Chapter 3

Application of MultiBac System to Large Complexes

Shuya Fukai

Abstract

Multisubunit protein complexes regulate numerous biologically important processes. Elucidation of their functional mechanisms based on their three-dimensional structures allows us to understand biological events at the molecular level. Crystallography and electron microscopy are powerful tools for analyzing the structures of biological macromolecules. However, both techniques require large-scale preparation of pure and structurally homogenous samples, which is usually challenging for large multisubunit complexes, particularly from eukaryotes. In this chapter, we describe the principles and methods of producing multisubunit complexes in insect cells using the MultiBac system.

Keywords Multisubunit protein complexes, Baculovirus, Insect cell expression, MultiBac system

1 Introduction

Multisubunit protein complexes play critical roles in numerous cellular processes, such as gene regulation, protein degradation, and intracellular signaling. Some complexes are abundant in cells and can be obtained from natural sources for use in biochemical and biophysical experiments. However, expressions of many others are spatiotemporally restricted, particularly in eukaryotes; therefore, overproduction of the recombinant complexes is often required for biochemical and biophysical analyses. Multisubunit protein complexes can be prepared in two different ways: recombinant subunits are either produced separately in cells and reconstituted in vitro, or coexpressed and reconstituted in cells. The first approach is effective when individual subunits themselves are stable and soluble in cells. Otherwise, the latter, the coexpression approach, is the only method of obtaining sufficient amounts of protein complexes for analyses. Unfortunately, most protein complexes in eukaryotes have subunits that are unstable and/or insoluble in isolation.

Techniques for recombinant protein production in the bacteria *Escherichia coli* can be rapidly and easily carried out. Several coexpression systems using *E. coli* have been developed. Insertion of several

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genes of interests with their upstream ribosome binding sites (i.e., the Shine-Dalgarno sequence) between one promoter in the 5′-end and one terminator in the 3′-end enables polycistronic expression of proteins. Furthermore, cotransformation with two or three expression plasmids carrying different antibiotic resistance markers can be used for the coexpression. However, many eukaryotic protein complexes have subunits that are difficult to produce in *E. coli*, possibly owing to problems on transcription, translation, folding, and/or posttranslational modifications (e.g., phosphorylation and glycosylation), resulting in the failure of efficient production in *E. coli*.

An excellent solution to this difficult protein production in *E. coli* is the use of a baculovirus expression system, which employs an engineered baculovirus genome derived from the *Autographa californica* nuclear polyhedrosis virus (AcNPV) and appropriate transfer vectors (Fig. 1). Polyhedrin and p10 are products from the

