baculoCOMPLETE

A Complete Laboratory Guide to the Baculovirus Expression System and Insect Cell Culture

User Guide
2019-20
This User Guide comprises two separate OET manuals that have been combined into one convenient document:

1. *flashBAC™* and BacPAK6 Baculovirus Expression Manual
2. Insect Cell Culture Manual
## Contents

1.0 Limited Use License  &nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbs...
1. Limited Use Licence for flashBAC™ Virus DNA

I. In the License the following expressions shall have the following meanings:

- **DNA**: shall mean deoxyribonucleic acid;
- **Fee**: shall mean the fee invoiced for the Materials by the Licensor to the Licensee;
- **Licensee**: shall mean the purchaser of the Materials;
- **Licensor**: shall mean Oxford Expression Technologies Ltd;
- **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 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.

II. The Licensor and the Licensee have agreed to enter into this Licence on the following terms and conditions.

III. The Licensee acknowledges and accepts that by opening and/or using the Materials it is agreeing to and accepting these terms and condition. If the Licensee does not agree to these terms and conditions it must immediately return all the Materials unused to the Licensor who shall issue a refund for the fee.

IV. The Licensor has certain know-how and has developed a product that can be used to produce recombinant proteins and/or viruses and has the right to exploit the product under, inter alia, patent applications numbered EP1144666, WO0112829 and AU6460800.

V. This Licence shall commence on the date hereof and continue until the DNA has been used or destroyed.
VI. The Licensor hereby grants to the Licensee and the Licensee hereby accepts a limited, non-exclusive, non-transferable, licence to use the Materials for the Purpose and as otherwise set out in this licence.

VII. The Licensee warrants to the Licensor that:
   a) it shall only use the Materials for the purpose of Research*; and
   b) it shall not alter, reverse engineer, produce, manufacture or amplify the DNA; and
   c) it shall not sell any protein and/or virus created pursuant to this Licence to any third party; and
   d) it shall not provide any services to any third party using the Materials; and
   e) if the Licensee desires to 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.

VIII. The Licensee shall keep the DNA in accordance with the directions contained in the User Guide.

IX. The Licensor shall raise an invoice to the Licensee for the Fee and the Licensee agrees to pay the same to the Licensor within thirty (30) day of receipt of the invoice (unless otherwise agreed in writing).

X. The Materials are provided as is and neither the Licensor nor any staff acting on its behalf accepts any liability whatsoever for any of the Materials or in connection with the Licensee’s possession, handling or use of the Materials.

XI. The Licensee’s remedy pursuant to this Licence shall be limited at the Licensor’s option to the replacement of the Materials or a refund of the Fee paid by the Licensee.

XII. Ownership of the Materials shall pass to the Licensee upon dispatch of the Materials by the Licensor to the Licensee.

XIII. The Licensee shall indemnify the Licensor for any loss suffered by the Licensor as a result of the Licensee’s breach of this licence and/or third party’s intellectual property rights.

XIV. This Licence is personal to the parties and shall not be assigned or otherwise transferred in whole or in part by either party.

XV. This Licence constitutes the entire agreement and understanding between the parties in respect of the Materials and supersedes all previous agreements, understandings and undertakings in this respect and all obligations implied by the law to the extent that they conflict with the express provisions of this Licence.

XVI. The invalidity, illegality or unenforceability of a provision of this Licence shall not affect or impair the continuation in force of the remainder of this Licence.
XVII. The Licensor reserves the right to revoke this permission and may require the Licensee to return or destroy any remaining DNA and/or the User Guide.

XVIII. Clauses 1, 3, 7, 9, 10, 13, 16, 18-20 shall survive any termination or expiry of this Licence.

XIX. The interpretation, construction and effect of this Licence shall be governed and construed in all respects in accordance with the laws of England and the parties hereby submit to the non-exclusive jurisdiction of the English courts.

XX. 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.

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

End of Limited Use Licence.

2. Kit Contents

All reagents and materials provided and referred to in this User Guide are for Research Purposes only.

a) flashBAC™ DNA (or BacPAK6 DNA). Store at 4°C.

b) Control transfer plasmid DNA (containing lacZ reporter gene). Store at -20°C. (flashBAC™ kits only)


3. Essential Information and Technical Assistance

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™ (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.

4. 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.
5. Product Ordering Information

*Prices are relevant for 2019-20

flashBAC™ Kits

<table>
<thead>
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<th>Product</th>
<th>Details</th>
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<th>Catalogue Number</th>
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BacPAK6 Kits

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<td>BacPAK6 Linearised DNA</td>
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<td>£279.00</td>
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<td>BacPAK6 Linearised DNA</td>
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## Transfection Reagents

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<td><em>baculoFECTIN II</em></td>
<td>150µL</td>
<td>£85.00</td>
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<td><em>baculoFECTIN II</em></td>
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## *baculoQUANT™* and *baculoCOMPLETE* Kits

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<th>Product</th>
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<td><em>baculoCOMPLETE</em> Protein Expression Kit &amp; <em>baculoQUANT™</em> All-In-One Kit</td>
<td>5 + 100 reactions</td>
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<td><em>baculoQUANT™</em> All-In-One Virus Extraction &amp; Titration Kit</td>
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## Transfer Plasmids

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<tr>
<td>pOET1</td>
<td>Polyhedrin gene promoter with multiple cloning site (MCS) (10µg)</td>
<td>£145.00</td>
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<tr>
<td>pOET1N 6xHis</td>
<td>Polyhedrin gene promoter, MCS with N-terminal 6xHis tag (10µg)</td>
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<td>pOET1C 6xHis</td>
<td>Polyhedrin gene promoter, MCS with C-terminal 6xHis tag (10µg)</td>
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<td>pOET2</td>
<td>As pOET1 but with reversed MCS (10µg)</td>
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<td>pOET2N/C 6xHis</td>
<td>Polyhedrin gene promoter, MCS with N- and C-terminal 6xHis tags and thrombin cleavage site (10µg)</td>
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<td>pOET3</td>
<td>P6.9 gene promoter for late phase expression (10µg)</td>
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<td>pOET4</td>
<td>As pOET3 but with reversed MCS (10µg)</td>
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<td>pOET5</td>
<td>Dual expression with polyhedrin and p10 gene promoters (10µg)</td>
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<td>pOET6 BacMAM</td>
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<td>pOET1 Gateway™</td>
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<tr>
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<td>pOET8.VE1</td>
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<td>pOET8.VE3</td>
<td>As pOET8.VE1 but with additional C-terminal 6x His-tag and Honey Bee Melittin signal sequence (10µg)</td>
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<tr>
<td>pOET Sequencing Primers</td>
<td>Can be used with any pOET transfer vector (2 x 100µL)</td>
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### Insect Cell Culture Media

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Insect Cell Lines

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<th>Live Culture</th>
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<td>On request</td>
<td>£195.00</td>
<td>Serum-free</td>
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<td><em>Sf21 Cells</em></td>
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<td>On request</td>
<td>£195.00</td>
<td>TC100 with 10% serum</td>
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<td><em>Super Sf9-1 Cells</em></td>
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<td>Serum-free</td>
<td>600104</td>
</tr>
</tbody>
</table>
6. Introduction to the Baculovirus Expression System and *flashBAC™*/BacPAK6 Technology

6.1 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 *flashBAC™* 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 2 A). 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 2 A & C**). These consist of multiple rod-shaped nucleocapsids (**Figure 2 B**) 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. 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.
6.2  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](image)

**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. (B) Cells expressing beta-galactosidase (blue) after staining with X-gal.

