

**Bac-to-Bac[®] Baculovirus
Expression System**

Version D

6 April 2004

10359

**Bac-to-Bac[®] Baculovirus Expression
System**

**An efficient site-specific transposition system to
generate baculovirus for high-level expression of
recombinant proteins**

Catalog nos. 10359-016, 10360-014, 10584-027, 10712-024



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Table of Contents

Kit Contents and Storage	v
Accessory Products	vii
Introduction	1
Overview.....	1
The Bac-to-Bac® Baculovirus Expression System	3
Experimental Outline	6
Culturing Insect Cells.....	7
General Guidelines	7
Generating the Recombinant pFastBac™ Vector.....	9
General Information.....	9
Cloning into pFastBac™1.....	10
Cloning into pFastBac™HT A, B, and C	11
Cloning into pFastBac™ Dual	15
Transformation and Analysis.....	17
Generating the Recombinant Bacmid.....	19
Transforming DH10Bac™ <i>E. coli</i>	19
Analyzing Recombinant Bacmid DNA by PCR	23
Producing Recombinant Baculovirus.....	26
Transfecting Insect Cells.....	26
Isolating P1 Viral Stock.....	29
Amplifying Your Baculoviral Stock.....	31
Performing a Viral Plaque Assay	33
Expressing Your Recombinant Protein.....	40
Troubleshooting	43
Appendix	49
Recipes.....	49
Isolating Recombinant Bacmid DNA.....	51
Map and Features of pFastBac™1.....	53
Map and Features of pFastBac™HT	55
Map and Features of pFastBac™ Dual	57
Map of pFastBac™1-Gus.....	59
Map of pFastBac™HT-CAT.....	60
Map of pFastBac™ Dual-Gus/CAT.....	61
Technical Service.....	62
Purchaser Notification	64
Product Qualification	66
References.....	69

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	Catalog no.
Bac-to-Bac [®] Baculovirus Expression System	1 kit	10359-016
Bac-to-Bac [®] Vector Kit	1 kit	10360-014
Bac-to-Bac [®] HT Vector Kit	1 kit	10584-027
pFastBac [™] Dual Vector Kit	1 kit	10712-024

Kit Components Each catalog number contains the components listed below. **Important:** Note that catalog numbers 10360-014, 10584-027, and 10712-024 contain pFastBac[™] vectors **only**. See the next page for a detailed description about the specific pFastBac[™] vector and other reagents supplied with each catalog number.

Component	Catalog no. 10359-016	Catalog no. 10360-014	Catalog no. 10584-027	Catalog no. 10712-024
pFastBac [™] Vectors	✓	✓	✓	✓
MAX Efficiency [®] DH10Bac [™] Competent <i>E. coli</i>	✓			
Cellfectin [®] Reagent	✓			

Shipping/Storage The Bac-to-Bac[®] Baculovirus Expression System is shipped in three 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	pFastBac [™] Vectors	Blue ice	+4°C
2	MAX Efficiency [®] DH10Bac [™] Competent <i>E. coli</i>	Dry ice	-80°C
3	Cellfectin [®] Reagent	Blue ice	+4°C

Catalog nos. 10360-014, 10584-027, and 10712-024 are shipped on blue ice. Upon receipt, store the vectors at +4°C.

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Kit Contents and Storage, continued

pFastBac™ Vectors

Each catalog number includes a specific pFastBac™ vector(s) and a corresponding expression control, and are supplied as detailed below. **Store at +4°C.**

Product	Catalog no.	pFastBac™ Vector	Expression Control
Bac-to-Bac® Baculovirus Expression System	10359-016	pFastBac™ 1 Supplied: 20 µl at 0.5 µg/µl in TE, pH 8.0 (10 µg total)	pFastBac™ 1-Gus Supplied: 20 µl at 0.2 ng/µl in TE, pH 8.0 (4 ng total)
Bac-to-Bac® Vector Kit	10360-014	pFastBac™ 1 Supplied: 20 µl at 0.5 µg/µl in TE, pH 8.0 (10 µg total)	pFastBac™ 1-Gus Supplied: 20 µl at 0.2 ng/µl in TE, pH 8.0 (4 ng total)
Bac-to-Bac® HT Vector Kit	10584-027	pFastBac™ HT A pFastBac™ HT B pFastBac™ HT C Supplied: 20 µl each at 0.5 µg/µl in TE, pH 8.0 (10 µg total of each vector)	pFastBac™ HT-CAT Supplied: 15 µl at 1 ng/µl in TE, pH 8.0 (15 ng total)
pFastBac™ Dual	10712-024	pFastBac™ Dual Supplied: 20 µl at 0.5 µg/µl in TE, pH 8.0 (10 µg total)	pFastBac™ Dual-Gus/CAT Supplied: 20 µl at 0.2 ng/µl in TE, pH 8.0 (4 ng total)

MAX Efficiency® DH10Bac™ Competent *E. coli* Reagents

MAX Efficiency® DH10Bac™ Chemically Competent *E. coli* are supplied with the Bac-to-Bac® Baculovirus Expression System **only**, and include the following items. Transformation efficiency is 1×10^8 cfu/µg DNA. **Store at -80°C.**

Item	Composition	Amount
MAX Efficiency® Chemically Competent DH10Bac™	--	5 x 100 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	100 µl

Genotype of DH10Bac™

F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*Δ*M15* Δ*lacX74* *recA1* *endA1* *araD139* Δ(*ara, leu*)7697 *galU* *galK* λ- *rpsL* *nupG*/bMON14272/pMON7124

Cellfectin® Transfection Reagent

Cellfectin® Reagent is supplied with the Bac-to-Bac® Baculovirus Expression System **only**.

Amount supplied: 1 ml

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

Storage conditions: +4°C

Accessory Products

Introduction

The products listed in this section may be used with the Bac-to-Bac[®] Baculovirus Expression System. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 62).

Additional Products

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

Item	Quantity	Catalog no.
Bac-to-Bac [®] Vector Kit	1 kit	10360-014
Bac-to-Bac [®] HT Vector Kit	1 kit	10584-027
pFastBac [™] Dual Vector Kit	1 kit	10712-024
Gateway [®] pDEST [™] 8 Vector	6 µg	11804-010
Gateway [®] pDEST [™] 10 Vector	6 µg	11806-015
Gateway [®] pDEST [™] 20 Vector	6 µg	11807-013
MAX Efficiency [®] DH10Bac [™] Competent <i>E. coli</i>	5 x 100 µl	10361-012
One Shot [®] TOP10 Chemically Competent <i>E. coli</i>	20 x 50 µl	C4040-03
One Shot [®] MAX Efficiency [®] DH10B [™] -T1 ^R Chemically Competent <i>E. coli</i>	20 x 50 µl	12331-013
Cellfectin [®] Reagent	1 ml	10362-010
Ampicillin	20 ml (10 mg/ml)	11593-019
Kanamycin Sulfate	100 ml (10 mg/ml)	15160-054
Gentamicin	10 ml (50 mg/ml)	15750-060
Tetracycline	5 g	Q100-19
Bluo-gal	1 g	15519-028
Isopropylthio-β-galactoside (IPTG)	1 g	15529-019
TEV Protease, Recombinant	1000 units	10127-017
Platinum [®] Taq DNA Polymerase	100 reactions	10966-018
Platinum [®] Taq DNA Polymerase High Fidelity	100 reactions	11304-011
	500 reactions	11304-029
PCR SuperMix High Fidelity	100 reactions	10790-020
M13 Forward (-40) Primer	2 µg	N540-02
M13 Reverse Primer	2 µg	N530-02
4% Agarose Gel	40 ml	18300-012
CAT Antiserum	50 µl	R902-25

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Accessory Products, continued

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, see our Web site (www.invitrogen.com) or contact Technical Service (see page 62). **Note:** Reagents are available in other sizes.

