Invitrogen™
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Instruction Manual

Bac-to-Bac® Baculovirus Expression Systems

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10608-016

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1.1 Important Information

This product is authorized for laboratory research use only. The product has not been qualified or found safe and effective for any human or animal diagnostic or therapeutic application. Uses for other than the labeled intended use may be a violation of applicable law.

1.2 Note for European Customers

DH10Bac™ competent cells are genetically modified and carry the pBR322-derived plasmid pMON7124 (*bom*$^{+}$*, tra*$^{-}$*, mob*$^{-}$). As a condition of sale, this product must only be used in accordance with all applicable local legislation and guidelines including EC directive 90/219/EEC on the contained use of genetically modified organisms.

1.3 Precautions

Warning: This product contains hazardous reagents. It is the end-user's responsibility to consult the applicable MSDS(s) before using this product. Disposal of waste organics, acids, bases, and radioactive materials must comply with all appropriate federal, state, and local regulations. If you have any questions concerning the hazards associated with this product, please call the Invitrogen Environmental Health and Safety Chemical Emergency hotline at (301) 431-8585.

1.4 Limited Label Licenses

Limited Label License No. 21:

This product is sold under patent license from Monsanto for research purposes only and no license for commercial use is included. Requests for licenses for commercial manufacture or use should be directed to Director, Monsanto Corporate Research, 800 N. Lindbergh, St. Louis, Missouri 63167.

Limited Label License No. 22:

Vectors are manufactured for Life Technologies, Inc. by QIAGEN, Inc. This product is provided with a license for research use only. Information in respect of licenses to use the product for purposes other than research may be obtained from F. Hoffmann-La Roche Ltd., Corporate Licensing, 4002 Basel Switzerland. Ni-NTA resin may be purchased from QIAGEN, Inc., 9600 De Soto Ave., Chatsworth, California 91311. (800-426-8157).

2 Overview

2.1 Principles of Baculovirus Expression Vectors

Recombinant baculoviruses have become widely used as vectors to express heterologous genes in cultured insect cells and insect larvae. Heterologous genes placed under the transcriptional control of the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) are often abundantly expressed during the late stages of infection. In most cases, the recombinant proteins are processed, modified, and targeted to their appropriate cellular locations, where they are functionally similar to their authentic counterparts (1–7).

A number of unique features distinguish the baculovirus expression vector system from other expression systems:

- High levels of heterologous gene expression are often achieved compared to other eukaryotic expression systems, particularly for intracellular proteins. In many cases, the recombinant proteins are soluble and easily recovered from infected cells late in infection when host protein synthesis is diminished.
- Expression of hetero-oligomeric protein complexes can be achieved by simultaneously infecting cells with two or more viruses or by infecting cells with recombinant viruses containing two or more expression cassettes.
- Baculoviruses have a restricted host range, limited to specific invertebrate species. These viruses are safer to work with than most mammalian viruses since they are noninfectious to vertebrates. Most of the susceptible insect cell lines are not transformed with pathogenic or infectious viruses and can be cared for under minimal containment conditions. Helper cell lines or helper viruses are not needed since the baculovirus genome contains all the genetic information needed for propagation in a variety of cell lines or larvae from different insect species.
- AcNPV is usually propagated in cell lines derived from the fall armyworm *Spodoptera frugiperda* or from the cabbage looper *Trichoplusia ni*. Prolific cell lines are available which grow well in suspension cultures, permitting the production of recombinant proteins in large-scale bioreactors.

AcNPV has a large (130 kb) circular double-stranded DNA genome with multiple recognition sites for many restriction endonucleases. As a result, recombinant baculoviruses are traditionally constructed in two steps. The gene to be expressed is first cloned into a plasmid transfer vector downstream from a baculovirus promoter that is flanked by baculovirus DNA derived from a nonessential locus, usually the polyhedrin gene. This plasmid is then introduced into insect cells along with circular wild-type genomic viral DNA. Typically, 0.1% to 1% of the resulting progeny are recombinant, with the heterologous gene inserted into the genome of the parent virus by homologous recombination *in vivo*. Recombinant viruses containing the heterologous gene inserted into the polyhedrin locus, for example, are identified by an altered plaque morphology which is characterized by the absence of occluded virus in the nucleus of infected cells. The desired occlusion-minus plaque phenotype is not always obvious against the background of > 99% wild-type parental viruses.

The fraction of recombinant progeny virus can be improved to nearly 30% by using a parent virus that is linearized at one or more unique sites located near the target site for insertion of the foreign gene into the baculovirus genome (8,9). A higher proportion of recombinant viruses (80% or higher) can be achieved using linearized viral DNA that is missing an essential portion of the baculovirus genome

downstream from the polyhedrin gene (10). Sequential plaque assays are required with each of these approaches to purify the recombinant virus away from the non-recombinant parental virus that contaminates the progeny virus after transfecting the plasmid and viral DNAs into insect cells. Plaque purifying the desired recombinant virus and confirming its DNA structure or using immunological methods to identify recombinant viruses expressing the desired protein can easily take more than a month to complete (5–7).

2.2 Summary of the Bac-to-Bac® Baculovirus Expression System

Recently, a rapid and efficient method to generate recombinant baculoviruses was developed by researchers at Monsanto (11) (figure 1). It is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli*. The bacmid (bMON14272) contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the *lac*Zα peptide from a pUC-based cloning vector. Inserted into the N-terminus of the *lac*Zα gene, is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-*att*Tn7) that does not disrupt the reading frame of the *lac*Zα peptide. The bacmid propagates in *Escherichia coli* DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a *lac*Z deletion present on the chromosome to form colonies that are blue (Lac$^+$) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG.

Recombinant bacmids (sometimes referred to as composite bacmids) are constructed by transposing a mini-Tn7 element from a pFastBac™ donor plasmid to the mini-*att*Tn7 attachment site on the bacmid when the Tn7 transposition functions are provided *in trans* by a helper plasmid (pMON7124). The helper plasmid confers resistance to tetracycline and encodes the transposase. A series of pFastBac™ donor plasmids are available which share common features (figure 2). Each vector has a baculovirus-specific promoter (*i.e*, the polyhedrin or p10 promoter from AcNPV) for expression of proteins in insect cells. The mini-Tn7 in a pFastBac™ donor plasmid (figure 2) contains an expression cassette consisting of a Gm^r gene, a baculovirus-specific promoter, a multiple cloning site, and an SV40 poly(A) signal inserted between the left and right arms of Tn7.

The plasmid pFastBac™ 1 (12) is used to generate viruses which will express unfused recombinant proteins. The pFastBac™ DUAL vector (14) has two promoters and cloning sites, allowing expression of two genes: one from the polyhedrin promoter and one from the p10 promoter. Genes to be expressed are inserted into the multiple cloning site of a pFastBac™ donor plasmid downstream from the baculovirus-specific promoter. Insertions of the mini-Tn7 into the mini-*att*Tn7 attachment site on the bacmid disrupts expression of the *lac*Zα peptide, so colonies containing the recombinant bacmid are white in a background of blue colonies that harbor the unaltered bacmid. Recombinant bacmid DNA can be rapidly isolated from small scale cultures and then used to transfect insect cells. Viral stocks (>10^7 pfu/ml) harvested from the transfected cells can then be used to infect fresh insect cells for subsequent protein expression, purification, and analysis.

Using site-specific transposition to insert foreign genes into a bacmid propagated in *E. coli* has a number of advantages over the generation of recombinant baculoviruses in insect cells by homologous recombination. Recombinant virus DNA isolated from selected colonies is not mixed with parental, nonrecombinant virus, eliminating the need for multiple rounds of plaque purification. As a result, this greatly reduces the time it takes to identify and purify a recombinant virus from 4 to 6 weeks (typical for conventional methods) to within 7 to 10 days. Perhaps the greatest advantage of this method is that it permits the rapid and simultaneous isolation of multiple recombinant viruses, and is particularly suited for the expression of protein variants for structure/function studies.

Overview

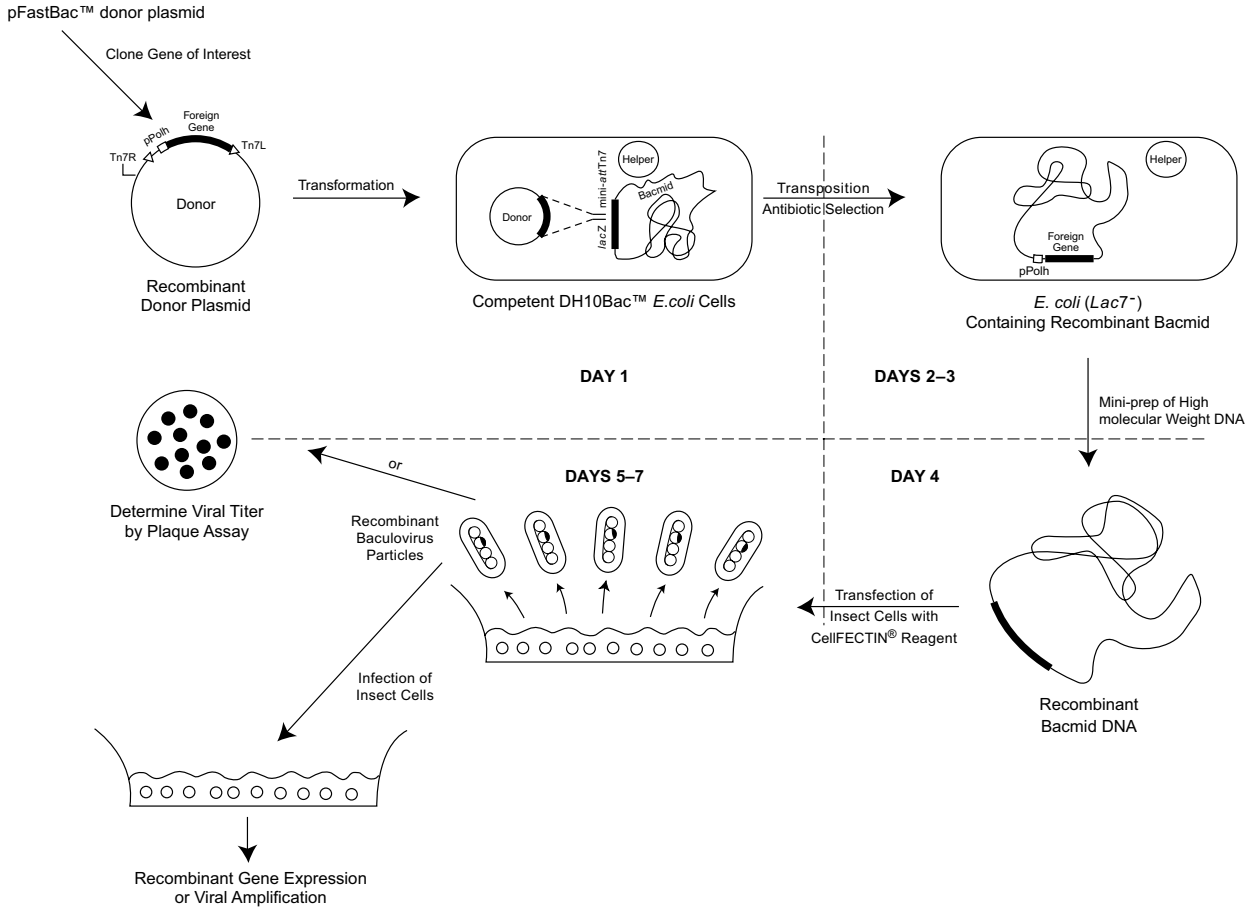
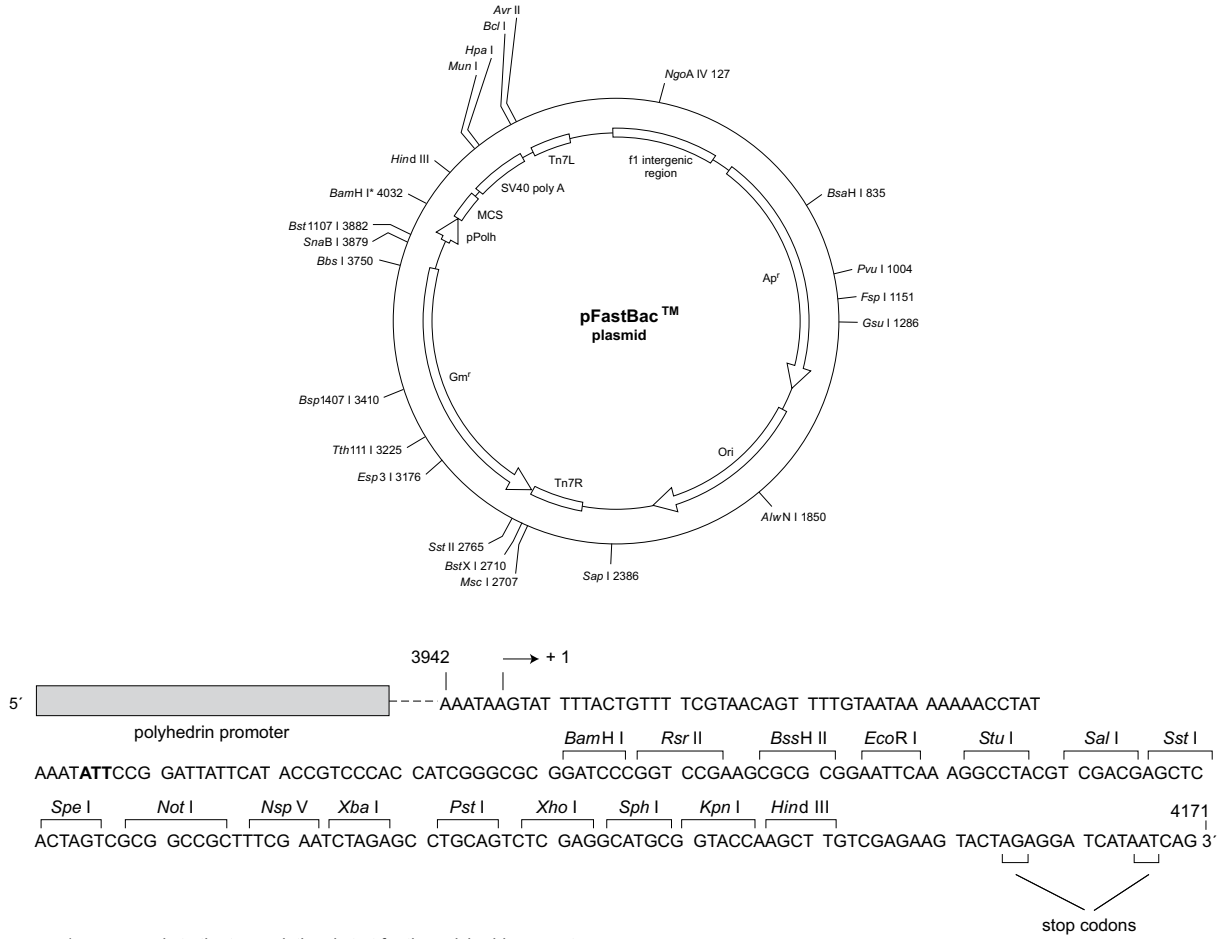