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 CO2 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 flashBAC™, 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 (cotransfection) 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 recombinant 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 \textit{lacZ} gene into the virus genome so that recombinant virus plaques would stain blue after the addition of X-gal\textsuperscript{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.

### 6.3 The BacPAK6 System

The efficiency with which recombinant viruses could be recovered was improved by the addition of a unique restriction enzyme site (\textit{Bsu36I}) at the \textit{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. \textit{LacZ} was then introduced into the parental virus genome to replace the \textit{polh} coding sequence, resulting in three \textit{Bsu36I} sites at the \textit{polh} locus\textsuperscript{12} (Figure 5).

Triple digestion of the resulting virus genome with \textit{Bsu36I} removed a section of virus DNA coding for \textit{lacZ} and part of the essential gene ORF 1629\textsuperscript{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 \textit{polh}, creates recombinant virus DNA in which ORF 1629 is restored and the re-circularised DNA can replicate to produce recombinant BV\textsuperscript{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.**

**NOTE**

The triple-cut linear BacPAK6 virus DNA is available from OET (see page 9). 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.
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.

6.4 The flashBAC™ System

The flashBAC™ system is the most advanced platform technology for the production and isolation of recombinant baculoviruses. Importantly, flashBAC™ 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 stage procedure that is fully amenable to high throughput manipulations (Figure 6) – multiple recombinant viruses can be made at one time using 24-well plates either manually or using simple robotic systems.

The flashBAC™ 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 flashBAC™ kits and co-transfections to make recombinant viruses (page 9).

A recombinant baculovirus is produced simply by co-transfecting insect cells with flashBAC™ 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 7, page 24).
Figure 6. Timescale for using the flashBAC™ system in protein production. The co-transfection mix comprises flashBAC™ DNA, transfer plasmid with gene to be expressed and transfection reagent.
The recombinant BV is harvested from the co-transfection medium and becomes the seed stock (P0) of recombinant virus. 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.

**NOTE**

The single step procedure greatly facilitates high throughput production of baculovirus expression vectors via automated systems. However, it is just as useful for a research lab making one or two viruses in individual dishes. It is very convenient for the novice.

The *flashBAC™* system is back-compatible 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.
Figure 7. Overview of how a recombinant baculovirus virus is produced using the flashBAC™ technology. (A) Partially deleted copy of the gene ORF 1629. (B) Bacterial artificial chromosome (BAC). (C) Sequence encoding the desired protein to be expressed is cloned into a suitable transfer vector. (D) polh promoter controls expression of the foreign gene. Other promoters can also be used. (E) Homologous recombination removes the BAC replicon and incomplete ORF 1629 gene, replacing them with the gene of interest and full length ORF 1629, respectively. (F) Restoration of ORF 1629 produces an infectious virus that is able to replicate within insect cells to make budded virus. The harvested recombinant budded viruses can now be used to synthesise the target protein.
Since the launch of the original flashBAC™ DNA, we have made further modifications to help express difficult to express proteins (Figure 8). The different flashBAC™ variants are:

**flashBAC™** 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.\(^{14-17}\)

**flashBAC GOLD** Backbone virus DNA has gene deletions for chiA and v-cath. This avoids production of chitinase and cathepsin, a viral protease that may otherwise degrade susceptible target proteins.

**flashBAC 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.

**flashBAC 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, page 27).

**Advantages of the flashBAC™ system:**

- Simple to use
- One step procedure that does not require plaque-purification (Figure 6, page 22)
- Amenable to making many viruses simultaneously – manually or using a robot in 24-well plates
- Amenable to high throughput systems
- Maximise secreted or membrane targeted proteins
- Maximise difficult 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

**Figure 8.** Comparative Western blot band intensities for the production of five recombinant proteins using either flashBAC™, flashBAC GOLD or flashBAC ULTRA. 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.

7. Making Recombinant Baculoviruses using either BacPAK6 or flashBAC™

7.1 Choice of Transfer Plasmid

Both the BacPAK6 and flashBAC™ 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:

<table>
<thead>
<tr>
<th>Promoter Type</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyhedrin gene</td>
<td>Simple vector such as pOET1 or 2 that has a multiple cloning site (MCS) to insert your gene under control of the strong, very late polyhedrin gene promoter.</td>
</tr>
<tr>
<td>P10 gene</td>
<td>Another strong, very late gene promoter, frequently used in vectors for multigene expression (see below).</td>
</tr>
<tr>
<td>Dual promoters</td>
<td>For dual expression of genes, usually one under polyhedrin and one under p10 promoters, such as pOET5.</td>
</tr>
<tr>
<td>Multiple promoters</td>
<td>A mix of copies of polh and p10 promoters. Careful construct plans are needed to insert genes according to MCS and restriction sites available.</td>
</tr>
<tr>
<td>BacMAM promoters</td>
<td>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).</td>
</tr>
<tr>
<td>Late gene promoters</td>
<td>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 4 use the p6.9 promoter.</td>
</tr>
<tr>
<td>Purification tags</td>
<td>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.</td>
</tr>
<tr>
<td>Fusion vectors</td>
<td>Some transfer plasmids allow the gene to be expressed as a fusion product with a fluorescent protein, for example, to allow visualisation by microscopy.</td>
</tr>
<tr>
<td>Signal peptides</td>
<td>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</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Start codons</th>
<th>Translation will start at the first ATG after the promoter so check constructs carefully to ensure there is no inadvertent additional ATG.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codon optimisation</td>
<td>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.</td>
</tr>
<tr>
<td>Membrane anchors</td>
<td>Many people try and secrete membrane protein domains by removing membrane anchor domains – this works sometimes but not always.</td>
</tr>
</tbody>
</table>

**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 transfrect 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.
7.2 Co-transfection of Insect Cells with BacPAK6 or flashBAC™ 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 7.6, page 52 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 MSDS data sheets where appropriate. We recommend wearing PPE such as lab coats and gloves at all times.

Provided in the kit:

- flashBAC™ DNA (any type) or BacPAK6 DNA (use 100ng [5µL] DNA per co-transfection)
- Positive control transfer plasmid DNA (expressing lacZ) (use 500ng [5µL] per co-transfection) [flashBAC™ kit only]

Also needed:

- 35mm tissue culture dish/6-well plate or 12-well plate seeded with a sub-confluent monolayer of Sf21 or Sf9 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 also 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. Expression Systems LLC Transfection Media). We recommend using TC100 medium as a transfection medium but most serum-free medium will also work.

