invitrogen™

Bac-to-Bac® HBM TOPO® Secreted Expression System

An efficient site-specific transposition system to generate recombinant baculovirus for high-level secreted protein expression

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User Manual

Contents

Kit Contents and Storage

System Components

Each Bac-to-Bac® HBM TOPO® Secreted Expression System contains the components listed below. See the next page for a detailed description of other reagents supplied with each system.

Component	Amount
Bac-to-Bac® HBM TOPO® Cloning Kit	1 kit
One Shot® Mach1-T1^{R} Chemically Competent *E. coli*	1 kit
MAX Efficiency® DH10Bac™ Competent *E. coli*	4 kits
Cellfectin® II Reagent	1 each
Bac-to-Bac® TOPO® Cloning Kit manual	1 each
Bac-to-Bac® HBM TOPO® Secreted Expression System manual	1 each

Shipping/Storage

The Bac-to-Bac® HBM TOPO® Secreted Expression System is shipped in four boxes as described below. Upon receipt, store each box as detailed below. All reagents are guaranteed for six months if stored properly.

Box	Item	Shipping	Storage
1	Bac-to-Bac® HBM TOPO® Cloning Kit	Dry ice	–20°C
2	One Shot® Mach1-T1^{R} Chemically Competent *E. coli*	Dry ice	–80°C
3	MAX Efficiency® DH10Bac™ Competent *E. coli*	Dry ice	–80°C
4	Cellfectin® II Reagent	Gel ice	4°C

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Bac-to-Bac® HBM TOPO® Cloning Kit

The cloning reagents for the Bac-to-Bac® HBM TOPO® Cloning Kit are listed below. **Store the contents at –20°C.**

Item	Concentration	Amount
pFastBac™/HBM-TOPO® vector	20 mL at 10 ng/µl in 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton® X-100 100 µg/mL BSA 30 µM bromophenol blue	20 µl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µL
dNTP Mix	12.5 mM each dATP, dCTP, dGTP, and dTTP; neutralized at pH 8.0 in water	10 µL
Salt Solution	1.2 M NaCl 60 mM $MgCl_2$	50 µL
Sterile Water	–	1 mL
Control PCR template	50 ng/µL in TE buffer*, pH 8.0	10 µL
Control PCR primers	100 ng/µL each in TE buffer, pH 8.0	10 µL
Polyhedrin forward sequencing primer	100 ng/µL in TE buffer, pH 8.0	20 µL
SV40 polyA reverse sequencing primer	100 ng/µL in TE buffer, pH 8.0	20 µL
pFastBac™ Gus control plasmid	0.2 ng/µL in TE buffer, pH 8.0	20 µL

***TE buffer, pH 8.0:** 10 mM Tris–HCl, 1 mM EDTA, pH 8.0

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One Shot® Mach1™ T1R Competent *E. coli*

The following reagents are included in the One Shot® Mach1™ T1R Chemically Competent *E. coli* kit. Transformation efficiency of One Shot® Mach1™ T1R *E. coli* cells is ≥ 1×10^9 cfu/µg DNA. **Store cells at –80°C.**

Reagent	Composition	Amount
One Shot® Mach1™ T1R Chemically Competent *E. coli*	–	21 × 50 µL
S.O.C. Medium (may be stored at room temperature or 4°C)	2% tryptone 0.5% yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 mL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µL

Genotype of Mach1™ T1R

F⁻ ϕ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R($r_K^- m_K^+$) Δ*rec*A1398 *end*A1 *ton*A

MAX Efficiency® DH10Bac™ Competent *E. coli*

MAX Efficiency® DH10Bac™ Competent *E. coli* (Box 3) have a transformation efficiency of 1×10^8 cfu/µg DNA. **Store at –80°C.**

Item	Composition	Amount
MAX Efficiency® DH10Bac™ Competent *E. coli*	–	4 kits (4 × 5 reactions)
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	100 µL

Genotype of DH10Bac™

F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 ΔlacX74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G/bMON14272/pMON7124

Cellfectin® II Transfection Reagent

Cellfectin® II Reagent is a proprietary cationic lipid formulation that offers the highest transfection efficiencies and protein expression levels on the widest variety of adherent and suspension insect cell lines.

Amount supplied: 1 mL

Composition: 1 mg/mL transfection reagent in membrane-filtered water

Storage conditions: 4°C **(do not freeze)**

Introduction

Description of the System

System Overview

- The Bac-to-Bac® HBM TOPO® Secreted Expression System provides a rapid, effective method of generating recombinant baculoviruses for secreted expression of your protein of interest. The Bac-to-Bac® HBM TOPO® Secreted Expression System combines the ease of **blunt-end** TOPO® cloning with the efficiency of site-specific transposition technology of the Bac-to-Bac® System.

Advantages of the Bac-to-Bac® HBM TOPO® Secreted Expression System

Using the Bac-to-Bac® HBM TOPO® Secreted Expression System to generate a recombinant baculovirus provides the following advantages over the traditional method using homologous recombination:

- Enables the cloning of the gene of interest as a **blunt-end PCR product** in a highly efficient one-step reaction, thus allowing the use of proofreading polymerases in the PCR amplification step
- Requires less than 2 weeks to identify and purify a recombinant baculovirus, compared to the 4–6 weeks required to generate a recombinant baculovirus using homologous recombination
- Reduces the need for multiple rounds of plaque purification, because the recombinant virus DNA isolated from selected colonies is not mixed with parental, non-recombinant virus
- Permits rapid and simultaneous isolation of multiple recombinant baculoviruses
- Allows secreted expression of protein variants for structure/function studies

Purpose of This Manual

This manual provides an overview of the Bac-to-Bac® HBM TOPO® Secreted Expression System, and provides instructions and guidelines to:

1. Transform the pFastBac™ construct containing your gene of interest into MAX Efficiency® DH10Bac™ competent *E. coli* to generate recombinant bacmid.
2. Transfect the recombinant bacmid DNA into the insect cell line of choice to produce recombinant baculovirus particles.
3. Amplify and titer the baculoviral stock, and use this stock to infect insect cells to express your recombinant protein.

Detailed instructions for cloning your gene of interest into the pFastBac™/HBM-TOPO® vector are provided in the Bac-to-Bac® TOPO® Cloning Kit manual (part no. A10605) supplied with the Bac-to-Bac® HBM TOPO® Secreted Expression System.

The Bac-to-Bac® TOPO® Cloning Kit manual is also available for downloading at www.invitrogen.com or from Technical Support (see page 56).

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Important

The Bac-to-Bac® HBM TOPO® Secreted Expression System is designed to help you create a recombinant baculovirus for high-level secreted expression of your gene of interest in insect cells. Although the system has been designed to help you to easily produce recombinant baculovirus and express your protein of interest, use of the system is geared towards those users who are familiar with baculovirus biology and insect cell culture. We highly recommend that users possess a working knowledge of viral and tissue culture techniques.

For more information about baculovirus biology, refer to published reference sources (King & Possee, 1992; Luckow, 1991; O'Reilly *et al.*, 1992). For more information about insect cell culture, refer to the *Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques* available from Invitrogen at www.invitrogen.com or by contacting Technical Support (see page 56).

Bac-to-Bac® HBM TOPO® Secreted Expression System Components

Components of the Bac-to-Bac® HBM TOPO® Secreted Expression System

The Bac-to-Bac® HBM TOPO® Secreted Expression System facilitates rapid and efficient generation of recombinant baculoviruses (Ciccarone *et al.*, 1997) by combining the ease of TOPO® cloning with the efficiency of the Bac-to-Bac® System. Based on a method developed by Luckow *et al*. (Luckow *et al.*, 1993), the Bac-to-Bac® HBM TOPO® Secreted Expression System takes advantage of the site-specific transposition properties of the Tn7 transposon to simplify and enhance the process of generating recombinant bacmid DNA. The following major system components are described in detail below:

- **pFastBac™/HBM-TOPO® plasmid**—allows the rapid generation of an expression construct containing the gene of interest under the control of a baculovirus-specific strong polyhedrin (P_H) promoter and in frame with the Honey Bee Mellitin (HBM) secretion signal coding sequence. One Shot® Mach1™ T1R Chemically Competent *E. coli* enable same-day isolation of recombinant pFastBac™ expression construct from the transformation mix.
- **An *E. coli* host strain, DH10Bac™**— contains a baculovirus shuttle vector (bacmid) and a helper plasmid to facilitate the generation of a recombinant bacmid following transposition of the pFastBac™ expression construct.
- **Cellfectin® II Reagent**—for fast, efficient transfection of insect cells to generate recombinant baculovirus particles.

Note: A control expression plasmid, pFastBac™ Gus allows production of a recombinant baculovirus which, when used to infect insect cells, constitutively expresses β-glucuronidase.

pFastBac™/HBM-TOPO® vector

The first major component of the System is the pFastBac™/HBM-TOPO® vector into which your gene of interest is cloned.

After you amplify your gene of interest using a proofreading polymerase and clone it into the pFastBac™/HBM-TOPO® vector as a blunt-end PCR product, you transform One Shot® Mach1™ $T1^R$ Chemically Competent *E. coli*. You then select and analyze transformants for the correct insertion of your blunt-end PCR products, and use the recombinant vector as a "donor plasmid" to generate a recombinant baculovirus.

The expression of the gene of interest is controlled by the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (P_H) promoter for high-level expression in insect cells. This expression cassette is flanked by the left and right arms of Tn7. The cassette also contains a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini Tn7.

The presence of the N-terminal Honey Bee Mellitin (HBM) secretion signal coding sequence on the plasmid facilitates the secretion of the cloned gene product into the extracellular medium; the C-terminal polyhistidine tag allows easy purification of the secreted protein.

Continued on next page

Bac-to-Bac® HBM TOPO® Secreted Expression System Components, Continued

DH10Bac™ *E. coli*

The second major component of the System is the DH10Bac™ *E. coli* strain that is used as the host for the pFastBac™/HBM construct containing your gene of interest. DH10Bac™ cells contain a baculovirus shuttle vector (bacmid) with a mini-*att*Tn7 target site and a helper plasmid (see the next page for details).

After the pFastBac™/HBM expression plasmid (the "donor plasmid") is transformed into DH10Bac™ cells, transposition occurs between the mini-Tn7 element on the pFastBac™/HBM vector and the mini-*att*Tn7 target site on the bacmid to generate a recombinant bacmid. This transposition reaction occurs in the presence of transposition proteins supplied by the helper plasmid.

After you have performed the transposition reaction, you isolate the high molecular weight recombinant bacmid DNA and transfect the bacmid DNA into insect cells using the Cellfectin® II reagent to generate a recombinant baculovirus that can be used for preliminary expression experiments. After the baculoviral stock is amplified and titered, this high-titer stock can be used to infect insect cells for large-scale expression of the recombinant protein of interest.

For a schematic representation of the Bac-to-Bac® HBM TOPO® Secreted Expression System, see the diagram on page 5.

Baculovirus Shuttle Vector

The baculovirus shuttle vector (bacmid), bMON14272 (136 kb), present in DH10Bac™ *E. coli* contains:

- A low-copy number mini-F replicon.
- Kanamycin resistance marker.
- A segment of DNA encoding the LacZα peptide from a pUC-based cloning vector into which the attachment site for the bacterial transposon, Tn7 (mini-*att*Tn7) has been inserted. Insertion of the mini-*att*Tn7 attachment site does not disrupt the reading frame of the LacZα peptide.

The bacmid propagates in *E. coli* DH10Bac™ as a large plasmid that confers resistance to kanamycin. This bacmid can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (Lac^+) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer, IPTG.

Recombinant bacmids (composite bacmids) are generated by transposing a mini-Tn7 element from a pFastBac™ donor plasmid to the mini-*att*Tn7 attachment site on the bacmid. The Tn7 transposition functions are provided by a helper plasmid (see below).

Helper Plasmid

DH10Bac™ *E. coli* also contain the helper plasmid, pMON7124 (13.2 kb), which encodes the transposase and confers resistance to tetracycline. The helper plasmid provides the Tn7 transposition function *in trans* (Barry, 1988).

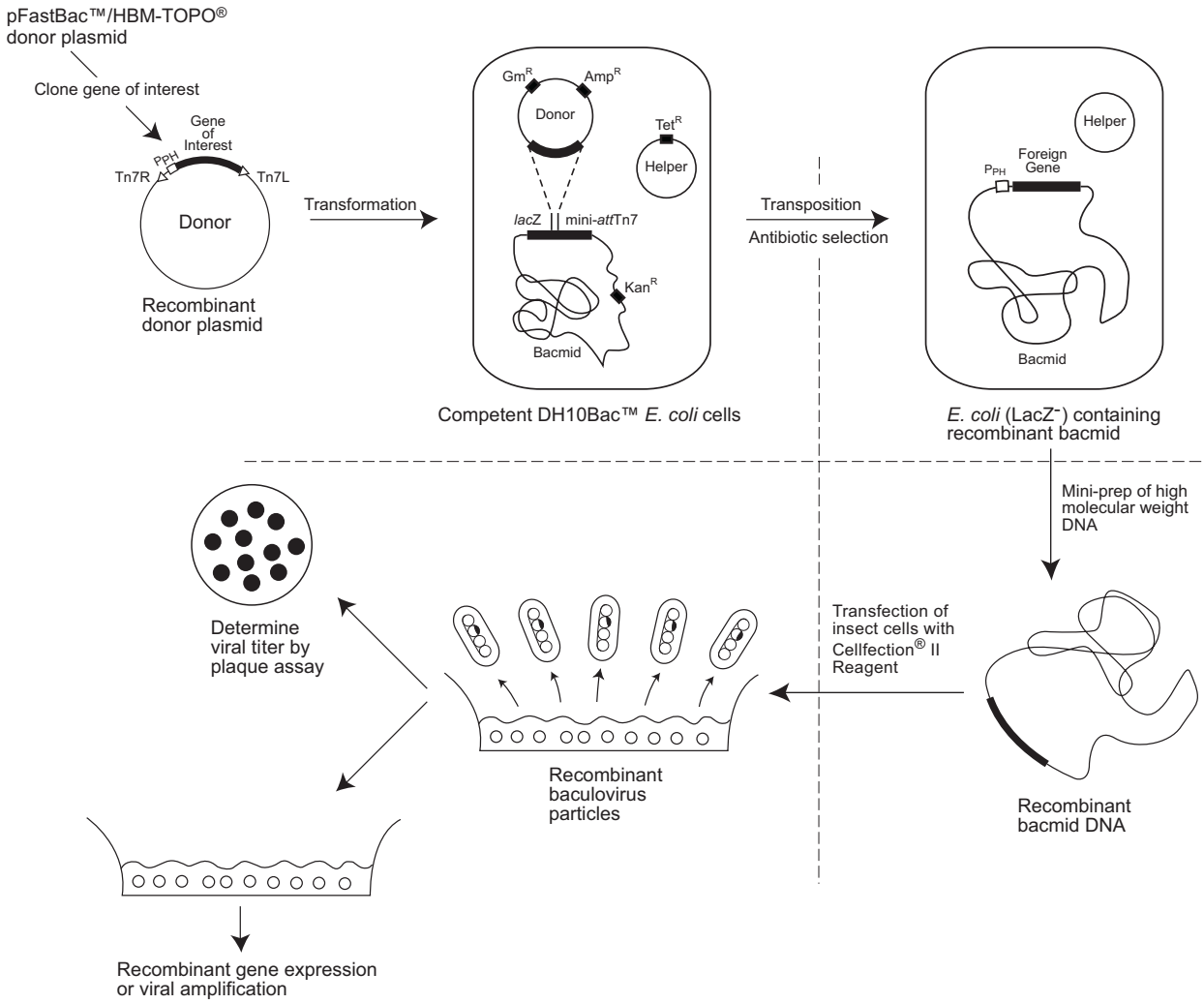
Cellfectin® II Reagent

Cellfectin® II Reagent is a proprietary cationic lipid formulation that offers the highest transfection efficiencies and protein expression levels on the widest variety of adherent and suspension insect cell lines, including Sf9 and Sf21 cells.

