invitrogen™

Bac-to-Bac® TOPO® Expression System

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

Catalog nos. A11101, A11100

Version A
15 December 2008
A10606

User Manual

Table of Contents

Kit Contents and Storage

Types of Products

This manual is supplied with the products listed below. For a list of the reagents supplied with each catalog number, see below and the next page.

Product	Quantity	Cat. no.
Bac-to-Bac® C-His TOPO® Expression System	1 kit	A11100
Bac-to-Bac® N-His TOPO® Expression System	1 kit	A11101

Kit Components

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

Component	Cat. no. A11100	Cat. no. A11101
Bac-to-Bac® C-His TOPO® Cloning Kit	✓	
Bac-to-Bac® N-His TOPO® Cloning Kit		✓
One Shot® Mach1-T1^{R} Chemically Competent *E. coli*	✓	✓
MAX Efficiency® DH10Bac™ Competent *E. coli*	✓	✓
Cellfectin® II Reagent	✓	✓
Bac-to-Bac® TOPO® Cloning Kit Manual	✓	✓
Bac-to-Bac® TOPO® Expression System Manual	✓	✓

Shipping/Storage

The Bac-to-Bac® C-His or N-His TOPO® 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® C-His TOPO® or Bac-to-Bac® N-His 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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Kit Contents and Storage, continued

Bac-to-Bac® TOPO® Cloning Kits

The cloning reagents for the Bac-to-Bac® N-His and C-His TOPO® Cloning Kits (Box 1) are listed below. **Store the contents of Box 1 at –20°C.**

Item	Concentration	Amount
pFastBac™/NT-TOPO® vector (Cat. no. A11101) or pFastBac™/CT-TOPO® vector (Cat. no. A11100)	20 µl 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 0.06 M $MgCl_2$	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	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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Kit Contents and Storage, continued

One Shot® Mach1™ T1^R Competent *E. coli*

The following reagents are included in the One Shot® Mach1™ T1^R Chemically Competent *E. coli* kit (Box 2). Transformation efficiency of One Shot® Mach1™ T1^R *E. coli* cells is ≥ 1 × 10^9 cfu/µg DNA. **Store cells at –80°C.**

Reagent	Composition	Amount
One Shot® Mach1™ T1^R 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™ T1^R

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 x 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)**

Accessory Products

Introduction

The products listed in this section may be used with the Bac-to-Bac® C-His and N-His TOPO® Expression Systems. For more information, refer to our website at www.invitrogen.com or contact Technical Support (see page 47).

Additional Products

All of the reagents supplied in the Bac-to-Bac® TOPO® Expression System as well as other products suitable for use with the Bac-to-Bac® TOPO® Expression System are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Cat. no.
Bac-to-Bac® C-His TOPO® Cloning Kit	1 kit	A10636
Bac-to-Bac® N-His TOPO® Cloning Kit	1 kit	A10637
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

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Accessory Products, continued

Additional Products, continued

All of the reagents supplied in the Bac-to-Bac® TOPO® Expression System as well as other products suitable for use with the Bac-to-Bac® TOPO® Expression System are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Cat. no.
Water, distilled (cell-culture grade)	500 ml	15230-162
BaculoTiter™ Assay Kit	30 titers	K1270
4% Agarose gel (optimal for insect cell growth)	40 ml	18300-012
Fetal Bovine Serum (FBS), Qualified, Heat Activated	100 ml	16140-063

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 our website at www.invitrogen.com or contact Technical Support (see page 47).

Note: Reagents are also available in other sizes.

Item	Quantity	Cat. no.
Sf9 Cells, SFM Adapted	1.5×10^7 cells	11496-015
Sf21 Cells, SFM Adapted	1.5×10^7 cells	11497-013
High Five™ Cells	3×10^6 cells	B855-02
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 l	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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Accessory Products, continued

Detecting Recombinant Fusion Protein

If you have cloned your gene of interest in frame with the N- or C- terminal polyhistidine tag, you may detect expression of your recombinant fusion protein using an antibody to the appropriate epitope. 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 IgG1 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™/CT-TOPO® and pFastBac™/NT-TOPO® vectors (supplied with the Bac-to-Bac® C-His and N-His TOPO® Expression Systems, respectively), you may use Invitrogen's 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

Introduction

Overview

Introduction

The Bac-to-Bac® TOPO® Expression System provides a rapid and highly effective method to generate recombinant baculoviruses by combining the ease of **blunt-end** TOPO® cloning with the efficiency of site-specific transposition technology of the Bac-to-Bac® System. The major components of the Bac-to-Bac® Baculovirus Expression System include:

- A choice of pFastBac™/CT-TOPO® or pFastBac™/NT-TOPO® donor plasmids that allow rapid generation of an expression construct containing the gene of interest under the control of a baculovirus-specific strong polyhedrin (P_H) promoter.
- One Shot® Mach1™ $T1^R$ Chemically Competent *E. coli* that enable same-day isolation of recombinant pFastBac™ expression construct from the transformation mix.
- An *E. coli* host strain, DH10Bac™, that contains a baculovirus shuttle vector (bacmid) and a helper plasmid, and allows generation of a recombinant bacmid following transposition of the pFastBac™ expression construct.
- Cellfectin® II Reagent for faster and more efficient transfection of insect cells to generate recombinant baculovirus particles.
- A control expression plasmid containing the Gus gene that allows production of a recombinant baculovirus which, when used to infect insect cells, expresses the *Arabidopsis thaliana* β-glucuronidase.

