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Membrane protein production in insect cells

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Abstract

Membrane proteins are an essential part of the machinery of life. They connect the interior and exterior of cells, play an important role in cell signalling and are responsible for the influx and efflux of nutrients and metabolites. For their structural and functional analysis high yields of correctly folded and modified protein are needed. Insect cells, such as *Sf9* cells, have been one of the major expression hosts for eukaryotic membrane proteins in structural investigations during the last decade, as they are easier to handle than mammalian cells and provide more natural post translation modifications than microbial systems. Here we describe general techniques for establishing and maintaining insect cell cultures, the generation and amplification of recombinant baculovirus stocks using the flashBAC™ or Bac-to-Bac™ systems, membrane protein production, as well as the production of membrane preparations for extraction and purification experiments.

Keyword

Membrane protein production, Sf9 insect cells, recombinant baculovirus

1 Introduction

Insect cells are often used for eukaryotic membrane protein production since they provide advantages over bacteria and yeast and usually give high protein yields. The advantages of their use include the fact that larger genes can be expressed, that they are not as prone to contamination as mammalian cells and that the post-translational modifications they provide only differ slightly from those in mammalian cells [1]. One example of these differences is the glycosylation pattern. Glycosylated proteins in yeast, insect and mammalian cells have a common sugar core containing two N-acetylglucosamine molecules and three mannose residues. Membrane proteins in mammalian cells are further modified with terminal fucose, sialic acid or galactose residues by the enzymes sialyltransferase and galactosyltransferase. Insect cell membranes contain higher amounts of unsaturated lipids and lower amounts of

cholesterol to ensure membrane fluidity [2]. Insect cell expression has been successfully used for the production of many membrane proteins, including GPCRs (G protein-coupled receptors) and ABC (ATP Binding Cassette) transporters [3-7]. Insect cells were used for approx. 31 % of the eukaryotic membrane protein structures obtained in the last 10 years [1].

The most commonly used cells lines are *Sf*9, *Sf*21 and Hi5. *Sf*21 and *Sf*9 cells were derived from the ovaries of a lepidopteran insect, the fall armyworm, *Spodoptera frugiperda*. The Hi5 (High five) cell-line originates from the ovarian cells of the cabbage looper, *Trichoplusia ni* [8]. The baculovirus used is derived from AcMNPV (*Autographica californica* multicapsid nucleopolyhedrovirus). Recombinant baculovirus lack the polyhedrin (coat protein) gene and carry the target gene of interest in its place. The recombinant baculovirus is used to infect insect cells in order to express recombinant proteins. Baculoviruses are essentially non-pathogenic to mammals and plants and their infection and replication is limited to specific *Lepidoptra* species such as looper moths and army worms. Recombinant baculovirus lacking the polyhedrin gene, are incapable of establishing a productive infection in a host organism and are only able to infect isolated cells. Thus, the baculovirus and insect cell system is safe and easy to use, harnessing the power of the viral infection for effective delivery of the target gene to the cells. The insect cells can be used both for amplifying the virus and protein production, and the ability to grow them in suspension, in incubators that do not require CO_2, makes them easy to scale up.

For production of recombinant baculovirus, two main systems are commonly used (Fig. 1). The first is the Bac-to-Bac™ system that initially involves the gene of interest being cloned into a pFastBac™ vector. The expression of the gene of interest is typically under the control of the polyhedrin promoter (which is a strong promoter and codes for many copies of the gene). The pFastBac vector, containing the gene of interest, is then transformed into DH10Bac™ *Escherichia coli* (*E. coli*) cells that contain a bacmid (baculovirus genome with a transposon) and a helper plasmid. Inside the DH10Bac™ cells the gene of interest is transferred from the pFastBac™ vector to the bacmid by site specific transposition. The

bacmid is isolated and purified and used to transfect insect cells which then produce and release the recombinant baculovirus into the medium [9,10]. In the second system, flashBAC™, the baculovirus genome lacks part of an essential gene, ORF1629, so that the virus cannot replicate in insect cells. Furthermore, it contains a Bacterial Artificial Chromosome (BAC) at the polyhedrin locus which replaces the polyhedrin coding gene. The insertion of BAC in the AcMNPV gene locus permits the viral DNA to be maintained and propagated as circular in bacteria and thus produce the flashBAC™ DNA which can be purified and isolated. Secondly, there is a transfer vector, such as pOET, containing the full ORF1629 gene and into which the target gene of interest is cloned. Both, the transfer vector containing your target gene and the flashBAC™ DNA, are co-transfected into insect cells where recombination occurs. This leads to the restoration of the ORF1629 function so that the viral DNA can be replicated. At the same time the gene of interest replaces the BAC sequence. The recombinant virus is harvested from the culture medium and further amplified for protein production. In comparison with the Bac-to-Bac™ system, the flashBAC™ simplifies the virus production as no recombinant bacmid needs to be produced and isolated [11,12].

In the following chapter, we describe how an *Sf9* insect cell culture is established and maintained, a recombinant baculovirus is produced using the Bac-to-Bac™ or flashBAC™ systems and how membrane proteins can be expressed in small and large scale monolayer and suspension cultures respectively. Figure 2 represents a visualization of the general workflow for this.