Item	Quantity	Catalog no.
Sf9 Cells, SFM Adapted	1.5 x 10 ⁷ cells	11496-015
Sf21 Cells, SFM Adapted	1.5 x 10 ⁷ cells	11497-013
High Five™ Cells	3 x 10 ⁶ cells	B855-02
Mimic™ Sf9 Insect Cells	1 x 10 ⁷ cells	12552-014
Sf-900 II SFM	500 ml	10902-096
Sf-900 Medium (1.3X)	100 ml	10967-0321
Express Five® SFM	1 L	10486-025
Grace's Insect Cell Culture Medium, Unsupplemented	500 ml	11595-030
Penicillin-Streptomycin	100 ml	15070-063
PLURONIC® F-68, 10% (100X)	100 ml	24040-032

Purifying Recombinant Fusion Proteins

If you use the pFastBac™ HT A, B, or C vector to express your gene of interest as a fusion with the 6xHis tag, 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	Catalog no.
ProBond™ Nickel-chelating Resin	50 ml	R801-01
	150 ml	R801-15
ProBond™ Purification System	6 purifications	K850-01
Ni-NTA Agarose	10 ml	R901-01
	25 ml	R901-15
	100 ml	R901-10
Ni-NTA Purification System	6 purifications	K950-01

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Introduction

Overview

Introduction

The Bac-to-Bac[®] Baculovirus Expression System provides a rapid and efficient method to generate recombinant baculoviruses (Ciccarone *et al.*, 1997). This method was developed by researchers at Monsanto, and is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli* (Luckow *et al.*, 1993). The major components of the Bac-to-Bac[®] Baculovirus Expression System include:

- A choice of pFastBac[™] donor plasmids that allow generation of an expression construct containing the gene of interest where expression of the gene of interest is controlled by a baculovirus-specific promoter.
 - 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.
 - A control expression plasmid containing the Gus and/or CAT gene that allows production of a recombinant baculovirus which, when used to infect insect cells, expresses β -glucuronidase and/or chloramphenicol acetyl-transferase.
-

Advantages of the Bac-to-Bac[®] Baculovirus Expression System

Using the Bac-to-Bac[®] Baculovirus Expression System to generate a recombinant baculovirus provides the following advantages over the traditional method using homologous recombination:

- 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
-

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Overview, continued

Choosing a pFastBac™ Vector

A number of pFastBac™ vectors are available for use with the Bac-to-Bac® Baculovirus Expression System (see table below). Choose the vector that best suits your needs.

Vector	Features	Reference
pFastBac™1	<ul style="list-style-type: none">• Strong AcMNPV polyhedrin (PH) promoter for high-level protein expression• Large multiple cloning site for simplified cloning	Anderson <i>et al.</i> , 1996
pFastBac™HT	<ul style="list-style-type: none">• Strong polyhedrin (PH) promoter for high-level protein expression• N-terminal 6xHis tag for purification of recombinant fusion proteins using metal-chelating resin and a TEV protease cleavage site for removal of the 6xHis tag following protein purification• Vector supplied in 3 reading frames for simplified cloning	Polayes <i>et al.</i> , 1996
pFastBac™ Dual	<ul style="list-style-type: none">• Two strong baculovirus promoters (PH and p10) to allow simultaneous expression of two proteins• Two large multiple cloning sites for simplified cloning	Harris and Polayes, 1997

Purpose of This Manual

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

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



Important

The Bac-to-Bac® Baculovirus 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 easily generate a baculovirus and express your recombinant 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 and 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 our Web site (www.invitrogen.com) or from Technical Service (see page 62).

The Bac-to-Bac[®] Baculovirus Expression System

Components of the Bac-to-Bac[®] Baculovirus Expression System

The Bac-to-Bac[®] Baculovirus Expression System facilitates rapid and efficient generation of recombinant baculoviruses (Ciccarone *et al.*, 1997). Based on a method developed by Luckow *et al.*, 1993, the Bac-to-Bac[®] Baculovirus 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 first major component of the System is a pFastBac[™] vector into which the gene(s) of interest will be cloned. Depending on the pFastBac[™] vector selected, expression of the gene(s) of interest is controlled by the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) or p10 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.
- The second major component of the System is the DH10Bac[™] *E. coli* strain that is used as the host for your pFastBac[™] vector. 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 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 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[®] Baculovirus Expression System, see the diagram on page 5.

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The Bac-to-Bac[®] Baculovirus 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 LacZ α 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 does not disrupt the reading frame of the LacZ α 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

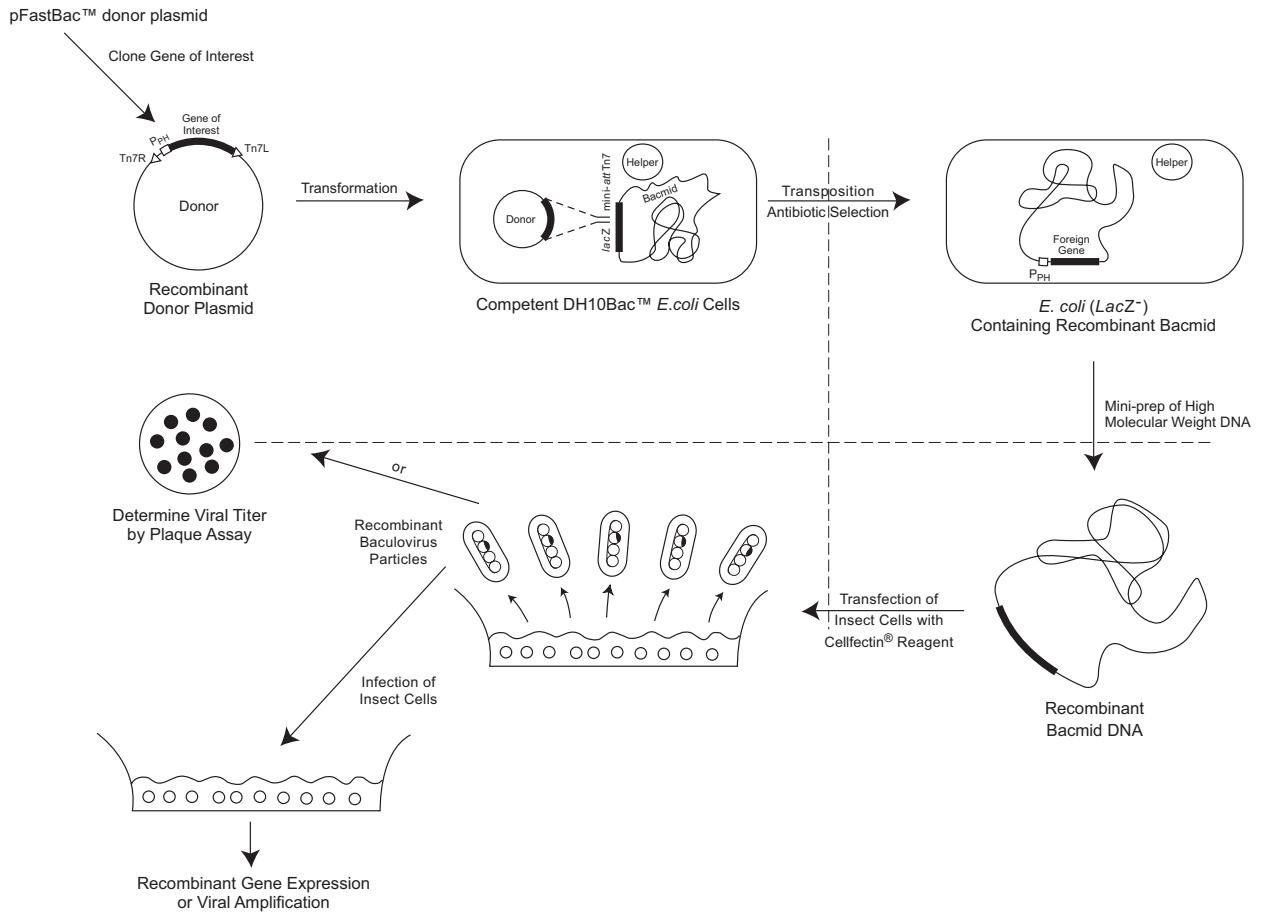
DH10Bac[™] *E. coli* also contain the helper plasmid, pMON7124 (13.2 kb), which encodes the transposase and confers resistance to tetracycline. The helper plasmid provides the Tn7 transposition function *in trans* (Barry, 1988).

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The Bac-to-Bac[®] Baculovirus Expression System, continued

Diagram of the Bac-to-Bac[®] System

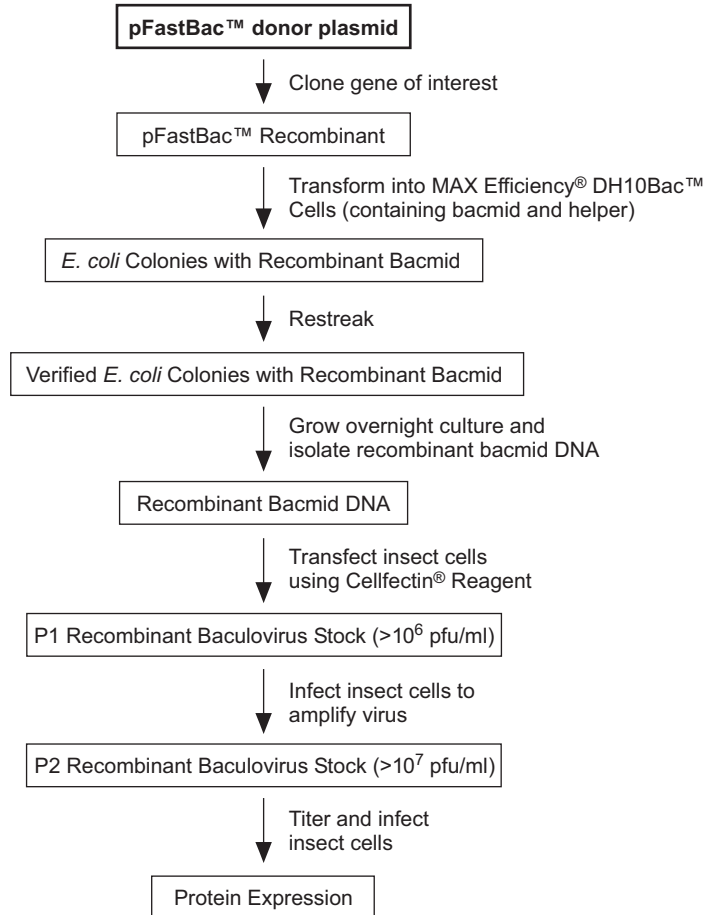
The figure below depicts the generation of recombinant baculovirus and the expression of your gene of interest using the Bac-to-Bac[®] Baculovirus Expression System.



