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Baculovirus Expression System

A Complete Laboratory and Guide to the Baculovirus Expression System

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A Complete Laboratory Guide to the Baculovirus Expression System

Contents

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* For the absence of doubt, 'Research' does not include any stage of commercialisation including clinical trials.

End of Limited Use Licence.

2. Essential Information and Technical Assistance

The information given in this Laboratory Guide is accurate to the best of our knowledge. It is a practical guide to allow researchers to use the *flash*BAC™ (and BacPAK6) technology to produce recombinant baculoviruses. It is not intended as a comprehensive guide to the baculovirus expression system or insect cell culture. Those experienced with the baculovirus expression system may find that they are already familiar with much of the information provided.

Users are reminded that they may require other licences to use the baculovirus expression system or types of insect cells and it is the responsibility of the user to ascertain and act on this information.

For additional help or guidance please refer to the Trouble Shooting Section of this Guide and/or the Frequently Asked Questions (FAQ) section of our website (www.oetltd.com). If these resources are unable to help, please contact us at info@oetltd.com and we will be pleased to help where possible. All technical assistance provided is given in good faith; we cannot take any responsibility whatsoever for any results you obtain by relying on our assistance. We make no warranties of any kind with respect to technical assistance or advice we provide.

3. Safety Requirements

These research products have not been approved for human or animal diagnostic or therapeutic use.

Procedures described within this User Guide should only be carried out by qualified persons trained in appropriate laboratory safety procedures.

Always use good laboratory practice when handling this product.

WARNING: SAFETY PRECAUTIONS MAY BE NECESSARY WHEN HANDLING SOME OF THE REAGENTS DESCRIBED IN THIS USER GUIDE. PLEASE REFER TO THE MATERIAL SAFETY DATA SHEETS SUPPLIED BY THE APPROPRIATE MANUFACTURER.

4. Product Ordering Information

Comprehensive details of all our products to support use of the baculovirus expression system can be found on our website: www.oetltd.com. This includes:

- *flash*BAC, and the ULTRA, GOLD and PRIME variants
- BacPAK6 DNA and variants
- Transfection medium and reagents (*baculo*FECTIN)
- Virus titration kits (*baculo*Quant)
- Insect cells
- Insect cell culture medium
- Transfer plasmids

The table below lists the transfer plasmids we supply.

Product	Details	Catalogue Number
pOET1.1	Polyhedrin gene promoter with multiple cloning site (MCS) (10µg)	**200101**
pOET1.1N 6xHis	Polyhedrin gene promoter, MCS with N-terminal 6xHis tag (10µg)	**2001011**
pOET1.1C 6xHis	Polyhedrin gene promoter, MCS with C-terminal 6xHis tag (10µg)	**2001012**
pOET2.1	As pOET1 but with reversed MCS (10µg)	**200103**
pOET2.1N/C 6xHis	Polyhedrin gene promoter, MCS with N- and C-terminal 6xHis tags and thrombin cleavage site (10µg)	**2001031**
pOET2.1C 6xHis	As pOET1C 6xHis but with reversed MCS (10µg)	**2001032**
pOET3	p6.9 gene promoter for late phase expression (10µg)	**200104**
pOET4	As pOET3 but with reversed MCS (10µg)	**200105**
pOET5.1	Dual expression with polyhedrin and p10 gene promoters (10µg)	**200106**
pOET6 BacMAM	*CMV* promoter for BacMam – mediated transduction of mammalian cells (10µg)	**200107**
pOET1 Gateway™	pOET1 transfer plasmid with Gateway® technology (6µg)	**200108**
pOET6 BacMAM Gateway™	pOET6 transfer plasmid with Gateway® technology (6µg)	**200109**
pOET8.VE1	Polyhedrin gene promoter with Vankyrin expression cassette for improved protein production (10µg)	**200121**
pOET8.VE2	As pOET8.VE1 but with additional N-terminal 8x His-tag and Honey Bee Melittin signal sequence (10µg)	**200122**
pOET8.VE3	As pOET8.VE1 but with additional C-terminal 6x His-tag and Honey Bee Melittin signal sequence (10µg)	**200123**
pOET Sequencing Primers	Can be used with any pOET transfer vector (2 x 100µL)	**200100**
pOET9$^{EF1\alpha}$	Enhanced for BacMam-mediated transduction of mammalian cells with *EF1α* promoter (10µg)	**200131**
pOET9^{CCAG}	Enhanced for BacMam-mediated transduction of mammalian cells with *CCAG* promoter (10µg)	**200132**
pOET9^{CMV}	Enhanced for BacMam-mediated transduction of mammalian cells with *CMV* promoter (10µg)	**200133**
pOET9^{SV40}	Enhanced for BacMam-mediated transduction of mammalian cells with *SV40* promoter (10µg)	**200134**
pOET9 Selection Box	Selection box of all four pOET9 varieties	**200135**

5. Introduction to the Baculovirus Expression System and *flash*BAC™/BacPAK6 Technology

5.1 Baculoviruses

Baculoviruses are insect viruses, predominantly infecting insect larvae of the order Lepidoptera (butterflies and moths)[1]. A baculovirus expression vector is a recombinant baculovirus that has been genetically modified to contain a foreign gene of interest, which can then be expressed in insect cells under control of a baculovirus gene promoter. The most commonly used baculovirus for foreign gene expression is Autographa californica nucleopolyhedrovirus (AcMNPV)[2,3]. AcMNPV has a circular, double-stranded, super-coiled DNA genome (133894 bp; Accession NC_001623)[4], packaged in a rod-shaped nucleocapsid. The nucleocapsid can be extended lengthways and thus the DNA genome can accommodate quite large insertions of DNA. The AcMNPV genome forms the basis of the *flash*BAC™ or BacPAK6 DNA provided in this kit.

AcMNPV has a bi-phasic life cycle (Figure 1) resulting in the production of two virus phenotypes: budded virus (BV) and occlusion-derived virus (ODV). BV contain single, rod-shaped nucleocapsids enclosed by an envelope, derived from the plasma membrane of insect cells, containing a membrane-fusion protein GP64 (Figure 2A). GP64 is acquired when the nucleocapsids bud through the host cell plasma membrane[5]. The BV form of the virus is 1000-fold more infectious for cultured insect cells[6], compared to the ODV phenotype, and is responsible for cell-cell transmission in the early stages of infection[7]. It is the BV form of the virus that delivers the foreign gene into the host insect cell for expression.

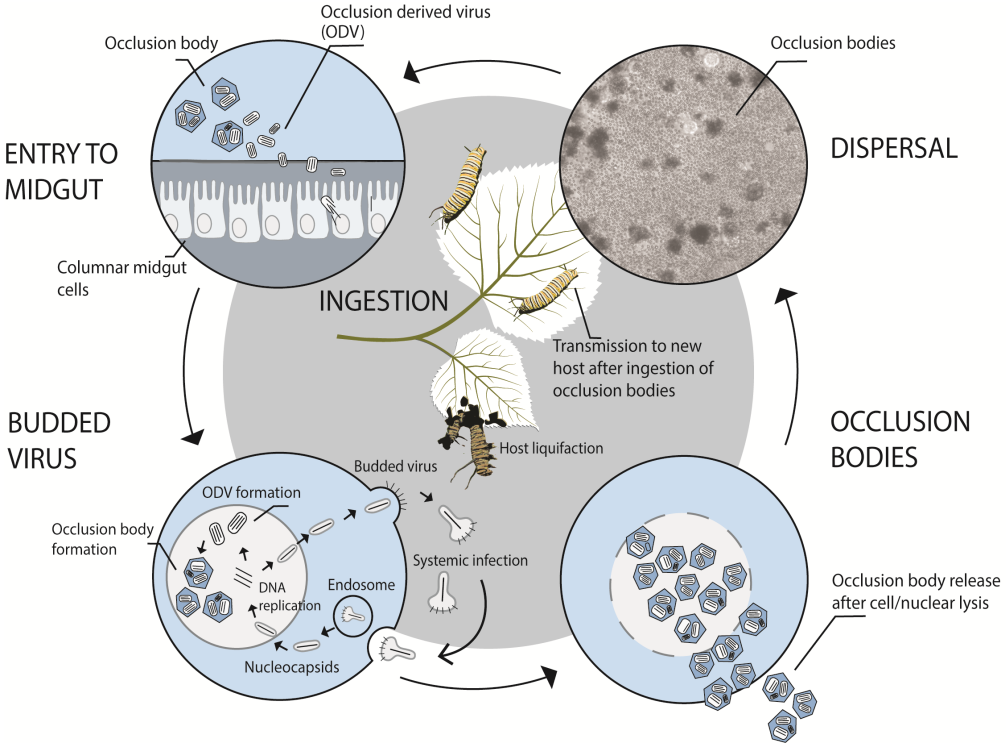


Figure 1. *A schematic representation of the bi-phasic life cycle of baculoviruses resulting in budded virus, occlusion bodies and occlusion-derived virus. Dr L. Graves.*

In the later stages of the infection cycle large numbers of occlusion bodies (OB) or polyhedra are formed inside the nuclei of cells (Figure 2A & C). These consist of multiple rod-shaped nucleocapsids (Figure 2B) enclosed within an envelope, acquired *de novo* in the nuclei of cells, which then become embedded within a para-crystalline matrix of the OB/polyhedra. The major component of the OB matrix is comprised of a single protein – polyhedrin (29 kDa)[8,9], which is produced by the powerful transcriptional activity of the polyhedrin gene (*polh*) promoter[10]. OBs protect the virus and allow them to survive between hosts in the environment.

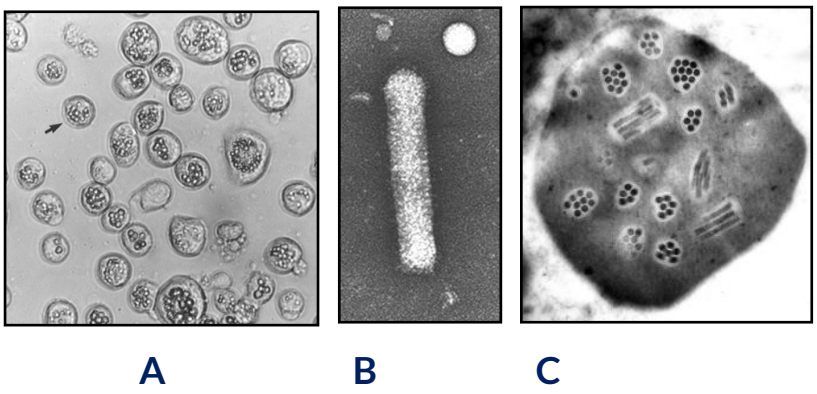


Figure 2. *(**A**) Infected cells in culture showing polyhedra in the enlarged nuclei. (**B**) Rod-shaped baculovirus particle. (**C**) Section through a polyhedron showing occlusion-derived virus particles embedded in a matrix of polyhedrin protein.*

Most baculovirus expression vectors do not produce polyhedra (see below for details), because the coding sequence for polyhedrin has been replaced by that of the foreign gene being expressed under control of the *polh* promoter. This is a useful safety feature because recombinant virus cannot persist in the environment in the absence of polyhedra.

Other non-essential genes within the baculovirus genome can be utilised in the same way by replacing the necessary coding sequence with that of the target protein. They include *p10*, *gp64*, *vp39*, *p6.9*, chitinase and cathepsin. These genes are expressed at specific time points during the virus replication cycle and allow for phases of target gene expression (Figure 3).