2 Materials

2.1 Equipment

1. HERA Safe laminar flow hood for cell culture work
2. 75 % Industrial methylated spirit (IMS) for disinfection of the work area and equipment
3. Haemocytometer for cell counting
4. Phase contrast microscope

5. Incubator at 27 – 28 °C, with orbital shaking platform and a stationary shelf.

2.2 General cell culture materials

1. Insect-XPRESS™ insect cell medium (Lonza) (*see* **Note 1**)
2. Foetal bovine serum (*see* **Note 2**)
3. Penicillin-streptomycin stock (*see* **Note 3**)
4. *Sf*9 insect cells
5. T25 and T75 monolayer cell culture flasks
6. 125 mL, 250 mL, 500 mL, 1 L and 2 L sterile shaker flasks

2.3 Cell freezing

1. Cryoprotective medium
2. Cryo-tubes

2.4 P0 virus stock production

2.4.1 Bac-to-Bac™

1. pFastBac™ Transfer vector (Invitrogen, ThermoFisher) (*see* **Note 4**)
2. Max Efficiency DH10Bac™ competent cells (Invitrogen, ThermoFisher)
3. TE buffer :10 mM Tris-HCl, 1 mM EDTA, pH 8
4. S.O.C. medium (provided with the DH10Bac™ cells).
5. LB agar plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL X-gal, 40 µg/mL IPTG
6. LB containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline
7. Microcentrifuge
8. Solution I:15 mM Tris-HCl pH 8, 10 mM EDTA, 100 µg/mL RNase A, filter sterilized and stored at 4 °C
9. Solution II:0.2 M NaOH, 1 % SDS, filter sterilized
10. 3 M potassium acetate, pH 5.5, filter sterilized

11. Isopropanol
12. Nanodrop or alternative spectrophotometer to measure absorbance at 260nm.
13. 6-well tissue culture plates
14. Grace's unsupplemented medium
15. Cellfectin or Expifectamine Sf transfection reagent (ThermoFisher)

2.4.2 FlashBAC™

1. 6-well tissue culture plates
2. Grace's unsupplemented medium
3. pOET Transfer plasmid (Oxford Expression Technologies) (*see* **Note 5**)
4. FlashBAC™ ULTRA system (Oxford Expression Technologies) (*see* **Note 6**)
5. BaculoFECTIN II transfection reagent (Oxford Expression Technologies)
6. X-Gal: 20 mg/mL in DMSO)

2.5 Protein production

1. P1 or higher virus stock
2. Ultracentrifuge (e.g. Optima™ XPN-100 Beckman Coulter)
3. Lysis buffer: 50 mM Tris-HCl, pH 7, 150 mM NaCl, 1 % Triton X-100
4. Detergent compatible protein assay kit (e.g. BIO-RAD DC or Pierce BCA)
5. Standard SDS-PAGE and Western blotting equipment.
6. 4639 Cell disruption vessel for nitrogen cavitation (Parr Instrument Company)
7. Nitrogen gas
8. Phosphate buffered saline (PBS)
9. Homogenization buffer: 50 mM Tris-HCl, pH 7.4, 250 mM Sucrose, 0.25 mM $CaCl_2$
10. MemPrep buffer: 50 mM Tris-HCl, pH 7.4, 250 mM Sucrose
11. cOmplete™ Mini EDTA-free protease inhibitor cocktail

3 Methods

3.1 Establishment and maintenance of a suspension cell culture

3.1.1 Cell thawing

1. Take a vial of *Sf*9 cells from -80 °C/liquid nitrogen storage and thaw as quickly as possible.
2. Transfer 1 mL of the cells to a T25 flask and add 9 mL growth medium (*see* **Note 7**).
3. Incubate at 27 – 28 °C for 1 hour.
4. Check if the cells have attached to the bottom of the flask using a phase contrast microscope.
5. Remove the medium and add 10 mL of fresh medium.
6. Incubate overnight at 27 – 28 °C. Refresh the medium again the next day.

3.1.2 Monolayer cell passage

1. Once cells reach approx. 80 – 90 % confluency they should be passaged. Detach the cells by hitting the side/bottom of the flask with your hand.
2. Transfer the cell suspension to a T75 flask and add 5 – 10 mL growth medium.
3. Once they reach 80 – 90 % confluency again, detach cells and transfer 2 mL to a new T75 flask and add 13 – 18 mL of growth medium.
4. Incubate at 27 – 28 °C (*see* **Note 8**).

3.1.3 Transferring cells to a shaker flask

1. When passaging cells from a T75 flask to another, instead of throwing away the remaining cells, they can be transferred to a 125 mL shaker flask.
2. Add approx. 20 mL of growth medium.
3. Incubate at 27 – 28 °C on a shaker platform at 100 rpm (*see* **Note 9**).

3.1.4 Maintaining a stock of cells in shaker flasks

1. Count the cells with a haemocytometer.
2. When cells reach a density of >3 x 10^6 cells/mL they need diluting into another flask (*see* **Note 10**).

3. Dilute the cells to a density of 0.4-0.5 x 10^6 cells/mL (*see* **Note 11**).
4. Incubate at 27 – 28 ˚C 100 rpm.

3.1.5 Cell freezing

1. Harvest 10 mL cells at 1.0 – 1.2 x 10^6 cells/mL.
2. Centrifuge at 500 x g for 4 minutes and discard the supernatant.
3. Resuspend cell pellet in 2 mL fresh growth medium and 2 mL freezing medium (*see* **Note 12**).
4. Aliquot in cryo-tubes (1 mL in each) and put the cryo-tubes in Mr. Freezy isopropanol bath at -80 °C for 2 – 3 days. Store long term in liquid nitrogen.