Experimental Outline

Flow Chart

The figure below illustrates the general steps required to express your gene of interest using the Bac-to-Bac[®] Baculovirus Expression System.



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 viii 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 available from Invitrogen (see page viii for ordering information). Sf-900 II SFM is a protein-free medium optimized for the growth and maintenance of Sf9 and Sf21 cells, and for large-scale production of recombinant proteins expressed using the Bac-to-Bac® System. For more information, see our Web site (www.invitrogen.com) or call Technical Service (see page 62).

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*. This guide is available for downloading from our Web site at www.invitrogen.com or from Technical Service (see page 62), 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, and 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 does **not** require addition of shear force protectants.

Cells for Transfection

You will need log-phase cells with >95% viability to perform a successful transfection. Refer to page 27 to determine how many cells you will need for transfection.

Generating the Recombinant pFastBac™ Vector

General Information

Introduction

To generate a recombinant plasmid containing your gene(s) of interest for use in the Bac-to-Bac® Baculovirus Expression System, you will use restriction enzyme digestion and ligation to clone your gene(s) into one of the pFastBac™ vectors. For recommendations and guidelines to help you design your cloning strategy, refer to the appropriate section on pages 10-16 depending on the pFastBac™ vector you are using.

General Molecular Biology Techniques

For help with restriction enzyme digestion, ligation, DNA sequencing, and other general molecular biology techniques, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Propagation and Maintenance of Plasmids

The pFastBac™ vectors and their corresponding expression control plasmids contain the ampicillin resistance gene to allow for selection in *E. coli* using ampicillin. To propagate and maintain the pFastBac™ vectors and the pFastBac™ control plasmids, use the following procedure:

1. Use the stock solution of vector provided to transform a *recA*, *endA* *E. coli* strain such as TOP10, DH10B™, or DH5α (see page 17 for more information).
 2. Select transformants on LB agar plates containing 100 µg/ml ampicillin.
 3. Prepare a glycerol stock from each transformant containing plasmid for long-term storage (see page 18).
-

Cloning into pFastBac™ 1

Introduction

To help you design a strategy to clone your gene of interest into pFastBac™ 1, see the recommendations and diagram below.

Cloning Considerations

The pFastBac™ 1 vector is a non-fusion vector (*i.e.* no fusion tags are present in the vector). To ensure proper expression of your recombinant protein, your insert must contain:

- An ATG start codon for initiation of translation
- A stop codon for termination of the gene

Note: Stop codons are included in the multiple cloning site in all three reading frames.

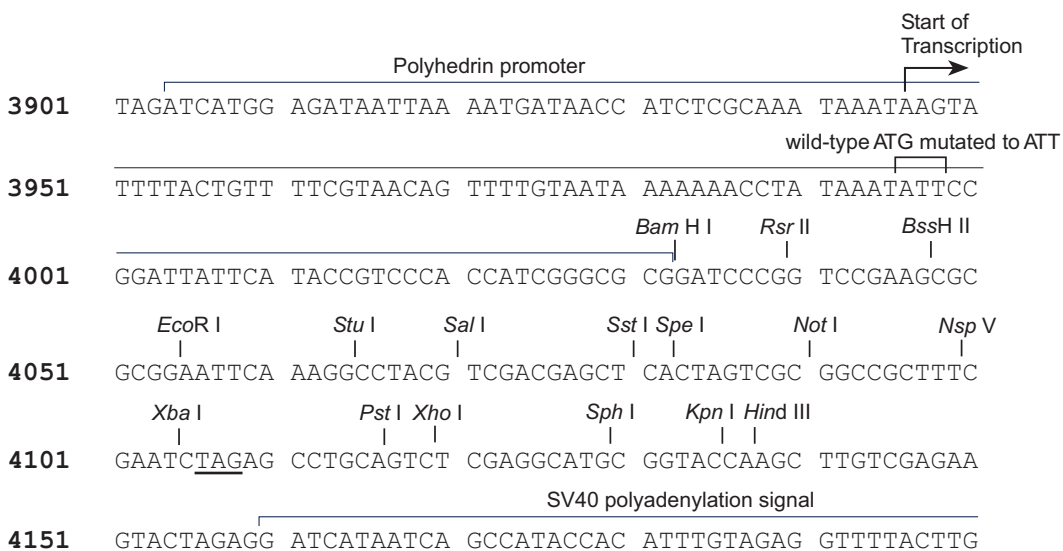


Note

The production of recombinant proteins requires that your insert contain a translation initiation ATG. Generally, transfer vectors that contain intact polyhedrin (PH) leader sequences (*e.g.* pFastBac™ vectors) may yield higher levels of expression than vectors that contain interrupted leader sequences. Protein translation can initiate at the mutated ATG (ATT) upstream of the multiple cloning site; however, initiation from this site is inefficient and generally does not interfere with expression and detection of recombinant protein.

Multiple Cloning Site of pFastBac™ 1

Below is the multiple cloning site for pFastBac™ 1. Restriction sites are labeled to indicate the actual cleavage site. Potential stop codons are underlined. The complete sequence of pFastBac™ 1 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62). For a map and a description of the features of pFastBac™ 1, refer to the **Appendix**, pages 53-54.



Cloning into pFastBac™ HT A, B, and C

Introduction

The pFastBac™ HT vector is supplied with the multiple cloning site in three reading frames (A, B, and C) to facilitate cloning your gene of interest in frame with the N-terminal 6xHis tag. See the recommendations below and the diagrams on pages 12-14 to help you design a cloning strategy.

Cloning Considerations

The pFastBac™ HT vectors are fusion vectors. To ensure proper expression of your recombinant protein, you must:

- Clone your gene in frame with the initiation ATG at base pairs 4050-4052. This will create a fusion with the N-terminal 6xHis tag and the TEV protease cleavage site
 - Include a stop codon with your insert
-



Note

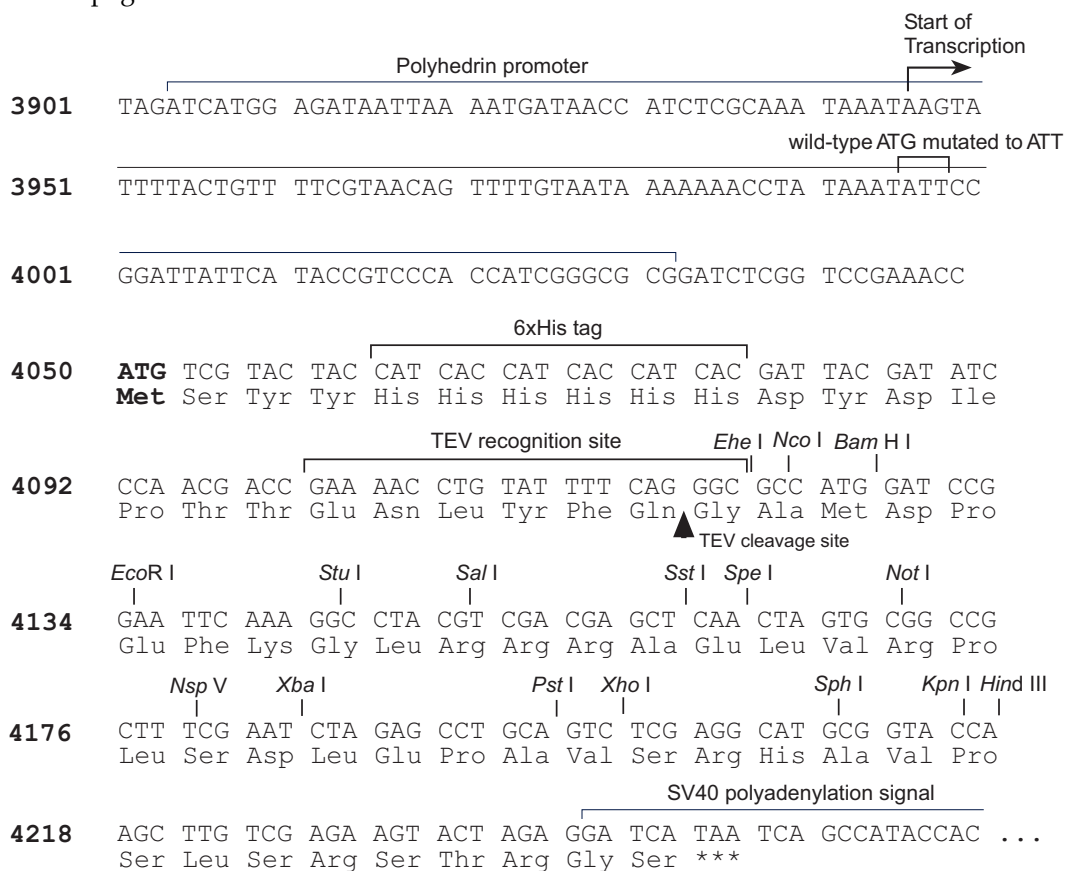
The production of recombinant proteins requires that your insert contain a translation initiation ATG. Generally, transfer vectors that contain intact polyhedrin (PH) leader sequences (*e.g.* pFastBac™ vectors) may yield higher levels of expression than vectors that contain interrupted leader sequences. Protein translation can initiate at the mutated ATG (ATT) upstream of the multiple cloning site; however, initiation from this site is inefficient and generally does not interfere with expression and detection of recombinant protein.

