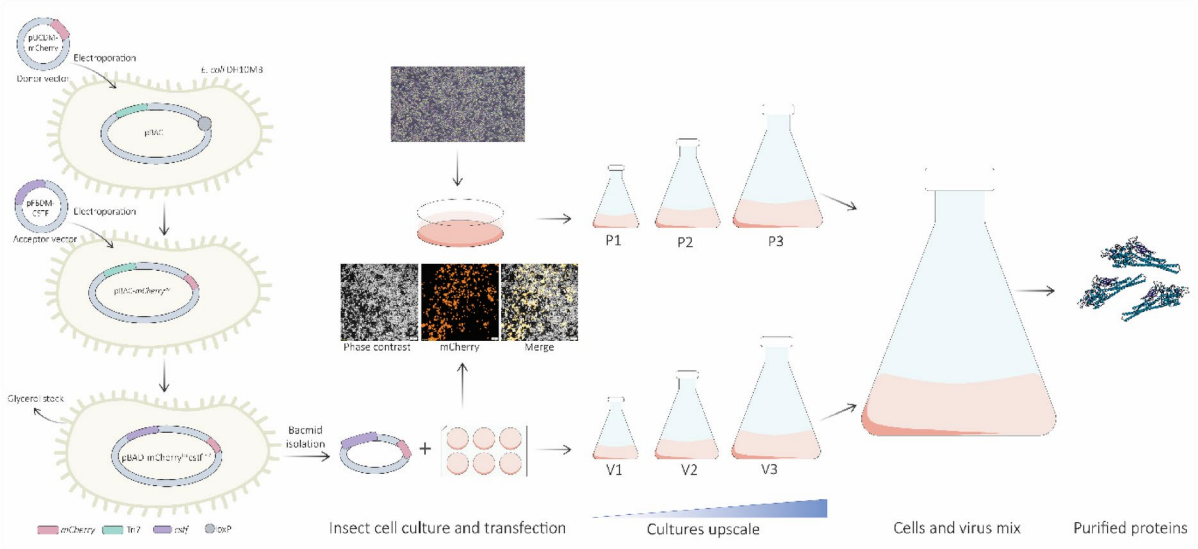

Fig. 1. Summary figure presenting crucial steps for described protocol. Photographs used present Sf21 cell lines, described in detail in the text.

colonies are picked, and antibiotic selection is used. Pay special attention while looking for the white clones, restreak colonies to fresh plate even 3 times, to confirm their color. Please note that colonies grow slowly due to the number of antibiotics used with resistance deriving from DH10MB itself as well as from vectors. Bacmid preparation might take a week. To verify if sequence of interest was incorporated within the bacmid DNA PCR should be performed, followed by product size analysis on an agarose gel. Design sequence-specific primers or perform PCR with M13 primer pair (flanking the *att*Tn7 site within *lacZ* gene). Transfect Sf9/Sf21 insect cells with the bacmid. Remember to prepare also the negative control—mock transfection mixture composed of transfection reagent with medium and without recombinant bacmid. If it is possible, after 3 days check the plate for positive transfection using a fluorescent microscope (or plate reader, imager). Congratulations! You just obtained the V0 virus generation. To estimate the progeny of virus, perform the plaque assay. Infect the cells with the required virus dilution. Add solidify agent, such as agarose, and cover the cells with medium. Immobilization of the cells restricts the virus infection only to surrounding cells. Dead cells will detach and create the clear space, so called plaque. Incubate 6-well plate with solidified cells for 4 days at 27 °C. You can prolong the incubation up to the week. In the next step stain the cells with Neutral Red solution. Then, you can read the plaques. The optimum number of plaques should be ranged from 10 to 30. It is extremely hard to count plaques when there are more than 80 of them. Moreover, if the number of plaques is too high, they will fuse with each other preventing accurate virus titer estimation—PFU (*Plaque Forming Units*). Values between 0.5×10^8 and 5×10^8 PFU/mL are expected. Estimation of PFU/mL allows for calculation of MOI factor (*Multiplicity of Infection*). MOI should range from 0.01 to 5. You will need to amplify the virus to higher generations. The V0 is not sufficient for protein synthesis, e. g. due to its low MOI factor, further generations are required. To do so, dilute the cells to 1×10^6 cells/mL concentration. Use low MOI (0.01–0.1) and remember that minimal cell passage should be used. You can perform a few batches of amplification in parallel. Incubate cells in suspension at 27 °C for 3–5 days. Monitor the cells daily and look for signs of infection: increased diameter of the cells (typically bigger than 18 µm), slower growth potential, disability for dividing, swollen look and decreased viability. Additionally, if the virus possesses a fluorescent reporter gene marker (e. g. mCherry), it will produce a colorimetric product. While you are producing the next virus generation remember to set a control noninfected culture, it would be helpful to spot the post-infection changes. Once you obtain the required virus generation, you are ready to start the protein synthesis. Inoculate 800 mL of log-phase Sf9 or Sf21 cells freshly diluted to $1–3 \times 10^6$ cells/mL V1 (or higher generation). Use MOI ranging from 2 to 10. Then incubate cells for 3–4 days with daily monitoring. If your recombinant baculovirus possesses fluorescent marker you can observe the change of culture color intensity. Importantly, we usually stop the synthesis when the cells reach 60–70% viability. You can perform a few syntheses in parallel. Spin the cells at 4 °C/10,000 rpm/10 min. Discard the supernatant and freeze the Sf cells pellet in liquid nitrogen, store at − 80 °C. Interestingly, Sf cells can be infected with more than one virus. Remember that the total volume of used recombinant viruses should not exceed 4% of the culture volume. Now, you can analyze the protein expression via Western-blot or standard SDS-PAGE electrophoresis with Laemmli buffer-treated samples. General workflow for insect cells/baculovirus expression system is presented in Fig. 1.

Results

Our protocol describes the way to synthesize recombinant protein in insect cells-baculovirus expression system. Obtained protein can be then purified using different chromatography techniques. As an example, we synthesized CSTF subcomplex of CPA machinery. Firstly, the protein undergoes nickel-affinity chromatography using Ni–NTA beads (Qiagen). Results showed that proteins included in the CSTF subcomplex—CSTF77, CSTF64, CSTF50—were eluted with success (elution fractions: E1–E6) (Fig. 2a). In the second step, the sample after Ni-NTA chromatography was further purified using anion exchange chromatography on ResourceQ column (Cytiva). One more time—proteins were eluted from the column successfully (elution fractions: E4–E7) (Fig. 2b).

We believe that our protocol can be implemented and successfully used in many different laboratories with various scientific profiles. Following our advice will allow for mammalian protein complexes as well as single proteins synthesis.

Discussion

Insect cell expression system is a powerful tool for synthesis of recombinant proteins as well as multiprotein complexes. In order to optimize the protein synthesis in Sf cells we had to adapt exhaustive existing protocols[7,9,10] to our laboratory conditions and reality. Here, we present applied tips and results of troubleshooting that allowed us for maintaining the Sf cells culturing, virus amplification and protein production. We hope that our publication will serve as a complete hands-on protocol for beginners.

Limitations

Technically, we recommend having separate laminar hood and incubators for culturing insect cells, as they are extremely fragile to bacterial and fungal infections. Insect cells produce less complex *N*-glycans with terminal mannose, compared to mammalian cells which generate complex *N*-glycans with terminal sialic acids, and different termini of *N*-glycans can impact bioactivity of glycoproteins[15,16]. However, to overcome this limitation many labs worked on engineering transgenic insect cell lines that are able to produce mammalian glycosylation enzymes[17–19].