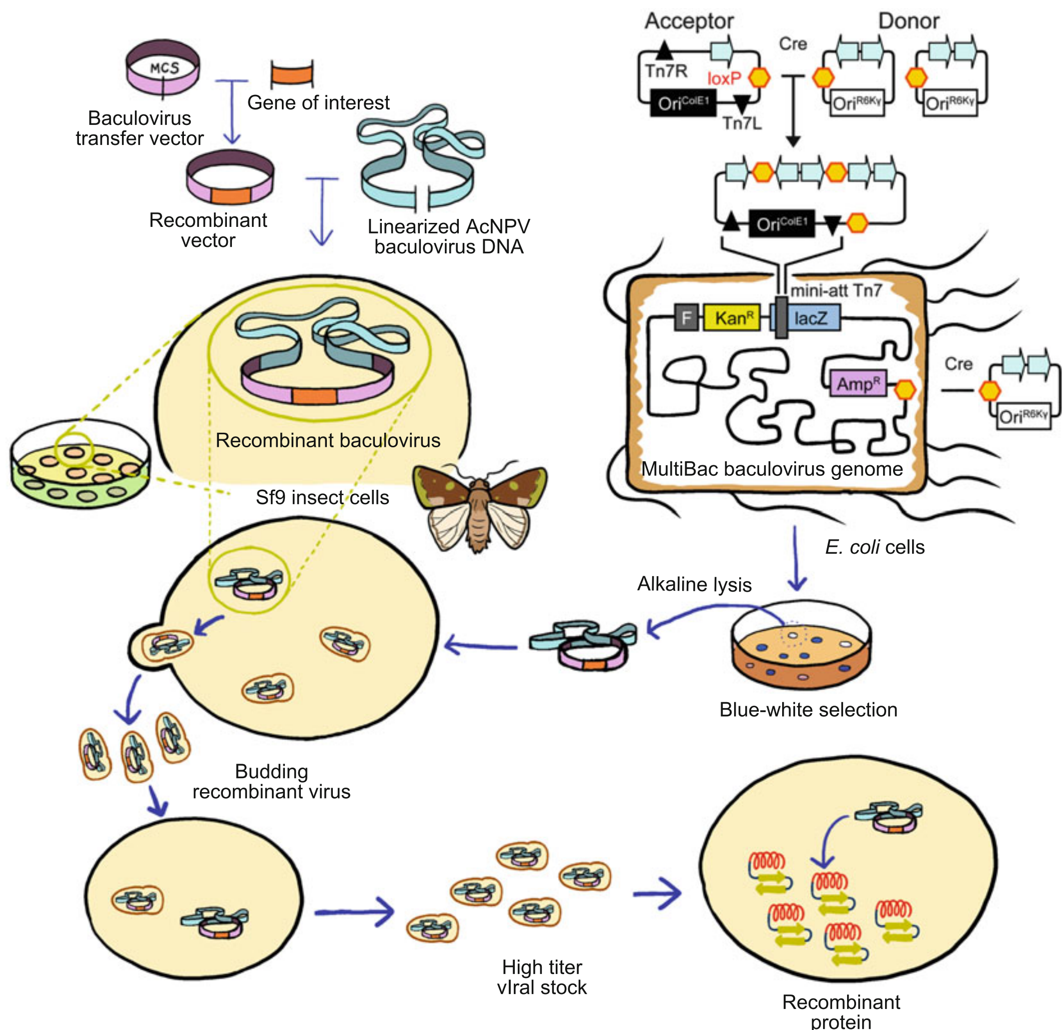


Fig. 1 Schematic drawing of MultiBac baculovirus-insect cell expression system

AcNPV genome and highly accumulated in the late stage of infection [1]. Their transcription promoters allow overproduction of proteins in insect cells. Genes of interest are first inserted between one of these strong transcription promoters (polyhedrin or p10 promoter) and a polyadenylation signal (SV40 or HSVtk polyadenylation signal) in the transfer vector and then integrated into the engineered AcNPV genome. The engineered AcNPV genome containing genes of interest is transfected to cells of the caterpillar *Spodoptera frugiperda* Sf9 or Sf21 cell line. The transfected Sf cells produce viruses that can infect insect cells to overexpress genes of interest. Suspension culture of the transfected Sf cells in shaker flasks is convenient and effective for large-scale protein production.

The genes of interest can be integrated to the engineered AcNPV genome in either insect cells or *E. coli* through the transfer vector. In the first case, both the linearized AcNPV genome containing a lethal deletion and the transfer vector are cotransfected into insect cells, and recombination occurs between the engineered AcNPV genome and the transfer vector. Because the transfer vector can rescue the lethal deletion of the engineered AcNPV genome, viruses that are generated from the genome fused with the transfer vector propagate. However, practically, this strategy might generate nonrecombinant viruses, which could apparently decrease virus titer (infection efficiency) and gene expression level. In such a case, a virus species with higher titer should be isolated from a single plaque by a plaque assay, which may require additional time and effort. This step can be bypassed when genes of interest are integrated to the engineered AcNPV genome in *E. coli* cells, applying the transposing reaction of the Tn7 transposon [2].

The Tn7 transposon is a relatively large DNA segment (14 kb) from the Tn7 phage, which can be integrated into a specific position called the Tn7 attachment site (attTn7) in the *E. coli* genome with high frequency. Tn7 transposon encodes a Tn7 transposase complex, which catalyzes the insertion of DNA elements containing the specific sequences Tn7L and Tn7R into the attTn7 site of another DNA molecule. As the transfer vector containing Tn7L and Tn7R is introduced to specific *E. coli* strains with a copy of the engineered AcNPV genome containing attTn7 and a helper plasmid encoding the Tn7 transposase complex, the transfer vector is transposed to the attTn7 site of the engineered AcNPV genome in the *E. coli* cells. Because the attTn7 site in the engineered AcNPV genome is located in the middle of *lacZα*, positive clones (i.e., *E. coli* cells containing the transposed AcNPV genome) are easily selected by standard blue-white selection in the presence of X-Gal and IPTG. The integrated AcNPV genome can then be isolated from the positive clones by a standard alkaline lysis protocol and used for the subsequent transfection to *S. frugiperda* cells.

In the baculovirus-insect cell system, coinfection by several viruses is one of two options for multigene expression: viruses

expressing each subunit of the complex are first prepared and then simultaneously applied to infect *S. frugiperda* cells for coexpression of the appropriate subunit combination. However, this strategy can only be used under specific conditions because it is difficult to achieve a uniform expression of individual subunits owing to the differences in virus titer and gene expression level. Differences in the expression level among individual subunits may result in the failure of homogeneous complex formation. Another versatile option is multigene incorporation to a single AcNPV genome to generate a single species of virus that can produce multisubunit proteins. The MultiBac expression system was designed and developed for this purpose by Berger and colleagues [3–5].

Multigene incorporation to a single AcNPV genome requires a transfer vector containing a set of genes of interest. The MultiBac system enables two distinct methods for easier construction of the transfer vector for this purpose: one is the use of a multiplication module (Fig. 2) and the other is recombinase-mediated vector fusion (Fig. 3).

Transfer vectors for the "first- and second-generation (old)" MultiBac systems (i.e., pKL, pFL, pUCDM, pSPL, pFBDM, and pKDM) [3, 5] (Fig. 2a) have two multicloning sites, flanked by the restriction sites *Pme*I and *Avr*II. In addition, between these two multicloning sites, there is the multiplication module M to be digested by the restriction enzymes *Bst*Z17I and *Spe*I, which generate cohesive ends compatible with those generated from *Pme*I and *Avr*II, respectively. Therefore, a multigene expression cassette withdrawn from one transfer vector by *Pme*I-*Avr*II digestion can easily be incorporated into the *Bst*Z17I-*Spe*I-digested multiplication module M in another transfer vector (Fig. 3a). Iteration of the incorporation using the multiplication module can multiply expression cassettes in the transfer vector. Similarly, in the "third-

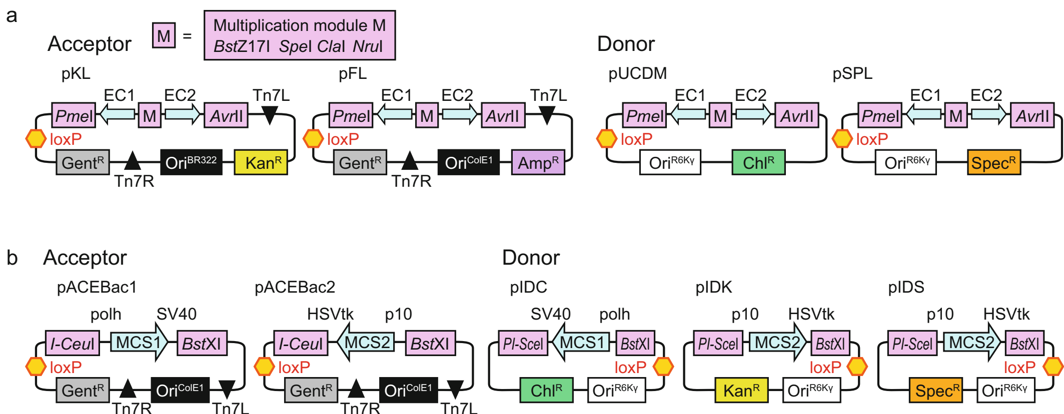


Fig. 2 Transfer vectors for MultiBac system. (**a**) Old set of transfer vectors. (**b**) New set of transfer vectors

