*flash*BAC™ Baculovirus Expression Systems

*flash*BAC and *flash*BAC ULTRA

Protocol for MIR 6115, 6120, 6135, 6140

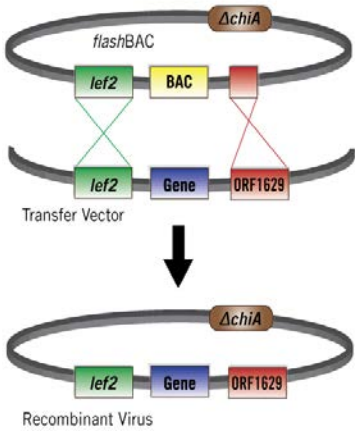
Quick Reference Protocols, SDS, Product Data Sheets and Certificate of Analysis available at mirusbio.com/6115

INTRODUCTION

The *flash*BAC™ System is a streamlined platform for the production of recombinant baculoviruses. Deletion of the essential gene ORF1629 from the *Autographa californica* nucleopolyhedrovirus (AcMNPV) genome prevents non-recombinant virus from replicating within insect cells, thus eliminating the need to plaque-purify recombinant virus from parental virus. Secreted or membrane-targeted recombinant protein yields are greatly increased by the deletion of the chitinase gene (*chiA*). Further nonessential gene deletions in *flash*BAC™ ULTRA provide enhanced quality and yield for difficult to express proteins. The insertion of a bacterial artificial chromosome (BAC) at the AcMNPV polyhedrin gene locus allows viral DNA to be maintained and propagated as a circular genome within bacterial cells. The genomic DNA can then be purified and is the *flash*BAC™ DNA provided with this kit.

To generate recombinant baculovirus using the *flash*BAC™ System, insect cells are transfected with *Trans*IT®-Insect Transfection Reagent, *flash*BAC™ DNA, and a transfer plasmid (e.g. pOET1, pOET1C_6xHIS, or pOET6) containing the gene of interest. Homologous recombination within the insect cells (see schematic at right) inserts the gene of interest under the control of the polyhedrin promoter, and restores the function of ORF1629 allowing viral DNA to replicate and produce virus particles. The recombinant virus genome replicates to produce baculovirus that can be harvested directly from the culture medium of transfected insect cells. Because the *flash*BAC™ System has effectively reduced recombinant baculovirus production to a one-step procedure, it is fully amenable to high throughput and automated production platforms. More details on the *flash*BAC™ and *flash*BAC™ ULTRA Systems are available at www.mirusbio.com/flashbac.

*flash*BAC™ Overview



CAUTION: Standard safe laboratory practices should be maintained when using baculovirus-generating reagents. ***Please refer to product SDS for full safety precautions.***

SPECIFICATIONS

Storage	Store *flash*BAC™ DNA at 4°C and Control Transfer Plasmid at 4°C or -20°C.
Product Guarantee	1 year from the date of purchase, when properly stored and handled.

MATERIALS SUPPLIED

The *flash*BAC™ System DNA is supplied in the following formats:

Product Number	Product Name	Components	Concentration	Volume
MIR 6115	*flash*BAC™ 5 reaction kit	*flash*BAC™ DNA	20 ng/µl	25 µl
		Control Transfer Plasmid (pAcRP23-lacZ)	100 ng/µl	5 µl
MIR 6120	*flash*BAC™ 24 reaction kit	*flash*BAC™ DNA	20 ng/µl	120 µl
		Control Transfer Plasmid (pAcRP23-lacZ)	100 ng/µl	25 µl
MIR 6135	*flash*BAC™ ULTRA 5 reaction kit	*flash*BAC™ ULTRA DNA	20 ng/µl	25 µl
		Control Transfer Plasmid (pAcRP23-lacZ)	100 ng/µl	5 µl
MIR 6140	*flash*BAC™ ULTRA 24 reaction kit	*flash*BAC™ ULTRA DNA	20 ng/µl	120 µl
		Control Transfer Plasmid (pAcRP23-lacZ)	100 ng/µl	25 µl

For Materials Required but Not Supplied, see Sections I-III on TRANSFECTION, AMPLIFICATION, and PLAQUE ASSAY

For Research Use Only

*flash*BAC™ Systems and pOET vectors are sold by Mirus Bio through partnership with Oxford Expression Technologies, Oxford, UK.