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

Using the Bac-to-Bac® TOPO® 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 as compared to the 4–6 weeks required to generate a recombinant baculovirus using homologous recombination
- Reduces the need for multiple rounds of plaque purification as 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, and is suited for the expression of protein variants for structure/function studies

Continued on next page

Overview, continued

Purpose of This Manual

This manual provides an overview of the Bac-to-Bac® TOPO® 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™ TOPO® vector of choice (pFastBac™/CT-TOPO® or pFastBac™/NT-TOPO®) are provided in the Bac-to-Bac® TOPO® Cloning Kit manual (part no. A10605) supplied with the Bac-to-Bac® TOPO® Expression System.

The Bac-to-Bac® TOPO® Cloning Kit manual is also available on our website at www.invitrogen.com or from Technical Support (see page 47).

Important

The Bac-to-Bac® TOPO® Expression System is designed to help you create a recombinant baculovirus for high-level 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 http://tools.invitrogen.com/content/sfs/manuals/bevtest.pdf or by contacting Technical Support (see page 47).

The Bac-to-Bac® TOPO® Expression System

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

The Bac-to-Bac® TOPO® 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® TOPO® 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 major components of the system are described below.

pFastBac™ TOPO® vector

The first major component of the System is a pFastBac™ TOPO® vector into which your gene of interest will be cloned.

Once you amplify your gene of interest using a proofreading polymerase and clone it into the pFastBac™ TOPO® vector as a blunt-end PCR product, and transform One Shot® Mach1™ $T1^R$ Chemically Competent *E. coli*. You will 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, and also contains a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini Tn7.

DH10Bac™ *E. coli*

The second major component of the System is the DH10Bac™ *E. coli* strain that is used as the host for your pFastBac™ 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).

Once the pFastBac™ expression plasmid (the "donor plasmid") is transformed into DH10Bac™ cells, transposition occurs between the mini-Tn7 element on the pFastBac™ 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.

Once you have performed the transposition reaction, you will 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® TOPO® Expression System, see the diagram on page 5.

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The Bac-to-Bac® TOPO® Expression System, continued

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 and 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

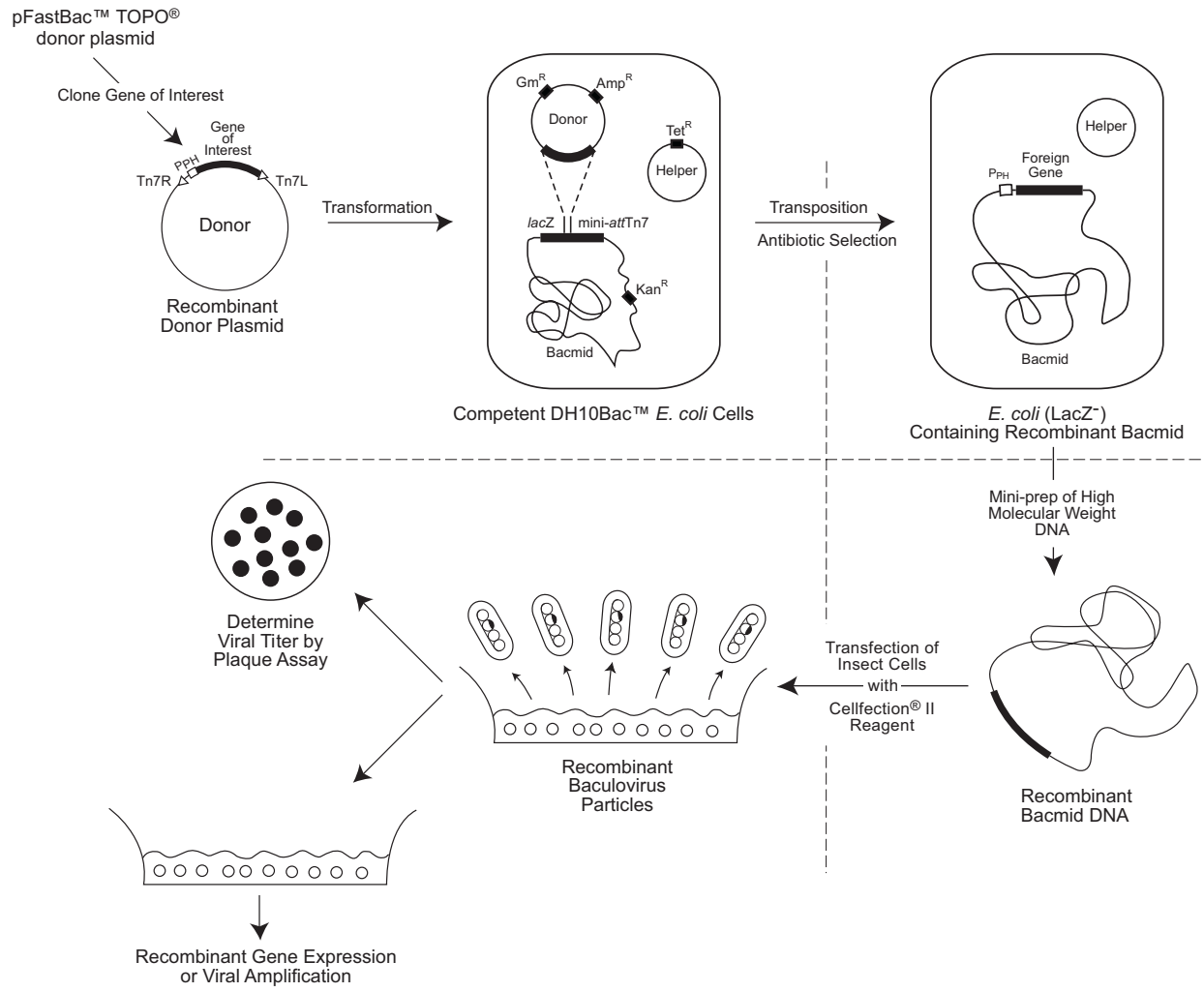
DH10Bac™ *E. coli* also contain the helper plasmid, pMON7124 (13.2 kb), which encodes the transposase and confers resistance to tetracycline. The helper plasmid provides the Tn7 transposition function *in trans* (Barry, 1988).

