OPPF-UK Standard Protocols:

Insect Expression

Last Updated 6th October 2016

Joanne Nettleship

joanne@strubi.ox.ac.uk

OPPF-UK

OXFORD PROTEIN PRODUCTION FACILITY - UK

Table of Contents

1. Materials

BacMax kit	Cambio - BMAX044
Bsu36I	New England Biolabs - R0524S
FugeneHD	Promega
Sf900II medium	Invitrogen - 10902-104 (6 x 1,000 ml case)
Vector	pTriEx-based vector (e.g. pOPINF, pOPINE etc)
Ian Jones bacmid	
LB	
Trypan blue	Invitrogen
Countess slide	Invitrogen
Bambanker	Anachem
TubeSpin (or other)	Sigma
NiNTA magnetic beads	GE Healthcare
NiNTA Spin columns	Qiagen
24-well plates (Flat TC)	Greiner BioOne
24-well plates (deep)	Qiagen round bottomed

2. Cell Maintenance

Passaging cells

1. Take 10 µl of Sf9 cell stock from a shake flask and pipette into an Eppendorf.
2. Add 10 µl of trypan blue, mix well and pipette into a reservoir of a Countess slide.
3. Count the cells and calculate the ratio of cell stock to Sf900II needed for a given dilution (cells are often split to $5*10^5$ cells/ml every 3-4 days).
4. Pipette the media and cell stock into a new sterile flask and incubate at 27°C, 120rpm.
5. Record the passage number on the flask (add 1 to the number on the feeder flask).

NB. If running transfection or infection experiment set up cells at $8*10^5$ cells/ml the day before.

Freezing cells

1. Spin the cells at 100g for 5-10 minutes and remove the supernatant.
2. Resuspend cells in the volume to give $12*10^6$ cells/ml of Bambanker or use a mix of 70% Sf900II/20% FBS/10%DMSO.
3. Add 1 ml of suspension to each cryovial.
4. Either freeze in a Mr. Frosty container (Nalgene) or place straight into -80°C but this does kill more cells (slower freezing, moving from 4°C to -20°C to -80°C over few hours or days is better.)
5. Either keep in the -80°C freezer or transfer to liquid nitrogen for long term storage.

Thawing cells

1. Prepare 25 ml of Sf900II in a 125 ml shake flask.
2. Rapidly thaw 1 vial of cells in a water bath at 37°C.
3. Add all the thawed cells to the prepared flask (~$5*10^5$ cells/ml).
4. Incubate at 27°C, 120rpm.
5. When the culture reaches a density of around $2*10^6$ viable cells/ml, passage the cells (should take about 5 days).
6. Passage the cells at least two times before using in other applications.

OR

1. Prepare 10ml of Sf900II in a T25 flask (static culture).
2. Rapidly thaw a vial of cells in a water bath at 37°C.
3. Add the full contents of the vial into the prepared flask.
4. Incubate at 27°C.
5. When the flask has reached 100% confluency, usually after 5-7 days, add an extra 20 ml of Sf900II media.
6. Resuspend cells into the media and do a cell count.
7. Transfer the cells to 125ml shaker flask in total of 25 ml of Sf900II, so the concentration is about 4-$5*10^5$ cells/ml.
8. Incubate at 27°C, 120rpm.
9. When the culture reaches a density of around $2*10^6$ viable cells/ml, passage the cells.
10. Passage the cells at least two times before using in other applications.

(Can keep cells in static T75 flask as back up, splitting 1 in 5 every 4-7 days.)