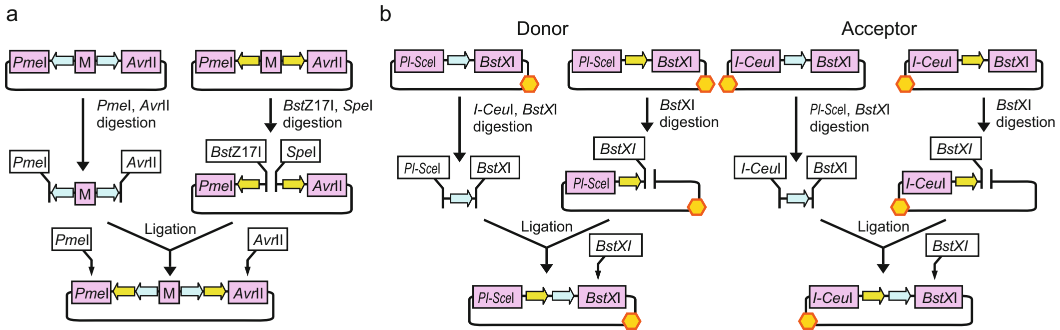


Fig. 3 Concept of multiplication module. The expression cassette can be excised from one vector by restriction digestion and transferred to another transfer vector. (**a**) Multiplication for old set of transfer vectors. (**b**) Multiplication for new set of transfer vectors

generation (new)" MultiBac system [4] (http://www.epigenesys.eu/images/stories/protocols/pdf/20120313121202_p54.pdf), transfer vectors (i.e., pACEBac1, pACEBac2, pIDC, pIDK, and pIDS) (Fig. 2b) have one multicloning site flanked by a homing endonuclease site (I-CeuI for pACEBac1 and pACEBac2 or PI-SceI for pIDC, pIDK, and pIDS) and a compatible *Bst*XI site, which enable iterative incorporation of expression cassettes to the transfer vector, generating a multigene transfer vector (Fig. 3b).

Multiplication of an expression cassette using the multiplication module should enable the incorporation of unlimited numbers of genes to the transfer vector in principle. However, practically, iterative incorporation of the expression cassette generates DNA plasmids that are too large to handle. To address this issue, for the MultiBac system, an additional concept is applied; that is, a number of transfer vectors can be fused by recombination to generate a multigene transfer vector that contains larger numbers of genes (Fig. 4).

Transfer vectors used for the MultiBac system are classified into two groups: "acceptors" and "donors" (Fig. 2). Both acceptors and donors have a short imperfect inverted repeat (LoxP) for the recombination reaction catalyzed by Cre recombinase [6]. Only acceptors contain Tn7L and Tn7R for integration to the engineered AcNPV genome. Acceptors have a ColE1 DNA replication origin and can propagate in standard *E. coli* cloning strains, whereas donors have an R6Kγ DNA replication origin and require a *pir* gene product for their propagation. Therefore, donors can be retained in *pir*-negative *E. coli* strains only when they are fused with acceptors. Propagation of donors in *pir*-negative *E. coli* strains indicates that the donors are appropriately fused with acceptors. This is an important feature because *E. coli* cells can retain two or more independent (i.e., unfused) plasmids with distinct antibiotic markers simultaneously in the presence of the antibiotics. One acceptor can be fused with one or two donors by in vitro Cre recombinase reaction

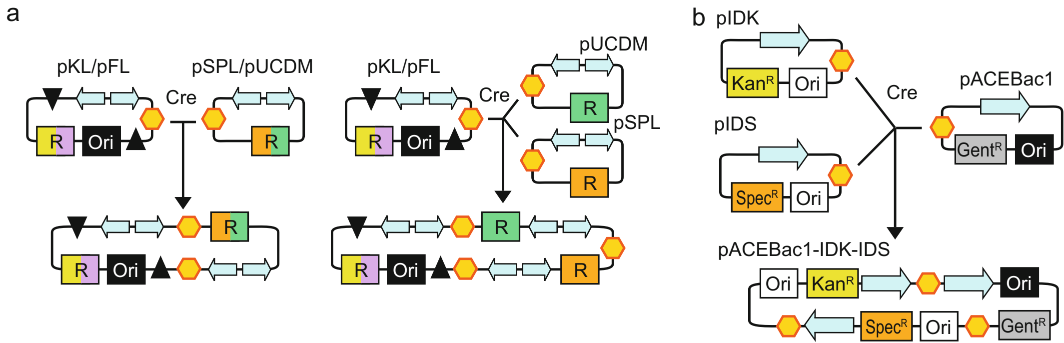


Fig. 4 Concept of recombination-mediated multiplication of expression modules. Cre-mediated recombination allows fusion of two to four transfer vectors. (**a**) Cre-mediated fusion for old set of transfer vectors. (**b**) Cre-mediated fusion for new set of transfer vectors

in one step, followed by transformation of standard *pir*-negative *E. coli* strains with the resultant multigene transfer vector in the presence of the appropriate combination of antibiotics. In the new MultiBac system, the Cre-mediated fusion with three donors can be practically performed in two steps.