Generating Recombinant Baculovirus

BEFORE YOU START:

Important Tips for Insect Cell Culture Maintenance

- **Cells for virus production.** Sf9 or Sf21 cells are generally used for co-transfections, virus amplification and plaque assays. *T. ni* cells are often used to maximize protein yields but are not recommended for virus production and amplification.
- **Subculturing Sf9 or Sf21 insect cells.** Maintain insect cells used for recombinant virus production in log-phase growth and discard after passage 30. Subculture cells before they become overgrown and enter stationary phase, generally every 3-4 days. For example:

  Cells in serum-free medium in shake flasks: Seed cells at 0.3-0.5 x 10^6 cells/ml and do not exceed a density of 6.0 x 10^6 cells/ml before passaging.

  Cells in serum-containing medium in spinner flasks: Seed cells at 0.1-0.2 x 10^6 cells/ml and do not exceed a density of 2.5 x 10^6 cells/ml before passaging.
- **Insect cell growth conditions.** The optimal temperature for Sf9 and Sf21 growth and infection is 27-29°C. If the insect culture medium used employs a phosphate buffering system, a CO2 incubator is not required (e.g. Grace's, Sf-900™ III). Use vented shake, spinner, or tissue culture flask caps to ensure adequate dissolved oxygen content.

Important Tips for Optimal Plasmid DNA Transfection

- **Cell density at transfection.** Use healthy cells from a log-phase culture**. S**eed cells such that the resulting monolayer is even and sub-confluent. Use 1.5 x 10^6 Sf21 or 1 x 10^6 Sf9 cells in a 2 ml volume of medium per well of a 6-well plate.
- **DNA purity.** Use high quality transfer vector DNA for transfection. Plasmid preparations that are sterile, endotoxin-free, and have $A_{260/280}$ absorbance ratio of 1.8–2.0 are desirable.
- ***flash*BAC™ vector compatibility.** The *flash*BAC™ system is compatible with all baculovirus transfer vectors that are based on homologous recombination in insect cells at the polyhedrin gene locus. Compatible transfer plasmids available through Mirus Bio LLC are as follows:
  - pOET1 (MIR 6150) and pOET1C_6xHis (MIR6151) for high titer baculovirus production
  - pOET6 BacMam (MIR 6152) for high titer BacMam virus production

  Other compatible examples include pBacPAK8/9, pAcUW31 and pBacPAK-His1/2/3 (BD Biosciences Clontech). pFastBac™ vectors, which are designed for site-specific transposition in *E. coli* using the Bac-to-Bac® system (Life Technologies) are NOT compatible.
- **Ratio of *Trans*IT®-Insect Reagent to DNA.** *Trans*IT®-Insect Transfection Reagent is a novel transfection formulation for high-performance transfection of plasmid DNA into insect cells. *Trans*IT®-Insect is composed of animal-origin free components and is serum compatible, which eliminates the need for any culture medium change after transfection. The *Trans*IT®-Insect Reagent:DNA ratio has been optimized for use with the *flash*BAC™ System. **Table 1** on page 3 provides recommended volumes based on cell culture vessel size.
- **Complex formation conditions.** Prepare *Trans*IT®-Insect Reagent:DNA complexes in serum-free growth medium. Mirus recommends Grace's Insect Basal Medium.
- **Cell culture conditions.** Culture cells in the appropriate medium, with or without serum. There is no need to perform a medium change to remove the transfection complexes.
- **Presence of antibiotics.** Antibiotics will inhibit transfection complex formation and therefore should be excluded from the complex formation step. Transfection complexes can be added directly to cells grown in complete culture medium containing serum and low levels of antibiotics (0.1–1X final concentration of penicillin/streptomycin mixture).
- **Post-transfection incubation time.** Harvest P0 seed stock of recombinant baculovirus 5 days post-transfection. Store in the dark at 4°C.

