BacMagic™ DNA Kits

I. About the System

BacMagic™ Transfection Kit	5 rxn	71546-3
BacMagic DNA Kit	5 rxn	71545-3
BacMagic-2 Transfection Kit	5 rxn	72158-3
BacMagic-2 DNA Kit	5 rxn	72157-3
BacMagic-3 Transfection Kit	5 rxn	72351-3
BacMagic-3 DNA Kit	5 rxn	72350-3

Description

BacMagic™ DNAs generate recombinant baculoviruses for target protein expression in insect cells without the tedious, time-consuming plaque purification steps. Derived from an AcNPV genome, BacMagic DNAs have a deletion of a portion of the essential open reading frame (ORF) 1629, preventing nonrecombinant virus from replicating in insect cells. The nonessential chitinase (*chiA*) gene is deleted to improve production of secreted and membrane-targeted proteins. BacMagic-2 has an additional deletion of the *v-cath* gene, a cathespsin-like cysteine protease, resulting in significantly improved quality and yield for most target proteins. BacMagic-3 has three further deletions to nonessential genes (p10, p24, and p26) that result in improved protein expression. BacMagic DNAs contain a bacterial artificial chromosome (BAC) in place of the polyhedrin (polh) coding sequencing. A compatible transfer plasmid (e.g., pIEx/Bac™, pBAC™, pTriEx™ vectors) containing the target coding sequence is cotransfected with BacMagic DNA into insect cells. In the cells, homologous recombination restores the viral ORF1629 and the target coding sequence replaces the BAC sequence. Only recombinant baculovirus can replicate, producing a homogeneous population of recombinants.

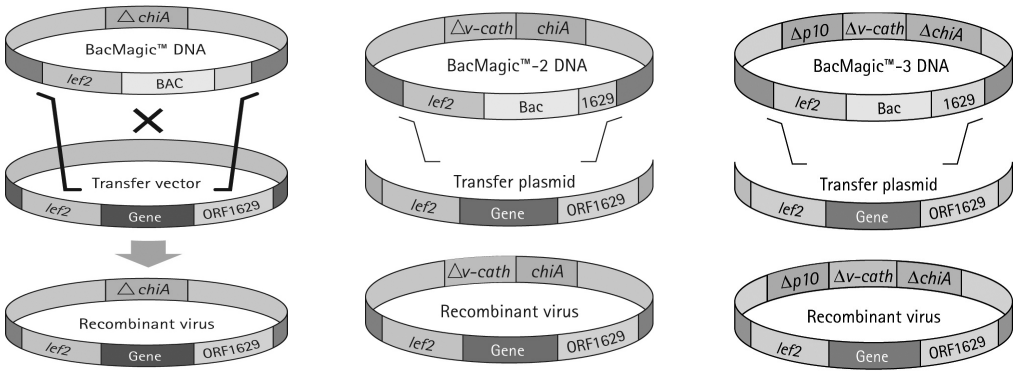

Figure 1. Construction of baculovirus recombinants with three BacMagic systems

Components

BacMagic™ Transfection Kits

- 0.5 µg BacMagic, BacMagic-2, or BacMagic-3 DNA
- 50 µl Insect GeneJuice® Transfection Reagent
- 2 µg Transfection Control Plasmid
- 2 x 1 ml Sf9 Insect Cells
- 1 L BacVector® Insect Cell Medium

BacMagic DNA Kits

- 0.5 µg BacMagic, BacMagic-2 or BacMagic-3 DNA
- 50 µl Insect GeneJuice Transfection Reagent
- 2 µg Transfection Control Plasmid

Storage

Store Transfection Control Plasmid at –20°C. Store BacMagic DNA, BacVector Insect Cell Medium, and Insect GeneJuice Transfection Reagent at 4°C. Remove Sf9 Insect Cells from the foil pack. Recover immediately, or place at -70°C if using cells within two weeks. For long term storage, place cells in liquid nitrogen.