Experiment Outline

Diagram of the Bac-to-Bac® System

The figure below depicts the generation of recombinant baculovirus and the expression of your gene of interest using the Bac-to-Bac® HBM TOPO® Secreted Expression System.



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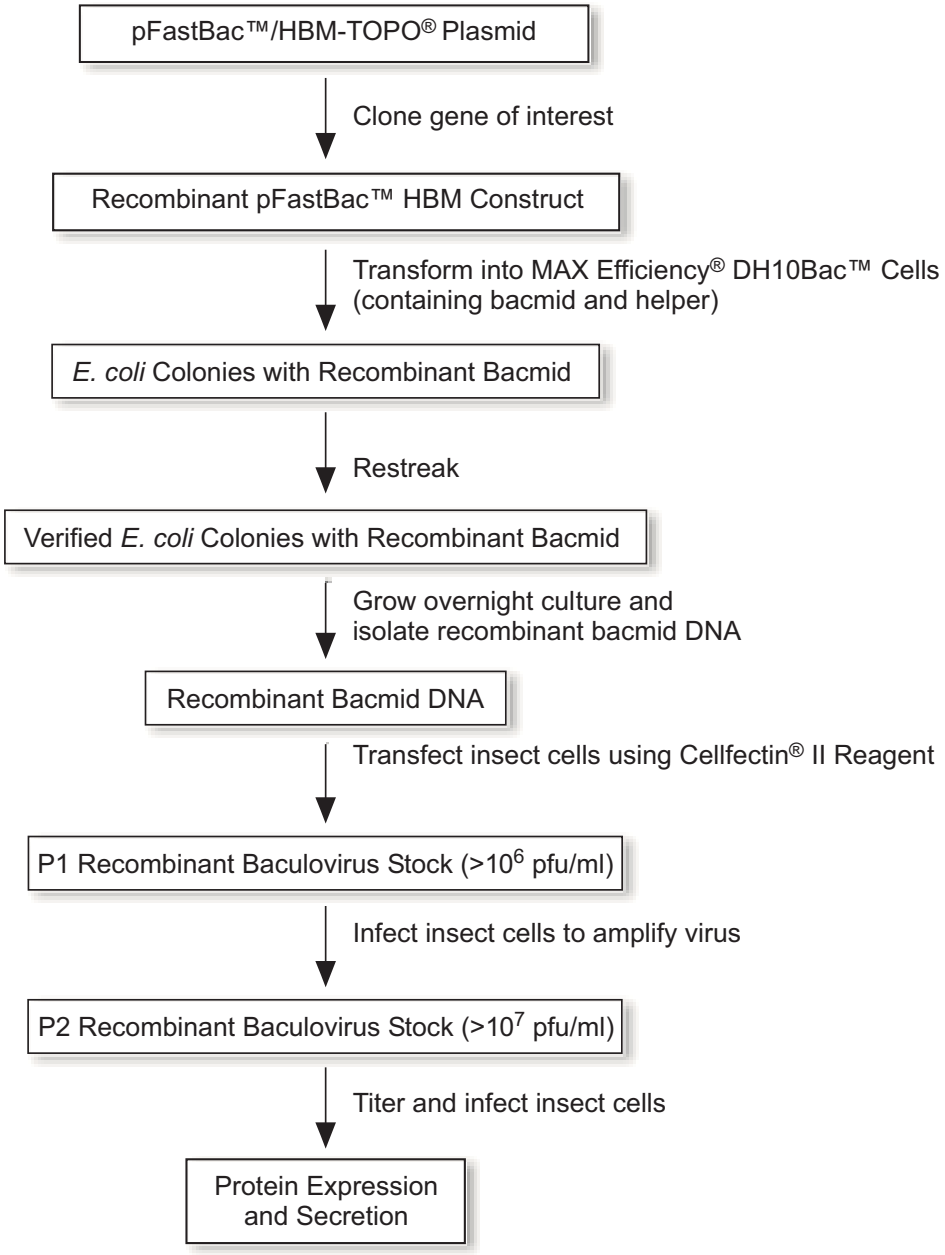
Flow Chart

The figure below illustrates the general steps required to express your gene of interest using the Bac-to-Bac® HBM TOPO® Secreted Expression System.



Methods

Culturing Insect Cells

General Guidelines

Introduction

We recommend using *Spodoptera frugiperda* Sf9 or Sf21 insect cells as the host for your baculovirus transfer vector when you produce your recombinant bacmid.

We recommend using High Five™ cells for secreted expression of your protein from the recombinant bacmid, because they are particularly well suited for expression of secreted recombinant proteins.

Before you start your transfection and expression experiments, be sure to have cultures of Sf9 or Sf21 and High Five™ cells growing, and have frozen master stocks available. Sf9, Sf21, and High Five™ cells and cell culture reagents are available separately from Invitrogen (see page 54 for ordering information).

Note: High Five™ and Mimic™ Sf9 insect cells are suitable for use for expression only.

Using Serum-Free Medium

Insect cells may be cultured under serum-free conditions. We recommend using Sf-900 II SFM or Sf-900™ III SFM available from Invitrogen (see page 54) for culturing Sf9 and Sf21 cells. Sf-900 II SFM and Sf-900™ III SFM are protein-free media optimized for the growth and maintenance of Sf9 and Sf21 cells. For culturing High Five™ cells under serum-free conditions, use Express Five® SFM (see page 54 for ordering). Express Five® SFM is optimized for the growth and maintenance of High Five™ cells, as well as for the large-scale production and secretion of recombinant proteins expressed using the Bac-to-Bac® HBM TOPO® Secreted Expression System. For more information, refer to www.invitrogen.com or contact Technical Support (see page 56).

Insect Cell Culture Reference Guide

For guidelines and detailed information on insect cell culture, refer to the *Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques*, available for downloading at www.invitrogen.com or by contacting Technical Support (see page 56). This guide contains information on:

- Maintaining and passaging insect cells in adherent and suspension culture
- Freezing cells
- Using serum-free medium (includes protocols to adapt cells to serum-free medium)
- Scaling up cell culture

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General Guidelines

Insect cells are very sensitive to environmental factors. In addition to chemical and nutritional culture factors, physical factors can also affect insect cell growth; therefore optimization is required to maximize cell growth. Consider the following when culturing insect cells:

- **Temperature:** The optimal range to grow and infect cultured insect cells is 27°C to 28°C.
- **pH:** A range of 6.1 to 6.4 works well for most culture systems. Sf-900 II SFM will maintain a pH in this range under conditions of normal air and open-capped culture systems.
- **Osmolality:** The optimal osmolality of medium for use with lepidopteran cell lines is 345 to 380 mOsm/kg.
- **Aeration:** Insect cells require passive oxygen diffusion for optimal growth and recombinant protein expression. Active or controlled oxygenated systems require dissolved oxygen at 10% to 50% of air saturation.
- **Shear Fforces:** Suspension culture generates mechanical shear forces. Growing insect cells in serum-containing media (10% to 20% FBS) generally provides adequate protection from cellular shear forces. If you are growing insect cells in serum-free conditions, supplementation with a shear force protectant such as PLURONIC® F-68 may be required.

  Note: Growing cells in Sf-900 II SFM or Sf-900™ III SFM does **not** require addition of shear force protectants.

Cells for Transfection

You need log-phase Sf9 or Sf21 cells with >95% viability to perform a successful transfection. Refer to page 19 to determine how many cells you will need for transfection.

Generating the Recombinant pFastBac™ HBM Vector

Introduction

To generate a recombinant plasmid containing your gene of interest for use in the Bac-to-Bac® HBM TOPO® Secreted Expression System, perform the following steps:

1. Generate a **blunt-end** PCR product containing your gene of interest with a thermostable **proofreading DNA polymerase** such as the Platinum® *Pfx* or the AccuPrime™ *Pfx* DNA Polymerase.
2. TOPO® Clone your blunt-end PCR product into the pFastBac™/HBM-TOPO® vector, and use the reaction to transform One Shot® Mach1™ T1^R Chemically Competent *E. coli*. **Do not transform the ligation reaction into DH10Bac™ cells.**
3. Pick colonies, isolate plasmid DNA (see below), and screen for insert directionality by sequencing expression clones with the primers provided in the kit.

For detailed instructions, refer to the Bac-to-Bac® TOPO® Cloning Kit manual (part no. A10605) supplied with this kit. The Bac-to-Bac® TOPO® Cloning Kit manual is also available at www.invitrogen.com or from Technical Support (see page 56).

Guidelines for Isolating DNA

1. Pick 10 overnight-grown colonies from the selective plates and culture them overnight in LB medium containing 100 µg/mL ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using Invitrogen's PureLink™ HiPure Mini Plasmid Purification Kit (see page 53 for ordering information).

   You need 1 ng of purified recombinant plasmid (5 µL at 200 pg/µL) to transform into DH10Bac™ *E. coli* for transposition into the bacmid (see page 11).

Note: If you have used One Shot® Mach1™ T1^R Chemically Competent *E. coli* for your transformation, you can prepare plasmid DNA 4 hours after inoculating a single, overnight-grown colony in the selective media of choice. Note that this feature is not limited to ampicillin selection.

Important

When generating the recombinant plasmid containing your gene of interest for use in the Bac-to-Bac® HBM TOPO® Secreted Expression System, you **must** follow certain design parameters for your PCR insert and the recommendations for the transformation procedure outlined in the Bac-to-Bac® TOPO® Cloning Kit manual.

To ensure proper expression of your recombinant protein, it is imperative that you read the sections on generating the blunt-end PCR product, blunt-end TOPO® Cloning, transforming One Shot® Mach1™ T1^R Chemically Competent *E. coli*, and analyzing transformants in the Bac-to-Bac® TOPO® Cloning Kit manual before beginning.

pFastBac™/HBM-TOPO® vector

For a vector map of pFastBac™/HBM-TOPO®, see page 51. For more instructions on generating your recombinant plasmid containing your gene of interest, refer to the Bac-to-Bac® TOPO® Cloning Kit manual (part no. A10605) supplied with this kit, also available at www.invitrogen.com or by contacting Technical Support (see page 56).

Generating the Recombinant Bacmid

Transforming DH10Bac™ *E. coli*

Introduction

After you have generated your pFastBac™ HBM construct containing your gene of interest, transform purified plasmid DNA into DH10Bac™ *E. coli* for transposition into the bacmid. Use blue/white selection to identify colonies containing the recombinant bacmid.

MAX Efficiency® DH10Bac™ chemically competent cells are supplied with the Bac-to-Bac® HBM TOPO® Secreted Expression System. These cells are also available separately from Invitrogen (see page 53). Guidelines and instructions for transforming DH10Bac™ cells are provided in this section.

Positive Control

The pFastBac™/HBM-TOPO® vector is supplied with the control plasmid pFastBac™ Gus for use as a positive transfection and expression control. We recommend including the control plasmid in your DH10Bac™ transformation experiments. For a map of the control plasmid, see page 52.

Materials Needed

- Your purified pFastBac™/HBM construct (200 pg/mL in TE, pH 8.0; see page 9)
- Positive expression control (i.e., pFastBac™ Gus; use as a control for transposition)
- MAX Efficiency® DH10Bac™ chemically competent cells (supplied with the Bac-to-Bac® HBM TOPO® Secreted Expression System; use 1 tube of competent cells for every transformation)
- pUC19 (supplied with the MAX Efficiency® DH10Bac™ *E. coli*; use as a control for transformation, if desired)
- LB agar plates containing kanamycin, gentamicin, tetracycline, Bluo-gal, and IPTG (3 freshly prepared plates for each transformation; see below)
- LB agar plate containing 100 µg/mL ampicillin (for plating pUC19 transformation control)
- S.O.C. Medium
- 42°C water bath
- 37°C shaking and non-shaking incubator

Prepare LB agar plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL IPTG to select for transformants. See page 50 for instructions to prepare plates.

If you are preparing LB plates using a pre-mixed formulation, we use Luria Broth Base (see page 53) instead of Lennox L (LB). Using Lennox L plates reduces the color intensity and may lower the number of colonies obtained.

Note: Use Bluo-gal instead of X-gal for blue/white selection. Bluo-gal generally produces a darker blue color than X-gal.