Continued on next page

Experimental Outline

Diagram of the Bac-to-Bac® System

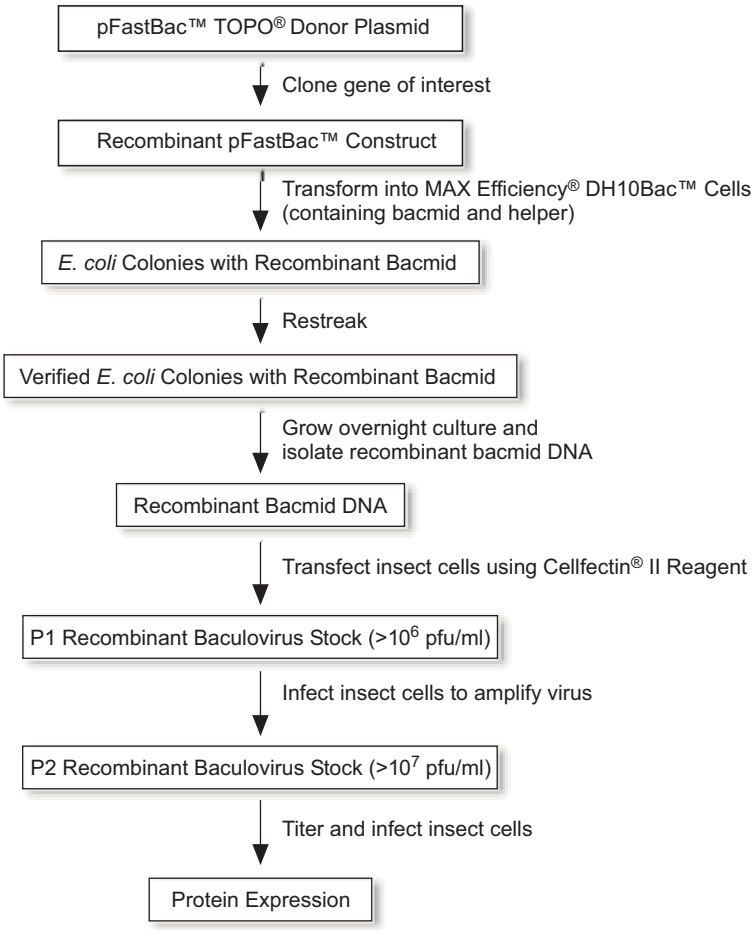
The figure below depicts the generation of recombinant baculovirus and the expression of your gene of interest using the Bac-to-Bac® TOPO® Expression System.



Experimental Outline, continued

Flow Chart

The figure below illustrates the general steps required to express your gene of interest using the Bac-to-Bac® TOPO® 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. Before you start your transfection and expression experiments, be sure to have cultures of Sf9 or Sf21 cells growing and have frozen master stocks available. Sf9 and Sf21 cells and cell culture reagents are available separately from Invitrogen (see page ix 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 ix). Both Sf-900 II SFM and Sf-900™ III SFM are protein-free media optimized for the growth and maintenance of Sf9 and Sf21 cells, as well as for the large-scale production of recombinant proteins expressed using the Bac-to-Bac® TOPO® Expression System. For more information, refer to www.invitrogen.com or contact Technical Support (see page 47).

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 from our website at http://tools.invitrogen.com/content/sfs/manuals/bevtest.pdf or by contacting Technical Support (see page 47), and 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, continued

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 Forces:** 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 will need log-phase Sf9 or Sf21 cells with >95% viability to perform a successful transfection. Refer to page 20 to determine how many cells you will need for transfection.

Generating the Recombinant pFastBac™ Vector

Introduction

To generate a recombinant plasmid containing your gene of interest for use in the Bac-to-Bac® TOPO® Expression System, you will 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™/CT-TOPO® or pFastBac™/NT-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, 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 47).