Additional Required Components

- Baculovirus transfer plasmid DNA
- Reagents and supplies for insect cell culture including BacPlaque™ Agarose (Cat. No. 70034)
- Incubator (28°C)
- Inverted phase-contrast microscope

Baculovirus transfer plasmids

The Novagen baculovirus transfer vectors provide convenient cloning of target genes for homologous recombination with BacMagic™ DNA to produce recombinant virus for protein expression. The pBAC™, pIEx/Bac™, and pTriEx™ transfer vectors offer options in type of promoter and fusion tags, shown below. Cloning sites are shown on the detailed vector maps available at http://www.emdchemicals.com.

Vector(s)	His•Tag®	S•Tag™	Strep•Tag® II	GST•Tag™	Protease	Insect promoter	Time of Exp. (h)	Secretion	Special Features
pBAC™-1[1], pBACgus-1[1, 3]	C					polh	24–72		"Classic" transfer plasmids for high-level expression; choice of fusion tags and cloning sites.
pBAC-2cp[4], pBAC-2cp Ek/LIC, pBACgus-2cp[3, 4], pBACgus-2cp Ek/LIC[2, 3]	N, C	I			Tb, Ek	polh	24–72		
pBAC-3[4], pBACgus-3[3, 4]	N, C	I			Tb, Ek	polh	24–72	●	Signal sequence fusions facilitate secretion of target proteins to the medium very late post-infection.
pBAC4x-1, pBACgus4x-1[3]	C					polh, p10	24–72		Four promoters/cloning sites for high-level expression of multiple target genes in same cell.
pBAC-5[4], pBACgus-5[3, 4]	N, C	I			Tb, Ek	gp64	4–48		Mid level expression during the early and late phase post-infection for more complete processing, with options for secretion.
pBAC-6[4], pBACgus-6[3, 4]	N, C	I			Tb, Ek	gp64	4–48	●	
pBACsurf-1						polh	24–72		For display of target proteins as gp64 fusions on the virion surface.
pIEx/Bac™-1, pIEx/Bac-1 Ek/LIC[2]	C		N		Ek	hr5 enhancer ie1 p10	0-72		Combination of immediate early and late/very late promoters enables continuous expression of the target gene.
pIEx/Bac-3, pIEx/Bac-3 3C/LIC[2]	N		C		3C, Tb	hr5 enhancer ie1 p10	0-72		
pIEx/Bac™-4, pIEx/Bac-4 Ek/LIC[2]	C			N	Ek	hr5 enhancer ie1 p10	0-72		
pIEx/Bac-5, pIEx/Bac-5 3C/LIC[2]				N	3C, Tb	hr5 enhancer ie1 p10	0-72		
pTriEx™-1.1, pTriEx-1.1 Hygro, pTriEx-1.1 Neo	C					p10	24–72		Three promoters for high level expression in bacterial, insect, and mammalian cells
pTriEx-2, pTriEx-2 Hygro, pTriEx-2 Neo	N, C	I			Tb, Ek	p10	24–72		
pTriEx-3, pTriEx-3 Hygro, pTriEx-3 Neo	C					p10	24–72		
pTriEx-4, pTriEx-4 Ek/LIC[2] pTriEx-4 Hygro, pTriEx-4 Neo	N, C	I			Tb, Ek	p10	24–72		
pTriEx-5, pTriEx-5 Ek/LIC[2]	C		N		Ek	p10	24–72		
pTriEx-6, pTriEx-3C/LIC[2]	C		N		Tb, 3C	p10	24–72		
pTriEx-7, pTriEx-7 Ek/LIC[2]	C		I		Ek	p10	24–72		

Tags: I = internal tag; N = N-terminal tag; C = optional C-terminal tag protease cleavage sites: Tb = thrombin; Ek = enterokinase; 3C = HRV 3C

1. Inserts cloned into pBAC-1 or pBACgus-1 must provide a translation initiation codon
2. LIC vectors are supplied as linearized plasmids ready for ligation-independent cloning of appropriately prepared PCR products.
3. Vectors having a gus designation carry the *E. coli* ß-glucuronidase gene under the control the the baculovirus late basic promoter ($P_{6.9}$) as a reporter gene to identify recombinants.
4. The *Stu* I site in these plasmids ovrlaps an *E. coli dcm* methylation site. When plasmids are produced in dcm^+ hosts, methylation at this site inhibits digestion with *Stu* I. To use the *Stu* I site, the plasmids must be grown in dcm^- hosts.