- 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 baculoFECTIN 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 Sf9 or Sf21 cells (Figure 9, page 34). If you are making a virus with the control vector provided in the flashBAC™ 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.

   **NOTE**

   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

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 $1.5 \times 10^6$ Sf21 or $1 \times 10^6$ Sf9 cells per 35mm dish/6-well plate to form a sub-confluent monolayer. For 12-well plates, add $0.4 \times 10^6$ Sf9/Sf21 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.

2. 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 (flashBAC™ or BacPAK6) [5µL]
- 500ng transfer plasmid (5µL lacZ transfer vector from flashBAC™ kit) or your own transfer vector
- 1.2µL baculoFECTIN 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.

**NOTE**

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

3. 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 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.

4. 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.

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

6. Harvest the culture medium containing budded recombinant virus into a sterile container and store in the dark at 4°C.

**NOTE**
Cells in which virus has replicated appear different from mock-transfected cells (Figure 8) 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.

**NOTE**
If you prepared a control virus with the lacZ transfer plasmid in the flashBAC™ kit, you can check for transfection success by staining the monolayer of cells left after harvesting the P0 virus; add 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.
**Figure 9.** 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. ➔ Indicates virus-infected cells.
7. The next step depends on whether you have used BacPAK6 or flashBAC™ DNA.

**flashBAC™ DNA:** Your 1-2mL stock of virus is your seed stock (P0), you now need to amplify this to obtain a 50mL P1 stock of virus for experimental work and freezing down (go to 7.4, page 42).

**BacPAK6 DNA:** You now need to plaque-purify your recombinant virus to obtain your seed stock (P0) (go to 7.3).

### NOTE
You can also plaque-purify virus produced using flashBAC™ DNA if required.

#### 7.3 Plaque-purification of Recombinant BacPAK6 Virus

The BV harvested after the co-transfection with BacPAK6 virus DNA 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. As long as 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.

The OET Cell Culture Manual has details of insect cell culture.
**Required:**

- Virus harvested from a co-transfection (see 7.2)
- 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 Sf21 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 Sf21 cells at about $4 \times 10^5$ cells/well (or Sf9 cells if Sf21 are not available). See Cell Culture Manual for more details. Allow the cells to settle for at least 1 hour.

   You need 12 dishes per virus.

   **NOTE**

   *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 7). *Sf9* 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 *Sf9* cells and serum free medium produce plaques that quickly fade after staining with Neutral Red.

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.

NOTE
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.

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

NOTE
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 (15mL is convenient).
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.

**NOTE**

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!

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. 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.
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 10).

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.

**NOTE**
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).

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.
Figure 10. Plaque-assay in Sf21 cells stained with Neutral Red to show (A) well isolated plaques, (B) crowded plaques (C) and merged plaques.

15. Amplify the plaque-picked virus by inoculating either a 35mm dish or a T25 flask of Sf21 or Sf9 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 7.4).

NOTE
The cell monolayers from the dish or flask used to amplify virus can be harvested and used to test for gene expression or to isolate DNA to do a PCR to check that the gene has gone into the virus genome.
7.4 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 serum to 2.5% 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 enough.

To have the best chance of producing a good high titre stock of P1 or P2 virus, use Sf9 cells growing in shake suspension cultures in serum-free growth medium, or Sf21 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) 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.

Read through the method before starting and use aseptic technique throughout.

**Required:**

- Stock of virus to be amplified (e.g. P0 from method 7.2, page 30 or 7.3, page 35)
- 50-200mL of healthy log phase Sf9 or Sf21 cells at no more than 2 x 10^6 cells/mL
- Shake flask appropriate to the volume of Sf9 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 Sf21 (see page 62) 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

**Method:**

1. Prepare 50-200mL log phase Sf9 cells or Sf21 cells in a shake or stirred culture as appropriate to the medium being used. Generally Sf9 cells in serum-free medium in a shake culture should not be more than 2 x 10^6 cells/mL and Sf21 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 flashBAC™ virus (go to 7.2, page 30) or BacPAK6 (go to 7.3,
page 35), 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.

You can titre your virus by plaque-assay – the gold standard (see 7.5) or by qPCR. OET has a convenient qPCR titration kit (baculoQUANT™ All-In-One) or we provide a fast and cost-

**NOTE**

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 $1 \times 10^8$ pfu/mL and you wanted to amplify 500mL P2 virus, you would need to add 1mL of P1 virus to 500mL cells at $2 \times 10^6$ cells/mL (MOI = 0.1).
effective virus titration service. Contact us at info@oetltd.com for more details.

**NOTE**

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.

**7.5 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 11). However, titres can also be obtained by qPCR and OET sells a convenient baculoQUANT™ 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.

**Required:**

- Virus to be titrated (see 7.4, page 42)
- 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 Sf21 cells (best; or Sf9 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 Sf21 cells (or Sf9 cells if Sf21 are not available). See 7.2, page 30 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, you can seed 35mm dishes with cells – see protocol 7.3, page 35 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.

**NOTE**

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!

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.

**NOTE**

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

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.
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 x 10^6 x 10 = 1.5 x 10^8 pfu/mL.

**NOTE**

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, *baculoQUANT All-In-One*, for determining virus titre in less than 3 hours. Alternatively our plaque assay service is also available.
Figure 11: Schematic diagram outlining the plaque assay procedure of recombinant baculovirus produced from flashBAC™ DNA.
7.6 A Guide to Using flashBAC™ in 24-well Plate Systems

The following is a guide to making recombinant flashBAC™ 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 Sf9 cells in serum free medium at a density of $5 \times 10^5$ cells/mL and dispense 0.4mL ($2 \times 10^5$ 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 flashBAC™ 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 baculofECTIN 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 $28^\circ$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 $4^\circ$C in the dark. Long-term stocks of virus should be stored at $-80^\circ$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 7.4, page 42 – as a guide use 250µL to infect 50-100mL of Sf9 cells.
8. 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 flashBAC™ systems. It is not intended to be prescriptive, simply a guide to help you get started.

8.1 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.

8.2 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 7.2, page 30 / 7.4, page 42) 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 flashBAC™ kit, you could make a recombinant virus with the control lacZ transfer vector and use this to set control infections to look for beta-galactosidase production (Figure 4, page 17).

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 8.3). It is well worth testing expression in both Sf9 or (Sf21) 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.

8.3 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
- Cell line (Sf9, Sf21, T. ni Hi-5™, SuperSf9 cells
- Time to harvest. E.g. – 0, 24, 36, 72, 96 hpi
- flashBAC™ variant (page 25)

8.4 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.
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](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. 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 flashBAC™ 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 9))? 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 fell out when inverting, or the plaques are smeared or only around the edges of 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. 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.

