Baculoviruses are insect-specific viruses widely used for the production of many thousands of recombinant proteins, ranging from membrane-bound proteins to cytosolic enzymes. The baculovirus expression system was initially developed over twenty years ago (1), and since then has undergone numerous technological improvements to optimise expression of foreign genes within insect cell lines.

The expression system is based on the replacement of a very late, non-essential virus gene-coding region (polyhedrin, \textit{polh}) with the gene of interest. As the baculovirus genome is generally considered too large for direct insertion of a foreign gene, the gene of interest is first cloned into a transfer vector containing sequences that flank the \textit{polh} gene in the viral genome. Insect cells are then co-transfected with the virus DNA and transfer vector. Homologous recombination, between the flanking sequences common to both DNA molecules, facilitates the insertion of the gene of interest into the viral genome at the \textit{polh} locus, resulting in the production of a recombinant virus genome. The genome then undergoes replication within the host nucleus, generating recombinant virus containing the foreign gene-coding region under the control of the powerful \textit{polh} promoter, which then drives expression of the foreign gene.

Considerable efforts have been made in recent years to increase both the speed and ease of recombinant virus production, and this has resulted in the development of a number of unique baculovirus expression systems. There have also been novel modifications made to the virus genome to improve both quantity and quality of expressed proteins. This article gives a short overview of the most prominent of these systems, focusing in particular on the development and use of bacmids.

**CONVENTIONAL BACULOVIRUS EXPRESSION SYSTEMS**

Recombinant baculovirus expression vectors were originally produced relatively inefficiently by homologous recombination between a transfer vector containing the gene of interest and circular wild-type virus DNA. Insect cells were co-transfected with the baculovirus and transfer plasmid DNAs, producing a mixture of both recombinant and parental viruses, with recombinant viruses comprising less than 0.1 per cent of total virus produced. Isolation of recombinant virus was then achieved by tedious rounds of plaque purification, where recombinant clones were identified by their characteristic occlusion-negative plaque phenotypes.

To improve the efficacy of recombinant virus production, a unique \textit{Bsu}361 restriction enzyme site was engineered into the \textit{polh} locus of the baculovirus genome to permit linearisation of the viral genome prior to co-transfection, giving rise to a higher frequency of recombinant virus detection (2). Further improvements employed multiple \textit{Bsu}361 sites, with digestion of the viral DNA, and resulted in more effective linearisation and a partial

By Richard Hitchman at Oxford Expression Technologies Ltd and Linda King at Oxford Brookes University

Dr Richard Hitchman has been Research Manager at Oxford Expression Technologies (OET) Ltd since its spin-out from Oxford Brookes University. He obtained a PhD in Baculovirology from CEH-Oxford (formerly the Institute of Virology and Environmental Microbiology, Oxford, UK), and subsequently carried out research into the development of baculovirus expression vectors in the Insect Virus Research Group at Oxford Brookes University.

Professor Linda King is Dean of the School of Life Sciences and Professor of Virology at Oxford Brookes University. She is a Founding Director of OET Ltd and heads the Insect Virus Research Group within the University. Professor King obtained her DPhil in molecular insect virology from the University of Oxford and is co-inventor of the patented flashBAC™ technology.
Conversely, in Bac-N-BlueTM (Invitrogen) the linear viral Bac-N-Blue one round of plaque purification (see Figure 1D). but isolation of recombinant virus still required at least BaculoDirectTM (Invitrogen) is a further development of BaculoDirect recombinant virus population. required, however, to be sure of a homologous blue and easy to identify. Plaque purification is still expresses recombination occurs, the resulting recombinant virus vectors contain a 5' fragment of the lacZ gene and when 

BacPAK6 In BacPAK6, a Bsu361 restriction enzyme site was located within the lacZ gene along with two additional sites situated in the two genes flanking either side of lacZ (see Figure 1A). Digestion of BacPAK6 with Bsu361 removed the lacZ gene and a fragment of ORF1629, resulting in linear virus DNA incapable of replicating within insect cells (see Figure 1B). Co-transfection of insect cells with BacPAK6 DNA and a transfer vector containing the gene of interest restored ORF1629 and re-circularised the virus DNA by allelic replacement (see Figure 1C). Restoration of the essential virus gene permits replication within insect cells. Although the additional Bsu361 sites in BacPAK6 led to an increased proportion of recombinants compared with previous baculovirus expression systems, by reducing parental background, restriction enzyme digestion of virus DNA is never 100 per cent efficient and co-transfection still resulted in a mixture of both parental and recombinant virus. The presence of lacZ allowed the selection of colourless, recombinant virus plaques against a background of parental, blue plaques in the presence of X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopranoside) but isolation of recombinant virus still required at least one round of plaque purification (see Figure 1D).