Another problem that may occur is proteolysis of the target product. As *polh* and *p10* promoters are active during late and very late stages of infection, the expression may cooccur with the lysis of cells mediated by viral proteases. In order to prevent possible degradation of the expressed proteins, some constructs were created

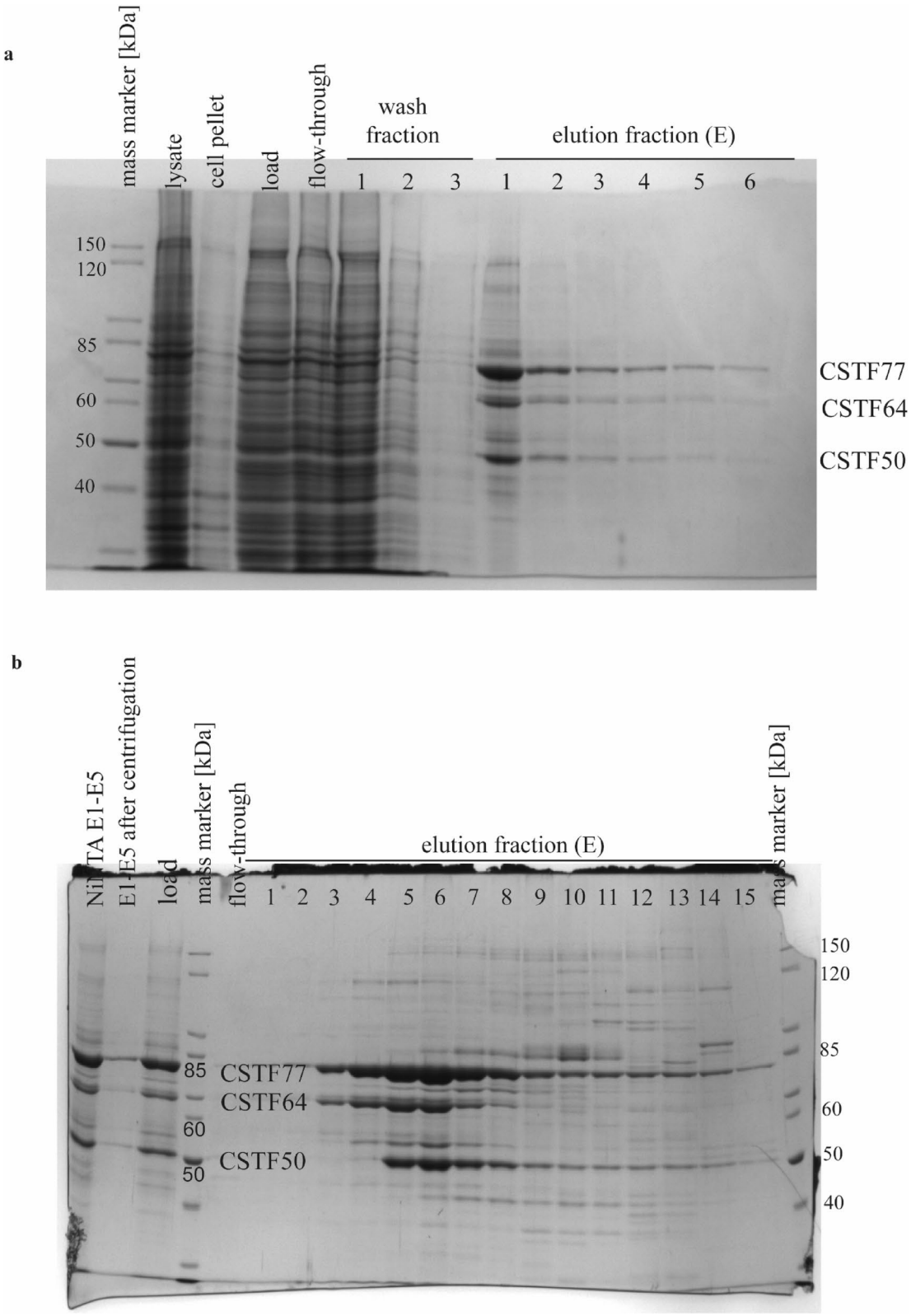

Fig. 2. SDS-PAGE analyses of purified subcomplex CSTF. (**a**) NiNTA chromatography. Lysate stands for centrifuged sample after sonication, pellet—cells debris after sonication, load—fraction loaded to NiNTA beads, flow-through—portion of unbound protein, wash fractions–portion of the protein eluted in low imidazole concentration and elution fraction (E)—fraction eluted in high imidazole concentration. (**b**) Anion exchange chromatography on ResourceQ column. NiNTA E1-E5 stands for pooled fractions after dialysis, load—sample loaded on ResourceQ, flow-through—portion of unbound protein, elution fraction (E)—eluted form the column in the gradient of KCl.

with the deletion of these proteases, for example, *chiA* and *v-cath* (deleted genes encoding for the chitinase and cathepsin)[7].

To further optimize the BEVS, steps towards minimization of the baculoviral genome are also being taken. There are estimations that almost 40 genes of AcMNPV are dispensable in the context of a heterologous protein expression system and, possibly, can be eliminated. These include, for example, some additional genes involved in occlusion body formation (*pp32*), genes regulating the behavior of a host (*egt, ptp*), and genes responsible for cell lysis[20]. Minimization of the baculoviral genome may enable the increase of the insert size and product yield for this expression system.

Comparison with other methods

There are a number of popular expression systems for recombinant protein production: bacterial, yeast, insect cells-baculovirus, and mammalian cell expression systems, as well as expression in plants. All of them have some advantages and drawbacks. Bacterial expression system is a method offering high productivity of expression while being the most cost-effective, due to rapid growth and extensively studied genetics (for *E. coli* as the most often used organism). On the other hand, disadvantages of this system involve improper post-translational modifications (PTMs) for eukaryotic proteins, the possibility of inclusion body formation, endotoxin contamination, and the need for codon optimization. Yeast expression systems, commonly using *Saccharomyces cerevisiae* and *Pichia pastoris*, may provide a good balance between productivity and the cost of expression on one hand, and the ability to achieve PTMs pattern close to native for the proteins expressed on the other. Expression of therapeutical proteins in plants has recently become popular due to scalability, relatively low cost, and, most importantly, because of safety—there is no risk of contamination with animal pathogens. The "golden standard" for the production of protein drugs remains still the mammalian cell expression system, as it allows for obtaining human proteins with native patterns of PTMs and proper folding, large proteins, as well as protein complexes. The drawback of mammalian expression is usually lower product yield, higher costs of expression and purification, and the risk of contamination with animal pathogens. Expression in insect cells with the baculovirus expression vector system (BEVS), which gained much interest and many users in recent decades, in many cases can offer an alternative to the mammalian system. While allowing for achieving proper folding and near-native PTMs for proteins produced, this method is characterized by higher production yield, lower overall cost, and easy scalability—in addition to low risk of product contamination with dangerous human pathogens[21,22].

Materials

Biological materials

Sf9 Insect Cells (Oxford Expression Technologies; cat. no. 600100)
Sf21 Insect Cells (Oxford Expression Technologies; cat. no. 600105)
DH10 MultiBac bacteria strain (kind gift of prof. Elmar Wahle)
pBAD-HisCre (Addgene; cat. no. 111187)

Reagents

Insect cell cultures

ESF 921 Insect Cell Culture Medium, Protein Free (Expression System; cat. no. 96-001-01)
EX-CELL® CD Insect Cell Medium (Merck; cat. no. 14380C)
Amphotericin B (250 µg/mL, Gibco; cat. no. 15290026)
Penicillin-streptomycin (5000 U/mL / 5000 µg/mL, Thermo Fisher; cat. no. 15140-163)
Gentamycin—for insect cell culture (50 mg/mL, Merck; cat. no. G1397-10ML)
Trypan Blue solution (0.4%, Sigma-Aldrich; cat. no. T8154-100ML)
Dulbecco's phosphate-buffered saline DBPS (Biowest; cat. no. L0615-500)
DMSO (Sigma-Aldrich; cat. no. 276855-250ML)
Fetal Bovine Serum (Gibco; cat. no. 10270106)
Fugene 4 K (Promega; cat. no. E5911)
Fugene HD (Promega; cat. no. E2311)
MycoStrip™ (Invivogen; cat. no. 50 REP-MYS-50)
Virkon™ Virucidal Disinfectant (cat. no. 12338667)

Bacterial cell cultures

Ampicillin (Bioshop; cat. no. AMP201.25)
Chloramphenicol (Bioshop; cat. no. CLR201.25)
Gentamycin (Bioshop; cat. no. GTA202.5)
Kanamycin (Bioshop; cat. no. KAN201.25)
Tetracycline (Bioshop; cat. no. TET701.25)
Bactotryptone (Bioshop; cat. no. TRP402.500)
Yeast Extract (Bioshop; cat. no. YEX401.500)
Glycerol (Bioshop; cat. no. GLY001.4)
Potassium phosphate monobasic (Bioshop; cat. no. PPM302.1)
Potassium phosphate dibasic (Bioshop; cat. no. PPD303.1)
IPTG (BioShop; cat. no. IPT001.25)