3.2 Virus production and amplification

3.2.1 P0 virus production using the Bac-to-Bac™ system (based on the Bac-to-Bac handbook [10]).

1. Clone your gene of interest into a pFastBac™ vector.
2. Thaw one vial of DH10Bac™ cells, keeping them on ice.
3. Dilute your purified pFastBac plasmid containing your gene of interest to 0.2 ng/µL in sterile TE buffer.
4. Transfer 100 µL DH10Bac™ cells to a 15 mL centrifuge tube, add 5 µL of your diluted plasmid and incubate on ice for 30 minutes.
5. Heat shock at 42 °C for 45 seconds. Then put back on ice for 2 minutes.
6. Add 900 µL room temperature S.O.C. medium. Incubate at 37 °C for 4 hours, shaking at 100 rpm.
7. Prepare 10-fold serial dilutions of the cells (10^{-1}, 10^{-2}, 10^{-3}) with S.O.C. medium, and plate 100 µL of each dilution on LB agar plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL X-gal and 40 µg/mL IPTG.
8. Incubate for 48 hours at 37 °C. The white colonies that appear are the ones to be used.

9. Pick 10 white colonies and re-streak them on fresh LB-agar plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL X-gal and 40 µg/mL IPTG to check the colonies really are white (*see* **Note 13**).
10. Incubate at 37 °C 48 hours.
11. From a single colony confirmed to be white on these plates, inoculate a 5 mL overnight culture in LB containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline.
12. Remove 1.5 mL of this overnight culture, and microfuge to pellet cells.
13. Remove the supernatant and resuspend the pellet in 300 µL solution I (*see* **Note 14**).
14. Add 300 µL of solution II, mix gently, and incubate 5 min at room temperature.
15. Slowly add 300 µL of 3 M potassium acetate pH 5.5, mixing gently, and incubate on ice for 5 – 10 min.
16. Microfuge at 13,000 rpm for 10 minutes to pellet the white precipitate.
17. Gently transfer supernatant to a fresh microcentrifuge tube containing 800 µL isopropanol (be sure not to transfer any of the white precipitate). Invert a few times and put on ice 5 – 10 min
18. Microfuge at 13,000 rpm, for 15 minutes.
19. Carefully remove the supernatant, taking care not to disturb the pellet.
20. Add 500 µL of 70 % ethanol and invert a few times to wash the pellet.
21. Microfuge at 13,000 rpm for 5 minutes.
22. Remove as much supernatant as possible and air dry the pellet for 5 – 10 min.
23. Dissolve the pellet in 40 µL TE buffer. Do not pipette or vortex to resuspend as this will shear the large bacmid DNA, instead just let sit with occasional tapping.
24. Store the isolated bacmid DNA at 4 °C (not -20 °C as thawing causes shearing).
25. Measure the DNA concentration using a Nanodrop.

To make a baculovirus you simply transfect *Sf*9 cells with the bacmid-gene of interest and after a few days harvest the medium which will contain the virus.

26. Dilute some *Sf*9 cells in log phase to 0.5 x 10^6 cells/mL in growth medium, and add 2 mL/well in a 6 well tissue culture plate (*see* **Note 15**).

27. Incubate for 1 hour at 27 – 28 °C for cells to adhere.

28. Dilute your bacmid DNA to 0.25 µg/µL in sterile TE buffer.

29. Add 4 µL of bacmid DNA to 100 µL Grace's unsupplemented medium.

30. Mix 6 µL Cellfectin reagent with 100 µL Grace's unsupplemented medium.

31. Add this medium/Cellfectin mixture to the bacmid DNA/medium. Mix gently and incubate at room temperature for 30 minutes,

32. Carefully remove the growing medium from the plated cells. Wash each well with 1 mL Grace's unsupplemented medium (*see* **Note 16**).

33. Add 800 µL Grace's unsupplemented medium to each tube of bacmid DNA/Cellfectin mixture. Then add this mixture to a well of cells.

34. Incubate at 27 – 28 °C for 5 hours.

35. Carefully remove the Cellfectin/medium mix from the cells and replace with 2 mL growing medium.

36. Incubate at 27 – 28 °C for 4 days (*see* **Note 17**).

37. To harvest virus, collect the media from the well. Spin at 500 x g, for 5 min to remove cell debris.

38. Put 1 mL of the supernatant in a sterile microcentrifuge tube and keep at 4 °C in the dark – this is the P0 virus stock (*see* **Note 18**). The remaining 300 – 500 µL are kept in another tube for long term storage at -80 °C.

3.2.2 P0 virus production using the flashBAC™ system

The protocol is based on the instructions by Oxford Expression Technologies (OET) [11].

1. Clone your gene of interest into a pOET vector.
2. Dilute *Sf9* cells grown to log phase to a cell density of 0.4 x 10^6 cells/mL using growth medium and add 2 mL per well to a 6-well plate (*see* **Note 15**).
3. Leave the plate at room temperature for 1 hour so that the cells attach.
4. Prepare the transfection mixture containing 100 µL Grace's unsupplemented medium, 5 µl flashBAC™ virus DNA, 500 ng of pOET plasmid (either positive control or containing your gene of interest), 1.2 µL baculoFECTIN II transfection reagent, and incubate at room temperature for 15 min (*see* **Note 19**).
5. Wash the cells in the 6 well plate twice with 1 mL Grace's unsupplemented medium (*see* **Note 16**).
6. Add the transfection mixture dropwise to each well and leave the plate in the incubator overnight at 27 – 28 °C without shaking.
7. Add 1 mL of growth medium to each well and leave the plate at 27 – 28 °C for another 4 days resulting in a total of 5 days (*see* **Note 17**).
8. To harvest virus, collect the medium from the well. Spin at 500 x g, for 5 min to remove cell debris and store it at 4 °C in the dark. This is the P0 virus (*see* **Notes 18 & 20**).