Fig. 1 Generation of recombinant boculovirus and gene expression with the Bac to Bac® Expression System.

Figure 1. Generation of recombinant baculoviruses and gene expression with the Bac-to-Bac® Expression System. The gene of interest is cloned into a pFastBac™ donor plasmid, and the recombinant plasmid is transformed into DH10Bac™ competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac™ donor plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the *lacZ*α gene. High molecular weight mini-prep DNA is prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA is then used to transfect insect cells.



+1 corresponds to the transcriptional start for the polyhedrin promoter.

ATT—site of original translational start. The ATG was mutated to an ATT.

Figure 2. Map and features of a pFastBac™ donor plasmid.

Figure 2. Map and features of a pFastBac™ donor plasmid. Due to differences in the multiple cloning site regions, see detailed maps of the vectors in the appendix for the locations of restriction endonucleases from *Bam*H I to *Avr* II.

3 Methods

3.1 Components

The components of the Bac-to-Bac® Baculovirus Expression Systems are as follows. Components are provided in sufficient quantities to perform 10 to 20 cloning reactions into the pFastBac™ donor plasmid, 5 transformations into DH10Bac™ Competent Cells, and ~200 transfections with CellFECTIN® Reagent. See the related products section for the specific combinations of components.

Component	Storage
pFastBac™ donor plasmid	–20°C
control plasmid (pFastBac™-Gus or pFastBac™ HT-CAT)	–20°C
MAX Efficiency® DH10Bac™ competent cells	–70°C
CellFECTIN® reagent	4°C
manual	

3.2 Additional Materials

The following items are required for use with the Bac-to-Bac® Baculovirus Expression System, but are *not* included in the system:

Equipment:

- microcentrifuge
- 37°C incubator
- water baths
- centrifuge

Reagents:

- restriction endonucleases
- T4 DNA ligase
- *E. coli* competent cells
- ampicillin
- gentamicin
- kanamycin
- tetracycline
- Bluo-gal
- IPTG
- RNase A
- NaOH
- SDS
- KOAc
- isopropanol
- 70% ethanol
- Luria Agar
- LB Medium
- LB agar plates
- S.O.C. Medium
- Tris-HCl (pH 8.0)

General Materials and Solutions:

- autoclaved microcentrifuge tubes
- 15-ml tubes (Falcon® 2059)
- autoclaved distilled water
- TE buffer

Cell Culture Materials:

- *Spodoptera frugiperda* Sf9 cells
- Sf-900 II Serum-Free Medium (SFM)
- plasticware (6- and 24-well tissue culture-treated plates)

An overview of the Bac-to-Bac® Baculovirus Expression System procedure is illustrated in figure 3.

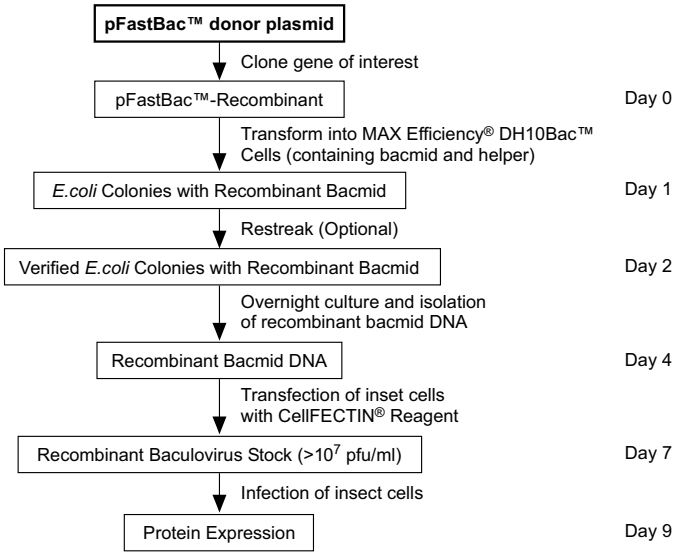


Figure 3. Outline of the Bac to Bac® Baculovirus Expression System procedure.

Figure 3. Outline of the Bac-to-Bac® Baculovirus Expression System procedure.

3.3 Cloning into pFastBac™ Donor Plasmids

A series of donor plasmids have been developed which are compatible with the Bac-to-Bac® Baculovirus Expression System. The first step in using the Bac-to-Bac® Baculovirus Expression System is to clone your gene of interest into a pFastBac™ donor plasmid. Care must be taken in selecting the appropriate restriction endonuclease for successful cloning. Refer to the product-specific documentation for information about cloning into each specific pFastBac™ donor plasmid.

In general, prepare pFastBac™ donor plasmid DNA and the foreign DNA fragment by digesting 500 ng to 1 µg DNA with the selected restriction endonuclease(s) under the appropriate conditions. If only one site in the vector is chosen as the cloning site, dephosphorylate the vector under the appropriate conditions. DNA fragments can be purified by agarose gel electrophoresis and the fragments of interest can be recovered from the gel by using a Concert™ DNA purification system or an equivalent purification. Ligate the prepared vector and insert fragments under the appropriate conditions.

Methods

For this initial cloning, *do not* use the DH10Bac™ cells included in the system. DH5α™ or DH10B™ competent cells can be used. Plate the transformation mix onto LB agar plates containing 100 μg/ml ampicillin.

For analysis of a directional cloning experiment, 6 colonies should be sufficient to screen; 12 or more may need to be analyzed for a nondirectional cloning strategy. Prepare plasmid DNA from overnight cultures using a mini-preparation procedure (16) and verify correct insertion of the gene of interest by restriction endonuclease digestion or PCR analysis.

After the recombinant pFastBac™ donor plasmid has been determined to be correct, the DNA is transformed into DH10Bac™ for transposition into the bacmid. The transposition assay and subsequent transfection steps are the same for all vectors.

Notes: Use of Lennox L (LB) agar instead of Luria agar will reduce color intensity and may reduce the number of colonies. Use of X-gal instead of Bluo-gal will decrease color intensity.

3.4 Transposition

1. Prepare Luria Agar plates containing:
   - 50 μg/ml kanamycin
   - 7 μg/ml gentamicin
   - 10 μg/ml tetracycline
   - 100 μg/ml Bluo-gal
   - 40 μg/ml IPTG
   - See additional protocols for formulations (Sections 5.1 and 5.2).
2. Thaw the DH10Bac™ competent cells on ice.
3. Dispense 100 μl of the cells into 15-ml round-bottom polypropylene tubes.
4. Add approximately 1 ng recombinant donor plasmid (in 5 μl) and gently mix the DNA into the cells by tapping the side of the tube.
5. Incubate the mixture on ice for 30 min.
6. Heat shock the mixture by transferring to 42°C water bath for 45 s.
7. Chill the mixture on ice for 2 min.
8. Add 900 μl S.O.C. medium to the mixture.
9. Place the mixture in a shaking incubator at 37°C with medium agitation (225 rpm) for 4 h.
10. Serially dilute the cells, using S.O.C. medium, to 10^{-1}, 10^{-2}, 10^{-3} *(i.e.,* 100 μl of transposition mix: 900 μl of S.O.C. medium = 10^{-1} dilution, use this to further dilute 10-fold to give 10^{-2} dilution, and similarly for 10^{-3} dilution).
11. Place 100 μl of each dilution on the plates and spread evenly over the surface.
12. Incubate for 24 to 48 h at 37°C (Colonies are very small and blue colonies may not be discernible prior to 24 h).

Hints: True whites tend to be large colonies and the selection of false positives can be avoided by selecting the largest, most isolated candidates thus avoiding possible cross contamination. By successively holding the plate over a dark and light background it is easier to determine whether a colony is blue or white (whites are more distinguishable against a dark background and blues against a light background).

3.5 Isolation of Recombinant Bacmid DNA

White colonies contain the recombinant bacmid, and therefore, are selected for isolation of recombinant bacmid DNA. Before isolating DNA, candidate colonies are streaked to ensure they are truly white.