XGAL (20 mg/mL, BioShop; cat. no XGA002.510)
LB (Miller) broth (Bioshop; cat. no. LBL407.1)
LB (Miller) agar (Bioshop; cat. no. LBA408.1)
Tris (Bioshop; TRS001.5)
EDTA (0.5 M, Invitrogen; cat no. AM9260G)
RNase A (Merck; cat. no. 10109169001)
NaOH (Stanlab; 011-002-00-6)
SDS (Bioshop; cat. no. SDS001.1)
Potassium acetate (Merck; cat. no. 60035-50G)
HCl 35–38% (Stanlab; cat. no 017-002-01-X)
Isopropanol (Stanlab; cat. no 603-117-00-0)
Ethanol 99.9% (Stanlab; cat. no. 603-002-00-5)

Equipments

Countess II FL Automated Cell Counter Assay Platform AMQAF1000 (New Life Technologies)
Countess™ Cell Counting Chamber Slides (Thermo Fisher; cat. no. C10312)
Neubauer chamber (cat. no. LLG-9161086)
Hand Tally Click Counter (cat. no. HS6594)
Plastic Cell Culture Flask 25 cm^2 50 mL (Greiner Bio-One; cat. no. 690175),
Glass Erlenmeyer Flasks, narrow mouth style: 25 mL (Duran; cat. no. 320/A10)
Glass Erlenmeyer Flasks, narrow mouth style 100 mL (Duran; cat. no. G-2052)
Glass Erlenmeyer Flasks, narrow mouth style 250 mL (Duran; cat. no. G-2053)
Glass Erlenmeyer Flasks, narrow mouth style 500 mL (Duran; cat. no. G-2055)
Glass Erlenmeyer Flasks, narrow mouth style 2000 mL (Duran; cat. no. G-2057)
Metal lids (Schuett-Biotec; cat. no. B-2964)
Falcon tubes 15 m L (Sarstedt; cat. no. 62.554.502)
Falcon tubes 50 mL (Sarstedt; cat. no. 62.548.004)
Cryo vials (Sarstedt; cat. no. J-1958)
Serological pipettes (Greiner Bio-one; cat. no. 1 mL 612–1011)
Serological pipettes (Greiner Bio-one; cat. no. 2 mL 612–1012)
Serological pipettes (Greiner Bio-one; cat. no. 5 mL 612–1013)
Serological pipettes (Greiner Bio-one; cat. no. 10 mL 612–1015)
Serological pipettes (Greiner Bio-one; cat. no. 25 mL 612–1017)
Serological pipettes (Greiner Bio-one; cat. no. 50 mL 612–1018)
Pipetboy SWIFT PRO (HTL; cat. no. 390)
Freezing container Mr. Frosty (Nalgene; cat. no. L-0388)
Centrifuges with rotors/adapters for 1 5 mL and 50 mL Falcon tubes and Eppendorf tubes
Incubator without CO_2
Incubator without CO_2 with shaking platform
Laminar flow hood
Fluorescent Microscope (or fluorescent plate reader, imager)
Neutral Red (Sigma Aldrich; cat. no. N2889-100ML)
Pipettes 1000 µL, 200 µL, 20 µL, 10 µL HTL
Vortex
Burner
Syringe filters 0.22 µm (Merck; cat. no. SLGP033RS)
Syringes (5 mL cat. no. 309 050; 10 mL cat. no. 309 110; 20 mL cat. no.300 296 BD)
Needles (cat.no. 0.8 × 40 4,657,527; 0.4 × 20 mm 4,657,705 Braun)
Bacterial plates (cat. no. BH900S25SQ)

Reagent setup

Ampicillin stock Prepare 100 mg/mL ampicillin stock by resuspending 1 g (Bioshop) in 10 mL MiliQ water. Filtrate the solution using 0.22 µM filter. Divide into 1 mL aliquots and store at – 20 °C.
Tetracycline stock Prepare 12.5 mg/mL tetracycline stock by resuspending 125 mg (Bioshop) in 10 mL ethanol (Stanlab). Filtrate the solution using 0.22 µM filter. Divide into 1 mL aliquots and store at – 20 °C.
Kanamycin stock Prepare 50 mg/mL kanamycin stock by resuspending 500 mg (Bioshop) in 10 mL MiliQ water. Filtrate the solution using 0.22 µM filter. Divide into 1 mL aliquots and store at – 20 °C.
Chloramphenicol stock Prepare 30 mg/mL chloramphenicol stock by resuspending 300 mg (Bioshop) in 10 mL ethanol (Stanlab). Filtrate the solution using 0.22 µM filter. Divide into 1 mL aliquots and store at – 20 °C.
Gentamycin stock Prepare 50 mg/mL gentamycin stock by resuspending 500 mg (Bioshop) in 10 mL MiliQ water. Filtrate the solution using 0.22 µM filter. Divide into 1 mL aliquots and store at – 20 °C.
IPTG stock Prepare 400 mM IPTG stock by resuspending 0.95 g (BioShop) in 10 mL MiliQ water. Divide into 1 mL aliquots and store at – 20 °C.
Supplemented ESF 921 medium In sterile conditions prepare supplemented ESF 921 medium by adding 20 µL of gentamicin (Merck), 1 mL of penicillin–streptomycin (Thermo Fisher), and 1 mL of amphotericin B (Gibco) to 100 mL ESF 921 Insect Cell Culture Medium, Protein Free (Expression System).

Supplemented EX-CELL medium In sterile conditions prepare supplemented EX-CELL medium by adding 20 µL of gentamicin (Merck), 1 mL of penicillin–streptomycin (Thermo Fisher), and 1 mL of amphotericin B (Gibco) to 100 mL EX-CELL® CD Insect Cell Medium (Merck).
0.1% Trypan Blue solution Prepare 12 mL of 0.1% Trypan Blue solution by resuspending 3 mL of 0.4% Trypan Blue (Sigma-Aldrich) in 9 mL of DPBS (Biowest).
Tris-HCl stock Prepare 1 M Tris–HCl stock by resuspending 12.1 g of Tris (Bioshop) in 80 mL MiliQ water. Adjust pH to 8 with appropriate volume of HCl (Stanlab). Fill with MiliQ water to the final volume of 100 mL.
RNaseA stock Prepare 10 mg/mL RNaseA stock by resuspending 10 mg (Merck) in 1 mL MiliQ water. Store in 4 °C.
NaOH stock Prepare 200 mM NaOH stock by resuspending 0.4 g (Stanlab) in 10 mL MiliQ water.
Buffer I Prepare Buffer I by mixing 1 mL of Tris–HCl, pH 8, 0.4 mL of EDTA, and 18.2 mL MiliQ water. Store the solution in 4–8 °C up to 3 months. Every time before use add an appropriate volume of RNaseA (in total you should add 200 µL of a 10 mg/mL stock).
Buffer II Prepare Buffer II by mixing 0.2 g of SDS (Bioshop), 4 mL NaOH and filling it up to 20 mL with MiliQ water. Store the solution in plastic falcon at room temperature for up to 3 months.
Buffer III Prepare Buffer III by resuspending 3.89 g potassium acetate (Merck) in 15 mL MiliQ water. Adjust pH to 5.5 with appropriate volume of HCl (Stanlab). Fill with MiliQ water to the final volume of 20 mL. Filtrate the solution using 0.22 µM filter.
SOC medium Prepare solution A by mixing 12 g of bactotryptone (Bioshop), 24 g of yeast extract (Bioshop), and 5 mL of glycerol (Bioshop), and fill to 900 mL with MiliQ water. Autoclave it. Prepare 10 × solution B by mixing 23.1 g of potassium phosphate monobasic (Bioshop) with 125.4 g of potassium phosphate dibasic (Bioshop) and fill to 1 L with MiliQ water. Filtrate solution B using 0.22 µM filter. To prepare any volume of SOC medium in sterile conditions mix solution A with solution B in 1:10 ratio.
LB medium Prepare 100 mL of LB medium by resuspending 2.5 g of LB (Miller) broth (Bioshop) in 100 mL of MiliQ water. Autoclave it.
LB with agar Prepare 100 mL of LB with agar by resuspending 4 g of LB (Miller) agar (Bioshop) in 100 mL of MiliQ water. Autoclave it.
10% glycerol solution Prepare 1 L of 10% glycerol solution by mixing 100 mL (Bioshop) with 900 mL of MiliQ water. Autoclave it, store in 4 °C.
60% glycerol solution Prepare 50 mL of 60% glycerol solution by mixing 30 mL (Bioshop) with 20 mL of MiliQ water. Autoclave it, store in 4 °C.
Neutral Red solution for plaque assay Prepare Neutral Red solution for plaque assay by mixing 1 mL of Neutral Red (Sigma Aldrich) with 12 mL of DPBS (Biowest).
Virkon stock Prepare 2% Virkon solution by resuspending 50 g of Virkon™ Virucidal Disinfectant in 2.5 L of water.