3. Bacmid Preparation

Using BacMax kit

1. Inoculate 100 ml of LB + Kan + Cm with the Ian Jones bacmid glycerol stock.
2. Grow O/N at 37°C.
3. Extract bacmid DNA using the Epicentre (Cambio) BacMax kit
4. Dilute bacmid DNA to ~100 ng/µl.
5. Add 10X NEB buffer 3 and 100X BSA to 1X final concentration.
6. Add 1 µl Bsu36I per 6 µg of DNA.
7. Incubate for 2h at 37°C.
8. Add another 1 µl per 6 µg of DNA.
9. Incubate another 2-3 hours at 37°C.
10. Heat at 72°C for 20 min.
11. No additional purification of the bacmid is required.
12. Aliquot into eppendorfs enough for 48 reactions per tube (you need 250ng bacmid per transfection in a 24-well plate) so 125µl per tube.
13. Store the cut bacmid at -20°C.
14. DO NOT re-freeze/thaw, i.e. once defrosted store in the fridge (it should be stable for 1-2 months).

NOTE: If you are transforming bacmid DNA, transform into any E. coli cloning strain and plate out onto agar plates containing Cm only (no Kan). This is due to the transformation frequency of the large bacmid being very low.

Using QIAgen MiniPrep Kit buffers

1. Inoculate 15ml of LB + Kan + Cm with the Ian Jones bacmid glycerol stock.
2. Grow O/N at 37°C.
3. Spin down 3x5ml (in Eppendorfs) of LB culture.
4. To each 5ml pellet add 0.3ml P1 from miniprep kit and resuspend by vortexing.
5. Add 0.3ml P2 and mix gently
6. Incubate at RT for 5mins.
7. Slowly add 0.3ml of 3M potassium acetate (pH5.5), mixing gently during addition
8. Place sample on ice for 5-10mins.
9. Spin at 14,000 g for 10mins at 4C.
10. Gently transfer supernatant (no white ppt) to clean tube and add 0.8ml isopropanol.
11. Mix by gently inverting tube few times.
12. Store on ice for 5-10mins
13. Spin at 14,000 g for 15mins at RT
14. Remove supernatant and add 0.5ml 70% ethanol to pellets.
15. Spin at 14,000 g for 5mins at RT
16. Remove supernatant.
17. Air dry pellet briefly - 5-10mins- at RT.
18. Dissolve DNA in 100ul TE buffer.
19. Leave in fridge overnight to dissolve.
20. Next day spin at top speed for 1min. Transfer liquid into 1 tube.
21. Follow steps 4-14 above to digest and aliquot bacmid.

4. Transfection

The protocol below is for insect cultures in 24-well plates. However, if using larger wells the following table indicates the amounts of each of the reagents that should be used:

Reagent	24 well plate (500 µl)	12 well plate (1 mL)	6 well plate (2 mL)
Sf9 cells at 5-7*10^5 cells/ml	500 µl	1000 µl	2000 µl
Transfection Mix (per well):			
Sf900II	50 µl	100 µl	200 µl
Linearised Bacmid	250 ng	500 ng	750 ng
Vector	100 – 500 ng	200 - 1000 ng	500 - 1500 ng
FugeneHD	1.5 µl	2 µl	4 µl

Protocol for 24-well plates

1. Prepare Sf9 cells in 24 well culture plates:
    a. Per well add 500 µl Sf9 cells @ 5*10^5 cells/ml in Sf900II.
    b. Leave to attach for 30 min – 1h at RT.
2. Mix (Note that mastermixes of Sf900II+bacmid+FugeneHD can be prepared as well):
    a. 2.5 µl bacmid (250 ng).
    b. 100-500 ng Vector DNA.
    c. 50 µl Sf900ii.
    d. Mix gently and add 1.5 µl FuGeneHD - **PIPETTE DIRECTLY INTO THE LIQUID** (Avoid pipetting against the plastic as this may reduce the transfection efficiency of FugeneHD).
3. Mix and incubate for 30 min at RT
4. Add transfection mix slowly (to avoid disrupting the monolayer) to the appropriate well.
5. Give it a gentle swirl to distribute the transfection mix across the well.
6. Incubate for 6-7 days at 27°C (check eGFP control for expression to make sure the transfection has worked).
7. Store viral supernatant in a 96-well storage block (strips of 8), sealed with plastic lids at 4°C in the dark. **THIS IS YOUR P0 VIRUS STOCK.**
8. Discard the 24-well plates.