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Transforming DH10Bac™ *E. coli*, Continued

Preparing for Transformation

For each transformation, you will need one vial of competent cells and three selective plates.

- Equilibrate a water bath to 42°C.
- Warm selective plates at 37°C for 30 minutes.
- Warm the S.O.C. Medium to room temperature.

Transformation Procedure

Follow the procedure below to transform MAX Efficiency® DH10Bac™ chemically competent *E. coli* cells with your recombinant pFastBac™ HBM construct. We recommend including positive controls for transposition (i.e., pFastBac™ Gus) and transformation (i.e., pUC19) in your experiment to help you evaluate your results.

1. Thaw **on ice** one vial of MAX Efficiency® DH10Bac™ competent *E. coli* cells for each transformation.
2. For each transformation, add the appropriate amount of plasmid DNA to 100 µL of DH10Bac™ cells and mix gently. **Do not pipet up and down to mix.**
   - Your recombinant pFastBac™/HBM construct: 1 ng (5 µL)
   - pFastBac™ Gus control plasmid: 1 ng
   - pUC19 control: 50 pg (5 µL)
3. Incubate the cells on ice for 30 minutes.
4. Heat-shock the cells for 45 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice and chill for 2 minutes.
6. Add 900 µL of room temperature S.O.C. Medium.
7. **For pFastBac™ transformations:** Shake the tubes at 37°C at 225 rpm for 4 hours.

   For pUC19 transformation: Shake the tube at 37°C at 225 rpm for 1 hour.
8. **For each pFastBac™ transformation:** Prepare 10-fold serial dilutions of the cells (10^{-1}, 10^{-2}, 10^{-3}) with S.O.C. Medium. Plate 100 µL of **each** dilution on an LB agar plate containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL IPTG.

   For the pUC19 transformation: Dilute the cells 1:100 with S. O.C. Medium. Plate 100 µL of the dilution on an LB agar plate containing 100 µg/mL ampicillin.
9. Incubate the plates for 48 hours at 37°C. Pick white colonies for analysis (see the next page for recommendations).

   Note: We do **not** recommend picking colonies earlier than 48 hours. Incubating the plates for less than 48 hours may create difficulty distinguishing between white and blue colonies.

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Important

Insertions of the mini-Tn7 into the mini-*att*Tn7 attachment site on the bacmid disrupt the expression of the LacZα peptide, so colonies containing the recombinant bacmid are white in a background of blue colonies that harbor the unaltered bacmid. **Select white colonies for analysis.** True white colonies tend to be large. To avoid selecting false positives, choose the largest, most isolated white colonies. Avoid picking colonies that appear gray or are darker in the center, because they can contain a mixture of cells with empty bacmid and recombinant bacmid.

Verifying the Phenotype

1. Pick 10 white colonies and restreak them on fresh LB agar plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL IPTG. Incubate the plates overnight at 37°C.
2. From a single colony confirmed to have a white phenotype on restreaked plates containing Bluo-gal and IPTG, inoculate a liquid culture containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/mL tetracycline.
3. Isolate recombinant bacmid DNA for analysis using the procedure provided on the next page.
4. Analyze the recombinant bacmid DNA to verify successful transposition to the bacmid. We recommend using PCR to analyze your bacmid DNA (see **Analyzing Recombinant Bacmid DNA by PCR**, page 15).

   Note: It is possible to verify successful transposition to the bacmid by using agarose gel electrophoresis to look for the presence of high molecular weight DNA. This method is less reliable than performing PCR analysis, because high molecular weight DNA can be difficult to visualize.

Note

You may also use other methods to prepare purified recombinant bacmid DNA for analysis and transfection. However, bacmid DNA must be clean and free from phenol and sodium chloride because contaminants may kill the insect cells, and salt interferes with lipid complexing, decreasing the transfection efficiency.

The PureLink™ HiPure Plasmid Prep Kits, available separately from Invitrogen, allow the purification of all types and sizes of plasmid DNA, including BAC, bacmids, and ssM13 DNAs. PureLink™ HiPure Plasmid Prep Kits are ideally suited for bacmid purification (see page 53 for ordering information).

Isolating Recombinant Bacmid DNA

Introduction

The PureLink™ HiPure Plasmid DNA Miniprep Kit allows you to purify high quality bacmid DNA from DH10Bac™ *E. coli* (see page 53 for ordering information). The isolated bacmid DNA is suitable for use in insect cell transfections.

Note: We do **not** recommend the PureLink™ HiPure Precipitator Module or the PureLink™ HiPure Plasmid Filter Mini/Midi/Maxiprep Kits for isolating bacmid DNA.

Before Starting

- Inoculate a single white bacterial colony into 2 mL LB medium with 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/mL tetracycline. Incubate the culture at 37°C in a shaking water bath at 250 rpm overnight.
- Verify that RNase A is added to the Resuspension Buffer (R3) and that the Lysis Buffer (L7) contains no precipitates.

Equilibrating the Column

Place the PureLink™ HiPure Mini column on the PureLink™ Nucleic Acid Purification Rack (see the manual supplied with the rack for more details). Apply 2 mL Equilibration Buffer (EQ1) to the column. Allow the solution in the column to drain by gravity flow.

Preparing the Cell Lysate

1. Harvest 1.5 mL bacterial cells by centrifuging at 9,000 × g for 15 minutes. Remove all medium.
2. Add 0.4 mL Resuspension Buffer (R3) containing RNase A to the pellet and resuspend the cells until homogeneous. Transfer the cell suspension to a centrifuge tube.
3. Add 0.4 mL Lysis Buffer (L7). Mix gently by inverting the capped tube five times. **Do not vortex.** Incubate at room temperature for 5 minutes.
4. Add 0.4 mL Precipitation Buffer (N3) and mix immediately by inverting the capped tube until the mixture is homogeneous. **Do not vortex**.
5. Centrifuge the mixture at >15,000 × g at room temperature for 10 minutes.

Note: If the pellet does not adhere to the bottom of the tube, incubate the tube at room temperature for 5 minutes to allow the separation of the lysate and gelatinous pellet. Pipet the clear lysate into a sterile tube and centrifuge at >15,000 × g for 5 minutes at room temperature to remove any remaining cellular debris.

Binding and Washing the DNA

1. Load the supernatant resulting from Step 5 (see above) onto the equilibrated column. Allow the solution in the column to drain by gravity flow.
2. Wash the column **twice** with 2.5 mL Wash Buffer (W8). Allow the solution in the column to drain by gravity flow after each wash. Discard the flow-through.

Continued on next page

Eluting and Precipitating DNA

1. Place a sterile centrifuge tube (elution tube) under the column.
2. Add 0.9 mL Elution Buffer (E4) to the column to elute DNA. Allow the solution to drain by gravity flow. Do not force out any remaining solution. **The elution tube contains the purified DNA.** Discard the column.
3. Add 0.63 mL isopropanol to the elution tube. Mix, then place the tube on ice for 10 minutes.
4. Centrifuge the mixture at >15,000 × g at 4°C for 20 minutes. Carefully remove and discard the supernatant.
5. Resuspend the DNA pellet in 1 mL 70% ethanol.
6. Centrifuge at >15,000 × g at 4°C for 5 minutes. Carefully remove and discard the supernatant.
7. Air-dry the pellet for 10 minutes.
8. Resuspend the DNA pellet in 40 µL TE Buffer (TE). Allow the pellet to dissolve for at least 10 minutes on ice. To avoid shearing the DNA, pipet only 1 or 2 times to resuspend.
9. Store the bacmid DNA at **4°C**.

Important

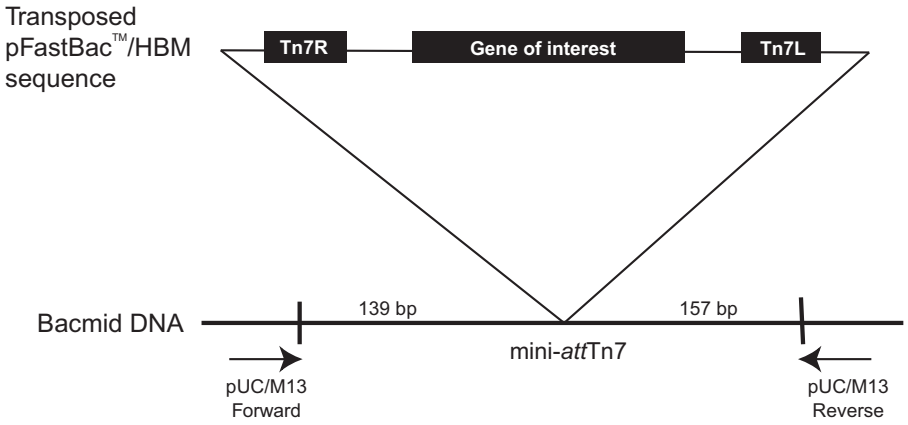
You may store your bacmid DNA at –20°C if you avoid frequent freeze/thaw cycles, which decrease the transfection efficiency. To store your purified bacmid DNA at –20°C, aliquot the bacmid DNA into separate tubes in TE Buffer, pH 8.0 to avoid more than one freeze/thaw cycle. Do **not** store the bacmid DNA in a frost-free freezer. You may also store the purified bacmid DNA for up to 2 weeks at 4°C in TE Buffer, pH 8.0.

You may prepare glycerol stocks of DH10Bac™ *E. coli* containing the bacmid DNA from mid-logarithmic phase culture grown from white colonies picked during the blue-white screening, and store the glycerol stocks at –80°C for future bacmid DNA isolation.

Analyzing Recombinant Bacmid DNA by PCR

Introduction

Recombinant bacmid DNA is greater than 135 kb in size. Since restriction analysis is difficult to perform with DNA of this size, we recommend using PCR analysis to verify the presence of your gene of interest in the recombinant bacmid. Use the pUC/M13 Forward and Reverse primers (sequences given below) that hybridize to sites flanking the mini-*att*Tn7 site (see figure below). This section provides guidelines and instructions for performing PCR using the pUC/M13 Forward and Reverse primers.



PCR Analysis with pUC/M13 Primers

To verify the presence of your gene of interest in the recombinant bacmid using PCR, you may:

- Use the pUC/M13 Forward and Reverse primers (see sequences below).
- Use a combination of the pUC/M13 Forward or Reverse primer and a primer that hybridizes within your insert.

Invitrogen does not supply the pUC/M13 Forward and Reverse primers; you must have these primers custom synthesized.

Primer	Sequence
pUC/M13 Forward	5′-CCCAGTCACGACGTTGTAAAACG-3′
pUC/M13 Reverse	5′-AGCGGATAACAATTTCACACAGG-3′

DNA Polymerase

You may use any DNA polymerase of your choice for PCR including Platinum® *Taq* DNA Polymerase. If the expected PCR product is > 4 kb, we recommend using a polymerase mixture such as Platinum® *Taq* DNA Polymerase High Fidelity for best results. See page 53 for ordering information.

Continued on next page

Generating the PCR Product

Use the procedure below to amplify your recombinant bacmid DNA using the pUC/M13 Forward and Reverse primers and Platinum® *Taq* polymerase. If you are using a combination of the pUC/M13 Forward or Reverse primers and a primer specific for your gene, determine the amplification conditions to use. If you are using another polymerase, follow the manufacturer's recommendations for the polymerase you are using.

Note: Amplification conditions may need to be optimized if your insert is > 4 kb.

1. For each sample, set up the following 50 µL PCR reaction in a 0.5 mL microcentrifuge tube:

Recombinant bacmid DNA (100 ng)	1 µL
10X PCR Buffer (appropriate for enzyme)	5 µL
10 mM dNTP Mix	1 µL
50 mM $MgCl_2$	1.5 µL
PCR Primers (1.25 µL each 10 µM stock)	2.5 µL
Sterile Water	38.5 µL
Platinum® *Taq* polymerase (5 units/µL)	0.5 µL
Total Volume	50 mL

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	3 minutes	94°C	1X
Denaturation	45 seconds	94°C	25–35X
Annealing	45 seconds	55°C	
Extension	5 minutes	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 5–10 µL from the reaction and analyze by agarose gel electrophoresis.

Continued on next page

What You Should See

If transposition has occurred and you have used the pUC/M13 Forward and Reverse primers for amplification, you should see a PCR product of the following size on the agarose gel:

Bacmid transposed with	Size of PCR Product
Bacmid alone	~300 bp
pFastBac™/HBM-TOPO®	~2,500 bp + size of your insert
pFastBac™ Gus	~4,200 bp

If you have used a combination of the pUC/M13 Forward or Reverse primer and a gene-specific primer for amplification, determine the expected size of your PCR product. Refer to the diagram on page 15 to help you calculate the expected size of your PCR product.

Producing Recombinant Baculovirus

Transfecting Insect Cells

Introduction

After you have confirmed that your recombinant bacmid contains the gene of interest, you are ready to transfect insect cells to produce recombinant baculovirus. This section provides guidelines and instructions for transfecting insect cells.

Cellfectin® II Reagent

We recommend using a cationic lipid such as Cellfectin® II Reagent for transfection. Cellfectin® II Reagent is a proprietary cationic lipid formulation that offers the highest transfection efficiencies and protein expression levels on the widest variety of adherent and suspension insect cell lines, including Sf9 and Sf21 cells.

Cellfectin® II Reagent is supplied with the Bac-to-Bac® HBM TOPO® Secreted Expression System, and is also available separately from Invitrogen. See page 53 for ordering information.

Insect Cell Lines for Transfection

We recommend using Sf9 or Sf21 cells for transfection and identification of recombinant plaques. High Five™ and Mimic™ Sf9 cells are not recommended because they generally transfect less efficiently. However, once you have generated your baculovirus stock, we recommend using High Five™ cells for secreted expression studies (see Secretion in High Five™ Cells, page 32).

Media for Transfection

For the highest transfection efficiency, we recommend performing the transfection in Grace's Insect Cell Culture Medium, Unsupplemented (see page 54 for ordering information). Note that the Grace's Insect Cell Culture Medium **should not** contain supplements or fetal bovine serum (FBS), because the supplements and the proteins in the FBS interferes with the Cellfectin® II Reagent, inhibiting the transfection.