Equipment setup

Environment sterilization
Before every initiation of cell culture work, spray the inside of the hood with 70% ethanol. While working with cells use a burner, work aseptically. After work, especially with virus infected cell cultures, sterilize the laminar hood with UV for at least 10 min.

Procedure

Insect cells culturing

Media preparation
Timing: 0.5 h.

1. For Sf9 cells culturing we chose to use ESF 921 Insect Cell Culture Medium Protein Free, and for Sf21 cells in EX-CELL® CD Insect Cell Medium, but when buying your own cell line pay attention to which medium is recommended by the supplier.
2. Warm medium to room temperature before use, thaw antibiotics stocks.
3. Spray the bottle with medium and tubes with antibiotics with ethanol and transfer them under the hood.
4. Pipette a volume of need to a clean flask and supplement it with gentamycin (final concentration: 10 µg/mL) and penicillin–streptomycin (end concentration: 100 U/mL, 100 µg/mL) antibiotics and amphotericin B (end concentration: 0.25 µg/mL) as an antifungal agent.

CAUTION: End concentrations of 10 µg/mL for gentamycin and 100 U/mL, 100 µg/mL for penicillin-streptomycin are usually sufficient for maintaining contamination-free culture, but with recurring fungal infections you can increase amphotericin B end concentration up to 2.5 µg/mL.
CAUTION: A good practice is maintaining cell lines antibiotics-free; however, this requires much more careful handling. Since protein overexpression requires long handling and often high volumes of media usage at final stages, the financial and time risk associated with antibiotic-free culture outweighs the benefits in our experience.
CAUTION: We prepare our media prior to use, and we do not store supplemented media, but there are no contraindications to do so. Just keep in mind the recommendations for storage of particular components, because they might differ in terms of stability at 4 °C.
CAUTION: The use of antibiotics and amphotericin B can be deleterious for cells growth.

Thawing and first passage of Sf9 and Sf21 cells
Timing: 1 day

Following different protocols for thawing you can come across subtle differences between them[14,19]. We generally adapted the protocol from Oxford Expression manual[14].

1. In sterile conditions in 15 mL falcon prepare 9 mL of proper medium supplemented with gentamycin, penicillin–streptomycin and amphotericin B.

   CAUTION: To minimize the risk of contamination we recommend using a burner to additionally sterilize the mouth of an opened flask with a flame.

2. Pick a vial of 1 mL of frozen Sf9/Sf21 cells from a cell bank and thaw it by keeping the vial in hands or expose it to running water.
3. Spray the tube with ethanol and transfer it under the working hood.
4. Aspirate the cells from tube and transfer the solution dropwise to the falcon with prepared medium.
5. Mix it by inverting the tube 4–5 times.
6. Divide the suspension in half and transfer each 5 mL to a sterile cell culture flask (25cm^2 50mL; Greiner).
7. Put the flasks into an incubator and allow cells to attach for few hours at 27 °C.
8. Medium used for freezing the cells contains toxic DMSO. After cells are attached remove the medium and gently change it for 5 mL of fresh one supplemented with antibiotics.

   CRITICAL STEP: Be careful not to detach the cells while aspirating old medium, new portion should be pipetted slowly above cells surface.

9. Incubate the cells for few days at 27 °C until they reach confluency suitable for further passage.

Second passage of Sf9 and Sf21 cells
TIMING: 0.5 day

1. If after few days of incubation in 27 °C Sf9 cells reach 100% confluency, and Sf21 > 80%[14] they are ready for detaching.

   CAUTION: There is a major difference between Sf9 and Sf21 cells regarding cell wellbeing and viability in high density cultures. In adherent cultures Sf9 cells behave well when overgrown, they can form layers on layers view (Fig. 3a). It might take even a week to reach this state, so remember to change the medium every 3–5 days. On the other hand, the confluency of Sf21 cells adherent culture should not exceed 80%[14] (Fig. 3b), it will take approximately 3–4 days to reach it.

2. In sterile conditions gently remove the old medium from the flask.

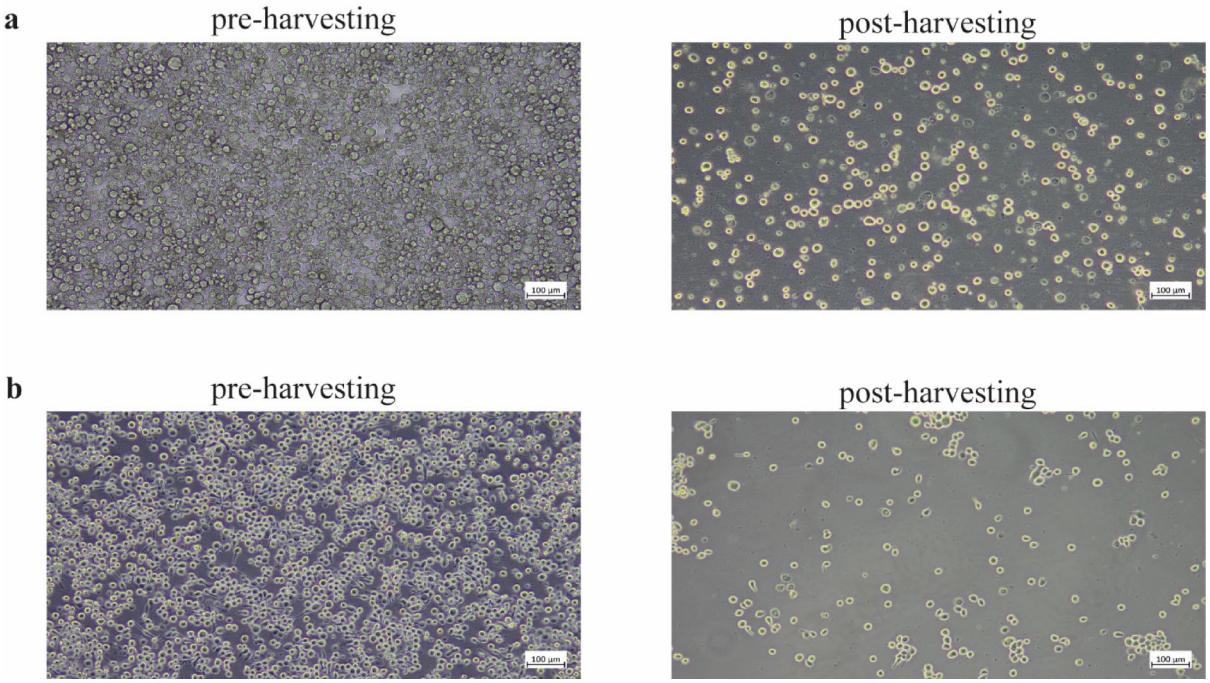

Fig. 3. Detaching Sf cells from adherent culture for the passage to suspension culture. Sf9 (**a**) and Sf21 (**b**) before and after harvesting, magnitude 10×.