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Cloning into pFastBac™ HT A, B, and C, continued

Multiple Cloning Site of pFastBac™ HT A

Below is the multiple cloning site for pFastBac™ HT A. The initiation ATG is indicated in bold. Restriction sites are labeled to indicate the actual cleavage site. The complete sequence of pFastBac™ HT A is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62). For a map and a description of the features of pFastBac™ HT, refer to the **Appendix**, pages 55-56.

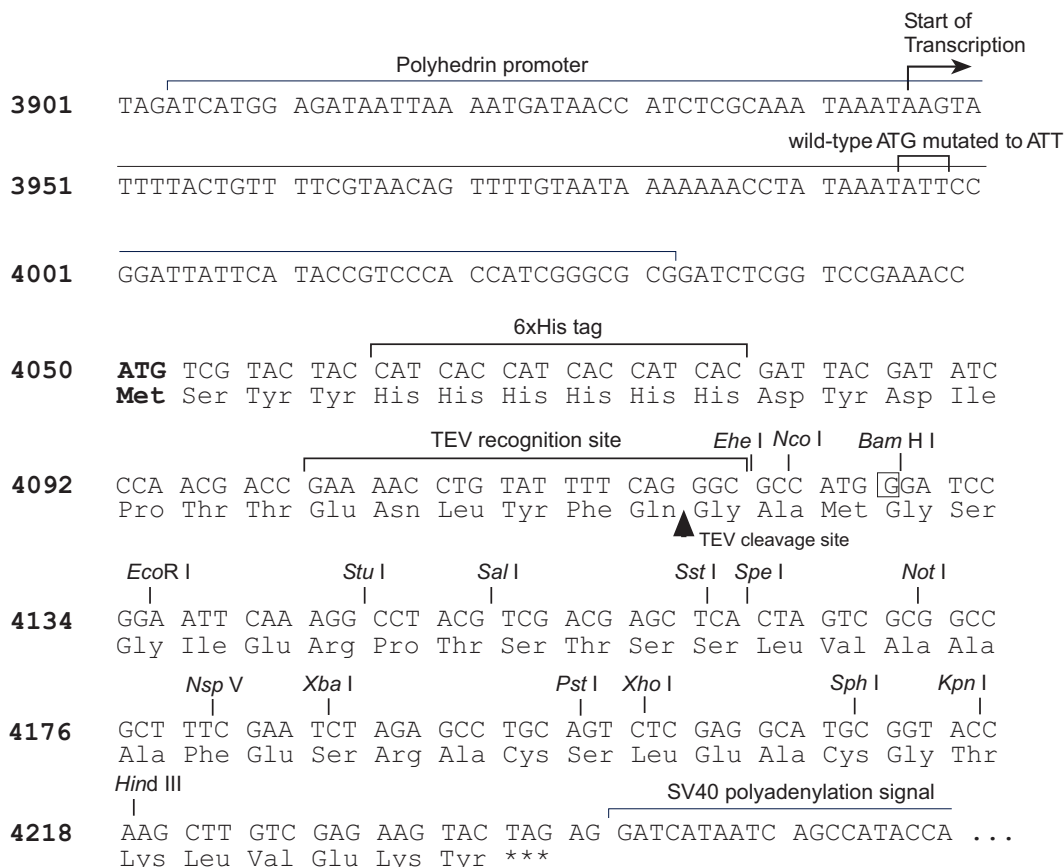


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Cloning into pFastBac™ HT A, B, and C, continued

Multiple Cloning Site of pFastBac™ HT B

Below is the multiple cloning site for pFastBac™ HT B. The initiation ATG is indicated in bold. Restriction sites are labeled to indicate the actual cleavage site. The boxed nucleotide indicates the variable region. The complete sequence of pFastBac™ HT B is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62). For a map and a description of the features of pFastBac™ HT, refer to the **Appendix**, pages 55-56.



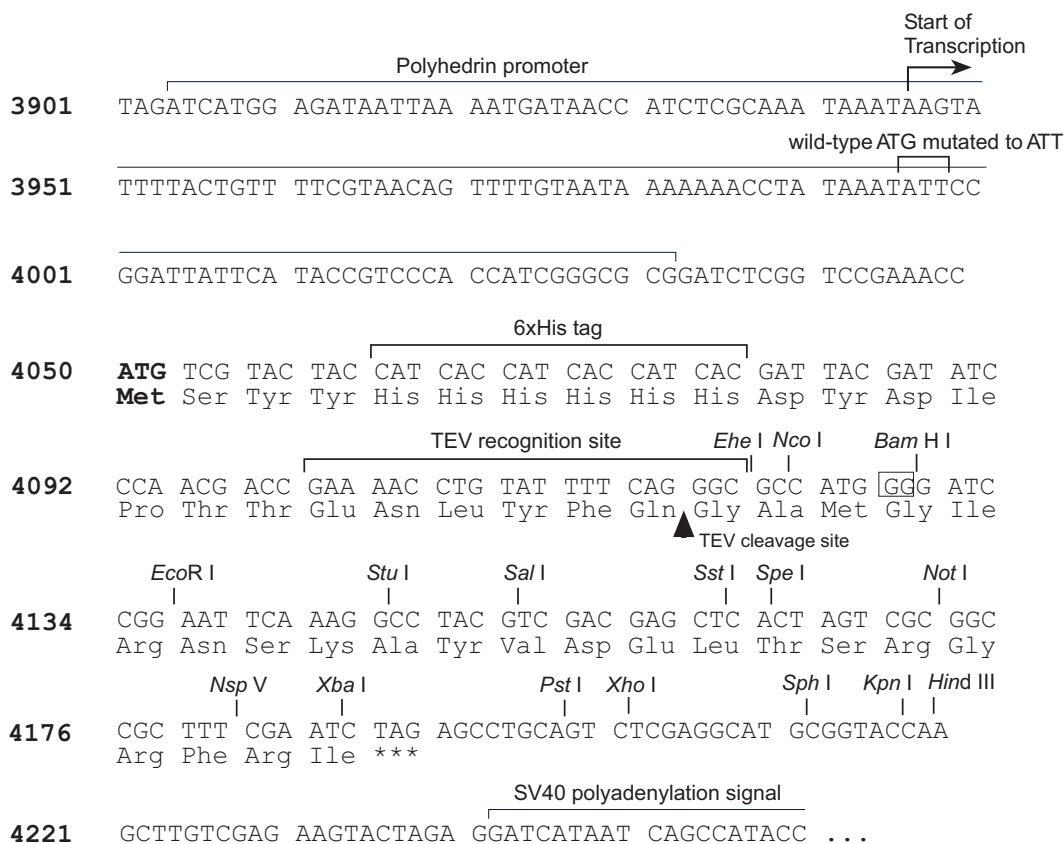
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Cloning into pFastBac™ HT A, B, and C, continued

Multiple Cloning Site of pFastBac™ HT C

Below is the multiple cloning site for pFastBac™ HT C. The initiation ATG is indicated in bold. Restriction sites are labeled to indicate the actual cleavage site. The boxed nucleotide indicates the variable region. The complete sequence of pFastBac™ HT C is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62). For a map and a description of the features of pFastBac™ HT, refer to the **Appendix**, pages 57-58.

Note: In pFastBac™ HT C, there is a stop codon within the *Xba* I site that is in frame with the N-terminal tag. Make sure that the 5' end of your gene is cloned upstream of the *Xba* I site.