Note: If you are culturing Sf9 or Sf21 cells in Sf-900 II SFM or Sf-900™ III SFM, you can perform the transfection in unsupplemented Grace's Medium, and then easily switch back to Sf-900 II SFM or Sf-900™ III SFM after transfection.

Positive Control

If you have generated a recombinant bacmid from the pFastBac™ Gus control plasmid, we recommend including this positive control in your transfection and expression experiments to help you evaluate your results. In this bacmid, the gene encoding β-glucuronidase is expressed under the control of the strong polyhedrin (P_H) promoter. After transfection, you may assay expression of β-glucuronidase as appropriate (see page 39).

Continued on next page

Materials Needed

- Purified recombinant bacmid DNA from your pFastBac™/HBM construct (500 ng/µL in TE Buffer, pH 8.0)
- Purified recombinant bacmid DNA from the pFastBac™ Gus control construct (if desired, 500 ng/µL in TE Buffer, pH 8.0)
- Sf9 or Sf21 cells cultured in the appropriate medium
- Cellfectin® II Reagent (store at 4°C until use)
- Grace's Insect Cell Medium, Unsupplemented (see page 54), **media should not contain supplements, FBS, or antibiotics**
- 6-well tissue culture plates and other tissue culture supplies
- 1.5 mL sterile microcentrifuge tubes
- Complete growth medium for culturing insect cells (*e.g.*, Sf-900 II SFM, Sf-900™ III SFM, TNM-FH, Grace's Supplemented Insect Cell Culture Medium, or other suitable medium)

Calculate the number of Sf9 or Sf21 cells that you need for your transfection experiment and expand cells accordingly. Make sure your cells are healthy with greater than 95% viability and are growing in the logarithmic phase with a density of 1.5×10^6–2.5×10^6 cells/mL before proceeding to transfection.

Transfection Conditions

We generally produce baculoviral stocks in Sf9 or Sf21 cells using the following transfection conditions. Use these conditions as a starting point for your transfection. To obtain the highest transfection efficiency and low non-specific effects, optimize transfection conditions by varying DNA and Cellfectin® II Reagent concentrations, and cell density.

Condition	Amount
Tissue culture plate size	6-well (35 mm) plate (one well/bacmid)
Number of Sf9 or Sf21 cells to transfect	8×10^5 cells
Amount of bacmid DNA	1 µg (can vary from 1 to 2 µg)
Amount of Cellfectin® II Reagent	8 µL (can vary from 1.5 to 9 µL)

Note: This procedure is for insect cells in a 6-well format. All amounts and volumes are given on a per well basis.

Important Guidelines for Transfection

- Use Grace's Insect Cell Culture Medium, Unsupplemented to seed all cells in plate for Sf9 and Sf21 cells grown in Grace's Insect Cell Culture Medium, Supplemented (with 10% FBS).
- With Cellfectin® II, you do not have to remove the medium from cells and wash cells prior to adding the DNA-lipid complex to cells.
- The DNA-lipid complex formation time is shorter (~15–30 minutes) when using Cellfectin® II as compared to Cellfectin® reagent.
- **Do not** add antibiotics during transfection. Adding antibiotics during transfection causes cell death.

Continued on next page

Transfection Procedure

For Sf9 or Sf21 insect cells cultured in Supplemented Grace's Insect Medium containing 10%FBS, use the following protocol to prepare your cells for transfection in a 6-well format. All amounts and volumes are given on a per well basis. To transfect cells in other tissue culture formats, first determine the optimal conditions to use.

1. Verify that the Sf9 or Sf21 cells are in the log phase (1.5×10^6–2.5×10^6 cells/mL) with greater than 95% viability.
2. Prepare transfection samples:
   - If the cell density is in range of 1.5×10^6–2.5×10^6 cells/mL and the culture is without antibiotics:
     a. Add 2 mL of Grace's Insect Medium, Unsupplemented (without antibiotics and serum) in each well.
     b. Seed 8×10^5 Sf9 or Sf21 cells from Step 1 per well. **Do not change medium or wash the cells. The medium carried over will enhance the transfection efficiency.**
     c. Allow cells to attach for 15 minutes at room temperature in the hood. Proceed to step 3.
   - If the cell density is **not** in this range or the cell culture contains antibiotics:
     a. Prepare 10 mL of plating medium by mixing 1.5 mL Supplemented Grace's Insect Medium containing 10% FBS (without antibiotics) and 8.5 mL Grace's Insect Medium, Unsupplemented (without FBS and antibiotics).
     b. Plate 8×10^5 Sf9 or Sf21 cells from Step 1 per well.
     c. Allow cells to attach for 15 minutes at room temperature in the hood.
     d. Remove the medium. Add 2.5 mL plating medium from step 2a per well. Proceed to step 3.
3. **For each transfection sample,** prepare complexes as follows:
   a. Mix Cellfectin® II before use, and dilute 8 µL in 100 mL Grace's Medium, Unsupplemented (without antibiotics and serum). Vortex briefly to mix.
   Note: You may leave this mixture at room temperature for up to 30 minutes.
   b. Dilute 1 µg baculovirus DNA in 100 µL Grace's Medium, Unsupplemented (without antibiotics and serum). Mix gently.
   c. Combine the diluted DNA with diluted Cellfectin® II (total volume ~210 µL). Mix gently and incubate for 15–30 minutes at room temperature.
4. Add ~210 µL DNA-lipid mixture or transfection mixture (Step 3c) **dropwise** onto the cells from Step 2a or 2c. Incubate cells at 27°C for 3–5 hours.
5. Remove the transfection mixture and replace it with 2 mL of complete growth medium (e.g., Grace's Insect Medium, Supplemented and 10% FBS). Using antibiotics is optional.
6. Incubate the cells at 27°C for 72 hours or until you see signs of viral infection.

Isolating P1 Viral Stock

Introduction

Budded virus should be released into the medium 72 hours after transfection. However, if your transfection efficiency was not optimal, cells may not show all of the signs of viral infection until 4 or 5 days post-transfection. Beginning at 72 hours after transfection, visually inspect the cells daily for signs of infection (see below).

Characteristics of Infected Cells

Insect cells infected with baculovirus typically display the following characteristics when visually observed using an inverted phase microscope at 250–400X magnification. The time points provided below assume that the transfection was successful (i.e., transfection efficiency was high).

Signs of Infection	Phenotype	Description
Early (first 24 hours)	Increased cell diameter	A 25–50% increase in cell diameter may be seen.
	Increased size of cell nuclei	Nuclei may appear to "fill" the cells.
Late (24–72 hours)	Cessation of cell growth	Cells appear to stop growing when compared to a cell-only control.
	Granular appearance	Signs of viral budding; vesicular appearance to cells.
	Detachment	Cells release from the plate or flask.
Very Late (>72 hours)	Cell lysis	Cells appear lysed, and show signs of clearing in the monolayer.

Preparing the P1 Viral Stock

1. When the transfected cells (from Step 6, previous page) demonstrate signs of late stage infection (e.g., 72 hours post-transfection), collect the medium containing the virus from each well (~2 mL) and transfer to sterile 15 mL snap-cap tubes. Centrifuge the tubes at 500 × g for 5 minutes to remove cells and large debris.
2. Transfer the clarified supernatant to fresh 15 mL snap-cap tubes. **This is the P1 viral stock**. Store at 4ºC, protected from light. See the next page for additional storage information.

   Note: To concentrate your viral stock to obtain a higher titer, filter your viral supernatant through a 0.2 µm, low protein binding filter after the low-speed centrifugation step, if desired.

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Isolating P1 Viral Stock, Continued

Storing Viral Stocks

- Store viral stock at 4ºC, protected from light.
- If medium is serum-free (e.g., Sf-900 II SFM, Sf-900™ III SFM), add fetal bovine serum to a final concentration of 2%. Serum proteins act as substrates for proteases.
- For long-term storage, store an aliquot of the viral stock at –80ºC for later reamplification.
- Do **not** store routinely used viral stocks at temperatures below 4ºC. Repeated freeze/thaw cycles can result in a 10- to 100-fold decrease in virus titer.

The Next Step

Once you have obtained your clarified P1 baculoviral stock, you may:

- Amplify the viral stock (see the next section for details). This procedure is recommended to obtain the highest viral titers and optimal results in your expression studies.
- Determine the titer of your viral stock (see **Performing a Viral Plaque Assay**, page 25).
- Plaque purify your recombinant baculovirus, if desired (see **Performing a Viral Plaque Assay**, page 25).
- Use the P1 viral stock to infect your Sf9 or Sf21 cells for preliminary expression experiments (see below).

Note

To perform small-scale or preliminary expression experiments, it is possible to proceed directly to expression studies by using the P1 viral stock to infect your cells. Note that the MOI is unknown if viral titer is not determined, and the amount of viral stock is limited without viral amplification; therefore, expression conditions may not be reproducible.

Amplifying Your Baculoviral Stock

Introduction

The P1 viral stock is a small-scale, low-titer stock. You may use this stock to infect cells to generate a high-titer P2 stock. The titer of the initial viral stock obtained from transfecting Sf9 or Sf21 cells generally ranges from 1×10^6 to 1×10^7 plaque forming units (pfu)/mL. Because amplification allows production of a P2 viral stock with a titer ranging from 1×10^7 to 1×10^8 pfu/mL, we generally recommend it. This section provides guidelines and protocols for amplifying the recombinant baculovirus to prepare a P2 viral stock.

Materials Needed

- Sf9 or Sf21 cells cultured in the appropriate growth medium
- P1 baculoviral stock
- Any appropriate tissue culture vessel (see **Important Note** below)
- Tissue culture reagents
- 27°C humidified incubator

Important

To amplify your P1 viral stock, you may infect Sf9 or Sf21 cells growing in suspension or monolayer culture. Depending on your needs, you may amplify your P1 viral stock at any scale, but remember that you may be limited by the amount of P1 viral stock available.

We generally amplify our P1 viral stock in a 10 mL suspension culture at 2×10^6 cells/mL or in 6-well tissue culture plates at 2×10^6 cells/well. Calculate the number of Sf9 or Sf21 cells that you need for infection and expand cells accordingly. Make sure that the cells are healthy, of low passage (5–20), and have >95% viability before proceeding to infection.

Multiplicity of Infection (MOI)

To amplify your viral stock, infect cells at a multiplicity of infection (MOI) ranging from 0.05 to 0.1. MOI is defined as the number of virus particles per cell. Use the following formula to calculate how much viral stock to add to obtain a specific MOI:

$$\text{Inoculum required (mL)} = \frac{\text{MOI (pfu/cell)} \times \text{number of cells}}{\text{titer of viral stock (pfu/mL)}}$$

Note: If you have not determined the titer of your P1 viral stock, you may assume that the titer ranges from 1×10^6 to 1×10^7 pfu/mL.

Example

To infect a 10 mL culture at 2×10^6 cells/mL with an MOI of 0.1 using a P1 viral stock at 5×10^6 pfu/mL:

$$\text{Inoculum required (mL)} = \frac{(0.1\,\text{pfu/cell}) \times (2 \times 10^7\ \text{cells})}{5 \times 10^6\ \text{pfu/mL}}$$

$$\text{Inoculum required (mL)} = 0.4\,\text{mL}$$

Continued on next page

Amplifying Your Baculoviral Stock, Continued

Important considerations

For successful amplification of your baculovirus, pay attention to several key points:

- Use Sf9 or Sf21 cells that are in excellent health, low passage (5–20), log-phase growth, and have >95% viability.
- Use sterile P1 baculoviral stock that is free of contaminants.
- Use a low MOI between 0.05–0.1. Higher MOI reduces baculovirus quality.
- Harvest the virus when 70–80% of cells are dead.
- You **cannot** amplify the baculovirus indefinitely, because the baculovirus acquires deleterious mutations with each passage. Usually, P3 is highest usable passage.

Amplification Procedure

Follow the guidelines below to amplify your P1 viral stock in a 6-well plate.

1. On the day of infection, prepare your Sf9 or Sf21 cell suspension and plate cells at 2×10^6 cells/well. Incubate cells at room temperature for 1 hour to allow attachment.
2. After 1 hour, inspect cells under an inverted microscope to verify attachment.
3. Add the appropriate amount of P1 viral stock to each well.
4. Incubate the cells for 48 hours in a 27ºC humidified incubator.
5. 48 hours post-infection, collect 2 mL of medium containing virus from each well and transfer the virus to sterile 15 mL snap-cap tubes. Centrifuge the tubes at 500 × g for 5 minutes to remove cells and large debris and to obtain clarified baculoviral stock.

   Note: It is possible to harvest virus at later times after infection (e.g., 72 hours). Because optimal harvest times can vary, determine them for each baculoviral construct. Remember that culture viability decreases over time as cells lyse.
6. Transfer the supernatant to fresh 15 mL snap-cap tubes. This is the **P2 viral stock.** Store at 4ºC, protected from light. For long-term storage, you may store an aliquot of the P2 stock at –80ºC, protected from light. See page 22 for storage guidelines.
7. Proceed to the next section to determine the titer of your P2 viral stock.

Scaling Up the Amplification Procedure

After you have generated a high-titer P2 baculoviral stock, you may scale-up the amplification procedure to any volume of your choice. To produce this high-titer P3 stock, scale up the amount of cells and volume of virus used appropriately, and follow the guidelines and procedure outlined in this section.

Generating High-Titer Stocks From Frozen Master Stock

If you have stored your viral master stock at –80ºC, we recommend amplifying this stock to generate another high-titer stock for use in expression experiments. Viral titers generally decrease over time when virus is stored at –80ºC. Follow the guidelines and amplification procedure detailed in this section.

Performing a Viral Plaque Assay

Introduction

We recommend you perform a plaque assay to determine the titer of your viral stock. You may also perform a plaque assay to purify a single viral clone, if desired. In this procedure, you infect cells with dilutions of your viral stock and identify focal points of infection (plaques) on an agarose overlay. You may also titer your viral stock by the end-point dilution method described in O'Reilly *et. al.*, 1992

Experimental Outline

To determine the titer of a baculoviral stock:

1. Plate Sf9 or Sf21 cells in 6-well plates.
2. Prepare 10-fold serial dilutions of your baculoviral stock.
3. Add the different dilutions of baculovirus to Sf9 cells and infect cells for 1 hour.
4. Remove the virus and overlay the cell monolayer with Plaquing Medium.
5. Incubate the cells for 7–10 days, stain (if desired), and count the number of plaques in each dilution.