5. Infection, Expression Testing and Optimisation

Although expression screening can be performed using the P0 virus if time is an issue, the most reliable route is to first amplify the P0 virus and then use the resulting P1 virus for small or medium scale expression screening.

If the experiment involves a reasonably large number of constructs, it is advisable to conduct small scale infections and expression screening in 24-well format first. This will give a very good idea which constructs express well, which will then need to be amplified and tested in large scale suspension. For small numbers of constructs, it may be better to go directly into medium scale suspension cultures.

Note that for virus amplification one infects with a small dose of virus and then harvests the amplified virus after a long incubation. These conditions are unlikely to be suitable for expression testing as cell death/lysis will have occurred as well as degradation of the protein. As such there is little point doing a Ni-NTA screen on the infected cells.

Finally, note that the dynamics of infection and protein expression differs between monolayer and suspension cultures, especially the time to optimal expression, which is always longer when using monolayer cultures.

5.1 VIRAL AMPLIFICATION (24-well static protocol)

1. Monolayer Infection with P0 virus to generate the P1 virus stock:To each well in a 24-well plate add 0.5 ml of Sf9 cells at $1*10^6$ cells/ml.
2. Attach for 30-60 min at RT.
3. Add 5µl P0 virus stock.
4. Incubate for 6-7 days at 27°C.
5. Check the eGFP signal to make sure the amplification has worked.
6. Harvest the supernatant. **THIS IS YOUR P1 VIRUS STOCK.**
7. Store at 4°C in the dark in same 96 well format as P0 virus.
8. For long term storage: Add FCS or BSA to 10% and store at -80°C.
9. Discard the 24-well plates.

5.2 EXPRESSION TESTING (24-well suspension protocol)

Small scale expression screen and P1 v's scaled P2 testing

1. Prepare 24 deep well plates with 3 ml of Sf9 cells at $1*10^6$ cells/ml in each well to be used (Plates need to have round bottoms).
2. Add 3 or 30 µl P1 virus to each well including a GFP control (for P1 v's scaled P2 testing, use the volume of virus that worked best in the initial screen).
3. Incubate for 3 days at 27°C, 250 rpm.
4. Transfer 1 ml from each well to a 96-well NiNTA deep well block.
5. Centrifuge for 15 min at 6,000g, remove the supernatant and freeze the cells at -80°C for at least 30 min.
6. Run the standard NiNTA Expression screen protocol on the Qiagen BioRobot 8000 (This is described in section 7).

5.3 EXPRESSION OPTIMISATION (TubeSpin protocol)

Suspension Expression Optimisation

Tests optimal MOI and TOI, using 50 mL TubeSpins. This is only run for projects which have failed in the standard pipeline.

1. Decide how many viral titres and time points you wish to investigate.
2. Prepare 10 ml Sf9 cells at $1*10^6$ cells/ml in TubeSpins for each viral titre you wish to investigate. An example would be to use 0.2, 1, 2, 5, 10 and 20 µl of P1 virus per tube.
3. Harvest 1 ml of culture at each time point. An example would be Day 2, 3, 4, and 5. Transfer to a 96-well block or use 2 ml tubes.
4. Spin for 10 min at 6000g at room temperature. Discard the supernatant and store the pellets at -80°C.
5. Run the standard NiNTA Expression screen protocol on the Qiagen BioRobot 8000 or run by hand if tubes were used (This is described in section 7).

6. Scale up Infection and Expression

6.1 VIRAL AMPLIFICATION (Shake flask protocol)

Suspension Infection with P1 virus to generate the scaled P2 virus stock

1. Prepare 50 ml of Sf9 cells at $1*10^6$ cells/ml.
2. Add 400 µl P1 virus stock.
3. Incubate for 6-7 days at 27°C.
4. Transfer to a fresh, sterile Falcon and spin for 10 min at 1,000g.
5. Transfer the supernatant to a fresh 50 ml Falcon.
6. Filter sterilise (virus will pass through a 0.2 µm filter) and store at 4°C in a black falcon tube (or wrap the tube in foil).
7. **THIS IS YOUR P2 VIRUS STOCK**
8. For long term storage: add FCS to 10% (you can also freeze at -80°C or liquid N_2)
9. Discard the cell pellet.