Do not use serum or antibiotics in the medium during transfection complex formation.

Transfection complexes can be added directly to cells cultured in complete growth medium containing serum and up to 0.1–1X antibiotics.

SECTION I: TRANSFECTION OF _flash_BAC™ + TRANSFER DNA

The following procedure describes DNA transfections using the *flash*BAC™ System and *Trans*IT®-Insect Transfection Reagent in 6-well plates. If using an alternate vessel, increase or decrease the amounts of serum-free medium, *Trans*IT®-Insect Transfection Reagent, *flash*BAC™ DNA, transfer vector DNA, and complete culture medium based on the surface area of the cell culture vessels (please refer to **Table 1**).

Table 1. Recommended starting conditions for *flash*BAC™ System transfections.

Culture vessel	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate	10-cm dish	T75 flask
Surface area	0.35 cm^2	1.0 cm^2	1.9 cm^2	3.8 cm^2	9.6 cm^2	59 cm^2	75 cm^2
Complete growth medium	73 µl	210 µl	0.4 ml	0.8 ml	2.0 ml	12.3 ml	15.6 ml
Serum-free medium	3.6 µl	10.4 µl	20 µl	40 µl	100 µl	614 µl	780 µl
*flash*BAC DNA (20 ng/µl stock)	0.2 µl	0.5 µl	1.0 µl	2.0 µl	5.0 µl	31 µl	39 µl
Transfer DNA (500 ng/µl stock)	0.04 µl	0.1 µl	0.2 µl	0.4 µl	1.0 µl	6.2 µl	7.8 µl
*Trans*IT-Insect Reagent	0.05 µl	0.12 µl	0.24 µl	0.48 µl	1.2 µl	7.4 µl	9.4 µl

If small volumes of *Trans*IT-Insect need to be pipetted, dilute the reagent in serum-free medium before each use to avoid pipetting errors. ***Do not*** store diluted *Trans*IT-Insect Reagent.

Additional Materials Required for Transfection

- Insect cell line (e.g. Sf9 or Sf21)
- *Trans*IT®-Insect Transfection Reagent
- pOET Transfer Plasmid or other compatible vector containing gene of interest.
- Tissue culture treated dishes for seeding insect cells
- Appropriate complete insect cell culture medium (e.g. SF900 III SFM for growth of Sf9 cells)
- Serum-free medium for complex formation (e.g. Grace's Basal Medium)
- Incubator at 28°C

Transient plasmid DNA transfection protocol per well of a 6-well plate

A. Plate cells

1. Approximately 1 hour prior to transfection, plate cells in 2 ml complete growth medium per well in a 6-well plate. The cell monolayer should be even and sub-confluent.

   For Sf21 cells: Plate 1.5 x 10^6 cells/dish in 2 ml volume of medium (0.75 x 10^6 cells/ml).

   For Sf9 cells: Plate 1 x 10^6 cells/dish in 2 ml volume of medium (0.5 x 10^6 cells/ml).

   NOTE: For comparison, set up one well as an untransfected control. If required, another well of cells can be used to make recombinant virus using a control transfer vector DNA (e.g. the pAcRP23.lacZ provided in the *flash*BAC™ kit).
2. Incubate cell cultures at room temperature for 1 hour.

B. Prepare *Trans*IT®-Insect Reagent:*flash*BAC™ DNA:transfer DNA complex

1. Warm *Trans*IT®-Insect Reagent to room temperature and vortex gently before using.
2. Place 100 µl of serum-free insect culture media (e.g. Grace's Basal Medium) into a sterile tube.
3. Add 500 ng (1 µl of 500 ng/µl stock) transfer vector or control DNA to tube. Add 100 ng (5 µl of 20 ng/µl stock DNA) of *flash*BAC™ DNA to same tube. Pipet gently to mix.
4. Add 1.2 µl *Trans*IT®-Insect Reagent to the diluted DNA mixture and pipet gently to mix.
5. Incubate at room temperature for 15–20 minutes to allow complexes to form.