Factors Affecting Viral Titer

A number of factors can influence viral titers including:

- **The size of your gene of interest:** Titers generally decrease as the size of the insert increases.
- **The transfection efficiency:** For the highest transfection efficiency, we recommend transfecting Sf9 or Sf21 cells using Cellfectin® II Reagent. Prepare DNA: lipid complexes in Grace's Insect Medium, Unsupplemented (see pages 18–20 for details).
- **The age of your baculoviral stock:** Viral titers may decrease with long-term storage at 4ºC or –80ºC. If your baculoviral stock has been stored for 6 months to 1 year, we recommend titering or re-titering your baculoviral stock prior to use in an expression experiment.
- **The number of freeze/thaw cycles:** If you are storing your viral stock at –80ºC, viral titers can decrease as much as 10% with each freeze/thaw cycle.
- **Improper storage of your baculoviral stock:** For routine use, baculoviral stocks should be aliquoted and stored at 4ºC, protected from light.

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Performing a Viral Plaque Assay, Continued

Materials Needed

- Your clarified baculoviral stock (store at 4ºC until use)
- Sf9 or Sf21 cells cultured in the appropriate medium (30 mL of log-phase cells at 5×10^5 cells/mL for each baculoviral stock to be titered)
- Sf-900 II SFM, Sf-900™ III SFM or other appropriate complete growth medium (see **Note** below)
- Sf-900 Medium (1.3X) (100 mL) or other appropriate plaquing medium (see **Note** below)
- 4% Agarose Gel (specifically formulated for optimal insect cell growth)
- Sterile, cell-culture grade, distilled water
- 100 mL sterile, glass bottle
- 6-well tissue-culture plates (2 plates for each viral stock to be titered)
- Sterile hood
- Waters baths at 40ºC and 70ºC
- Microwave oven (optional)
- 27ºC humidified incubator
- Neutral Red (Sigma, Cat. no. N7005)

See page 54 for ordering information.

Note

If you are culturing your Sf9 or Sf21 cells in serum-supplemented media (i.e., complete TNM-FH), you should have the following reagents on hand:

- Grace's Insect Cell Culture Medium, Supplemented
- Grace's Insect Cell Culture Medium (2X)
- Fetal Bovine Serum (FBS), Qualified, Heat-Inactivated

See page 54 for ordering information.

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Preparing the Plaquing Medium

Plaquing medium consists of a mixture of culture medium and agarose. Plaquing medium is used to immobilize the infected cells for the plaque assay. Prepare plaquing medium immediately before use, following the procedure below. If you are culturing the Sf9 cells in Sf-900 II SFM or Sf-900™ III SFM, prepare Sf-900 Plaquing Medium. If you are culturing cells in TNM-FH, prepare Grace's Plaquing Medium.

Note: Other Plaquing Media are suitable.

1. Melt the 4% Agarose Gel by placing the bottle in a 70ºC water bath for 20 to 30 minutes **or** heating the agarose in a microwave oven. While the 4% agarose gel is melting, place the following in the 40ºC water bath:
   - Empty, sterile 100 mL bottle
   - Sf-900 Medium (1.3X) or Grace's Insect Cell Culture Medium (2X), as appropriate
2. After the 4% agarose gel has liquefied, move the agarose gel, medium, and empty 100 mL bottle to a sterile hood.
3. Working quickly, prepare the plaquing medium as follows:

   Sf-900 Plaquing Medium: Combine 30 mL of Sf-900 Medium (1.3X) and 10 mL of the melted 4% Agarose Gel in the empty 100 mL bottle and mix gently.

   Grace's Plaquing Medium: Add 20 mL of heat-inactivated FBS to the 100 mL bottle of Grace's Insect Medium (2X) and mix. Combine 25 mL of the Grace's Insect Medium (2X) containing serum with 12.5 mL of cell-culture grade, sterile, distilled water and 12.5 mL of the melted 4% Agarose Gel in the empty 100 mL bottle and mix gently.
4. Return the bottle of plaquing medium to the 40ºC water bath until use.

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Plaque Assay Procedure

Use the procedure below to perform a plaque assay in 6-well plate format to determine the titer of your pFastBac™/HBM baculoviral stock. If you have generated a baculoviral stock of the expression control pFastBac™ Gus, we recommend titering this stock as well. Remember to include a negative control (no virus) in your experiment.

Note: The amounts provided in this procedure are suitable to titer one baculoviral stock (two 6-well plates per viral stock). To titer more than one baculoviral stock, scale up the reagent quantities accordingly.

1. On the day of infection, harvest Sf9 or Sf21 cells and prepare a 30 mL cell suspension at 5×10^5 cells/mL in Sf-900 II SFM (or other complete growth medium). Aliquot 2 mL of cell suspension into each well of two 6-well plates. If you are including a negative control, you need another 6-well plate.
2. Allow the cells to settle to the bottom of the plate and incubate, covered, at room temperature for 1 hour.
3. Following the 1 hour incubation, observe the cell monolayers using an inverted microscope. Sf9 cells should be attached and at 50% confluence.
4. Prepare an 8-log serial dilution (10^{-1} to 10^{-8}) of the clarified baculoviral stock in Sf-900 II SFM **or** Grace's Insect Cell Culture Medium, Supplemented, without FBS, as appropriate.

   Sequentially dilute 0.5 mL of the baculoviral stock or previous dilution in 4.5 mL of medium in 12 mL disposable tubes, finishing with 8 tubes of diluted viral stock (i.e., 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}). Use the dilutions 10^{-4} to 10^{-8} in your assay.
5. Move the 6-well plates containing Sf9 cells and the tubes of diluted virus to the sterile hood. Label the plates, in columns of 2 (1 sample well plus 1 duplicate) as follows: no virus (negative control), 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}.
6. Remove the medium from each well, discard it, and immediately replace it with 1 mL of the appropriate virus dilution. As a negative control, add the appropriate medium without virus.
7. Incubate the cells with the virus for 1 hour at room temperature.
8. Move the cells and the bottle of plaquing medium from the 40ºC water bath (Step 4, previous page) to a sterile hood.
9. Sequentially starting from the highest dilution (10^{-8}) to the lowest dilution (10^{-4}), remove the medium containing virus from the wells and replace it with 2 mL of plaquing medium. Work quickly to avoid desiccation of the cell monolayer.
10. Allow the agarose overlay to harden for 1 hour at room temperature before moving the plates.
11. Incubate the cells in a 27ºC humidified incubator for 7–10 days until plaques are visible and ready to count. To stain plaques to facilitate counting, see the next page. To calculate the titer, see page 30.

Continued on next page

Note

To improve the visualization of plaques, stain the plates using Neutral Red. **Crystalline Blue and other plaque staining dyes containing organic solvents are not recommended because they kill the host cells**. To stain plaques, you may do one of the following:

- Prepare an agarose solution containing Neutral Red and overlay this solution on the plates 4 days post-infection. Count plaques 7–10 days post-infection.

  or

- Prepare a Neutral Red solution and add it to plates for 1–2 hours just prior to counting plaques (7–10 days post-infection).

Important: If you plan to plaque purify your baculovirus, **do not** stain plaques, because Neutral Red is a known mutagen that can alter your recombinant virus.

Neutral Red Staining Procedure

Preparing a Neutral Red Agarose Overlay (for use on Day 4)

1. Prepare a 1 mg/mL Neutral Red solution in Sf-900 II SFM (or other appropriate complete growth medium). Filter-sterilize.
2. Combine the reagents below in a 50 mL tube and place in a 40ºC water bath.

1 mg/mL Neutral Red solution	1.5 mL
Sf-900 II SFM	16.5 mL

3. Microwave 4% Agarose Gel until melted, then place it in a 40ºC water bath for 5 minutes.
4. Move the 50 mL tube of Neutral Red solution and the 4% agarose gel to a sterile hood. Add 6 mL of 4% agarose gel to the Neutral Red solution.
5. Add 1 mL of the Neutral Red overlay to each well containing plaquing overlay. Once the agarose has hardened, return plates to a 27ºC humidified incubator until plaques are ready to count. Plaques appear as clear spots on a red monolayer.

Preparing a Neutral Red Stain (for use on Day 7–10 prior to counting plaques)

1. Prepare a 1 mg/mL Neutral Red solution in cell-culture grade, distilled water.
2. Add 0.5 mL of Neutral Red solution to each well containing plaquing overlay. Incubate for 1 to 2 hours at room temperature.
3. Gently remove excess stain with a pipette or blotter and count the plaques. Plaques will appear as clear spots in a nearly clear gel against a red background.

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Calculating the Titer

Count the number of plaques present in each dilution, then use the following formula to calculate the titer (plaque forming units (pfu)/mL) of your viral stock. Note that the optimal range to count is 3 to 20 plaques per well of a 6-well plate.

$$\text{titer (pfu/mL)} = \text{number of plaques} \times \text{dilution factor} \times \frac{1}{\text{mL of inoculum/well}}$$

Example

If you add 1 mL of inoculum and observe 20 plaques in the well containing the 10^{-6} viral dilution, the titer of the viral stock is:

$$\text{titer (pfu/mL)} = 20 \text{ plaques} \times 10^{6} \times \frac{1}{1 \text{ mL of inoculum/well}}$$

$$\text{titer (pfu/mL)} = 2 \times 10^{7} \text{ pfu/mL}$$

What You Should See

When titering pFastBac™/HBM baculoviral stocks, we generally obtain titers ranging from:

- 1×10^{6} to 1×10^{7} pfu/mL for P1 viral stocks
- 1×10^{7} to 1×10^{8} pfu/mL for P2 viral stocks

Note: If the titer of your baculoviral stock is less than 1×10^{6} pfu/mL or 1×10^{7} pfu/mL for a P1 or P2 viral stock, respectively, we recommend producing a new baculoviral stock.

For tips and guidelines to optimize your viral yield, see **Factors Affecting Viral Titer**, page 25, and the **Troubleshooting** section, page 47.

Continued on next page

Plaque Purification

You may generate a viral stock from a single viral clone by plaque purifying your baculovirus, if desired. Use a protocol of your choice or the procedure below.

Materials Needed

- Plate containing well-spaced viral plaques (from **Plaque Assay Procedure**, Step 11, page 28; **do not** stain plates with Neutral Red)
- Log phase Sf9 or Sf21 cells at greater than 95% viability
- Sterile Pasteur pipette and bulb

Procedure

1. Follow Steps 1–3 in the **Plaque Assay Procedure**, page 28, to seed Sf9 or Sf21 cells.
2. Using a sterile Pasteur pipette and bulb, carefully pick a clear plaque and transfer the agarose plug (containing virus) to a 1.5 mL microcentrifuge tube containing 500 µL of complete growth medium. Mix well by vortexing.
3. Add 100 µL of the agarose plug solution to each well.
4. Incubate the cells in a 27ºC humidified incubator for 72 hours.
5. Collect the medium containing virus from each well (~2 mL) and transfer to sterile 15 mL snap-cap tubes. Centrifuge the tubes at 500 × g for 5 minutes to remove cells and large debris.
6. Transfer the clarified supernatant to fresh 15 mL snap-cap tubes. This is your plaque-purified viral stock.
7. Proceed to **Amplifying Your Baculoviral Stock**, page 23.

Expressing Your Recombinant Protein

Introduction

Once you have generated a pFastBac™/HBM baculoviral stock with a suitable titer (e.g., 1×10^8 pfu/mL), you are ready to use the baculoviral stock to infect High Five™ insect cells and assay for expression of your recombinant protein.

The following guidelines and recommendations are provided for your convenience. If you need more details about the techniques discussed, refer to *Current Protocols in Molecular Biology*, Unit 16.9-16.11 (Ausubel, *et al.*, 1994), *The Baculovirus Expression System: A Laboratory Guide* (King and Possee, 1992), or *Baculovirus Expression Vectors: A Laboratory Manual* (O'Reilly, *et al.*, 1992).

We recommend that you:

- Use High Five™ cells adapted to suspension culture in serum-free medium for expression of secreted proteins
- Perform a time course of expression to determine the maximum point of expression
- Have a detection method for your protein

High Five™ Cells

High Five™ cells (see page 54 for ordering information) are particularly well suited for expression of secreted recombinant proteins. This cell line (BT1-TN-5B1-4) was originally developed by the Boyce Thompson Institute, Ithaca, NY and originated from the egg cells of the cabbage looper, *Trichoplusia ni*, the native host of AcMNPV (Davis *et al.*, 1992). This cell line has the following characteristics:

- Grows well in monolayer and doubles in less than 24 hours for ease of use
- Adaptable to suspension culture and serum-free medium for high-level protein expression and purification
- Provides 5–10 fold higher secreted expression than Sf9 cells (Davis *et al.*, 1993)

For more information about High Five™ cells or a protocol for adaptation to suspension culture, refer to the **Growth and Maintenance of Insect Cell Lines** manual (part no. 25-0127) available at www.invitrogen.com or from Technical Support (see page 56).

Secretion in High Five™ Cells

Expression of secreted alkaline phosphatase (SEAP) using its native secretion signal was evaluated in eight different cell lines including High Five™, Sf9, and Sf21. On a per cell basis, High Five™ cells produced 20-fold more protein than Sf21 cells and 23-fold more protein than Sf9.

Since High Five™ cells are larger than Sf9 or Sf21 cells and the assays were performed on adherent cells, the amount of SEAP was also determined per milliliter of culture medium. In this case, High Five™ cells produced 5-fold more SEAP than Sf9 cells and 8-fold more than Sf21 (Davis *et al.*, 1993).

Continued on next page

Guidelines for Expression

General guidelines are provided below to infect insect cells with the recombinant baculovirus to express your protein of interest. As with any expression system, optimal expression conditions depend on the characteristics of the protein being expressed.

- **Cell line:** We recommend using High Five™ cells (see page 54 for ordering information) for expression of secreted recombinant proteins.

