

*flash***BAC**

one-step baculovirus protein expression

User Guide

January 2008

flashBAC User Guide

Contents	Page
1 Limited use license	5
2 Kit contents	8
3 Essential information	8
4 Ordering information	9
5 Technical assistance and further information	9
6 Safety requirements	10
7 Introduction to the baculovirus expression system and the <i>flashBAC</i> system	11
8 Production of recombinant baculoviruses using the <i>flashBAC</i> system	21
8.1 Production of recombinant baculovirus by co-transfection of insect cells with <i>flashBAC</i> DNA and a transfer vector	21
8.2 Amplification of recombinant <i>flashBAC</i> virus	27
8.3 Plaque-assay to titre recombinant <i>flashBAC</i> virus	32
8.4 Guide to using <i>flashBAC</i> in robotic systems	38
9 Analysis of gene expression: a guide	41
10 A guide to insect cell culture	47
10.1 Maintaining insect cells in suspension or monolayer cultures	48
10.2 Sub-culturing suspension cultures	50
10.3 Sub-culturing monolayer cultures	53
11 Troubleshooting & FAQ	57
12 References	62

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Material	shall mean the Licensor's product known as flashBAC comprising either or both an agreed quantity of DNA and the relevant User Guide;
Purpose	shall mean the use by the Licensee of the Materials for the production of recombinant proteins and/or viruses for Research purposes only;
Research	shall mean the Licensor's systematic search or investigation towards increasing the sum of its knowledge in the production of recombinant proteins and/or viruses;
User Guide	shall mean the instructions provided with flashBAC to enable the Licensee to produce recombinant proteins and/or viruses from the DNA.

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7.2 it shall not alter, produce, manufacture or amplify the DNA; and

7.3 it shall not sell any protein and/or virus created pursuant to this Licence to any third party; and

7.4 it shall not provide any services to any third party using the Materials; and

7.5 if the Licensee desires to use the Materials for any purpose other than the Purpose, it shall notify the Licensor accordingly and procure a suitable licence prior to any such use.

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- 20 The Contracts (Rights of Third Parties) Act 1999 shall have no application to this Licence whatsoever and the parties do not intend hereunder to benefit any third party.

2 Kit Contents

All reagents and materials provided and referred to in this User Guide are for research purposes only:

- 1 *flashBAC* DNA. Store at 4 °C.
- 2 Control transfer vector DNA (containing *lacZ* reporter gene). Store at -20 °C.
- 3 *flashBAC* User Guide.
- 4 Certificate of Analysis.

NOTE: Transfection reagent and insect cells are **NOT** supplied with this kit.

3 Essential Information

The information given in this User Guide is accurate to the best of our knowledge. It is a practical guide to allow researchers to use the *flashBAC* 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 also reminded that they may require other licenses to use the baculovirus expression system and it is the responsibility of the user to ascertain this information.

4 Ordering Information

To order by post:

Oxford Expression Technologies

Oxford Brookes University

Headington Campus

Oxford

OX3 OBP

UK

To order by email: sales@oetltd.com

To order by Fax: +44 (0) 1865 483250

Fax form available at: www.oetltd.com

5 Technical Assistance and Further Information

For additional help or guidance please refer to the Troubleshooting section of this User Guide and/or the frequently asked questions (FAQ) section of our website at <http://www.oetltd.com>. If these resources are unable to help you, please contact us at info@oetltd.com. All technical assistance is provided without charge and 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 information we provide.

6 Safety Requirements

- 1 These research products have not been approved for human or animal diagnostic or therapeutic use.
- 2 Procedures described within this User Guide should only be carried out by qualified persons trained in appropriate laboratory safety procedures.
- 3 Always use good laboratory practice when handling this product.

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

7 Introduction to the Baculovirus Expression System and *flashBAC* Technology

7.1 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 foreign DNA. The AcMNPV genome forms the basis of the flashBAC DNA provided in this kit.

AcMNPV has a bi-phasic life cycle resulting in the production of two virus phenotypes: budded virus (BV) and occlusion-derived virus (ODV). BVs contain single, rod-shaped nucleocapsids enclosed by an envelope (Figure 1) containing a membrane-fusion protein (GP64). GP64 is acquired when the nucleocapsids bud through the host cell plasma membrane⁵. The BV form of the virus is 1000-fold more infectious for cultured insect cells⁶, compared to the ODV phenotype, and is responsible for cell-to-cell transmission in the early stages of infection⁷. It is the BV form of the virus that delivers the foreign gene into the host insect cell.

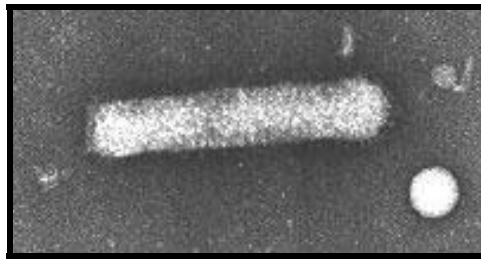


Figure 1. A rod-shaped baculovirus particle.

In the late stages of infection large numbers of occlusion bodies (OB) or polyhedra are formed. These consist of multiple rod shaped nucleocapsids enclosed by an envelope, acquired *de novo* in the nucleus, and embedded within the para-crystalline matrix of the OB/polyhedra. The major component of the OB matrix is polyhedrin^{8,9}, a protein that is produced by the powerful transcriptional activity of the polyhedrin gene (*polh*) promoter¹³. OBs protect the virus and allow them to survive between hosts, within the environment. Most baculovirus expression vectors do not produce polyhedra (see below for details), just the BV form of the virus. This is a useful safety feature because recombinant virus cannot persist in the environment in the absence of polyhedra.

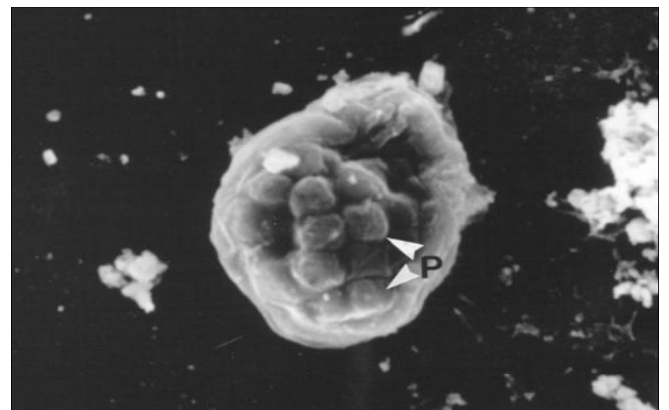
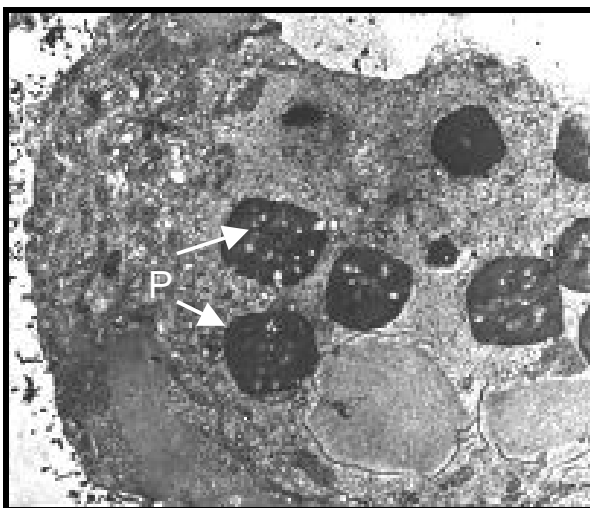


Figure 2. Infected insect cells showing polyhedra (P) within an enlarged nucleus.

7.2 The baculovirus expression system

The baculovirus polyhedrin gene is non-essential for virus replication in insect cells and this has led to the development of the widely-used baculovirus expression vector system, first described by Smith *et al.*³. The coding sequences of the polyhedrin gene are replaced by those of a foreign gene, to produce a recombinant baculovirus in which the powerful polyhedrin promoter drives expression of the foreign gene. Hence recombinant baculoviruses are sometimes referred to as polyhedrin/polyhedra-negative viruses.