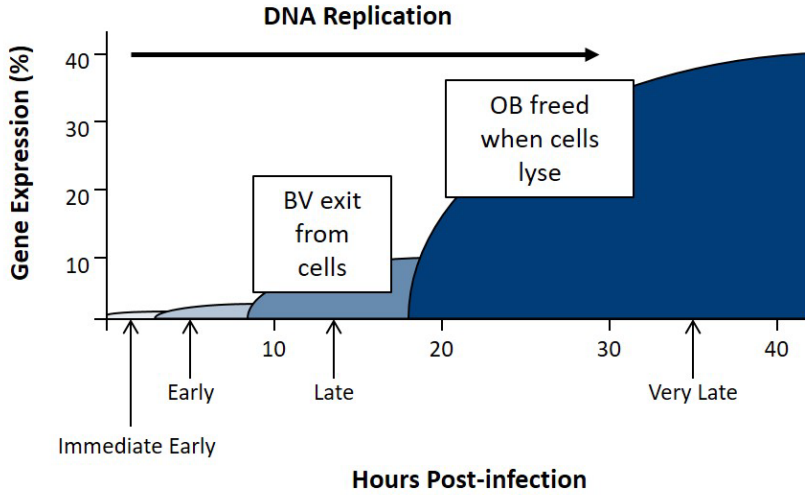


Figure 3. *The four key phases of baculovirus gene expression in vitro. This temporal progression has been confirmed by transcriptome analysis.*

5.2 The Baculovirus Expression System

The baculovirus *polh* is non-essential for virus replication in insect cells grown in culture and this has led to the development of the widely-used baculovirus expression vector system, first described in 1983[3]. The coding sequence of *polh* is replaced by the coding region of the gene to be expressed, to produce a recombinant baculovirus in which the powerful *polh* promoter drives expression of the foreign gene. Recombinant baculoviruses produced in this way are polyhedrin-negative (Figure 4).

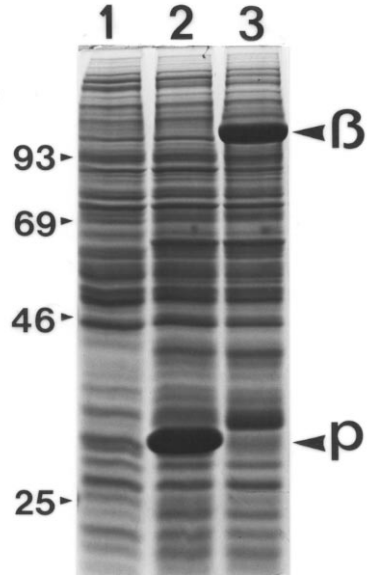


Figure 4. *(**A**) SDS-PAGE analysis of cell extracts from (1) non-infected insect cells (2) wild-type virus-infected cells showing polyhedrin protein at 29 kDa and (3) recombinant virus-infected cells expressing lacZ (beta-galactosidase) – note no polyhedrin protein is made.*

Expression of foreign genes in insect cells using recombinant baculoviruses has become one of the most widely used eukaryotic expression systems. The BEVS, as it is called, has several advantages over other expression systems:

- Safe to use – baculoviruses only infect insects and *polh*-negative viruses cannot survive in the environment
- Can accommodate large genes or multiple genes – as the rod shaped nucleocapsid can increase in length
- Wide variety of promoters can be used – not just *polh* – to control level of expression and/or temporal aspects of expression
- Proteins made are usually functional and are cleaved/processed correctly
- Can be used to transduce mammalian cells and achieve gene expression by replacing *polh* promoter with a mammalian-specific promoter (BacMAM)
- Insect cells are easy to grow and scale-up at lower temperatures than mammalian cells and without the need for CO_2 incubators

However, the BEVS is not without its disadvantages and these lie mainly in the labour intensive and technically demanding steps needed to produce and isolate recombinant viruses and the fact that glycosylation differs from mammalian cells; the latter often has no effect on function but is important in considering therapeutic proteins.

The following section outlines the development of the BEVS over time and the fine tuning that has been achieved to improve the system over the last few years. Our focus is on the improvements made

with the system we call *flash*BAC™, which was developed to make it easier and quicker to make recombinant viruses and to help achieve better expression with 'difficult' to express proteins.

Generally, the baculovirus genome is considered too large to insert genes directly (although one commercial product BaculoDirect™ achieves this). Instead, foreign genes are cloned into a transfer plasmid, which contains sequences that flank the *polh* in the virus genome. The virus genome and transfer plasmid are simultaneously introduced into insect cells (co-transfection) and homologous recombination, between the flanking sequences of *polh* in the plasmid and genome, results in exchange of DNA resulting in a recombinant baculovirus. The virus genome then replicates and produces recombinant virus which can be harvested as budded virus in the culture medium.

In most available BEVS using the homologous recombination method, this results in a mixture of recombinant virus and recirculation of the parental virus DNA to produce non-recombinant virus. These are separated by plaque-purification to produce a stock of pure recombinant virus. Plaque-purification is time consuming and technically demanding to the non-virologist. Many developments have attempted to improve the method by which recombinant and parental virus may be separated. The frequency of recombination efficacy in the BEVS is low, less than 1%, so recombinant virus plaques can often be obscured by parental virus plaques. This problem was partially addressed by inserting a copy of the *lac*Z gene into the virus genome so that recombinant virus plaques would stain blue after the addition of X-gal[11]. However, this did not address the fact that only 1% of plaques went blue and also resulted in contamination of the expressed protein with beta-galactosidase.

5.3 The BacPAK6 System

The efficiency with which recombinant viruses could be recovered was improved by the addition of a unique restriction enzyme site (*Bsu*36I) at the *polh* locus. Linearization of the virus genome prior to homologous recombination reduced the infectivity of the parental virus DNA; only recombinant virus genomes become circular and can replicate. This resulted in the recovery of about 30% recombinant virus. *Lac*Z was then introduced into the parental virus genome to replace the *polh* coding sequence, resulting in three *Bsu*36I sites at the *polh* locus[12] (Figure 5).

Triple digestion of the resulting virus genome with *Bsu*36I removed a section of virus DNA coding for *lac*Z and part of the essential gene ORF 1629[12], resulting in a linear virus DNA (BacPAK6) that cannot replicate in insect cells (Figure 5). Co-transfection of insect cells with linearised BacPAK6 DNA and a transfer plasmid with a foreign gene under control of *polh*, creates recombinant virus DNA in which ORF 1629 is restored and the re-circularised DNA can replicate to produce recombinant BV[12]. This reduced even further the chance of parental virus replicating and resulted in an increase in the recovery of recombinant virus to more than 90%*. It also introduced a useful blue-white selection system – with non-recombinant virus giving rise to blue plaques and recombinant virus to white plaques. It was thus easier to achieve purified virus with a single round of plaque-purification. *It is not 100% because it is impossible to ensure that every molecule of DNA is triple-digested and any circular DNA remaining can replicate and produce non-recombinant virus.

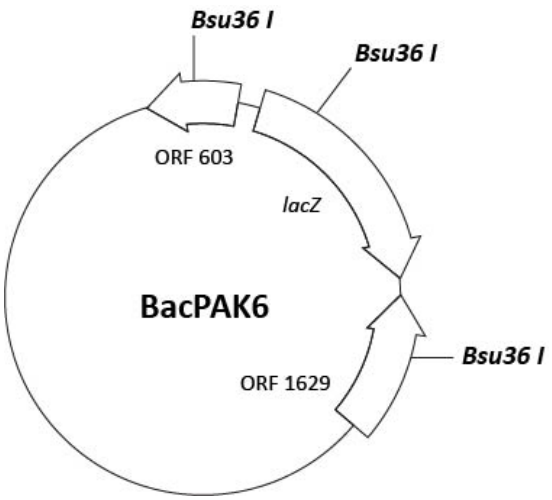


Figure 5. *Map of the BacPAK6 vector virus genome showing Bsu361 restriction sites and orientation of genes.*

Despite this fine tuning and optimisation of the system, a number of steps are still required to make recombinant baculoviruses, thus making it more time consuming than bacterial expression systems and less amenable to scale-up and high throughput automation.

NOTE

The triple-cut linear BacPAK6 virus DNA is available from OET (see website). We are also pleased to offer BacPAK6 *Sec+*, which has a deletion in the chitinase gene to aid expression of membrane targeted and secreted proteins. Practical techniques to make recombinant BacPAK6 viruses are included in this User Guide.

5.4 The *flash*BAC™ System

The *flash*BAC™ system is the most advanced platform technology for the production and isolation of recombinant baculoviruses. Importantly, *flash*BAC™ has been designed to remove the need for separation of recombinant virus from parental virus, so no plaque-purification steps are needed. The production of recombinant virus has been simplified to a single step procedure that is fully amenable to high throughput manipulations (Figures 6, 7) – multiple recombinant viruses can be made at one time using 24-well plates either manually or using simple robotic systems.

The *flash*BAC™ technology builds on the BacPAK6 technology. At the heart of the new system is an AcMNPV genome that lacks part of the essential gene ORF 1629 and contains a bacterial artificial chromosome (BAC) at the *polh* locus, replacing the *polh* coding sequence. The essential gene deletion prevents virus replication in insect cells and the BAC allows the virus genome to be maintained in bacterial cells as a bacmid. Circular virus DNA is isolated from bacterial cells and purified ready for use in *flash*BAC™ kits and co-transfections to make recombinant viruses (Figure 6).

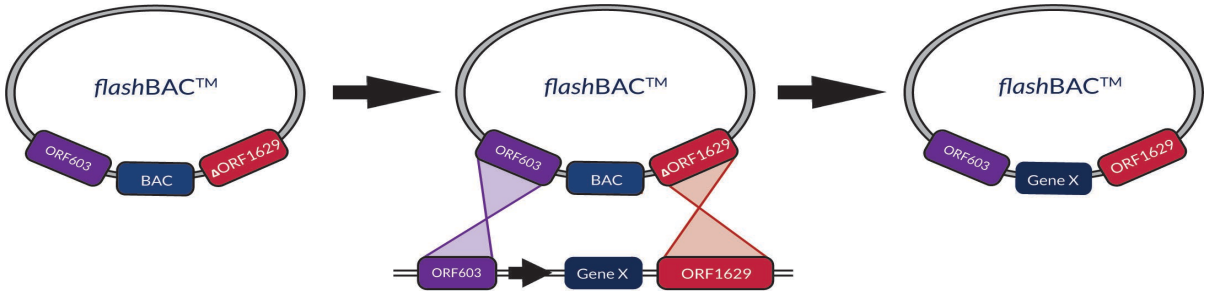


Figure 6.

Schematic to show production of recombinant baculoviruses using flashBAC™. Non-infectious flashBAC™ DNA with a deletion in ORF1629 is mixed with a transfer plasmid containing the gene to be expressed (gene X) in a suitable transfer vector. Within insect cells, homologous recombination replaces the Bac elements in flashBAC™ with gene X and the deletion in ORF1629 is restored to produce recombinant, infectious flashBAC™ DNA.