Cloning into pFastBac™ Dual

Introduction

The pFastBac™ Dual vector contains two multiple cloning sites to allow expression of two heterologous genes; one controlled by the polyhedrin (PH) promoter and one by the p10 promoter. To help you design a strategy to clone your genes of interest into pFastBac™ Dual, see the recommendations and the diagram below.

Cloning Considerations

The pFastBac™ Dual vector is a non-fusion vector. To ensure proper expression of your recombinant proteins, both of your inserts must contain:

- An ATG start codon for initiation of translation
- A stop codon for termination of the gene if you don't use one of the stop codons provided in the multiple cloning site

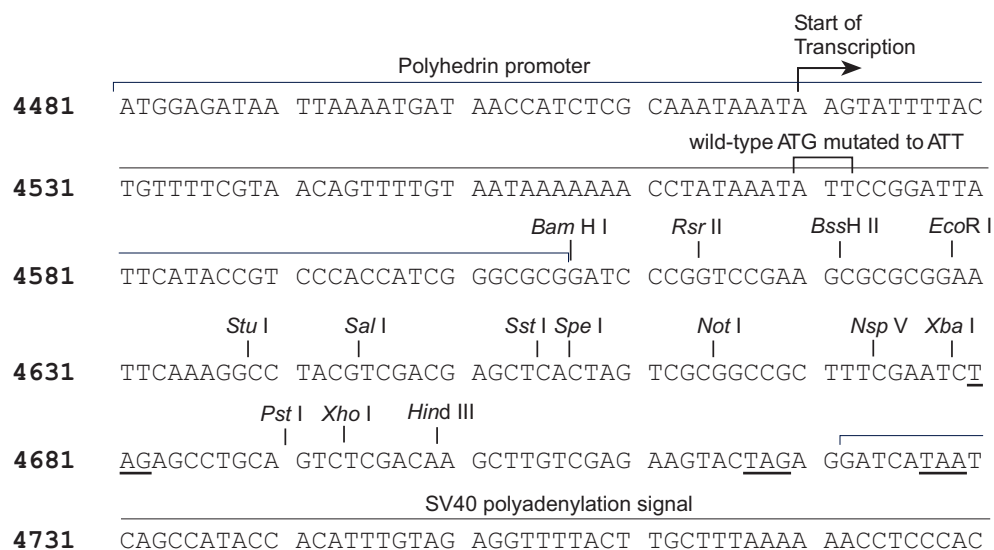


Note

The production of recombinant proteins requires that your insert contain a translation initiation ATG. Generally, transfer vectors that contain intact polyhedrin leader sequences (e.g. pFastBac™ vectors) may yield higher levels of expression than vectors that contain interrupted leader sequences. For inserts cloned downstream of the polyhedrin promoter, note that protein translation can initiate at the mutated ATG (ATT); however, initiation from this site is inefficient and generally does not interfere with expression and detection of recombinant protein.

Multiple Cloning Site Downstream of the PH Promoter

Below is the multiple cloning site located downstream of the PH promoter in pFastBac™ Dual. Restriction sites are labeled to indicate the actual cleavage site. Potential stop codons are underlined. The complete sequence of pFastBac™ Dual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62). For a map and a description of the features of pFastBac™ Dual, refer to the **Appendix**, pages 57-58.

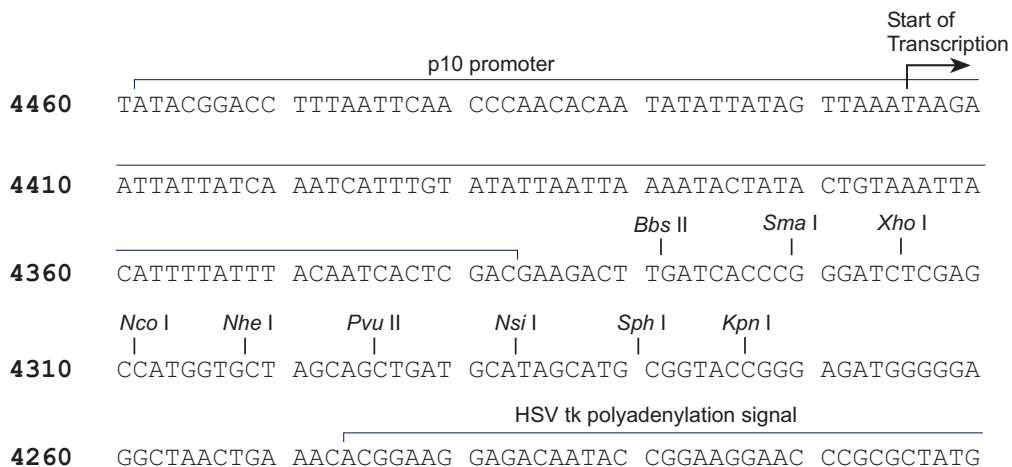


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Cloning into pFastBac™ Dual, continued

Multiple Cloning Site Downstream of the p10 Promoter

Below is the multiple cloning site located downstream of the AcMNPV p10 promoter in pFastBac™ Dual. Restriction sites are labeled to indicate the actual cleavage site. Potential stop codons are underlined. The complete sequence of pFastBac™ Dual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62). For a map and a description of the features of pFastBac™ Dual, refer to the **Appendix**, pages 57-58.



Transformation and Analysis

Introduction

Once you have completed your ligation reactions, you are ready to transform your pFastBac™ construct into *E. coli*. Many *E. coli* host strains and transformation procedures are suitable. General recommendations to transform *E. coli* and analyze transformants are provided in this section.

E. coli Host

Once you have cloned your insert into one of the pFastBac™ vectors, you will transform the ligation reaction into *E. coli* and select for ampicillin-resistant transformants. You may use any *recA*, *endA* *E. coli* strain including TOP10, DH10B™, or DH5α for transformation. **Do not transform the ligation reaction into DH10Bac™ cells.**

Note: Chemically competent TOP10 and DH10B™ *E. coli* are available from Invitrogen in a convenient One Shot® format (see table below).

Item	Quantity	Catalog no.
One Shot® TOP10 Chemically Competent <i>E. coli</i>	20 x 50 µl	C4040-03
One Shot® MAX Efficiency® DH10B™-T1 ^R Chemically Competent <i>E. coli</i>	20 x 50 µl	12331-013
One Shot® MAX Efficiency® DH5α-T1 ^R Chemically Competent <i>E. coli</i>	20 x 50 µl	12297-016

Transformation Method

You may use any method of choice to transform *E. coli*. Chemical transformation is the most convenient method, while electroporation is the most efficient and method of choice for large plasmids. To select for transformants, use LB agar plates containing 100 µg/ml ampicillin.

Analyzing Transformants

Once you have obtained ampicillin-resistant transformants, we recommend the following:

1. Pick 10 transformants and culture them overnight in LB or S.O.B. containing 100 µg/ml ampicillin.
 2. Isolate plasmid DNA using your method of choice. We recommend using the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) available from Invitrogen.
 3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.
-

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Transformation and Analysis, continued

Analyzing Transformants by PCR

You may also analyze positive transformants using PCR. Use the appropriate PCR primers and amplification conditions for your insert. If you are using this technique for the first time, you may want to perform restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed:

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 μ M each)

Procedure:

1. For each sample, aliquot 48 μ l of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 μ l each of the forward and reverse PCR primer.
 2. Pick 10 colonies and resuspend them individually in 50 μ l of the PCR SuperMix containing primers (remember to make a patch plate to preserve the colonies for further analysis).
 3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
 4. Amplify for 20 to 30 cycles.
 5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
 6. Visualize by agarose gel electrophoresis.
-

Sequencing

You may sequence your construct to confirm that your gene of interest is in the correct orientation for expression. If you have cloned your gene into one of the pFastBacTM HT vectors, verify that your gene is cloned in frame with the N-terminal tag.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing 100 μ g/ml ampicillin.
 2. Isolate a single colony and inoculate into 1-2 ml of LB containing 100 μ g/ml ampicillin.
 3. Grow until culture reaches stationary phase.
 4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
 5. Store at -80°C.
-

Generating the Recombinant Bacmid

Transforming DH10Bac™ *E. coli*

Introduction

Once you have generated your pFastBac™ construct, 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® Baculovirus Expression System, but are also available separately from Invitrogen (Catalog no. 10361-012). Guidelines and instructions to transform DH10Bac™ cells are provided in this section.