Expression of foreign genes in insect cells using recombinant baculoviruses has become one of the most widely used expression systems, and is often the first choice eukaryotic system.

The baculovirus expression system has several advantages over bacterial systems:

- Safe to use.
- Can accommodate large or multiple genes
- Uses a variety of promoters for early and/or late gene expression
- Uses very efficient gene promoters
- Proteins produced are almost always functional
- Proteins are processed: signal peptide cleavage, nuclear targeting, membrane targeting, secretion, phosphorylation, glycosylation, acylation

However, it is not without its disadvantages and these lie mainly in the labour-intensive and technically demanding steps needed to produce recombinant viruses. The following outlines the

development of the baculovirus expression system and the fine-tuning that has been used to improve the system over the years.

Generally, the baculovirus genome is considered too large in which to insert the foreign gene directly. Instead the foreign gene is cloned into a **transfer vector**, which contains sequences that flank the polyhedrin gene in the virus genome. The virus genome and the transfer vector are introduced into the host insect cell and **homologous recombination**, between the flanking sequences common to both DNA molecules, effects insertion of the foreign gene into the virus genome, resulting in a **recombinant virus genome**. The genome then replicates to produce recombinant virus (BV phenotype only, as the polyhedrin gene is no longer functional), which can be harvested from the culture medium.

In most baculovirus expression systems available that use homologous recombination to transfer the foreign gene into the virus genome, a mixture of recombinant and original parental virus is produced after the initial round of replication. Before using the virus as an expression vector, the recombinant virus has to be separated from the parental virus. Traditionally this has been achieved by **plaque-assay or plaque-purification**. This process is labour-intensive, technically demanding and time-consuming.

Many developments have attempted to improve the methods by which recombinant and parental virus may be separated. The frequency of recombination using this system is low (<1%) and recombinant virus plaques can be obscured by parental virus plaques. This problem was partially addressed by the insertion of the *Escherichia coli lacZ* gene into the virus genome, in

addition to the gene of interest. The recombinant virus plaques could then be stained blue by the addition of X-gal (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside) against a background of colourless parent plaques. However, this did not improve the low recombination efficiency and resulted in the contamination of recombinant protein with β -galactosidase.

The efficiency with which recombinant virus could be recovered was improved by the addition of a unique restriction enzyme site (*Bsu36I*) at the polyhedrin locus (AcRP6-SC). Linearization of the virus genome prior to homologous recombination reduced the infectivity of the virus DNA but increased the proportion of recombinant virus recovered to 30%. Homologous recombination between the transfer vector and the linear DNA re-circularised the virus genome, restoring infectivity and the production of virus particles. *LacZ* was then introduced at the polyhedrin gene locus, replacing the polyhedrin coding region, producing AcRP23.*lacZ*. A *Bsu36I* restriction site within *lacZ* allowed for more efficient restriction of the linear DNA prior to homologous recombination and the presence of *lacZ* allowed the selection of colourless recombinant virus plaques against a background of blue parental virus plaques in the presence of X-gal¹¹.

Greater than 90% recovery of recombinant virus plaques was achieved by further modifications to produce BacPAK6¹². BacPAK6 contains the *E. coli lacZ* gene inserted at the polyhedrin gene locus and *Bsu36I* restriction enzyme sites in two flanking genes on either side of *lacZ*. Digestion with *Bsu36I* removes the *lacZ* gene and a fragment of an essential gene (ORF 1629)¹⁰ producing linear virus DNA (BacPAK6) that is unable to replicate within insect cells. Co-transfection of insect cells with BacPAK6 DNA and a transfer vector containing

the gene of interest, under the control of the polyhedrin gene promoter, restores ORF1629 and re-circularises the virus DNA by allelic replacement. The recombinant baculovirus DNA is then able to replicate in insect cells and in the late phase of infection, virions are assembled and recombinant baculoviruses are produced. However, *Bsu36I* digestion is never 100% efficient and the final virus population will always contain a mixture of recombinant and parental virus that requires purification by plaque-assay.

Despite the fine-tuning and optimisation of the system, a number of steps are still required to produce and isolate recombinant virus. **Hence compared to bacterial expression systems, it has not been amenable to high throughput or automated systems.**

7.3 The *flashBAC* system

The *flashBAC* system¹³ is a **new platform technology** for the production of recombinant baculoviruses. Most importantly, *flashBAC* has been specifically designed to remove the need to separate recombinant virus from parental virus by plaque-purification or any other means. The production of recombinant virus has been reduced to a **one-step procedure** in insect cells and is thus **fully amenable to high throughput and automated production systems.**

The ***flashBAC* technology** has been developed by the same team that produced the triple-cut, linear DNA (BacPAK6) system that has been the stalwart of the baculovirus expression system for the past 10 years¹². At the heart of *flashBAC* technology is an AcMNPV genome that lacks part of an essential gene (ORF 1629) and contains a bacterial artificial chromosome (BAC) at the polyhedrin gene locus, replacing the

polyhedrin coding region (see Figure 3). The essential gene deletion prevents virus replication within insect cells but the BAC allows the viral DNA to be maintained and propagated, as a circular genome, within bacterial cells. Circular viral DNA is then isolated from the bacterial cells and purified. This is the *flashBAC* DNA provided in this kit.

A recombinant baculovirus is produced by simply transfecting insect cells with *flashBAC* DNA and a transfer vector containing 'the gene under investigation'. Homologous recombination within the insect cells (1) restores the function of the essential gene allowing the virus DNA to replicate and produce virus particles and (2) simultaneously inserts 'the gene under investigation' under the control of the polyhedrin gene promoter and **removes the BAC sequence**. The recombinant virus genome, with the restored essential gene, replicates to produce BV that can be harvested from the culture medium of the transfected insect cells (and forms a seed stock of recombinant virus). As it is not possible for non-recombinant virus to replicate there is no need for any selection system. **This system is outlined in Figure 3.**

This one-step procedure greatly facilitates the high throughput production of baculovirus expression vectors via automated systems. However, it is also of benefit to the small research group just requiring one or a few recombinant baculoviruses prepared in individual dishes of cells.

The *flashBAC* system is **back compatible** with all baculovirus transfer vectors based on homologous recombination in insect cells at the polyhedrin gene locus. This includes vectors using the polyhedrin promoter, dual, triple and quadruple expression vectors and those that use other gene promoters such as *p10*,

ie1 etc. Examples include pBacPAK8/9, pAcUW31 and pBacPAK-His1/2/3 (BD Biosciences Clontech) but not vectors such as pFastBac™, which are designed for site-specific transposition in *E. coli* using the Bac-to-Bac® system (Gibco-BRL)¹⁴.

The *flashBAC* system also **maximises protein secretion and membrane protein** targeting. Baculovirus genomes contain several auxiliary genes, which are non-essential for replication in insect cell culture. One of these is chitinase (*chiA*), which encodes an enzyme with exo- and endochitinase activity¹⁵. In an infected insect, chitinase (together with cathepsin) facilitates host cuticle breakdown and tissue liquefaction at the very late stages of infection, so releasing the virus to infect more hosts¹⁶. Confocal and electron microscopy observations of insect cells infected with AcMNPV have shown that chitinase is targeted to the endoplasmic reticulum (ER) where it is densely packed in a para-crystalline array, severely compromising the function and efficacy of the secretory pathway^{17,18}. Deletion of *chiA* from *flashBAC* has improved the efficacy of the secretory pathway and resulted in a greatly enhanced (up to 60-fold in some instances) yield of recombinant proteins that are secreted or membrane targeted (in comparison with recombinant viruses that synthesise chitinase)¹⁹.