6.2 SCALE UP EXPRESSION (Shake flask protocol)

2.5 L scale up for soluble targets and 1 L for membrane targets for FSEC

1. Prepare 2.5 L of Sf9 cells at $1*10^6$ cells/ml in a Thompson flask (or 2 x 500 ml in 2 L shake flasks).
2. Add 2.5 ml or 25 ml P2 virus stock per 2.5 L (depending on the expression screen results).
3. Incubate for 3 days at 27°C.
4. Take a 2 ml sample for analysis by NiNTA using the protocols in section 7.
5. Harvest by spinning at 6,000g for 15 minutes.
6. Freeze the pellet at -80°C ready for purification.

7. Small Scale Ni-NTA Expression Screen

7.1 FOR CULTURES HARVESTED IN 96-WELL FORMAT – SOLUBLE PROTEINS

Cell lysis

1. Resuspend defrosted pellets in 210 µl NPI-10-Tween containing protease inhibitors and 400 Kunitz units/ml DNaseI. Do this by placing the block on a shaker for 30 minutes at 1000 rpm.
2. Centrifuge at 6,000*g* for 30 minutes.

Protein binding

1. Add 20 µl of NiNTA magnetic beads to each well of a flat bottomed 96-well MTP plate.
2. Carefully transfer 200 µl of the supernatant from the 96-well block to the FB plate.
3. Gently resuspend the magnetic beads by pipetting up and down once.
4. Shake the plate for 30 minutes at 600 rpm to bind the protein to the magnetic beads.
5. Place the plate onto a Qiagen Magnet Type B for 2 minutes.
6. Remove the supernatant.

Washing and elution

1. Remove the plate from the magnet and add 200 µl of NPI-20 to each well. Gently resuspend the magnetic beads as before.
2. Place the plate back onto the magnet for two minutes before removing the supernatant.
3. Repeat the wash step by removing the plate from the magnet and adding 200 µl of NPI-20 to each well. Gently resuspend the magnetic beads as before. Place the plate back onto the magnet for two minutes before removing the supernatant.
4. Remove the plate from the magnet and add 50 µl of NPI-250 to each well, gently resuspending as before.
5. Shake the plate for 10 minutes at 600 rpm to elute the protein from the magnetic beads.
6. Transfer 50 µl of eluted protein solution to a new 96-well plate.
7. Run a gel of the samples to assess expression.

NPI-10: 50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole; 1 % Tween-20. Adjust to pH 8.0.
NPI-20: 50 mM NaH_2PO_4; 300 mM NaCl; 20 mM imidazole; 0.05 % Tween-20. Adjust to pH 8.0.
NPI-250: 50 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole; 0.05 % Tween-20. Adjust to pH 8.0.

7.2 FOR CULTURES HARVESTED IN 96-WELL FORMAT – MEMBRANE PROTEINS

Cell lysis

1. Resuspend defrosted pellets in 210 µl NPI-10-Tween containing protease inhibitors and 400 Kunitz units/ml DNaseI.
2. This is done by repeated aspirate/dispense with a suitable multi-channel pipette and then placed on an orbital MTP shaker (~1000 rpm for 10 minutes at 4°C).
3. To aid lysis of insect cells, refreeze cells for 20mins and then defrost for another 20mins at room temperature.
4. Add 1% n-Dodecyl β-D-maltoside (DDM) final concentration to lysates
5. Continue shaking (~1000rpm for 60mins at 4°C).
6. Clear the lysate by centrifuging the deep-well block at 6000*g* for 30 minutes at 4°C (the Beckman JS5.3 rotor for the Beckman *Avanti* centrifuges is ideal for this).