3.2.3 P0 to P1 virus amplification

1. Dilute *Sf*9 cells from log phase to 1 x 10^6 cells/mL in growth medium, and plate 2 mL cells per well in a 6-well plate. Allow 3 to 6 wells per strain of virus to amplify (*see* **Note 21**).
2. Incubate 1 – 2 hours, at 27 – 28 °C for cells to adhere.
3. Add 40 µL P0 virus to each well. Incubate at 27 – 28 °C for 3 days.
4. To harvest virus collect the media from each well. Combine the 3/6 wells of the same virus together (5 – 10 mL).
5. Spin at 500 x g for 5 minutes to pellet cells and debris.

6. Transfer to a sterile 15 mL centrifuge tube, wrap in aluminium foil and store at 4 °C. This is the P1 virus stock. Further amplification can be done using the usual shaker culture method.

3.2.4 P1 to P2 (or higher) virus amplification

1. Dilute *Sf*9 cells in a shaker flask to a density of 0.6 – 0.7 x 10^6 cells/mL. Grow overnight at 27 – 28 °C 100 rpm.
2. The next day the cells should be at approx. 1 x 10^6 cells/mL. Add virus at a MOI of 0.1 (*see* **Notes 22 & 23**).
3. Incubate at 27 – 28 °C, 100 rpm for 5 days.
4. Transfer the culture to sterile 50 mL centrifuge tubes and spin at 4,000 x g for 10 minutes at 4 °C.
5. Harvest supernatant in fresh sterile tubes. Wrap in aluminium foil and store at 4 °C.

3.3 Recombinant protein production

3.3.1 Small scale test expression

1. Dilute *Sf9* insect cells grown to log phase from a suspension culture to a cell density of 0.4 x 10^6 cells/mL in growth medium (*see* **Note 24**)
2. Plate 2 mL/well in 6 well plates and leave the plates at room temperature for 1 hour without shaking (*see* **Note 16**).
3. Add 500 µL P1 virus per well. As a negative control add 500 µL medium to some wells.
4. Incubate the wells for either 48 hours or 72 hours at 27 – 28 °C without shaking.
5. To harvest the cells, detach the cells by pipetting the medium at different positions up and down.
6. Transfer the cell suspension to a microcentrifuge tube and spin the cells at 16,000 x g for 1 minute.
7. Remove the supernatant and resuspend the cell pellet in 100 µL lysis buffer.
8. Measure the protein concentration using a detergent compatible protein assay.

9. Load 2 µg protein for each sample (including negative control) on SDS-PAGE gels and analyse target protein production by Western blot.

3.3.2 Large scale expression

1. Dilute *Sf*9 cells from a 1 L shaker flask to a 2 L shaker flask at a density 0.7 x 10^6 cells/mL. Grow overnight at 27 – 28 °C, 100 rpm (*see* **Note 24**).
2. The next day the cells should be approx. 1 x 10^6 cells/mL. Add virus at an MOI of 5 or 10 (*see* **Notes 22, 25, 26**).
3. Incubate at 27 – 28 ˚C, 100 rpm for 2 – 3 days (*see* **Note 26**).

3.3.3 Membrane preparation

1. Transfer the culture to centrifuge bottles.
2. Centrifuge at 7,000 x g for 10 minutes at 4 °C and discard the supernatant.
3. Resuspend each pellet in 10 mL PBS, and transfer to 50 mL centrifuge tubes.
4. Centrifuge at 3,220 x g for 10 minutes at 4 ˚C and discard the supernatant (*see* **Note 27**).
5. Resuspend the pellet in 20 mL homogenisation buffer supplemented with protease inhibitors.
6. Transfer the suspension into a pre-cooled cell disruption vessel and fill with nitrogen gas to a pressure of 500 PSI.
7. Put the vessel on ice for 15 minutes.
8. Release the suspension carefully into a 50 mL tube and repeat step 6 to 8 two additional times (*see* **Note 28**).
9. Centrifuge at 750 x g for 10 minutes at 4 °C (*see* **Note 29**).
10. Take the supernatant and ultracentrifuge at 100,000 x g for 20 minutes.
11. Discard the supernatant and weigh the pellet.
12. Resuspend the membrane pellet in MemPrep buffer to the concentration of choice (*see* **Note 30**).

4 Notes

1. There are multiple alternative media commercially available for growing *Sf*9 cells, but for us Insect Xpress gives the best growth and expression.
2. We buy foetal bovine serum (FBS) in 500 mL batches which we aliquot in 50 mL tubes for storage at -20 °C. This should be carried out using aseptic techniques in a laminar flow hood. Insect cells can be fairly easily adapted to grow in serum free media [13]. However, we found our cells grow better in the presence of FBS, and the cost of FBS is small compared to the media so we keep it in. However, if you wish to scale up a lot or have specific reasons to not want FBS they can be grown serum free.
3. The penicillin-streptomycin solution is a mixture of antibiotics and used to prevent contamination. Store the solution in 10 mL aliquots at -20 °C. This should be carried out using aseptic techniques in a laminar flow hood. As you scale up the insect cell cultures and have to transfer larger volumes between flasks, the risk of contamination increases, so the antibiotics can be very helpful.
4. There is a range of pFastBac™ vectors available. pFastBac1 is the basic vector, which includes a large MCS and the polyhedrin promotor, pFastBac-HT includes an N-terminal polyhistidine-tag, and pFastBac-Dual allows production of two proteins under the polyhedrin and P10 promotors respectively [10].
5. There is a wide range of different pOET vectors available. These include various promotors including polyhedrin, P10 and p6.9, as well as mammalian promotors for transduction of mammalian cells with baculovirus, and the option of affinity tags [11].
6. We tried producing the human voltage-gated potassium ion channel Kv1.3 using the normal flashBAC™ system, but it did not result in protein production. The flashBAC™ ULTRA system however did lead to successful production of Kv1.3 (Fig. 3).
7. Growth medium consists of 1 L Insect-XPRESS™ insect cell medium supplemented with 100 mL foetal bovine serum and 10 mL penicillin-streptomycin.