Advantages of the flashBAC system:

- Simple to use
- One step production of recombinant virus in insect cells
- No steps needed to purify recombinant virus
- Amenable to high throughput and automated systems
- Maximises production of secreted and membrane-targeted proteins
- Back-compatible with a huge range of transfer vectors

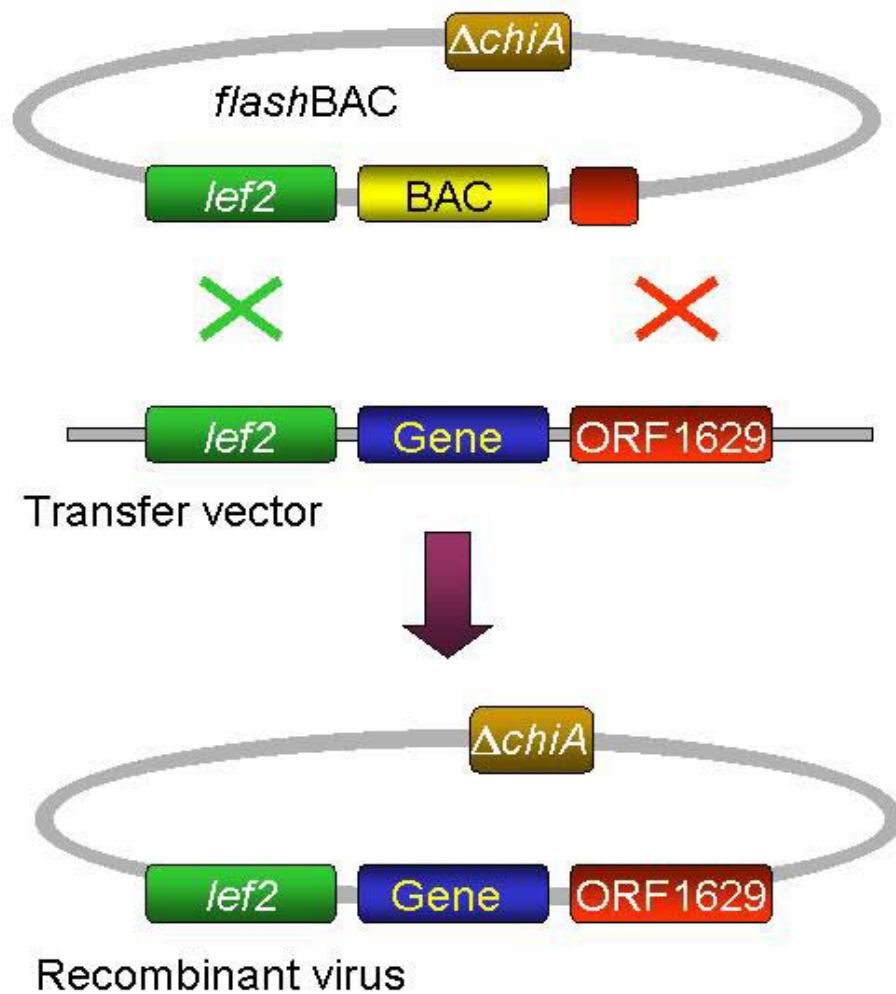


Figure 3. Schematic of the flashBAC system for making recombinant baculoviruses

8 Production of recombinant baculoviruses using the *flashBAC* system

Overview

Protocol 8.1 describes the method for making recombinant viruses in 35 mm dishes and protocol 8.4 gives guidance on adapting this method for use in automated or robotic systems.

The following procedures **must** be carried out using **aseptic technique**, as the DNA/liposome complexes will be introduced into insect cells maintained in antibiotic-free medium.

8.1 Production of recombinant baculovirus by co-transfection of insect cells with *flashBAC* DNA and a transfer vector (containing the gene under investigation)

Provided:

- *flashBAC* DNA
(use 100 ng [5 µl] per co-transfection [20 ng/µl]).
- Positive-control transfer vector DNA
(use 500 ng [5 µl] per co-transfection [100 ng/µl]).

Required:

- 35 mm tissue culture treated dishes seeded with insect cells in a sub-confluent monolayer (Sf9 or Sf21) [see section 10 for information about insect cells].
- Serum-free insect cell culture medium.
If using serum supplemented medium, you will need medium with and without 10% foetal bovine serum.

- Sterile baculovirus transfer vector DNA containing ‘the gene under investigation’ (500 ng per co-transfection)
Any vector designed for double crossover, homologous recombination with baculovirus DNA at the polyhedrin locus is suitable [see website for more details]. The DNA must be sterile and must be of a quality suitable for transfection into cells.
- Transfection reagent. *Reagents tested and found to be successful are Lipofectin[®] (Invitrogen), FuGENE 6 (Roche), GeneJuice[®] (Novagen), Tfx-20[™] (Promega) and CELLFECTIN[®] (Invitrogen).*
- Incubator set at 28°C.
- 1% Virkon (Amtec), or other suitable disinfectant.
- Inverted phase-contrast microscope.
- Plastic box to house dishes in the incubator.
- Sterile pipettes, bijoux or similar.
Note that plastic ware used to prepare the transfection mixture must be made from polystyrene and not from polypropylene.

Procedure:

- 1 **For each co-transfection**, to make a recombinant virus, you will require **one 35mm dish of insect cells** (Sf9 or Sf21). It is also advisable to set up one dish as a mock-transfected control. If required, a further dish of cells can be set up to make a recombinant virus using the control transfer vector DNA provided in the kit.
 - *Seed the dishes with insect cells at least 1 hour before use. It is extremely important to use*

healthy cells from a log-phase culture [see section 10] and to seed the cells at the correct cell density so the resulting monolayer is even and sub-confluent. Use 1.5×10^6 Sf21 or 1×10^6 Sf9 cells/dish in a 2 ml volume of medium.

- *Ensure that the cells are evenly distributed over the surface of the dish and leave to settle at room temperature for 1 hour on a flat surface.*
- 2 During the 1 hour incubation period above, prepare the co-transfection mix of DNA and liposome reagent. **For each co-transfection, pipette 1 ml serum-free, antibiotic-free medium into a sterile, disposable polystyrene container** (7 ml bijoux are convenient).
 - 3 **Add an appropriate volume of transfection reagent as directed by the manufacturer and mix.**
 - *As a guide, use 5 μ l Lipofectin[®] (or alternative transfection reagent.)*
 - 4 **Next, add 100 ng flashBAC DNA (5 μ l from the kit) and 500 ng transfer vector DNA** (one with the gene under investigation or the control provided in the kit [in which case use 5 μ l]). Mix with gentle agitation or vortexing.
 - *In the mock-transfection control, omit the DNA from the medium.*
 - 5 Incubate at room temperature for 15 - 30 minutes to allow the liposome-DNA complexes to form.

- 6 Just before the end of the incubation period in (5), remove the culture medium from the 35 mm dishes of cells using a sterile pipette; ensuring that the cell monolayer is not disrupted.
- ***If using cells maintained in serum-supplemented medium, wash the monolayer twice with serum-free medium before carrying out the co-transfection. This is to remove any residual serum, which inhibits liposome-mediated transfection of DNA into cells. Carefully add 1 ml serum-free medium then remove and discard medium. Repeat once more.***
 - *This washing step is not necessary when using cells maintained in serum-free medium.*
 - *When removing liquid from a dish of cells, tip the dish at a 30-60° angle so the liquid pools towards one side the dish.*
 - ***It is IMPORTANT not to allow the cell monolayer to dry out at this point.***
- 7 As soon as the medium has been removed from the cells, add the 1 ml of DNA + liposome complex dropwise and gently to the centre of each dish. Incubate in a plastic sandwich box at 28°C for a minimum of 5 hours or overnight.
- *Adding the mixture dropwise should not disturb the cell monolayer if it is done slowly and gently.*
- 8 After the incubation period add a further 1 ml of the appropriate insect cell culture medium to each dish. **Continue the incubation for 5 days in total.**

- *If the cells are normally maintained in serum-supplemented medium, at this step add 1 ml of medium containing 10% serum.*
- 9 Following the 5 day incubation period, harvest the medium containing the recombinant virus into a sterile bijou, and store in the dark at 4°C until required. **This is your seed stock of recombinant baculovirus.**
- 10 **As you have only limited stocks of this virus, the next step is to amplify the virus as described in the next set of protocols (8.2).**