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 Sf9 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!
10. References


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1. Introduction

1.1 Insect Cell Culture

This manual provides a guide to the growth and maintenance of insect cell cultures. It is extremely important that the insect cells used for the production and analysis of recombinant baculoviruses are of the highest quality. Insect cells can also be transfected with suitable plasmids to produce stable cell lines expressing a gene of choice. This manual provides tried and trusted protocols used within the labs at OET and by scientists with over thirty-years’ experience of working with insect cell cultures and baculoviruses. We hope it is useful to both beginners and more experienced researchers.

1.2 Choice of Cell Line

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); Sf9 cells, which are a clonal isolate of Sf21; or T. ni (T. ni Hi5™) cells, originally derived from the ovarian cells of Trichoplusia ni (cabbage looper). Generally, Sf21 or Sf9 cells are used for co-transfections, virus amplification and plaque-assays. Whilst many labs use Sf9 for all protocols, Sf21 cells are superior for plaque-assays and monitoring virus cytopathic effects and are more tolerant to sub-optimal conditions; and so ideal for those new to the system. Sf9 cells are usually better for amplification of large stocks of virus and protein production and grow very well in large-scale fermenters or shake flasks. T. ni Hi5™ cells are often used to achieve maximal protein production but should not be used to produce or amplify virus because of the increased possibility of generating virus mutants. For reasons that remain unclear, some genes are expressed much better in Sf cells than T. ni cells or vice versa, so testing expression in both cell lines at an early stage is recommended.
A number of engineered cell lines are available that may enhance expression of certain genes, particularly those that may be difficult to express in normal insect cells. For example, Super Sf9 cells are genetically modified to express a stabilising protein and have increased longevity after virus infection resulting in up to a 15-fold increase in protein yield compared to unmodified Sf9 cells. Table 1 provides a summary of the main characteristic of each cell line.

1.3 Culture Medium

Most insect cell culture medium utilizes a phosphate buffering system, rather than the carbonate-based buffers 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 is a large variety of insect cell culture media available and it is beyond the scope of this manual to list them all; Table 1 lists the media currently in use in our labs. Sf9, Sf21 and T. ni Hi5™ cells can all be grown in medium with serum or serum-free media. 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 penicillin G sodium and 5 μL/mL 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 later cause inefficient virus replication or protein production. Addition of antibiotics to plaque-assay medium is, however, recommended.

NOTE
Superscripts 1-5 in Table refer to references. *Foetal calf serum – batches vary; always test before using a new batch, some lots may require heat-inactivation at 60°C for 30 minutes.
Table 1. Insect cell lines and characteristics

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Appearance</th>
<th>Doubling Time</th>
<th>Uses</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sf21¹</td>
<td>Spherical, more irregular in size than Sf9 cells</td>
<td>24 hours</td>
<td>Plaque-assays (large, easy to see plaques form in 3-4 days) in serum-containing media</td>
<td>Serum-free (e.g. ESF 921)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monolayer cultures</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Suspension cultures</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Making recombinant viruses</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Amplification of virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Production of proteins</td>
<td></td>
</tr>
<tr>
<td>Sf9²</td>
<td>Spherical, more regular in size than Sf21 cells</td>
<td>24 hours</td>
<td>Suspension cultures</td>
<td>Serum-free (e.g. ESF 921)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monolayer cultures</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Making recombinant viruses</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Amplification of virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Production of proteins</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Plaque-assays (small plaques form in 4 days)</td>
<td></td>
</tr>
<tr>
<td>Super Sf9 1-3⁵</td>
<td>Spherical, more regular in size than Sf21 cells</td>
<td>24 hours +</td>
<td>Protein production for secreted or difficult to express proteins</td>
<td>Serum-free - adapted to SF900™ II (Invitrogen) or; TC100 plus 10% v/v serum*</td>
</tr>
<tr>
<td>T.niHi5™</td>
<td>Spherical, larger and more irregular in size than Sf9 cells</td>
<td>18 hours</td>
<td>Suspension cultures</td>
<td>Serum-free (e.g. ESF 921)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monolayer cultures (loose attachment)</td>
<td></td>
</tr>
</tbody>
</table>
1.4 General Requirements for Insect Cell Culture

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 from 25-30°C. The optimal temperature for cell growth and infection for insect cells is considered to be 27-28°C. Insect cells can also be cultured at room temperature (about 20-22°C) when a slower growth rate is required. Virus infection is usually carried out at 27-28°C. We recommend carrying out any cell culture work each day prior to handling virus and only using one cell line at a time.

1.5 Maintaining Cell Cultures

Insect cell lines can be maintained as either suspension cultures, in shake flasks or in stirred vessels (Figure 1), or in monolayer adherent cultures in T flasks or dishes. Generally, insect cells adapted to serum-free medium are cultivated in suspension shake cultures whilst cells adapted to serum-supplemented media are cultivated in monolayer cultures or stirred suspension cultures (as growing these cells in shake culture generates excessive foaming and subsequent cell damage). However, cells grown in serum-free medium may also be cultured in monolayers.
Figure 1. Examples of insect cell culture flasks. (A) Suspension culture shake flask from 125mL to 3L. (B) Suspension culture stirred vessel from 125mL to 1L

Shake flasks may be recyclable glass or disposable. Stirred flasks are usually glass and contain either a magnetic stirring bar or suspended magnetic stirring rod (Figure 1). Both types are available from a range of suppliers. To maintain optimum cell culture conditions in a suspension culture, cell densities should be kept within certain ranges, i.e. within the log phase of growth (see Table 2, page 67).

Sub-culturing (or passaging) of cells allows them to be maintained within log phase, preventing them from entering their stationary phase. 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. Far more important than passage number of the cell is the number of times the culture has been stressed! Sub-culturing of shaker or stirrer cultures requires the seeding density of each cell culture to be determined before sub-culturing of cells can commence. We tend to sub-culture cell lines continuously for approximately 30 passages before returning to stocks stored in liquid nitrogen; but this is a matter of preference for each lab.
2. General Cell Culture Techniques

2.1 Sterile Technique

All techniques must be carried out under sterile conditions either in a class II or laminar flow safety cabinet.

2.2 Passaging Cells

This is also referred to as sub-culturing cells and allows a stock of cells to be kept within log phase and optimal viability for experimental use. For example, if cells are not in log phase, they will not have available all the enzymes and molecules needed for effective and efficient virus replication, leading to poor virus titres or low level of expression. It is important that cell cultures are passaged before the culture conditions reach stationary phase – at this point cells have become ‘stressed’ and are starting to die, making recovery of passaged cells at this stage more difficult. Cultures that are continually left to reach stationary phase (stress) before passaging may suffer permanent problems and will not support virus replication. In this case, a new culture must be established from a frozen stock.

At each passage, record the passage number on the culture flask. Generally, insect cell cultures can be passaged for about 30 times before returning to frozen stocks of cells to initiate a new culture.