Bac-N-Blue Conversely, in Bac-N-Blue™ (Invitrogen) the linear viral DNA was manipulated to contain the 3' sequence of the lacZ gene. Custom pBlueBac and pMelBac transfer vectors contain a 5' fragment of the lacZ gene and when recombination occurs, the resulting recombinant virus expresses β-galactosidase and recombinant plaques are blue and easy to identify. Plaque purification is still required, however, to be sure of a homologous recombinant virus population.

BaculoDirect BaculoDirect™ (Invitrogen) is a further development of the linear DNA system. This utilizes linear DNA but is based on Gateway® technology (4), which allows the direct transfer of a gene into the baculovirus genome, without the need for the propagation of recombinant bacmid DNA. It is based on the site-specific recombination properties of the bacteriophage lambda, which facilitates the integration of lambda into the E. coli chromosome. Integration is mediated by a mixture of enzymes and occurs via intermolecular DNA recombination at specific attachment (att) sites. BaculoDirect™ linear DNA contains attR1 and attR2 recombination sites located at the polh locus, along with a Herpes simplex virus thymidine kinase gene (HSV1 tk) and a lacZ gene located between attR sites. During the in vitro recombination reaction, attR sites within the virus DNA undergo recombination with the attL sites within a transfer vector, resulting in integration of the foreign gene into the virus genome. Recombinant virus DNA is then used to directly transfect insect cells and recombinant viruses are isolated by the use of the selective nucleoside analog ganciclovir. Ganciclovir is enzymatically phosphorylated by HSV1 tk (present in non-recombinant genomes), and results in the incorporation of this nucleoside into DNA, thereby inhibiting DNA replication. However, recombinant viruses that have lost the counter-selectable marker via homologous recombination are able to replicate within insect cells.
This system appears to offer significant time-savings compared with other systems, but is restricted to the production of recombinant single gene baculoviruses preventing co-expression of multiple proteins. In addition, the necessity to selectively isolate recombinant from parental virus via the addition of ganciclovir is detrimental to total cell numbers. This may reduce final viral titres, ultimately requiring multiple virus amplification steps before a high titre working virus stock is produced.

**BacVector-3000**

BacVector-3000 (Novagen) is a traditional ‘triple-cut system’ that has two gene deletions designed to maximise protein production. Chitinase (chiA) blocks the endoplasmic reticulum, severely compromising the function and efficacy of the secretory pathway (5,6). Removal of chitinase also facilitates chitin-affinity chromatography for purification without interference from the chiA gene product. Cathepsin (v-cath) is a protease and degrades susceptible recombinant proteins, particularly in the latter stages of infection when the polyh promoter is most active (7). Complex secretory or membrane-bound glycoproteins are often more difficult to express and are generally produced at lower levels by the baculovirus system, compared with cytoplasmic or nuclear proteins. However, deletion of chiA and v-cath has been shown to greatly enhance the production of these proteins.

**BACMID-BASED EXPRESSION SYSTEMS**

There are now several systems available commercially which have incorporated bacterial artificial chromosomes (BACs) into the baculovirus genome. These BAC replicons allow the virus DNA to be maintained and amplified within bacterial cells as bacmids, in a similar manner to a large plasmid. These systems have sought to remove the time-consuming and technically difficult step of plaque purification and in doing so have facilitated the use of automation for the generation of multiple recombinant viruses in multi-well plate formats.

**Bac-to-Bac**

Invitrogen initially developed a bacmid system – Bac-to-Bac® – based on in vivo bacterial site-specific transposition of a gene from a transfer vector into a bacmid within *E. coli* (8). The bacmid contains a low-copy number mini-F replicon, a kanamycin resistance gene and the lacZα gene. Located at the N-terminus of the lacZα gene is an attachment site for the bacterial transposon Tn7 that does not interrupt the reading frame of the lacZα peptide. Prior to the production of a recombinant baculovirus, the gene of interest is cloned into a transfer vector containing a baculovirus promoter. The plasmid is flanked by the left and right arms of the Tn7 transposon and contains a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini Tn7. The recombinant transfer vector is then transformed into *E. coli* cells containing the bacmid and a helper plasmid encoding a transposase. The mini Tn7 element from the recombinant transfer vector is transposed to the mini-attTn7 attachment site on the bacmid DNA, aided in trans by helper plasmid-encoded transposase. Insertion of the gene of interest into the bacmid disrupts the lacZα peptide resulting in white recombinant colonies when exposed to X-gal and IPTG (Isopropyl-β-D-thiogalactopyranoside) compared with blue parental colonies containing non-recombinant bacmid DNA. Having created the recombinant bacmid, the viral DNA must then be purified from bacterial cells prior to transfection into insect cells.