  Note: You may also use other cell lines such as Sf9, Sf21, or Mimic™ Sf9, but your secreted expression levels will be lower.

- **Culture Conditions:** We generally culture High Five™ cells in serum-free conditions using in Express Five® SFM (see page 54 for ordering information). You may grow your cells either in adherent or suspension culture using your culture vessel of choice.

  Depending on your application and the protein of interest, note that it may be necessary to supplement the culture post-infection with 0.1% to 0.5% FBS or BSA to protect the recombinant protein from proteolysis. Protein-based protease inhibitors are generally less expensive and more effective than many synthetic protease inhibitors.

- **Infection Conditions:** We recommend infecting cultures while cells are in the mid-logarithmic phase of growth at a density of 1×10^6 to 2×10^6 cells/mL. Make sure that the culture is not rate-limited by nutritional (*i.e.*, amino acid or carbohydrate utilization) or environmental factors (*i.e.*, pH, dissolved O_2, or temperature) during infection.

- **MOI:** Optimal MOI will vary between cell lines, and the relative infection kinetics of the virus isolate or clone used. Establish a dose for each virus, medium, reactor, and cell line employed to determine the optimal infection parameters to use for protein expression. As a starting point, infect cells using an MOI of 5 to 10.

  Note: This MOI recommendation is unlike the generation of a high-titer stock, where a low MOI of 0.5–1.0 is recommended.

- **Time course:** We recommend performing a time course to determine the expression kinetics for your recombinant protein as many proteins may be degraded by cellular proteases released in cell culture. Maximum expression of secreted proteins is generally observed between 30 and 72 hours.

Positive Control

If you have generated a high-titer viral stock from pFastBac™ Gus control plasmid, include this recombinant baculovirus in your experiments for use as an expression control. After you have infected cells with the control virus, the gene encoding β-glucuronidase is constitutively expressed. The molecular weight of β-glucuronidase is 68.5 kDa. For a rapid but qualitative assay for β-glucuronidase expression, see page 39.

Continued on next page

Seeding Densities and Volumes for Infections

The table below gives approximate seeding densities and volumes for typical vessel sizes. Infection at these densities in the minimal volumes listed yields optimal infection.

Minimal Volumes: The total volumes used are lower than those used in general cell culture and maintenance, so that the virus added is concentrated and can infect cells more readily.

Cell Density: Cell density in adherent culture is approximately 50% confluent to allow maximal cell surface area for contact with virus and subsequent infection. However, to maximize the infection efficiency, we recommend that you determine the optimal cell density for the specific cell type you are using.

MOI: Use an MOI of 5–10 for a time course of protein expression or a large-scale protein preparation.

Amount of Virus to Add: The amount of virus to add depends on MOI.

Type of Vessel	Cell Density	Final Volume (culture medium + added virus)
96-well plate	3.0×10^4 cells/well	100 µL
24-well plate	2.0×10^5 cells/well	500 µL
12-well plate	4.0×10^5 cells/well	750 µL
6-well plate	1.0×10^6 cells/well	1 mL
60 mm^2 plate	2.5×10^6 cells/plate	3 mL
25 cm^2 flask	2.0×10^6 cells/flask	5 mL
75 cm^2 flask	6.0×10^6 cells/flask	10 mL
150 cm^2 flask	1.2×10^7 cells/flask	15–20 mL
spinners (all)	2.0×10^6–2.5×10^6 cells/mL	no more than half of the total volume of the flask

Calculating Virus Volumes

To calculate the volume of viral stock needed to achieve a given MOI;

$$\text{Volume of virus} = \frac{(\text{MOI desired})\ (\text{Total number of cells})}{\text{Titer of viral stock}}$$

For example, to infect a spinner with 50 mL of culture at a cell density of 2×10^6 cells/mL (i.e., a total of 1×10^8 cells) at an MOI of 5 using a high-titer virus stock at 1×10^8 pfu (virions)/mL, you need:

$$\frac{(5 \text{ virions/cell})\ (1 \times 10^8 \text{ cells})}{1 \times 10^8 \text{ virions/mL})} = 5 \text{ mL of your viral stock}$$

Continued on next page

Determining Time Points for Optimal Secreted Protein Expression

When expressing a secreted protein, analyze the **supernatant** for the presence of **secreted, recombinant protein** and the lysates from the **cell pellet** for the presence of **unprocessed recombinant protein**. Compare the supernatant sample and the lysate sample to determine if recombinant protein is being secreted, how much protein has been secreted, and how much protein remains intracellular at different times during secretion. Using this data, optimize your time points for maximal secreted protein expression levels (see **Optimizing Protein Expression**, page 41).

Note: β-glucuronidase expression from the pFastBac™ Gus positive control baculovirus is intracellular (i.e., not secreted), because the gene product lacks the HBM secretion signal sequence.

Protocol for Cells in Suspension Culture

The following procedure is designed to allow expression analysis from 50 mL of High Five™ cells at a density of 2×10^6 cells/mL cultured in a 100 mL spinner flask.

Note: Use cells with a doubling time of 18–24 hours and a viability of 95%. Cells should be at a passage number less than 30, and they should not have been in spinner culture for more than 2 months.

Materials needed:

- High-titer pFastBac™/HBM baculoviral stock of known titer (≥ 10^8 pfu/mL)
- High Five™ cells
- Express Five® SFM
- One 100 mL spinner flask per sample
- Microcentrifuge tubes

Protocol:

1. Add the pFastBac™/HBM baculoviral stock to the spinner flask at the desired MOI. Include the appropriate controls [mock-infected (uninfected) cells, pFastBac™ Gus positive control baculovirus, previously characterized recombinant baculoviruses]. To calculate the amount of virus to add, see **Calculating Virus Volumes**, page 34.
2. Incubate spinners at 27°C with a spin rate of 80 to 90 rpm.
3. Remove 1 mL aliquots of cells at designated time point(s) (see **Determining Time Points for Optimal Secreted Protein Expression**, above), and transfer each sample to a microcentrifuge tube.
4. Pellet cells at 800 × g for 10 minutes at 4°C. Keep samples at 4°C or on ice to prevent proteolysis.
5. Transfer supernatant to a new tube. Do not discard the cell pellet. Label each tube containing the supernatant and cell pellet.
6. Proceed to **Analyzing Recombinant Protein**, page 37, or store the cell pellet **and** the supernatant at –80°C for analysis at a later time.

   Note: Storage at –80°C will reduce proteolysis of the recombinant protein. If you are performing a time course, lyse the cell pellet later when you have collected all time points.

Continued on next page

Protocol for Adherent Cells

The following procedure, adapted from Luckow and Summers , is designed to allow expression analysis in a 24-well format from recombinant baculovirus infected adherent cells harvested 24 to 96 hours post-infection. For other plate and/or flask sizes, adjust the cell seeding densities and volumes. Other protocols are also suitable.

Materials needed:

- High-titer pFastBac™/HBM baculoviral stock of known titer (≥ 10^8 pfu/mL)
- High Five™ cells
- Express Five® SFM
- One 24-well plate per sample
- Microcentrifuge tubes

Protocol:

1. Seed 2 × 10^5 High Five™ cells per well in a 24-well plate. Let cells attach for at least 30 minutes.
2. Remove the media and rinse the cells once with fresh growth media. Replace with 300 mL of fresh media.
3. Add the pFastBac™/HBM baculoviral stock to each well at the desired MOI. Include the appropriate controls [mock-infected (uninfected) cells, pFastBac™ Gus positive control baculovirus, previously characterized recombinant baculoviruses]. To calculate the amount of virus to add, see **Calculating Virus Volumes**, page 34.
4. Incubate cells in a 27ºC humidified incubator.
5. Harvest each well at the designated time point. Scrape the cells from each well and transfer the entire solution from each well to a microcentrifuge tube.
6. Pellet the cells at 800 × g for 10 minutes at 4°C. Keep the samples at 4°C or on ice to prevent proteolysis.
7. Transfer supernatant to a new tube. Label each tube.
8. Proceed to **Analyzing Recombinant Protein**, page 37, or store the cell pellet and the supernatant at –80°C for analysis at a later time.

Analyzing Recombinant Protein

Introduction

The next step after harvesting baculovirus infected insect cells is to analyze the secreted expression of your protein by SDS-PAGE or western blot. In addition to analyzing the supernatant, we recommend that you analyze the cell lysate to determine if all of your protein is being secreted. Analyzing cell lysate can assist you in optimizing your MOI and time course for expression (see **Optimizing Protein Expression**, page 41). After you determine the optimal experimental parameters for secreted expression, you may proceed with **Large-Scale Expression** (page 42).

Protease Inhibitors

We recommend that you add one or more protease inhibitors to each of the lysis buffers that are described in the protocol below. The following table summarizes recommended protease inhibitors, their method of action, and working concentrations.

Protease Inhibitor	Method of Action	Stock Solution	Working Concentration
PMSF	Serine protease inhibitor	10 mg/mL in isopropanol	100 µg/mL
Leupeptin	Serine and thiol protease inhibitor	50 µg/mL in deionized water	0.5 µg/mL
Aprotinin	Serine protease inhibitor	50 µg/mL in deionized water	0.5 µg/mL
Pepstatin A	Acid protease inhibitor	100 µg/mL in methanol	1 µg/mL

You can store all of the above protease inhibitor solutions at –20°C except for PMSF. Store PMSF at room temperature in isopropanol. PMSF is not stable in aqueous solution; add it to the lysis buffer just before use.

PMSF (phenylmethylsulfonylfluoride) is very harmful if inhaled, swallowed, or contacted by the skin. Wear protective clothing and gloves when handling.

Preparing Cell Lysates

You may use any method to prepare cell lysates for analysis, including detergent lysis, sonication, or freeze-thaw lysis. The protocol on the next page, **Detergent Lysis**, provides a quick procedure for preparing lysates suitable for analyzing the secretion level of your recombinant protein. If you do not want to use detergent to lyse your cell samples (e.g., your protein is sensitive to detergent), lyse your cells by sonication and/or freeze-thawing.

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Detergent Lysis

Detergent lysis is a quick and efficient way to lyse cells and extract intracellular protein. The protocol below uses Triton® X-100, but you may also use Nonidet P-40 (NP-40).

1. Place all cell pellets from the time course on ice. Be sure to include the control sample.
2. Make up 2–5 mL of lysis buffer (0.1% Triton® X-100 in PBS or TBS). Use 100 µL of lysis buffer for each 10^6 cells.
3. Add each of the protease inhibitors (Leupeptin, Aprotinin and Pepstatin A) at the working concentrations described on the previous page. Perform this step on ice. **Add PMSF just after adding the lysis buffer to the cell pellet (Step 5).**
4. Add 100 µL of lysis buffer for each 10^6 cells in the pellet.
5. Add PMSF to each sample to a final concentration of 100 µg/mL.
6. Vortex each cell sample to break up the cell pellet and begin lysis.
7. **Lysis/Incubation:** Incubate all samples on ice for 30–45 minutes, then vortex them at 10 minute intervals to assist lysis.
8. After all samples have been lysed, pellet cellular debris at 1,000 × g for 10 minutes at 4°C.
9. **Check for lysis efficiency:** To check for cell lysis, take a 10 µL sample, add 1 µL of Trypan Blue and load onto a hemacytometer. See **Growth and Maintenance of Insect Cell Lines** manual for protocols. All cells stain blue when lysis is complete.
10. Transfer supernatant (lysate) to a new tube. Keep on ice. Proceed to **Detecting Recombinant Protein,** below.

Note: We recommend that you save the pellet from the lysate (the insoluble portion), because it may be useful for analysis if you cannot detect proteins in the lysate or the supernatant.

Detecting Recombinant Protein

You may use any method of choice to detect your recombinant protein of interest including functional analysis or western blot. If you perform western blot analysis, you will need to have an antibody to your protein of interest.

The pFastBac™/HBM-TOPO® vector allows the expression of your recombinant protein of interest as a C-terminal 6×His fusion. You can use the antibodies listed on page 55 to detect your recombinant protein.

Note

If you are using polyacrylamide gel electrophoresis to detect your recombinant protein, you should note that the presence of the C-terminal 6×His tag, and the Tobacco Etch Virus (TEV) recognition site will increase the size of your protein by at least 3 kDa.

The HBM secretion signal coding sequence is cleaved upon secretion, and thus does not change the size of your protein.

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Glycosylation

When expressing and purifying a glycosylated protein in a heterologous expression system, quickly determine whether the protein is glycosylated properly. Refer to published protocols for carbohydrate analysis of proteins to characterize glycosylated proteins of interest (Ausubel *et al.*, 1994). Further information about glycosylation in eukaryotes is also available in published literature (Varki & Freeze, 1994).

Purifying Recombinant Protein

The presence of the C-terminal 6×His tag in the pFastBac™/HBM-TOPO® vector allows the purification of your recombinant protein with a metal-chelating resin such as ProBond™ and Ni-NTA available from Invitrogen (see page 55 for ordering information). Refer to the manual included with each product for guidelines to purify your fusion protein. These manuals are available for downloading at www.invitrogen.com or by contacting Technical Support (page 56).

Removing the 6×His Tag Using AcTEV™ Protease

The pFastBac™/HBM-TOPO® vector contains a Tobacco Etch Virus (TEV) recognition site that allows the removal of the 6×His tag from your recombinant fusion protein using AcTEV™ Protease available separately from Invitrogen (see page 53 for ordering information). Instructions for digestion are included with the product. For more information, refer to www.invitrogen.com or contact Technical Support (see page 56).

Assay for β-glucuronidase

If you include the pFastBac™ Gus baculoviral control construct in your expression experiment, you may assay for β-glucuronidase expression. To assess β-glucuronidase expression in a rapid manner, mix a small amount of media from the infected cells with the chromogenic indicator X-glucuronide and observe development of blue color.