1. Select white colonies from a plate with approximately 100 to 200 colonies. **Note:** This number facilitates differentiation between blue and white colonies.
2. Pick ~10 white candidates and streak to fresh plates to verify the phenotype. Incubate overnight at 37°C.
3. From a single colony confirmed as having a white phenotype on plates containing Bluo-gal and IPTG, set up a liquid culture containing antibiotics (kanamycin, gentamicin, and tetracycline) for isolation of recombinant bacmid DNA.

Note: DH10Bac™ colonies may also be screened by PCR for the presence of the desired sequence(s). This may be used as an alternative to blue/white screening or to confirm the insert sequence.

The following protocol developed for isolating large plasmids (>100 kb)(17) was adapted for isolating bacmid DNA. Alternatively, the CONCERT High Purity Plasmid Isolation System can be used to isolate the Bacmid DNA (24).

1. Using a sterile toothpick, inoculate a single, isolated bacterial colony into 2 ml LB medium supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline. A 15-ml snap-cap polypropylene tube is suitable. Grow at 37°C to stationary phase (up to 24 h) shaking at 250 to 300 rpm.

Notes: Solutions I and II should be filter-sterilized. Store Solution I at 4°C. 3 M potassium acetate should be autoclaved and stored at 4°C.

2. Transfer 1.5 ml of culture to a 1.5-ml microcentrifuge tube and centrifuge at 14,000 × *g* for 1 min.
3. Remove the supernatant by vacuum aspiration and resuspend (by gently vortexing, or pipetting up and down, if necessary) each pellet in 0.3 ml of Solution I [15 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A]. Add 0.3 ml of Solution II (0.2 N NaOH, 1% SDS) and gently mix. Incubate at room temperature for 5 min. **Note:** The appearance of the suspension should change from very turbid to almost translucent.
4. Slowly add 0.3 ml of 3 M potassium acetate (pH 5.5), mixing gently during addition. A thick white precipitate of protein and *E. coli* genomic DNA will form. Place the sample on ice for 5 to 10 min.
5. Centrifuge for 10 min at 14,000 × *g*. During the centrifugation, label another microcentrifuge tube and add 0.8 ml absolute isopropanol to it.
6. Gently transfer the supernatant to the tube containing isopropanol. Avoid any white precipitate material. Mix by gently inverting tube a few times and place on ice for 5 to 10 min.

   At this stage, the sample can be stored at –20°C overnight.
7. Centrifuge the sample for 15 min at 14,000 × *g* at room temperature.
8. Remove the supernatant and add 0.5 ml 70% ethanol to each tube. Invert the tube several times to wash the pellet. Centrifuge for 5 min at 14,000 × *g* at room temperature. (Optional: repeat step 8.)

Note: To avoid shearing, do not *mechanically* resuspend the DNA.

9. Remove as much of the supernatant as possible. **Note:** The pellet may become dislodged from the bottom of the tube, so it is better to carefully aspirate the supernatant than to pour it.
10. Air dry the pellet briefly, 5 to 10 min, at room temperature and dissolve the DNA in 40 µl TE. Allow the solution to sit in the tube with occasional gentle tapping of the bottom of the tube. The DNA is generally ready for use within 10 min, as long as the pellets are not overdried.
11. Store the DNA at –20°C. However, avoid repeated freeze/thaw cycles to avoid a drastic reduction in transfection efficiency.

    PCR analysis is recommended to verify successful transposition to the bacmid. See section 5.3.2 for protocol. Alternatively, preparations of bacmid DNA may be analyzed by agarose gel electrophoresis to confirm the presence of high molecular weight DNA. Use the protocol in Section 5.3 for best results.

3.6 Transfection of Sf9 Cells with Recombinant Bacmid DNA

Notes: If using other medium, use medium without sera, supplements or antibiotics for making DNA-lipid complexes and during transfection.

1. Seed 9 × 10^5 cells per 35-mm well (of a 6-well plate) in 2 ml of Sf-900 II SFM containing penicillin/streptomycin at 0.5X final concentration (50 units/ml penicillin, 50 µg/ml streptomycin). Use only cells from a 3- to 4-day-old suspension culture in mid-log phase with a viability of >97%.
2. Allow cells to attach at 27°C for at least 1 h.
3. Prepare the following solutions in 12 × 75-mm sterile tubes:

   Solution A: For each transfection, dilute ~5 µl of mini-prep bacmid DNA into 100 µl Sf-900 II SFM without antibiotics.

   Solution B: For each transfection, dilute ~6 µl CellFECTIN® Reagent into 100 µl Sf-900 II SFM without antibiotics. **Note**: CellFECTIN® Reagent is a lipid

Notes: The amount of bacmid DNA and CellFECTIN® Reagent should be optimized for each cell line. The recommended amounts are guidelines *only*.

suspension that may settle with time. Mix thoroughly by inverting the tube 5-10 times before removing a sample for transfection to ensure that a homogeneous sample is taken.

4. Combine the two solutions, mix gently, and incubate for 15 to 45 min at room temperature.

Note: After collecting the viral supernatant at 72 h, the cells in the well may be analyzed for protein expression *in situ* or in cell lysates.

5. Wash the cells once with 2 ml of Sf-900 II SFM without antibiotics.
6. For each transfection, add 0.8 ml of Sf-900 II SFM to each tube containing the lipid-DNA complexes. Mix gently. Aspirate wash media from cells and overlay the diluted lipid-DNA complexes onto the cells.
7. Incubate cells for 5 h in a 27°C incubator.
8. Remove the transfection mixtures and add 2 ml of Sf-900 II SFM containing antibiotics. Incubate cells in a 27°C incubator for 72 h.
9. Harvest virus from cell culture medium at 72 h post-transfection.

3.7 Harvest/Storage of Recombinant Baculovirus

1. When harvesting virus from the transfection, transfer the supernatant (2 ml) to a sterile, capped tube. Clarify by centrifugation for 5 min at 500 × *g* and transfer the virus-containing supernatant to a fresh tube.
2. From the initial transfection, viral titers of 2×10^7 to 4×10^7 pfu/ml can be expected.
3. Store the virus at 4°C, protected from light. For long term storage of virus, the addition of fetal bovine serum (FBS) to a final concentration of at least 2% FBS is recommended. Storage of an aliquot of the viral stock at –70°C is also recommended.
4. Determine the viral titer before amplifying the virus stock or analyzing protein expression. See Section 5.13 for plaquing procedures.
5. For amplifying viral stocks, infect a suspension or monolayer culture at a Multiplicity of Infection (MOI) of 0.01 to 0.1. Use the following formula:

$$\text{Inoculum required (ml):} \quad \frac{\text{desired MOI (pfu/ml)} \times \text{(total number of cells)}}{\text{titer of viral inoculum (pfu/ml)}}$$

For example, infect a 50-ml culture at 2×10^6 cells/ml with 0.5 ml of a viral stock that is 2×10^7 pfu/ml, for an MOI of 0.1.

Harvest virus at 48 h post-infection. This will result in approximately 100-fold amplification of the virus. Infections over 48 h at an appropriate MOI will yield lower quality of virus stocks.

3.8 Infection of Insect Cells with Recombinant Baculovirus Particles

Optimal infection conditions for insect cells can vary. A starting point for infection is an MOI of 5 to 10. For more information, please refer to reference 2. It is recommended that several experiments be performed for each protein to be expressed.

- ***MOI optimization:*** Infect a population of cells at varying MOIs (*e.g.,* 1, 2, 5, 10) and assay protein expression upon harvesting the cells (or media, if the protein is secreted).
- ***Time course:*** Infect cells at a constant MOI. Harvest cells (or media) at the following time intervals: 24 h, 48 h, 72 h, and 96 h. Assay for expression.

Troubleshooting Guide

4

4.1 General Troubleshooting Guidelines

Problem	Possible Cause	Suggested Solution
Cloning into Donor Plasmids:		
Recombinant donor plasmid lacks insert	Incomplete digestion of pFastBac™ donor plasmid or insert DNA	Use additional restriction endonuclease. Purify insert DNA.
	Incomplete or excessive phosphatase treatment of pFastBac™ donor plasmid	Adjust phosphatase conditions to manufacturer's recommendations.
	Poor recovery of pFastBac™ donor plasmid or insert DNA from agarose gel	Use Concert™ High Purity Plamid Purification System to purify DNA.
	Incomplete ligation reactions	Adjust ligation conditions to manufacturer's recommendations.
		Optimize ligation reaction by adjusting vector:insert molar ratios (*e.g.,* 1:3, 1:1, 3:1).
	Insert contains unstable DNA sequences such as LTR sequences and inverted repeats	Grow transformed cells at lower temperatures (30°C). Use Stbl2™ Competent Cells.
No or few colonies obtained when subcloning insert fragment	Transformation efficiency of *E. coli* is low	Use cells that transform at a higher efficiency.
	Impurities in DNA	Remove phenol, proteins, detergents, and ethanol from DNA solution.
	Excess DNA	For competent cells, add 1 to 10 ng of DNA in a volume of 5 µl or less per 100 µl of cells. For ElectroMAX™ cells, add 10 to 50 ng of DNA in a volume of 1 µl or less per 20 µl of cells.
	Incomplete ligation reaction	Optimize ligation reaction. To check, transform cells with vector DNA that has been digested with a restriction endonuclease and ligated. Check ligation reaction on a gel. Note: Ligated products are 10-fold less efficient in transformation reactions than super-coiled DNA. Linear DNA is 100-fold to 1,000-fold less efficient in transformation reactions than supercoiled DNA (18).
	Ligation reaction mix inhibits transformation of competent cells	Reduce amount of ligation mix used in the transformation. Dilute ligation mix 5-fold with TE buffer prior to transformation.
	Problem with antibiotic	Confirm use of correct antibiotic; confirm antibiotic concentration; and use fresh stock of antibiotic.
		Verify that antibiotic solutions are not degraded (*i.e.,* a change in color or precipitate indicate degradation).
	Improper heat shock procedure	Follow the recommended incubation conditions and use recommended tubes.
	Competent cells handled improperly	Thaw cells on ice; use immediately after thawing; and do not vortex.
	Competent cells stored improperly	Store at –70°C. Do not store in liquid nitrogen.
	Inadequate incubation period for cells after heat shock	Use S.O.C. medium for 1 h recovery/expression; check pH; and use highly purified water.

Problem	Possible Cause	Suggested Solution
Generating Recombinant Bacmid DNA:		
No white (recombinant) colonies obtained	Insufficient time for color development	Wait at least 24 h before identifying colony phenotypes.
	Used X-gal instead of Bluo-gal in agar plates	Use Bluo-gal in agar plates to increase contrast between blue and white colonies.
	Insufficient growth after transposition	Grow in S.O.C. medium for a minimum of 4 h before plating.
	Poor quality recombinant donor used to transform DH10Bac™ competent cells	Check DNA preparation for degraded DNA by gel electrophoresis.
	Too many colonies on the plate	Plate dilutions to give well-separated colonies.
	Gentamicin and/or tetracycline omitted from plates.	Prepare fresh plates.
	Plates too old or stored in light	Prepare fresh plates.
All colonies are white	Bluo-gal and IPTG omitted from agar plates	Prepare fresh plates.
	Kanamycin omitted from agar plates	Prepare fresh plates.
	Incubation period too short or temperature too low	Increase incubation time to >24 h and verify 37°C temperature.
Too few colonies obtained	Used LB medium for expression/ transposition	Use S.O.C. Medium for >4 h growth time.
Low percentage of white colonies obtained	Growth time after transformation of DH10Bac™ competent cells too short	Increase growth time to >4 h at 37°C or 6 h at 30°C.
	Too many colonies on plate	Adjust dilution of cells (10^{-2} to 10^{-4}) to obtain optimal number of cells.
Poor blue/white colony differentiation	Agar not at correct pH	Adjust Luria agar to pH 7.0.
	Intensity of blue color too weak	Use Bluo-gal at 100 to 300 µg/ml, not X-gal. Use dark and light backgrounds to view plates.
	Bluo-gal and IPTG added to agar surface	Add substrates throughout the agar.
	Too many or too few colonies on plate	Adjust dilution to give an optimal number of colonies.
	Incubation period too short or temperature too low	Increase incubation time to >24 h and temperature to 37°C.
	IPTG concentration not optimal	Optimize the IPTG concentration. A range of 20 to 60 µg/ml has been found to give optimal color development.
Isolation of Bacmid DNA:		
DNA is degraded	Improper storage conditions	Store mini-prep bacmid DNA aliquotted at –20°C. Do not freeze-thaw repeatedly.
	Improper handling of high molecular weight DNA	Do not vortex DNA preparation. Resuspend DNA pellets by gently mixing, not mechanically.
Poor yield	Improper antibiotic concentrations	Verify antibiotic concentrations for overnight, liquid cultures.