Positive Control

Each pFastBac™ plasmid is supplied with a corresponding control plasmid for use as a positive transfection and expression control (see table below). Depending on the pFastBac™ vector you are using, we recommend including the corresponding control plasmid in your DH10Bac™ transformation experiment (see table below). For maps and a description of the features of each control plasmid, see the **Appendix**, pages 59-61.

pFastBac™ Vector	Control Plasmid
pFastBac™ 1	pFastBac™ 1-Gus
pFastBac™ HT	pFastBac™ HT-CAT
pFastBac™ Dual	pFastBac™ Dual-Gus/CAT

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Transforming DH10Bac™ *E. coli*, continued

Materials Needed

Have the following materials on hand before beginning:

- Your purified pFastBac™ construct (200 pg/μl in TE, pH 8)
 - Positive expression control (*i.e.* pFastBac™1-Gus, pFastBac™HT-CAT, or pFastBac™ Dual-Gus/CAT; use as a control for transposition)
 - MAX Efficiency® DH10Bac™ chemically competent cells (supplied with the Bac-to-Bac® Baculovirus 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 plates for each transformation; use freshly prepared plates; see recommendation below)
 - LB agar plate containing 100 μg/ml ampicillin (for plating pUC19 transformation control)
 - S.O.C. Medium (Invitrogen, Catalog no. 15544-034)
 - 15 ml round-bottom polypropylene tubes
 - 42°C water bath
 - 37°C shaking and non-shaking incubator
-



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 DH10Bac™ transformants. See page vii to order antibiotics, Bluo-gal, and IPTG, and page 50 for instructions to prepare plates. If you are preparing LB plates using a pre-mixed formulation, we recommend using Luria Broth Base (Invitrogen, Catalog no. 12795-027) 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 cells with your pFastBac™ construct. We recommend including positive controls for transposition (*i.e.* pFastBac™ 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 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 pFastBac™ construct: 1 ng (5 µl)
 - pFastBac™ 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 LacZ α 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.

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Transforming DH10Bac™ *E. coli*, continued

Verifying the Phenotype

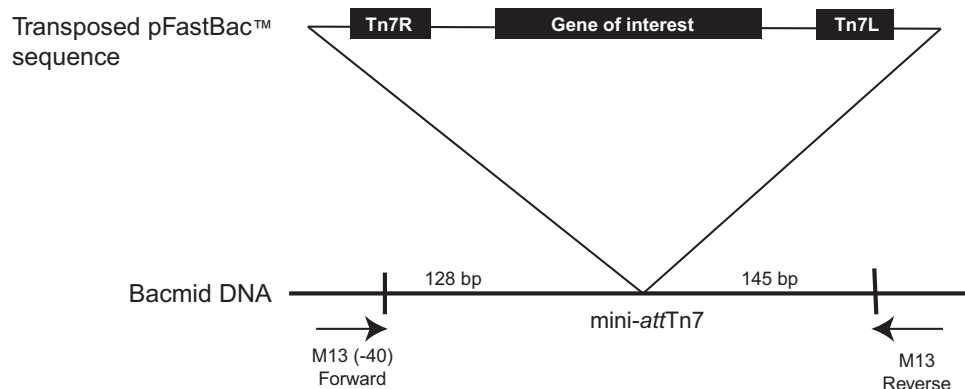
1. Pick 10 white colonies and restreak them on fresh LB agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml IPTG. Incubate the plates overnight at 37°C.
2. From a single colony confirmed to have a white phenotype on restreaked plates 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 S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) available from Invitrogen. Alternatively, you may use the procedure provided in the **Appendix**, page 51. This procedure was originally developed to isolate large plasmids (>100 kb) (P. deJong, personal communication) and has been adapted to allow isolation of bacmid DNA.
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**, next page for details).

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.

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. The bacmid contains M13 Forward (-40) and M13 Reverse priming sites flanking the mini-*att*Tn7 site within the *lacZ* α -complementation region to facilitate PCR analysis (see figure below). Guidelines and instructions are provided in this section to perform PCR using the M13 Forward (-40) and M13 Reverse primers.



PCR Analysis with M13 Primers

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

- Use the M13 Forward (-40) and M13 Reverse primers
- Use a combination of the M13 Forward (-40) or M13 Reverse primers and a primer that hybridizes within your insert.

The M13 Forward (-40) and M13 Reverse primers are available from Invitrogen (see below).

Primer	Sequence	Catalog no.
M13 Forward (-40)	5'd[GTTTTCCAGTCACGAC]3'	N540-02
M13 Reverse	5'd[CAGGAAACAGCTATGAC]3'	N530-02

DNA Polymerase

You may use any DNA polymerase of your choice for PCR including Platinum® *Taq* DNA Polymerase (Invitrogen, Catalog no. 10966-018). If the expected PCR product is > 4 kb, we recommend using a polymerase mixture such as Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Catalog no. 11304-011) for best results.

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Analyzing Recombinant Bacmid DNA by PCR, continued

Producing the PCR Product

Use the procedure below to amplify your recombinant bacmid DNA using the M13 Forward (-40) and M13 Reverse primers and Platinum[®] *Taq* polymerase. If you are using a combination of the M13 Forward (-40) or M13 Reverse 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
<u>Platinum[®] <i>Taq</i> polymerase (5 units / µl)</u>	<u>0.5 µl</u>
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	3 minutes	93°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.

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Analyzing Recombinant Bacmid DNA by PCR, continued

What You Should See

If transposition has occurred and you have used the M13 Forward (-40) and M13 Reverse primers for amplification, you should see a PCR product of the following size on the agarose gel:

Sample	Size of PCR Product
Bacmid alone	~300 bp
Bacmid transposed with pFastBac™1	~2300 bp + size of your insert
Bacmid transposed with pFastBac™1-Gus	~4200 bp
Bacmid transposed with pFastBac™HT	~2430 bp + size of your insert
Bacmid transposed with pFastBac™HT-CAT	~3075 bp
Bacmid transposed with pFastBac™ Dual	~2560 bp + size of your insert
Bacmid transposed with pFastBac™ Dual-Gus/CAT	~5340 bp

If you have used a combination of the M13 Forward (-40) or M13 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 23 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 S.N.A.P.[™] MidiPrep Kit (Catalog no. K1910-01) available from Invitrogen or the procedure provided in the **Appendix**, page 51.

Transfection Method

We recommend using a cationic lipid such as Cellfectin[®] Reagent for transfection. Cellfectin[®] Reagent is supplied with the Bac-to-Bac[®] Baculovirus Expression System and is available separately from Invitrogen (Catalog no. 10362-010).

Cellfectin[®] Reagent

Cellfectin[®] Reagent is a 1:1.5 (M/M) liposome formulation of the cationic lipid N₁, N₂, N₃-Tetramethyl-N, N₁, N₂, N₃-tetrapalmitylspermine (TM-TPS) and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water. Cellfectin[®] Reagent has been found to be superior for transfection of Sf9 and other insect 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.

Note: For convenience, we refer to Sf9 cells in the transfection procedure; however Sf21 cells may be substituted and used at the same densities.

Media for Transfection

For the highest transfection efficiency, we recommend performing the transfection in unsupplemented Grace's Insect Cell Culture Medium (Invitrogen, Catalog no. 11595-030). Note that the Grace's Insect Cell Culture Medium **should not** contain supplements or fetal bovine serum (FBS) as the proteins in the FBS and supplements will interfere with the Cellfectin[®] Reagent, inhibiting the transfection.

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

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Transfecting Insect Cells, continued

Positive Control

If you have generated a recombinant bacmid from one of the pFastBac™ control plasmids (*i.e.* pFastBac™1-Gus, pFastBac™HT-CAT, or pFastBac™ Dual-Gus/CAT), we recommend including this positive control in your transfection and expression experiments to help you evaluate your results. In these bacmids, the gene encoding β -glucuronidase (Gus) and/or chloramphenicol acetyltransferase (CAT) will be expressed under the control of the polyhedrin (PH) or p10 promoter. After transfection, expression of β -glucuronidase or CAT may be assayed, as appropriate.

Materials Needed

Have the following materials on hand before beginning:

- Purified recombinant bacmid DNA from your pFastBac™ construct (500 ng/ μ l in TE Buffer, pH 8.0)
 - Purified recombinant bacmid DNA from the appropriate pFastBac™ control construct (if desired, 500 ng/ μ l in TE Buffer, pH 8.0)
 - Sf9 or Sf21 cells cultured in the appropriate medium
 - Cellfectin® Reagent (store at +4°C until use)
 - Grace's Insect Cell Medium, Unsupplemented (Invitrogen, Catalog no. 11595-030; **media should not contain supplements, FBS, or antibiotics**)
 - 6-well tissue culture plates and other tissue culture supplies
 - 12 x 75 mm sterile tubes
 - Complete growth medium for culturing insect cells (*e.g.* Sf-900 II SFM, TNM-FH, or other suitable medium)
-



Calculate the number of Sf9 cells that you will need for your transfection experiment and expand cells accordingly. Make sure that the cells are healthy and >97% viability before proceeding to transfection.

Transfection Conditions

We generally produce baculoviral stocks in Sf9 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® Reagent concentrations, and cell density.