Notes:

- *Cell monolayers in which recombinant virus has been produced will appear very different from mock-transfected control cells under the inverted microscope. Control cells will have formed a confluent monolayer whilst virus-infected cells will not have formed a confluent monolayer and will appear grainy with enlarged nuclei.*
- *If the instructions above have been followed and the insect cells are in good condition, the titre of recombinant virus produced after the co-transfection will normally be high (extensive testing indicates an average titre of about 1×10^7 pfu/ml at 5 days).*
- *The cells remaining from the co-transfection may be used to test for foreign gene expression by Western blot analysis, for example.*
In our experience, the titre of recombinant virus produced during the co-transfection is not adversely

affected by using semi-pure transfer plasmid DNA (e.g. resin-based mini-prep DNA protocols), however, the levels of foreign gene expression in these initial infected cells is far higher if good quality transfer vector DNA is used. Subsequent levels of expression, when using the recombinant virus to infect fresh cultures of cells, is of course not affected by the quality of transfer vector DNA

- *To test for expression of lacZ in a co-transfection using the positive control transfer vector provided, simply add 2 ml fresh medium containing 15 μ l 2% v/v X-gal to the cell monolayer, after you have harvested the recombinant virus. After a short time the cell monolayer will turn blue.*

8.2 Amplification of recombinant *flashBAC* virus

The recombinant virus produced in 8.1 must be amplified for experimental work. We strongly recommend amplifying virus in cells grown in suspension culture, and the following gives a protocol for amplifying 100-200 ml virus using the seed stock of virus harvested from the co-transfection as inoculum. Virus may also be amplified in monolayer cultures and some guidance on this is given in the notes at the end of this protocol.

The following procedures **must** be carried out using **aseptic technique**.

Required:

- Seed stock of recombinant virus prepared in Protocol 8.1.
- 100-200 ml culture of log-phase insect cells (Sf21 or Sf9) in appropriate medium [*see section 10 for more details*].
- Shake culture flask (e.g., 1L sterile glass flask or disposable Erlenmeyer flask) or spinner flask (e.g. 1L Bellco Glass spinner flask) [*see section 10 for more details*].
- Incubator set at 28°C.
- Inverted phase-contrast microscope.
- Sterile pipettes.
- Disinfectant for discard.

Procedure:

- 1 Prepare a 100-200 ml culture of Sf9 or Sf21 cells at an appropriate cell density (in log growth phase). See Figure 4.
 - *The cell density will vary with the cell type and the method of culture. As a guide, use Sf9/Sf21 cells in shake culture, in serum-free medium, at 2×10^6 cells/ml or Sf21 cells, in serum-supplemented medium, in spinner culture at 0.5×10^6 cells/ml. See section 10 for more information.*
 - *It is important that the cells are healthy and in log phase of growth to ensure that virus replication occurs efficiently to amplify high titre stocks of virus for subsequent use in expression studies. **You should check the cell density and cell viability of your culture before using it to amplify virus (see section 10 for details).***
 - *It is also vitally important that the cells are infected at a very low multiplicity of infection (moi) (< 1 pfu/cell). This means that initially few cells are infected; the virus replicates to release BV, which then infects more cells and so on. In this way multiple rounds of replication occur and high virus titres are obtained; it also reduces the chances of any defective virus particles occurring. If the cells are infected at high moi (>1 pfu/cell), all the cells will be infected initially and only one round of replication will occur, giving a poor virus amplification and low titre virus stock.*

2 Using aseptic technique, **add 0.5 ml (no more) of the recombinant virus seed stock** (from 8.1) to the cell culture and incubate with shaking or stirring (as appropriate) until the cells are well infected (normally 4-5 days).

- *Virus-infected cells become uniformly rounded and enlarged, with distinct enlarged nuclei. They appear grainy when compared with healthy cells under the phase-contrast inverted microscope.*
- *Virus-infected cells have an increased need for oxygen and therefore the contents of the shake flasks should be shaken at quite high speeds to maximize aeration. The surface area to volume ratio should also be as large as possible for maximum gas exchange – do not overfill flasks!*

3 When the cells appear well infected with virus, harvest the culture medium by centrifugation at 3000 rpm, at 4°C for 15 minutes. Decant aseptically and store the recombinant virus in the dark at 4°C.

- *The virus inoculum may be stored for 6-12 months or longer in the dark at 4°C. The titre of the virus will start to fall after a time and after storage for more than 3-4 months it is recommended to titre the virus before using it – it may require re-amplification. The titre seems to drop more quickly in serum-free medium and the addition of serum to 2-5% may help to prevent this.*
- *Virus may also be frozen at –80°C for a longer period of time. If frozen, avoid multiple freeze and thaw*

cycles. Upon freezing, the viral titre may decrease and should be re-amplified when thawed. Do not store virus at -20°C or in liquid nitrogen.

- 9 Before using the virus in experiments, it is strongly recommended that it is titrated by plaque-assay to determine an accurate titre (see Protocol 8.3).**



Figure 4. A shake culture infected with recombinant baculovirus

- *It is important that the titre of the recombinant virus is known so that, in expression studies, cells can be infected with a known, high moi. This will ensure that all the cells are infected simultaneously to produce a synchronous culture for accurate optimisation studies.*
- *It also maximises the chance of detecting the expressed protein – especially where the levels of expression are at the lower end of the scale! It also minimises the chances of degradation being a problem*
- *Sometimes, for unknown reasons, virus amplifications do not work (although the reason is normally that the cells were not healthy or not in log phase, or the cells*
- *were infected at too high an moi). If this happens and*

the virus titre has not been checked, there may be disappointment when gene expression is very low or undetectable.

- The most common cause of failure to detect foreign gene expression is using a stock of virus in which the titre is assumed to be high but, when titrated by plaque-assay, turns out to be very low!*
- For most purposes a titre of 5×10^7 pfu/ml or higher is adequate. A titre of less than 10^7 pfu/ml will not normally be sufficient for expression studies.*
- Virus may also be amplified in monolayer cultures, with the cells seeded in T75 or T150 flasks to form sub-confluent monolayers. The medium is removed and the cells infected with virus (use 100 –200 μ l of the seed stock virus from the co-transfection diluted to 0.5 – 1.0 ml with medium). Allow the virus to adsorb for 1 hour and then remove. Replace with fresh medium (10-15 ml for a T75 and 30 ml for a T150). Allow the virus to replicate until the cells are well infected – 3-5 days. Harvest the medium as the source of amplified virus inoculum and store/titrate as described above. The titre of virus amplified in this way is not usually as high as virus amplified in shake culture.*

8.3 Plaque assay to titre recombinant *flashBAC*

The following procedures **must** be carried out using **aseptic technique**.

Required:

- 35 mm tissue culture treated dishes (8 dishes per virus to be titrated).
- Insect cells (Sf9 or Sf21 cells).
The use of Sf21 cells in serum-supplemented medium is strongly recommended for plaque-assays, as they produce distinct, large plaques in 3 days, compared to smaller less distinct plaques in 4 days for Sf9 or Sf21 cells grown in serum-free medium.
- Virus to be titrated (from 8.2).
- Appropriate culture medium for the cells being used (see section 10).
Serum-free or serum-supplemented medium can be used.
- Low Gelling Temperature Agarose for cell culture (use 2% w/v in sterile dH₂O, sterilized by autoclaving).
Small aliquots of 10 ml are convenient and can be prepared in advance and stored solidified at room temperature. Melt in a microwave oven just prior to use.
- Antibiotics (optional) (penicillin and streptomycin prepared with 5 units/ml⁻¹ penicillin G sodium and 5 µl/ml⁻¹ streptomycin sulphate in 0.85% saline; 1:100 final dilution).
Antibiotic use is optional but if used should be added to all medium.
- Incubator at 28°C and a plastic sandwich box.