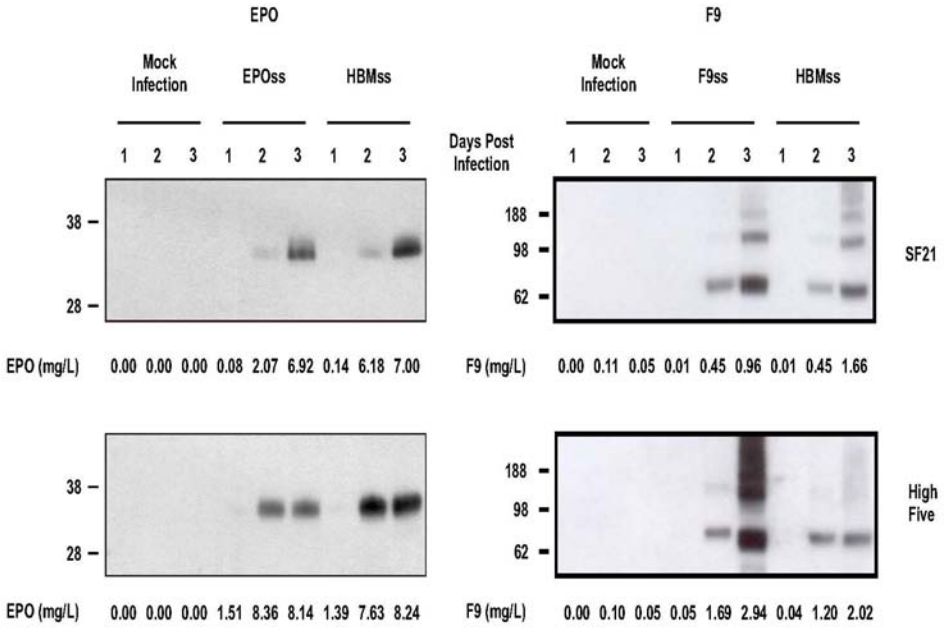
1. Mix 5 µL of 20 mg/mL X-glucuronide solution (in DMSO or dimethylformamide) with 50 µL of cell-free medium.
2. Monitor for development of blue color within 2 hours.

Note: Other methods are also suitable.

Expected Results

Introduction

In the following experiments (see figures below), the relative secretion efficiency of proteins fused to the HBM signal sequence was compared to two ordinarily secreted proteins, human coagulation factor IX (F9) and erythropoietin (EPO). To asses the relative secretion efficiency, SF21 and High Five™ cells were infected with recombinant viruses coding for EPO and F9 fused to their own or to HBM signal sequences as indicated. The culture media were collected at the indicated time points. Proteins were detected by Western blots using anti-his antibody. Protein yield (mg/L) was estimated by enzyme-linked immunosorbent assays using VisuLize™ Factor IX Antigen Kit (Affinity Biologicals Inc, Ancaster, Ontario, Canada) and Quantikine® IVD® (R&D Systems, Minneapolis, MN).



Optimizing Protein Expression

Introduction

A number of factors can influence determination of optimal expression conditions including the cell line, MOI, your application of interest, and the nature of your gene of interest. Use the following guidelines to determine the optimal conditions for expressing your recombinant protein of interest.

Time Course

Infect cells at a constant MOI and assay for recombinant protein expression at different time points post-infection (e.g., 24, 48, 72, 96 hours). We recommend that you assay for protein expression at 24-hour intervals initially, to get a general idea of when the protein is being expressed.

Once you have determined a time frame where optimal protein expression occurs (e.g., between 48 and 72 hours), perform a second time course with selected intermediate time points (e.g., 52, 60, and 68 hours) to further optimize your expression levels.

Synchronous Infection

Synchronous infection is defined as the infection of all cells in a culture at the same time point. Therefore, a true time = zero is established. Achieving synchronous infection results in the maximum amount of protein being harvested at a given time point post-infection, because all cells in the culture are expressing protein at the same time. You must determine the maximum time point for each protein and for each cell line used. You may test different MOIs after the initial time course to achieve synchronous infection.

Multiplicity of Infection (MOI)

Infect a population of cells at varying MOIs (*e.g.*, 1, 2, 5, 10, 20) and assay for protein expression. Use the MOI that provides the optimal level of recombinant protein expression.

For example, if an MOI of 5 gives you protein over a wide range of times, but an MOI of 10 lyses all infected cells before sufficient protein can accumulate, try an MOI of 6 and/or an MOI of 8. The objective in trying different MOIs is to find the MOI that yields the highest protein levels and the least loss due to lysis and proteolysis.

Using Suspension Culture vs. Adherent Culture

The use of suspension culture (spinner or shake flask) versus adherent culture can increase the cell density per mL of culture, and therefore can potentially increase the relative yield of protein per mL of culture.

Large-Scale Expression of Recombinant Protein

Introduction

After successfully optimizing expression and secretion levels, you may proceed to large-scale expression of your recombinant protein. You may move up to larger vessels (1 liter or more) or go larger still and use airlift bioreactors and/or fermenters. This section summarizes the requirements and options that exist for large-scale secreted expression of protein using the baculovirus expression system.

Large-Scale Expression in Spinner Flasks

If you are scaling up your suspension culture up to 1 liter spinner flasks, see the sections on Suspension Cell Culture in the **Growth and Maintenance of Insect Cell Lines** manual. This manual provides information on how to adapt Sf9, Sf21, or High Five™ insect cell lines to suspension culture, and protocols for maintaining and scaling up suspension cultures. It is available for downloading at www.invitrogen.com or by contacting Technical Support (see page 56).

To scale up your culture to 1 liter spinner flasks (500 mL total culture volume), we recommend the following:

1. Generate a large-scale, high-titer stock of the desired recombinant virus (see page 23). This stock will allow you to infect many large-scale cultures and ensure consistency in protein expression.
2. Start with 100 or 250 mL spinner flasks (50–125 mL of insect cell culture) and scale up to 1 liter spinners with 500 mL of cell suspension.
3. Seed cultures at 1×10^6 cells/mL and subculture cell suspension when the density reaches 2.0×10^6–2.5×10^6 cells/mL.
4. Check cell viability daily to ensure the culture is >95% viable.
5. Add Pluronic® F-68 to a final concentration of 0.1% in your spinner culture. Adding Pluronic® F-68 protects the cells from shearing forces, and it allows you to increase the impellar speed to 120 rpm for larger cultures. Increasing the impeller speed increases aeration of the culture for better growth.

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Large-Scale Expression of Recombinant Protein, Continued

Large-Scale Expression Options

The following table summarizes other methods, requirements, benefits and references for scale-up production of recombinant protein using the baculovirus expression system.

Method	Requirements	Benefits	References
Stirred Bioreactor	For a 5-liter Bioreactor: • 5-liter bioreactor. • Sterilized tubing. • Microbial air filters. • High purity nitrogen, oxygen, and air. • pH, dissolved oxygen and temperature probes. • External dissolved oxygen controller. • External pH controller. • Peristaltic pump for acid/base lines. • Linear recorder to monitor dissolved oxygen and pH control. • Laminar flow hood in close proximity to the bioreactor.	• Addresses increased oxygen needs of large-scale culture. • Controlled growth and optimization of variables in the culture. • Increased cell densities. • Elevated protein production. • Reproducible results for batch production of protein.	(Tom *et al.*, 1995) (Murhammer & Goochee, 1988) (Maiorella *et al.*, 1988) (O'Reilly *et al.*, 1992)
Airlift Fermentor	For a 5-liter Airlift Fermentor: • 5-liter airlift fermentor system. • Dissolved oxygen control module. • Dissolved oxygen electrode. • Microbial air filters. • 1/4" stainless-steel tubing. • Silicone tubing. • Circulating water bath.	• Addresses increased oxygen needs of large-scale culture. • Control over environmental variables in the culture. • Increased cell densities. • Elevated protein production.	(Maiorella *et al.*, 1988) (Murhammer & Goochee, 1988) (Onken & Weiland, 1983) (O'Reilly *et al.*, 1992)
Insect Larvae	• *Trichoplusia ni* larvae and facilities to grow larvae.	• More accurate posttranslational modification of recombinant protein – does not rely on one cell type only. • Higher levels than in cell culture. • Inexpensive.	(Medin *et al.*, 1990) (Wood *et al.*, 1993)

Troubleshooting

Cloning into the pFastBac™/HBM-TOPO® Vector

For troubleshooting any problems you may encounter when generating your pFastBac™/HBM-TOPO® construct, refer to the Bac-to-Bac® TOPO® Cloning Kit manual (part no. A10605) supplied with this kit. The Bac-to-Bac® TOPO® Cloning Kit manual is also available at www.invitrogen.com or by contacting Technical Support (see page 56).

Generating Recombinant Bacmid DNA

The table below lists some potential problems that you may encounter when generating the recombinant bacmid following transformation into DH10Bac™ *E. coli*.

Problem	Reason	Solution
No blue colonies (non-recombinant) obtained (i.e., all colonies are white) **Note:** Although you will pick white colonies, you should expect to see some blue colonies. Blue colonies contain non-recombinant bacmids.	Insufficient time for color development.	Wait at least 48 hours before identifying colony phenotypes.
	Used X-gal instead of Bluo-gal in agar plates.	Use Bluo-gal in selective plates to increase the contrast between blue and white colonies.
	Insufficient growth after transposition.	Grow transformed cells in S.O.C. Medium for a minimum of 4 hours before plating.
	Bluo-gal and IPTG omitted from plates.	Prepare fresh selective plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL IPTG.
	Too many colonies on the plate.	• Serially dilute the transformation mixture and plate to give well-separated colonies. • Adjust the serial dilutions of cells (10^{-2} to 10^{-4}) to obtain well-spaced colonies.
	Plates too old or stored in light.	• Do not use plates that are more than 4 weeks old. • Store plates protected from light.
	Incubation period too short or temperature too low.	Wait at least 48 hours before picking colonies. Incubate plates at 37°C.

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Generating Recombinant Bacmid DNA, continued

Problem	Reason	Solution
All colonies are blue	DNA from your pFastBac™/HBM-TOPO® construct used for transformation was of poor quality.	• Use purified plasmid DNA for transformation. • Check the quality of your plasmid DNA; make sure that the DNA is not degraded.
	Gentamicin omitted from plates.	Prepare fresh selective plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL IPTG.
Few colonies obtained	Used LB medium for recovery/expression period.	Use S.O.C. Medium for the 4 hours growth time.
	Recovery/expression time too short.	Increase the recovery time to > 4 hours at 37°C or 6 hours at 30°C.
Poor blue/white colony differentiation	Agar not at correct pH.	Adjust pH of LB agar to 7.0.
	Intensity of the blue color too weak.	• Use Bluo-gal, not X-gal. • Increase the concentration of Bluo-gal to 300 µg/mL. • Use dark and light backgrounds to view plates.
	Too many or too few colonies on plate.	Adjust the serial dilutions of cells to obtain an optimal number of colonies.
	Incubation period too short or temperature too low.	• Do not pick colonies until 48 hours after plating. • Incubate plates at 37°C.
	IPTG concentration not optimal.	Optimize the IPTG concentration. A range of 20–60 µg/mL IPTG generally gives optimal color development.

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Isolating Bacmid DNA

The table below lists some potential problems and possible solutions to help you troubleshoot recombinant bacmid DNA isolation.

Problem	Reason	Solution
Bacmid DNA is degraded	DNA stored improperly.	• Store purified bacmid DNA in aliquots at 4°C for no more than 2 weeks. • Do not freeze/thaw the bacmid DNA. • For long term storage of bacmid DNA, prepare glycerol stocks of DH10Bac™ *E. coli* containing the verified bacmid DNA.
	High molecular weight bacmid DNA handled improperly.	• When isolating bacmid DNA, do not vortex the DNA solution. • Do not resuspend DNA pellets mechanically; allow the solution to sit in the tube with occasional gentle tapping of the bottom of the tube.
Poor yield	Used incorrect antibiotic concentrations.	Grow transformed DH10Bac™ cells in LB medium containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/mL tetracycline.
Bacmid DNA contains a mixture of recombinant bacmid and empty bacmid	Picked a colony that was gray or dark in the center.	Analyze more white DH10Bac™ transformants and choose one that contains recombinant bacmid DNA only.

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Transfecting Insect Cells

The table below lists some potential problems and possible solutions that may help you troubleshoot insect cell transfection.

Problem	Reason	Solution
Low yield of virus	Low transfection efficiency.	• Use Invitrogen's Cellfectin® II Reagent for transfection. • Perform transfection in unsupplemented Grace's Medium; make sure that no supplements, FBS, or antibiotics are present during transfection. • Harvest viral supernatant when signs of infection are visible (i.e., >72 hours post-transfection).
	Cells plated too sparsely.	Plate insect cells at the recommended cell density.
	Used too much or too little Cellfectin® II or other lipid reagent.	Optimize the amount of Cellfectin® II or other lipid reagent used.
	Time of incubation with DNA:lipid complexes too short or too long.	Optimize the incubation time (e.g., 3 to 8 hours).
	Recombinant bacmid DNA is degraded.	• Check the quality of your recombinant DNA by agarose gel electrophoresis prior to transfection. • Prepare bacmid DNA using Invitrogen's PureLink™ HiPure Miniprep or Maxiprep Kit (see page 53 for ordering information). • Store purified bacmid at 4°C; **do not** freeze, because freezing the baculovirus decreases transfection efficiency.
	Bacmid DNA is not pure (i.e., contains recombinant bacmid and empty bacmid).	• Screen other DH10Bac™ transformants and choose one that contains only recombinant bacmid. • Perform plaque purification to isolate recombinant baculovirus.

Continued on next page

Expressing Your Protein

The table below lists some potential problems and possible solutions that may help you troubleshoot your expression experiments.

Problem	Reason	Solution
Low protein expression	Viral stock contains a mixture of recombinant and non-recombinant baculovirus.	Perform plaque purification to isolate recombinant baculovirus.
	Baculovirus not recombinant.	• Verify transposition of bacmid DNA by PCR analysis, using the pUC/M13 Forward and Reverse primers. • Re-transfect insect cells with new recombinant bacmid DNA.
	Used too low or too high viral titer.	Optimize infection conditions by varying the MOI.
	Time of cell harvest not optimal.	Perform a time course of expression to determine the optimal time to obtain maximal protein expression.
	Cell growth conditions and medium not optimal.	• Optimize culture conditions based on the size of your culture vessel and expression conditions. • Culture High Five™ cells in Express Five® SFM for optimal cell growth and protein expression.
	Cell line not optimal.	Use High Five™ Cells for highest level of secreted expression.
	Protein expression is not optimal.	Optimize protein expression by varying such parameters as incubation temperature and oxygenation.