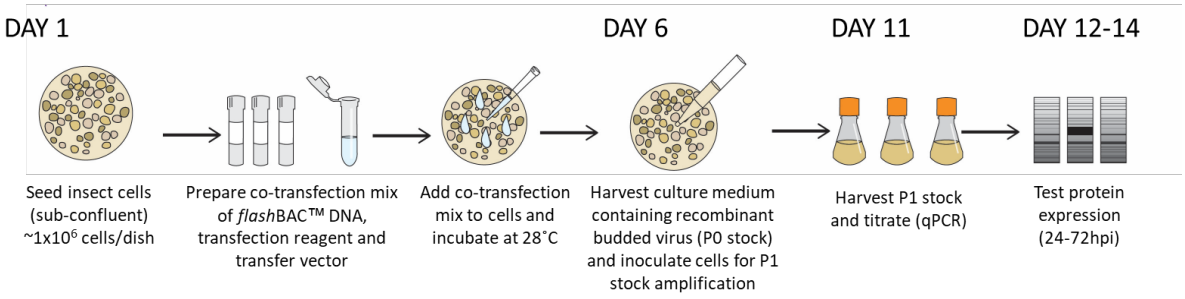


Figure 7. The simple process and timeline of making a recombinant baculovirus using flashBAC™.

A recombinant baculovirus is produced simply by co-transfecting insect cells with *flash*BAC™ DNA and a transfer plasmid containing the gene to be expressed. Homologous recombination within the insect cells (1) restores ORF 1629 allowing the recombinant virus to replicate (2) removes the BAC sequences and (3) inserts the foreign gene under control of the *polh* promoter (or other promoter present in the transfer plasmid) (Figure 6).

The recombinant BV is harvested from the co-transfection medium and becomes the seed stock (P0) of recombinant virus (Figure 7). No selection systems are needed. However, the virus stock is not homogeneous in the way plaque-purified virus is and for very large-scale applications or for work that may be taken through regulatory processes, we recommend a single round of plaque-purification. For most purposes, however, plaque-purification is not necessary. In addition for GMP use, we recommend using *flash*BAC™ GOLD (as no bacterial sequences remain in the final recombinant expression vector).

The *flash*BAC™ system is backcompatible with all transfer plasmids based on homologous recombination at the *polh* locus. The OET website has details of most of these and they include single, dual, triple and quadruple expression plasmids, those with purification tags at N and C termini, and other promoters including *p10*, *p6.9*, *ie-1* and, *CMV* (for mammalian cells). It is not compatible with pFASTBac™ vectors and the Bac-to-Bac® system[13].

Since the launch of the original *flash*BAC™ DNA, we have made further modifications to help express difficult to express proteins. The different *flash*BAC™ variants are shown below and Figure 8 shows comparative expression data.

_flash_BAC™ Backbone virus DNA has a *chiA* deletion which prevents production of virus chitinase. This enzyme blocks the secretory pathway and its absence helps improve membrane and secreted protein production[14-17].

_flash_BAC GOLD Backbone virus DNA has gene deletions for *chiA* and *v-cath*[18]. This avoids production of chitinase and cathepsin, a viral protease that may otherwise degrade susceptible target proteins. There are no remaining bacterial sequences in recombinant viruses made using FB GOLD so this is the variant of choice for GMP work.

_flash_BAC ULTRA Backbone virus DNA has deletions of *chiA*, *v-cath* and *p10/p26/p74*. Deletion of *p10* results in delayed cell lysis (particularly noticeable) in *T. ni* Hi5™ cells and thus can extend protein production times. It also reduces the metabolic burden on the cell of producing high levels of P10 protein.

_flash_BAC PRIME No gene deletions in the virus back bone. Useful if the proteins being expressed form complexes inside the cytoplasm or nucleus that need to be purified. However, target proteins must be resistant to cellular proteases. We find that the relatively early cell lysis associated with PRIME makes it easier to purify these complexes e.g. VLPs (**Figure 9**). As with GOLD, there are no remaining bacterial sequences in the final recombinant virus construct.

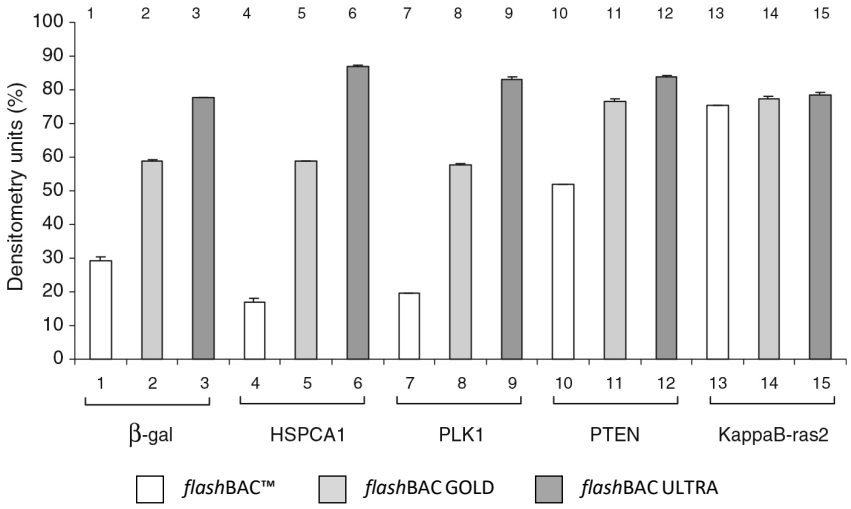


Figure 8. *Comparative expression analysis (Western blot intensities) of five target proteins using flashBAC™ (open boxes), GOLD (light grey boxes) and ULTRA (dark grey boxes). Modified from Ref 19.*

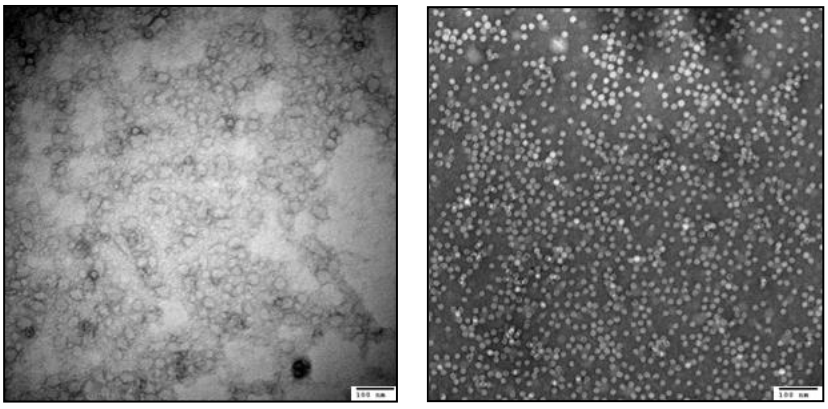


Figure 9. *Electron microscopy images of semi-purified circovirus VLP, produced using recombinant flashBAC™ ULTRA (left) or flashBAC™ PRIME (right) viruses. Circovirus does not bud from the membrane so can only be harvested once it has been released into the supernatant during cell lysis, a process that occurs more readily after infection with flashBAC™ PRIME.*

SUMMARY: Advantages of the *flash*BAC™ system:

- ✓ Simple to use
- ✓ One step procedure that does not require plaque-purification (**Figures, 6 and 7**)
- ✓ Amenable to making many viruses simultaneously – manually or using a robot in 12 or 24-well plates
- ✓ Maximise secreted or membrane targeted proteins
- ✓ Maximise difficult to express protein production
- ✓ Maximise VLP production and release from cells
- ✓ Back compatible with a large range of transfer plasmids
- ✓ Now compatible with Invitrogen™ Gateway™ cloning system

6. Making Recombinant Baculoviruses using either BacPAK6 or *flash*BAC™

6.1 Choice of Transfer Plasmid

Both the BacPAK6 and *flash*BAC™ systems use transfer plasmids to mediate transfer of the gene/s to be expressed into the virus genome at the *polh* locus. A large number of transfer plasmids are available from OET and other suppliers (Table 1). Please see the OET website for a full list of compatible vectors.

Table 1. Transfer plasmids can be grouped as follows:

Promoter Type	Features
Polyhedrin gene	Simple vector such as **pOET1.1** or **pOET2.1** that has a multiple cloning site (MCS) to insert your gene under control of the strong, very late polyhedrin gene promoter.
P10 gene	Another strong, very late gene promoter, frequently used in vectors for multigene expression (see below).
Dual promoters	For dual expression of genes, usually one under polyhedrin and one under *p10* promoters, such as **pOET5.1**.
Multiple promoters	A mix of copies of *polh* and *p10* promoters. Careful construct plans are needed to insert genes according to MCS and restriction sites available.
BacMAM promoters	These contain a mammalian promoter in place of *polh* so that the final recombinant virus can be used to effect gene expression in mammalian cells e.g. **pOET6** (*CMV* promoter), **pOET9** (*CMV/SV40/CCAG/EF1α* promoters).
Late gene promoters	Use baculovirus gene promoters that are expressed earlier than *polh* and *p10*, in the late phase of gene expression. Useful for secreted or membrane targeted protein where *polh/p10* has not worked, or for proteins that need processing before the virus shuts off host cell protein production. e.g. **pOET3** and **pOET4** use the *p6.9* promoter.
Purification tags	Several transfer plasmids will give the option for N- or C- terminal tags such as His, Strep, HA to aid protein purification. These may also have a cleavage site to release the final product from the tag.
Fusion vectors	Some transfer plasmids allow the gene to be expressed as a fusion product with a fluorescent protein, for example, to allow visualisation by microscopy.
Signal peptides	Generally, the natural signal peptide of a protein will work in insect cells but if you want to add a signal peptide or use an insect virus one, then the signal peptides of either AcMNPV GP64 or chitinase work very well. Adding a signal peptide to a protein that is not normally secreted may not work.
Start codons	Translation will start at the first ATG after the promoter so check constructs carefully to ensure there is no inadvertent additional ATG.
Codon optimisation	There is no general data to show that codon optimisation is needed, however, if you are getting your gene synthesised then it makes sense to optimise for insect cells.
Membrane anchors	Many people try and secrete membrane protein domains by removing membrane anchor domains – this works sometimes but not always.

When cloning genes into transfer plasmids note:

- Check the gene is in the correct orientation with respect to the promoter
- Check that the first ATG after the promoter is the start codon you want to initiate translation in the mRNA
- Check you have a stop codon

- Check that any fusion or purification tags are in frame and after any signal peptide sequence (that will be cleaved off)
- Sequence any gene that has been cloned via PCR or gene synthesis. Check cloning junctions of genes cloned in using RE digestion and ligation.
- Ensure final plasmid is sterile as it will be used to transfect insect cells – you don't want your cells getting bugged
- Mini-prep type DNA works OK in transfections

Contact us on info@oetltd.com if you need advice or help with transfer plasmids.

6.2 Co-transfection of Insect Cells with BacPAK6/BacPAK6 *MT* or *flash*BAC™ DNA and a Transfer Plasmid to make a Seed Stock (P0) of Recombinant Baculovirus

This method uses cells prepared in individual 35mm cell culture dishes, 6-well plates, or 12-well plates. Protocol **6.6**, provides an adaptation of this method for making multiple viruses using 24-well plates. This method must be carried out using aseptic technique as the DNA complexes will be introduced in insect cells in the absence of antibiotics. Read through the whole protocol before starting to check you have all the reagents and equipment needed.

Check safety advice and SDS data sheets where appropriate. We recommend wearing PPE such as lab coats and gloves at all times.