- Phosphate-Buffered Saline (PBS, sterilized by autoclaving), pH 6.2.
- Neutral Red (e.g. from Sigma).
Prepare a stock solution at 5 mg/ml in water, filter through a 0.2 µm filter and store at room temperature. For use, dilute 1:20 in PBS (do not store after dilution).
- Optional: 2% (w/v) X-gal in dimethylformamide (DMF) (only required if titrating a recombinant virus expressing *lacZ*).
- Sterile pipettes, tips, bijoux to make serial dilutions.
- Discard for virus waste e.g., 1% Virkon (Amtec) or other suitable disinfectant.
- Inverted phase-contrast microscope.

Procedure:

- 1 To assay the titre of a recombinant virus, prepare **ten** 35 mm dishes with Sf21 (recommended) or Sf9 cells. Seed the dishes with an appropriate number of insect cells to form a sub-confluent monolayer (normally 1.4×10^6 Sf21 cells or 0.9×10^6 Sf9 cells/dish). Leave the dishes for 1 hour, on a level surface, at room temperature for the cells to recover.
 - *Sf21 cells are preferred for plaque-assays as they give more distinct, larger plaques in a shorter period of time.*
 - *The cells must be healthy and taken from a log-phase culture (see section 10 for more details).*
- 2 During this incubation period prepare serial log (1 in 10) dilutions of the virus to be titrated, from 10^{-1} to 10^{-7} . It is

convenient to prepare 0.5 ml dilutions by placing 0.45 ml appropriate medium into each of 7 sterile bijoux (or micro centrifuge tubes).

Add 50 μ l of undiluted recombinant virus from Protocol 8.2 to the first bijoux (this will be 10^{-1}) and mix thoroughly by vortex or inversion. Using a fresh pipette tip, remove 50 μ l from these bijoux and transfer it to the next one (this will be 10^{-2}) and vortex/mix. Continue diluting the virus in this way to 10^{-7} . You will also need 0.5 ml medium for the control dishes.

- 3 About 1 hour after seeding the dishes (in 1), check that the cells have formed an even, sub-confluent monolayer. When ready to add the virus dilutions to the cells, remove the culture medium and discard into 1% Virkon or other disinfectant.

- *Ensure that the cell monolayer is not disrupted during this process and **that it is does not dry out.***
- *It is best to leave a small amount of medium to just cover the cells – if the monolayer dries out this will give rise to a large shiny pink area, devoid of live cells, after staining the plaque assay.*

- 4 Add **100 μ l amounts** from each of the dilutions from **10^{-4} to 10^{-7}** to duplicate dishes (**8 dishes in total, 4 dilutions to be plated**). Add the diluted virus dropwise to the centre of each dish, using a fresh sterile pipette for each, and label accordingly. **Also include 2 dishes as controls** where 100 μ l of the appropriate insect cell culture medium is added to each dish, in place of a virus dilution.

- 5 Incubate the dishes at room temperature for 1 hour on a level surface for virus adsorption. Do not leave the dishes for longer than 1 hour (40 minutes is the minimum).
- *It is important to ensure that the cell monolayer does not dry out at this stage. If working within a Class II microbiological cabinet, remove the dishes to the bench at this stage to prevent the cells drying out.*
- 6 About 15 minutes prior to the end of the virus adsorption period, prepare the LGT agarose overlay. Completely melt 1 x 10 ml aliquot of ready-prepared and solidified 2% (w/v) LGT agarose (in a microwave oven or boiling water bath, taking appropriate safety precautions) and, after cooling to about 50°C (hand-hot), add an equal volume (10 ml) of appropriate insect cell culture medium. Mix thoroughly but gently, avoiding air bubbles.
- *You need 2 ml of this agarose/medium overlay for each dish.*
 - *Use immediately or keep warm at 45°C to prevent solidification. If using a water bath, ensure it is clean and wipe the bottle of overlay with alcohol before use to prevent fungal/bacterial contamination of the plaque-assay.*
 - *If using antibiotics, add to the culture medium before preparing the overlay.*
 - *Should the agarose set before using, do not re-melt it; prepare a fresh batch.*
- 7 After the virus adsorption period and after preparing the agarose overlay carefully remove the virus inoculum

from each of the 35 mm dishes, by tipping the dish to one side and using a sterile Pasteur pipette. Discard into 1% Virkon or other disinfectant.

- *Take care not to disturb the cell monolayer or allow it to dry out during this process.*
- 8 Gently pipette 2 ml of the agarose-overlay down the side of each dish, allowing it to roll over the cells, so as not to disturb the monolayer. Incubate at room temperature for 15 minutes or until the agarose is solid.
 - *The time taken for the agarose to solidify depends on the temperature of the room.*
 - 9 When the agarose overlay has set, add 1 ml of appropriate insect cell culture medium to each dish, as a liquid feed overlay. Antibiotics may be added to the medium if required.
 - 10 Place the dishes into a secure container (e.g., a sandwich box) and incubate at 28°C for 3 days (Sf21 cells) or 3-4 days (Sf9 cells), by which time the cell monolayer should be confluent (with no gaps between cells).
 - 11 Once the cells have reached confluence, the dishes can be stained with Neutral Red in order to visualise the plaques.
 - *Plaques are clear areas against a red background as only live cells take up the stain.*

Remove the liquid overlay from the dishes and replace with 1 ml diluted Neutral Red stain (see list at the start). Incubate for 3-4 hours at 28°C.

Tip off the stain (into disinfectant) and invert dishes (place on tissue paper which can then be discarded by autoclaving). Replace lids.

Leave the dishes in the dark, in the inverted position, for the plaques to clear. This may take a few hours or may occur very rapidly, depending on the strength of the Neutral Red.

- *LacZ-positive virus plaques (e.g. virus produced using the control transfer vector) can be stained using X-gal rather than Neutral Red. Add 1 ml of appropriate insect cell culture medium containing 15 µl (2%w/v) X-gal (in DMF) and incubate for at least 5 hours (may need overnight) at 28°C. Plaques will appear blue in colour.*

- 12 After staining, select one set of duplicate dishes with between 10 and 30 plaques (ideally) and count them. Calculate the average number of plaques for that dilution. To determine the virus titre use the following calculation:

Titre of virus (pfu/ml) = average plaque count x dilution factor* x 10**

**multiply by the inverse of the dilution used to count the plaques*

*** multiply by 10 because only 0.1 ml was applied to each*

Example: 25 plaques (average) on the 10⁻⁶ dilution

plates give a titre of:

$$25 \times 10^6 \times 10 = 25 \times 10^7 = 2.5 \times 10^8 \text{ pfu/ml}$$

8.4 A guide to using *flashBAC* in robotic systems

Preparing recombinant baculoviruses using the *flashBAC* system involves simply transfecting cells with a transfer vector, containing the gene to be expressed, and *flashBAC* DNA. The culture medium harvested after the co-transfection constitutes a seed stock of the recombinant virus. Because of this, it is possible to prepare recombinant viruses using simple robotic or automated systems.

As each robotic system will be different, the following is not a detailed protocol but guidance on adapting the protocol in 8.1, for use in 35 mm dishes, for use in a 24 well plate format. In this way up to 24 recombinant viruses can be made simultaneously.

The robotic system must be capable of pipetting under sterile conditions. Smaller robots may be placed in a Class II hood.

Preparing the cell monolayers

Prepare a master mix of Sf9 cells in serum-free medium at a cell density of 5×10^5 cell/ml. Use the robot to aliquot 400 μ l (2×10^5 cells/well) into each well of a 24 well plate (for tissue culture). Allow the cells to settle and attach for 1 hour before use.

Preparing the co-transfection mix of transfer vector DNA and *flashBAC* DNA

The robotic system can be programmed to prepare the 24 co-transfection mixes using standard liquid handling protocols.

The co-transfection mixes can conveniently be prepared in the wells of a 96-well plate (made from polystyrene and with U- or V-shaped well – flat-bottomed plates do not work well).