8. We always keep a monolayer culture of cells as a backup, in case the suspension culture gets contaminated.
9. If the shaker flask has a lid with a filter, then it can be fully closed, but if not, the lid should only be loosely closed to make the exchange of oxygen possible.
10. The cells only grow well within a narrow range of cell densities. If they are too dilute they grow slowly, if they are too dense they start to die. At the start, check their growth daily and do not let them grow higher than 5 x 10^6 cells/mL. Typically we split them 3 times a week.
11. To scale up the suspension culture, dilute the cells in the same way just for larger volumes. The flasks should not be full, to allow for oxygenation. The highest volumes we would recommend in the different sized shaker flask are: 80 mL cells in a 250 mL flask, 150 mL cells in a 500 mL flask, 300 mL cells in a 1 L flask and 500 mL cells in a 2 L flask.
12. For freezing the cells, we typically use freezing medium, but simply supplementing your growth medium with DMSO works as well.
13. It is important you do wait the full 48 hours or more, as it can be difficult to distinguish the blue colonies at a shorter time point.
14. This method works well for the bacmid DNA which has a much higher molecular weight than standard plasmids, but an alternative to this method is to use a commercial kit such as PureLink HiPure plasmids miniprep kit.
15. Rock the plate carefully on the bench to ensure that the cells are distributed equally across the surface. Do not shake the wells as this results in the distribution of the cells to the sides of the wells. After 1 hour, check the confluency of the cells using a phase contrast microscope before continuing the experiment.
16. Washing removes excess serum which might disturb the transfection.
17. The cells change in appearance when virus replicates in them. You can observe this change using a phase contrast microscope (Fig. 4).

18. P0 denotes the first generation of your virus stock. With every amplification the number increases. The P0 stock is usually not used for protein production because the amount of stock solution and its titre is too low. Amplification of a virus generally results in higher titres. However, with higher virus generations the risk of unwanted mutations in the gene of interest increases. Therefore, you should ideally not go above a P4 virus stock. If you are using serum-free medium, supplement your viral stocks with 5 % FBS.
19. A positive control should always be run next to the negative control (mock transfection) and the transfection of the target transfer plasmid. This consumes an additional aliquot of flashBAC virus DNA. Therefore, if possible, multiple different transfer plasmids should be transfected at the same time. No virus or template DNA is added to the mock transfection. Instead water is added until the final volume is reached.
20. If you have included a positive control using the lacZ transfer plasmid in the flashBAC™ kit, you can check for transfection success by staining the monolayer of cells left after harvesting the P0 virus. Add 1 mL of growth medium containing 15 µL 2 % v/v X-gal to the cell monolayer and leave for a few hours to overnight for the blue colour to develop. The blue colour indicates that the transfection and virus production was successful. The transfer plasmids used as the positive control contains the gene for a β-galactosidase which removes the sugar moiety from x-gal and turns it into 5,5'-dibromo-4,4'-dichloro-indigo. This Indole dimerizes and is oxidized to a blue dye.
21. The scale up from P0 to P1 virus can alternatively be carried out with a small volume shaker culture, but we prefer to use the 6 well plate due to the small volume of P0 virus.
22. MOI stands for multiplicity of infection, and is the ratio of virus particles to cells.
23. For viral amplification a low MOI is used, typically in the region of 0.1, meaning there are 10 times more cells than virus particles present. However, the infection is carried out for a long period of time, typically 5 days, to maximise the amount of virus released into the media. To know how much virus to add to meet the recommended MOI you need to know the titre of your virus. There are many methods to do this, including

plaque assays or qPCR [10,11,5]. Typically, the titre of a P1 virus is 1×10^6 - 1×10^7 pfu/mL, so an estimated titre of 5×10^6 pfu/mL can be used for viral amplification.

24. Hi5 cells can be used instead of *Sf*9 for protein production (though not for viral amplification). For some proteins Hi5 cells give higher yields of protein production [6]. However, we stick with *Sf*9 because of the ease of having to maintain just a single cell line, for most proteins we have tested it has made little difference on expression, and the medium for Hi5 cells is even more expensive.
25. For protein production an MOI of 5 – 10 is used, meaning an excess of virus over the cells, to maximise infection efficiency. Infection is carried out for shorter periods of time as you are looking for recombinant protein production prior to cell lysis and release of virus. Again, you need to titre your virus to know exactly how much to add (a P2 virus is typically 1×10^7 to 1×10^8 pfu/mL). However, plaque assays can be time consuming and unreliable, so it can be quicker and easier to just test a range of different amounts of virus in expression screening trials to find the optimal conditions.
26. Protein synthesis can be optimised by changing variables such as cell density, MOI (amount of virus) or time of incubation [7]. As an example, Figure 5 shows a Western blot for the production of human ABCC4. After 48 hours the full-length protein is expressed, however after 72 hours the protein is cleaved, making 48 hours optimal for this protein. In contrast human Kv1.3 production after 72 hours is good, and the protein is not cleaved (Fig. 3). Furthermore, it can be seen, that changing the amount of virus added to the culture, can affect the yield of Kv1.3 (Fig. 3).
27. The cell pellet can be frozen at -80 °C at this point rather than progressing directly to the membrane preparation.
28. We routinely use nitrogen cavitation for breaking our insect cells. This technique allows high pressure N_2 to equilibrate within the cells. Then upon release of suspension from the vessel and release of pressure, the cells are burst. This technique has been shown to be effective yet relatively gentle on membrane protein function [14]. However, alternative methods include freeze-thaw cycles and/or manual homogenization.