Problem	Possible Cause	Suggested Solution
Transfection of Insect Cells:		
Low virus yield	Used low cell density	Use recommended cell density for infection.
	Used too much or too little lipid reagent	Optimize the amount of lipid reagent.
	Incubation of cells with lipid-DNA complex too short or too long	Optimize the time (*e.g.,* 3 to 8 h).
	DNA is degraded	Check DNA on gel before transfection.
Infection of Insect Cells:		
Low protein yield	Used too low or too high viral titer	Optimize viral titer for infection.
	Time of cell harvest not optimal	Determine optimal time for maximum protein expression.
	Cell line not optimal	Try other insect cell lines.
	Cell growth conditions and medium not optimal	Optimize conditions. Refer to the *Insect Cell Culture Guide* and Sections 5.7–5.9. Use Sf-900 II SFM for improved cell growth and protein expression.
	Virus not recombinant	Verify transposition by PCR of bacmid DNA with M13 forward and reverse primers.

Troubleshooting Guide

4.2 Using the Control DNA

Each Bac-to-Bac® Baculovirus Expression System contains a control DNA. The control DNA contains either β–glucuronidase (*gus*) or chloramphenicol acetyltransferase (*CAT*) as the reporter gene. Refer to the product-specific documentation for information about the control DNA. The control DNA can be used as a standard for transposition and transfection experiments. The following series of experiments is recommended:

1. Transform 100 µl of DH10Bac™ Competent Cells with 1 ng control DNA (see Section 3.4).
2. Select a white colony (it has undergone transposition and thus carries the recombinant bacmid containing the encoding reporter gene), and innoculate a 2-ml liquid culture. (See Section 3.5).
3. Isolate recombinant bacmid DNA from overnight cultures (See Section 3.5) and transfect into Sf9 or another insect cell line (see Section 3.6).
4. At 48 to 72 h post-transfection, cells are assayed for the reporter gene. For gus activity, cells may be stained *in situ* using the protocol in Section 5.5. **Expected results**: By 48 h, islands of transfected cells expressing gus are blue. By 72 h, more extensive cell staining is observed as a result of infection of new cells by budded virus.

   For CAT activity, cells may be prepared and assayed using the protocol in Section 5.6. **Expected results**: Measurable CAT activity is observed 48 h post-transfection.
6. Recombinant baculovirus may be harvested from transfected cell supernatants (See Section 3.7) and titered (See Section 5.13) and/or used for re-infection of insect cells. **Expected results**: The titer of virus isolated from transfected Sf9 cells is $>1 \times 10^7$ pfu/ml.

Data obtained using pFastBac™-*gus* are illustrated in figures 4 and 5.

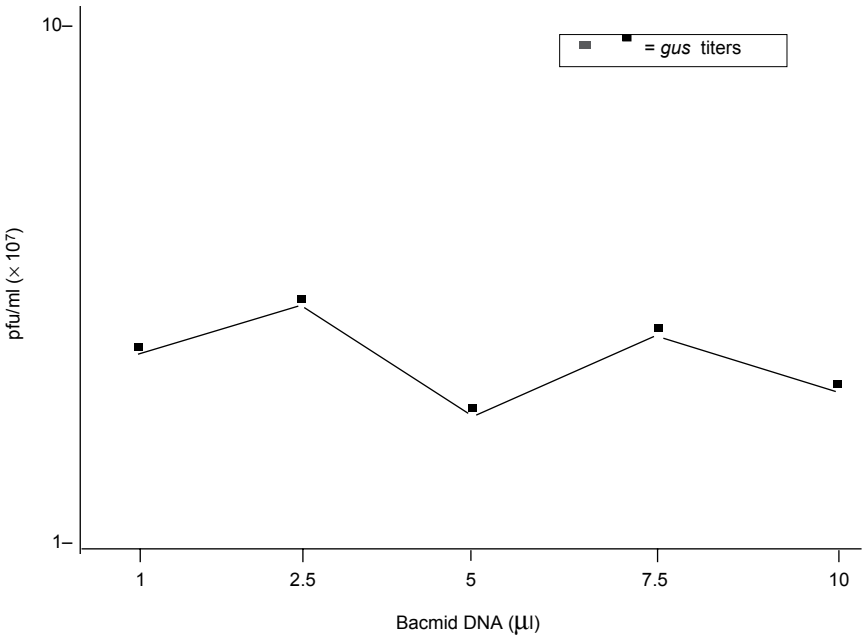


Figure 4. Yield of β-glucuronidase-recombinant virus following transfection of Sf9 cells with varying amounts of recombinant bacmid DNA. Recombinant bacmid DNA containing the *gus*A gene encoding β-glucuronidase was isolated from transposed DH10Bac™ colonies as described in Section 3.5 and transfected into Sf9cells using CellFECTIN® Reagent as described in Section 3.6. Viral supernatant was collected from the cells at 72 h post-transfection, and titer was determined by plaque assay (See Section 5.13). Results are indicated as pfu/ml of supernatant in one 35-mm well (2 ml total volume).

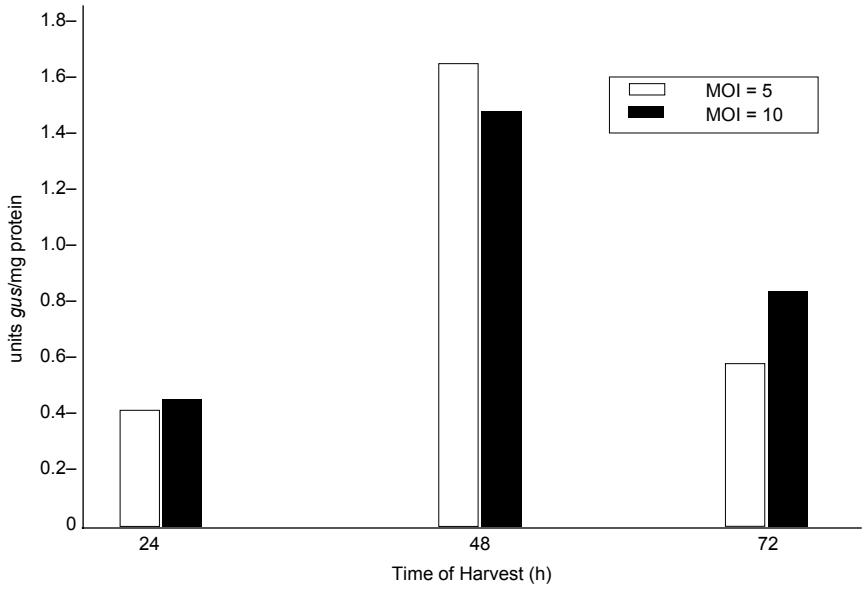


Figure 5. Effect of MOI and time of harvest on virus yield. Sf9 cells were infected with β-glucuronidase-recombinant virus at an MOI of 5 or 10. Cells were lysed at 24, 48, or 72 h post-infection and assayed for β-glucuronidase (*gus*) activity using MUG (methylumbelliferyl-β-D-galactopyranoside) as a substrate.

5 Additional Information

5.1 Preparation of Luria Agar Plates

(Recommended premixed formulation: Cat. No. 12945-036)

Component	Amount
Peptone 140	10 g
Yeast Extract	5 g
sodium chloride	10 g
SELECT Agar	12 g
distilled water	to a volume of 1 L

Autoclave. Cool solution to 55°C. Add to cooled solution:

Component	Amount
kanamycin	to 50 µg/ml
gentamicin	to 7 µg/ml
tetracycline	to 10 µg/ml
IPTG	to 40 µg/ml
Bluo-gal	100 to 300 µg/ml

Mix the agar solution before pouring plates under sterile conditions.

5.2 Preparation of Stock Solutions

Antibiotics can be ordered in either dry powered form or as a stabilized, sterile, premixed solution. These solutions should be stored according to the manufacturer's recommendations. Stock solutions of antibiotics dissolved in water should be sterilized by filtration through a 0.22-micron filter. Antibiotics dissolved in ethanol need not be sterilized. Store stock solutions in light-tight containers. Magnesium ions are antagonists of tetracycline. Use media without magnesium salts for selection of bacteria resistant to tetracycline.

Notes: Plates containing antibiotics should be stored at 4°C and are stable for up to four weeks. Plates containing tetracycline and/or Bluo-gal should be stored at 4°C in the dark as these compounds are light sensitive.

Antibiotic	Stock Solution Concentration	Storage
ampicillin	50 mg/ml in water	–20°C
kanamycin	10 mg/ml in water	–20°C
tetracycline	10 mg/ml in ethanol	–20°C
gentamicin	7 mg/ml in water	–20°C

Bluo-gal solutions are made by dissolving the dry powder in dimethylformamide or dimethyl sulfoxide (DMSO) to make a 20 mg/ml stock solution. Care must be taken when using dimethylformamide, dispense solutions in a vented chemical hood only. Use a glass or polypropylene tube. The tube containing the solution should be wrapped in aluminum foil to prevent damage by light and stored at –20°C. It is not necessary to sterilize the solution by filtration.

A 200 mg/ml stock of IPTG is made by mixing 2 g of IPTG with 8 ml of water until dissolved. Adjust the volume of the solution to 10 ml with water and sterilize by filtration through a 0.22-micron filter. Dispense the solution into several 1-ml aliquots and store at –20°C.

5.3 Analysis of Bacmid DNA

5.3.1 Agarose Gel Electrophoresis

1. Pour a 0.5% agarose gel containing 0.5 µg/ml ethidium bromide. Prepare the gel using TAE buffer.
2. Load 5 µl of the bacmid mini-prep onto the gel.
3. Electrophorese at a constant voltage of 23 V for 12 h for a 6 × 8-cm gel.
4. Photograph the gel. Presence of the bacmid species is indicated by the appearance of a band that migrates more slowly than the 23.1 kb fragment of the λ DNA/*Hin*d III fragments.

Note: λ DNA *Hin*d III Fragments or the 1 Kb DNA Ladder are recommended for use with Step 2.

Figure 6 shows the typical appearance of a bacmid mini-prep after agarose gel electrophoresis.

Note: If the expected amplification product is >4 kb, use an enzyme mix such asElongase™ enzyme mix.