The robot needs to be programmed to add the following to each of 24 wells of a 96 well plate. Add in the following order, the exact volumes will depend on the actual reagents being used, however, the final volume needs to be 20 μ l:

- 1 serum-free medium (8 μ l)
- 2 transfection reagent (for example, 2 μ l Lipofectin™)
- 3 *flashBAC* DNA (5 μ l; 100 ng)
- 4 transfer vector DNA (5 μ l; 500 ng)

The robot should be programmed to mix the reagents by pipetting up and down 3 times.

Adding the co-transfection mix to the cell monolayers

After the cell monolayers have settled, programme the robot to add the 20 μ l co-transfection mix to the appropriate wells of the 24 well plate. There is no need to change the medium, simply add the co-transfection mix to the medium and mix by pipetting up and down 3 times.

Replace the lid and cover with parafilm to prevent evaporation. Incubate at 28°C for 5 days.

Harvest the culture medium from each well and store in individual sterile containers at 4°C in the dark. This is the seed stock of recombinant virus. Use to amplify further stocks of virus using the protocols in section 8.2. As a guide use 250 μ l seed stock virus to infect 100 ml Sf9 cells.

9 Analysis of gene expression: a guide

This section gives guidance on analysing gene expression from the recombinant virus made using the protocols in the previous sections. It is not comprehensive nor a list of protocols as each laboratory will have its own methods for analysing proteins.

A quick check for gene expression

After the co-transfection and harvesting the seed stock of virus for further amplification, it is possible to harvest the remaining cells from the dish and prepare these for SDS-PAGE/Western blotting. This will give a quick check for gene expression although in our experience the levels of expression in these cells is very variable depending on the quality of the transfer vector DNA used. This suggests that at least some of the expression may be transient from the transfer vector.

Infecting cells to amplify further stocks of recombinant virus

With the exception of the quick analysis of cells used in the transfection, as described above, most analyses of gene expression will require infecting fresh cells with the recombinant virus. As only 2 ml of recombinant virus are produced initially, it is important to amplify a stock of high titre virus for experimental work as soon as possible. This can be done at various levels. Protocol 8.2 gives a method for infecting 100-200 ml cells grown in shake or spinner culture. This is a very simple and cost-effective way of producing reasonable stocks of virus to work with.

If you do not have access to shake or spinner cultures, further stocks of virus can be amplified in monolayer cultures in T75 or T125 flasks. Simply seed the cells so an even, sub-confluent

monolayer is formed, remove the medium, add 0.1 –0.2 ml seed stock virus (diluted to 1-2 ml with medium) and allow the virus to adsorb for an hour (rock the cells periodically to ensure the medium covers the cells). After the adsorption period, add 10 ml (T75) or 30 ml (T125) medium and allow the virus to replicate until all the cells are well infected (3-5 days). Remove the medium and store in the dark at 4°C. If setting up multiple flasks, pool the medium before titrating the virus. Titre the virus stock using the protocols in section 8.3. Higher virus titres will almost always be produced in shake or spinner culture so it is well worth setting one of the options up in the lab. Shake cultures can be set up very cheaply using similar equipment designed for bacterial cultures.

The key to obtaining high virus titres is to use healthy cells in log phase of culture and to infect cells at a low multiplicity of infection (less than 0.1 pfu/cell). It is also important to know the titre of the recombinant virus stock before setting out on too much experimental work to examine gene expression. If the virus has not amplified for some reason, you need to know this!

Infecting cells to monitor gene expression

Gene expression can be monitored very simply by infecting 35 mm dishes of cells with the recombinant virus at a **high multiplicity of infection** (5-10 pfu/cell) to ensure a synchronous infection of every cell. Seed the dishes with cells to form an even, just sub-confluent monolayer. After allowing the cells to attach, remove the medium and add the appropriate amount of virus in a volume of 200 µl medium. If you have not yet titrated your virus and do not know the titre, use 200 µl of undiluted virus. Leave the virus to adsorb at room temperature

for 1 hour, remove the inoculum and replace with 2 ml fresh medium.

You should also prepare **non-infected or mock-infected** cells as a control for host cell proteins and, if possible, a **control recombinant virus infection** (use the *lacZ* transfer vector in the kit to make a control recombinant virus expressing β -galactosidase) to examine virus proteins.

Harvest the cells and/or culture medium for analysis of proteins by SDS-PAGE/Western (etc.) at 48 hours post-infection (hpi). This is a good single time point to use, as the polyhedrin promoter is maximal at this time.

If you do not detect expression from your gene:

Were the cells in good condition and in log phase of growth when used?

Have you titrated the virus? If not, this is important as the virus may not have amplified for some reason and the cells in the test for expression may not have been infected with sufficient virus to enable detection of recombinant protein.

Has the virus been stored for some time before use? If so, check the titre.

Does the control recombinant *lacZ* virus give good levels of β -galactosidase? If not, you may need to revise your cell culture and cell infection protocols.

Is the coding region of the gene downstream of the polyhedrin gene promoter inserted in such a way that the gene's AUG start codon is the first AUG after the promoter sequences?

If you have added tags or other sequences, are they in frame?

Has the gene transferred from the transfer vector to the baculovirus genome? Check this by extracting DNA from virus-infected cells and analysing by PCR. It is very, very rare that this is a problem.

Optimising gene expression

Carrying out the simple test of expression described above may not produce optimal expression levels for your gene. Gene expression may be optimised by considering the following:

Cell line

Although viruses must be made and amplified using Sf21/Sf9 cells, other cell lines may produce better yields of protein. A commonly quoted cell line that often produces good yields of protein is the *T. ni* Hi5™ cell line available from Invitrogen or ECACC, for example.

Time to harvest

If cells are infected at a high moi, a synchronous infection results and the best time to harvest the recombinant protein can be examined by taking samples at different times after infection. The most commonly used times are at 24, 48, 72 and 96 hpi. Some proteins may be very stable and accumulate to high levels by 96 hpi, others may start to degrade and thus need to be harvested much earlier.

Setting up a time course experiment can either be done in 35 mm dishes (one dish per time point to be harvested) or by setting up a shake or spinner culture and harvesting a sample (typically 2 ml) at the required time points. Do not forget to include non-infected and control recombinant virus-infected samples.

Multiplicity of infection

In order to achieve synchronous infection of cells, a high moi is needed. This is normally 5-10 pfu/cell. However, it is well worth optimising the best moi to use for your particular virus, especially if you are considering scaling up protein production and the costs of producing large quantities of virus inoculum are considerations. There are also some instances where optimal protein synthesis is obtained by infecting cells with a low moi (1 pfu/cell) and harvesting at a later time point (96 hpi or later).

To optimise the moi, set up dishes of cells or shake cultures and infect with different moi (2, 5 and 10 moi are recommended as a guide). Harvest samples at different times after infection (24, 48, 72 and 96 hpi) and examine protein synthesis.

You may achieve good levels of protein synthesis at 48 hpi with a very high moi (10 moi) but if you wait until 72 hpi, you may achieve the same levels with only 2 or 5 moi; thus having to use less virus inoculum!

Scaling-up protein production

Whilst fermenters and bio-reactors are now routinely used to produce recombinant proteins in insect cells, quite reasonable yields can be achieved in shake cultures at a fraction of the cost, if this a consideration. Large disposable shake flasks can be obtained that will take up to 1.5 L insect cell culture on a shaking platform in a warm room or incubator.

10 A Guide to Insect Cell Culture

It is extremely important that the insect cells used for the production of recombinant viruses are of the highest quality. This can be achieved by sub-culturing cells before they become overgrown (too far into stationary phase) and by using cells which have been sub-cultured no more than thirty or so times. The insect cells most commonly used for the baculovirus expression system are Sf21 cells, originally derived from the pupal ovarian cells of *Spodoptera frugiperda* (fall army worm)¹⁸, Sf9 cells, which are a clonal isolate of Sf21²⁰ and *T. ni* cells, originally derived from the ovarian cells of *Trichoplusia ni* (cabbage looper)²¹. Generally, Sf21 or Sf9 cells are used for co-transfections, virus amplification and plaque assays. *T. ni* cells are often used to achieve maximal protein production.