The MultiBac system also applies new technologies for the engineered AcNPV genome, where genes encoding viral protease and apoptotic activities are deleted to avoid protein degradation and delay lysis of the infected insect cells. Furthermore, in addition to the attTn7 site, the MultiBac AcNPV genome contains the LoxP site, which is useful for additional functionalities. For example, when a yellow fluorescence protein (YFP) gene is integrated to the LoxP site of the MultiBac AcNPV genome, the produced YFP provides information about protein production and virus performance through its fluorescence almost in real time. This YFP-integrated MultiBac AcNPV genome (EMBacY) is included in the new MultiBac system kit.

Many of the recent structural studies of biologically important eukaryotic multisubunit complexes utilize the MultiBac expression system for their production [7–9] (Fig. 4). All of such complexes were challenging targets of structural studies because of their large size and/or complicated subunit composition.

The anaphase-promoting complex (APC/C) is a cell cycle regulator and composed of 13 subunits with a total molecular weight of ~1.1 MDa. The gene assembly encoding the complete APC/C was inserted into two MultiBac baculoviruses, one encoding eight subunits and the other five. Coinfection by two viruses enables the production of the entire 1.1 MDa APC/C complex. The purified complex was subjected to electron microscopy analysis, revealing the complicated subunit architecture of this huge complex [9] (Fig. 5).

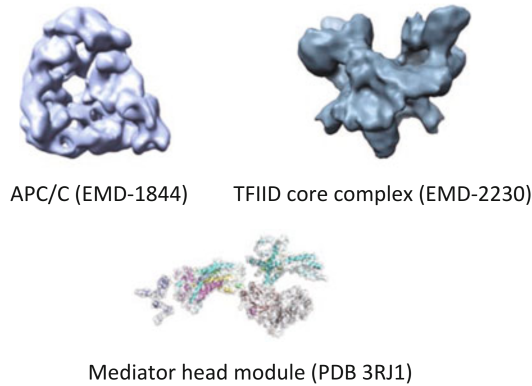


Fig. 5 Three-dimensional structures of large multisubunit complexes analyzed using MultiBac expression system. For APC/C, two subcomplexes (named TPR5 and SC8) were also analyzed besides the entire APC/C

The transcription mediator controls transcription through its interactions with RNA polymerase and transcriptional activators. The mediator consists of 25 or more subunits with a total molecular weight of ~1.2 MDa. The head module of the yeast mediator (seven subunits, Mw 223 kDa) was produced using the MultiBac system and subjected to cryo-EM analysis and X-ray crystallography, revealing the architecture and dynamics at the atomic level [8].

A general transcription factor, TFIID, binds gene promoters and regulates the initiation event of transcription. TFIID is composed of the TATA-box-binding protein (TBP) and 13 TBP-associated factors (TAFs) with a total molecular weight of over 1 MDa. The TFIID core complex (five subunits, Mw 650 kDa) was produced using the MultiBac system and subjected to cryo-EM analysis, revealing its subunit stoichiometry and architecture [7]. For the production of this TFIID core complex, an additional technology was applied for the uniform expression of the individual subunits. Coexpression of the TFIID core subunits by the original MultiBac system showed imbalanced production of the individual subunits, which hampered the purification of the complexes. This imbalanced expression problem was solved by a polyprotein strategy, where a number of proteins are encoded in one large open reading frame (ORF) and generated by proteolysis with a highly specific protease in a manner similar to protein production by RNA viruses such as the coronavirus. The TAF-encoding genes are concatenated into a single ORF, spaced by cleavage sites for a protease NIa from the tobacco etch virus (TEV). The TEV protease gene precedes the TAF genes in the ORF. A cyan fluorescent protein (CFP) gene is also inserted in the 3′ end of the ORF for checking the expression of all conjoined proteins.

These structural studies of large protein complexes including those with molecular weights of over 1 MDa demonstrate that the

MultiBac system is a powerful tool for the overproduction of challenging multisubunit protein complexes. Higher-resolution analysis of complex structures requires more homogeneous samples in terms of size, composition, and posttranslational modification and conformation, which typically require the removal of specific subunits and/or modification of the individual subunits by site-directed mutations and/or trimming regions that are predicted or experimentally shown to be flexible or disordered. The modular concept of the MultiBac system is highly compatible with this optimization process. It has been announced that the MultiBac system is still under development and that new technologies are planned to be included. This powerful tool for the production of multiprotein complexes will further accelerate the study of the structural biology of challenging targets to elucidate more complex biological processes.

2 Materials

2.1 Bacteria Work

Escherichia coli strains: DH10Bac, DH10MultiBac, a standard *pir*-negative strain (e.g., DH5α), *PirHC, *PirLC (see Note 1)

Transfer vectors: pKL, pFL, pUCDM, pSPL (old set), pACEBac1, pACEBac2, pIDC, pIDK, pIDS (new set)

Antibiotics (1000×): 50 g/L kanamycin, 10 g/L tetracycline, 10 g/L gentamicin, 50 g/L ampicillin, 30 g/L chloramphenicol

Enzymes: Cre recombinase (NEB), high-fidelity DNA polymerase (Toyobo KOD plus neo), in-fusion cloning kit (Clontech)

Medium: Luria broth (LB) medium (Nacalai Tesque)

Equipment: Shaker incubator (temperature controlled at 37 °C; INNOVA 44R, TAITEC BR-23FP•MR), electroporator (BioRad MicroPulser), toothpick

Chemicals: X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), IPTG

2.2 Insect Cell Work

Insect cell lines: Sf9, Sf21

Antibiotics (200×): Penicillin-streptomycin mixed solution (Nacalai Tesque)

Medium: Sf-900II SFM serum-free medium (Invitrogen), fetal bovine serum (Gibco)