Important

When generating the recombinant plasmid containing your gene of interest for use in the Bac-to-Bac® TOPO® 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, transformation of One Shot® Mach1™ T1^R Chemically Competent *E. coli*, and analysis of transformants in the Bac-to-Bac® TOPO® Cloning Kit manual before beginning.

pFastBac™ TOPO® vectors

For the features and vector maps of pFastBac™/CT-TOPO® and pFastBac™/NT-TOPO®, refer to the Bac-to-Bac® TOPO® Cloning Kit manual (part no. A10605) supplied with this kit, and also available on our website (www.invitrogen.com) or by contacting Technical Support (see page 47).

Generating the Recombinant Bacmid

Transforming DH10Bac™ *E. coli*

Introduction

Once you have generated your pFastBac™ construct containing your gene of interest in the correct orientation, you are ready to transform purified plasmid DNA into DH10Bac™ *E. coli* for transposition into the bacmid. You will use blue/white selection to identify colonies containing the recombinant bacmid.

MAX Efficiency® DH10Bac™ chemically competent cells are supplied with the Bac-to-Bac® TOPO® Expression System, but are also available separately from Invitrogen (see page viii). Guidelines and instructions to transform DH10Bac™ cells are provided in this section.

Positive Control

Both the pFastBac™/CT-TOPO® and the pFastBac™/NT-TOPO® vectors are 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 and a description of the features of the control plasmid, refer to the Bac-to-Bac® TOPO® Cloning Kit manual (part no. A10605) supplied with this kit, also available for downloading at www.invitrogen.com or by contacting Technical Support (see page 47).

Materials Needed

- Your purified pFastBac™ construct (200 pg/µl in TE, pH 8.0)
- 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® TOPO® 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 recommendation below)
- LB agar plate containing 100 µg/ml ampicillin (for plating pUC19 transformation control)
- S.O.C. Medium (see page ix for ordering information)
- 15 ml round-bottom polypropylene tubes
- 42°C water bath
- 37°C shaking and non-shaking incubator

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Transforming DH10Bac™ *E. coli*, continued

You will need to 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 TE Buffer, pH 8.0. See page viii to order antibiotics, Bluo-gal, and IPTG, and page 43 for instructions to prepare plates.

If you are preparing LB plates using a pre-mixed formulation, we recommend using Luria Broth Base (see page ix) instead of Lennox L (LB). Using Lennox L plates will reduce the color intensity and may reduce 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.

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.
- Pre-chill one 15 ml round-bottom polypropylene tube for each transformation.

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Transforming DH10Bac™ *E. coli*, continued

Transformation Procedure

Follow the procedure below to transform MAX Efficiency® DH10Bac™ chemically competent *E. coli* cells with your recombinant pFastBac™ construct. We recommend including the positive controls for transposition (*i.e.*, pFastBac™ Gus expression plasmid) 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, gently mix and transfer 100 µl of the DH10Bac™ cells into a pre-chilled, 15 ml round-bottom polypropylene tube.
3. Add the appropriate amount of plasmid DNA to the cells and mix gently. **Do not pipet up and down to mix.**
   - Your recombinant pFastBac™ construct: 1 ng (5 µl)
   - pFastBac™ Gus control plasmid: 1 ng
   - pUC19 control: 50 pg (5 µl)
4. Incubate cells on ice for 30 minutes.
5. Heat-shock the cells for 45 seconds at 42°C without shaking.
6. Immediately transfer the tubes to ice and chill for 2 minutes.
7. Add 900 µl of room temperature S.O.C. Medium.
8. **For pFastBac™ transformations:** Shake tubes at 37°C at 225 rpm for **4** hours.
   For pUC19 transformation: Shake tube at 37°C at 225 rpm for 1 hour.
9. **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.
10. Incubate 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 as it may be difficult to distinguish between white and blue colonies.

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; therefore, to avoid selecting false positives, choose the largest, most isolated white colonies. Avoid picking colonies that appear gray or are darker in the center as they can contain a mixture of cells with empty bacmid and recombinant bacmid.

Continued on next page

Transforming DH10Bac™ *E. coli*, continued

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 using the procedure provided on the next page for analysis. You may also use the procedure for the PureLink™ HiPure Plasmid Maxiprep Kit provided in the **Appendix**, page 44, for increased recombinant bacmid yield.
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 16).

   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 as 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 as contaminants may kill the insect cells, and salt will interfere with lipid complexing, decreasing the transfection efficiency.

The PureLink™ HiPure Plasmid Prep Kits available separately from Invitrogen are ideally suited for bacmid purification (see page viii 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 viii 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 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. Pipette 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 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

Isolating Recombinant Bacmid DNA, continued

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.
3. **The elution tube contains the purified DNA.** Discard the column.
4. Add 0.63 ml isopropanol to the elution tube. Mix and place on ice for 10 minutes.
5. Centrifuge the mixture at >15,000 × g at 4°C for 20 minutes. Carefully remove and discard the supernatant.
6. Resuspend the DNA pellet in 1 ml 70% ethanol.
7. Centrifuge at >15,000 × g at 4°C for 5 minutes. Carefully remove and discard the supernatant.
8. Air-dry the pellet for 10 minutes.
9. Resuspend the DNA pellet in 40 µl TE Buffer (TE). Allow pellet to dissolve for at least 10 minutes on ice. To avoid shearing the DNA, pipette only 1–2 times to resuspend.
10. Store the bacmid DNA at **4°C**.