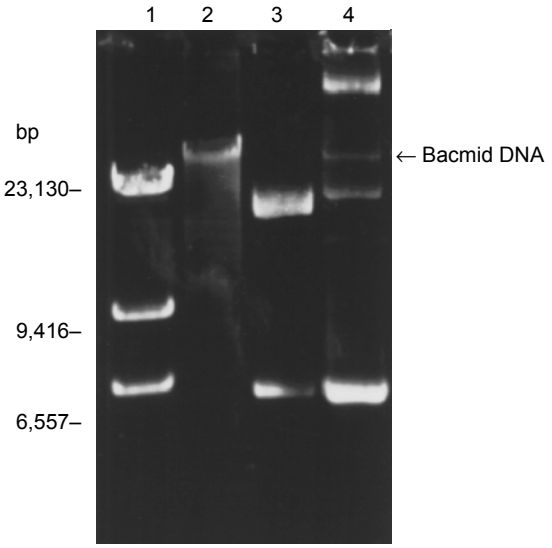


Figure 6. Agarose gel analysis of mini-prep bacmid DNA. Lane 1: λ DNA/*Hin*d III Fragments. Lane 2: Purified bacmid DNA. Lane 3: Mini-prep of Helper DNA. Lane 4: Mini-prep of high-molecular-weight (bacmid) DNA.

5.3.2 PCR Analysis of Recombinant Bacmid

The bacmid DNA is >135 kb. Verification of the insertion of the gene of interest is difficult using classical restriction endonuclease digestion analysis. It is better to use PCR to confirm that the gene of interest has transposed to the bacmid. The pUC/M13 amplification primers are directed at sequences on either side of the mini-*att*Tn7 site within the *lac*Zα-complementation region of the bacmid (see figure 7). If transposition has occured, the PCR product produced by these primers is 2,300 bp plus the size of the insert. Alternatively, one can amplify a product using one gene-specific primer and one pUC/M13 primer. Use of two gene-specific primers for PCR will produce an amplification product whether or not transposition has occured.

1. Label the appropriate number of 0.5-ml microcentrifuge tubes. Place on ice.
2. To each tube, add: 5 µl of 10X PCR buffer, 1 µl of 10 mM dNTP mix, 1.5 µl of 50 mM $MgCl_2$, 2.5 µl of primer mix (1.25 µl each 10 µM stock), 1 µl template DNA and 0.5 µl *Taq* Polymerase (2.5 U). Add distilled water to a final volume of 50 µl.
3. Mix the contents of the tube by gently tapping the tube.

Note: If the expected PCR product is >4 kb, use a polymerase mixture such as Platinum® *Taq* DNA Polymerase High Fidelity for best results.

Additional Information

Notes: For larger inserts (>4 kb), use long PCR conditions.

4. Add 20 µl of silicone oil.
5. After incubation at 93°C for 3 min, perform 25-35 cycles of PCR as follows:

   94°C for 45 s

   55°C for 45 s

   72°C for 5 min
6. For colony and viral DNA amplification, the colony or viral DNA is picked directly into the reaction mix and the PCR is performed as above for forty cycles.
7. Electrophorese the samples on a 0.7% agarose gel in 1X TAE containing 0.5 µg/ml ethidium bromide at 100 V for 90 min.
8. For colony and viral DNA amplification products, load 10-15 µl of each sample on the gel; for miniprep DNA amplification products, load 5 µl of sample.
9. The expected results from the PCR are as follows:

Sample	PCR Product
Bacmid alone	~300 bp
Bacmid transposed with pFastBac™1	~2300 bp
Bacmid transposed with pFastBac™ HT	~2430 bp
Bacmid transposed with pFastBac™-gus	~4200 bp
Bacmid transposed with pFastBac™ HT-CAT	~3075 bp
Bacmid transposed with pFastBac™ DUAL	~2562 bp
Bacmid transposed with pFastBac™-gus-CAT	~5337 bp

Insertion of your gene into any of the pFastBac™ donor plasmids will result in an

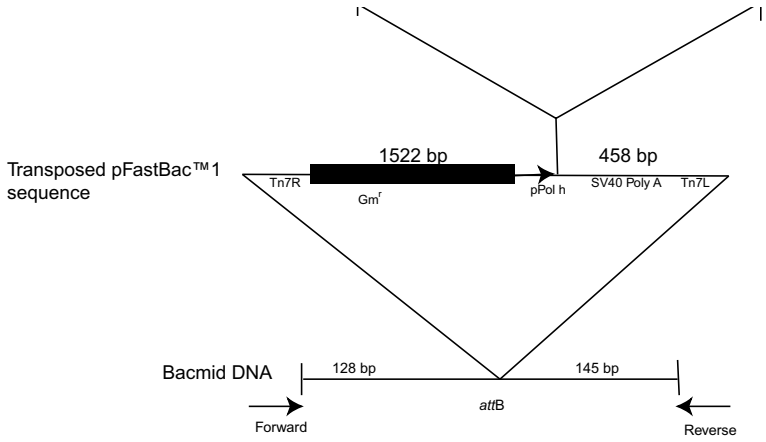


Figure 7. Transposition Region. For PCR, the sense primer is the M13/pUC Forward Amplification Primer (Forward). The anti-sense primer is the M13/pUC Reverse Amplification Primer (Reverse). For gene-specific amplification, use the M13/pUC forward primer and your gene-specific anti-sense (non-coding) primer.

increase in the size of the PCR product. This increase in PCR product corresponds to the size of your gene of interest.

5.4 Analyzing Expression by Recombinant Viruses

Analysis of recombinant virus expression can be carried out in 24-well plates using the virus stocks harvested 72-h post-infection. The following protocol is adapted from Luckow and Summers (1988) (19).

Notes: Recombinant viruses expressing gus can be easily identified as blue plaques on agarose plates containing the chromogenic indicator X-glucuronide (17). To assess the expression of the gus gene of the recombinant viruses in a rapid but qualitative manner, a small amount of media from the transfected cells may be mixed with X-glucuronide, a chromogenic substrate for β-glucuronidase. (*e.g.,* Incubate 5 µl of 20 mg/ml solution of X-glucuronide in DMSO or DMF with 50 µl of cell-free medium. A blue color should develop within 2 h.)

1. Seed 6×10^5 *Spodoptera frugiperda* (Sf9) cells per well in a 24-well plate. Let cells attach for at least 30 min.
2. Rinse the cells once with fresh (serum-supplemented or serum-free) media and replace with 300 µl of fresh media.
3. Add 200 µl of virus stock to each well. Include several extra wells as controls that contain uninfected (mock-infected) cells, wild-type AcNPV-infected cells, and (optionally) one or more wells with cells infected with previously characterized recombinant baculoviruses.
4. Incubate the plate at 27°C for 48 h.
5. Save the viral supernatant, if desired, and rinse cells once with serum-free medium. Lyse cells with 400 µl of 1X SDS-PAGE protein disruption buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS]. Freeze samples, if desired, at –20°C. Boil samples for at least 3 min and separate proteins by SDS-PAGE.
6. Repeat the infection and analysis by SDS-PAGE to determine the time course of expression and the optimal time to harvest cells for maximal expression. DNA dot-blot hybridizations and all other routine cell culture methods are described (11). Radiolabel infected cells with 10 µCi/well ^{35}S-methionine or ^{35}S-cysteine in methionine or cysteine-deficient media, as described in reference 15.

Notes: Use a glass pipet or polypropylene pipet tip to measure dimethylformamide solutions. Dimethylformamide will dissolve polystyrene (DMSO will not).

5.5 Assay for β-Glucuronidase Expression *In Situ*

Solutions:

20 mg/ml X-glucuronide in DMSO or dimethylformamide
Store at –20°C in a polypropylene tube, in the dark.

Fixative: PBS containing 2% formaldehyde, 0.05% glutaraldehyde

Store at 4°C. Prepare as follows:

85 ml water
10 ml of 10X PBS (cat. no. 70013-016)
5 ml formalin (37% formaldehyde solution)
0.2 ml glutaraldehyde (25% solution)

Stain solution: PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$

Store at 4°C. Prepare as follows:

70 ml water
10 ml of 10X PBS
10 ml of 50 mM potassium ferricyanide
10 ml of 50 mM potassium ferrocyanide
0.2 ml of 1 M $MgCl_2$.

Substrate/Stain solution: 1 mg/ml X-glucuronide in stain solution. Prepare immediately before using as follows:

20 ml Stain solution
1 ml X-glucuronide (20 mg/ml in dimethylformamide)

1. Wash the cells in each well once with 2 ml of phosphate-buffered saline (PBS) containing calcium and magnesium.
2. Fix lightly in 1 ml of fixative for 5 min at room temperature.

3. Wash the cells in each well twice with 2 ml of PBS.
4. Add 1 ml/well of substrate/stain solution and incubate 2 h to overnight at 37°C.
5. Rinse each well with 2 ml of PBS. Observe the cells on an inverted microscope and calculate the percent of gus-positive, blue cells.
6. To store the plates, fix each well with 1 ml of 10% formalin in PBS for 10 min at room temperature, rinse with PBS, and store in PBS at 4°C.

5.6 Assay for Chloramphenicol Acetyltransferase

Solutions:

1 M Tris-HCl (pH 8.0)
0.1 M Tris-HCl (pH 8.0)
0.1 M Tris-HCl (pH 8.0) containing 0.1% Triton X-100. Store at 4°C.
250 mM chloramphenicol in ethanol (100%). Store aliquotted at -20°C.

CAT enzyme standards: Prepare standard solutions of 0.2, 1, 2, 4 and 10 U/ml in CAT Dilution Buffer [0.1 M Tris-HCl (pH 8.0), 50% glycerol, 0.2% BSA]. Store at 4°C.

5.6.1 Procedure for 6-Well, 35-mm Dishes

Harvest transfected or infected cells 24 to 72 h post-transfection or infection as follows:

1. Wash cells in each well once with PBS.
2. Put plates on ice. Add 1 ml of 0.1 M Tris-HCl (pH 8.0) containing 0.1% Triton X-100 to each well. Freeze 2 h at -70°C.
3. Thaw plates at 37°C, then chill on ice. Transfer the cell lysates to a microcentrifuge tube and centrifuge the samples at 12,000 x *g* for 5 min.
4. Transfer the supernatants to a second tube and heat at 65°C for 10 min to inactivate deacetylases and other inhibitors of the CAT reaction. Centrifuge at 12,000 x *g* for 3 min and transfer the supernatants to a third tube. This material will be referred to as the “cell extract”. Store the cell extracts at -70°C.

5.6.2 CAT Enzyme Assay [Modification of Neumann (23)]

1. For each sample, add the following items to a 3.5-ml polypropylene scintillation vial:

Volume of cell extract	1-10 µl
0.1 M Tris-HCl (pH 8.0)	bring to 150 µl

2. Negative control: 150 µl 0.1 M Tris-HCl (pH 8.0).
3. Positive control:
   Add 150 µl of 0.1 M Tris-HCl (pH 8.0) to each of 5 vials.
   Add 5 µl of each CAT standard solution to give a standard curve of 1, 5, 10, 20 and 50 milliunits of CAT.
4. To each sample and to each control, add 100 µl of a cocktail containing:
   84 µl deionized, distilled water
   10 µl 1 M Tris-HCl (pH 8.0)
   1 µl 250 mM Chloramphenicol (in ethanol)
   5 µl (50 nCi) [^{14}C]-butyryl Coenzyme A (0.010 µCi/µl)
5. Cap all samples and incubate in a waterbath or incubator at 37°C for 2 h.
6. Add 3 ml Econofluor™ solution to all tubes and cap tightly. Invert the tube one time.