29. A low speed spin is used to remove cell debris and any unbroken cells, with the membranes remaining in the supernatant. The supernatant is then ultracentrifuged to pellet the membranes. This produces a crude membrane preparation of all cellular membranes. If you want a pure sample of plasma membranes, an additional sucrose cushion step is needed [14].
30. 60 mg/mL (wet pellet weight) or 10 mg/mL (total protein concentration) are typical concentrations used. If yields are low, increasing the membrane concentration might be helpful.

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Figure legends

Figure 1. Comparison of the Bac-to-Bac™ and flashBAC™ system for virus production. With the Bac-to-Bac™ system a recombinant bacmid with the gene of interest has to be produced in DH10Bac™ *E. coli* cells first which is then isolated and purified so that it can be transfected into insect cells for virus production. The generation of a bacmid is not needed in the flashBAC™ system where the flashBAC™ virus DNA is co-transfected with a transfer vector, containing the gene of interest, into the insect cells directly, and recombination occurs within the insect cells.

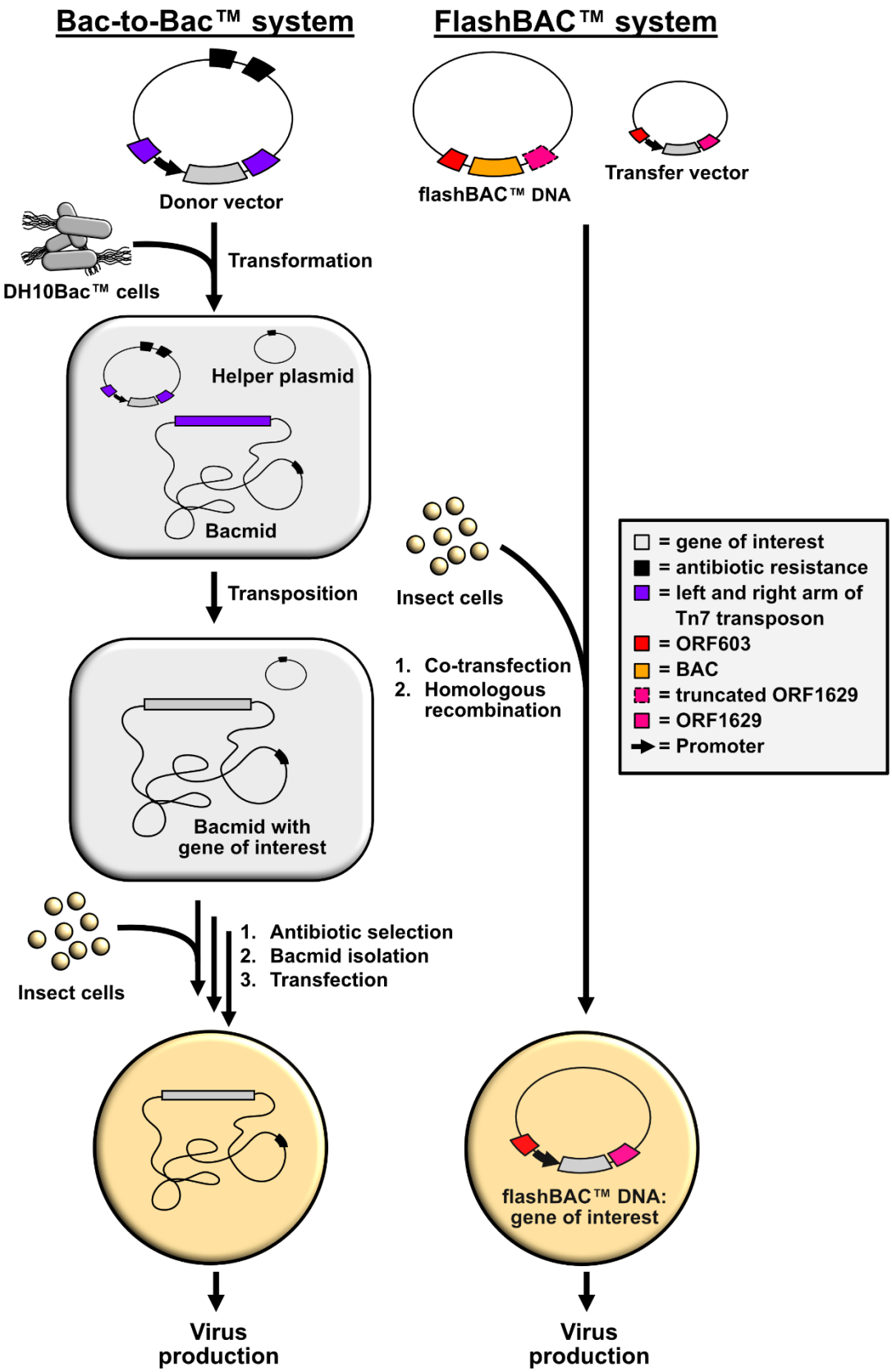
Figure 2. General workflow for establishing an insect cell culture and production of a recombinant protein. At first the insect cells are thawed and grown as monolayers before being transferred into suspension cultures. A monolayer culture should always be kept as a back-up. Once the cells are growing satisfactorily in suspension cultures, aliquots should be frozen away as back-up and for future use (1). P0 followed by P1 or higher virus stocks are produced (2) before test (3) and high scale (4) protein productions.