3. Detach the cells by 6–7 harsh strikes of the culture flask on the surface of the hood.
4. Add 3 mL of fresh medium and wash out the cells by gentle pipetting.
5. Aspirate the suspension and transfer it to a sterile 15 mL falcon.
6. Count the cells. At this point cells might not have 90% viability, they will recover during next passages.
7. You can culture insect cells either in adherent culture or in suspension.
8. Passage to adherent culture: dilute Sf9 cells to $2–5 \times 10^4$ viable cells/mL and Sf21 to 0.5×10^6 cells/mL.
9. Passage to suspension: dilute Sf9 cells to 1×10^6 viable cells/mL and Sf21 to 0.5×10^6 viable cells/mL.
10. Place the glass flask with suspension culture (Erlenmeyer Flasks) in the shaker (27 °C/110 rpm). To maintain the proper aeration the culture volume should not exceed 1/5 of the volume of the glass flask. Put flasks with adherent culture into an incubator at 27 °C.

CAUTION: It is worth keeping the culture flask with remaining attached cells as a backup culture, add 5 mL of fresh medium to the flask and incubate the cells in 27 °C until they reach desired confluency.
CAUTION: We recommend using glass flasks for insect cells culturing, not only for environmental reasons, but also for the fact, that insect cells tend to adhere to plastic more, and form a bigger cell ring at the bottom of the flask compared to glass ones (See *Flask cleaning* in Troubleshooting section). A good way to close Erlenmayer flask is to use metal lids. Avoid total closure of the lid to enable gas exchange.

Cell counting
TIMING: 0.5h.

You can count cells using an automatic counter e.g. Countess™ or manually using Neubauer chamber. Dead cells are labelled with Trypan Blue solution.

1. Prepare 0.1% Trypan Blue in PBS solution.
2. In sterile conditions pipette ~ 100 µL of a cell culture to an Eppendorf tube.
3. In fresh Eppendorf tube mix 50 µL of cells and 50 µL of 0.1% Trypan Blue solution.
4. Take 10 µL of the mixture and transfer it on the slide or in the Neubauer chamber and calculate the number of living cells.

CRITICAL STEP: We noticed that sometimes the automatic counter underestimated the number of live cells. Manual counting allows more accurate calculation of cell quantity and their viability estimation. While manual cell counting is more time-consuming, using the right number of insect cells for overexpression experiments is critically important for their success therefore we suggest avoiding the temptation of taking shortcuts here.

Routine maintenance of Sf in suspension culture

1. Monitor Sf9/Sf21 cells concentration and viability daily.
2. For Sf9 cells: when cells reach $2.5–3.5 \times 10^6$ cells/mL, at least 95% alive with 15–17 µm Ø, pass them to $0.3–0.5 \times 10^6$ cells/mL dilution. Compared to Sf21, Sf9 cells are not as susceptible to overgrowing.

CAUTION: When cells reach concentration of $3.8–4 \times 10^6$ cells/mL, remember about *cell density effect*.

3. For Sf21 cells: when cells reach $1.5–2 \times 10^6$ cells/mL, at least 95% alive with 15–17 µm Ø, pass them to $0.2–0.4 \times 10^6$ cells/mL dilution.

CAUTION: General recommendation for passing Sf9 cells is dilution to 0.5×10^6 cells/mL and Sf21 to 0.2×10^6 cells/mL[14]. However, we noticed, most likely due to use of antibiotics in media, that cells grow slowly, and they do not double every second day as it is described in protocols. That is why we sometimes pass them to even 1×10^6 cells/mL and 0.8×10^6 cells/mL for Sf9 and Sf21 cells, respectively. Remember to first exchange medium if you want to passage the cells to such high concentration.

4. If cells will not reach desired density within 4–5 days, spin down the half of a culture 1000 g/5 min), aspirate the old medium, and complement with fresh one.

CRITICAL STEP: We did not conduct cultures for longer than 15 passages.

5. For transfection, virus production or protein synthesis the viability of cells should be at least 90–95%.

Cells freezing
TIMING: 1 h.

CRITICAL STEP: Freeze cells when they are not older than passage 5. Freeze down the cells in 1 mL portions of 1×10^7 cells/mL.

1. Mix 45% (450 µL) of the culture, 45% fresh medium (450 µL) and 10% DMSO (100 µL) in a cryo vial.
2. Place vial into the Mr. Frosty freezing container filled with isopropanol and keep it for 3 days at − 80 °C.
3. If you do not have the Mr. Frosty freezing container, transfer the vials to − 20° for 1–2 h, then move them to − 80 °C for 3 days.

PAUSE POINT

4. For long-term store the vials in liquid nitrogen cell bank.

Bacmid preparation
This part of the protocols chapter is based on preparation of pBAC-mCherry^{lox}-CSTF^{Tn7} bacmid. However, the instruction can be transplanted to many different bacmids.
Preparation of DH10 MultiBac electrocompetent cells.
TIMING: 3–5 days.

1. Prepare: LB agar plate with ampicillin, kanamycin, tetracycline (Table 2) for bacteria selection blue/white colonies:

    a. Prepare LB with agar and autoclave it.
    b. Cool down the autoclaved medium until it is cool enough to handle (~ 50 °C).
    c. In sterile conditions (best to work under the hood, but it's also ok to work on bench with a burner) add ampicillin (end concentration: 100 µg/mL), kanamycin (end concentration: 50 µg/mL), tetracycline (end concentration: 12.5 µg/mL), (Table 2) into the flask with gelling LB medium.
    d. Add X-gal (2 µg/mL), and IPTG (end concentration: 320 µM).

2. Prepare 100 mL of LB medium in 500 mL flask (without antibiotics), 900 mL of LB medium in 2 L flask (without antibiotics); 1 L of 10% glycerol (sterile, cold).
3. Streak a chunk of the ice from the DH10 MultiBac (kind gift of prof. Elmar Wahle) frozen stock to LB agar plate with antibiotics. Incubate overnight at 37 °C.
4. On the second day inoculate a single colony of *E. coli* from the agar plate into a flask containing 100 mL of LB medium (no antibiotics). Incubate the culture overnight at 37°C in a rotary shaker (~ 200 rpm).
5. Inoculate 100 mL of overnight culture into 900 mL of pre-warmed LB (no antibiotic). Incubate at 37 °C, 200 rpm. When OD_{600} reaches 0.4–0.5, cool down the culture on ice for 15 min.
6. Centrifuge at 4000 rpm, 4 °C, 15 min.
7. Decant supernatant. Resuspend cells in 500 mL ice cold sterile 10% glycerol solution.
8. Centrifuge at 4000 rpm, 4 °C, 15 min.
9. Decant supernatant. Resuspend cells in 250 mL ice cold sterile 10% glycerol solution.
10. Centrifuge at 4000 rpm, 4 °C, 15 min.
11. Decant supernatant. Resuspend cells in 10 mL ice cold sterile 10% glycerol solution.
12. Centrifuge at 4000 rpm, 4 °C, 15 min.
13. Decant supernatant. Resuspend cells in 1 mL ice cold sterile 10% glycerol solution.
14. Prepare 40 µL aliquots, freeze them in liquid nitrogen and store at − 80 °C.

CRITICAL STEP: Competent cells are very fragile, avoid unnecessary pipetting and work on ice.
Preparation of DH10 MultiBac^{Cre} electrocompetent cells.
TIMING: 2 days.

1. Electrocompetent DH10 MultiBac *E. coli* cells were electroporated with pBADZ-HisCre (Addgene) plasmid to prepare DH10 MultiBac^{Cre} cells[10].
2. Prepare 40 µL aliquots and store them at liquid nitrogen.

Electroporation of DH10 MultiBac^{Cre} *E. coli* cells with donor vector.
TIMING: 1 day.
DH10 MultiBac^{Cre} *E. coli* cells were electroporated with pUCDM-mCherry donor vector (kind gift of prof. Elmar Wahle) as follow:

1. Prepare the SOC medium, pre-warm it to 37 °C.
2. Prepare selective LB agar plates with X-gal, IPTG and required antibiotics, in our case: ampicillin, kanamycin, tetracycline and chloramphenicol (Table 2).
3. Place electroporation cuvettes and 2 mL Eppendorf tube on ice.
4. Use 40 µL of electrocompetent DH10 MultiBac cells and add 250 ng of pUCDM-mCherry. Incubate on ice for 2 min. Transfer to a chilled electroporation cuvette.

    CRITICAL STEP: Avoid air bubbles while pipetting. Also make sure that you pipette the solution across the bottom of the cuvette.