Transfection reagent: X-Treme GENE HP transfection reagent (Roche)

Flasks: Erlenmeyer flasks (125, 250, 500, 1000, 2000 mL; Corning)

Biological safety hood with UV illumination

Six-well (35-mm-diameter) tissue culture plates (Falcon)

Shaker incubator (temperature controlled at 27 °C; TAITEC G•BR200)

3 Methods

3.1 Cloning of Genes of Interest for Construction of MultiBac Transfer Vectors

The old set of MultiBac plasmids contains two multicloning regions, whereas the new set contains a single multicloning region. Conventional cloning methods using restriction enzymes and ligases are applicable. However, sequence- and ligation-independent cloning (SLIC) methods [10] are highly convenient for large DNA insertions, which typically have multiple restriction sites. Therefore, we use SLIC for the construction of MultiBac transfer vectors. In-fusion cloning kits (Clonetech) are commercially available. The Clonetech website (https://www.takara-bio.co.jp/infusion_primer/) is highly convenient for the design of PCR primers for SLIC.

1. Linearize 3–5 μg of vectors (for ten or less SLIC reactions) by either PCR or restriction enzyme digestion and purify them by agarose gel electrophoresis (see Note 2).
2. Set up SLIC reaction (10 μL for each), as shown in Table 1.
3. Incubate the reaction mixture at 50 °C for 15–20 min and stop the reaction by placing the mixture on ice.
4. Transform chemically competent *E. coli* cells with 1 μL of the reaction. Typical *E. coli* cloning strains (e.g., DH5α) are available for vectors harboring the ColE1 or pBR322 replication origin (i.e., pFL, pKL, pACEBac1, and pACEBac2), whereas a special strain (PirHC or PirLC) is required for vectors harboring the *pir* replication origin (i.e., pUCDM, pSPL, pIDC, pIDK, and pIDS) (see Table 2).
5. Plate the transformed cells on LB agar plates containing the appropriate antibiotics for selection (see Table 2) and incubate them at 37 °C for 12–15 h.

Table 1
SLIC reaction

5 X reaction mix (enzyme included)	2 μL
PCR-amplified insert	~50 ng
Linearized vector	~50 ng
Pure water	to 10 μL

Table 2
Antibiotics and host strains of transfer vectors for selection

Vectors	Antibiotics	Host strains
pFL	Ampicillin, gentamicin	Standard
pKL	Kanamycin, gentamicin	Standard
pACEBac1, pACEBac2	Gentamicin	Standard
pUCDM, pIDC	Chloramphenicol	PirHC, PirLC
pSPL, pIDS	Spectinomycin	PirHC, PirLC
pIDK	Kanamycin	PirHC, PirLC

6. Select positive colonies by PCR analysis. A premixed PCR solution (e.g., Promega GoTaq Master Mix) is convenient for this purpose. Each colony is picked with a sterilized toothpick, and the toothpick is briefly dipped into a tube containing a PCR reaction mix. The reaction products are separated by agarose gel electrophoresis. We use the 5′ and 3′ primers derived from the insert and vector sequences, respectively, to confirm that the insert is integrated into the vector. Typically, four to eight colonies are sufficient to obtain two or more positive clones.
7. Isolate vectors from positive clones using a commercially available mini-prep kit (e.g., Promega Wizard Plus SV Minipreps DNA Purification System) and verify the nucleotide sequences of the insert by DNA sequencing. Promoter and terminator regions should also be verified when vectors are linearized by PCR in Step 1.
8. Store the verified vectors at −20 °C until use.

3.2 Multiplication of Expression Cassettes

Multigenes can be assembled in a single vector by using a multiplication module (Fig. 3). Genes inserted into the old vectors (i.e., pKL, pFL, pUCDM, and pSPL) can be excised as a cassette by restriction enzyme digestion with *Pme*I and *Avr*II. This cassette can be transferred to another vector digested with *Bst*Z17I and *Spe*I. Similarly, genes inserted into the new vectors (i.e., pACEBac1, pACEBac2, pIDC, pIDK, and pIDS) can be excised by *Bst*XI digestion and either I-*Ceu*I or PI-*Sce*I digestion and transferred to another vector digested with *Bst*XI within either of the donor vectors or acceptor vectors.

1. Prepare the cassette and linearized vector by restriction enzyme digestion of 2–3 μg of vectors containing gene(s) of interest and purify them by agarose gel electrophoresis before ligation (see Note 3).

2. Set up the ligation reaction with the purified cassette and linearized vector.
3. Transform the appropriate *E. coli* strains with 1 μL of the ligated reaction product. The efficiency of transformation may decrease if the ligated vector is longer than 10 kb. Therefore, we use electrocompetent cells for longer vectors, instead of chemically competent cells. For electroporation, we use a MicroPulser (BioRad) with the conditions preset for *E. coli*.
4. Select positive clones as in Step 3.1.6, except that the 5′ and 3′ primers derived from the insert are used for colony PCR analysis because the upstream and downstream sequences are the same in the cassettes.
5. Isolate the vector from the positive clone using a commercially available mini-prep kit (e.g., Promega Wizard Plus SV Minipreps DNA Purification System) and store it at −20 °C until use.

3.3 Cre-mediated Fusion

Cre-mediated fusion is also available for generating multigene transfer vectors. In both the new and old systems, it is guaranteed that one acceptor vector can be fused with one or two donor vectors. Furthermore, fusion between one acceptor and three donors is possible in the new system.