Important

You may store your bacmid DNA at –20°C if you avoid frequent freeze/thaw cycles as it decreases the transfection efficiency. To store your purified bacmid DNA at –20°C, aliquot into separate tubes in TE Buffer, pH 8.0 to avoid more than one freeze/thaw cycle and do **not** store 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 at –80°C for future bacmid DNA isolation.

Note

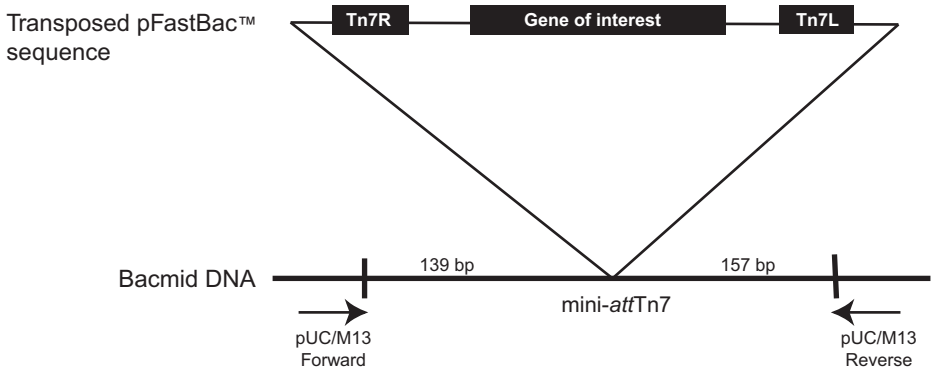
You may also use the procedure for PureLink™ HiPure Plasmid Maxiprep Kit provided in the **Appendix**, page 44, for increased recombinant bacmid yield.

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, and are ideally suited for bacmid purification (see page viii for ordering information).

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 within the *lac*Zα-complementation region to facilitate PCR analysis (see figure below). Guidelines and instructions are provided in this section to perform 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 viii for ordering information.

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Analyzing Recombinant Bacmid DNA by PCR, continued

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 primer and a primer specific for your gene, you will need to 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 µl

2. Overlay with 50 µl (1 drop) of mineral or silicone oil, if necessary.
3. 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

4. Remove 5–10 µl from the reaction and analyze by agarose gel electrophoresis.

Continued on next page

Analyzing Recombinant Bacmid DNA by PCR, continued

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	~350 bp
pFastBac™/CT-TOPO®	~2420 bp + size of your insert
pFastBac™/NT-TOPO®	~2440 bp + size of your insert
pFastBac™ Gus	~4220 bp

If you have used a combination of the pUC/M13 Forward or Reverse primer and a gene-specific primer for amplification, you will need to determine the expected size of your PCR product. Refer to the diagram on page 16 to help you calculate the expected size of your PCR product.

Producing Recombinant Baculovirus

Transfecting Insect Cells

Introduction

Once you have confirmed that your recombinant bacmid contains the gene of interest, you are ready to transfect insect cells to produce recombinant baculovirus. Guidelines and instructions to transfect insect cells are provided in this section.

Plasmid Preparation

You may use any method to prepare purified recombinant bacmid DNA for transfection. Bacmid DNA must be clean and free from phenol and sodium chloride as contaminants may kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating bacmid DNA using the PureLink™ HiPure Plasmid Miniprep Kit (available separately from Invitrogen; see page viii for ordering information) following the procedure provided on page 14.

Transfection Method

We recommend using a cationic lipid such as Cellfectin® II Reagent for transfection. Cellfectin® II Reagent is supplied with the Bac-to-Bac® TOPO® Expression System and is also available separately from Invitrogen. See page viii for ordering information.

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.

Insect Cell Lines

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, you may use High Five™ or Mimic™ Sf9 cells for expression studies.

Media for Transfection

For the highest transfection efficiency, we recommend performing the transfection in Grace's Insect Cell Culture Medium, Unsupplemented (see page ix for ordering information). Note that the Grace's Insect Cell Culture Medium **should not** contain supplements or fetal bovine serum (FBS) as the supplements and the proteins in the FBS will interfere 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.

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Transfecting Insect Cells, continued

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 (Gus) will be expressed under the control of the strong polyhedrin (P_H) promoter. After transfection, expression of β-glucuronidase may be assayed as appropriate.

Materials Needed

- Purified recombinant bacmid DNA from your pFastBac™ 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 ix), **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 will 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 1.5–2.5 × 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. Note that these conditions should be used as a starting point for your transfection. To obtain the highest transfection efficiency and low non-specific effects, you may 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.

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Transfecting Insect Cells, continued

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 as this causes cell death.

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. If you wish to transfect cells in other tissue culture formats, you will need to determine the optimal conditions to use.