Bacteria strain/vectors	Feature	Antibiotic resistance (end concentration)
DH10 MultiBac strain	Bacterial strain	Ampicillin (100 µg/µL) Kanamycin (50 µg/µL) Tetracycline (12.5 µg/µL)
pUCDM-mCherry	Donor vector	Chloramphenicol (25 µg/µL)
pFBDM-CSTF	Acceptor vector	Ampicillin (100 µg/µL) Gentamicin (50 µg/µL)

Table 2. Antibiotic resistance of used bacterial strain and vector.

5. Electroporate using following conditions: V = 1.8 kV.
6. Transfer the solution to a chilled 2 mL Eppendorf tube, add 1 mL of SOC medium.
7. Incubate for at least 6 h with vigorous shaking (180 rpm).
8. Centrifuge solution (3000 g), aspirate 800 µL of the supernatant. Dilute the cells pellet in remaining ≈250 µL of supernatant. Spread the solution on pre-warmed selective plate.
9. Incubate the plate overnight at 37 °C.

Preparation of DH10^{Cre} MultiBac-mCherry^{lox} electrocompetent cells.

1. Prepare all media and plate as described in *Preparation of DH10 MultiBac electrocompetent cells* (points 1–2), despite the plate should possess in our case additionally chloramphenicol (Table 2).
2. Select a single blue colony of DH10 MultiBac^{Cre}-mCherry^{lox} cells (obtained in point 9th of *Electroporation of DH10 MultiBac^{Cre} E. coli cells with donor vector*) and inoculate 100 mL of LB medium (without antibiotics). Incubate overnight at 37 °C, then follow points 4–14 from *Preparation of DH10 MultiBac electrocompetent cells*.
3. Prepare 40 µL aliquots, freeze them in liquid nitrogen and store at − 80 °C

Electroporation of DH10 MultiBac^{Cre}-mCherry^{lox} *E. coli* cells with acceptor vector.
TIMING: 2–4 days.

1. In order to incorporate the gene of interest within DH10 MultiBac^{Cre}-mCherry^{lox} bacterial genome via Tn7-transposition, perform the electroporation with pFBDM/pKL/pFL/pSPL acceptor vector[10], for this we used pFBDM-CSTF vector.
2. Procced with instructions from *Electroporation of DH10 MultiBac^{Cre} E. coli cells with donor vector* (points 1–9).
3. Select a single white colony from the plate and streak it to fresh LB plate with X-gal, IPTG, and required antibiotics (in our case ampicillin, tetracycline, kanamycin, chloramphenicol, gentamycin).

   CRITICAL STEP: It is very difficult to distinguish between white and blue colonies. Re-streaking the white colony up to three times to the fresh plate will ensure that the selection is positive.

4. Congratulation, you have just obtained the bacmid!

Bacmid isolation by alkaline lysis
TIMING: 2 days.

1. Prior, prepare 6 mL of LB medium with all required antibiotics, in our case ampicillin, kanamycin, tetracyclin, chloramphenicol and gentamycin; buffers I, II, III, and 60% glycerol.
2. Inoculate 6 mL LB medium with antibiotics with single, white colony from the plate (obtained in point 3rd of *Electroporation of DH10 MultiBac^{Cre}-mCherry^{lox} E. coli cells with acceptor vector*).
3. Incubate overnight at 37 °C.
4. Next day harvest the overnight culture (5 min/14 000 g).

   CRITICAL STEP: Remember to make a backup stock. Mix 150 µL of a 60% glycerol and 750 µL of the bacterial culture, freeze at liquid nitrogen, store at − 80 °C.

5. Resuspend cell pellet in 250 µL of buffer I.

   CRITICAL STEP: Do not use vortex, you can gently pipette up and down to avoid shearing of bacmid DNA.

6. Add 250 µL of buffer II, mix and invert 4–6 times.
7. Add 350 µL of buffer III, mix by inverting 4–6 times.
8. Centrifuge the sample for 10 min at 16 000 g, room temperature.

   CRITICAL STEP: Repeat this step since all precipitates from the supernatant are spin down.

9. Transfer the clear supernatant to a fresh 2 mL Eppendorf tube.
10. Add isopropanol in 0.8 volume of supernatant (≈640 µL) and gently invert, place on ice for 15 min to precipitate DNA.
11. Spin down the sample for no longer than 20 min at maximum speed in room temperature to precipitate DNA. Aspirate the supernatant without disturbing the pellet.
12. Wash the pellet in 80% EtOH by inverting the tube a few times.
13. Centrifuge for 5 min at 16,000 g, room temperature and remove the supernatant.
14. Air-dry pellet for 5–10 min.

    CRITICAL STEP: Do not over dry the pellet!

15. Resuspend the pellet in 50 µL of sterile H_2O or 10-mM Tris pH 8.0 plus 0.1 mM EDTA by gentle tapping the bottom of the tube. Try to avoid pipetting.
16. Estimate the bacmid concentration using A_{260} spectrophotometric method.

CRITICAL STEP: Store bacmid at − 20 °C up to one year. Avoid multiple freezing and thawing cycles.

17. You have just isolated the pBAC-mCherry^{lox}-CSTF^{Tn7} bacmid.

Sf cells transfection with recombinant bacmid DNA
TIMING: 5 days.

1. Prior to the transfection, culture Sf9 or Sf21 insect cells. Count the cells, they have to be at least 95% viable.
2. Seed them in 6-well plate in number of $0.8–0.9 \times 10^6$ cells per well (2 mL of $0.4–0.45 \times 10^6$ cells/mL per well.

   CRITICAL STEP: Do not exceed cell concentration of 0.6×10^6 cells/mL.

3. Attach the cells for 1 h at 27 °C.
4. In the meantime, prepare the transfection mixture: mix 200 µL of insect cell medium without antibiotics, add 5 µL of FUGENE HD or FUGENE 4 K (Promega) transfection reagent and 2 µg of bacmid DNA.

   CRITICAL STEP: Remember to pipette the FUGENE directly to the solution, not to an empty Eppendorf tube.
   CAUTION: We found transfection with FUGENE 4 K more efficient than with FUGENE HD.

5. Pipette the transfection mixture up and down.
6. Incubate at room temperature for 15–45 min.

   CRITICAL STEP: Do not exceed 45 min.

7. Aspirate the old medium and slowly add 2 mL of fresh one to each well by pipetting it by the wall.
8. Add each transfection mixture drop wise to the well with seeded cells.

   CRITICAL STEP: As a control prepare the mock transfection—mixture composed of FUGENE reagent with medium and without recombinant bacmid DNA.

9. Seal the plate with parafilm and incubate for 4–5 days at 26 °C.
10. If it is possible, monitor the plate for positive transfection using a fluorescent microscope (Fig. 4).
11. After the required time, aspirate the supernatant. Centrifuge for 3 min, 3 000 g, room temperature, transfer the fresh supernatant to a 2 mL Eppendorf tube and store at 4 °C and protect from light. You have just obtained the V0 virus generation.

    CRITICAL STEP: To increase the stability of the virus, add 10% FBS to the stock.

Plaque assay
TIMING: 5 days

1. Pre-warm insect cell medium at 27 °C. Use the medium recommended for the insect cell line.
2. Place 4% low melting agarose and a portion of the cell culture medium supplemented with FBS at 60 °C.
3. Seed 2 mL of 0.9×10^6 cells on 6-well plate.

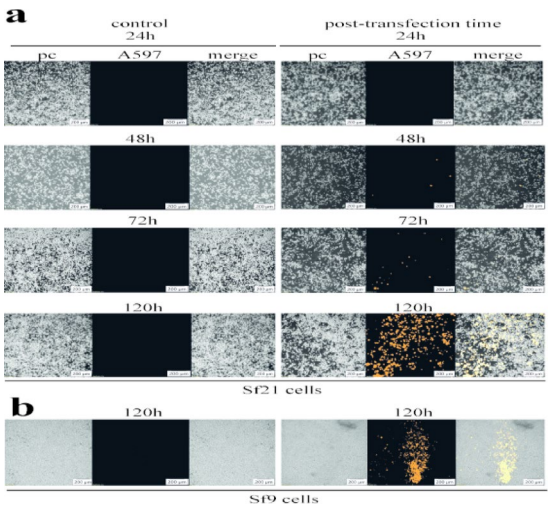

Fig. 4. Expression of CSTF complex and mCherry protein. In Sf21 (**a**) and Sf9 (**b**) cells transfected with pBAC-mCherry^{loxP}-CSTF^{Tn7} bacmid. Images taken 24 h, 48 h 72 h, 120 h post transfection, magnitude 10×. Abbreviation pc stands for phase contrast.