***T. ni* cells should not be used to produce or amplify virus because of the increased possibility of generating virus mutants²⁰.**

Most insect cell culture medium utilizes a phosphate buffering system, rather than the carbonate-based buffers which are commonly used for mammalian cells. This means that CO₂ incubators are not required. Serum is required for the maintenance of certain cell lines, but many have now been adapted to serum-free conditions. There are large numbers of insect cell culture media available and it is beyond the scope of this booklet to list them. The only point to remember is that most transfection reagents work in serum-free medium, so if using serum-supplemented medium, you must carry out the transfection and wash the cell monolayer in medium without added serum (see section 8.1).

Insect cells have a relatively high dissolved oxygen content (DOC) requirement, particularly when infected with virus. Maintaining the appropriate DOC is important for cell growth and virus replication, and this can be achieved in shake, spinner and tissue culture flasks by using vented caps and not over-tightening lids. Most insect cells can be cultivated over a temperature range of 25-30°C. The optimal temperature for cell growth and infection for Sf21 and Sf9 cells is considered to be 27-28°C.

We recommend carrying out any cell culture work prior to handling virus and only handling one cell line at a time. Always use a different bottle of cell culture medium for each cell line. The addition of antibiotics is optional (penicillin and streptomycin prepared with 5 units/ml⁻¹ penicillin G sodium and 5 µl/ml⁻¹ streptomycin sulphate in 0.85% saline can be used) but generally it is not recommended for virus amplification or protein production. Certainly it is best to maintain stock cultures without antibiotics; otherwise you may be maintaining a low-level contaminant that may cause inefficient virus replication or protein production.

The following is a general guide to preparing insect cells for the protocols listed in section 8.

10.1 Maintaining insect cells in suspension or monolayer cultures

Insect cell lines can be maintained as either suspension cultures, in shake or stirred flasks, or in monolayer cultures in T flasks or dishes. Generally, insect cells adapted to serum-free medium are cultivated in shake cultures with the aid of an

orbital shaker platform, whilst cells adapted to serum-supplemented media are cultivated in stirrer flasks or monolayer cultures (as growing these cells in shake culture generates excessive foaming and subsequent cell damage). Shake flasks may be glass or disposable and stirrer flasks are glass and contain either a magnetic stirring bar or suspended magnetic stirring rod. Both types are available from a range of suppliers.

To maintain optimum cell culture conditions in a shake or stirrer flask, cell densities should be kept within certain ranges, i.e. within the log-phase of growth (see Table 1). This is achieved by counting the number of cells, using either a Neubauer counting chamber or an automatic cell counter.

Sub-culturing (or passaging) of cells allows them to be maintained within log-phase, preventing them from entering their stationary phase. Sub-culturing of shaker cultures or stirrer cultures requires the seeding density of each cell culture to be determined before sub-culturing of cells can commence. These cell lines can be sub-cultured continuously for approximately 30 passages before returning to stocks stored in liquid nitrogen.

Table 1. Insect cell densities in suspension culture

Cell line & Medium	Seeding cell density (cells/ml⁻¹)	Maximum cell density (cells/ml⁻¹) before passaging
Sf9 in serum-free medium	0.3 - 0.5 x 10 ⁶	6.0 x 10 ⁶
Sf21 in serum-containing medium	0.1- 0.2 x 10 ⁶	2.0 - 2.5 x 10 ⁶

10.2 Sub-culturing cells in suspension cultures

Use aseptic technique throughout and work in a Laminar Flow Cabinet or Class II Hood.

Counting cells and determining cell viability

Before passaging cells, take a sample (about 1 ml into a 35 mm dish) to observe under the phase-contrast microscope using x10 and x40 objectives. Healthy cells should look bright, round and refractile. Many cells should also be in the process of dividing into daughter cells.

Using a second sample, count the cells using an electronic cell counter or using a Neubauer counting chamber (Figure 5), as follows. Using a Pasteur pipette or capillary tube, load a sample into the counting chamber using capillary action only, i.e. avoid damaging the cells by forcing them through the pipette. Count all the cells within the 5 x 5 square grid on the

counting chamber (Figure 5), using the phase-contrast microscope (x10 objective). Count cells touching the triple line on the top and left of the squares. Do not count cells touching the triple lines on the bottom or right side of the squares.

The 5 x 5 square gives the number of cells present in 0.1 μl of culture. Repeat the count to give a more statistically correct estimate of cell density. To calculate the number of cells in 1 ml of culture, multiply the average number of cells from the 5 x 5 square by 10^4 . If the cells were diluted before counting, remember to also multiply by the dilution factor.

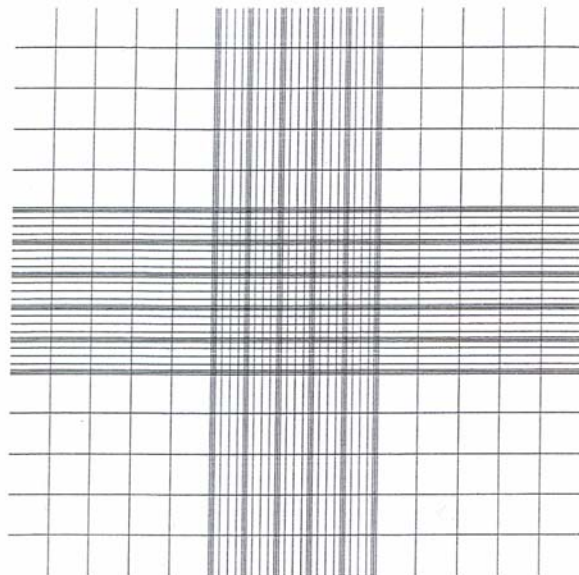


Figure 5. Illustration of the Neubauer 5 x 5 counting grid as seen under a phase contrast microscope using the x10 objective.

For example, cells are diluted 1:5, three 5 x 5 squares (three grids in total) are counted and cell numbers of 49, 52 and 54 are obtained. Calculate the mean number of cells counted = 51.66 cells. Multiply this number by 10^4 and then by the dilution

factor, in this case 5, to give the total number of cells per ml. In this case 2,583,000 cells/ml (2.583×10^6 cells/ml).

Trypan blue staining can also be used to determine the percentage viability of the cells in culture. Trypan blue is the most commonly used vital stain to distinguish viable cells from non-viable cells; only non-viable cells adsorb the dye and appear blue. Conversely, live and healthy cells appear bright, round and refractile and exclude the blue-coloured dye. Prepare dilutions just prior to counting as viable cells adsorb Trypan blue over time, and this can affect counting and viability results. Healthy, log-phase cultures should contain more than 90% unstained, viable cells. Into a bijou mix equal volumes of cell suspension and Trypan blue (2% w/v) (1 in 2 dilution of cells). Load the cells into a counting chamber and count the cells as described above.

To calculate the percentage viability of a culture:

$$\% \text{ dead cells} = \frac{\text{total blue cells counted} \times 2^*}{\text{total cells counted} \times 2^*} \times 100$$

$$\% \text{ viable cells} = 100 - \% \text{ dead cells}$$

* to take into account 1 in 2 dilution of cells when staining with Trypan blue.

Passaging suspension cultures of cells

To passage the cells, calculate how much of the existing culture needs to be transferred to the new flask, and how much fresh medium needs to be added to obtain the required cell density and volume of the new culture. Each time this cell stock is transferred in this way, the passage number of the culture increases by 1.

The following information should be recorded on the flask that the cell culture is maintained in:

- Cell Line
- Passage Number
- Date of each passage
- Density the culture has been split to
- Medium used

In a lab book record the following:

- Liquid nitrogen batch
- Date and passage when cells were raised from liquid nitrogen

Discard any unused cells into sodium dodecyl sulphate (5% w/v SDS) or an autoclavable discard container.

10.3 Sub-culturing cells in monolayer culture

Aseptic technique should be used throughout and culturing should be carried out in a Laminar Flow Cabinet or Class II Hood.