During complex formation the *Trans*IT-Insect: DNA solution may appear slightly opaque. This is normal and does not negatively impact transfection performance.

C. Distribute the complexes to cells in complete growth medium

1. Remove 1 ml of culture medium from each well of cells using a sterile pipette, ensuring that the cell monolayer is not disturbed (leaving 1 ml in the dish).
2. Add the *Trans*IT®-Insect Reagent:DNA complexes (prepared in Step B) drop-wise to different areas of the wells.
3. Gently rock the culture vessel back-and-forth and from side-to-side to evenly distribute the *Trans*IT®-Insect Reagent:DNA complexes. Do not disrupt the cell monolayer.
4. Incubate cell cultures overnight at 28°C.

   NOTE: To prevent evaporation, wrap the edges of the cell culture vessel with parafilm or store in a sterile plastic box. In addition, a tray of sterile water may be placed inside the incubator to increase humidity.

*Trans*IT-Insect is a low-toxicity reagent. There is no need to change culture medium after transfection. If required, perform a medium change at least 4 hours post-transfection.

D. Incubate and harvest the P0 seed stock

1. The following day, add 1 ml of complete insect culture medium back to each well for a total of 2 ml medium per well. NOTE: The growth medium added can contain antibiotics (0.1–1X final concentration of penicillin/streptomycin mixture) if required.
2. Continue to incubate cultures at 28°C for 4 more days, protected from evaporation.

   NOTE: Observe cells for signs of infection. Untransfected control cells will continue to divide and will form a confluent monolayer. Baculovirus-infected cells will not reach confluency and will appear grainy, rounded and slightly enlarged, with enlarged nuclei.
3. At 5 days post-transfection, transfer the 2 ml culture medium containing recombinant virus to a sterile tube or conical and centrifuge at 300 x g for 5 minutes to remove cellular debris.
4. Transfer the supernatant to a new sterile tube and store at 4°C, protected from light. This is your P0 seed stock.

E. Expression Verification (optional)

1. If the pAcRP23.lacZ positive control transfer vector was used to make recombinant virus, the infected cells can be stained for expression verification using X-gal.
   - To do so, add 1 ml of appropriate insect cell culture medium (or PBS) containing 15 µl X-gal (2% w/v in N, N-Dimethylformamide) and incubate at 28°C.
   - If the cells and culture medium appear blue after approximately 5 hours, recombinant virus expressing lacZ was produced.
2. Alternatively, Western blot analysis can be performed on the remaining cells to verify foreign gene expression.

A titer of approximately 1 x 10^7 pfu/ml at day 5 is expected when healthy cells are used for transfection and the steps in this protocol are followed.

SECTION II: AMPLIFICATION OF RECOMBINANT VIRUS

The recombinant P0 virus produced in the previous section must be further amplified for experimental work. The following workflow will amplify 50-100 ml virus using the seed stock of virus harvested from the co-transfection as inoculum. Use proper aseptic technique for the following procedures.

Additional Materials Required for Amplification

- Seed stock of recombinant virus (prepared in Section I, page 3)
- 50-100 ml insect cell culture (per gene of interest) in log-phase growth (Sf9 or Sf21)
- Appropriate insect culture medium
- Erlenmeyer shake flasks (e.g. Corning® Cat. No. 431143 or Thomson Cat. No. 931110)
- Orbital shaker (e.g. New Brunswick Innova 2000)
- Incubator at 28°C
- Sterile pipettes

A. Maintain Sf9 or Sf21 cell cultures at an appropriate cell density

1. Maintain insect cells to ensure that they are in log growth phase prior to baculovirus amplification. The following instructions are typical for insect culture maintenance:

   Cells in serum-free medium in shake flasks: Seed at 0.3-0.5 x 10^6 cells/ml and do not exceed a density of 6.0 x 10^6 cells/ml before passaging.