1. Set up the Cre reaction (10 μL for each), as shown in Table 3, and incubate the reaction mixture at 37 °C for 30 min to 1 h (see Note 4). Optionally, the reaction can be stopped by heating at 65 °C for 5 min. To avoid the integration of more than one acceptor, the amount of the acceptor vector should be lower than those of the donor vectors.
2. Transform the *pir*-negative *E. coli* strain (e.g., DH5α) with 1 μL of the Cre reaction mixture. Cre fusion generates longer vectors, particularly in the old system. Therefore, we use electrocompetent cells for transformation with the Cre-fused vectors.
3. Select positive clones as in Step 3.1.6.

Table 3
Cre reaction

10 X Cre reaction buffer (NEB)	1 μL
Acceptor vector	~400 ng
Donor vector(s)	~500 ng each
Cre recombinase (NEB)	1 μL
Pure water	to 10 μL

4. Isolate the vector from the positive clones using a commercially available mini-prep kit (e.g., Promega Wizard Plus SV Minipreps DNA Purification System).
5. Confirm that all genes are present in the vector by PCR analysis after the vector isolation (see Note 5) and store the vector at −20 °C until use.

3.4 Preparation of Multigene-Integrated Engineered AcNPV Genome

Multigene transfer vectors constructed in Steps 3.2 and/or 3.3 are integrated into the engineered AcNPV genome retained in special *E. coli* strains such as DH10Bac and DH10MultiBac (see Note 6). DH10MultiBac is deficient in protease and chitinase to reduce proteolysis and extend cell viability. For the transformation, we use electrocompetent cells (see Note 7).

1. Prepare electrocompetent DH10Bac or DH10MultiBac cells that retain the engineered AcNPV genome and helper plasmid.
   (a) Inoculate 0.5 L of LB medium containing 50 mg/L kanamycin and 10 mg/L tetracycline in a 2 L flask with 5 mL of a fresh overnight culture in the same medium. Prepare 1 L of sterilized deionized water and 5 mL of sterilized 10 % glycerol and keep them in a refrigerator or a cold room.
   (b) Grow cells at 37 °C with shaking to OD_{600} of 0.5–0.8.
   (c) Chill the flask on ice for 15–30 min. Keep the cells as close to 0 °C as possible in the steps below.
   (d) Centrifuge the culture in a cold rotor at 4000 × *g* for 15 min.
   (e) Remove as much of the supernatant as possible. Do not be concerned about the loss of a few cells while removing the supernatant.
   (f) Gently suspend the obtained cell pellet in 500 mL of the cold sterilized deionized water from Step (a).
   (g) Centrifuge the suspension as in Step (d) and remove the supernatant as in Step (e).
   (h) Repeat Steps (f) and (g).
   (i) Gently suspend the obtained cell pellet in 10 mL of the cold sterilized 10 % glycerol from Step (a).
   (j) Repeat Step (g).
   (k) Gently suspend the cell pellet in 2 mL of the cold sterilized 10 % glycerol from Step (a).
   (l) Flash-freeze this suspension in 50–200 μL aliquots in liquid N_2 and store them at −80 °C until use.
2. Mix 1 μL of 100–500 ng/μL multigene transfer plasmid with 50 μL of electrocompetent cells and incubate it on ice for 5 min.

3. Transfer the cells to an electroporation cuvette and electroporate them using the appropriate electroporation equipment (e.g., MicroPulsor, BioRad).
4. Suspend the cells with 500 μL of LB medium (or richer medium such as SOC) in the cuvette and transfer them to a 1.5 mL tube.
5. Incubate the cells at 37 °C with shaking for 6 h.
6. Transfer 10 μL of the culture to 1 mL of LB medium in a new tube. Then, 10 μL of the diluted culture is transferred to 90 μL of LB medium in another new tube.
7. Streak 100 μL of these two diluted cultures on LB plates containing 50 mg/L kanamycin, 7 mg/L gentamicin, 10 mg/L tetracycline, X-Gal (or an equivalent indicator), and IPTG. We supply 50 μL of 2 % X-Gal and 25 μL of 0.2 M IPTG for each antibiotic-containing plate just before streaking the cells. The culture dilution is important for optimal colony separation.
8. After the incubation at 37 °C for 2 days, larger white colonies appear if the genes in the transfer plasmid are successfully integrated to the engineered AcNPV genome in *E. coli* cells. Restreaking on a fresh plate is recommended to confirm the white phenotype.
9. Pick a white colony for each construct and inoculate it to 5 mL of LB medium supplemented with 50 mg/L kanamycin, 7 mg/L gentamicin, and 10 mg/L tetracycline in a culture tube.
10. Incubate the culture with shaking at 37 °C for 15–17 h.
11. Collect the cells by centrifugation and suspend them in 300 μL of Solution 1 (15 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 mg/L RNase A, see Note 8).
12. Add 300 μL of Solution 2 (0.2 N NaOH, 1 % SDS, see Note 8) and gently mix the solution by inverting the tube upside down several times, followed by incubation at room temperature for 5 min.
13. Slowly add 300 μL of Solution 3 (3 M potassium acetate, pH 5.5, see Note 8) and gently mix the solution by inverting the tube upside down several times, followed by incubation on ice for 5 min. A thick white precipitate of *E. coli* proteins and genomic DNA appears.
14. Clear the solution by centrifugation at 14,000 × *g* for 10 min. During the centrifugation, label another fresh 2 mL tube and add 0.8 mL of 2-propanol to it.
15. Carefully transfer the supernatant to the 2 mL tube containing 2-propanol to avoid contamination of the white precipitate as much as possible and mix it gently by inverting the tube upside down a few times.

16. Place the tube on ice for 10 min and centrifuge it at 14,000 × *g* for 10 min at room temperature. The multigene-integrated bacmid is precipitated as a translucent pellet in this step.
17. Remove the supernatant and add 500 μL of 70 % ethanol.
18. Centrifuge the tube at 14,000 × *g* for 5 min at 4 °C or room temperature .
19. Remove the supernatant as much as possible and air-dry the pellet (the integrated AcNPV genome) in a sterile hood to avoid contamination of microorganisms in the transfected cells.
20. Store the dried pellet at −20 °C until use (Note 9).