When initiating a new culture from a frozen stock, we strongly recommend starting the culture as an adherent culture and once the cells are growing well (1-2 passages), transfer them into a suspension culture. It is also easier to monitor cells visually under the microscope when growing in monolayer culture. It is possible to go straight from a frozen vial to a suspension culture but this requires a high density and high viability of the frozen cells. See notes later on in this manual.
2.3 Adherent Culture

Cells can be maintained in T25 or T75 flasks and grown at 27-28°C until the cells just reach confluency. Cells can be maintained in serum-free media or in media containing 10% serum (Table 1, page 60). Confluency means the cells have just reached the point where they are touching each other, covering the surface of the culture vessel (Figure 2). Cells that are passaged repeatedly when the culture has past confluency will suffer from increased cell growth time and the appearance of grainy and ‘sausage’ shaped cells that have arrested mid cell division (Figure 2). Overgrown cells also have increasing numbers of cells floating in the medium; healthy cultures have very few floating cells. Attempting to passage cells before a culture reaches confluency means it is harder to dislodge cells as they are firmly attached to the flask surface and thus the chances of damaging the cells increase.

To sub-culture adherent cells, they should be detached from the surface by tapping the flask sharply on the bench top – this is the method we routinely use. Cells can also be detached by repeatedly pipetting the culture media over the monolayer to dislodge cells – use a Pasteur pipette.

**NOTE**

Cell scrapers can be used but in our experience this method leads to an excess of dead cells – home-made scrapers consisting of a bent glass rod with an attached piece of soft silicon tubing, and sterilised by autoclaving, are more gentle on cells than commercial scrapers.

Usually a 1 in 5 (Sf9) or 1 in 5-1 in 10 (Sf21/ T. ni Hi-5™) dilution with 1 part old culture and 4 (or 9) parts fresh medium is sufficient to keep cells in a log phase culture. A typical 75cm² flask contains 10mL of culture medium and so 2mL would be transferred into 8mL of fresh medium for a 1 in 5 split (1mL plus 9mL for 1 in 10 split). These values are guides and will need to be adapted to suit the actual growth parameters in each lab.
It is also important not to make the density of cells too low when passing, as cells need to be in reasonable proximity with other cells to promote growth. Some cells are more tolerant of this than others: Sf21 cells are the most tolerant, Sf9 cells are the least. **Figure 2** shows an example of cell density immediately after passaging and at confluent levels for Sf21, Sf9 and T. ni Hi5™ cells. If the cells in the newly seeded culture are too sparse, they may not divide and the culture will not become confluent.

It is essential to monitor cells under an inverted microscope prior to and after passing to check for confluency, detachment after tapping, or any signs of poor health or over growth. Signs of poor health include: grainy cells, ‘sausage’ shaped cells, floaters, longer doubling time, failure to reach confluence. Grainy cells with refractive cuboidal-like structures in the nucleus are a sign of wild-type baculovirus contamination.

Maintain a log book of passaging and record the passage number, date and split ratio on the culture flask. After about 30 passages of being maintained in log phase, cultures start to lose viability and virus replication can be impaired. The old culture should be discarded and a new one established from a frozen stock. The log book can also be used to record any observations about the culture and this is sometimes very helpful when trouble shooting.
<table>
<thead>
<tr>
<th></th>
<th>Sub-confluent</th>
<th>Confluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sf9</td>
<td><img src="Sf9_subconfluent.png" alt="Image" /></td>
<td><img src="Sf9_confluent.png" alt="Image" /></td>
</tr>
<tr>
<td>Sf21</td>
<td><img src="Sf21_subconfluent.png" alt="Image" /></td>
<td><img src="Sf21_confluent.png" alt="Image" /></td>
</tr>
<tr>
<td>T. ni Hi5™</td>
<td><img src="T.ni_Hi5_subconfluent.png" alt="Image" /></td>
<td><img src="T.ni_Hi5_confluent.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 2.** Images to illustrate insect cells at various stages of culture. (A) Sf21 cells - sub-confluent (B) Sf21 cells - confluent (C) Sf9 cells - sub-confluent (D) Sf9 cells - confluent (E) T. ni cells - sub-confluent (F) T. ni cells – confluent.
Whilst it is not essential, some labs prefer to count cells at each passage and seed a certain number of cells per flask each time. Table 2 provides an indication of seeding density for passaging cells grown in monolayer culture.

When harvesting cells for use in experimental work, always count the number of cells and determine their viability so that the correct seeding density can be achieved for transfections or virus amplification.

Table 2. Seeding density for passaging monolayer cultures

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cells to Seed a T25 Flask</th>
<th>Cells to Seed a T75 Flask</th>
<th>Cells to Seed a T150 Flask</th>
<th>Split Ratio Guide (Culture : Fresh Medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sf21</td>
<td>$1 \times 10^6$</td>
<td>$3 \times 10^6$</td>
<td>$5-6 \times 10^6$</td>
<td>1:5 to 1:10</td>
</tr>
<tr>
<td>Sf9/Super Sf9</td>
<td>$1.5 \times 10^6$</td>
<td>$5 \times 10^6$</td>
<td>$1 \times 10^7$</td>
<td>1:5</td>
</tr>
<tr>
<td>T. ni Hi5™</td>
<td>$0.9 \times 10^6$</td>
<td>$2-3 \times 10^6$</td>
<td>$4-6 \times 10^6$</td>
<td>1:5 to 1:10</td>
</tr>
<tr>
<td>Culture Volume</td>
<td>5-7mL</td>
<td>10-15mL</td>
<td>30-40mL</td>
<td>-</td>
</tr>
</tbody>
</table>

Key points when culturing cells:

- Check cells each day under the microscope until a confluent monolayer has formed
- Passage cells when confluent or shortly after
- Do not allow cells to become overgrown
- Do not split at too high a ratio
- Keep a record of passage number, date and split ratio
- Start a new culture from frozen stocks after about 30 passages
- Do not use antibiotics in routine cultures
2.4 Suspension Culture

Maintaining insect cells in suspension culture is very easy and provides a ready source of cells for amplifying recombinant viruses and infecting cells for protein production. There are two main methods for small-scale suspension cultures – shake flasks or stirred flasks (Figure 1, page 62).

Shake flasks require the use of serum-free medium as otherwise serum creates excess froth that results in cells bursting as their membranes fuse with the bubbles. Most serum-free medium contain surfactants to reduce frothing. A surfactant such as Pluronic®F-68 can be added to media to reduce frothing but in our experience even adding surfactant to serum-containing medium does not prevent damage to cells.

There are commercial disposable shake flasks in a range of sizes that permit cultures from 10mL to 1.5L. As the culture volume increases attention must be given to aeration since insect cells, particularly those infected with virus, have a high oxygen requirement for metabolism. This can be achieved by selecting an appropriate rpm, not over filling the flask to maximise the surface area for gas exchange and ensuring lids are vented.