   Cells in serum-containing medium in spinner flasks: Seed at 0.1-0.2 x 10^6 cells/ml and do not exceed a density of 2.5 x 10^6 cells/ml before passaging.

2. Check the density and viability of cultures before seeding for amplification. Cells should be at a density of at least 5 x 10^5 viable cells/ml with a viability of >95%.
3. Seed 50-100 ml of Sf9 or Sf21 cells at an appropriate cell density for amplification. The cell density will vary with each cell type and method of culture. For example:

   Cells in serum-free medium: Seed in shake flasks at 2 x 10^6 cells/ml.

   Cells in serum-supplemented medium: Seed in spinner flasks at 0.5 x 10^6 cells/ml.

   NOTE: Do not exceed 50% of the recommended volume for the flask. For example, if using a 250-ml Erlenmeyer shake flask, do not use a culture volume greater than 125 ml.

B. Inoculate insect cell cultures with P0 baculovirus stock.

1. Add 0.5 ml of the recombinant virus seed stock (P0) to the flask containing 50-100 ml of cells at 2 x 10^6 cells/ml (if in serum-free conditions).

   NOTE: Do not add more than 0.5 ml virus. Cells should be inoculated at a low multiplicity of infection (MOI) (1 pfu/cell), which allows multiple rounds of replication to occur for generating high virus titers.

2. Incubate the cells at 28°C, shaking at 130-140 RPM. Proper infection typically requires 3-4 days. Baculovirus-infected cells will appear grainy, rounded and enlarged, with enlarged nuclei.
3. When the cells appear infected with virus, harvest the culture medium by centrifugation at 3000 rpm, at 4°C for 15 minutes. In a laminar flow hood, pour the supernatant into a sterile container. Store the recombinant virus at 4°C, protected from light.

   NOTE: Baculovirus stocks may be stored for up to 12 months at 4°C, though loss of titer can occur earlier. To minimize titer loss, add of 2-5% serum. If the baculovirus stock has been stored for greater than 3 months, titer the virus before use and re-amplify if necessary. For long-term storage, make aliquots of virus and store at –80°C. Due to reduction of viral titer by freezing, multiple freeze thaws should be avoided and virus should be re-amplified before use. Storage at –20°C or in liquid nitrogen is not recommended.

Virus-infected cells have an increased need for oxygen. The surface area to volume ratio should be as large as possible for maximum gas exchange – do not overfill flasks!

SECTION III: PLAQUE ASSAY TO DETERMINE VIRUS TITER

To ensure that all cells are infected simultaneously for optimization and/or expression studies, it is important that the titer of the virus be determined. A titer of 5 x 10^7 pfu/ml or higher is generally adequate for gene expression; a titer of less than 10^7 pfu/ml is not generally sufficient. This assay utilizes Neutral Red to visualize plaques, which appear as clear areas against a red background, as only live cells take up the stain. Depending on the type of cells used, this assay will take 3 or 4 days to complete.

Materials Required for Plaque Assay

- Recombinant virus to be titrated (prepared in Section II: Amplification of Recombinant Virus)
- Insect cells in log-phase growth (Sf9 or Sf21)
- 6-well tissue culture treated dishes (10 wells per virus to be titrated)
- Appropriate culture medium for the cells (serum-free or serum-supplemented)
- Low Gelling Temperature Agarose for cell culture (e.g. Sigma, A9045)
  NOTE: Use 2% w/v in sterile dH_2O, sterilized by autoclaving. Small aliquots of 10 ml are convenient and can be prepared in advance and stored solidified at room temperature. Melt in a microwave oven just prior to use.
- Neutral Red (e.g. Sigma Aldrich, 861251).
- Sterile Phosphate-Buffered Saline, pH 6.2.
- Incubator at 28°C.
- Inverted phase-contrast microscope.
- Antibiotics (optional). If antibiotics are used, they should be used similarly for all conditions. (0.1–1X final concentration of penicillin/streptomycin mixture).
- Optional: 2% (w/v) X-gal in dimethylformamide (DMF) (if titrating a virus expressing lacZ).