Appendix

Recipes

Antibiotic Stock Solutions

Antibiotics can be ordered in either dry powdered form or as a stabilized, sterile, premixed solution. Store these solutions according to the manufacturer's recommendations. For the antibiotics below, prepare and store the stock solutions as directed:

Antibiotic	Stock Solution Concentration	Storage
Ampicillin	50 mg/mL in water; filter-sterilize	–20°C, protected from light
Kanamycin	10 mg/mL in water; filter-sterilize	–20°C, protected from light
Tetracycline*	10 mg/mL in 100% ethanol; filter-sterilize	–20°C, protected from light
Gentamicin	7 mg/mL in water; filter-sterilize	–20°C, protected from light

IPTG

Follow the procedure below to prepare a 200 mg/mL stock solution of IPTG.

1. Dissolve 2 g of IPTG in 8 mL of sterile water.
2. Adjust the volume of the solution to 10 mL with sterile water.
3. Filter-sterilize through a 0.22 micron filter.
4. Dispense the stock solution into 1 mL aliquots.
5. Store at –20°C.

Bluo-gal

Follow the guidelines below to prepare a 20 mg/mL stock solution of Bluo-gal.

1. Dissolve the Bluo-gal in dimethylformamide or dimethylsulfoxide (DMSO) to make a 20 mg/mL stock solution. Use a glass or polypropylene tube. **Important:** Exercise caution when working with dimethylformamide. Dispense solutions in a vented chemical hood only.
2. Do not filter the stock solution.
3. Store at –20°C protected from light.

Continued on next page

LB (Luria-Bertani) Medium

Composition:

1.0% Tryptone (casein peptone)
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic if needed.
4. Store at room temperature or at 4°C.

LB (Luria-Bertani) Plates

Follow the procedure below to prepare LB agar plates.

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add antibiotic(s) and pour into 10 cm plates.
4. Let harden, then invert and store at 4°C, in the dark. Plates containing antibiotics are stable for up to 4 weeks.

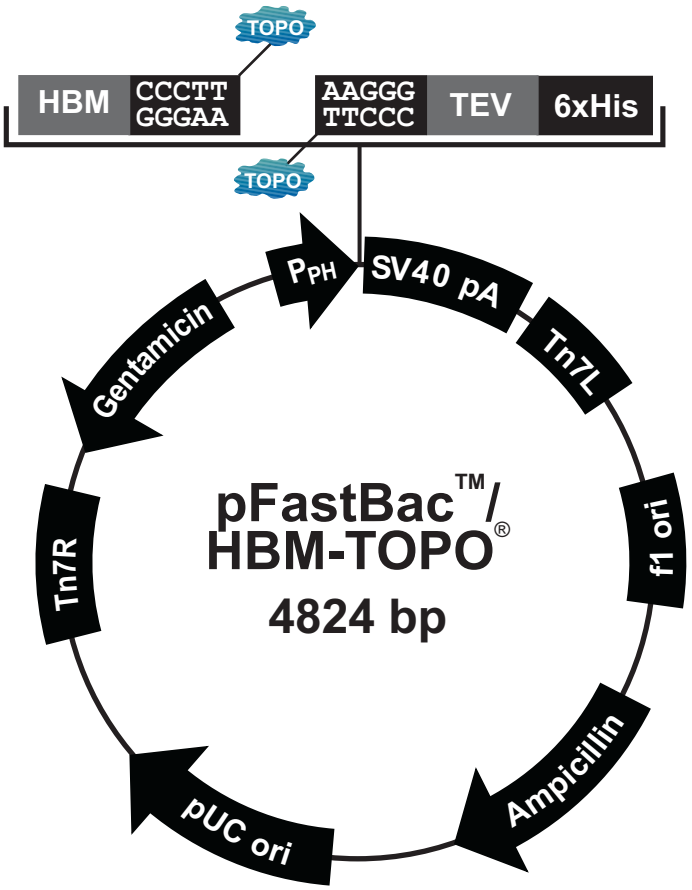
LB agar selective plates for DH10Bac™ transformation

1. Follow Steps 1–2 in the procedure above.
2. After autoclaving, cool to ~55°C, and add the following:
   - 50 µg/mL kanamycin
   - 7 µg/mL gentamicin
   - 10 µg/mL tetracycline
   - 100 µg/mL Bluo-gal
   - 40 µg/mL IPTG
3. Let harden, then invert and store at 4°C, in the dark. Tetracycline and Bluo-gal are light sensitive, so make sure that plates are stored protected from light.

Map of pFastBac™/HBM-TOPO®

Description

The map below shows the elements of pFastBac™/HBM-TOPO® vector. **The vector sequence is available for downloading from www.invitrogen.com or by contacting Technical Support (page 56).**



Comments for pFastBac™/HBM-TOPO® vector 4824 nucleotides

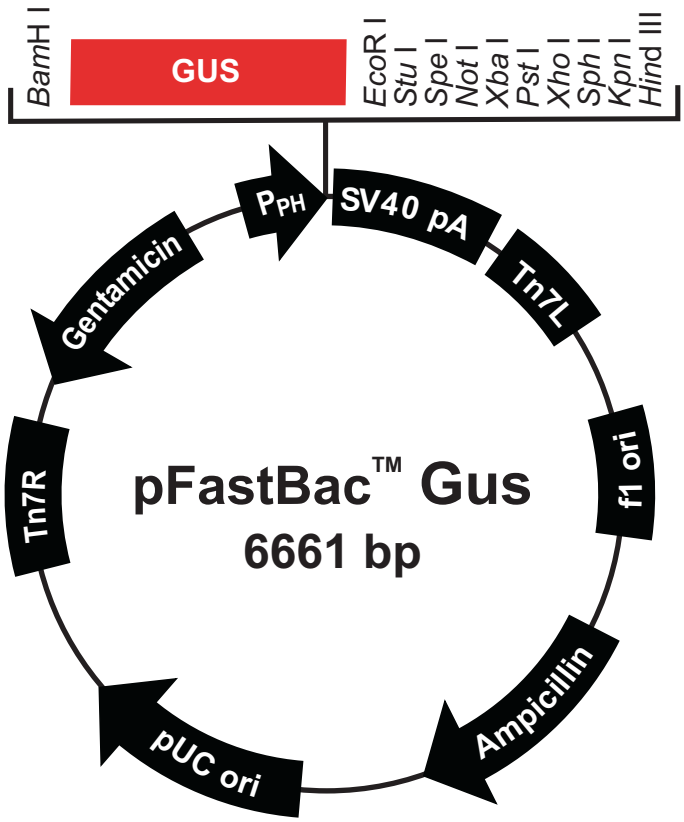
Polyhedrin promoter (P_{PH}): bases 1-129
Honey Bee Mellitin (HBM) secretion signal: 141-210
TOPO cloning site: bases 215-216
TEV recognition site: bases 222-242
6xHis tag: bases 243-260
SV40 polyadenylation signal: bases 305-545
Tn7L: bases 574-739
f1 origin: bases 923-1377
Ampicillin resistance gene: bases 1508-2368
pUC origin: bases 2513-3186
Tn7R: bases 3432-3656
Gentamicin resistance gene: bases 3723-4256 (complementary strand)

Map of pFastBac™ Gus Control Plasmid

Description

pFastBac™ Gus is a 6,661 bp control vector that contains the *Arabidopsis thaliana* gene for β-glucuronidase (Gus) (Kertbundit *et al.*, 1991). The molecular weight of β-glucuronidase is 68.5 kDa.

The map below shows the elements of pFastBac™ Gus control plasmid. **The vector sequence is available for downloading from www.invitrogen.com or by contacting Technical Support (page 56).**



Comments for pFastBac™ Gus vector 6661 nucleotides

f1 origin: bases 2-457
Ampicillin resistance gene: bases 589-1449
pUC origin: bases 1594-2267
Tn7R: bases 2511-2735
Gentamicin resistance gene: bases 2802-3335 (complementary strand)
Polyhedrin promoter (P_{PH}): bases 3904-4032
GUS ORF: bases 4081-5892
SV40 polyadenylation signal: bases 6047-6287
Tn7L: bases 6315-6480

Accessory Products

Additional Products

All of the reagents supplied in the Bac-to-Bac® HBM TOPO® Secreted Expression System and other products suitable for use with the Bac-to-Bac® HBM TOPO® Secreted Expression System are available separately from Invitrogen. Ordering information for these reagents is provided below. For more information, refer to our website at www.invitrogen.com or contact Technical Support (see page 56).

Item	Quantity	Cat. no.
Bac-to-Bac® HBM TOPO® Cloning Kit	1 kit	A11338
MAX Efficiency® DH10Bac™ Competent *E. coli*	5 × 100 µL	10361-012
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	21 × 50 µL	C8620-03
Cellfectin® II Reagent	1 mL	10362-100
Platinum® *Pfx* DNA Polymerase	100 units	11708-013
AccuPrime™ *Pfx* DNA Polymerase	200 reactions	12344-024
Pfx50™ DNA Polymerase	100 reactions	12355-012
Platinum® *Taq* DNA Polymerase High Fidelity	100 reactions	11304-011
PureLink™ PCR Purification Kit	50 preps	K3100-01
PureLink™ Quick Gel Extraction System	1 kit	K2100-12
PureLink™ HiPure Plasmid Miniprep Kit	25 preps 100 preps	K2100-02 K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps 50 preps	K2100-04 K2100-05
PureLink™ HiPure Plasmid Maxiprep Kit	10 preps 25 preps	K2100-06 K2100-07
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
Kanamycin Sulfate	5 g 25 g	11815-024 11815-032
Kanamycin Sulfate (100X), liquid	100 mL	15160-054
Gentamicin Reagent Solution, liquid (50 mg/mL)	10 mL 10 × 10 mL	15750-060 15750-078
Bluo-gal	1 g	15519-028
Isopropylthio-β-galactoside (IPTG)	1 g	15529-019
S.O.C. Medium	10 × 10 mL	15544-034
(Miller's LB Broth Base)® Luria Broth Base, powder	500 g	12795-027
Water, distilled (cell-culture grade)	500 mL	15230-162
4% Agarose gel (optimal for insect cell growth)	40 mL	18300-012
Fetal Bovine Serum (FBS), Qualified, Heat Activated	100 mL	16140-063

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Insect Cell Culture Products

A variety of insect cell lines and GIBCO® cell culture products are available from Invitrogen to facilitate baculovirus-mediated expression of your recombinant protein in insect cells. For more information about the insect cell lines and GIBCO™ cell culture products, refer to www.invitrogen.com or contact Technical Support (see page 56).

Note: Reagents are also available in other sizes.

Item	Quantity	Cat. no.
High Five™ Cells, SFM adapted	3×10^6 cells	B855-02
Sf9 Cells, SFM Adapted	1.5×10^7 cells	11496-015
Sf21 Cells, SFM Adapted	1.5×10^7 cells	11497-013
Mimic™ Sf9 Insect Cells	1×10^7 cells	12552-014
Sf-900 II SFM	500 mL	10902-096
Sf-900™ III SFM	500 mL	12658-019
Sf-900 Medium (1.3X)	100 mL	10967-032
Express Five® SFM	1 liter	10486-025
Grace's Insect Cell Culture Medium, Unsupplemented	500 mL	11595-030
Grace's Insect Cell Culture Medium, Supplemented	500 mL	11605-094
Grace's Insect Cell Culture Medium (2X)	100 mL	11667-037
Penicillin-Streptomycin	100 mL	15070-063
PLURONIC® F-68, 10% (100X)	100 mL	24040-032

PLURONIC® is a registered trademark of BASF Corporation.

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Detecting Recombinant Fusion Protein

If you have cloned your gene of interest in frame with C-terminal polyhistidine tag of the pFastBac™/HBM-TOPO® vector, you may detect expression of your recombinant fusion protein using the following antibodies. The amount of antibody supplied is sufficient for 25 western blots.

Product	Epitope	Cat. no.
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6×His) tag: HHHHHH-COOH (requires the free carboxyl group for detection (Lindner *et al.*, 1997)	R930-25
Anti-His (C-term)-HRP Antibody		R931-25
Anti-His (C-term)-AP Antibody		R932-25
Penta-His™ mouse IgG_1 monoclonal Antibody	Detects both N- and C-terminal polyhistidine (6×His) tag	P21315

Purifying Recombinant Fusion Proteins

If you express your gene of interest as a fusion with the polyhistidine tag from the pFastBac™/HBM-TOPO® vector, you may use ProBond™ or Ni-NTA resins to purify your recombinant fusion protein. See the table below for ordering information.

Item	Quantity	Cat. no.
ProBond™ Nickel-chelating Resin	50 mL 150 mL	R801-01 R801-15
ProBond™ Purification System	6 purifications	K850-01
Ni-NTA Agarose	10 mL 25 mL 100 mL	R901-01 R901-15 R901-10
Ni-NTA Purification System	6 purifications	K950-01
Purification Columns (10 mL polypropylene columns)	50 columns	R640-50
AcTEV™ Protease	1,000 Units 10,000 Units	12575-015 12575-023

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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Tech Fax: 44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

MSDS

Material Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/msds.

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product and is searchable by product lot number, which is printed on each box. CofAs are .available on our website at www.invitrogen.com/support.

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Introduction

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Purchaser Notification, Continued

Information for European Customers Using Mach1™-T1^R Cells

The Mach1™-T1^R *E. coli* strain is genetically modified to carry the *lac*ZΔM15 *hsd*R *lac*X74 *rec*A *end*A *ton*A genotype. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

Information for All Non-U.S. Customers Using Mach1™-T1^R Cells

The parental strain of Mach1™-T1^R *E. coli* is the non-K-12, wild-type W strain (ATCC #9637, S. A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

Information for European Customers Using DH10Bac™ cells

The DH10Bac™ strain is genetically modified and carries the pBR322-derived plasmid, pMON7124 (*bom*$^+$, *tra*$^-$, *mob*$^-$). As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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