3.5 Initial Virus Preparation (P1)

The following steps should be performed in a sterile hood to avoid contamination of insect cell culture:

1. Add 30 μL of sterile pure water to the tube containing the isolated AcNPV genome pellet.
2. Dissolve the pellet by gently tapping the tube and incubate the tube at room temperature for 5–10 min to completely dissolve the pellet.
3. Add 200 μL of an antibiotic-free insect cell medium (e.g., Grace's insect cell medium) to the tube containing the bacmid solution (Tube A).
4. Add 100 μL of an antibiotic-free insect cell medium to another fresh tube (Tube B).
5. Add 8 μL of the transfection reagent, X-Treme GENE HP (Roche), to Tube B.
6. Transfer the mixture containing the transfection reagent in Tube B to Tube A containing the isolated AcNPV genome, followed by incubation at room temperature for 15–30 min.
7. During the incubation, seed 0.5–1.0 million cells to each well of a 6-well tissue culture plate (see Note 10) and add a supplemented insect cell medium [containing antibiotics and/or 4 % fetal bovine serum (FBS)]) with a final volume of 3 mL for each well (Fig. 6).
8. Add one-half of the mixture containing the isolated AcNPV genome and transfection reagent dropwise to each well.
9. Incubate the plate at 27 °C for 60–72 h.
10. Collect the supernatant from the wells and transfer it to sterile 15 mL tubes (P1 virus).
11. Store the P1 virus at 4 °C (or −80 °C after flash-freezing in liquid N_2 for long-term storage) protected from light (see Note 11).

a

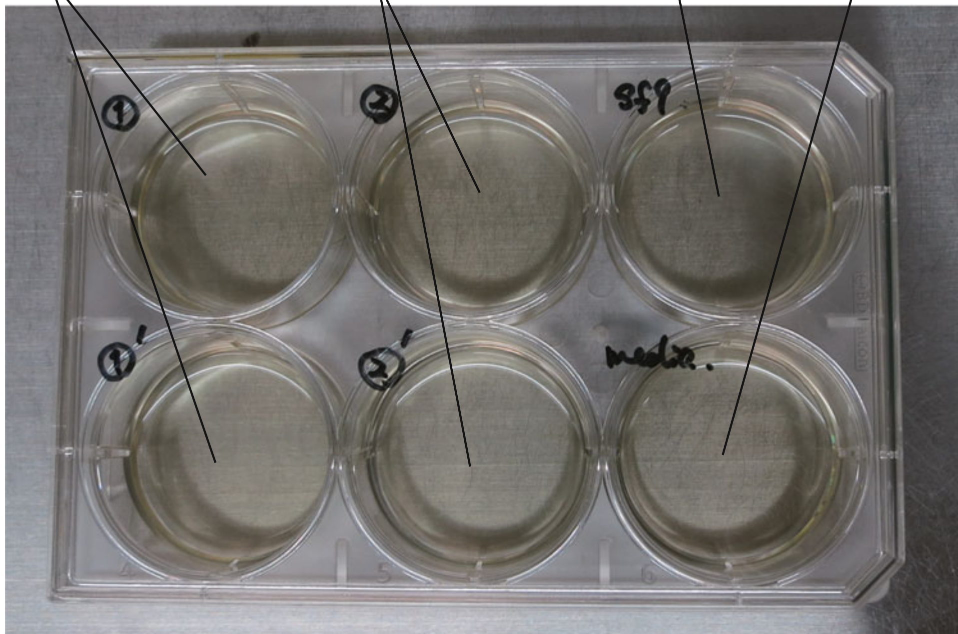
Sf9 cells (~1.0 million cells/mL)

b DH10Bac-derived samples

DH10MultiBac-derived samples

Uninfected Sf9 cells

Medium only



Six-well plate for P1 virus preparation

Fig. 6 Sf9 cells and P1 virus preparation. (**a**) Sf9 cells at a density of ~1.0 million/mL. (**b**) Six-well plate for P1 virus preparation

3.6 Virus Amplification (P2) and Expression Check

1. Prepare 9 mL of cell culture (containing 4 % FBS and antibiotics) at a density of 0.5–1.0 million cells/mL in 125 or 250 mL Erlenmeyer flasks (see Note 12). For virus amplification, we use flasks whose volumes are 10- to 25-fold larger than the culture volume.
2. Inoculate 1 mL of the P1 virus to a 9 mL cell culture for infection.
3. Culture the infected cells at 27 °C for 60–72 h with shaking. We recommend the monitoring of cell growth every 12–24 h. We suggest that cell density should be lower than 1.5 million cells/mL to avoid insufficient aeration. When the density is over 1.5 million cells/mL, dilute the culture to a density of 0.5–1.0 million cells/mL, keeping the culture volume at 1/10–1/25 of the vessel.
4. Centrifuge the culture for 5 min at 2000 × *g* and collect the supernatant (P2 virus). The cell pellet after the centrifugation is used in Step 6.
5. Store the P2 virus at 4 °C (or −80 °C after flash-freezing in liquid N_2 for long-term storage) protected from light (see Note 13).
6. Check the protein expression using the cell pellet collected by centrifugation (see Note 14).
   (a) Suspend the cell pellet in 300 μL of an appropriate buffer for tag-affinity beads (e.g., 50 mM Tris-Cl, pH 8.0, containing 150 mM NaCl and 20 mM imidazole for Ni-chelating beads) and transfer it to a 1.5 mL tube.
   (b) Disrupt the cells by sonication (e.g., Branson Sonifier 450A equipped with a microtip) for 15 s on ice. Take 5 μL as the whole extract for SDS-PAGE analysis (Sample A).
   (c) Centrifuge the disrupted cell suspension at 20,000 × *g* for 15 min at 4 °C. During the centrifugation, pre-equilibrate 15 μL of affinity beads with a sonication buffer in a fresh 1.5 mL tube, following the manufacturer's instruction.
   (d) Take 5 μL of the supernatant as the soluble extract (Sample B) and pick a very small amount of the resulting pellet with a micropipette tip as the insoluble extract (Sample C) for SDS-PAGE analysis.
   (e) Transfer the remaining supernatant to the fresh 1.5 mL tube containing pre-equilibrated affinity beads.
   (f) Incubate the sample for 0.5–1 h at 4 °C.
   (g) Centrifuge the sample at 500 × *g* for 3 min and remove the supernatant.