Figure 3. Expression trials to produce the human potassium ion channel Kv1.3. (a) Kv1.3 forms a tetramer where each monomeric unit consists of a cytoplasmic T1-domain and a voltage sensing domain (VSD) and pore forming region in the membrane. The figure shows the homologous structure of Kv1.2-2.1 (PDB code: 6EBK) for illustrative purposes and was created using ChimeraX [15,16]. (b,c) Test expressions were performed in six well plates. Multiple constructs were transfected using either the flashBAC™ or flashBAC™ ULTRA system. Only viruses created with the flashBAC™ ULTRA system resulted in protein production as seen by Western blotting (b). Production of Kv1.3 was seen after 48 h, and this remained after 72h (c). Western blot with α-His (primary) and α-mouse-HRP (secondary) antibodies.

Figure 4. Changes in appearance of *Sf*9 cells during generation of a baculovirus. *Sf*9 cell growth over five days untreated (b) and transfected with FlashBAC™ virus and plasmid DNA containing the gene of interest (c) on a 6 well plate. At the start of the experiment the normal sized cells are forming a confluent monolayer (a). After five days, the amount of non-transfected cells has increased significantly and cell lysis has become visible across the whole well surface (b). The number of cells in the well with transfected cells has not increased as much but the cells swelled by a considerable amount (c). Cell swelling is an indication for the successful production of a virus.

Figure 5. Optimization of production of the ABC transporter ABCC4 (MRP4). (a) Model of the structure of ABCC4 (MRP4). Transmembrane Domain 1 (TMD1) is presented in purple, TMD2 in yellow, Nucleotide Binding Domain 1 (NBD1) is in red and NBD2 in blue. The model was generated using I-TASSER service [17-19] and visualized by UCSF Chimera software [15]. (b) Expression of ABCC4 (recombinant baculovirus generated using Bac-to-Bac) was carried out for 48 hours and 72 hours. As it is shown, the protein is cleaved after 72 hours of viral incubation. Western blot with α-MRP4 M_4I-10 (primary) and α-rat-HRP (secondary) antibodies. Reprinted from Methods, 180, Kesidis *et al*, 3-18, 2020 [1], with permission from Elsevier.

536 **Figure 1**



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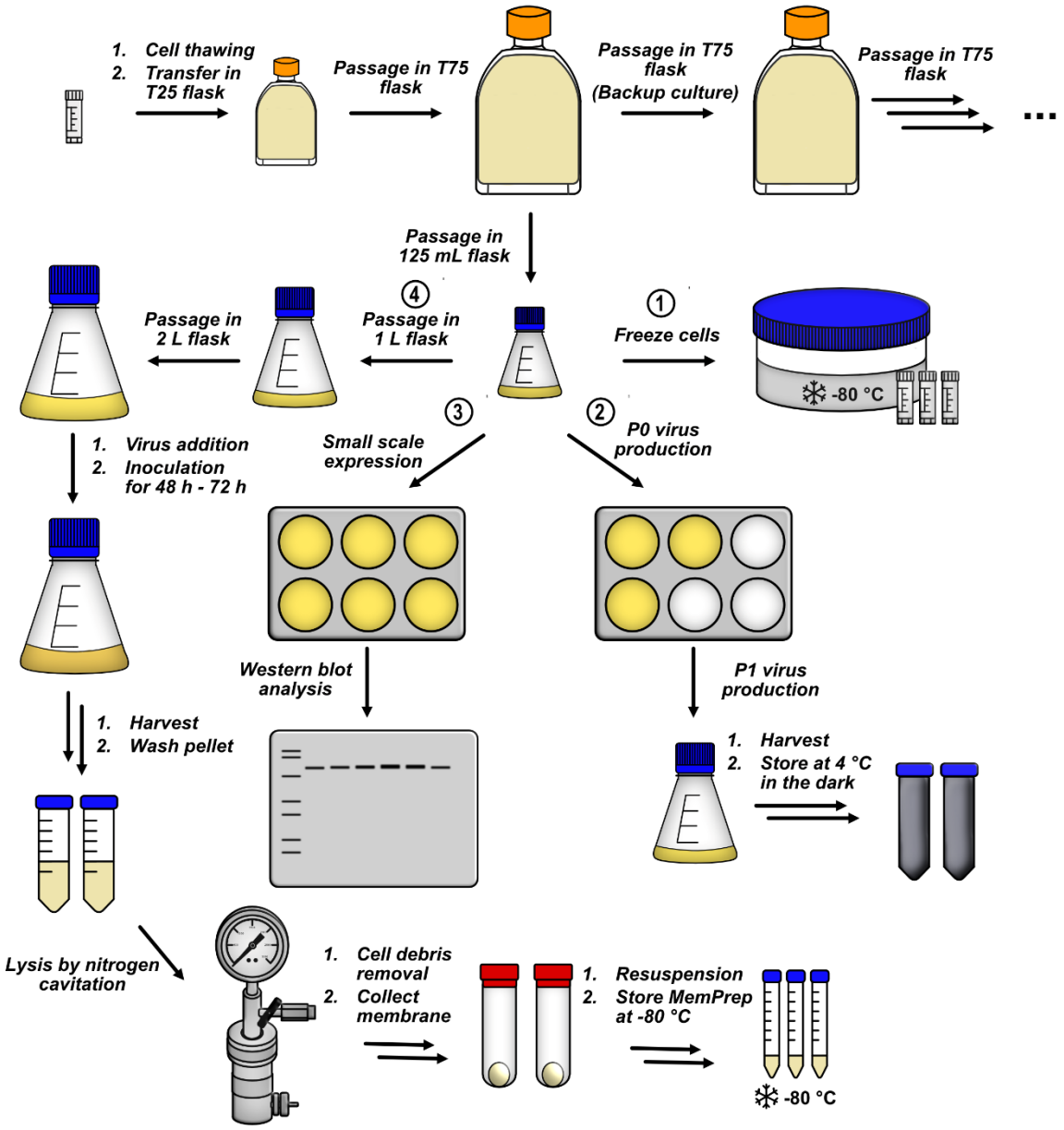
538 **Figure 2**