II. Getting Started

Sf9 Insect Cell culture

BacVector® Insect Cell Medium

BacVector® Insect Cell Medium is serum-free and optimized for growth of Sf9 Insect Cells. This medium is recommended for transfection, plaque assays, virus production, and protein expression. Cells can be transferred directly from serum-containing medium into BacVector Insect Cell Medium without any noticeable lag in growth. For applications requiring serum (e.g., agarose overlays), use 5% (v/v) heat-inactivated, sterile filtered fetal bovine serum (FBS) in BacVector Insect Cell Medium.

Antibiotics may be used with BacVector Insect Cell Medium, except where noted in the procedures. *Certain lots of antibiotics may severely inhibit cell growth.* We recommend testing new lots for compatibility with the cells prior to routine use.

Thawing Sf9 Insect Cells

1. Place bottle of medium in 28°C water bath and retrieve vial of Sf9 Insect Cells from liquid nitrogen tank or –70°C freezer.
2. Thaw cells quickly by immersing vial about half way into 28°C water bath. Gently swirl vial until cells are fully thawed (approximately 2 min).
3. Immediately sterilize outside of vial with 70% ethanol.
4. Under laminar flow hood, carefully open vial and slowly pipet contents of vial into sterile 15-ml polypropylene centrifuge tube (e.g., Falcon).
5. Add 9 ml pre-warmed 28°C medium drop-wise to cells. Do NOT add the thawed cells to the medium.
6. Gently pipet cell suspension 3–5 times.
7. Centrifuge the cells at 400 x *g* for 3 minutes. (e.g. 1500 rpm in GH 3.8 rotor)
8. Remove medium using a pipet or by aspiration.
9. Add 10 ml fresh medium, prewarmed to 28°C. Gently pipet the cells to resuspend.
10. Transfer the cells to a 125-ml shake flask (recommended) or a T-75 flask.
11. Incubate at 28°C. For shake flasks, set shaker to 150 rpm. After 48 hours, determine growth and viability by Trypan Blue dye exclusion method. (see p 6). Cell number will usually be above 1 x 10^6 cells/ml with viability above 85%. Proceed to passage cells as described in the following sections. Recently thawed cells often grow slowly and have low viability, but after one week, viability should be above 95% and cell growth should be steady with a doubling time of about 1–1.4 days.

Suspension cultures

Sf9 Insect Cells can be conveniently grown and maintained in suspension as shaker cultures. Exponentially growing cells are incubated in a temperature-controlled orbital shaker operating at 150 rpm and 28°C. Use sterile, disposable polycarbonate Erlenmeyer culture flasks. To ensure proper aeration, the liquid culture should not comprise more than 20% of the vessel volume, and threaded caps should be slightly loose.

To initiate shake culture from monolayer cultures, dislodge cells from flask by pipetting medium over cells. Count cells and dilute with pre-warmed 28°C medium to final concentration of 0.75 x 10^6 cells/ml. Transfer to shake flask.

Insect cell densities in suspension culture

Cell line	Medium	Cell seeding density (cells/ml)	Maximum cell density (cells/ml) before passaging
Sf9	Serum-free medium	0.3–0.75 x 10^6	6.0 x 10^6

1. Incubate at 28°C with shaker set to 150 rpm.
2. Passage cells every 2-3 days. Count cells and determine viability by Trypan Blue dye exclusion method. (see p 6).

   Note:To maintain viability and for successful virus and protein production keep the cell concentration between 0.3 x 10^6 and 5 x 10^6 cells/ml. The cells are usually passaged when the density reaches 2–4 x 10^6 cells/ml.

3. Dilute with pre-warmed 28°C medium to desired seeding density. Transfer to shake flask and incubate at 28°C shaking at 150 rpm. Suspension cells are usually seeded at 0.75 x 10^6 cells/ml in a total volume of 50 ml in a 250-ml disposable polycarbonate Erlenmeyer flask and passaged every other day. To scale up, seed Sf9 cells to larger or multiple new flasks according to the application needs. A 1L–shake flask can be used for 150–250 ml culture.