Analysis

1. If GFP fusions are being tested a sample of the cleared lysate can be analysed by in-gel fluorescence.
    a. Transfer 10 µl of the cleared lysate to a microtitre plate and add 10 µl of SDS PAGE gel loading buffer (100 mM Tris, pH 6.8, 4% w/v SDS,0.2% w/v Bromophenol blue, 10% v/v β-mercaptoethanol, and 20% v/v glycerol). **CAUTION! DO NOT BOIL THE SAMPLE.**
    b. Load 10 µl of this onto SDS PAGE gel(s) and run at 100-120 V (constant voltage) at 4°C until the dye front reaches the bottom (2-2.5 hr).
    c. Place the gel onto an imager (e.g. with a blue-light filter to detect the GFP fusion proteins). The exposure time is chosen to ensure that the brightest bands are not saturated.
2. If there is no GFP tag run a Western blot – gels can be run as normal (200V at room temperature) but **DO NOT BOIL THE SAMPLE.** (OPPF routinely use anti-His monoclonal antibody from R+D MAB050 as most of our suite of vectors are His-tagged.)

7.3 FOR 2 ML CULTURES IN TUBES - USING Qiagen NiNTA Spin Columns

Cell lysis

1. Resuspend the defrosted pellet in 450 µl NPI-10-Tween containing protease inhibitors and 400 Kunitz units/ml DNaseI. Do this by placing the tubes on a shaker for 30 minutes. During cell lysis, prepare the Spin columns.
2. Centrifuge the tubes at 12,000*g* for 10 minutes.

Spin column preparation

3. Equilibrate the Qiagen NiNTA Spin Column by adding 600 µl NPI-10 and centrifuging for 2 minutes at 890*g* (approx 2900 rpm).

Protein purification

4. Carefully transfer 400 µl of the supernatant from the spun down tubes to the equilibrated NiNTA Spin column.
5. Centrifuge the tubes for 5 minutes at 270*g* (approx 1600 rpm). Discard the flow through.
6. Wash the spin column by applying 600 µl NPI-20 and centrifuging for 5 minutes at 890*g* (approx 2900 rpm). Discard the flow through.
7. Repeat the wash step (Step 6).
8. Elute the protein using 100 µl NPI-250 and centrifuging for 2 minutes at 890*g* (approx 2900 rpm).
9. Run a gel.

NPI-10: 50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole; 1 % Tween-20. Adjust to pH 8.0.
NPI-20: 50 mM NaH_2PO_4; 300 mM NaCl; 20 mM imidazole; 0.05 % Tween-20. Adjust to pH 8.0.
NPI-250: 50 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole; 0.05 % Tween-20. Adjust to pH 8.0.

NB. Can also follow protocol on page 10 for 2ml samples in tubes where we use a magnet like – MagRack6 (28-9489-64 GE Healthcare)

8. Baculovirus Titre Estimation Using Q-PCR

Brief outline

The method involves two steps: extraction of virus DNA and Q-PCR. The extraction of viral DNA is facilitated in a buffer containing GnHCl and proteinase K, dissolving the virus capsid at elevated temperature. The viral DNA is then purified using standard Qiagen spin columns. Q-PCR is performed using primers for specific regions of the baculovirus genome.

The protocol determines the quantity of baculovirus DNA in a given sample. It does NOT determine the actual number of infectious particles. Nevertheless, we have found that when extracted DNA from a pre-titered virus is included as a reference, one gets a reasonably accurate idea of the titers of unknown samples when the sybr green readings are normalised with respect to this reference. Therefore, it is recommended to include DNA extracted from a titered virus in all Q-PCR experiments.

One could, in principle, use purified IE1 PCR product (see below) of known concentration to make, for instance, a concentration curve and calculate the amount of viral DNA in unknown samples using this curve. However, we have found that this approach consistently over-estimates the titers by at least an order of magnitude.