1. Verify that the Sf9 or Sf21 cells are in the log phase ($1.5–2.5 \times 10^6$ cells/ml) with greater than 95% viability.
2. If the cell density is in range of $1.5–2.5 \times 10^6$ cells/ml and the culture is without antibiotics, proceed to step 2a. If the cell density is **not** in this range or the cell culture contains antibiotics, follow steps 2b–2c:
   a. Add 2 ml of Grace's Insect Medium, Unsupplemented (without antibiotics and serum) in each well. 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.** Allow cells to attach for 15 minutes at room temperature in the hood. Proceed to step 3.
   b. Prepare 10ml 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).
   c. Plate 8×10^5 Sf9 or Sf21 cells from Step 1 per well. Allow cells to attach for 15 minutes at room temperature in the hood. Remove the medium. Add 2.5 ml plating medium from step 2b 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 µl 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 µl 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 with 2 ml of complete growth medium (*e.g.*, Grace's Insect Medium, Supplemented and 10% FBS). Using antibiotics is optional.
6. Incubate 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, you should visually inspect the cells daily for signs of infection (see below). Once the cells appear infected (*i.e.*, demonstrate characteristics typical of late to very late infection), harvest the virus from the cell culture medium using the procedure 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. Once 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: If you wish to concentrate your viral stock to obtain a higher titer, you may 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 stocks as follows:

- 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 **Error! Bookmark not defined.**).
- Plaque purify your recombinant baculovirus, if desired (see **Performing a Viral Plaque Assay**, page 26).
- Use the P1 viral stock to infect your Sf9 or Sf21 cells for preliminary expression experiments (see below).

Note

If you wish 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 Sf9 or Sf21 cells. Note that the amount of viral stock is limited and expression conditions may not be reproducible (*i.e.*, MOI is unknown if titer is not determined).

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. Amplification allows production of a P2 viral stock with a titer ranging from 1×10^7 to 1×10^8 pfu/ml and is generally recommended. Guidelines and protocols are provided in this section to amplify 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 will 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

We wish to infect a 10 ml culture at 2×10^6 cells/ml using an MOI = 0.1. We assume that the titer of our P1 viral stock is 5×10^6 pfu/ml.

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

$$\text{Inoculum required (ml)} = 0.4\ \text{ml}$$

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Amplifying Your Baculoviral Stock, continued

Important considerations

For successful amplification of your baculovirus, you should 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 as higher MOI will reduce baculovirus quality
- Harvest the virus when 70–80% of cells are dead
- You **cannot** amplify the baculovirus indefinitely, as they acquire 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 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). Optimal harvest times can vary and should be determined for each baculoviral construct. Remember that culture viability will decrease 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 23 for storage guidelines.
7. Proceed to the next section to determine the titer of your P2 viral stock.

Scaling Up the Amplification Procedure

Once 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 will 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

BaculoTiter™ Assay Kit

We recommend using the BaculoTiter™ Assay Kit, available separately from Invitrogen, to determine the titer of your baculoviral stock. The BaculoTiter™ Assay Kit rapidly determines the titer of an unknown baculovirus sample with minimal handling steps, providing both accuracy and convenience in an easy-to-use kit format in two days as opposed to ten days with the serial dilution assays. See page ix for ordering information. For more information, refer to our website at www.invitrogen.com or contact Technical Support (page 47).

Alternatively, you may follow the guidelines and instructions provided below to:

- Determine the titer of your baculoviral stock
- Plaque purify the virus (optional)

Experimental Outline

To determine the titer of a baculoviral stock, you will:

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 will 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 19–21 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.
- **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.

Continued on next page

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 ix 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 ix for ordering information.

Continued on next page

Performing a Viral Plaque Assay, continued

Preparing the Plaquing Medium

Plaquing medium consists of a mixture of culture medium and agarose, and will be 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. Once 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.

Continued on next page

Performing a Viral Plaque Assay, continued

Plaque Assay Procedure

Use the procedure below to perform a plaque assay in 6-well plate format to determine the titer of your pFastBac™ baculoviral stock. If you have generated a baculoviral stock of the pFastBac™ 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). If you wish 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 will 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. To do this, sequentially dilute 0.5 ml of the baculoviral stock or previous dilution in 4.5 ml of medium in 12 ml disposable tubes. You should finish 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}). You will 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, and immediately replace with 1 ml of the appropriate virus dilution. As a negative control, add the appropriate medium without virus.
7. Incubate cells with virus for 1 hour at room temperature.
8. Following the 1 hour incubation, 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 with 2 ml of plaquing medium. Work quickly to avoid desiccation of the cell monolayer.
10. Allow 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. If you wish to stain plaques to facilitate counting, see the next page. To calculate the titer, see page 31.

Continued on next page

Performing a Viral Plaque Assay, continued

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 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, you should **not** stain plaques as 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 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 will 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 pipet or blotter and count the plaques. Plaques will appear as clear spots in a nearly clear gel against a red background.

Continued on next page

Performing a Viral Plaque Assay, continued

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.
ml of inoculum/well

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

Example

In this example, we add 1 ml of inoculum and observe 20 plaques in the well containing the 10^{-6} viral dilution. Using the formula above, the titer of this 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™ 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 26, and the **Troubleshooting** section, page 40.

Continued on next page

Performing a Viral Plaque Assay, continued

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 29; **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 29, 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 24.