The maintenance of cells in monolayer culture does not normally require counting of cell densities. Cells in these cultures are observed under the phase-contrast microscope

(x10, x20 and x40 objectives) until they begin to become confluent. When confluence is observed under the microscope, the cells are sub-cultured (see Table 2). The exception to this is when setting up a monolayer culture flask (T flask) from cells growing in a suspension culture (when a known number of cells are used to seed a T flask, Table 3.).

Healthy insect cells attach well to the bottom of the T flask forming a monolayer and double every 18–24 h. In monolayer cultures, you may notice loosely attached cells or cells floating in the medium. These ‘floaters’ are especially frequent in cultures that are overgrown, whereas in healthy cultures only a few floaters will be visible. However, some *T. ni* cell lines adapted to grow in suspension culture will not attach and grow well in monolayer cultures. Many of the cells will grow quite well floating in the medium; in this case it is not a sign that the culture is overgrown.

Table 2. Typical ratios employed to sub-culture insect cells maintained in T flasks

Cell Line	Estimated ratio of existing culture to fresh medium
Sf9	1:5
Sf21	1:10
<i>T. ni</i>	1:10

Table 3. Seeding densities of insect cells in T flasks

Cell Line	T flask size (cm ²)	Seeding density
Sf9 or Sf21	T25	1.0 - 1.5 x 10 ⁶
Sf9 or Sf21	T75	3.0 – 5.0 x 10 ⁶
<i>T. ni</i>	T25	0.5 – 0.9 x 10 ⁶
<i>T. ni</i>	T75	2.0 – 3.0 x 10 ⁶

Passaging cells grown in monolayer culture (T-flasks)

Using an inverted phase-contrast microscope (x10, x20 and x40 objectives), observe the condition of the cells. When the cells are confluent dislodge them from the surface of the flask by banging the flask down firmly on a solid surface. Do not hold the neck of the flask when carrying out this operation and minimize foaming. Trypsin and other enzymes are not recommended to dislodge insect cells. Cell scrapers should be used only if absolutely necessary, as they may damage the cells. This is particularly true for Sf9 cells cultivated in serum-free medium. Then, using a graduated pipette, gently pipette the culture up and down to break up clumps of cells into a single cell suspension. Avoid causing bubbles and frothing in the culture.

Split the culture into a new T flask using the appropriate medium and ratios shown in Table 2. Each time a culture is split its passage number increases by 1.

The following information should be recorded on each T flask:

Cell line

Passage number

Date of each passage

Density the culture has been split to (i.e. 1:5 or 1:10)

Medium used

In a lab book record the following:

Liquid nitrogen batch

Date and passage when cells were raised from liquid nitrogen

11 Troubleshooting & FAQ

Q) Why are my cells not growing well or why are they enlarged and floating?

A) The cells may have been left too long between passages, and have overgrown, which can result in some floating. They can be sub-cultured like this to produce a fresh healthy culture, but should not be used for making or amplifying viruses. The cells may also be old, i.e. have undergone more than 30 continuous passages since being raised from liquid nitrogen. You will need to raise a fresh batch of low passage number cells from liquid nitrogen.

Enlarged, floating cells can also be indicative of virus contamination of stock cells, particularly if the nuclei are enlarged. Always handle stock cells and medium before handling virus. Other problems may include the use of incorrect medium or incubation conditions.

Q) Why has my co-transfection become contaminated?

A) Your transfer vector DNA may have microbial contamination. Phenol/chloroform the DNA, ethanol-precipitate and re-suspend in sterile TE buffer. Alternatively, your lipofection reagent or your medium may be contaminated. Sterility test a sample of each by adding to a small volume of culture medium and incubating at 28°C and 37°C for 48 hours. Then monitor for signs of contamination using a phase-contrast microscope or plate out onto bacterial plates.

The *flashBAC* and control transfer vector DNA provided in the kit has been purified using CsCl density gradients and has been tested for sterility prior to packing.

Q) Why has my co-transfection failed to produce recombinant virus?

A) Check that the cells are healthy using a phase-contrast microscope and that they did not dry out during the co-transfection procedure. If using cells normally cultivated in serum-supplemented medium ensure that you wash the cells with serum-free medium before using them for a co-transfection. Remember to add the extra 1 ml of appropriate culture medium to the co-transfected cells after incubating them for 5 - 24 h. Is the transfection reagent working?

Q) Why has my virus amplification failed to produce high titre recombinant virus?

A) Check the condition and density of your stock insect cells. If the cells are seeded too dense they will inhibit virus replication. The virus titre from the co-transfection may be too low for the volume of cells being infected. You will need to leave the infection for longer than 5 days and monitor virus amplification using a phase-contrast microscope to determine the optimal harvest time. Conversely, you may have added too much virus to the culture of cells so that only one round of amplification has occurred. See section 8.2 for more advice.

Q) Why do I not see any plaques on my plaque assay?

A) Ensure you are using healthy cells and avoid dislodging cells when replacing medium. The cells should adhere to tissue

culture dishes within 1 h after plating, with very few floaters present. Otherwise, discard dishes and obtain fresh cells. Check the cell concentration was not too high or too low when seeding the dishes. If the cells are seeded too densely then the virus does not replicate properly and plaques will be very small – so small that they can only be seen under the microscope. *This is a very common problem with plaque-assays!* Seeding cells too thinly will require a longer incubation period to produce confluent monolayers and also results in large, ill-defined and diffuse plaques.

Ensure that the temperature of the agarose overlay was not too high ($>37^{\circ}\text{C}$) when poured over the cells or that the cells have not been allowed to dry out at any stage. The latter is characterised by large areas of bright pink stain with a glassy appearance.

Remember to add serum-supplemented medium to the agarose overlay when using cells that require serum and don't forget the 1 ml liquid feed overlay when the agarose overlay has set.

The virus titre may be too low to be detected on the dilutions that you have plated out (try plating out the lower dilutions too from 10^{-1} to 10^{-7}). Or the virus titre may be so high that it has lysed all the cells and plaques have merged into each other (plate out higher dilutions). A common error that results in this problem is to forget to change tips when preparing the virus dilutions – so that virus carry-over occurs and gives a false high virus titre.

Always prepare freshly diluted Neutral Red for each plaque assay.

Q) My agarose overlay has cracks and/or my plaques look smeared and/or plaques are all located around the edge of the plate. Or my agarose overlay came away from the dish when I inverted them. Why?

A) If the virus inoculum is not completely removed from the cells before adding the agarose overlay, it will interfere with the gelling process and produce cracks. This may also cause the overlay to fall away when the dishes are inverted! It may also cause the plaques to look smeared by allowing the virus to spread randomly, rather than being contained within foci of cells.

Always seed the cells uniformly in the dish and ensure that the virus inoculum is added to the centre of the dish dropwise to cover the cells evenly or it can also cause smearing and may result in plaques predominating around the edge of the dish.

Q Why can I not detect expression of my gene?

A) Were the cells in good condition and in log phase of growth when used for the infection? If not, the virus may not have been able to replicate and the polyhedrin gene promoter may not have been activated (see section also 10).

Have you titrated the virus to know that the cells have actually been infected? If not this is important, as the virus may not have amplified for some reason (see section also 8.3).

Has the virus been stored for some time before use? If so, check the titre (see also section 8.3).

Does the control recombinant *lacZ* virus give good levels of β -galactosidase? If not, you may need to revise your cell culture and cell infection protocols.

Is the coding region of the gene downstream of the polyhedrin gene promoter inserted in such a way that the gene's AUG start codon is the first AUG after the promoter sequences? This is important as translation occurs at the first AUG in the mRNA.

If you have added tags or other sequences, are they in frame? Have you checked your construct by DNA sequencing?

Has the gene transferred from the transfer vector to the baculovirus genome? Check this by extracting DNA from virus-infected cells and analysing by PCR. It is very, very rare that this is a problem.

Have you optimised expression conditions – cell line, time to harvest, moi? See section 9 for more details.

Finally, unfortunately a very few proteins simply do not express well in the baculovirus system. But check all of the above advice before coming to this conclusion.

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