7. Incubate at room temperature for 2 h.
8. Count each sample for 0.5 min in a liquid scintillation counter.

5.7 Insect Cell Culture Media

Invertebrate cell cultures are very sensitive to environmental factors. In addition to chemical and nutritional culture factors, physical factors can also affect invertebrate cell growth and require optimization to maximize *in vitro* cell growth. Some important general areas include:

- ***Temperature.*** The optimal range for growth and infection of cultured insect cells is 27°C to 30°C.
- ***pH.*** A range of 6.1 to 6.4 appears to work well for most culture systems. Sf-900 II SFM will maintain a pH in this range under conditions of normal air and open-capped culture systems.
- ***Osmolality.*** The optimal osmolality of medium for use with lepidopteran cell lines is 345 to 380 mOsm/kg.
- ***Aeration.*** Invertebrate cells are dependent upon passive oxygen diffusion for optimal proliferation and expression of recombinant proteins. Active or controlled oxygenated systems require dissolved oxygen at 10% to 50% of air saturation.
- ***Shear Forces.*** Suspension culture techniques generate mechanical shear forces. Serum concentration ranges of 5% to 20% in medium provides adequate protection from cellular shear forces. In the absence of serum, the medium may require supplementation with a shear force protectant such as Pluronic® F-68.

Note: Sf-900 II SFM does *not* require the addition of shear force protectants.

5.7.1 Serum-Supplemented Culture

While a number of insect cell lines have been successfully adapted to BEVS technology, this section highlights the most commonly employed cell line. *Spodoptera frugiperda* (Sf9) cultured cells (ATCC No. CRL 1711) have a doubling time of 20 to 30 h in IPL-41 containing either 2.6 g/L of tryptose phosphate and 5% to 10% heat-inactivated (HI) fetal bovine serum (FBS) or 4 g/L of yeastolate and 5% to 10% HI-FBS. Alternatively, cells may be cultured in Grace's Insect Cell Culture Medium containing 3.3 g/L of yeastolate, 3.3 g/L lactalbumin hydrolysate, and 5% to 10% HI-FBS or in TC-100 Insect Medium containing 5% to 10% FBS.

Notes: Sf-900 II SFM as almost all liquid media, contains photolabile ingredients. It should be stored in the DARK! Only cell-culture-grade quality water should be used in the preparation of media and the cleaning of culture glassware to minimize experimental variability.

5.7.2 Serum-Free Culture

For serum-free cultures, Sf-900 II SFM is recommended for optimal cell growth and product yield. It is a complete medium requiring no supplementation. Cells cultured in SFM may be cultured essentially the same as described in Section 5.8.1 and Section 5.8.2. Protocols for adapting cells from serum-supplemented media to SFM are described in Sections 5.9.1 and 5.9.2.

5.8 Insect Cell Culture Techniques

General Equipment and Supplies:

- Incubator capable of maintaining 28°C ± 0.5°C and large enough to accommodate the desired culture configuration apparatus.
- Inverted and light microscope for morphologic examination of cell cultures.
- Hemacytometer chamber.
- Low speed centrifuge.
- Laminar flow hood suitable for cell culture.

Additional Specific Materials and Equipment:

Monolayer Culture

- Cell culture "T"-flasks, 25- and 75-cm^2

Shaker Culture

- Orbital shaker fitted for 100- to 500-ml Erlenmeyer flasks.
- Disposable 125-, 250- and 500-ml Erlenmeyer flasks.

5.8.1 Monolayer Culture Procedure

A monolayer should reach confluency in two to four days without requiring a medium change. In serum-supplemented monolayer cultures, the resident cellular population is primarily loosely attached with some free-floating cells. Most established insect cell lines are not anchorage dependent and may be transferred between monolayer and suspension culture without alterations of viability, morphology, or growth rate. Some cultures are passage number dependent. Therefore, fresh cultures should be established periodically (*e.g.,* every three months) from frozen seed stocks. Antibiotics or antimycotics are not specifically recommended for SFM cultures. The popular prophylactic antibiotic concentrations (per ml) for serum-supplemented cultures are generally near 0.25 µg amphotericin B, 100 U penicillin and 100 µg streptomycin.

Note: If SFM culture conditions are used and antibiotics are being considered, antibiotic concentrations should be reduced by 50% or more to avoid toxicity.

1. Aspirate medium and floating cells from a confluent monolayer and discard.
2. Add 4 ml of room temperature complete growth medium to each 25-cm^2 flask (12 ml to a 75-cm^2 flask).
3. Resuspend cells by pipetting the medium across the monolayer with a Pasteur pipet. *Enzymatic dissociation is not recommended.*
4. Observe cell monolayer using an inverted microscope to ensure adequate cell detachment.
5. Perform viable cell count on harvested cells (*e.g.,* using trypan blue dye exclusion).
6. Inoculate cells at 2×10^5 viable cells/ml into respective culture vessels.
7. Incubate cultures at 28°C ± 0.5°C with loose caps to allow gas exchange.
8. On day four post-planting, aspirate the spent medium from one side of the monolayer and subculture the flask as described above.
9. With slower growing cell lines, it may be necessary to feed the flasks on day(s) three to four post-planting. Aspirate spent medium from one side of the monolayer and gently refeed with fresh medium.
10. Subculture the flasks when the monolayer reaches 80 - 100% confluency, approximately two to four days post-planting (and up to seven days if the culture had to be fed).

Notes: Serum-supplemented cultures of Sf9 cells do not adhere tightly to most glass or plastic substrates, therefore, care must be taken during movement. By maintaining a minimal media depth and loose caps, adequate oxygenation especially critical during the post-infection period with wild-type or recombinant virus will occur.

Insect cells attach very tightly to substrates under serum-free conditions and require additional detachment effort. It may be necessary to shake the flask vigorously two to three times using a wrist-snap (motion) to dislodge the cells.

Reminder: ALWAYS tighten caps before shaking to avoid contamination.

5.8.2 Adaptation to Suspension Culture Procedure

Insect cells are not generally anchorage dependent and can be adapted to suspension culture. The following protocol will optimize the adaptation of most invertebrate cell lines to suspension culture and reduce/eliminate cell clumping over a short period of time.

1. Six to 10 confluent 75-cm^2 monolayer flasks are required to initiate a 100-ml suspension culture.
2. Dislodge cells from the bottom of the flasks as previously described.
3. Pool the cell suspension and perform viability count.
4. Dilute the cell suspension to approximately 5×10^5 viable cells/ml in room temperature complete serum-supplemented or serum-free growth medium.
5. Incubate vessels at 28°C ± 0.5°C at a constant stirring rate of 100 rpm.

6. Subculture when the viable cell count reaches 1×10^6 to 2×10^6 cells/ml (three to seven days post-planting). Increase stirring speed 5 rpm. If cell viability drops below 75%, decrease stirring speed 5 rpm until culture viability recovers (>80%).
7. Repeat step 6 until you have reached a constant stirring speed of 130 rpm to 140 rpm. At this point reduce the seeding density to 3×10^5 cells/ml when you subculture.
8. If large clumps (>10 cells per clump) persist, let the shaker flask culture sit two to three min prior to subculturing. This will allow the larger clumps to settle to the bottom of the flask. Pull samples for counting and seeding new cultures from the upper one-third of the suspension culture. This technique selects for a cell population that grows as single cells.
9. It may be necessary to repeat step 8 two to three times until clumping is reduced.
10. Cryopreserve a quantity of cells adapted to suspension culture for future use. (Refer to Section 5.10).

Note: Sf-900 II SFM does *not* require the addition of shear force protectants.

5.8.3 Shaker Culture Procedure

A shaker method which works well uses 250 ml shaker flasks containing 50 to 125 ml total volume (see table 1) with loosened caps. Oxygen tensions are not rate limiting under these conditions. If desired, add 0.1% Pluronic® F-68 to serum-supplemented media formulations.

Table 1. Useful Medium Volumes.

Flask Size (ml)	Shaker Flask Size Culture (ml)
125	25 – 50
250	50 – 125
500	125 – 200
1,000	200 – 400

1. Orbital shaker apparatus must have the capacity for 50- to 500-ml Erlenmeyer flasks and a shaking speed of up to 140 rpm.
2. The standard flask employed is the 250-ml disposable sterile Erlenmeyer for a 100-ml volume. Glass flasks without baffles may be used, but thorough cleaning after each use is essential.
3. The orbital shaker flask assembly should be maintained in a 27°C ± 0.5°C, non-humidified, ambient air regulated incubator or warm room.
4. Aeration is accomplished by loosening the cap. Under these conditions, there is no oxygen limitation to the cells and cultures will achieve maximum population doubling times and densities.
5. Inoculate a 250-ml Erlenmeyer flask with 100 ml of complete medium containing 3×10^5 viable cells/ml.
6. Set the orbital shaker at 135 rpm for cultures adapted to and maintained in suspension culture.
7. Incubate the culture until it reaches 2×10^6 to 3×10^6 viable cells/ml. Subculture the shaker flask cultures to approximately 3×10^5 viable cells/ml. For consistent optimal cell growth, the culture should be in mid-log-phase of growth when subcultured. Do not allow the cultures to reach stationary phase at any time (for cells in Sf-900 II SFM this is a density of 5 x 10^6 cells/ml). For consistent growth, the cells *must* be subcultured when they are in mid-log (exponential) growth.
8. Once every three weeks, cell suspension from shaker cultures may be gently centrifuged ($100 \times g$) for 5 min and the cell pellet resuspended in fresh medium to reduce accumulation of cell debris and metabolic by-products.

Note: Although shaker type culture is scalable to a variety of vessels and volumes, relative flask fill volumes and orbital shaker speeds must be optimized for each configuration. (Consult Table 1 for useful medium volumes.)

Note: Monolayer cells should first be adapted to suspension conditions! Refer to Section 5.8.2.

Additional Information

Note: The chief advantage to this method is time. Insect cultures can be adapted to SFM in five to eight passages (approximately three weeks). If viabilities decrease to <50% or if cultures are growing slowly (population doubling times of >72 h) for more than three to four consecutive passages, use the sequential adaptation (weaning) method as described.

Note: Cells must be in mid-exponential growth with a viability of at least 90% to use this procedure.

5.9 SFM Adaptation Methods

The simultaneous adaption of cells to SFM using both methods as described below may save valuable time if one of the methods does not work.

5.9.1 Direct Adaptation to SFM Procedure

1. Cells growing in medium containing 5% to 10% FBS are transferred directly into SFM prewarmed to 28°C ± 0.5°C, at a density of 5×10^5 cells/ml.
2. When the cell density reaches 2×10^6 to 3×10^6 cells/ml (four to seven days post-seeding), subculture to a density of 5×10^5 cells/ml.
3. When the cells are completely adapted to serum-free culture, they should reach maximum densities and have population doubling times that are comparable to growth in serum-supplemented medium.
4. Stock cultures of SFM adapted cells should be subcultured once to twice weekly when the viable cell count reaches 2×10^6 to 3×10^6 cells/ml with at least 80% viability.

5.9.2 Sequential (Weaning) Adaptation to SFM Procedure

1. Subculture cells grown in serum-containing medium into a 1:1 ratio of SFM and the original serum-supplemented media.
2. Incubate cultures until viable cell count exceeds 1×10^6 cells/ml (about one population doubling). Subculture by mixing equal volumes of conditioned medium and fresh SFM (1:1).
3. Continue to subdivide the culture in this manner until the serum concentration falls below 0.1%, cell viability > 80%, and a viable cell concentration of $>1 \times 10^6$ cells/ml is achieved.
4. Subculture when the viable cell concentration reaches 2×10^6 to 3×10^6 cells/ml, which is approximately four to seven days post-planting.
5. After several passages, the viable cell counts of most insect lines should exceed 2×10^6 to 4×10^6 cells/ml with viabilities >85% after approximately four to seven days of culture. At this stage the culture is adapted to SFM, and consideration should be given to the cryopreservation of a quantity for future use.