For those on a restricted budget, it is cheaper to use reusable glass flasks with cotton wool and loosely covered foil caps that have been sterilised by autoclaving and then dried. However, insect cells are very susceptible to contaminants in flasks and so any washing-up regime must be very stringent.

At OET our washing up regime is as follows:

- Disinfect flask with Virkon
- Soak in hot water with mild detergent (washing up liquid) and scrub internal surface with nylon bottle brush to remove adherent cell debris
- Rinse five times with hot water
- Soak in hot water for 2 hours
- Rinse in deionised water twice
• Soak in deionised water overnight
• Rinse in deionised water, dry and sterilise in an autoclave
• Dry in warm cabinet prior to use

As insect cells do not require CO\(_2\), a shaking platform can be placed inside a standard incubator maintained at 27-28°C or even a clean cupboard/room maintained at this temperature.

Cells can also be grown in stirred cultures using commercial systems that have vessels with either a vertical impeller or hanging stirring bar that sit on a bespoke stirring device (Figure 1, page 62). There are often side ports to take samples or add media/cells. Again, the caps should be vented or left loose and the vessels must not be overfilled to ensure good aeration. Cost effective home-made stirred flasks can be made using a flat-bottomed round flask with a stirring bar sat on a conventional magnetic stirrer. Again attention must be given to ensure the flasks are properly cleaned and sterilised before use.

Cells detached from a healthy adherent culture should be counted and used to set up a suspension culture according to the guidance in Table 3. It is important that the cells have a high viability – at least 95%. When establishing a suspension culture for the first time, set up a relatively modest scale flask (50-100mL culture). Once the suspension culture is established, a larger culture can be established if required.

Cells should be monitored daily by taking a small sample for counting and determining cell viability. In this way a growth curve can be plotted and monitored. Cells should be passaged before they reach stationary phase. An example of a typical growth curve is shown in Figure 3. Whilst we provide guidance on this matter, each lab should establish the optimal conditions for their cells and media combination.

Passaging cells in suspension is very easy, after counting and determining the viability, remove all the excess cells and, to the cells remaining in the flask, add fresh medium to establish a new culture at the correct cell density (Table 3). This can continue for up to about 30 passages (see notes under
adherent cultures). After this time, the culture needs to be set up from a fresh adherent culture at low passage number.

The excess cells removed can be used to set up further suspension cultures for virus infection – either to amplify stocks of recombinant virus or infect cells for protein production. Cells can also be used to seed monolayer cultures for experimental use e.g., 30mm dishes or multiwall plates for plaque assay, co-transfections to make recombinant viruses or to test expression levels.

![Figure 3](image_url)

**Figure 3.** Typical growth curve for Sf9 cells grown in suspension culture in ESF 921 medium.
Table 3. Seeding density for passaging suspension cultures

<table>
<thead>
<tr>
<th></th>
<th>Sf21 Cells</th>
<th>Sf9 Cells</th>
<th>T. ni Hi5™ Cells</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeding density for spinner culture (80-90rpm)</td>
<td>2-3 x 10⁵ cells/ml</td>
<td>Not usually grown in spinners</td>
<td>Not usually grown in spinners</td>
<td>Do not overfill flasks – maximum 50% of total volume</td>
</tr>
<tr>
<td>Passage spinner culture when density reaches</td>
<td>1-2 x 10⁶ cells/ml</td>
<td>N/A</td>
<td>N/A</td>
<td>Sf9 and T. ni cells are not usually grown in spinners</td>
</tr>
<tr>
<td>Seeding density for shake culture (135rpm)</td>
<td>4-5 x 10⁵ cells/ml</td>
<td>4-5 x 10⁵ cells/ml</td>
<td>2-3 x 10⁵ cells/ml</td>
<td>Do not overfill flasks – have maximum surface area to volume ratio 25% of total volume</td>
</tr>
<tr>
<td>Passage shake culture when density reaches:</td>
<td>3-6 x 10⁶ cells/ml*</td>
<td>3-6 x 10⁶ cells/ml*</td>
<td>3-6 x 10⁶ cells/ml*</td>
<td>*Cells may reach higher density if required.</td>
</tr>
</tbody>
</table>

Key points when setting up a new suspension culture:

- Use healthy log phase cells from an adherent culture that is at least 95% viable
- Count the cells and seed a culture no larger than 100mL using the guidelines in Table 3 using serum-free medium
- Ensure good aeration by not over filling flasks, maintaining optimal surface area to volume ratio, using an appropriate rpm and vented lids
- Monitor cells daily and set up a growth curve
- Passage cells by removing excess cells and adding fresh media to achieve correct cell density (Table 3) before cells reach stationary phase
- Use excess cells to start cultures for virus amplification, protein production, or experimental use in monolayer cultures
• Progress to larger volumes once cells established in culture
• Always note passage number, date and cell count/viability data on flask and in log book
• After 30 passages (from retrieval of frozen stock – include passage number data from adherent cultures), start a new adherent culture from a frozen stock
• Only use log phase cells for virus infections
• Do not use T. ni Hi5™ cells for making recombinant viruses or amplifying viruses
3. Freezing and Thawing Cells

3.1 Freezing Cells

Once a cell line has been established in either adherent or suspension culture, after two-three passages, stocks should be frozen in ampoules in liquid nitrogen so that new cultures can be established when required, for example, after 30 passages or if the culture fails for some reason. It is important to freeze cells at as lower passage number as possible.

There are many variations in the protocols published for the freezing down and thawing of insect cells. The following is one that we find works well.

**Protocol:**

1. Set up a culture (suspension or adherent but suspension is better) that will provide sufficient cells for freezing down (1 x 10^7 cells per vial). Freeze down several vials in one batch – at least 20.

2. Harvest cells from a log phase culture (mid log phase for suspension or just prior to confluency for adherent cells). Count and determine viability. Cells need to be at least 95% viable.

3. Place and label the required number of cryovials on ice.

4. Pellet required number of cells very gently at 500rpm for 5 minutes. Remove and use the conditioned growth medium to prepare the freezing mixture as follows:

<table>
<thead>
<tr>
<th>Serum-free medium*</th>
<th>Serum-containing medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>45% conditioned growth medium</td>
<td>40% conditioned medium</td>
</tr>
<tr>
<td>45% fresh medium</td>
<td>10% FBS (serum)</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>10% DMSO</td>
</tr>
<tr>
<td></td>
<td>40% fresh medium</td>
</tr>
</tbody>
</table>

*use same freezing mix as serum-containing medium if preferred
5. Re-suspend the required number of cells in freezing mixture by very gently pipetting up and down to achieve a density of $1 \times 10^7$ cells/mL. Place 1mL aliquots into cryovials.

6. Place the vials in a freezing chamber (e.g., we use Mr Frosty by Nalgene®) containing isopropanol and immediately place the chamber at -80°C overnight before transferring to liquid nitrogen.

Or, place the vials at -20°C for one hour and then at -80°C overnight before placing in liquid nitrogen.