Provided in the kit:

- *flash*BAC™ DNA (any type) or BacPAK6 DNA (any type) (use 100ng [5µL] DNA per co-transfection)
- Positive control transfer plasmid DNA expressing *lac*Z (*flash*BAC™ kit) or GFP (BacPAK kit) (use 500ng [5µL] per co-transfection)

Also needed:

- 35mm tissue culture dish/6-well plate or 12-well plate seeded with a sub-confluent monolayer of *Sf*21 or *Sf*9 cells – one dish/well for each co-transfection and one for a control

> **NOTE**
> **It is vital for transfection success that cells used are taken from a culture that is in log phase growth – virus can only replicate when cells are in log phase! Cells must be seeded as a sub-confluent monolayer in which there are spaces around each cell so there is room for each cell to divide in the 24 hours after co-transfection.**

- Serum-free insect cell culture medium or specialist transfection media (e.g., OET or Expression Systems LLC Transfection Media). TC100 medium also works as a transfection medium (do not add serum)
- Growth medium (serum-free or TC100 with 10% serum, as preferred)
- Sterile transfer plasmid DNA containing gene to be expressed (see **7.1** for details) (500ng per transfection)

- Transfection reagent such as OET's *baculo*FECTIN II (volume as indicated by the manufacturer)
- Incubator set at 28°C
- 1% Virkon (Amtec) or other suitable disinfectant
- Plastic box to house dishes in the incubator
- Sterile pipettes and bijoux or other polystyrene containers to make up the transfection mix; do not use micro-centrifuge tubes made of polypropylene

Method:

1. For each co-transfection you require one 35mm dish/one well of a 6-well plate or one well of a 12-well plate, containing sub-confluent *Sf*9 or *Sf*21 cells (Figure 10 - day 1 panel). If you are making a virus with the control vector provided in either the *flash*BAC™ kit or BacPAK6 kit, add an extra dish/well of cells. It is also good practice to have one dish/well for a mock-transfection in which no DNA is added.

2. Seed the dishes/wells with cells at least 1 hour before use to allow cells to attach and recover. Cells should be taken from a log phase culture that were at least 90% viable. As a rough guide, you need about 1.5 x 10^6 *Sf*21 or 1 x 10^6 *Sf*9 cells per 35mm dish/6-well plate to form a sub-confluent monolayer. For 12-well plates, add 0.4 x 10^6 *Sf*9/*Sf*21 cells per well. The volume of medium should be 2mL in 35mm dishes/6-well plates and 1mL in 12-well plates. Observe cells under either a phase contrast or brightfield microscope to ensure cells are evenly distributed over the surface of the dish/well.

3. During the 1 hour incubation period, prepare the co-transfection mix of DNA and transfection reagent. For each co-transfection in either a 35mm dish/6-well plate or well of a 12-well plate, you need to mix in a polystyrene tube, in the following order:

   - 100µL serum-free medium (TC100 preferably)
   - 100ng virus DNA from the kit (*flash*BAC™ or BacPAK6) [5µL]
   - 500ng of your own transfer vector or control plasmid from the *flash*BAC™ kit (*lac*Z) or BacPAK6 kit (GFP) [5µL]
   - 1.2µL *baculo*FECTIN II

   Mix (total volume = 111.2µL) and leave at room temperature for 15 minutes.

   Set up a control transfection mix by omitting the DNAs, if wished.

4. If the plated cells were maintained in serum-containing medium, wash the monolayers twice with TC100 without serum and then add 1mL of TC100 without serum (or OET/ES Transfection Medium) to each 35mm dish/6-well plate or well of a 12-well plate. If the cells were maintained in serum-free medium, there is no need to wash at this step; simply remove and discard 1mL of medium from only the 35mm dishes/6-well plate. All the 35mm dishes/6-well plate or wells of a 12-well plate should at this stage contain 1mL of medium without any serum.

5. Pipette the 111.2µL transfection mix from stage 2 into each 35mm dish/well of a 6-well plate/well of a 12-well plate as appropriate. Place in a sandwich box and incubate overnight at 28°C.

6. After overnight incubation, add an extra 1mL of growth medium to the 35mm

Notes:

Do not use *T. ni* Hi5™ cells to make recombinant viruses or for amplifying virus as they are prone to mutations that affect gene expression from *polh* promoter.

It is vital for transfection success that cells used are taken from a culture that is in log phase growth – virus can only replicate when cells are in log phase. Cells must be seeded as a sub-confluent monolayer in which there are spaces around each cell so there is room for each cell to divide in the 24 hours after co-transfection.

This protocol is optimised for using *baculo*FECTIN II. If using a different reagent, consult the protocol supplied by the manufacturer.

dishes/6-well plate or replace the 1mL medium in the 12-well plates with 1mL growth medium (may either be serum-free medium or TC100 with 10% serum). Continue the incubation for 4 more days (5 days in total).

7. Harvest the culture medium containing budded recombinant virus into a sterile container and store in the dark at 4°C.
8. If you prepared a control virus with the *lacZ* transfer plasmid in the *flash*BAC™ kit, you can check for transfection success by staining the monolayer of cells left after harvesting the P0 virus; add 1mL of growth medium containing 15µL 2% v/v X-gal to the cell monolayer and leave for a few hours to overnight for the blue colour to develop. If you prepared a control virus with the GFP transfer plasmid in the BacPAK6 kit you can measure transfection success by observing the cells under a suitable fluorescent microscope.

Cells in which virus has replicated appear different from mock-transfected cells (**Figure 10**) so comparing mock-transfected cells with experimental dishes can be a useful indicator that the transfection has worked; infected cells appear more grainy with swollen nuclei.

9. The next step depends on whether you have used BacPAK6 or *flash*BAC™ DNA.

   ***flash*BAC™ DNA:** Your 1-2mL stock of virus is your seed stock (P0), you now need to amplify this to obtain a 50 -100mL P1 stock of virus for experimental work and freezing down (go to **6.4**).

   BacPAK6: You now need to plaque-purify your recombinant virus to obtain your seed stock (P0) (go to **6.3**).

You can also plaque-purify virus produced using *flash*BAC™ DNA if required.

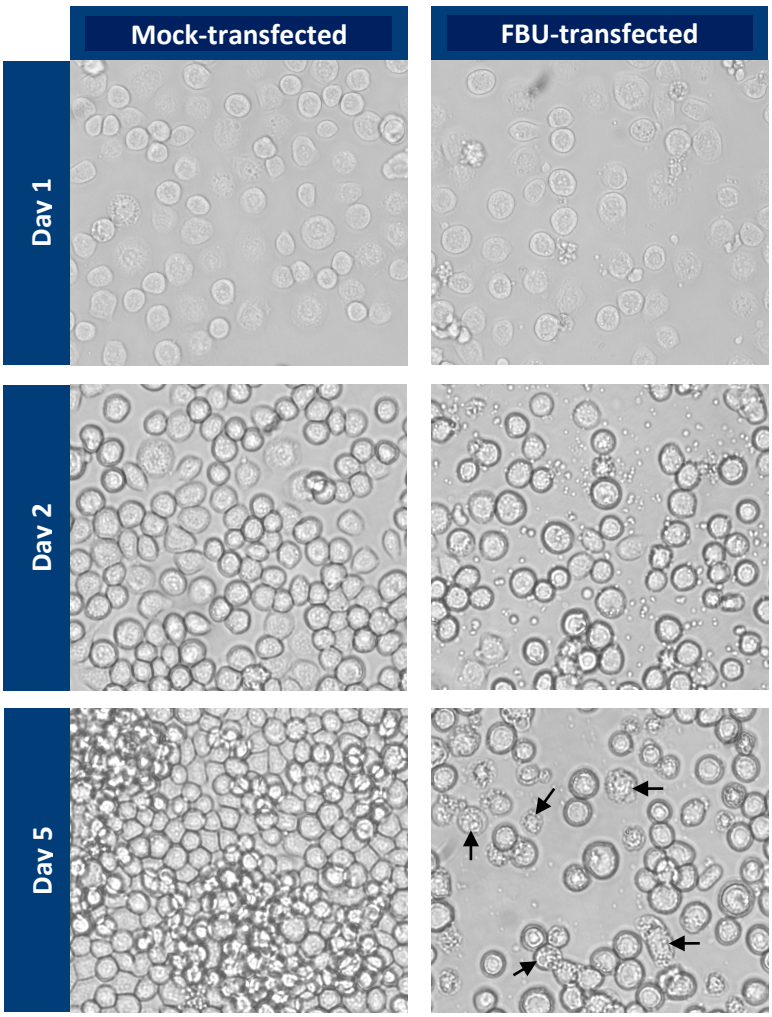


Figure 10. *Comparison of mock cells and virus-transfected cells using flashBAC ULTRA (FBU). Images were taken at 1 day, 2 days and 5 days post-transfection.*

Note appearance of sub-confluent monolayer of cells in Day 1 cells.

→ *Indicates virus-infected cells.*

6.3 Plaque-purification and Amplification of P0 Recombinant BacPAK6 Virus

This section refers to both BacPAK6/BacPAK6^{sec+} linear DNA and BacPAK6 *Maximum Transduction* (*MT*) DNA. The budded virus harvested after the co-transfection with BacPAK6 virus DNA (Protocol 6.2) will contain a mixture of parental virus (about 10% blue) and recombinant virus (about 90% clear/colourless). These need to be separated by performing a plaque-assay and picking individual plaques to amplify pure virus stocks. If well isolated plaques are picked from wells without parental blue plaques present, only one round of plaque-purification is needed.

This is a multi-step method that enables you to isolate plaques and then amplify plaque-picked virus to produce a P0 seed stock of virus. Read through the whole method before starting to ensure you are aware of time scales and reagents/equipment needed at each stage.

Required:

- Virus harvested from a co-transfection (see **6.2**)
- TC100 growth medium with serum (best; or serum free growth medium) - antibiotics (Penicillin, 10000 units/mL and Streptomycin, 10000µg/mL in 0.85% saline; dilute 1 in 50 for use) may be added to plaque-assay medium to help reduce the chance of contamination
- Culture of *Sf*21 cells (preferred; or *Sf9* cells) that are in log phase of growth and at least 90% viable
- 35mm dishes and T25 flasks
- Low temperature gelling (Sea-plaque®) agarose for overlay (Sigma-Aldrich®; 2% w/v solution in deionised water). It is convenient to make up small batches (15mL) of agarose overlay by melting the agarose using a boiling water bath or microwave oven (take care). Solidified agarose can be stored and re-melted prior to use. (Larger volumes may also be prepared and melted multiple times). Cool to 'hand hot' before making up final overlay.
- Sterile pipettes and bijoux or similar containers for making virus dilutions
- Sterile Pasteur pipettes
- Beaker with hand hot water as a temporary water bath
- Plastic box
- Incubator at 28°C
- Phosphate-buffered saline (PBS)
- Neutral Red stain (Sigma-Aldrich®; 5mg/mL in deionised water, filter sterilize and store at room temperature). For use dilute 1 in 20 with PBS solution. Do not store diluted stain.
- X-gal (2% v/v in dimethylformamide (DMF)) to stain for blue plaques
- 1% Virkon (Amtec) or similar disinfectant
- Inverted phase contrast light microscope
- Lightbox to view plaques

Method:

1. Seed 35mm cell culture dishes with a sub-confluent monolayer of healthy log phase *Sf*21 cells at about 4 x 10^5 cells/well (or *Sf*9 cells if *Sf*21 are not available). See OET's Insect Cell Culture Manual for more details. Allow the cells to settle for at least 1 hour.