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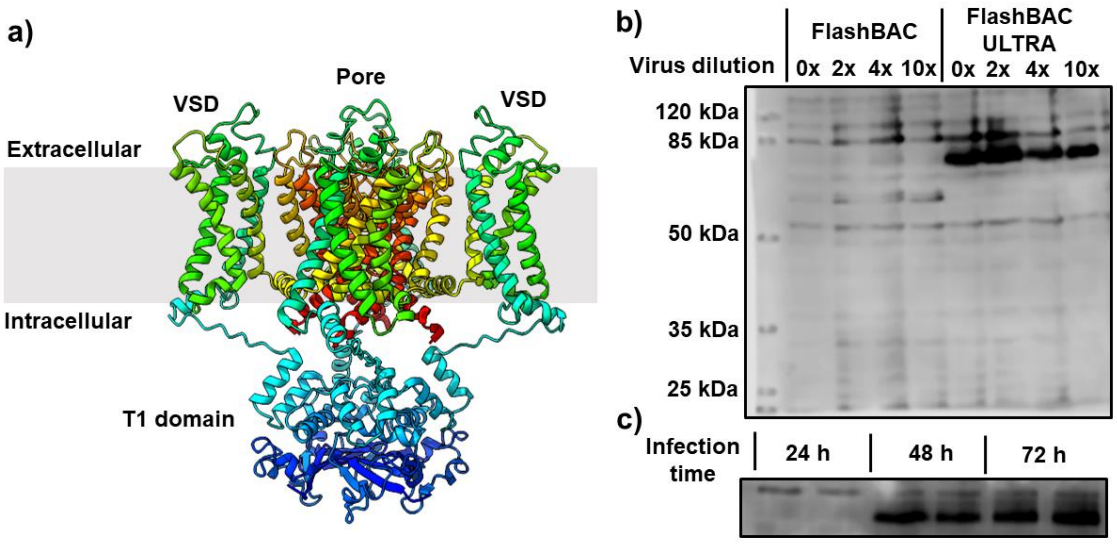

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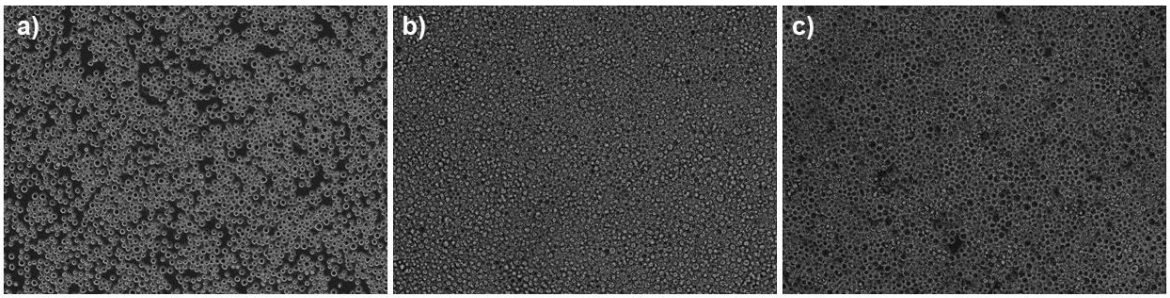

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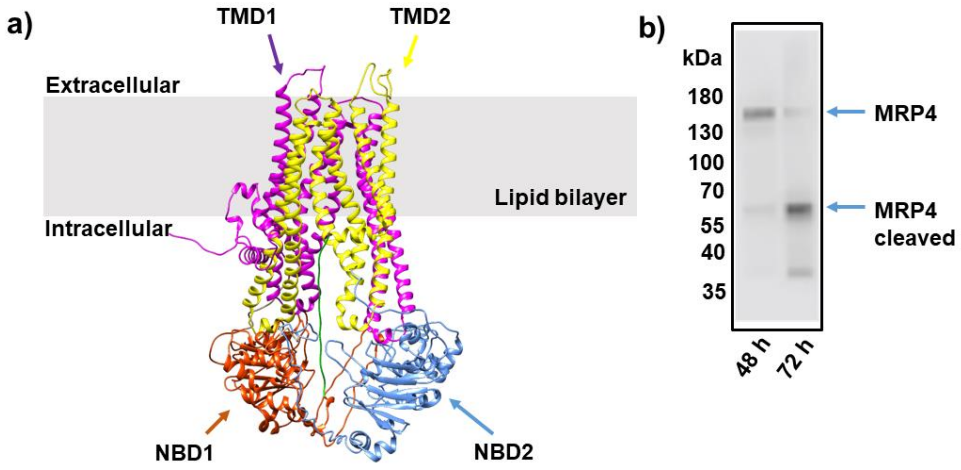
544 **Figure 5**



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