5.10 Cryopreservation of Insect Cell Cultures Procedure

Note: Frozen cells are stable indefinitely in liquid nitrogen storage. Viability and recovery of cryopreserved cells should be checked 24 h after storing vials in liquid nitrogen by following the recovery procedures described in Section 5.11.

1. Grow desired quantity of cells in suspension using either spinner or shaker culture. Harvest cells in mid-log phase of growth with a viability >90%.
2. Determine the viable cell count and calculate the required volume of cryopreservation medium required to yield a final cell density of 1×10^7 to 2×10^7 cells/ml.
3. Prepare the required volume of cryopreservation medium. For serum-free cultures, prepare cryopreservation medium consisting of 7.5% DMSO in 50% fresh SFM and 50% conditioned medium (2- to 3-day conditioned SFM from the cell line to be frozen; sterile filtered). Alternatively, one may use a serum-free freezing medium consisting of fresh SFM containing 10% BSA and 7.5% DMSO. For serum-supplemented cultures prepare a cryopreservation medium consisting of fresh medium supplemented with 7.5% DMSO and 10% FBS. Chill the prepared freeze medium and hold at 4°C until use.
4. Centrifuge cells from suspension or monolayer culture medium at $100 \times g$ for 5 min. Resuspend cell pellet in the determined volume of chilled cryopreservation medium.
5. Dispense well mixed aliquots of cell suspension into cryovials according to volumes recommended by manufacturer's specifications.
6. Refrigerate cryovials at 0°C to 4°C for 30 min.
7. Cryopreserve in an automated or manually controlled rate freezing apparatus following standard procedures and a temperature reduction rate of 1°C per min.

5.11 Recovery of Cryopreserved Cultures Procedure

1. Recover cultures from frozen storage by rapidly thawing vials in a 37°C ± 0.5°C water bath.
2. Wipe or spray ampule exterior with 70% ethanol.
3. Transfer the entire contents of the vial into a shaker or spinner flask containing complete, prewarmed and equilibrated medium. It is essential to innoculate cultures at a minimal viable cell density at 3×10^5 to 5×10^5 cells/ml.
4. Maintain the culture between 0.3×10^6 and 1×10^6 cells/ml for the first two subcultures after recovery before returning to the normal maintenance schedule.

5.12 Amplification of Viral Stocks

For amplification of viral stocks, infect a suspension or monolayer culture at an MOI (multiplicity of infection) of 0.01-0.1 according to the following formula:

$$\text{Inoculum required (ml):} \quad \frac{\text{desired MOI (PFU/cell)} \times \text{(Total number of cells)}}{\text{Titer of viral inoculum (pfu/ml)}}$$

For example, for an MOI of 0.1, infect a 50-ml culture at 2×10^6 cells/ml with 0.5 ml of a viral stock which is 2×10^7 pfu/ml.

Harvesting virus 48 h post-infection usually results in a 2-log amplification.

5.13 Viral Plaque Assay

Determination of the infectious potency of a stock of baculovirus may be accomplished by plaque formation in immobilized monolayer culture.

Equipment:

sterile hood

water baths at 40°C and 70°C

27°C humidified incubator

inverted microscope

Materials:

30 ml of exponential culture of Sf9 or Sf21 cells at 5×10^5 cells/ml

6-well plates (2 each)

1 bottle 4% Agarose Gel

1 bottle Sf-900 (1.3X) or Grace's Insect Plaquing Medium, (2X)

1 bottle sterile, cell-culture-grade, distilled water

100 ml (empty) sterile glass bottle (1 each)

0.5 ml of clarified, cell-free, sterile baculovirus supernatant

100 ml of Sf-900 II SFM

20 ml of qualified, heat-inactivated FBS

1. Under sterile conditions dispense 2 ml of cell suspension per well.
2. Allow cells to settle to bottom of plate and incubate, covered, at room temperature for 1 h.

Note: In serum-containing medium, transport the plates gently because cells do not adhere tightly to the plate surface.

3. Place the bottle of agarose gel in the 70°C water bath. Place the empty 100 ml bottle and the bottle of Sf-900 II Insect Medium, 1.3X, or Grace's Insect Medium, 2X, in the 40°C bath.
4. Following a 1 h incubation of the plates at room temperature, observe monolayers under the inverted microscope to confirm cell attachment and 50% confluence.
5. Produce an eight-log serial dilution of the harvested viral supernatant by sequentially diluting 0.5 ml of the previous dilution in 4.5 ml of Sf-900 II SFM

(or Grace's Insect Cell Culture Medium, Supplemented, without FBS) in 12-ml disposable tubes. You should conclude with eight tubes containing 10^{-1} to 10^{-8} dilutions of the original virus stock.

6. Move the six well plates and the tubes of diluted virus to the hood. Label the plates, in columns of two, "10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}".
7. Sequentially remove the supernatant from each well, discard, and immediately replace with 1 ml of the respective virus dilution to each duplicate well. Incubate for 1 h at room temperature.

*****To prepare Sf-900 plaquing overlay:***

8a. Move bottles from waterbaths (from step 3) to sterile hood when agarose has liquified (20 to 30 min). Quickly dispense 30 ml of the Sf-900 Insect Plaquing Medium (1.3X) and 10 ml of the 4% Agarose Gel to the empty bottle and mix gently. Return the bottle of plaquing overlay to the 40°C water bath until use.

*****To prepare the Grace's plaquing overlay:***

8b. Move bottles from waterbaths (from step 3) to sterile hood when agarose has liquified (20 to 30 min). Aseptically add 20 ml of qualified, heat-inactivated FBS to the Grace's Insect Plaquing Medium, 2X, and mix. Combine 25 ml of the Grace's Insect Medium, 2X supplemented with FBS, 12.5 ml of cell-culture grade sterile water and 12.5 ml of the melted 4% Agarose Gel into the sterile empty bottle and mix gently. Return the plaquing overlay to the 40°C water bath until use.

Continuation:

9. Following this second 1 h incubation, return the bottle of diluted agarose and the 6-well plates to the hood.
10. Sequentially (from high to low dilution) remove the virus inoculum from the wells and replace with 2 ml of the diluted agarose. Work quickly to avoid desiccation of the monolayer. A Pasteur pipet connected to a vacuum pump easily removes inoculum traces.
11. Allow gel to harden for 10 to 20 min before moving.
12. Incubate at 27°C in a humidified incubator for 4 to 10 days.
13. Recombinant virus produces milky/gray plaques of slight contrast visible without staining or other detection methods.
14. Monitor plates daily until the number of plaques counted does not change for two consecutive days.
15. To determine the titer of the inoculum employed, an optimal range to count is 3 to 20 plaques per well of a six well plate. The titer (pfu/ml) may be calculated by the following formula:

pfu/ml (of original stock) = 1/dilution factor × number of plaques × 1/(ml of inoculum/plate).

5.13.1 Neutral Red Staining of Viral Plaques

If the plaques are allowed to incubate for 6 to 7 days, one can usually visualize the plaques without difficulty. However, staining with Neutral Red may be helpful for initial plaque identification. Other plaque staining dyes such as Crystalline Blue are not recommended, because they contain organic solvents which will kill the host cells.

Materials

- distilled water, cell-culture grade
- neutral red staining solution (3.3 g/L)
- plates with developed plaques

1. Freshly prepare a 0.1% (w/v) neutral red stain solution in cell-culture grade water.
2. To each well containing 2 ml of plaquing overlay, add 0.5 ml of 0.1% neutral red solution. Incubate for 1 to 2 h at room temperature.

3. Gently remove excess stain with pipette or blotter.
4. Plates yield clear plaques in a nearly clear gel against a red russet background.

5.14 Storage of Recombinant Baculovirus

1. When harvesting virus from transfection or post-infection supernatants, transfer 1.5 to 2 ml to a sterile, capped tube. Clarify (centrifuge, 5 min at 500 × *g*) and transfer virus-containing supernatant to fresh tube.

   Virus may be sterile filtered through a 0.2-μm, low protein binding filter with minimal loss in titer (<10%).
2. From initial transfections, viral titers should range from 2×10^7 to 4×10^7 pfu/ml.
3. Store virus stocks at 4°C. Protect from light! For long-term storage of virus, the addition of FBS to a final concentration of 2% FBS is recommended. Storage of the viral stock at –70°C is also recommended.

5.15 Production of of rAcNPV and Heterologous Proteins

Points to Consider

1. The first step towards the successful infection of insect cells with either wild-type or recombinant baculovirus is that the culture not be rate limited by nutritional *(i.e.,* amino acid or carbohydrate utilization) or environmental factors (*i.e.,* pH, dissolved O_2, or temperature). Cultures should be infected while in the mid-logarithmic phase of growth with an established MOI.
2. Optimal MOI will vary between cell lines, and the relative infection kinetics of the virus isolate or clone employed. A dose response (or MOI) should be established for each virus, medium, reactor and cell line employed. This will enable determination of optimal infection parameters for production of virus or recombinant product.
3. When producing non-occluded virus stock, recombinant or wild-type, it is recommended that the culture be infected at a cell density of 1×10^6 to 2×10^6 cells/ml and an MOI of 0.01 to 0.1. For the expression of recombinant gene products, MOIs of 0.5 to 10 are commonly employed.
4. The BEVS recombinant gene product may or may not be secreted. Maximum expression of secreted proteins is usually observed between 30 and 72 h and nonsecreted between 48 and 96 h post-infection. It is important to determine the expression kinetics for each recombinant DNA product, as many proteins (secreted or nonsecreted) may be degraded by cellular proteases released in cell culture.
5. Although many insect cell lines have been adapted to serum-free culture it may be necessary to supplement the culture post-infection with 0.1% to 0.5% FBS or BSA for expression of some recombinant products and/or rAcNPV to protect the recombinant product or virus from proteolysis. Protein-based protease inhibitors are generally less expensive and more effective than many synthetic protease inhibitors.
6. To purify recombinant proteins produced, refer to references 21 and 22 for general guidelines on protein purification methods.