   Important: Over-dilution will result in cell death; avoid densities less than 0.3 x 10^6 cells/ml.

Monolayer Cultures

Typically, cells grown at 28°C in a monolayer are split 1:5–1:8 every 3–4 days when confluency is between 85–95%. Depending on needs, nearly confluent monolayer cells can be split at any ratio between 1:2 and 1:20.

Typical ratios used to subculture and seeding densities of insect cells in T flasks

Cell line	Ratio of existing culture to fresh medium	Flask size	Seeding density (cells/flask)	Medium Volume
Sf9	1:5	T-25	2.0–2.5 x 10^6	5 ml
		T-75	3.0–5.0 x 10^6	10 ml
		T-150	6.0–10.0 x 10^6	30 ml

1. Based on cell count, seed Sf9 cells to flask, according to needs (see table, above).
2. Incubate at 28°C.
3. Examine monolayer Sf9 insect cells under microscope every 2–4 days to determine if cells are healthy and if the monolayer confluency is between 85–95%.

   Note: Healthy Sf9 cells should appear rounded and bright with distinct cell boundaries, whereas unhealthy Sf9 cells appear "blebby", dark, and granular. A large number of floating cells is usually a sign of an unhealthy culture. However, when over-confluent, Sf9 cells will start to float and divide in medium because Sf9 cells are not subject to contact inhibition.

4. Gently aspirate medium from flask.
5. Add 5 ml prewarmed (28°C) medium by pipetting from side of flask.
6. Gently rinse cells off by pipetting repeatedly, or by gently using a sterile scraper.
7. Transfer dislodged cells into sterile 50-ml polypropylene centrifuge tube.
8. Count cells using Trypan Blue exclusion method. (see p 6).
9. Based on cell count, seed Sf9 cells to new flasks according to needs.

Preparation of Sf9 cells for storage in liquid nitrogen

For long term storage, Sf9 cells should be frozen and stored in liquid nitrogen. Aliquots of Sf9 cells frozen in liquid nitrogen also provide a source of fresh Sf9 cells when the working stocks become too old or have been passaged more than 20–25 times. Freeze cells in 1 ml aliquots at 1 x 10^7 cells/ml in BacVector® Insect Cell Medium, 30% FBS, 10% DMSO.

1. Prepare exponentially growing Sf9 cells as described above for suspension cultures.
2. Count cells to ensure viability is greater than 90% by Trypan Blue exclusion. (see p 6).
3. Centrifuge cells at 400 x *g* for 3 minutes. Remove medium using a pipet.
4. Resuspend cells in BacVector Insect Cell Medium at 2 x 10^7 viable cells per ml.
5. Label cryogenic vials.
6. For each ml resuspended cells, add 0.2 ml BacVector Insect Cell Medium, 0.6 ml heat-inactivated sterile filtered FBS, and 0.2 ml DMSO.

   Note:It is important to use a high-grade DMSO

7. Gently pipet cell suspension to ensure complete mixing.
8. Aliquot 1 ml cell suspension into each cryogenic vial. Close cap tightly.
9. Freeze vials by decreasing the temperature 1°C per minute until temperature reached -70°C. Rate of temperature decrease can be controlled by using a suitable device (e.g. "Mr. Frosty:").
10. Transfer vials as rapidly as possible to liquid nitrogen tank for long term storage.

    Important: After one or two weeks, retrieve a vial and test viability of stored cells by following the protocols for cell recovery, and Trypan Blue exclusion (see p 6).

Cell viability by Trypan Blue exclusion

1. Add 100 µl cells to 100 µl Trypan Blue solution (0.4% Trypan Blue in 0.85% saline).
2. Mix by pipetting up and down 5 times.
3. Immediately apply small aliquot to both sides of a hemacytometer with a cover slip on.
4. Count cells. For an accurate assessment, count several samples. Trypan Blue stains only dead cells. It is important to count live cells soon after Trypan Blue dye is added because live cells lose their capacity to exclude dye with time.