Plaque formation will require a 3 day (Sf21) or 4 day (Sf9) incubation after cells are exposed to the virus to be titrated.

Virus Titration Protocol

A. Prepare a stock solution of Neutral Red (5 mg/ml)

1. Add 5 mg of Neutral Red powder to 1 ml of water to make a concentrated stock.
2. Filter sterilize through a 0.2 um filter and store at room temperature. NOTE: This stock will need to be diluted 1:20 in PBS for use in the assay. Do not store diluted stock long-term.

B. Prepare 10 wells in 6-well plates (or 10 x 35 mm dishes) of cells

1. Approximately 1 hour prior to virus addition, plate cells in 2 ml complete growth medium per well in a 6-well plate. The cell monolayer should be even and sub-confluent.

   For Sf21 cells: Plate 1.4 x 10^6 cells/dish in 2ml volume of medium (0.7 x 10^6 cells/ml).

   For Sf9 cells: Plate 0.9 x 10^6 cells/dish in 2ml volume of medium (0.45 x 10^6 cells/ml).

   NOTE: Cells should be healthy and taken from a log-phase culture.
2. Incubate cell cultures at room temperature for 1 hour to establish a cell monolayer.

C. Prepare and distribute serial dilutions of virus to be titrated

1. While cells are incubating, prepare serial log dilutions of the virus to be titrated from 10^{-1} to 10^{-7}. To do this, place 450 µl of the appropriate cell culture medium into each of 7 sterile microfuge tubes labeled 1-7. Add 50 µl of undiluted recombinant virus to tube 1 and mix thoroughly. Using a fresh pipette tip, transfer 50 µl from tube 1 to tube 2 and mix thoroughly. Continue diluting the virus this way through tube 7. Dilutions 10^{-4} through 10^{-7} (tube #'s 4-7) will be used for the assay. NOTE: You will also need 500 µl medium for control dishes.
2. Confirm with a microscope that the cells have formed an even, sub-confluent monolayer.

3. Remove ~1.7 ml of the culture media from each well and discard. NOTE: Do not disrupt the cell monolayer. Leaving a small amount of medium in wells will prevent drying of cells.
4. Using a fresh sterile pipette for each, add 100 µl volumes from dilutions 10^{-4} through 10^{-7} dropwise to the center of duplicate wells (4 dilutions plated in 8 wells total) and label accordingly. For the remaining 2 wells, add 100 µl of insect cell culture medium in place of a virus dilution. These are the negative controls.
5. Incubate the plates at room temperature for 40-60 minutes on a level surface for virus absorption. NOTE: You may want to move the culture vessels to the bench top to prevent drying out in a laminar flow hood.

D. Prepare and apply the LGT (Low Gelling Temperature) agarose overlay

1. About 15 minutes prior to the end of the virus absorption period, prepare the LGT agarose overlay by melting a 10 ml aliquot of ready-prepared 2% (w/v) LGT agarose in a microwave or boiling water bath. After the aliquot has cooled to ~50°C (tolerable but still hot to touch), add an equal volume (10 ml) of insect cell culture medium. Mix thoroughly but gently, avoiding air bubbles.

   NOTE 1: You will need 2 ml for each dish. Use immediately or keep warm at 45°C to prevent solidification. If using a water bath, wipe the bottle with alcohol prior to use to prevent contamination. If the agarose sets, do not re-melt (prepare a fresh batch).