7. After a few days, retrieve one vial to ensure that the freezing process has been successful.

8. Keep a log book/e-record of where and when each cell line is frozen, and when vials are recovered.

**Key points when freezing cells:**

- Use healthy log phase cells with 95% or greater viability
- Once DMSO has been added, cells must be cooled immediately to avoid damage
- Freeze slowly to avoid damage to cells
- Check process has been successful after a few days of storage
- Check liquid nitrogen levels regularly to ensure cells don’t start to thaw during storage
- Take all the normal H&S precautions when handling liquid nitrogen

### 3.2 Thawing Cells

It is important that cells are rapidly thawed and transferred into fresh growth medium as soon as possible. DMSO is cytotoxic when cells are thawed. We recommend recovering cells into an adherent culture for ease of replacing the freezing mixture with fresh medium and monitoring the cells under the microscope.
Protocol:

1. Rapidly defrost the cells e.g., in a water bath at 30°C until just thawed.

2. Decontaminate the outside of the cryovial by misting with 70% ethanol spray before transferring the contents to a sterile 30mL universal tube containing 10mL of pre-warmed (~28°C) culture medium. Perform a cell count and record the initial viability.

3. Divide the 10mL between two T25 flasks to give a final volume of 5mL in each flask.

4. Transfer the flasks to a 28°C incubator and allow the cells to attach to the flask for 45-60 minutes.

5. Monitor the cells under the microscope. Live cells should have attached firmly within the incubation period; dead cells will float. Remove the freezing medium and any floating cells. Replace with 5mL of fresh medium.

6. Continue to incubate the cells until a confluent monolayer is formed and then passage/sub-culture as previously described, setting up a suspension culture after 2-3 passages if required.

7. Alternatively, the whole 1mL contents of the cryovial can be placed in a 125mL shake/stirred flask containing 25mL pre-warmed fresh medium and incubated as required for shake/stirred cultures. Remove 5mL as a back-up into a T25 monolayer culture flask (and treat as above-steps 4-5). Continue the culture until the cell density is $2 \times 10^6$ cells/mL and passage as normal.

It is recommended that suspension cultures set up in this way are passaged 2-3 times before being used for experimental or virus work.
**Key points when thawing cells:**

- Thaw quickly in a clean water bath
- Sterilise the outside of the vial before opening
- Set up a monolayer culture first so it is easy to remove dead cells
- When cells are growing well, establish suspension cultures
4. Counting Cells and Determining Cell Viability

4.1 Counting Cells

Before passaging cells or using cells for virus infection or transfections, cells should be counted to establish an accurate count per mL. This can be achieved using a commercial cell counter or by using a standard haemocytometer. When establishing a new culture, new cell line or using a new medium, it is well worth setting up a growth curve and monitoring cell density every 24 hours to establish the growth pattern.

The following provides a protocol for counting cells using a Neubauer haemocytometer.

Protocol:

1. Take a sample of cells from a shake or spinner culture or cells harvested from a monolayer culture and using a Pasteur pipette, load the prepared Neubauer chamber using capillary action (attach cover slip firmly to form the counting chamber).

2. Count all the cells within the central 5 x 5 square grid (Figure 4) on the counting chamber using a phase-contrast microscope (x 10 objective). Count cells touching the etched triple line on the top and left of squares. Do not count cells touching the triple lines on the bottom or right of the squares.

3. Count cells on both 5 x 5 grids and average the results. If the cells are too dense to count accurately, dilute the sample an appropriate amount to get a countable number of cells. If the cells are clumped, they should be dispersed by gently pipetting up and down to get single cells; otherwise the cell count will be inaccurate. Ideally you need at least 30 cells and no more than 100.
4. The 5 x 5 square gives the number of cells present in 0.1µL of culture. To calculate the number of cells per mL, multiply by 10,000 \((10^4)\). If the cells were diluted before counting, remember to multiply the answer by the dilution factor.

**Key points when counting cells:**

- Ensure cells are in a single cell suspension and not clumped
- Count at least in duplicate to get an average
- Don’t forget to multiply answer by any dilution factor
Figure 4. Typical field of view when counting Sf9 cells using a Neubauer counting chamber.

The number of cells counted in this field of view = 56 so the cell density for this sample would be 56 x 10⁴ x 5 (as sample was diluted 1 in 5 to count) = 2.8 x 10⁶ cell/mL. Note the sample was not stained with Trypan Blue so this count represents total cell numbers. It is recommended you take an average cell count across a minimum of 3 samples.
4.2 Cell Viability

Cell viability should be tested from time to time and every time cells are used for virus work (transfections or virus infection) as poor viability is a common reason for failure of virus to amplify to high titres or ensure high yield of protein production.

The easiest method is Trypan Blue exclusion and this can conveniently be performed when counting cells. Trypan Blue is a vital stain that is actively excluded from live cells; therefore, dead cells take up and retain the stain appearing blue under the microscope. Healthy cells appear re-fractile, bright and clear. The percentage of dead cells can be calculated and used to determine the overall viability of the culture. A good culture should be at least 90% and preferably 95% viable.

Protocol:

1. Prepare a 2% (w/v) preparation of Trypan Blue (e.g. Sigma-Aldrich®) in PBS.

2. Dilute a sample of cells 1 in 1 with the stain (final concentration 1%) and view cells under a phase contrast microscope immediately. It is convenient to count the cells at this stage (see above protocol).

3. % viability = 100 - % dead cells
   % dead cells = total blue cells counted/total cells counted x 100

Key points when determining viability:

- Ensure cells are in a single cell suspension and not clumped
- Count at least in duplicate to get an average
- Don’t forget to multiply answer by two to take account of adding stain
- Don’t leave cells in stain for more than few minutes before determining viability
5. Establishing a New Culture from Living Cells

If cells are received from another lab or from a supplier (e.g., the baculoCOMPLETE kit from OET), you will need to establish a new culture as soon as the cells arrive. You will need to ensure that you have a supply of medium (see page 58), access to a laminar flow hood or class II safety cabinet, a supply of sterile flasks and pipettes, access to a cell counter or a Neubauer haemocytometer to count cells, an incubator set at 27-28°C (no CO₂ required), access to a phase contrast inverted microscope and Trypan Blue stain (e.g. from Sigma-Aldrich®)

Protocol:

1. View cells under an inverted light microscope and note if majority of cells are floating or attached as a monolayer.

2. Decontaminate the outside of the flask by spraying with 70% alcohol.

3. Place flask containing cells in a laminar flow hood or class II safety cabinet and use aseptic technique throughout all procedures.

4. If many cells are still attached as a monolayer, decant the medium and any floating cells into a discard container and replace with 5-6mL fresh medium (e.g. ESF 921).

If majority of cells are floating, decant medium and cells into a sterile centrifuge tube and gently pellet cells at 1000rpm for 5-10 minutes in a bench top centrifuge. Also add 5-6mL fresh medium to the attached cells in the original flask and incubate at 27-28°C until just about confluent (~80%).