Expressing Your Recombinant Protein

Introduction

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

Positive Control

If you have generated a high-titer viral stock from the pFastBac™ Gus control plasmid, you may want to include this recombinant baculovirus in your experiments for use as an expression control. Once you have infected cells with the FastBac™ Gus control virus, the gene encoding β-glucuronidase (Gus) will be constitutively expressed and can be easily assayed (see page 35).

Guidelines for Expression

General guidelines are provided below to infect insect cells with the recombinant baculovirus to express your protein of interest.

- **Cell line:** Depending on your application and gene of interest, you may use any insect cell line including Sf9, Sf21, High Five™, or Mimic™ Sf9 for expression. You may grow your cells either in adherent or suspension culture using your culture vessel of choice.

  Note: If you are expressing a secreted protein, you may improve expression by using High Five™ cells.

- **Culture Conditions:** We generally culture cells in serum-free conditions using Sf-900 II SFM, Sf-900™ III SFM, or Express Five® SFM (see page ix for ordering information) as appropriate. 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.
- **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.

  Note: Maximum expression of secreted proteins is generally observed between 30 and 72 hours and non-secreted proteins between 48 and 96 hours post-infection.

Continued on next page

Expressing Your Recombinant Protein, continued

Optimizing Expression

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. You may follow the following guidelines to determine the optimal conditions to use for expressing your recombinant protein of interest:

- **Cell line:** Infect Sf9, Sf21, High Five™, or Mimic™ Sf9 cells at a constant MOI. Assay for recombinant protein expression at different times post-infection (*e.g.*, 24, 48, 72, 96 hours post-infection). Choose the cell line that provides the optimal level of recombinant protein expression.
- **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.
- **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). Choose the time point at which the recombinant protein expression is optimal.

Harvesting Baculovirus Infected Insect Cells

Use the following procedure for harvesting recombinant baculovirus infected insect cells to analyze expression of your recombinant protein of interest. This procedure is adapted from Luckow and Summers and is designed to allow expression analysis in a 24-well format from cells harvested 24 to 96 hours post-infection. Other protocols are also suitable.

1. Seed 6×10^5 Sf9 or Sf21 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 µl of fresh media.
3. Add the pFastBac™ baculoviral stock to each well at the desired MOI. Include the appropriate controls (*e.g.*, mock-infected (uninfected) cells, pFastBac™ positive control baculovirus, previously characterized recombinant baculoviruses).
4. Incubate cells in a 27ºC humidified incubator.
5. Harvest cells (or media, if the recombinant protein is secreted) at the appropriate time (*i.e.*, 24, 48, 72, 96 hours post-infection). If harvesting cells, remove the media and rinse the cells once with serum-free medium.
6. Lyse the cells with 400 µl of 1X SDS-PAGE Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS).
7. Freeze samples at –20ºC or boil samples for at least 3 minutes and separate proteins by SDS-PAGE.

Analyzing Recombinant Protein

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™/CT-TOPO® and pFastBac™/NT-TOPO® vectors (included in the Bac-to-Bac® C-His and N-His TOPO® Expression Systems, respectively) allow the expression of your recombinant protein of interest as a 6×His fusion. You can use the antibodies listed below (available separately from Invitrogen) to detect your recombinant protein. See page x for ordering information.

For use with	Product	Epitope
pFastBac™/CT-TOPO®	Anti-His (C-term) Antibody Anti-His (C-term)-HRP Antibody Anti-His (C-term)-AP Antibody	Detects the C-terminal polyhistidine (6×His) tag: HHHHHH-COOH (requires the free carboxyl group for detection)
pFastBac™/CT-TOPO®, pFastBac™/NT-TOPO®	Penta-His™ mouse IgG1 monoclonal Antibody	Detects both C- and N-terminal polyhistidine (6×His) tag

Note

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

Assay for β-glucuronidase

If you include the baculoviral control created using the pFastBac™ Gus control construct in your expression experiment, you may assay for β-glucuronidase expression using the following methods. Other methods are also suitable.

- Identify blue plaques on agarose plates containing the chromogenic indicator, X-glucuronide.
- To assess β-glucuronidase expression in a rapid but qualitative manner, mix a small amount of media from the infected cells with X-glucuronide and observe development of blue color. Briefly, mix 5 µl of a 20 mg/ml X-glucuronide solution (in DMSO or dimethylformamide) with 50 µl of cell-free medium. Monitor for development of blue color within 2 hours.

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Analyzing Recombinant Protein, continued

Purifying Recombinant Protein

The presence of the C- and N-terminal 6×His tag in the pFastBac™/CT-TOPO® and pFastBac™/NT-TOPO® vectors, respectively, allows the purification of your recombinant protein with a metal-chelating resin such as ProBond™ and Ni-NTA available from Invitrogen (see page x 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 (see page 47).

Removing the 6×His Tag Using AcTEV™ Protease

pFastBac™/CT-TOPO® and pFastBac™/NT-TOPO® vectors contain 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 viii 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 47).

Troubleshooting

Cloning into the pFastBac™ TOPO® Vectors

For troubleshooting any problems you may encounter when generating your pFastBac™ 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 on our website (www.invitrogen.com) or by contacting Technical Support (see page 47).

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*. Possible solutions that may help you troubleshoot the transposition reaction are provided.

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.