Site-specific transposition of genes into bacmid DNA has a number of advantages over homologous recombination within insect cells for the production of recombinant baculoviruses. Primarily, the viral DNA isolated from selected bacterial colonies is not mixed with parental DNA, eliminating the need to isolate recombinant from non-recombinant virus by multiple rounds of plaque-purification. This substantially reduces the time required to isolate and purify recombinant virus. The Bac-to-Bac® expression system is also compatible with Invitrogen’s Gateway® cloning vectors, a recombinatorial system that facilitates rapid transfer of heterologous sequences between vectors, making Bac-to-Bac® a highly versatile system for multi-parallel protein expression. However, the bacmids generated require several passages to obtain a high titre working stock, and there have been reports of inherent instability of recombinant baculovirus propagated via bacmid transposition, resulting in spontaneous deletions of the transposed bacmid sequences.

**MultiBac**

MultiBac (ETH Zurich/Redbiotec AG) is a bacmid-based system that incorporates two cloning sites for Tn7 site-specific transposition and Lox-P site-specific recombination in *E. coli* (9). Both chiA and v-cath have also been disrupted by the incorporation of the Lox-P into these sites. The system utilises custom vectors that contain multiplication modules allowing the incorporation of heterologous genes with a minimum requirement for unique restriction sites. This facilitates the integration and expression of multiprotein complex subunits and also allows the integration of enzymes for modifying the proteins being expressed. This system offers the ability to express complex subunits from a single expression vector.
**flashBAC**

At Oxford Expression Technologies, we have fused the advantages of the traditional homologous recombination system within insect cells to those of the bacmid-based systems described above. **flashBAC** (10) is a recent technology, which was developed primarily to remove the requirement for a selection system when isolating recombinant from parental virus – that is, by plaque purification or use of antibiotics or nucleoside analogs. Consequently, this has resulted in a simple one-step purification or use of antibiotics or nucleoside analogs. The system is based on a modified bacmid, where the **bac** replicon has replaced the **polh**. This allows the virus genome to be maintained and propagated within bacterial cells from which the circular DNA is then isolated and purified. The bacmid also contains a deletion in the essential gene ORF1629, which prevents the purified virus DNA from replicating within insect cells (see Figure 2A). Homologous recombination between **flashBAC** DNA and a suitable transfer vector containing the foreign gene, following transfection into insect cells, restores the deletion within – and thus the function of – the essential ORF1629 gene. Simultaneously, the foreign gene is inserted into the viral genome at the **polh** locus under the control of the **polh** promoter, concomitantly removing the **BAC** replicon (see Figure 2B).

Removal of the bacmid sequences is unique to **flashBAC** and ensures genomic stability within insect cells. The recombinant virus genome, with the restored essential gene, is then capable of replication within insect cells, producing recombinant budded virus particles that can be harvested from the culture medium of the transfected cells. The deletion within ORF1629 prevents replication of any non-recombinant, parental virus; therefore, there is no requirement for recombinant virus isolation. Following transfection, virus can be added directly to insect cells to produce a high-titre working recombinant virus stock (see Figure 2C). This one-step process greatly facilitates the use of automation for the generation of multiple expression vectors. Consequently, the increased use of robotic systems for high throughput production is resulting in more laboratories employing baculoviruses as their expression system of choice.

The authors can be contacted at r.hitchman@oetltd.com and laking@brookes.ac.uk

**CONCLUSION**

Manipulation of the baculovirus genome provides a powerful tool for the expression of recombinant proteins in insect cells. Advances in vector design and the availability of a range of baculovirus expression systems has resulted in many more user-friendly recombinant virus production systems. **flashBAC** represents a recent advance in the field; it negates the requirement for virus isolation and purification, incorporates deletions to dramatically improve protein yields and facilitates the use of automation for the generation of multiple expression vectors. Consequently, the increased use of robotic systems for high throughput production is resulting in more laboratories employing baculoviruses as their expression system of choice.

**References**


**Figure 2:** Schematic representation of recombinant virus production using the flashBAC™ system

The **flashBAC™** expression system is based on a modified baculovirus genome containing a bacterial artificial chromosome (BAC) at the polyhedrin (polh) locus and a partial deletion of the essential ORF1629 viral gene (A). Homologous recombination within insect cells between the **flashBAC™** DNA and a transfer vector containing a foreign gene and the complete ORF1629 gene coding region, results in the insertion of the foreign gene at the polh locus and restoration of ORF1629 (B). As only recombinant viruses with a restored ORF1629 can replicate, it results in a monoclonal recombinant virus population. This is then used to infect a larger insect cell culture (50-200ml) producing a high-titre working stock (C).