   You need 12 dishes per virus.

2. Make 1 in 10 dilutions of your transfection virus stock from 1 in 10 (10^{-1}) to 1 in 10^6 (10^{-6}). Use 50µL virus and 450µL growth medium as diluent at each step. Mix the virus and diluent between each step and change tip/pipette each time to avoid carry-over.

3. Remove the medium from the dishes of cells using aseptic technique and add 100µL of diluted virus drop wise to the centre of each dish. Plate a range of dilutions and two plates per dilution – the aim is to get well isolated plaques on at least one dilution. We normally plate the 1 in 100 (10^{-2}) to 10^{-6} dilutions in duplicate dishes and use two dishes as mock-infected controls (use medium only).

4. Allow the virus to adsorb and be taken up into the cells at room temperature for 45-60 minutes. Rock the dishes every few minutes to ensure even coverage of the inoculum. Do not put the cells in the incubator as they will dry out.

5. During this time prepare the overlay. Dissolve agarose in water to 2% w/v by boiling (water bath or microwave oven – take appropriate safety precautions). You need 1mL per dish of cells. Cool the agarose overlay to hand hot (about 50-55°C) and add an equal volume of pre-warmed growth medium (28°C). Keep warm to prevent setting (we use a temporary clean water bath comprising a beaker of hot tap water). You need 2mL final overlay per dish.

6. At the end of the incubation period (4), remove the inoculum using a pipette and discard into Virkon or other disinfectant. Working quickly, add 2mL warm overlay to each dish allowing the agarose to flow down the side of the dish and spread slowly over the monolayer of cells. Do not pipette into the centre of the dish.

7. Allow the agarose overlay to set at room temperature. Then add 1mL liquid overlay of growth medium to feed the cells and prevent them from drying out.

8. Place the dishes in a plastic box and incubate at 28°C for 4 days.

9. Add 1mL growth medium containing 15µL 2% v/v X-gal in Dimethylformamide (DMF) to each dish to stain for blue (parental) plaques. Incubate at 28°C for 5-6 hours.

   Conveniently, this is done in the course of a normal working day.

Notes:

*Sf*21 cells in TC100 with 10% serum give the largest, easy-to-spot plaques because these cells have a well-defined cytopathic effect (CPE) (**Figure 11**). *Sf*9 cells will also yield plaques but they are smaller, take longer to develop and are not quite so easy to define. We have also noted that plaque assays conducted with *Sf*9 cells and serum free medium produce plaques that quickly fade after staining with Neutral Red.

It is important that the cell monolayers do not dry out during this process of virus inoculation. Do not leave lids off dishes for long periods. If working in a class II hood be aware the air flow can dry plates very quickly. If, after staining, your monolayer appears with a shiny red patch devoid of cells, you have allowed the monolayer to dry out.

If the agarose in water sets, it is easy to melt again by boiling. If the agarose overlay with growth medium sets, you cannot re-melt. You need to start again. We often prepare several small batches of agarose in water and let them set and then melt each aliquot as we need it (15mL is convenient).

Process one set of dishes per virus sample at a time. If working in a hood, keep the agarose overlay in a beaker or sandwich box filled with warm water to delay solidification. If the agarose sets prematurely, you can leave the dishes with virus inoculums for longer than 60 minutes without adverse effects.

If you have removed the virus and then find that your overlay medium has set, just add 0.1-0.2mL fresh medium to each plate to prevent drying of cells. Prepare more agarose overlay medium and carry on, but don't forget to remove the extra medium you added to each dish!

Blue plaques should start to develop during this time.

10. Prepare the Neutral Red stain in water to 5mg/mL deionised water and filter sterilize or purchase ready made from Sigma-Aldrich®, for example. Dilute 1 in 20 with sterile PBS for use.

Different batches of Neutral Red may differ in their efficacy. Sometimes 1 in 40 dilutions give better results. Do not store diluted stain, it will form a precipitate. The concentrated stock is stable at room temperature for several months (if sterile).

11. Add 1mL diluted neutral red stain to each dish. Do not remove the X-gal already added. Incubate at 28°C for 16 hours (overnight).

12. Decant all liquid and view plaques on a light box. Recombinant virus plaques will appear clear in a sea of red healthy cells. Parental, non-recombinant plaques will stain blue with X-gal.

13. You need to pick 3-6 plaques for each virus. Select well isolated plaques from a dish where there are no blue plaques (**Figure 11**).

If the dilutions were unsuitable (i.e. too few or too many plaques per dish), you may have to repeat the plaque assay adjusting the dilutions to obtain dishes with well isolated plaques and no blue plaques. With experience you can cut down the range of dilutions plated once you know the general titre of virus that you obtain from a co-transfection. We recommend starting with a wide range, as transfection efficiency can vary considerably.

14. To pick a plaque, you need to take up a plug of agarose from the centre of a plaque using a Pasteur pipette or Gilson® tip. Disperse the plug of agarose into 500µL growth medium in a micro centrifuge tube and vortex to release the virus from the agarose into the medium. Store in the dark at 4°C.

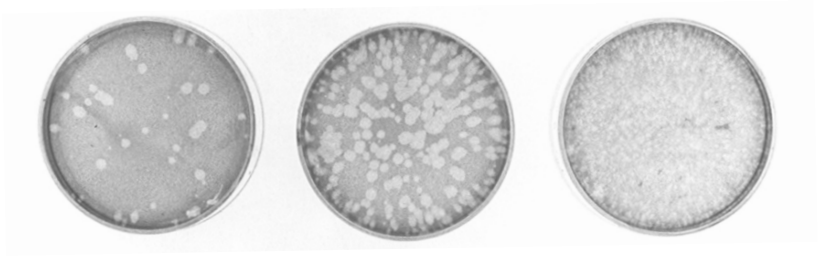
15. Amplify the plaque-picked virus by inoculating either a 35mm dish or a T25 flask of *Sf*21 or *Sf*9 cells using 100µL (35mm dish) or 250µL (T25 flask) of your 500µL as inoculum.

    To do this, seed a 35mm dish or T25 flask with cells to form a sub-confluent monolayer and after an hour or so, remove the medium and replace with the inoculum for 45-60 minutes. Then add 2mL (dish) or 5mL (T25 flask) growth medium (no need to remove the inoculum) and incubate for 4-5 day at 28°C.

    The cells should appear well infected when viewed under the microscope at the end of the infection period (**Figure 8**).

16. Harvest the 2mL or 5mL of medium containing your P0 seed stock virus. Store at 4°C in the dark. Use this to amplify a P1 working stock of recombinant virus to test gene expression (see **6.4**).

The cell monolayers from the dish or flask used to amplify virus can be harvested and tested for gene expression or to isolate DNA to do a PCR to check that the gene has gone into the virus genome.



A B C

Figure 11*. Plaque-assay in Sf21 cells stained with Neutral Red to show (**A**) well isolated plaques, (**B**) crowded plaques (**C**) and merged plaques.*

6.4 Amplification of Recombinant Baculoviruses to Produce High Titre Stocks

This is a generic method to amplify recombinant baculovirus from P0 to P1, or P1 to P2 etc. We do not recommend serial passage of the virus stock because mutations can and do arise. These can sometimes lead to reduced expression levels or loss of the gene. Good practice is to amplify a 50-200mL P1 stock for initial test of gene expression and optimisation of expression. Some of this virus should also be frozen down at -80°C for long-term storage. Do not store virus at -20°C. Virus can be stored in the dark at 4°C for a few months but in the absence of serum, the titre can start to drop after a few weeks. We recommend adding serum to 5% for all viruses stored at 4°C. If you cannot do this, then freeze aliquots of P1 virus at -80°C after adding sterile glycerol to 5% and use these to establish new P2 stocks when needed.

If you are planning to scale-up protein production beyond a few hundred mL, you will need to produce some P2 virus to use for experiments – even P3. Again, you may need to think about storing some of this at -80°C.

Most recombinant baculoviruses will amplify to titres in the region of 1-2 x 10^8 pfu/mL. Sometimes a foreign protein inhibits/affects BV formation or is toxic and virus titres will be lower. Anything above 1-2 x 10^7 pfu/mL should be good enough.

To have the best chance of producing a good high titre stock of P1 or P2 virus, use *Sf*9 cells growing in shake suspension cultures in serum-free growth medium, or *Sf*21 cells growing in TC100 with serum in stirred cultures. See the OET Cell Culture Manual for more details on insect cell culture. Whichever cells are used, they must be harvested in log phase and be at least 90% viable when used to set up a new culture ready to infect. This is because the virus needs cells in a dividing state to be able to replicate.

To avoid accumulating mutants, always infect cultures at very low multiplicity of infection (MOI of 0.1 plaque-forming unit/cell) and allow the virus to undergo multiple rounds of replication – this also achieves the highest titres possible.

If you infect cells at high MOI, all the cells in the culture will be infected at the start and the virus will undergo one round of multiplication with a higher chance of cross-over or other mutation events occurring and giving a lower overall titre.

Read through the method before starting to understand timescales and materials required and use aseptic technique throughout.

Required:

- Stock of virus to be amplified (e.g., P0 from method **6.2** or **6.3**)
- 50-200mL of healthy log phase *Sf*9 or *Sf*21 cells at no more than 2 x 10^6 cells/mL
- Shake flask appropriate to the volume of *Sf*9 cells to be used – you need maximum surface area for oxygen exchange as when cells are infected they have a high O_2 requirement
  OR: Stirred flask e.g., Techne for *Sf*21 cells in medium containing serum
- Growth medium (serum-free or TC100 with 10% serum)
- Incubator at 28°C with shaking platform or stirred culture platform
- Phase contrast light microscope
- Disinfectant for waste
- Sterile pipettes

See OET Insect Cell Culture Handbook for more information on culturing insect cells.

Method:

1. Prepare 50-200mL log phase *Sf*9 cells or *Sf*21 cells in a shake or stirred culture as appropriate to the medium being used. Generally, *Sf*9 cells in serum-free medium in a shake culture should not be more than 2 x 10^6 cells/mL and *Sf*21 cells in serum-containing culture should not be more than 1 x 10^6 cells/mL.

2. To amplify virus, simply add the appropriate volume of inoculum to give a low MOI of 0.1 pfu/cell. When amplifying the seed stock (P0) of *flash*BAC™ or BacPAK6 virus, we recommend adding no more than 0.5mL virus into 100mL culture (we do not normally titrate the seed stock virus before P1 amplification).

3. Ensure the cells are shaking at the appropriate rpm for the platform being used. If cells are not rotated fast enough, they will not be oxygenated and the virus will not replicate. Infected insect cells have a high requirement for oxygen. Allow the virus to amplify for 3-5 days.

4. When the cells appear well infected under the light microscope, harvest the culture and remove cells by centrifugation at 3000 rpm for 15 minutes in a bench top or other slow speed centrifuge.

5. Aseptically, decant the clarified culture medium into storage containers and store in the dark at 4°C. Add serum to 5% for longer term storage. We also recommend storing aliquots of 1-2mL at -80°C.

6. Titrate your P1, P2 or P3 virus stock before using – the most common reason for poor expression levels is that the virus used to infect the cells had not actually amplified and so the cells were not infected.