Alternative insect cell lines

Insect cell densities in suspension culture

Cell line	Medium	Cell seeding density (cells/ml)	Maximum cell density (cells/ml) before passaging
Sf21	Serum-containing medium	0.1–0.2 x 10^6	2.0–2.5 x 10^6

Typical ratios used to subculture and seeding densities of insect cells in T flasks

Cell line	Ratio of existing culture to fresh medium	Flask size	Seeding density (cells/flask)	Medium Volume
Sf21 or *T. ni*	1:10	T-25	1.0–1.5 x 10^6	5 ml
		T-75	3.0–5.0 x 10^6	10 ml

Preparation of transfection quality plasmid DNA

After cloning the target gene into an appropriate transfer plasmid, it is necessary to prepare plasmid DNA from the *E. coli* cloning host. For co-transfection into insect cells the DNA should be low in endotoxins. For consistent DNA quality, use a product specifically intended for producing transfection quality plasmid DNA. Alternatively, plasmid DNA may be prepared on CsCl gradients using standard methods. Prepare working solution for transfection at a final DNA concentration of 0.1 µg/µl in TlowE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

III. Production of Recombinant Baculovirus

To produce recombinant baculovirus, insect cells are co-transfected with transfer plasmid DNA and BacMagic™ DNA. Homologous recombination between these two molecules yields a baculovirus genome with the promoter and target sequence from the transfer plasmid located between the *ORF1629* and the *lef2* loci. The recombination also restores the function of the essential viral *ORF1629*, enabling the recombinant baculovirus to replicate and produce a population of recombinant virus, which are released into the medium.

Preparation of cell cultures for transfection

For each co-transfection, prepare one 35-mm plate. We recommend also including plates for positive and negative transfection controls.

1. Seed dishes with insect cells at least 1 h before use. Use 1 x 10^6 cells/dish for Sf9 cells in 2 ml BacVector® Insect Cell Medium. Gently rock plates in a side-to-side and back-and-forth pattern to ensure an even monolayer. ***Do not swirl plates*** because cells will cluster into the center. Allow cells to attach to plates (about 20 min at 28°C).
2. During 1 h incubation period, prepare a co-transfection mix of DNA and Insect GeneJuice® Transfection Reagent as follows:

   For each transfection, assemble the following components in the order listed in a sterile tube.

   1 ml BacVector Insect Cell Medium
   5 µl Insect GeneJuice
   5 µl BacMagic DNA (100 ng total)
   5 µl transfer vector DNA (500 ng total)
   1.015 ml Total volume

   Note:The following transfections are optional, but highly recommended:

   Negative control: Instead of the recombinant transfer plasmid, use a corresponding amount of medium or TlowE.

   Positive control: Instead of the recombinant transfer plasmid, use 500 ng of the supplied Transfection Control Plasmid.

3. Mix with gentle agitation or vortexing.
4. Incubate at room temperature for 15–30 min to allow complexes to form.

   Co-transfection
5. Just prior to the end of the transfection mixture incubation period, remove culture medium from 35-mm plate(s) using sterile pipet. Do not disturb cell monolayer. When removing liquid from a dish of cells, tip the dish at a 30–60° angle, so the liquid pools to one side of the dish.
   Do not let monolayer dry out.

   Note: For cells maintained in serum-supplemented medium, wash the monolayer two times, each with 1 ml serum-free medium, before proceeding with co-transfection.
6. Immediately after medium has been removed from cells, add 1 ml transfection mixture drop wise to center of dish. Incubate in humidified container at 28°C overnight (minimum 5 h).
7. After initial incubation period, add 1 ml BacVector® Insect Cell Medium to each dish. Serum can be added to medium at this point, if desired. Continue incubation 5 days total.
8. After 5 days incubation, harvest medium containing recombinant baculovirus. The expected titer of this initial viral seed stock is generally about 1 x 10^7 pfu/ml. Cells in the negative control will have formed a confluent monolayer. Virus-infected cells will appear grainy with enlarged nuclei and will not have formed a confluent monolayer.

   Note:If a gus-containing transfer plasmid (such as the Transfection Control Plasmid) was used, expression of β–glucuronidase can be evaluated to detect presence of recombinant virus. Three to five days post-transfection, remove a 100 µl sample of the medium from the dish and combine with 5 µl X-Gluc (20 mg/ml). Incubate overnight at room temperature for the X-Gluc staining. Recombinant gus-containing viruses express β–glucuronidase (gus) and the medium will stain blue.