Continued on next page

Troubleshooting, continued

Generating Recombinant Bacmid DNA, continued

Problem	Reason	Solution
All colonies are blue	DNA from your pFastBac™ 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.

Continued on next page

Troubleshooting, continued

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.

Continued on next page

Troubleshooting, continued

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 viii for ordering information). • Store purified bacmid at 4°C; **do not** freeze at –80°C as it 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

Troubleshooting, continued

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 by PCR analysis of bacmid DNA 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 cells in Sf-900 II SFM or Sf-900™ III SFM for optimal cell growth and protein expression.
	Cell line not optimal	Try other insect cell lines.
	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

Recipes, continued

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.

Bacmid DNA Isolation Using PureLink™ HiPure Maxiprep Kit

Introduction

After you have transformed your pFastBac™ TOPO® construct containing your gene of interest into DH10Bac™ *E. coli* and performed the transposition reaction, use the PureLink™ HiPure Plasmid Maxiprep Kit to purify recombinant bacmid DNA from DH10Bac™ transformants (see page viii for ordering information). Bacmid DNA purified by this method is suitable for use in PCR analysis or 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.

For more information on PureLink™ HiPure purification products, visit our website at www.invitrogen.com or contact Technical Support (see page 47).

Growing bacmid DNA stock

Growing bacmid DNA stock from DH10Bac™ transformants in LB medium requires three days.

Day 1:

- Pick a single white bacterial colony from among the DH10Bac™ transformants (see page 12) and inoculate 4 ml of LB medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin and 10 µg/ml tetracycline. Alternatively, you can thaw glycerol stocks of DH10Bac™ cells harboring your verified recombinant bacmid and use 100 µl to inoculation.
- Incubate the culture at 37°C in a shaking water bath at 250 rpm overnight.

Day 2:

- Transfer the entire 4 ml of overnight culture into 50 ml of fresh LB medium with antibiotics (as above) and incubate at 37°C in a shaking water bath at 250 rpm overnight.

Day 3:

- Transfer the entire 50 ml of overnight culture into 500 ml of fresh LB medium with antibiotics (as above) and incubate at 37°C in a shaking water bath at 250 rpm overnight.

On Day 4, proceed with the PureLink™ HiPure bacmid DNA isolation procedure as described on the next page.

Continued on next page

PureLink™ HiPure Bacmid DNA Isolation, continued

Before Starting

Before beginning, verify that RNase A has been added to the Resuspension Buffer (R3) and that no precipitate has formed in the Lysis Buffer (L7).

Equilibrating the Column

Place the PureLink™ HiPure Maxi column on the PureLink™ Nucleic Acid Purification Rack (see the manual supplied with the rack for more details). Apply 30 ml Equilibration Buffer (EQ1) to the column. Allow the solution in the column to drain by gravity flow. Proceed to **Preparing the Cell Lysate** (next page) while the column is equilibrating.

Preparing the Cell Lysate

1. Harvest 250-500 ml of the overnight culture by centrifuging at 4,000 × g for 10 minutes in a bucket. Remove all medium.
2. Add 20 ml Resuspension Buffer (R3) with RNase A to the pellet and resuspend the cells until homogeneous. Transfer cell suspension to a 50-ml centrifuge tube.
3. Add 20 ml Lysis Buffer (L7). Mix gently by inverting the capped tube five times. **Do not vortex.** Incubate at room temperature for 5 minutes.
   Note: Do not allow lysis to proceed for more than 5 minutes.
4. Add 20 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 >12,000 × 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. Pipette the clear lysate into another 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 from Step 5 (see above) onto the equilibrated column. Allow the solution in the column to drain by gravity flow.
2. Wash the column with 60 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

PureLink™ HiPure Bacmid DNA Isolation, continued

Eluting and Precipitating the DNA

1. Place a sterile 30 ml centrifuge tube (elution tube) under the column.
2. Add 15 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 10.5 ml isopropanol to the elution tube. Mix well.
4. Centrifuge the mixture at >15,000 × g at 4°C for 30 minutes. Carefully remove and discard the supernatant.
5. Add 1 ml 70% ethanol to the pellet in the 30 ml elution tube, displace the pellet from the side of the tube, and transfer all the pellet fragments into a 1.5 ml microcentrifuge tube.
6. Centrifuge at >15,000 × g at 4°C for 10 minutes. Carefully remove and discard the supernatant.
7. Add another 1 ml fresh 70% ethanol to the pellet in the microcentrifuge tube, and centrifuge at >15,000 × g at 4°C for another 10 minutes (second wash). Carefully remove and discard the supernatant.
8. Air-dry the pellet at room temperature until the appearance of the pellet changes from white-opaque to translucent and crystalline.
9. Resuspend the DNA pellet in 200–500 µl TE Buffer, pH 8.0 by vortexing.
10. Measure the concentration of the purified bacmid DNA. The concentration should be in range of 150–300 ng/ml
11. Store the tube at **4°C**.

Important

You may store your bacmid DNA at –20°C if you avoid frequent freeze/thaw cycles as it decreases the transfection efficiency.

To store your purified bacmid DNA at –20°C, aliquot into separate tubes in TE Buffer, pH 8.0 to avoid more than one freeze/thaw cycle and **do not** store 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 at –80°C for future bacmid DNA isolation.

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

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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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