6

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Related Products

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Product	Size	Cat. No.
Bac-to-Bac® Products:		
Plasmid pFastBac™1 Expression Vector pFastBac™ DNA pFastBac™-gus DNA (4 ng) Manual	10 µg	10360-014
pFastBac™ DUAL Expression Vector pFastBac™ DUAL DNA pFastBac™ DUAL Control DNA (4 ng) Manual	10 µg	10712-024
MAX Efficiency® DH10Bac™ Competent Cells	0.5 ml	10361-012
CellFECTIN® Reagent	1 ml	10362-010
Bac-to-Bac® Expression Systems:		
Bac-to-Bac® Baculovirus Expression System pFastBac™1 Expression Vector (1 each) MAX Efficiency® DH10Bac™ Competent Cells (5 x 0.1 ml) CellFECTIN® Reagent (1 ml)	5 reactions	10359-016
Bac-to-Bac® HT Baculovirus Expression System pFastBac™ HT Expression Vectors (1 each) MAX Efficiency® DH10BAC Competent Cells (5 x 0.1 ml) CellFECTIN® Reagent (1 ml)	5 reactions	10608-016
Molecular Genetics Media and Antibiotics:		
Ampicillin Sodium Salt, lyophilized	20 ml	11593-019
Gentamicin Reagent Solution (10 mg/ml), liquid	10 ml	15710-064
Kanamycin Sulfate (100X), liquid	100 ml	15160-054
LB Agar, powder	500 g	22700-025
LB Broth (1X), liquid	500 ml	10855-021
Luria Agar, powder	500 g	12945-036
S.O.C. Medium	10 × 10 ml	15544-034
Terrific Broth	500 g	22711-022
Molecular Biology Products:		
1 Kb DNA Ladder	250 µg	15615-016
λ DNA/*Hin*d III Fragments	500 µg	15612-013
Subcloning Efficiency™ DH5α™ Competent Cells	2 ml	18265-017
Taq DNA Polymerase, Recombinant	100 units	10342-053
Platinum® *Taq* DNA Polymerase	100 units	10966-018
Platinum® *Taq* DNA Polymerase, High Fidelity	100 units	11304-011
PCR SuperMix High Fidelity	100 reactions	10790-020
M13/pUC Forward and Reverse Amplification Primers	1 set (2 × 3.5 µg)	18430-017
Reagents:		
4% Agarose Gel	40 ml	18300-012
Agarose	100 g	15510-019
Buffer-Saturated Phenol	100 ml	15513-039
Concert™ High Purity Plasmid MiniPrep System	25 reactions	11449-014
Distilled Water	500 ml	15230-162
	1 L	15230-147

Related Products

Ethylenediaminetetraacetic Acid	500 g	15576-078
IPTG	1 g	15529-019
Neutral Red Solution	100 ml	15330-079
Phosphate-Buffered Saline (PBS), pH 7.4 (1X)	500 ml	10010-023
Phosphate-Buffered Saline (PBS), pH 7.4 (10X)	500 ml	70011-044
10% SDS Solution	4 × 100 ml	15553-027
10X TAE Buffer	1 L	15558-042
1 M Tris-HCl, pH 8.0	1 L	15568-025
Insect Media:		
Fetal Bovine Serum, qualified, heat-inactivated	100 ml	16140-063
IPL-41 Insect Medium (1X), liquid	1000 ml	11405-081
Penicillin-Streptomycin, liquid	100 ml	15070-063
Pluronic® F-68, 10% (100X)	100 ml	24040-032
Sf-900 II SFM (1X), liquid with L-glutamine	500 ml	10902-096
Sf-900 II SFM (1.3X), liquid with L-glutamine	100 ml	10967-032
TC-100 Insect Medium, powder	10 × 1 L	11600-061
Sf9 cells, Serum-Free Medium Adapted	3 ml	11496-015
Sf21 Cells, Serum-Free Medium Adapted	3 ml	11497-013

Appendix A:
Maps and Restriction Endonuclease Sites for pFastBac™ Expression Vectors

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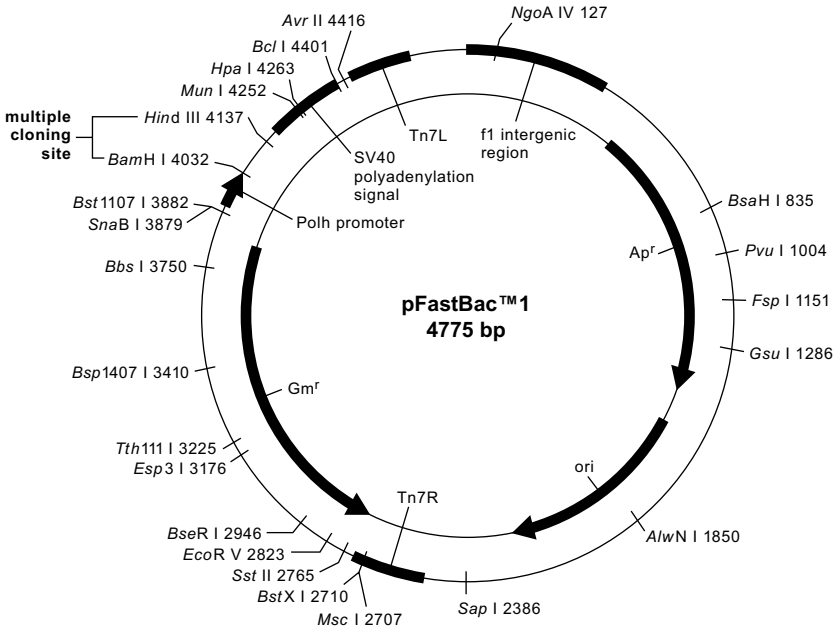


pFastBac™1 multiple cloning site and primer binding region: 3942 – 4169

4000 suggested sequencing primer 5´– TATTCC GGATTATTCA TACC – 3´ 4050

+1 BamH I Rsr II BssH II EcoR I

5´– AAATAAGTA TTTTACTGTT TTCGTAACAG TTTTGTAATA AAAAAACCTA TAAATATTCC GGATTATTCA TACCGTCCCA CCATCGGGCG CGGATCCCGG TCCGAAGCGC GCGGAATTCA

polyhedrin promoter

Stu I Sal I Sst I Spe I Not I Nsp V Xba I Pst I Xho I Sph I Kpn I Hind III 4150

AAGGCCTACG TCGACGAGCT CACTAGTCGC GGCCGCTTTC GAATCTAGAG CCTGCAGTCT CGAGGCATGC GGTACCAAGCTT GTCGAGAAGT AC**TAG**AGGAT CA**TAA**TCAG – 3´

stop codons

ATT was the position of the original start codon. The original ATG was mutated to ATT.

Restriction endonucleases that do not cleave pFastBac™1 DNA:

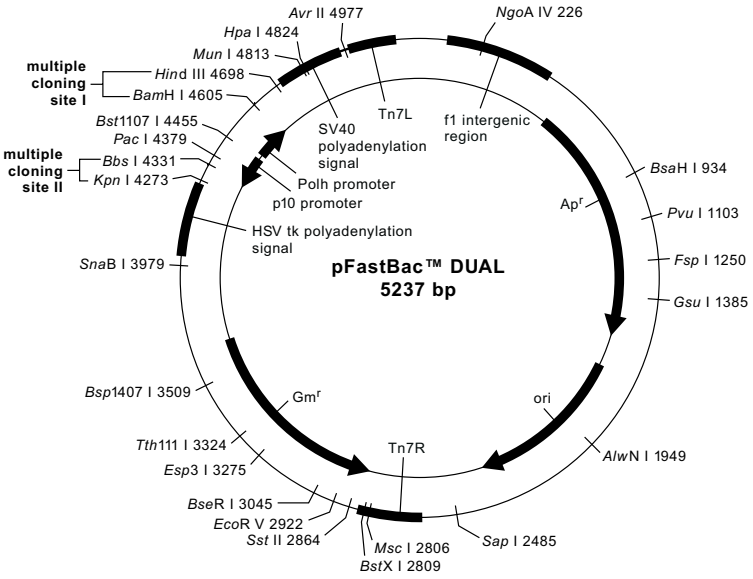
Aat II	*Cla* I	*Nco* I	*Pme* I	*Sse* I
Afl II	*Cvn* I	*Nde* I	*Psp*5 II	*Sse*8387 I
Apa I	*Eco*47 III	*Nhe* I	*Pvu* II	*Sun* I
Asc I	*Eco*72 I	*Nru* I	*Sex*A I	*Swa* I
*Bpu*1102 I	*Eco*N I	*Nsi* I	*Sfi* I	*Xcm* I
Bsg I	*Eco*O109 I	*Pac* I	*Sgr*A I	
*Bsp*M I	*Mlu* I	*Pfl*M I	*Sma* I	
*Bst*E II	*Nar* I	*Pin*A I	*Srf* I	

Restriction endonucleases that cleave pFastBac™1 DNA twice:

Acc I	3882	4070	*Bsm* I	4247	4346	*Rca* I	536	1544
Afl III	2264	3246	*Bsm*F I	4015	4775	*Sca* I	893	4150
Ban II	157	4076	*Dra* III	230	3578	*Tfi* I	2290	4101
Bgl II	2547	3017	*Eam*1105 I	1371	4651	*Xmn* I	772	3797
Bsa I	1304	3661	*Kpn*2 I	3888	3998			

Figure 8. Map and restriction endonuclease sites for pFastBac™1. Restriction endonucleases that cleave pFastBac™1 once are shown on the outer circle. The nucleotide position refers to the 5´ base of the recognition sequence.

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. Vector sequences, restriction information, and maps can be found in the Vector Data area of our web site, www.invitrogen.com.



pFastBac™ DUAL polyhedrin promoter region and MCS I:

4515 +1

5´ - - -AAATAAGTAT TTTACTGTTT TCGTAACAGT TTTGTAATAA AAAAACCTAT AAATATTCCG GATTATTCAT

polyhedrin promoter

*Bam*H I | *Rsr* II | *Bss*H II | *Eco*R I | *Stu* I | *Sal* I | *Sst* I | *Spe* I | *Not* I | *Nsp* V | *Xba* I

ACCGTCCCAC CATCGGGCGC GGATCCCGGT CCGAAGCGCG CGGAATTCAA AGGCCTACGT CGACGAGCTC ACTAGTCGCG GCCGCTTTCG AATCTAGAGC

Pst I | *Hind* III

CTGCAGTCTC GACAAGCTTG TCGAGAAGTA C**TAG**AGGATC A**TAA**TC 3´

+1 corresponds to the transcriptional start for the polyhedrin promoter.
ATT corresponds to the original translational start codon. The ATG was mutated to an ATT.
An in-frame ATG codon must be provided by the cloned gene to initiate translation.
Stop codons are shown in bold.

pFastBac™ DUAL p10 promoter region and MCS II:

4417 +1

5´ - AAA TAAGAATTAT TATCAAATCA TTTGTATATT AATTAAAATA CTATACTGTA AATTACATTT TATTTACAAT

p10 promoter

Bbs I | *Sma* I | *Xho* I | *Nco* I | *Nhe* I | *Pvu* II | *Nsi* I | *Sph* I | *Kpn* I

CACTCGACGA AGACTTGATC ACCCGGGATC TCGAGCCATG GTGCTAGCAG CTGATGCATA GCATGCGGTA CCGGGAGATG GGGGAGGC**TA AC****TGA**AACAC 3´

+1 corresponds to the transcriptional start for the p10 promoter and corresponds to position 4420 on the map.
Digestion at the *Bbs* I site generates a *Bam*H I compatible overhang. An in-frame ATG codon must be provided by the cloned gene to initiate translation when the *Bbs* I, *Sma* I or *Xho* I sites are used for cloning. When cloning into the *Nco* I site, or sites downstream of the *Nco* I site, make sure the reading frame of the cloned gene is in frame relative to the ATG sequence of the *Nco* I site.
Stop codons are shown in bold.

Restriction endonucleases that do not cleave pFastBac™ DUAL DNA.

Aat II	*Asc* I	*Bsp*M I	*Cvn* I	*Eco*O109 I	*Nde* I	*Pin*A I	*Sex*A I	*Srf* I
Afl II	*Bpu*1102 I	*Bst*E II	*Eco*72 I	*Mlu* I	*Nru* I	*Pme* I	*Sfi* I	*Sse*8387 I
Apa I	*Bsg* I	*Cla* I	*Eco*N I	*Nar* I	*Pfl*M I	*Psp*5 II	*Sgr*A I	*Sun* II

Restriction endonucleases that cleave pFastBac™ DUAL DNA twice:

Afl III	2363	3345	*Bsp*LU 11	2363	3345	*Tfi* I	2389	4674
Bcl I	4324	4962	*Bss*S I	806	2190	*Xmn* I	871	3896
Bgl II	2646	3116	*Dra* III	329	3677			
Bsm I	4808	4907	*Sca* I	992	4711			

Figure 9. Map and restriction sites for pFastBac™ DUAL expression vector. Restriction endonucleases that cleave pFastBac™ DUAL once are shown on the outer circle. The nucleotide position refers to the 5´ base of the recognition sequence.

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. Vector sequences, restriction information, and maps can be found in the Vector Data area of our web site, www. invitrogen.com.

Notes

Notes

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India 11 577 3282
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Japan 03 3663 7974
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