IV. Amplification of Recombinant Virus

Amplification of the recombinant virus is necessary before proceeding with experimental work. The following provides a protocol for amplification of virus in cells grown in suspension culture.

1. Prepare 100–200 ml culture of Sf9 cells at an appropriate cell density (e.g. 2 x 10^6 Sf9 cells/ml in log phase growth). Cells should be infected at a low multiplicity of infection (MOI) of < 1 pfu/cell.

   Note: The surface area to volume in shake flasks should be as large as possible. Shake flasks should be shaken at speeds to maximize aeration.
2. Add 0.5 ml recombinant virus seed stock to cell culture. Incubate with shaking until cells are well infected (usually 4–5 days).

   Note: Under a phase-contrast inverted microscope, cells infected with virus appear grainy when compared to healthy cells. The infected cells become uniformly rounded and enlarged, with distinct enlarged nuclei.
3. When cells appear to be well infected with virus, harvest cell culture medium by centrifugation at 1000 x g for 20 min at 4°C. Remove supernatant aseptically. Store supernatant (recombinant virus stock) in dark at 4°C.

   Note: The virus stock can be stored in the dark at 4°C for 6–12 months, although the titer will begin to drop after 3–4 months. Titer the virus before use and reamplify if necessary. The addition of 2–5% serum when using serum-free medium can be helpful in avoiding a drop in titer. Virus may be frozen at –80°C for longer periods of time. Avoid multiple freeze thaw cycles.
4. A plaque assay to determine accurate titer is strongly recommended before proceeding with use of the virus in subsequent expression experiments.

V. Plaque Assay to Titer Recombinant Virus

The following provides a protocol for a plaque assay to accurately determine the titer of the virus. An alternative method for quickly determining titer is the FastPlax™ Titer Kit (Cat. No. 70850).

Preparation of BacPlaque™ Agarose stock

BacPlaque™ Agarose is used for all agarose overlays including virus titration. To prepare 10 ml stock of 3% BacPlaque Agarose, add 0.3 g agarose to 10 ml sterile deionized water in a 100 ml Pyrex media bottle, cap loosely and autoclave. Mix well as soon as the autoclave cycle has finished (being careful to avoid splashing of the hot liquid). Allow the bottle to cool, tighten the cap, and store at room temperature.

Plaque assay

Use a plaque assay to determine the titer of a virus stock. For most applications, a titer of 5 x 10^7 pfu/ml or higher is adequate. A titer of less than 1 x 10^7 pfu/ml will generally not be sufficient for expression studies.

1. Label ten 35-mm plates, two each of "10^{-4}", "10^{-5}", "10^{-6}", "10^{-7}", and "control". Add Sf9 cells (0.9 x 10^6 cells/plate). Leave plates at room temperature for 1 h on a level surface.
2. During incubation period, label 7 sterile tubes from 10^{-1} to 10^{-7} for serial dilutions. Add 0.45 ml room temperature BacVector® Insect Cell Medium (no antibiotics; if using cells grown in serum-supplemented medium, serum must be added) to each tube. Add 0.5 ml BacVector Insect Cell Medium in another tube for control.
3. Add 50 µl undiluted recombinant virus to the tube labeled 10^{-1}. Mix thoroughly.
4. Using a fresh pipet tip, transfer 50 µl from this tube to the next. Mix thoroughly. Continue this process, diluting the virus until the 10^{-7} is reached.
5. Once cells have attached and formed (sub-confluent) monolayer, remove the media and add 100 µl virus dilution (10^{-4} to 10^{-7} in duplicate) to each dish of cells (8 dishes total). Include 2 control dishes, using 100 µl BacVector Insect Cell Medium on each dish, in place of virus dilution.
6. Incubate plates at room temperature for 1 h, gently rocking the liquid across them twice during the hour to prevent cells from drying out. Rock stacked plates by tipping them just enough for the liquid to pool to the side of dish. Hold the position for 15–30 s, then tip the liquid across the dish. Rotate stack 90° and repeat process, ensuring entire surface is covered with the mixture.
7. Approximately 15 min prior to completion of incubation period, prepare agarose overlay. Loosen cap of 10 ml 3% BacPlaque Agarose stock. Microwave until agarose is completely melted and there are no remaining lumps. Place bottle in 37°C water bath, cool to less than 50°C and add prewarmed (37°C) BacVector Insect Cell Medium containing 5% FBS to a total volume of 30 ml. The final agarose concentration is 1% (w/v) agarose at 2/3 strength medium containing serum. The overlay can be used immediately, or kept at 37°C for at least 1 day without solidifying.