Notes:

If we are amplifying P1 to P2 or P2 to P3, we always use a defined amount of inoculum based on a virus infectivity titration. If your P1 virus titre was 1 x 10^8 pfu/mL and you wanted to amplify 500mL P2 virus, you would need to add 1mL of P1 virus to 500mL cells at 2 x 10^6 cells/mL (MOI = 0.1).

Virus can also be amplified in monolayer cultures in T75 or T150 flasks. Simply seed the flasks to provide a sub-confluent monolayer of cells. Remove the medium and add the inoculum to give the correct MOI (0.1 pfu/cell) (use 100 or 200µL P0 virus from 7.2 (page 30) or 7.3 (page 35) diluted in medium to 500µL (T75) or 1mL (T150) per flask). After 45 minutes incubation, add 10-15mL medium (T75) or 30mL medium (T150) and allow the virus to replicate for 3-5 days until all the cells are well infected. The titre of virus amplified in this way is not usually as high as that amplified in shake cultures.

You can titre your virus by plaque-assay – the gold standard (see **6.5**) or by qPCR. OET has a convenient qPCR titration kit (*baculo*QUANT™ ALL-IN-ONE) or we provide a fast and cost-effective virus titration service. Contact us at info@oetltd.com for more details.

6.5 Titration of Recombinant Virus by Plaque-assay

This is the acknowledged gold standard for determining accurate virus titres. The protocol below is one that we have adapted for 12-well plates and is convenient and easy to follow (see Figure 12). However, titres can also be obtained by qPCR and OET sells a convenient *baculo*QUANT™ ALL-IN-ONE kit for this purpose. Alternatively, OET offers a service to titrate your viruses by qPCR or plaque-assay from as little as £60 per virus – contact us on info@oetltd.com. See overview in Figure 12.

Required:

- Virus to be titrated (see **6.4**)
- TC100 growth medium with serum (best; or serum free growth medium) - antibiotics (Penicillin, 10000 units/mL and Streptomycin, 10000 µg/mL in 0.85% saline; dilute 1 in 50 for use) may be added to plaque-assay medium to help reduce the chance of contamination
- Culture of *Sf*21 cells (best; or *Sf*9 cells) that are in log phase of growth and at least 90% viable
- 12-well plate (or 35mm dishes/6-well plate)
- Sterile pipettes and a 48-well plate to make dilutions. Alternatively, dilutions can be made in sterile 1.5-2mL tubes.
- Beaker with hand hot water as a temporary water bath
- Plastic sandwich box
- Incubator at 28°C
- Low temperature gelling (Sea-plaque®) agarose for overlay (Sigma-Aldrich®; 2% w/v solution in deionised water). It is convenient to make up small batches (7mL) of agarose overlay by melting the agarose using a boiling water bath or microwave oven (take care). Solidified agarose can be stored and re-melted prior to use. Cool to 'hand hot' before making up final overlay.
- Phosphate-buffered saline (PBS)
- Neutral Red stain (Sigma-Aldrich®; 5 mg/mL in deionised water, filter sterilize and store at room temperature). For use dilute 1 in 20 with PBS solution. Do not store diluted stain.
- 1% Virkon (Amtec) or similar disinfectant
- Inverted phase contrast light microscope
- Lightbox to view plaques

Method:

1. Seed wells of a 12-well plate with a sub-confluent monolayer of healthy *Sf*21 cells (or *Sf*9 cells if *Sf*21 are not available). See **6.2** for more details. About 4 x 10^5 cells/well. Allow the cells to settle for at least one hour.

   You need 1 x 12-well plate per virus to be titrated. Alternatively,

Notes:

Sf21 cells in TC100 with 10% serum give the largest easy-to-spot plaques because these cells have a well-defined cytopathic effect (CPE) (Figure 11). Sf9 cells will also yield plaques but they are smaller, take

you can seed 35mm dishes with cells – see protocol **6.3** for doing plaque-assays in 35mm dishes.

2. Make 1 in 10 dilutions of your virus stock from 1 in 10 (10^{-1}) to 1 in 10^7 (10^{-7}). Use 50µL virus and 450µL growth medium as diluent at each step. Mix the virus and diluent between each step and change tip/pipette each time to avoid carry-over. It is convenient to do this in a 12/48-well plate.

3. Remove the medium from the dishes of cells using aseptic technique and add 100µL of diluted virus drop wise, gently to the centre of each dish. Plate a range of dilutions from 10^{-4} to 10^{-7} and three wells per dilution = 12 wells. The aim is get at least one set of wells with a countable number of plaques.

4. Allow the virus to adsorb and be taken up into the cells at room temperature for 45-60 minutes. Rock the dishes every few minutes to ensure even coverage of the inoculum. Do not put the cells in the incubator as they will dry out.

5. During this time prepare the overlay. Dissolve agarose in water to 2% w/v by boiling (water bath or microwave oven – take appropriate safety precautions). You need 0.5mL per dish of cells. Cool the overlay to hand hot (about 50-55°C) and add an equal volume of pre-warmed growth medium (28°C). Keep warm to prevent setting (we use a temporary clean water bath comprising a beaker of hot tap water). You need 1mL final overlay per dish.

6. At the end of the incubation period (4), remove the inoculum using a pipette and discard into Virkon or other disinfectant. Working quickly, add 1mL warm overlay to each dish allowing the agarose to flow down the side of the dish and spread slowly over the monolayer of cells. Do not pipette into the centre of the dish.

7. Allow the agarose overlay to set at room temperature. Then add a 0.5mL liquid overlay of growth medium to feed the cells and prevent them from drying out.

8. Place the dishes in a plastic box and incubate at 28°C for 4 days.

9. Prepare the stain by dissolving Neutral Red in water to 5mg/mL deionised water and filter sterilise, or purchase ready made from Sigma-Aldrich®, for example. Dilute 1 in 20 with sterile PBS for use.

10. Add 0.5mL diluted neutral red stain to each dish and incubate for 3-4 hours at 28°C.

11. Decant the remaining liquid and view plaques on a light box. Recombinant virus plaques will appear clear in a sea of red healthy cells. It sometimes takes a few hours for plaques to be really visible.

12. Count the plaques from wells where there are a countable number of plaques (10-20). Average the plaque count from the triplicate dishes and note the dilution that gave rise to these plaques.

longer to develop and are not quite so easy to define.

It is important that the cell monolayers do not dry out during this process of virus inoculation. Do not leave lids off dishes for long periods. If working in a class II hood be aware the air flow can dry plates very quickly. If, after staining, your monolayer appears with a shiny red patch devoid of cells, you have allowed the monolayer to dry out.

Process one set of dishes per virus sample at a time. If working in a hood, keep the agarose overlay in a beaker or sandwich box filled with warm water to delay solidification. If the agarose sets prematurely, you can leave the dishes with virus inoculum for longer than 60 minutes without adverse effects. If you have removed the virus and then find that your overlay medium has set, just add 0.1-0.2ml fresh medium to each plate to prevent drying of cells. Prepare more agarose overlay medium and carry on, but don't forget to remove the extra medium you added to each dish!

If the agarose in water sets, it is easy to melt again by boiling. If the agarose overlay with growth medium sets, you cannot re-melt. You have to start again. We often prepare several small batches of agarose in water and let them set and then melt each aliquot as we need it - 7mL is convenient).

Some batches of Neutral Red may work better at 1 in 40 dilution – do not store diluted stain as it precipitates.

13. Determine the virus titre as follows:

    Average number plaques x dilution factor* x 10** = plaques/mL in the original virus stock.

    *Inverse of dilution; **because only 0.1mL was added to dish

    For example, if the average number of plaques was 15 taken from the 10^{-6} dilution wells, the virus titre would be:

    $15 \times 10^6 \times 10 = 1.5 \times 10^8$ pfu/mL.

Virus titres will drop after storage at 4°C and so we recommend re-titrating virus before use if it has been stored for more than 3-4 months. OET offers a convenient virus extraction and titration kit, *baculo*QUANT ALL-IN-ONE, for determining virus titre in less than 3 hours. Alternatively, our plaque assay service is also available.

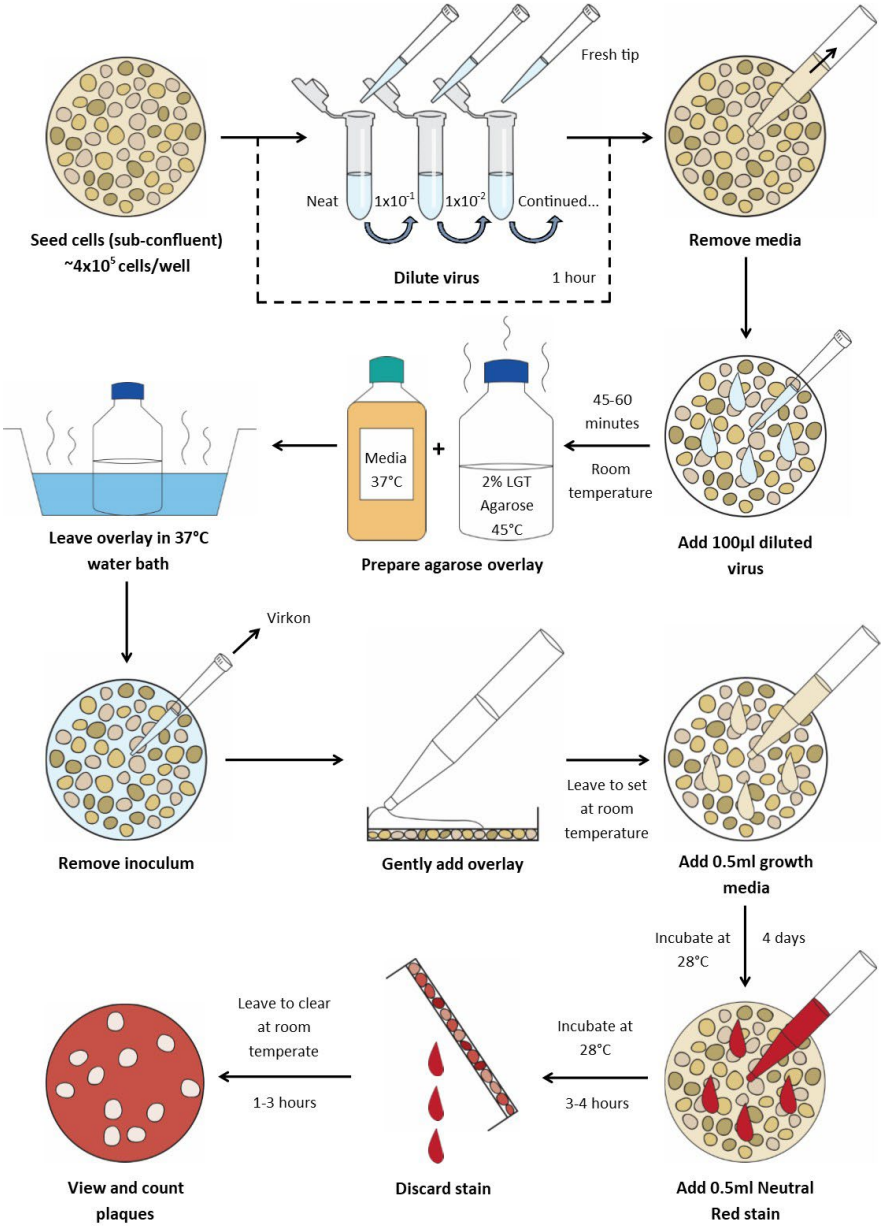


Figure 12: *Schematic diagram outlining the plaque assay procedure of recombinant baculovirus produced from flashBAC™ DNA.*

6.6 A Guide to Using *flash*BAC™ in 24-well Plate Systems

The following is a guide to making recombinant *flash*BAC™ viruses in a 24-well plate format. This can be achieved manually or the protocol can be adapted to use in a simple robotic system for liquid handling. In this way it is relatively straightforward to make 24 recombinant viruses at one time.