Condition	Amount
Tissue culture plate size	6-well (35 mm) plate (one well per bacmid)
Number of Sf9 cells to transfect	9×10^5 cells
Amount of bacmid DNA	1 μ g (can vary from 1 to 2 μ g)
Amount of Cellfectin® Reagent	6 μ l (can vary from 1.5 to 9 μ l)

continued on next page

Transfecting Insect Cells, continued

Transfection Procedure

Use the following procedure below to transfect Sf9 cells in a **6-well format**. If you wish to transfect cells in other tissue culture formats, you will need to determine the optimal conditions to use. Remember to use **unsupplemented** Grace's Medium that does not contain FBS or antibiotics for transfection.

1. In a 6-well or 35 mm tissue culture plate, seed 9×10^5 Sf9 cells per well in 2 ml of growth medium containing antibiotics (*e.g.* 2 ml of Sf-900 II SFM containing 50 units/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin final concentration).
 2. Allow cells to attach at 27°C for at least 1 hour.
 3. **For each transfection sample**, prepare bacmid DNA:Cellfectin® Reagent complexes as follows in 12 x 75 mm sterile tubes.
 - a. Dilute 1 μg of purified bacmid DNA in 100 μl of unsupplemented Grace's Medium.
 - b. Mix Cellfectin® Reagent thoroughly before use by inverting the tube 5-10 times. Remove 6 μl of Cellfectin® Reagent and dilute in 100 μl of unsupplemented Grace's Medium.
 - c. Combine the diluted bacmid DNA with the diluted Cellfectin® Reagent (total volume is ~210 μl). Mix gently and incubate for 15 to 45 minutes at room temperature.
 4. While DNA:lipid complexes are incubating, remove the media from the cells and wash once with 2 ml of unsupplemented Grace's Medium. Remove the wash media.
 5. Add 0.8 ml of unsupplemented Grace's Medium to each tube containing the DNA:lipid complexes. Mix gently and add the DNA:lipid complexes to each well containing cells.
 6. Incubate the cells in a 27°C incubator for 5 hours.
 7. Remove the DNA:lipid complexes and add 2 ml of complete growth media (*e.g.* Sf-900 II SFM containing antibiotics) to the cells.
 8. Incubate the cells in a 27°C humidified incubator for 72 hours or until you start to see signs of viral infection. Proceed to **Isolating P1 Viral Stock**, next page.
-

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

Virally-infected insect cells typically display the following characteristics as observed from visual inspection 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 8, previous page demonstrate signs of late stage infection (*e.g.* 72 hours post-transfection), collect the medium containing virus from each well (~2 ml) and transfer to sterile 15 ml snap-cap tubes. Centrifuge the tubes at 500 x 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 +4°C, 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 +4°C, protected from light.
 - If medium is serum-free (e.g. Sf-900 II 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 -80°C for later reamplification.
 - Do not store routinely used viral stocks at temperatures below +4°C. 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 33).
 - Plaque purify your baculoviral construct, if desired (see **Performing a Viral Plaque Assay**, page 33).
 - Use the P1 viral stock to infect Sf9 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 Sf9 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 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

You should have the following materials on hand before beginning:

- 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 cells that you will need for infection and expand cells accordingly. Make sure that the cells are healthy and >97% 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:

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

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.

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

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

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Amplifying Your Baculoviral Stock, continued

Amplification Procedure

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

1. On the day of infection, prepare an 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 27°C 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 $500 \times g$ for 5 minutes to remove cells and large debris.

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 +4°C, protected from light. For long-term storage, you may store an aliquot of the P2 stock at -80°C, protected from light. See page 30 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 -80°C, 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 -80°C. Follow the guidelines and amplification procedure detailed in this section.

Performing a Viral Plaque Assay

Introduction

We recommend using a plaque assay to:

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

Guidelines and instructions are provided in this section.

Experimental Outline

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

1. Plate Sf9 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 cells using Cellfectin[®] Reagent. Prepare DNA: lipid complexes in unsupplemented Grace's Insect Medium (see pages 26-28 for details).
 - The age of your baculoviral stock. Viral titers may decrease with long-term storage at +4°C or -80°C. 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 -80°C, 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 aliquotted and stored at +4°C, protected from light.
-

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Performing a Viral Plaque Assay, continued

Materials Needed

You should have the following materials on hand before beginning:

- Your clarified baculoviral stock (store at +4°C 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 or other appropriate complete growth medium (see **Note** below)
 - Sf-900 Medium (1.3X) (100 ml; Invitrogen, Catalog no. 10967-032) or other appropriate plaquing medium (see **Note** below)
 - 4% Agarose Gel (Invitrogen, Catalog no. 18300-012)
 - Sterile, cell-culture grade, distilled water (Invitrogen, Catalog no. 15230-162)
 - 100 ml sterile, glass bottle
 - 6-well tissue-culture plates (2 plates for each viral stock to be titered)
 - Sterile hood
 - Waters baths at 40°C and 70°C
 - Microwave oven (optional)
 - 27°C humidified incubator
-



Note

If you are culturing your Sf9 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 (Invitrogen, Catalog no. 11605-094)
 - Grace's Insect Cell Culture Medium (2X) (100 ml, Invitrogen, Catalog no. 11667-037)
 - Fetal Bovine Serum (FBS), Qualified, Heat-Inactivated (Invitrogen, Catalog no. 16140-063)
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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, 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 70°C 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 40°C 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 40°C water bath until use.

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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, we recommend titrating 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 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}).
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: 10^{-3} , 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.
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 40°C water bath (Step 4, previous page) to a sterile hood.
9. Sequentially starting from the highest dilution (10^{-8}) to the lowest dilution (10^{-3}), remove the medium containing virus from the wells and replace with 2 ml of plaquing medium. Work quickly to avoid dessication of the cell monolayer.
10. Allow agarose overlay to harden for 10-20 minutes at room temperature before moving the plates.
11. Incubate the cells in a 27°C 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 38.

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Performing a Viral Plaque Assay, continued



Note

To improve the visualization of plaques, stain the plates using Neutral Red. Other plaque staining dyes such as Crystalline Blue are not recommended because they contain organic solvents that 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.
- 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.

Materials Needed

You should have the following materials on hand before beginning:

- Neutral Red (Sigma, Catalog no. N7005)
 - Cell-culture grade, distilled water
 - Sf-900 II SFM or other appropriate complete growth medium (if preparing the agarose solution)
 - 4% Agarose Gel (if preparing the agarose solution)
 - 40°C water bath (if preparing the agarose solution)
-

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 40°C 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 40°C 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 27°C 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. Count plaques. Plaques will appear as clear spots in a nearly clear gel against a red background.
-

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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.

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

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:

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

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

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. See page 33 and the **Troubleshooting** section, page 47 for more tips and guidelines to optimize your viral yield.

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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 36; o 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 36 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 μ l of complete growth medium. Mix well by vortexing.
 3. Add 100 μ l of the agarose plug solution to each well.
 4. Incubate the cells in a 27°C 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 x 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 31.
-

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™ control baculoviral construct (i.e. pFastBac1-Gus, pFastBac™ HT-CAT, pFastBac™ Dual-Gus/CAT), you may want to include this construct in your experiments for use as an expression control. Once you have infected cells with the FastBac™ control virus, the gene encoding β -glucuronidase (Gus) and/or chloramphenicol acetyltransferase (CAT) will be constitutively expressed and can be easily assayed (see page 42).

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. Cells may be grown in adherent or suspension culture in the 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 (Catalog no. 10902-096) or Express Five® SFM (Catalog no. 10486-025) 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. A dose response should be established 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 1 to 5.
 - **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.
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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 perform the following to determine the optimal conditions to use to express 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 times post-infection (*e.g.* 24, 48, 72, 96 hours post-infection). Choose the time point at which optimal recombinant protein expression is obtained.
-

Analyzing Recombinant Protein Expression

Use the following procedure to analyze protein expression from your recombinant baculovirus. This procedure is adapted from Luckow and Summers 1988) and is designed to allow expression analysis in a 24-well format from cells harvested 24 to 96 hours post-infection. Other protocols are suitable.

1. Seed 6×10^5 Sf9 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 μ l 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 27°C 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 media and rinse the cells once with serum-free medium. Lyse cells with 400 μ l of 1X SDS-PAGE Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS).
 6. Freeze samples at -20°C or boil samples for at least 3 minutes and separate proteins by SDS-PAGE.
-

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.

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Expressing Your Recombinant Protein, continued



Note

If you are expressing your recombinant from pFastBac™HT, the presence of the N-terminal 6xHis tag and the TEV recognition site will increase the size of your protein by at least 3 kDa.

Assay for β -glucuronidase

If you include the pFastBac™1-Gus or pFastBac™ Dual-Gus/CAT baculoviral construct in your expression experiment, you may assay for β -glucuronidase expression using the following methods. Other methods are 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 μ l of a 20 mg/ml X-glucuronide solution (in DMSO or dimethylformamide) with 50 μ l of cell-free medium. Monitor for development of blue color within 2 hours.
-

Assay for CAT Protein

If you include the pFastBac™HT-CAT or pFastBac™ Dual-Gus/CAT baculoviral construct in your expression experiment, you may assay for CAT expression using your method of choice. There are commercial kits available for assaying CAT expression as well as a rapid radioactive assay (Neumann *et al.*, 1987). For western blot analysis, the CAT Antiserum is available from Invitrogen (Catalog no. R902-25) to facilitate detection.