4. Attach them for 1–2 h at 27 °C.

   CRITICAL STEP: Cells should be equally distributed in the bottom of the well.

5. In the meantime, prepare the virus dilution according to Fig. 5.
6. Prepare 6 1.5 mL Eppendorf tubes, add 270 µL of pre-warmed medium at 27 °C to each.
7. Add to the first tube 30 µL of V0. Vortex.
8. Transfer 30 µL of first dilution to the second tube. Vortex. You obtained 10^{-1} dilution.
9. Repeat step 8th as long as you reach tube number 6.
10. Prepare dilutions in two replicates.

    CRITICAL STEP: Use fresh tip every time and to vortex properly each virus dilution.

11. Add 100 µL of dilutions 10^{-4}, 10^{-5}, 10^{-6} drop-wise to the center of the wells with attached cells.
12. Incubate for 1 h at room temperature.

    CRITICAL STEP: Rotate the plate from time to time to evenly distribute the virus.

13. Prepare 12 mL of 4% agarose-medium mixture to obtain 1% agarose: add 3 mL of agarose and 9 mL of medium (use reagents warmed to 65 °C).
14. Cool down the solution to 37–40 °C.
15. Aspirate the virus from each well.
16. Add 2 mL of 1% agarose to each well.

    CRITICAL STEP: Add the agarose slowly by the wall. Avoid air bubbles formation. Make sure the agarose solution is properly cooled. Do not let the cells dry.

17. Incubate the agarose for 15–30 min and add 1 mL of medium with FBS per well.
18. Cover the plate with parafilm and incubate for 4 days at 27 °C.

Time: 4–7 days

19. Prepare Neutral Red solution for plaque assay.
20. Remove the medium from agarose and add 1 mL of dye.
21. Incubate for 2 h at 27 °C.
22. Aspirate the dye, incubate the plates upside down overnight in a dark place at room temperature.
23. Next day count the near-white plaques.
24. Estimate the virus titer according to formula:

$$\frac{PFU}{ml} = \frac{plaques\ number}{dillution \times inococulum},$$

where PFU means *Plaque Forming Units*. E.g. if 40 plaques were counted when 0.1 mL of virus was added to dilution 10^{-5}, PFU number of undiluted virus stock is 4×10^7.

25. Calculate the MOI factor (*Multiplicity Of Infection*):

$$MOI = \frac{virus\ titer \times virus\ volume}{total\ cells\ number},$$

E.g. when virus titer is 4×10^7, virus volume is 100 µL and total cells number is equal to 25×10^6, MOI is 0.16.

26. Transformation of the above formula will allow for calculation of how much virus is needed to be add to the culture, as follow:

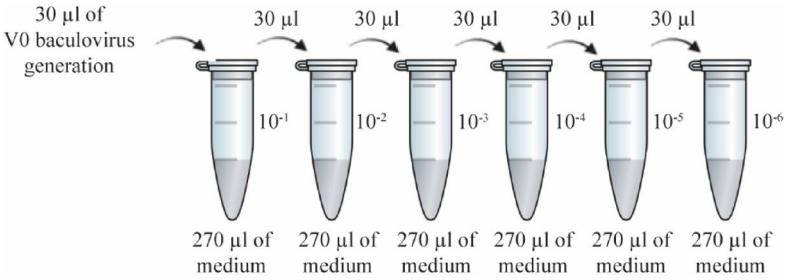

Fig. 5. Preparation of viral dilution.

$$virus\ volume = \frac{MOI \times total\ cells\ number}{virus\ titer}.$$

Virus amplification
TIMING: 5 days.

The V0 generation shouldn't be used for protein expression. Further generations are required. To have a higher titer and better-quality viral particles follow the instruction:

1. Estimate the MOI of V0 virus generation according to the formula given in *Plaque assay* section.

   CAUTION: Low MOI's (from 0.01 to 0.1) should be used.
   CAUTION: You can perform a few batches of amplification in parallel. Remember to prepare a control culture lacking viruses.

2. Prepare cells suspension in low density—1×10^6 cells/mL.

   CAUTION: Minimal cell passage should be used for this purpose.

3. Add appropriate volume of V0 calculated based on formula given in *Plaque assay* section.
4. Incubate cells suspension at 27 °C for 3–5 days.

   CAUTION: Monitor the cells daily and look for signs of infection: increased diameter of the cells (typically bigger than 18 μm), slower growth potential, impaired dividing, swollen phenotype and decreased viability. Additionally, if the virus possesses a fluorescent reporter gene marker (eg. mCherry), it will produce a colorimetric product.

5. Harvest the cells—5 min / 1000 g at 4 °C.
6. Store the V1 virus at 4 °C for a short period of time or freeze in liquid nitrogen for longer term storage.
7. For the production of higher virus generations perform titration of V1 in analogues way to one described for V1 production from V0.

   CAUTION: Our experience showed that produced viruses are integral and have infectious potential for months at 4 °C (up to 4–6 months), and for years at − 80 °C. However, we never start the protein synthesis from a frozen stock. After virus stock thawing perform virus amplification to the next generation. Importantly, viruses should be protected from light.

Protein synthesis in a medium scale
TIMING: 3 days.

Once you obtain the required virus generation (V1 or higher), you are ready to start the protein synthesis in suspension culture.

1. Inoculate 800 mL of log-phase Sf9 or Sf21 cells freshly diluted to $1–3 \times 10^6$ cells/mL with V1 generation (or higher) Calculate the PFU and MOI factors (as described in section Plaque assay). Use MOI ranging from 2 to 10 PFU/ml.
2. Incubate cells for 3–4 days with daily monitoring.

   CRITICAL STEP: If your recombinant baculovirus possesses fluorescent marker, you can observe the change of culture color intensity (Fig. 6a).

3. Importantly, we usually stop the synthesis when the cells reach 60–70% viability. You can perform a few syntheses in parallel.
4. Spin the cells at 4 °C/10 000 rpm/10 min (Fig. 6b).
5. Discard the supernatant and freeze the Sf cells pellet in liquid nitrogen, store at − 80 °C.

   CRITICAL STEP: Interestingly, Sf cells can be infected with more than one virus.
   CAUTION: You can analyze the protein expression via Western-blot or standard SDS-PAGE electrophoresis with Laemmli buffer-treated samples. You can also directly proceed with the protein purification using ÄKTA Pure chromatography systems.

Troubleshooting

Contamination of the cell culture
It is crucial to monitor the cell culture daily. If any aggregates, abnormal culture color, intensive smell are noticed, stop the culture. Clean the incubator, autoclave everything that can be autoclaved, and start the culture from scratch. Antibiotics together with proper glass cleaning should minimize the risk of bacterial or fungal infection. Additionally, remember to work sterile under the hood, always wash your hands before work, wear gloves, spray them with ethanol, and clean the hood regularly. Moreover, it helps to sterilize the lids and the mouth of the flasks using a flame under the hood.

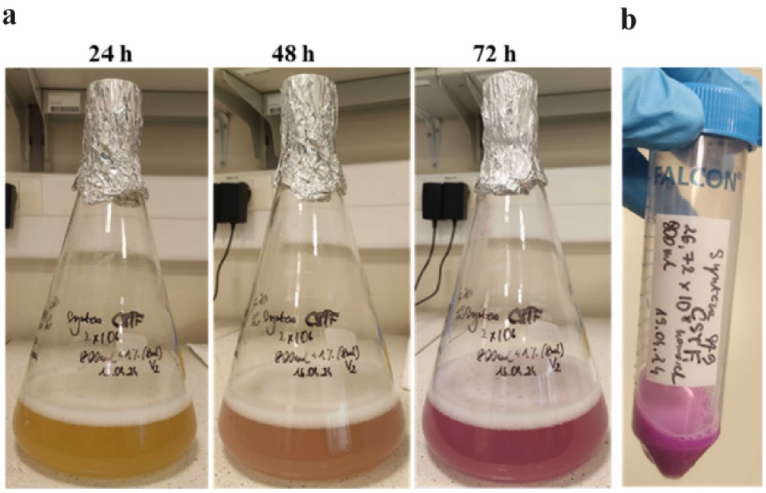

Fig. 6. Co-expression of CSTF complex and mCherry protein in Sf9 cells. (**a**) The change in culture color upon expression of fluorescent mCherry protein. (**b**) Cell pellet after harvesting.