Cells: Prepare a master mix of *Sf*9 cells in serum free medium at a density of 5 x 10^5 cells/mL and dispense 0.4mL (2 x 10^5 cells) per well. Allow cells to settle for 1 hour.

Transfection master mix: It is convenient to make this in the wells of a 96-well plate. Make up a master mix of 220µL TC100 medium w/o serum (or other serum-free medium) and 120µL *flash*BAC™ DNA (5µL per virus). Dispense 14µL into 24-wells of a 96-well plate. Then add 5µL of the correct transfer plasmid (500ng DNA) and 1.2µL *baculo*FECTIN II to each of the 24-
wells as appropriate. Mix by pipetting up and down a few times and allow to stand for 15 minutes.

Add transfection mix to cells: Simply add the 20µL transfection mix into each of the wells containing cells in the 24-well plate. Seal to prevent evaporation and incubate at 28°C for 5 days.

Harvest recombinant virus by transferring the culture medium containing budded virus to the wells of a new 24-well plate, seal and store.

Short-term storage (1-3 months) of harvested virus should be at 4°C in the dark. Long-term stocks of virus should be stored at -80°C and can be kept for a number of years. However, virus stocks should always be re-titrated after long-term storage.

To amplify virus, follow protocol **6.4** – as a guide use 250µL to infect 50-100mL of *Sf*9 cells.

7. Analysis and Optimisation of Gene Expression

This section provides a guide to the analysis of gene expression from recombinant virus made using either the BacPAK6 or *flash*BAC™ systems. It is not intended to be prescriptive, simply a guide to help you get started.

7.1 Quick Check for Gene Expression

After the co-transfection or after amplification of P0 virus to give P1, remaining cells in the monolayer can be harvested and used to test for gene expression by SDS-PAGE and/or Western blotting. However, the expression levels are variable at these stages so many people prefer to wait until they have a known high titre stock of virus (P1 or P2). Some of the expression after the co-transfection will also be transient expression from the transfer plasmid itself.

7.2 Test Expression by Infecting Cells with High Titre Virus Stocks

It is always best to test expression using a virus with a known titre. That way you can control the MOI. Normally the best levels of expression are obtained with high MOIs (5-10 pfu/cell) so that all the cells

are infected simultaneously and a synchronous infection is established. However, for a few proteins, best expression is obtained at lower MOI. We therefore recommend that expression testing includes a range of MOI (1, 3 and 10 is a good starting point).
It is convenient to monitor gene expression by setting up small-scale monolayer cultures in either 35mm dishes or the wells of a 12-well plate. Set up monolayers in dishes/wells as described for co-transfections/plaque-assays (see **6.2** /**6.4**) and leave the cells to recover for an hour. Always take cells from log phase cultures to ensure that virus can infect the cells and replicate – otherwise the *polh* promoter (or other virus promoter) will not be turned on and expression levels will be very low.

Infect cells in 35mm dishes with 200µL virus inoculum or in 12-well plates with 100µL per well. Simply remove the medium, add the inoculum drop wise and gently to the centre of the dish and leave to adsorb for 45-60 minutes, with occasional rocking of the dishes. Then replace the growth medium (2mL for 35mm dishes and 1mL for 12-well plates). Incubate at 28°C.

Always include a negative control (mock-infected cells) for comparison. If you have a known recombinant baculovirus, you can add a positive control. If you purchased a *flash*BAC™ kit, you could make a recombinant virus with the control *lac*Z transfer vector and use this to set control infections to look for beta-galactosidase production (**Figure 4**).

We normally test expression by harvesting the cells and/or culture medium (as needed) at 72 hours post infection (hpi) initially. If you want to test the culture medium for secreted protein, harvest the culture medium, centrifuge to remove any floating cells and decant into a fresh tube. If expression levels are expected to be on the low side, treat 1mL of medium with StrataClean™ resin, which concentrates the protein ready for SDS-PAGE and/or Western blot analysis.

If the protein is intracellular, scrape the cells into the culture medium with a blue Gilson® tip, pellet the cells in a microcentrifuge tube. If liked, you can wash the dish with TE buffer to remove the last few cells, and add these to the tube with the main bulk of cells. Wash the cell pellet with TE buffer and re-suspend the cell pellet in SDS-PAGE loading buffer and boil samples in the usual way.

We may later optimise expression by testing expression at multiple time points (see **7.3**). It is well worth testing expression in both *Sf*9 or (*Sf*21) cells and *T. ni* Hi-5™ cells. See the OET Cell Culture handbook for details of culturing *T. ni* cells. Sometimes there can be a large difference in the expression levels between these two cell lines. Whilst *T. ni* cells should not be used for virus amplification (due to accumulation of mutations), they can be an excellent cell line for protein production and grow well in serum-free medium in shake cultures.

7.3 Optimisation of Expression

Sometimes it is necessary to optimise expression levels. This is particularly important if you are going to scale-up production of protein – work here can save litres of medium and hard work later on. You can either set up multiple 35mm dishes or 12-well plates (one dish/well per condition) or set up small (20mL) shake cultures and take samples (2mL) at various time points. The latter is better if you are planning on scaling up in future. You may also need to do pilot protein purification and small-scale shake cultures can work well for this too. Always do control mock-infected dishes or take samples prior to infecting shake cultures.

Parameters to optimise include:

- MOI – start by comparing 1, 3 and 10 pfu/cell (for some proteins a lower MOI works best even below 1 – try 0.1 and 0.3 pfu/cell)
- Cell line (*Sf*9, *Sf*21, *T. ni* Hi-5™, Super*Sf*9 cells
- Time to harvest, e.g. – 24, 36, 48, 72, 96 hpi
- *flash*BAC™ variant (e.g., GOLD, ULTRA, PRIME)

7.4 Scaling-up Production

There are many ways to scale-up insect cell culture and hence virus or protein production. The simplest is to use large-scale shake flasks. In this way up to 1.25L cells can be infected at one time. The key to success is to ensure that flasks are not overfilled (aim for maximum surface area) and that cells are shaken at a high rpm to ensure good aeration. GE Healthcare's cellbag™ are also relatively easy to use but are expensive and require access to a Wave Bioreactor™. The OET Cell Culture Handbook has more information on this topic.

8. Using the Baculovirus Expression Vector System to Produce Recombinant Proteins in Mammalian Cells

8.1 An Introduction to Mammalian Cell Transductions

The baculovirus expression vector system is not just limited to insect cells. Despite being an arthropod-specific virus, baculoviruses can express foreign DNA within mammalian cell lines by insertion of a mammalian specific promoter (e.g., CMV) to drive target gene expression. The resulting recombinant virus is referred to as a BacMAM. BacMAMs enter the cell as budded virus and are taken up by receptor-mediated endocytosis before being released into the cytoplasm as nucleocapsids. The nucleocapsids are then directed to the nucleus where viral DNA is made available for transcription (Figure 13). This process of gene delivery is known as transduction.

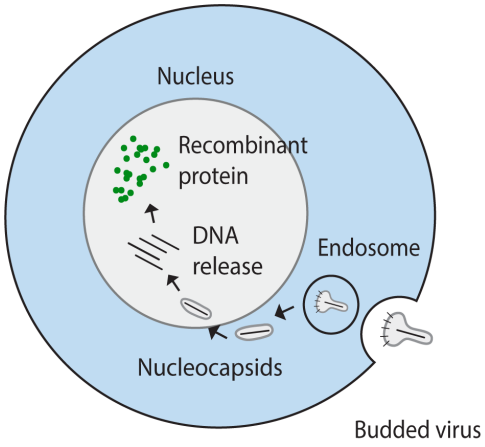


Figure 13: A schematic representation showing recombinant protein production in mammalian cells following transduction with a BacMAM in the form of budded virus.

8.2 Applications

BacMAMs are primarily adopted for protein expression in mammalian cells as they ensure the desired processing of the protein is achieved, resulting in a final structure that is fully functional. BacMAMs are also prime candidates for vaccine development as VLP production is uncontaminated by budded virus. However, BacMAM viruses are increasingly being used in the application of gene therapy. The delivery of foreign genetic material via a viral vector allows host cells to manufacture proteins that provide advantageous effects to the health and function of the cell or organism. This may include increased production of anti-inflammatory and anti-apoptotic factors to prolong cell health during transplantation; genes towards increased tumour regression or suppression of tumour growth; inducing an immune response such as T-cell activation; production of apoptotic promoters; gene silencers. Unlike transfection, the use of chemical reagents to facilitate virus entry into target cells is not essential for transduction. This feature can provide useful if working with highly sensitive cell lines that are susceptible to toxic damage.

Advantages of BacMAMs:

Transduction of mammalian cells with a viral expression vector is not new, however the BacMAM system has many advantages over conventional methods involving mammalian derived viruses (e.g. adeno-associated, retro and herpes viruses). These include:

- Safety; baculoviruses are unable to produce further virus particles in cell cultures sourced from non-insect cells and thus cannot replicate in mammalian cell lines.
- The viral nucleocapsid has the capacity to accommodate large or multiple genes meaning there is little restriction on the size of the genes you can express.
- Easy and cost-effective production. The recombinant BacMAM virus is generated in insect cells which are cheaper and easier to maintain than mammalian cell cultures. They also have the added benefit of being readily available for large-scale production and high throughput systems.
- Wide variety of mammalian promoters can be incorporated into the baculovirus genome.
- Virus DNA does not integrate itself within the host cell genome therefore prevents potential negative side effects during cell division.
- Capable of transducing a broad range of cell lines and cell types including single cells to whole organs and tissues.
- Is transient expression an advantage? Transduction success can be measured quickly and early on, can help to control expression/act as a control mechanism

8.3 Producing BacMAM Expression Vectors for Transduction

Generation of BacMAM viruses can be achieved in the same way as that for a recombinant baculovirus using *flash*BAC™, BacPAK6, BacPAK6^{sec+}, BacPAK6 *Maximum Transduction* (*MT*), or your own expression system and following the manufacturer's instructions. The only difference is that the target gene must be cloned into a transfer plasmid containing a suitable mammalian promoter. OET's pOET9 range of transfer plasmids come with the option of four mammalian promoters - Cytomegalovirus (CMV), Chicken Beta-Actin (CCAG), Human Elongation Factor-1 Alpha (EF1α) and Simian Virus 40 (SV40). With a choice of promoter, pOET9 is suitable for a wide range of cells and can allow adaptability in the strength of gene expression.

As the necessary insect promoters required for viral replication are not present within mammalian cells, the BacMAM virus is unable to replicate and target gene expression is dependent solely on the number of genomes that initially enter the cell. Therefore, the virus will need to be amplified to allow

for the high MOI required for successful transduction. Advice on how to titrate and amplify viruses can be found on page xx. It is not recommended that you use chemical reagents to enhance viral titre as these can have negative effects on cell metabolism. A simpler/alternative solution to avoid repeated rounds of virus amplification is the BacPAK6 *Maximum Transduction* (*MT*) kit from OET. By making additional modifications to our BacPAK6 virus DNA, the BacPAK6 *MT* expression vector permits increased formation of infectious budded virus that, when applied to the transduction of mammalian cells, helps to improve gene delivery and transduction efficiency.