Materials

Item	Manufacturer/preparation
Proteinase K	2x250 mg, Roche applied science (Cat. No: 03115801001) Or: 200 mg bioline. Resuspend 250 mg in 12.5 ml H_2O. Aliquot in 0.5 ml aliquots, store at -20µ°C.
PCR clean-up plate or spin columns	Qiagen
PB buffer	Qiagen
PE buffer	Qiagen
EB buffer	Qiagen
KOD HotStart polymerase	Novagen (Cat. No 71086)
SYBR green I or Sybr Gold	Invitrogen
QPCR plates, white	Abgene
QPCR seal	Abgene
IE1 PCR product, quantified (OPTIONAL)	
Extracted virus DNA from a titered virus stock	

Lysis solution

Per well:

200 µl PB buffer, 50 µl proteinase K.

E.g. for 48 samples: 9.6 ml PB buffer, 2.4 mL proteinase K

Virus DNA extraction Protocol

High throughput method

1. Transfer 200 µl of virus to a fresh 1.1 ml 96-well PCR plate
2. Add 250 µl of lysis solution to each well
3. Seal the plate
4. Incubate at 72°C for 10min
5. Add 600 µl PB to each well
6. Transfer to the PCR cleanup plate
7. Apply vacuum until the liquid has gone through
8. Wash 2x with 750 µl PE buffer
9. After final wash, remove any residual PE by tapping the nozzles on absorbent paper
10. Empty wash tray and re-assemble the manifold
11. Apply vacuum for 10 min (to fully dry the plate)
12. Place a collection microtiter plate in the vacuum manifold
13. Return the filter plate to the manifold
14. Add 75 µl EB to each well
15. Apply vacuum for 5 min

Low throughput method

1. Transfer 200 µl of virus to a 1.5 ml eppendorf tube
2. Add 250 µl of lysis solution
3. Incubate at 72°C for 10 min
4. Add 600 µl PB
5. Transfer to the PCR clean-up spin column (Qiagen)
6. Spin for 1 min at max speed
7. Wash 2x with 750 µl PE buffer
8. After final wash (and removal of the flow through), centrifuge for 2 min to remove residual ethanol
9. Then remove any remaining residual PE by tapping on absorbent paper
10. Transfer spin column to a fresh eppendorf tube
11. Add 75 µl EB
12. Spin for 1 min at max speed

QPCR Protocol

Adapted from Lo & Chao, *Biotechnol. Prog.* **2004**, *20*, 354-360

IE1 FWD PRIMER: 5'-CCCGTAACGGACCTCGTACTT-3'
IE1 REV PRIMER: 5'-TTATCGAGATTTATTTGCATACAACAAG-3'

IE1 amplicon Sequence (141 bp):
5'CCCGTAACGGACCTCGTACTTTTGGCTTCAAAGGTTTTGCGCACAGACAAAATGTGCCACACTTGCAGC
TCTGCATGTGTGCGCGTTACCACAAATCCCAACGGCGCAGTGTACTTGTTGTATGCAAATAAATCTCGATA
A-3'
(MW = 86876.4 g/mol)

Prepare the following PCR mix for each reaction (for multiple samples prepare a master stock to minimise variations between different reactions):

5 µl extracted virus or 5 µl H2O for the blanks
1 µl or 10 pmol of IE1 FWD primer
1 µl or 10 pmol of IE1 REV primer
5 µl 10X KOD HotStart Buffer
5 µl dNTPs (2 mM each)
2 µl 25 mM MgSO4
1 µl 50X solution of Sybr Green I or Sybr Gold
1.5 µl KOD HotStart polymerase
29.5 µl H2O

Seal the PCR plate (avoid smudging the surface of the seal as much as possible as this might interfere with the readings).

Spin the plate briefly to remove any air bubbles (as this will interfere with the readings).

Then cycle as follows:

Step 1 97°C/7 min
Step 2 97°C/20 sec
Step 3: 60°C/20 sec
Step 4: 68°C/1 min
Step 5: 80°C/10s ec
Step 6: Read plate (sybr green I fluorescence)
Step 7: Goto 2 for 39 more times (i.e. 40 cycles in total)
Step 8: Hold at 4°C