   NOTE 2: If using antibiotics, add to the culture medium before preparing the overlay.
2. After preparing the overlay, carefully remove the virus inoculum from each well with a sterile pipette. DO NOT allow cells to dry completely.
3. Gently pipette 2 ml of the agarose-overlay down the side of each well without disrupting the monolayer. Incubate at room temperature until the agarose is solid (about 15 minutes).
4. When the overlay has solidified, add 1 ml of complete insect cell culture medium to each dish as a liquid feed overlay. (Antibiotics may be added to medium if desired.)
5. Place the dishes in a secure container and incubate at 28°C for 3 days (Sf21 cells) or 4 days (Sf9 cells). By the end of the incubation, the cell monolayer should be confluent.

E. Neutral Red visualization of plaques

1. Remove the liquid overlay from the dishes and replace with 1 ml diluted Neutral Red stain (see Section A for instructions on preparing dilution).
2. Incubate for 3-4 hours at 28°C. After the incubation, remove the stain from the wells.
3. Invert the culture vessels and place in the dark to allow plaques to clear. This may take several hours.
4. Select one set of duplicate dishes with 10-30 plaques and count them.
5. Determine the virus titer (pfu/ml) using the following calculation:

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

   * Dilution factor = the inverse of the dilution used on counted plate

   ** Multiply by 10 because 0.1 ml was applied to each dish

   Example: If 25 plaques were counted on the 10^{-6} dilution plate, the titer is calculated as:

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

If the LacZ positive control transfer vector was used, LacZ-positive virus plaques can be stained using X-gal rather than Neutral Red. Follow the procedure outlined above, but add 1 ml insect cell culture medium containing 15 µl (2%w/v) X-gal (in DMF) and incubate > 5 hours (to overnight) at 28°C. Plaques will appear blue in color.

TROUBLESHOOTING GUIDE

Problem	Solution
LOW P0 BACULOVIRUS TITERS FROM CO-TRANSFECTION	
Incorrect vector sequence	If target insert expression is not observed, verify the sequence of the plasmid DNA. If expressing a fusion protein, verify that all components are in the correct reading frame. Verify that your gene of interest has been cloned in the correct orientation to the promoter and that the coding region downstream of the promoter is inserted such that the gene's AUG start codon is the first AUG after the promoter sequences. This is important as translation occurs at the first AUG in the mRNA.
Suboptimal DNA concentration	Determine the DNA concentration accurately. Use plasmid DNA preps that have an $A_{260/280}$ absorbance ratio of 1.8–2.0. The optimal DNA ratio for the *flash*BAC system is typically 1:5 (100 ng *flash*Bac™ DNA + 500 ng transfer vector DNA in a 6-well plate).
Low-quality plasmid DNA	Use highly purified, sterile, endotoxin- and contaminant-free DNA for transfection. We recommend using Mirus MiraCLEAN Endotoxin Removal Kit (MIR 5900) for removal of endotoxin from your DNA preparation. Alternatively, use cesium chloride gradient or anion exchange purified DNA which contains levels of endotoxin that do not harm most cells. **Do not** use DNA prepared using mini-prep kits as it might contain high levels of endotoxin.
Cells not actively dividing at the time of transfection	Maintain insect cells used for the production of recombinant viruses in log-phase growth and discard after passage 30. Subculture cells before they become overgrown and enter stationary phase, generally every 3-4 days. Cells used for transfection and amplification should be in log-phase growth with a viability of >95%.
Cell health compromised during co-transfection	Check that the cells are healthy using a phase-contrast microscope and that they did not dry out during the co-transfection procedure. Remember to add the appropriate additional volume of culture medium to the co-transfected cells after incubating them for 5 - 24 hours.
Transfection incubation time	Determine the optimal transfection incubation time for each cell type and experiment. Test a range of incubation times. For baculovirus production, the best incubation time is generally 4-5 days.
*Trans*IT®-Insect was not mixed properly	Warm *Trans*IT®-Insect Reagent to room temperature and vortex gently before each use.
Precipitate formation during transfection complex formation	During complex formation, scale all reagents according to **Table 1** on Page 3 including serum-free media, *Trans*IT®-Insect Reagent, transfer plasmid, and *flash*BAC DNA. Precipitation may be observed when excess DNA is used during complex formation. This may negatively impact transfection efficiency. To avoid precipitation when using high concentrations of DNA, increase the volume of serum-free medium during complex formation by two-fold.
Transfection complexes distributed unevenly	Add transfection complexes drop-wise to the cells. Gently rock the dish back-and-forth and from side-to-side to distribute the complexes evenly. Do not swirl or rotate the dish, as this may cause uneven distribution.
Proper experimental controls were not included	To verify efficient transfection, use *Trans*IT®-Insect Reagent to deliver the positive control plasmid provided in the flashBAC™ kit (pAcRP23-lacZ). LacZ-positive virus plaques can be stained by following the plaque assay procedure outlined in Section III using X-gal rather than Neutral Red.