   Note: If agarose sets before use, do not remelt. Prepare fresh batch.
8. After incubation period, remove virus inoculum.
9. Add 2 ml BacPlaque Agarose-medium-serum mixture to each plate, by pipetting it slowly down side of plate. Do not move plate again until agarose has solidified (usually 20 min at room temperature is sufficient). Keep plates covered.
10. When agarose overlay has set, add 1 ml BacVector Insect Cell Medium to each dish. The additional medium equilibrates medium in the agarose that is at 2/3 strength in order to promote optimal cell and virus growth.
11. Carefully transfer 35-mm dishes to a flat-bottomed covered humid container so monolayer does not dry out. Incubate at 28°C for 3–4 days (72–96 h), by which time cell monolayer should be confluent.

Plaque staining

After incubation for 3–4 days, plaques in the monolayer can be visualized and counted. Successful plaque identification is dependent on careful evaluation of several dilutions of virus with appropriate staining techniques. Plaques can be seen on unstained monolayers but are most easily observed by staining for live cells or β-glucuronidase (gus) reporter gene activity. Neutral Red stains live cells red, leaving unstained plaques visible in the monolayer. X-Gluc stains plaques blue that contain recombinant *gus* virus. X-Gluc staining can be used only with pBAC™gus transfer plasmids (including Transfection Control Plasmid). Neither staining method harms the virus. For staining, always remove the medium from the plates or wells by tipping slightly so the agarose and cell monolayer is not disturbed. Do not pour off medium because this may cause monolayer to slip. Stain with one of the following methods.

1. *Neutral Red Staining:* Dilute a 0.33% (w/v) Neutral Red stock solution 1:13 with sterile phosphate-buffered saline (PBS: 43 mM Na_2HPO_4, 15 mM KH_2PO_4, 137 mM NaCl, 27 mM KCl, pH 7.4) just before use. To avoid precipitation, do not store diluted stain in the light at room temperature for more than 8 h. Carefully remove liquid overlay from plates, and pipet 1 ml freshly diluted staining solution onto center of each plate. Incubate plates at 28°C for 2 h. Carefully remove stain and store plates in the dark at room temperature for 3 h or more. During this time cells take up stain, making the plaques more visible. Leaving plates at room temperature overnight may make plaques more distinct.
2. *Gus activity and Neutral Red staining*: Prior to staining, thaw the X-Gluc solution (20 mg/ml) at room temperature (do not allow contact with polystyrene surfaces). Unused solution should be stored at –20°C. To visualize gus-producing plaques, carefully aspirate the liquid overlay from the wells or dishes. Return the plates or dishes to a flat surface and replace the medium with 2.0 ml (60-mm dish) or 1.0 ml (6-well plate) freshly diluted Neutral Red solution in PBS (see above). Add 15 µl X-Gluc Solution per ml Neutral Red solution and incubate at 28°C for 2 h. Carefully remove the staining solution and incubate at room temperature an additional 3 h to overnight. The gus gene is under the control of the late basic promoter ($P_{6.9}$). Due to the low level expression with this promoter, pBAC™gus and Transfection Control Plasmid recombinants could take overnight to develop blue plaques.

Plaque identification

Hold the plate up to a light source and observe the monolayer from underneath. Alternatively, place the plate on a light box. Do not disturb the monolayer by jarring or inclining the plate. On unstained monolayers plaques appear cloudy against a more transparent background. Plaques may become more clearly defined with increasing incubation time and form large areas of cleared cells; however, because the monolayer will overgrow and plaques will expand and overlap, it is best to identify plaques at an early stage. On stained monolayers, the plaques appear as clear (or blue) circles on the pink monolayer of stained cells. It is useful to examine several plates of different dilutions to obtain an accurate titer.