(h) Wash the beads with 500–1000 μL of a sonication buffer (or a more stringent buffer of choice) three times by iteration of buffer addition, centrifugation, and buffer removal. Take 5 μL of each wash solution as the wash samples (Samples D–F) for SDS-PAGE analysis.

(i) Add 20 μL of an SDS-PAGE loading buffer as the purified sample (Sample G) for SDS-PAGE analysis.

(j) Analyze Samples A–G by SDS-PAGE with standard Coomassie brilliant blue and/or immunostaining.

3.7 Large-Scale Protein Production

For large-scale protein production, we use 2 L Erlenmeyer flasks containing 400–500 mL of cell culture (see Note 15). For example, two flasks are used for 0.8–1 L cell culture. The number of flasks depends on the expression level of your target protein. We use a fresh virus preparation propagated from the stock P2 or P3 virus for large-scale protein production.

1. Inoculate 250 μL of the stock P2 or P3 virus to 25 mL of cell culture (containing antibiotics and 4 % FBS) for infection at a density of 0.5–1 million cells/mL in 0.5 L flask to obtain fresh virus preparations for large-scale expression.
2. Culture the infected cells at 27 °C for 60–72 h.
3. Seed cells to 400–500 mL media containing antibiotics and 4 % FBS at a final density of 0.3 million cells/mL for each flask 1 day after Step 1.
4. Add 25 mL of the infected cell culture (from Steps 1–2) to a fresh 400–500 mL cell culture at a density of one to two million cells/mL for each flask (from Step 3).
5. Culture the infected cells from Step 4 at 27 °C for 60–72 h (see Note 16).
6. Collect the cells by centrifugation at 2000 × *g*.
7. Flash-freeze the cells in liquid N_2 and store them at −80 °C until use.

4 Notes

1. PirHC and PirLC are strains required for preparation of donor plasmids (i.e., pIDC, pIDK, pIDS, pUCDM, pSPL). These two strains contain the *pir* gene in their genomes and can propagate plasmids that have *pir* replication origins. LC and HC mean low copy and high copy, respectively. PirLC strains are used when inserted genes make the propagation of donor plasmids difficult in PirHC.
2. We favor restriction enzyme digestion for the linearization of vectors for SLIC in order to avoid PCR-associated mutations.

3. The uncut original circular vector sometimes migrates similarly to the cassette and cannot be removed by gel extraction. To avoid this problem, we recommend using vectors with two different antibiotic resistance makers for the cassette-mediated multiplication (e.g., the cassette from pUCDM is transferred to pFL).
4. Although we never tried fusion with more than two donors, an online manual for the new system describes in detail the protocol for fusion with more than two donors. Following such a protocol, the Cre reaction is performed at 30 °C for 1–2 h with ~500 ng of each donor and ~400 ng of one acceptor (http://www.epigenesys.eu/images/stories/protocols/pdf/20120313121202_p54.pdf).
5. According to the online manual (http://www.epigenesys.eu/images/stories/protocols/pdf/20120313121202_p54.pdf), restriction analysis using appropriate restriction enzymes is highly recommended for confirming the presence of all genes in the vector.
6. We try both DH10Bac and DH10MultiBac and compare them.
7. Protocols for chemically competent cells are described in the online manual for the new system (http://www.epigenesys.eu/images/stories/protocols/pdf/20120313121202_p54.pdf).
8. Solutions 1, 2, and 3 are our own preparations, but solutions from commercially available plasmid mini-prep kits can be used for the bacmid isolation.
9. We quickly move to Step 3.5 after isolating the integrated AcNPV genome.
10. A 35 mm culture dish is available for this purpose. However, we recommend a 6-well plate because media in the culture dish seem to evaporate much faster than those in the 6-well plate.
11. We quickly move to Step 3.6 after preparing P1 virus.
12. We reuse plastic Erlenmeyer flasks supplied by Corning after autoclaving.
13. Baculoviruses can be stored in liquid N_2 in the form of baculovirus-infected insect cells (BIICs), which is widely adopted in many laboratories [11] (http://www.epigenesys.eu/images/stories/protocols/pdf/20120313121202_p54.pdf). This BIIC storage is recommended in terms of space saving in refrigerators and almost no loss of titer. Some viruses lose their transfection activity within a month or less when stored in the form of a virus solution at 4 °C, while others retain the activity for more than a 2–3 years.

14. Protein expression level may depend on incubation time. You can determine when cells stop proliferation after infection and analyze time-dependent changes in expression level by sampling one million cells every 12 or 24 h after the proliferation arrest.
15. For protein production, High Five cells (derived from the caterpillar *Trichoplusia ni*) may be more suitable than Sf cells in some cases.
16. Optionally, 1 day after infection, the infected cells are cultured at 20 °C for 72 h. This change in culture temperature increases the expression level and/or protein solubility in some cases.

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