TROUBLESHOOTING GUIDE continued

Problem	Solution
VIRUS AMPLIFICATION FAILED TO PRODUCE HIGH TITER RECOMBINANT BACULOVIRUS	
Cell density not optimal for amplification	See Section II for starting recommendations on amplification plating densities. Baculovirus replication is inhibited when cells are seeded too densely. Depending on the cell type, higher or lower densities may be required for optimal amplification.
Cells not actively dividing when inoculated	Subculture cells before they become overgrown and enter stationary phase, generally every 3-4 days. Cells used for transfection and amplification should be in log-phase growth with a viability of >95%.
Cell morphology has changed	Mycoplasma contamination can alter cell morphology and affect transfection efficiency. Check cells for mycoplasma contamination. Use a fresh frozen stock of cells or use appropriate antibiotics to eliminate mycoplasma. NOTE: For baculovirus production, cell morphology changes are expected as the cells become infected with virus. A high or low cell passage number can make cells more sensitive and refractory to transfection. Maintain a similar passage number between experiments to ensure reproducibility. It is not recommended that insect cells be used beyond passage 30.
P0 stock had insufficient titer for amplification	The virus titer from the co-transfection may be too low for the volume of cells being infected. To determine the optimal harvest time, leave the infection for longer than 5 days and monitor virus amplification using a phase-contrast microscope. Conversely, only one round of amplification may have occurred if too much virus was added to the culture of cells. If the P0 stock has been stored for longer than 3 months, you may need to re-titer. If the titer has decreased, increase the volume of P0 used for amplification.
INADEQUATE PLAQUE ASSAY RESULTS	
No visible plaques on plaque assay plates	Ensure that cells are healthy when seeding and avoid dislodging cells when replacing medium. Cells should adhere to the tissue culture dishes within 1 hour after plating. If cells do not adhere or there are many floating cells, discard plates and obtain fresh cells. The cell concentration was too high when seeding the dishes. If the cells are seeded too densely, the virus does not replicate properly and plaques may be too small to visualize without a microscope. This is a very common problem with plaque assays.
Plaques are poorly defined and diffuse	Cells seeded too thin for the plaque assay will require a longer incubation to produce a confluent monolayer. Resulting plaques are often large, ill-defined and diffuse. If plaques appear smeared, the virus inoculum may not have been removed completely prior to adding the agarose overlay. This allows the virus to spread rather than remain contained within the foci of the cell.
There are too many or too few plaques on the plates	If few plaques are visible, the virus titer may be low to detect with the dilutions plated. Plate out the lower dilutions (10^{-1} to 10^{-3}). If all the cells have lysed and plaques have merged, the virus titer may be too high and further dilutions may be required. Always change tips when preparing the virus dilutions, as virus carry-over can result in inaccurately high titers.
The agarose overlay cracked	If the virus inoculum is not completely removed from the cells before adding the agarose overlay, it will interfere with the gelling process and produce cracks.
Plaques are clustered on the perimeter of the plate	Seed cells uniformly in the dish and add the virus inoculum to the center of the dish dropwise to cover the cells evenly. Uneven plating or addition of inoculum will cause smearing and may result in plaques predominating around the edge of the dish.

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