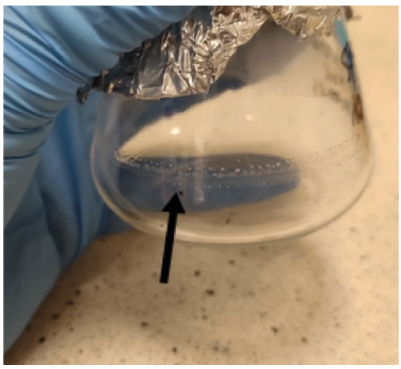

Fig. 7. The picture of the ring of cell aggregates attached to the glass wall. The ring is indicated by an arrow.

Mycoplasma testing

Mycoplasmas are common contaminants of eukaryotic cell cultures. Resistant to most antibiotics used in culturing, those small self-replicating organisms affect most of culture parameters, including cell growth, viability, cell metabolism, and virus propagation[23]. It is crucial to control if the ongoing culture is infected by mycoplasma, to prevent its further transfer. For mycoplasma testing we employ MycoStrip™ cassette detection kit from InvivoGen.

Flask cleaning

An appropriate flask cleaning routine is essential for experimental success when employing re-usable glass flasks. Not only remains of previous culture but also residual detergent used for washing can inversely impact the cell culture health. After removing cells from the flask, rinse it with 2% Virkon solution, put some crushed ice inside, and swirl toss firmly. This helps to remove the ring of cell aggregates attached to the glass wall after culture (Fig. 7). Cover the flask with aluminum foil and autoclave it. Incubate the glass with a splash of 2% Virkon overnight and clean it carefully with a brush. Then wash it again using a dishwasher. To get rid of residual detergent rinse the flask 3–5 times with MilliQ water. Metal lids used for securing the glass flask wash manually using dish soap and rinse them 3–5 times with MilliQ water. Cover the flask with a lid and 2 layers of aluminum foil and autoclave it. Using metal lids is not obligatory; instead, you can use 4 layers of aluminum foil. After the second autoclaving round bake the glass at 180 °C for at least 5 h. An alternative to this stringent washing routine is using plastic dispensable flasks. We do not recommend this for environmental reasons.

Data availability

All datasets generated or analyzed in this publication are available from the corresponding author upon reasonable request.

Received: 9 December 2024; Accepted: 23 April 2025

Published online: 06 June 2025

References

1. Dimitrov, D. S. Therapeutic proteins. In *Therapeutic Proteins: Methods and protocols* (eds Voynov, V. & Caravella, J. A.) 1–26 (Humana Press, 2012). https://doi.org/10.1007/978-1-61779-921-1_1.
2. Hong, Q., Liu, J., Wei, Y. & Wei, X. Application of baculovirus expression vector system (BEVS) in vaccine development. *Vaccines* **11**, 1218 (2023).
3. Hong, M. et al. Genetic engineering of baculovirus-insect cell system to improve protein production. *Front. Bioeng. Biotechnol.* **10**, 994743 (2022).
4. Murphy, C. I. & Piwnica-Worms, H. Overview of the baculovirus expression system. *Curr. Protoc. Neurosci.* **10**, 4–18 (2000).
5. Williams, T., Virto, C., Murillo, R. & Caballero, P. Covert infection of insects by baculoviruses. *Front. Microbiol.* **8**, 1337 (2017).
6. Smith, G. E., Summers, M. D. & Fraser, M. J. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* **3**, 2156–2165 (1983).
7. Contreras-Gómez, A., Sánchez-Mirón, A., García-Camacho, F., Molina-Grima, E. & Chisti, Y. Protein production using the baculovirus-insect cell expression system. *Biotechnol. Prog.* **30**, 1–18 (2014).
8. Berger, I., Fitzgerald, D. J. & Richmond, T. J. Baculovirus expression system for heterologous multiprotein complexes. *Nat. Biotechnol.* **22**, 1583–1587 (2004).
9. Possee, R., Chambers, A., Lissina, O. & King, L. *Insect cell culture: A complete laboratory guide to insect cell culture* (Oxford Expression Technologies ltd, 2024).
10. Fitzgerald, D. J. et al. Protein complex expression by using multigene baculoviral vectors. *Nat. Methods* **3**, 1021–1032 (2006).
11. Pichard, S. et al. Insect cells-baculovirus system for the production of difficult to express proteins: From expression screening for soluble constructs to protein quality control. In *Insoluble proteins* Vol. 2406 (eds Garcia Fruitós, E. & Arís Giralt, A.) 281–317 (Springer, 2022).
12. Zhang, Y., Sun, Y., Shi, Y., Walz, T. & Tong, L. Structural insights into the human pre-mRNA 3'-end processing machinery. *Mol. Cell* **77**, 800-809.e6 (2020).
13. Boreikaite, V., Elliott, T. S., Chin, J. W. & Passmore, L. A. RBBP6 activates the pre-mRNA 3' end processing machinery in humans. *Genes Dev.* **36**, 210–224 (2022).
14. Schmidt, M. et al. Reconstitution of 3' end processing of mammalian pre-mRNA reveals a central role of RBBP6. *Genes Dev.* **36**, 195–209 (2022).
15. Harrison, R. L. & Jarvis, D. L. Protein *N*-glycosylation in the baculovirus-insect cell expression system and engineering of insect cells to produce "Mammalianized" recombinant glycoproteins. In *Advances in Virus Research* Vol. 68 159–191 (Academic Press, USA, 2006).
16. Felberbaum, R. S. The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. *Biotechnol. J.* **10**, 702 (2015).
17. Harrison, R. L. & Jarvis, D. L. Transforming lepidopteran insect cells for improved protein processing. *Methods Mol. Biol.* **388**, 341–356 (2007).
18. Okada, T. et al. *N*-Glycosylation engineering of lepidopteran insect cells by the introduction of the beta1,4-*N*-acetylglucosaminyltransferase III gene. *Glycobiology* **20**, 1147–1159 (2010).
19. Aumiller, J. J., Hollister, J. R. & Jarvis, D. L. A transgenic insect cell line engineered to produce CMP-sialic acid and sialylated glycoproteins. *Glycobiology* **13**, 497–507 (2003).
20. van Oers, M. M., Pijlman, G. P. & Vlak, J. M. Thirty years of baculovirus–insect cell protein expression: From dark horse to mainstream technology. *J. Gen. Virol.* **96**, 6–23 (2015).
21. Owczarek, B., Gerszberg, A. & Hnatuszko-Konka, K. A brief reminder of systems of production and chromatography-based recovery of recombinant protein biopharmaceuticals. *Biomed. Res. Int.* **2019**, e4216060 (2019).
22. Tripathi, N. K. & Shrivastava, A. Recent developments in bioprocessing of recombinant proteins: Expression hosts and process development. *Front. Bioeng. Biotechnol.* **7**, 420 (2019).
23. Drexler, H. G. & Uphoff, C. C. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. *Cytotechnology* **39**, 75–90 (2002).

Acknowledgements

We would like to thank prof. Elmar Wahle for providing resources and constant support. We thank Anne-Katrin Hoffmeister, Moritz Schmidt and Florian Kluge for their technical and intellectual support.

Author contributions

Conceptualization, J.K., E.St. and K.K.-G.; Investigation, J.K. and E.St.; writing—original draft, J.K. and A.R.; writing—review & editing, J.K., A.R., E.St., M.P.-G., M.P-B, E.Sz, U.K., K.K.-G.; figure preparation, J.K. (Figs. 2–7) and M.P.-G. (Fig. 1); funding acquisition, K.K.-G.; resources, U.K.; supervision, J.K. and K.K-G.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to J.K.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.