Calculation of virus titer

1. To count plaques, remove lid from plate and invert plate on a light box. It is helpful to touch one edge of the plate on a paper towel (while inverted) to catch any remaining dye that drains off the plate. The plate should then be kept in a level position on the light box while plaques are marked. Use a marker pen to circle the locations of well-isolated plaques on the underside of plate. Set plates in an upright position, replace lid and let them remain at room temperature overnight. Since small plaques will be more visible after an overnight incubation, previously unmarked plaques should be counted the following day to obtain a more accurate titer.
2. To calculate the titer (number of plaque forming units per ml in the original undiluted sample) multiply the number of plaques observed on a plate by the dilution factor and divide by the volume plated. For example, if 22 plaques occur on the plate of 0.1 ml of the 10^{-6} dilution, the titer is 2.2×10^8 pfu/ml ($22 \times 10^6/0.1$).

VI. Expression

For expression, exponentially growing cells should be infected at a high MOI to ensure all cells are infected simultaneously and the culture is synchronous. The optimal MOI is usually 5–10 pfu/cell, but should be determined for each particular virus. To optimize the MOI, use various MOIs to infect prepared plates or shake cultures. Examples of recommend MOIs to test are 2, 5, and 10. Harvest cells and/or culture medium at different time points after infection (24, 48, 72, and 96 h) and evaluate protein expression using SDS-PAGE or Western analysis.

VII. Troubleshooting Guide

Problem	Probable cause	Solution
Co-transfection failed to produce recombinant virus	Cells not in good condition	Use cells in good condition.
	Cells dried out	Do not let monolayer dry out.
	Serum present	If cells were grown in serum-supplemented medium, wash cells with serum-free medium before transfection.
	Transfection reagent not working	Evaluate transfection reagent with positive control.
Amplification failed to produce high titer recombinant virus	Cell density too high; virus amplification inhibited	Seed plates according to recommendations on p 5.
	Virus titer too low	Incubation beyond 5 days necessary. Monitor infection with phase-contrast microscope to determine optimal harvest time.
	Cells infected at too high an MOI; only one round of amplification occurred	Cells should be infected at low (MOI) of < 1 pfu/cell.
No plaques on plaque assay	Cells not in good condition	Use cells in good condition.
	Cell density too high; plaques too small to see with microscope	Seed plates according to recommendations on p 5.
	Cell density too low; large, poorly defined plaques	Seed plates according to recommendations on p 5.
	Agarose overlay too hot	Agarose overlay should be < 37°C
	Cells not conditioned to serum-free medium	Add serum-supplemented medium to agarose overlay for cells that require serum.
	Virus titer too low	Plate lower dilutions
	Virus titer too high; all cells lysed	Plate higher dilution
	Neutral Red not fresh	Always prepare freshly diluted Neutral Red for plaque assays.
Agarose overlay has cracks	Virus inoculum not completely removed from cells before addition of agarose overlay	Remove inoculum to avoid interference with gelling process.
Plaques appear smeared	Virus inoculum not completely removed from cells before addition of agarose overlay	Remove inoculum to keep virus contained within foci of cells, and avoid virus spreading randomly.
Plaques around edge of plate	Virus inoculum not added uniformly	Add inoculum drop-wise to center of dish
No expression	Cells not in good condition	Use cells in good condition.
	Cells not in log phase	Use cells in log phase.
	Cells not infected at adequate MOI	Titer virus. Infect at adequate MOI.
	Virus stored for extended time period.	Titer virus. Amplify if necessary.
Low protein yield	Sf9 or Sf21 cells must be used for co-transfection and virus amplification, but other cells may produce higher protein yields.	Use *T. ni* for protein expression. (ECACC)
	Harvest time not optimal	Set up time course to determine highest protein yield while avoiding proteolytic degradation.
	MOI not optimal	Optimal MOI is usually 5–10 pfu/cell, but should be optimized for each particular virus.