5. Decant the old medium from the pelleted cells into discard and gently re-suspend the cells in 5-6mL of fresh medium to obtain a single cell suspension. Place the cells into a fresh T-flask, ensure
they are evenly dispersed over the surface, and incubate overnight. Any live cells will attach overnight. The following day, remove floating dead cells and replace with 5-6mL fresh medium and incubate as above until cells are just about confluent.

6. We recommend an initial 1 in 4 to 1 in 5 split of cells when just confluent. For example, harvest the cells by tapping the flask sharply on the bench to detach cells, and gently pipette up and down to get a single cell suspension. For example, place 1.25mL cells with 4.75mL fresh medium into a new T-flask.

Passage the cells through at least one further round of monolayer culture before setting up shake cultures.

7. The cells can be maintained like this in monolayer culture, expanding into larger T-flasks as required, or the cells can be grown in suspension/shake culture if cultured in serum-free medium.

8. To establish a shake culture, harvest cells from 2 x T25 monolayer cultures and transfer 8mL into a 125mL shaker flask containing 12mL fresh medium.

Ensure the lid is not on tightly to allow for aeration and shake at 100-110rpm at 27-28°C for 3-4 days and then count the cells.

9. Set up a fresh shake culture at 1 x 10^6 cells/mL with the appropriate amount of cell culture and fresh medium. Once the suspension culture is established, seed the newly passaged cells at 0.8 x 10^6 cells/mL or as required. Cells will grow to a density of 5-8 x 10^6 cells/mL.

10. Do not allow cells to overgrow in either monolayer or shake culture. For optimal virus infections, cells should be used in log growth phase. Undertaking a growth curve is useful to understand the growth characteristics of the culture.
11. We recommend that as soon as possible a batch of expanded cells are frozen down in liquid nitrogen to act as a source of low passage number cells for long term use. We normally passage cells for 30 passages before retrieving fresh stocks from liquid nitrogen.

12. For general information about insect cell culture read pages 63 to 72.
6. Establishing a New Culture of Cells from a Frozen Ampoule

On receipt, it is essential that the ampoule of frozen cells is either transferred to liquid nitrogen for storage or thawed to initiate a live cell culture. Do not freeze insect cells at -80°C.

You must use aseptic technique throughout and work in a class II safety hood or tissue culture laminar flow hood. Rinse or mist the vial of cells with 70% alcohol before opening.

Read through the general information about insect cell culture on pages 63 to 72 before starting and have the following materials available: suitable growth medium (e.g. ESF 921 from OET), T25/T75 monolayer flasks, 1mL and 10mL sterile pipettes, incubator at 27-28°C, water bath at ~37°C (best to use a ‘temporary bath’ such as a clean beaker with warm clean water rather than a dirty water bath).

Protocol:

1. On receipt, using aseptic technique, defrost the cells rapidly in a clean water bath at 37°C until just thawed.

2. Rinse or mist the outside of the vial with 70% alcohol and then transfer the contents of the ampoule between 2 x T25/T75 flasks containing 10-15mL fresh culture medium (e.g. ESF 921). Incubate the cells overnight at 27-28°C.

3. Check the cells under the microscope. Dead cells will be floating. Live cells will have attached.

4. Decant medium containing floating cells into discard. Replace with an appropriate amount of fresh media and continue to incubate cells until they form a just confluent monolayer. Check daily.
5. When ready, passage cells and set up further monolayer or suspension cultures as described

6. Cells can be used to prepare recombinant viruses as soon as they have recovered from shipping and are doubling approximately every 24 hours with a high viability (90% or more). This may take 2-3 passages of cells.

It is important that cells are not used to make recombinant viruses until they are growing well in a log phase culture.

7. As soon as possible prepare a stock of cells for freezing down for long term storage in liquid nitrogen (see page 73).
7. Adapting Cells to New Media

On occasion it is necessary to transfer cells into a new culture medium. Sometimes this can be achieved by simply using the new medium in place of the old. However, more often a period of adaption is required.

The accepted standard protocol is to culture the cells in 75% old medium: 25% new for one to two passages, then 50% old: 50% new for 1-2 passages followed by 75% new: 25% old before finally culturing the cells in the new medium. This takes time but ensure the cells adapt well to a new medium.

A quicker version of this method can often be successful. It simply involves culturing the cells in 50:50, old: new medium for one passage (monolayer or suspension culture) and then continuing to grow the cells in the new medium for at least one passage before using them for experimental work (e.g. amplifying virus or making recombinant viruses).

For this quicker protocol to be successful, it is important that the cells are in log phase of growth (80% confluent for monolayer or 3-5 x 10^6 cells/mL for suspension cultures) and that you monitor the cells each day to ensure they are still growing well.

The cells should be passaged as soon as they are confluent (monolayer cultures) or when they reach a density of 4-5 x 10^6 cells/mL (suspension culture). If the cells take a very long time to reach confluency or a density of 4-5 x 10^6 cells/mL, or cell viability drops very low, the longer adaption process described above will need to be followed.

7.1 Adapting Cells to ESF 921

We have successfully adapted Sf9 cells to ESF 921 (from a variety of media) using the quick adaption protocol described below.
Protocol:

1. Take a log phase culture of the cells to be adapted (80% confluent monolayer culture) or cells at 3-5 x 10^6 cells/mL for suspension cultures. Ensure the culture is at least 90% viable.

2. For suspension cultures, dilute the culture with an equal amount of the new medium and continue growing the cells until they reach 4-5 x 10^6 cells/mL.

   For monolayer cultures, passage the cells using 50% old medium saved from the previous culture and 50% new medium. Grow until the cells are just confluent.

3. Passage the cells as normal using the new medium.

4. Use cells for experimental work after 1-2 passages in the new medium.
8. Mycoplasma

Mycoplasmas are bacteria that can often be present in cell cultures and affect their growth. As they lack a cell wall around their membrane they are resistant to many antibiotics such as penicillin and are very small and difficult to detect using conventional microscopes. While contamination with mycoplasma is less common in insect cells, mammalian cell cultures are particularly susceptible.

If you obtain any cell cultures from a source that cannot certify them as mycoplasma-free you should initially quarantine them until you can perform the appropriate test. There are a variety of methods to do this but in our experience one based on quantitative PCR works best. Cells should be tested a minimum of every 6 months to ensure they are clean of mycoplasma.

Should your cultures become contaminated it is possible to treat with specialised antibiotics, but our advice would be to discard the infected cells and obtain a new sample from a reputable source.
9. References

2. Sf9 was cloned by G. E. Smith and C. L. Cherry from the parent line, IPLB-SF 21 AE, by Vaughn *et al.*, in 1977\(^1\).

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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 flashBAC™ baculovirus expression vectors and baculovirus qPCR titration kit, to transfection reagents and transfer plasmids. In addition we also stock a variety of Spodoptera frugiperda insect cell lines and culture media.

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