8.4 Transducing Mammalian Cells with Recombinant BacMAM Viruses

Required:

- Appropriate mammalian cell line
- Cell culture growth medium pre-warmed to 28°C e.g., DMEM with 10% foetal bovine serum (FBS)
- Foetal bovine serum (FBS)
- Sterile phosphate-buffered saline (PBS)
- Trypsin-EDTA (0.05%)
- 1% Virkon or other suitable disinfectant
- 70% alcohol for surface disinfecting
- BacMAM virus vectors (MOI of 100-500 pfu/cell)
- Sterile cell culture disposables – 6/12/24 multi-well plates or 35mm dishes
- Sterile pipettes
- Sterile 15ml conical tubes and 7ml bijoux
- Haemocytometer with cover slips or cell counter
- Inverted phase contrast microscope
- Incubator at 37°C, 5% CO_2 (humidity settings)

Before starting:

- Check under microscope that the mammalian cells are healthy and in a log-phase of growth.
- All sterile work is to be performed in a class II safety cabinet or laminar flow hood. If possible, use a hood with UV lights. Spray everything that enters the hood, including gloved hands, with 70% alcohol. Wear specific cell culture lab coat and gloves. Mammalian cells are highly susceptible to contamination, especially by mycoplasma (see OET's Insect Cell Culture Guide) which can reduce cell growth and inhibit protein production.

Method

1. For each transduction/expression test seed one well of a 6 or 12 well plate containing healthy, log-phase mammalian cells at a density of $0.5x10^5$ - $2x10^5$ cells/ml.

   Ideally set up duplicate expression tests for each BacMAM vector, which includes one mock-transduced and one null-transduced control for each cell line. After seeding the cells, ensure they are evenly distributed over the surface of the well and leave to settle overnight at 37°C in a 5% CO_2 humidified

Notes:

It is extremely important to use healthy cells from a log-phase culture and to seed the cells at the correct density for the specific mammalian cell line being used. The null-transduced control is performed with a recombinant BacMAM that contains no target gene under the control of a mammalian promoter. Meanwhile the mock-

incubator.

2. To attain the high MOI necessary for successful transduction, a volume of BacMAM virus that corresponds to the required MOI may need to be concentrated into a suitable working volume. For example, 2ml may need to be concentrated into 200µl. This can be done by high-speed centrifugation, 14,000 rpm in a microcentrifuge for 30 minutes at 4°C. If high speed or ultra-centrifugation facilities are available this can be used to concentrate higher volumes.

3. Following centrifugation remove the culture medium and soak the virus pellet in the required volume of either phosphate-buffered saline (PBS) (100µl per virus) or mammalian cell culture medium (without FBS) (250µl per virus) overnight at 4°C.

4. Re-suspend the virus pellet with gentle but thorough pipetting to form the high titre BacMAM virus stock. The MOI can be calculated assuming 100% recovery of virus.

5. Observe the cells under the microscope and remove the medium carefully from the wells without disturbing the cell monolayer and wash with 1-2ml of PBS. Remove PBS and add the BacMAM virus in a volume of 250µl at a MOI of ~150 pfu/cell.

6. Leave the virus to adsorb for 1 hours at room temperature (if using BacMAM virus stock suspended in 100µl PBS) or 5 hours at 37°C in a 5% CO_2 humidified incubator (if using BacMAM virus stock suspended in 250µl mammalian cell culture media).

7. After incubation, remove the inoculum and wash wells with cell culture medium or PBS and replace with 1-2ml of fresh growth medium. Incubate at 37°C in a 5% CO_2 humidified incubator. Time to harvest for BacMAM protein production should be optimised by time course in a process comparable to that suggested for insect cells (see page **54**). It is suggested to use a range of 24-72 hours post-transduction in the first instance.

8. After incubation, harvest the culture media from each well and store in a 1.5ml eppendorf tube. If the target recombinant protein is secreted the medium is kept and processed for analysis. It is important to remove any cells/cell debris from the culture media to prevent false positives when screening for the target recombinant protein. This can be achieved by microcentrifuge at 5000 rpm for 3 minutes at 4°C. To harvest the cells, wash wells with 0.5-1.0ml of PBS and add 150µl trypsin. When cells are observed to detach, re-suspend with the previously collected cell culture medium for that sample. Harvest the culture into a 1.5ml microcentrifuge tube and pellet cells using a microcentrifuge at 5000 rpm for 3 minutes at 4°C. Discard the waste into Virkon.

9. Samples can be kept at -20°C or -80°C prior to analysis.

For detection of target protein it is recommended to perform an SDS PAGE gel or western blot. However, other methods of analysis include fluorescent microscopy or flow cytometry.

transduced control will be absent of BacMAM virus and is useful for observing cell growth.

The virus does not need to be concentrated if the required volume is less than 50µl.

Overnight soaking of the virus pellet followed by thorough resuspension is important to produce a predominantly single particle suspension, rather than 'clumps' of viruses.

The wash step can be omitted for mammalian cell lines that are considered to be semi-adherent, to prevent dislodging cells from the monolayer. Optimal MOI for transduction will vary between cell lines, so should be reviewed on a case by case basis. Commonly used MOIs for BacMAM work are in the 100-500 pfu/cell range, we recommend initially using a series of MOIs within this range to establish optimum conditions for BacMAM expression.

Check the monolayer under the microscope to ensure majority of cells have been harvested.

A fluorescent reporter gene can be used to help measure and observe transduction efficiency of the BacMAM viruses.

9. Trouble Shooting and FAQ

We hope the information below will be able to help answer your questions. However, if not please visit our blog 'Bac to the Future' (https://oetltd.wordpress.com) for more detailed articles on working with the baculovirus expression system. In addition you can contact us directly at info@oetltd.com or calling +44(0)1865 483 236.

Q Why are my cells not growing well?

A The most likely problem with cells occurs when they have been allowed to reach stationary phase before passaging. If this 'stress' happens to a culture 2 or 3 times, then the cells no longer grow properly. Always check cells on a regular basis and do not let cultures overgrow. If this happens, go back to liquid nitrogen stocks and set up a new culture. Far more important than passage number of the cell is the number of times the culture has been stressed!

Cells that are not growing well should never be used to make recombinant viruses, amplify virus, or test for protein production because each of these techniques requires the virus to infect and replicate inside cells and it can only do this is the cells are actively replicating – i.e. in log phase of growth.

Q My cells are not growing and have enlarged nuclei?

A See above, but they also may be contaminated with baculovirus. Start with a fresh cell culture. Never use virus and stock cells in the same class II hood. Always do cell culture work before virus work.

Q My co-transfection has not worked or become contaminated?

A See Q on cells above. If your cells are too dense or not in log phase it will impair virus replication and be unable to produce budded virus. Have you followed the protocol exactly? Try a different transfection reagent. The plasmid DNA used in the co-transfection must be sterile – try precipitating with alcohol and re-suspending in sterile TE. Check your medium is not contaminated. The *flash*BAC™ and BacPAK6 DNA is quality checked to ensure it is sterile.

Q My virus has not amplified to high titre?

A See Q on cells above – high cell density and not being in log phase will be the most likely problem. Did you infect cells with low MOI (0.1 pfu/cell)? High MOI will lead to lower titres and very low MOI will work but you may need to leave the cells longer to achieve high titres. Did the cells look infected (grainy and swollen nuclei under the microscope (Figure 10))? Could the foreign gene product be affecting budded virus production?

Q Why don't I see plaques in my plaque-assay?

A Were the cells in good condition – see Q on cells above. Double check the cell density that was plated – too high and cells cannot undergo enough rounds of replication to form a plaque (they will be like pin pricks and hard to see) – too low and the cells do not close up to form a monolayer so edges of plaques can be ragged and hard to spot. Are there any cells left at all – look under the microscope – if the dishes dried out at any time there will be no cells left and no

plaques. Was the agarose overlay too hot – which may have killed the cells? Did you remember the liquid overlay (with 10% for TC100)? Was the virus titre too low to be detected try again with lower dilutions or even neat virus plated out. Or was titre too high and you need to plate out higher dilutions to see plaques – they may have merged together and be hard to see (did you change tips between dilutions to avoid carry over?). Was the Neutral Red freshly diluted ready for use?

Q My plaque-assay overlay has cracks or the agarose layer fell out when inverting dishes, or the plaques are smeared or only around the edges of the dish, or the cell monolayer has a glassy red/pink appearance and no cells can be seen or only around the edgesof the dish

A If the virus inoculum is not removed before adding the agarose overlay, it interferes with the gelling process and can produce cracks. It may even cause the overlay to fall out when you tip off the stain as they will be a liquid layer under the agarose layer. It may also allow the virus to spread under the overlay and so the plaques appear smeared and diffuse. Always add inoculum to the centre of the dish and rock the dish a few times during the incubation period to ensure even coverage of the virus. Ensure the cells are also evenly distributed over the dish. Do not use a swirling motion at any time as this simply distributes cells and virus to the edges of the dish. Occasionally, multi-well plate wells do not have perfectly flat surfaces – in our experience the worst culprits for this are 6-well plates and so we always use individual 35mm dishes. A central glassy red area with no cells means the cell monolayer was allowed to dry out and the cells have all died.

Q I cannot detect any gene expression?

A Were the cells used for test expression in good condition – see answer above about cells. Did you use a virus with a known titre (by plaque-assay or qPCR) – there may not have been any virus if you didn't. Has the virus been stored for some time before use - did you add serum to maintain the titre? If not re-titrate your virus and try again. Only use qPCR on fresh virus. If you have a control virus, did that work? Very occasionally, the gene is not stable – check that the gene is actually in the virus genome by PCR. Harvest cellular/virus DNA from a 35mm dish and use for PCR analysis. Is the gene properly under control of the polyhedrin gene promoter – is the first ATG the ATG of your gene? If not, you need to address the construct and make a new virus. If you are using tags to detect the gene, check they are in frame. Did you try optimising expression (see above). In particular, sometimes *T. ni* cells yield protein when *Sf*9 do not. Finally, if you have exhausted all avenues, there are a very, very few genes that for unknown reasons do not express. Most have been found to be toxic to the cell. But check all of the above before thinking this!

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OET Kits & Products

All our baculovirus products have been optimised to ensure rapid and straightforward generation of recombinant virus, gene expression, and protein production. We offer everything you might need, from the revolutionary *flash*BAC™ baculovirus expression vectors and baculovirus qPCR titration kit, to transfection reagents and transfer plasmids. In addition we also stock a variety of *Spodotptera frugiperda* insect cell lines and culture media.

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Oxford Expression Technologies Ltd is a name you can trust when it comes to baculovirus services and contract work. Our team of experts have over 30 years' experience with recombinant protein production, having not only worked on it but helped to develop new and improved systems. From concept to completion, all projects are performed using the most up to date methods to ensure the highest quality throughout. OET also understand that every project is important, which is why we offer assistance at every step of the way, with all our services tailored to individual needs and requirements.

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Recombinant Virus Production
Optimising Gene Expression
Protein Purification
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