Purifying Recombinant Protein

You may use any method of choice to purify your recombinant protein of interest. Refer to published references (Deutscher, 1990; Janson and Ryden, 1989) for general guidelines on protein purification methods.

Note: If you have cloned your gene of interest in frame with the 6xHis tag in pFastBac™HT, you may purify your recombinant protein using a metal-chelating resin such as ProBond™ or Ni-NTA available from Invitrogen (see page viii for ordering information). Refer to the manual included with each product for guidelines to purify your fusion protein. These manuals are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62).

Removing the N-Terminal Fusion Tag Using TEV Protease

If you have expressed your recombinant fusion protein from pFastBac™HT, you may use recombinant TEV protease available from Invitrogen (Catalog no. 10127-017) to remove the N-terminal fusion tag. Instructions for digestion are included with the product. For more information, contact Technical Service (see page 62).

Note: Depending on which restriction enzymes are used for cloning, additional amino acids may be present at the N-terminus of your protein (refer to the diagrams on pages 12-14 for more help).

Troubleshooting

Cloning into the pFastBac™ Vectors

The table below lists some potential problems that you may encounter when generating your pFastBac™ construct. Possible solutions that may help you troubleshoot your cloning are provided.

Problem	Reason	Solution
Recombinant pFastBac™ construct lacks insert	Incomplete digestion of pFastBac™ plasmid or insert DNA	<ul style="list-style-type: none"> Use additional restriction enzyme for digestion. Purify insert DNA.
	Incomplete or excessive phosphatase treatment of pFastBac™ plasmid	Optimize dephosphorylation conditions according to the manufacturer's recommendations for the phosphatase you are using.
	Poor recovery of pFastBac™ plasmid or insert DNA from agarose gel	Use the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) to purify DNA.
	Incomplete ligation reactions	<ul style="list-style-type: none"> Follow ligation conditions according to the manufacturer's recommendations for the ligase you are using. Optimize ligation reaction by varying vector:insert molar ratios (e.g. 1:3, 1:1, 3:1).
	Insert contains unstable DNA sequences such as LTR sequences and inverted repeats	<ul style="list-style-type: none"> Grow transformed cells at lower temperatures (30°C). Use MAX Efficiency® Stbl2™ Competent Cells available from Invitrogen (Catalog no. 10268-019) for transformation. Stbl2™ <i>E. coli</i> are specifically designed for cloning unstable inserts.
No or few colonies obtained after transformation	Low transformation efficiency of competent <i>E. coli</i>	<ul style="list-style-type: none"> If stored incorrectly, prepare or obtain new competent cells. Use Invitrogen's One Shot® TOP10 (Catalog no. C4040-03) or One Shot® MAX Efficiency® DH10B™-T1^R (Catalog no. 12331-013) Chemically Competent <i>E. coli</i> for transformation.
	Impurities in DNA	Purify insert DNA. Make sure to remove excess phenol, proteins, detergents, and ethanol from the DNA solution.

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Troubleshooting, continued

Cloning into the pFastBac™ Vectors, continued

Problem	Reason	Solution
No or few colonies obtained after transformation, continued	Too much DNA transformed	<ul style="list-style-type: none"> For chemically competent cells, add 1 to 10 ng of DNA in a volume of 5 µl or less per 100 µl of cells. For electrocompetent cells, add 10 to 50 ng of DNA in a volume of 1 µl or less per 20 µl of cells. If you have purchased competent cells, follow the manufacturer's instructions.
	Incomplete ligation reaction	<ul style="list-style-type: none"> Optimize the ligation reaction. Include a ligation control (<i>i.e.</i> digested pFastBac™ vector + ligase; no insert). Check the ligation reaction on a gel. <p>Note: Ligated products and linear DNA transform 10X and 100-100X less efficiently, respectively than super-coiled DNA (Hanahan, 1983).</p>
	Ligation reaction mix inhibits transformation of competent cells	Reduce the amount of ligation reaction transformed. Dilute ligation reaction 5X with TE Buffer prior to transformation.
	Problem with antibiotic	<ul style="list-style-type: none"> Confirm use of the correct antibiotic; confirm antibiotic concentration. Check that the antibiotic is not degraded (<i>i.e.</i> change in color of solution or the appearance of precipitate). Use fresh antibiotic.
	Competent cells stored improperly	Store competent cells at -80°C.
	Competent cells handled improperly	Thaw cells on ice; use immediately after thawing; do not vortex.
	Cells not heat-shocked or incubated properly during transformation	Follow the recommended transformation procedure for the cells you are using.

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Troubleshooting, continued

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 (non-recombinant) colonies obtained (<i>i.e.</i> 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	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> 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.
All colonies are blue	pFastBac™ DNA used for transformation was of poor quality	<ul style="list-style-type: none"> 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.

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Troubleshooting, continued

Generating Recombinant Bacmid DNA, continued

Problem	Reason	Solution
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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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.

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	<ul style="list-style-type: none"> • Store purified bacmid DNA in aliquots at -20°C. • Do not freeze / thaw repeatedly.
	High molecular weight bacmid DNA handled improperly	<ul style="list-style-type: none"> • 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.

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Troubleshooting, continued

Isolating Bacmid DNA, continued

Problem	Reason	Solution
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.

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	<ul style="list-style-type: none"> • Use Invitrogen's Cellfectin® 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>i.e.</i> >96 hours post-transfection).
	Cells plated too sparsely	Plate insect cells at the recommended cell density.
	Used too much or too little Cellfectin® or other lipid reagent	Optimize the amount of Cellfectin® or other lipid reagent used.
	Time of incubation with DNA:lipid complexes too short or too long	Optimize the incubation time (<i>e.g.</i> 3 to 8 hours).
	Recombinant bacmid DNA is degraded	<ul style="list-style-type: none"> • Check the quality of your recombinant DNA by agarose gel electrophoresis prior to transfection. • Prepare bacmid DNA using Invitrogen's S.N.A.P. MidiPrep Kit (Catalog no. K1910-01) or use the procedure provided on page 51.

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Troubleshooting, continued

Transfecting Insect Cells, continued

Problem	Reason	Solution
Low yield of virus, continued	Bacmid DNA is not pure (<i>i.e.</i> contains recombinant bacmid and empty bacmid)	<ul style="list-style-type: none"> • Screen other DH10Bac™ transformants and choose one that contains only recombinant bacmid. • Perform plaque purification to isolate recombinant baculovirus.

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 yield	Viral stock contains a mixture of recombinant and non-recombinant baculovirus	Perform plaque purification to isolate recombinant baculovirus.
	Baculovirus not recombinant	<ul style="list-style-type: none"> • Verify transposition by PCR analysis of bacmid DNA using the M13 forward (-40) and M13 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	<ul style="list-style-type: none"> • Optimize culture conditions based on the size of your culture vessel and expression conditions. • Culture cells in Sf-900 II SFM for optimal cell growth and protein expression.
	Cell line not optimal	Try other insect cell lines.

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 water; filter-sterilize	-20°C, protected from light
Gentamicin	7 mg/ml in water; filter-sterilize	-20°C, protected from light

*If you are using tetracycline from Invitrogen (Catalog no. Q100-19), prepare in water. If you are using the free-base form of tetracycline, prepare in 100% ethanol.

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.

- Dissolve the Bluo-gal in dimethylformamide or dimethyl sulfoxide (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.
- Do not filter the stock solution.
- 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.
-

Isolating Recombinant Bacmid DNA

Introduction

After you have transformed your pFastBac™ construct into DH10Bac™ *E. coli* and performed the transposition reaction, use the procedure below to purify recombinant bacmid DNA from DH10Bac™ transformants. Purified bacmid DNA is suitable for use in PCR analysis or transfection.

Materials Needed

Have the following materials on hand before beginning.

- LB medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline
 - Solution I (15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A; filter-sterilize and store at +4°C)
 - Solution II (0.2 N NaOH, 1% SDS; filter-sterilize)
 - 3 M potassium acetate, pH 5.5 (autoclave and store at +4°C)
 - Isopropanol
 - 70% ethanol
 - 1X TE Buffer, pH 8.0
-

DNA Isolation Procedure

1. Using a sterile toothpick, inoculate a single, isolated bacterial colony in 2 ml of LB medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline.
 2. Grow culture in a 37°C shaking incubator until cells reach stationary phase.
 3. Transfer 1.5 ml of bacterial culture to a 1.5 ml microcentrifuge tube and centrifuge at 14,000 x g for 1 minute to pellet cells.
 4. Remove the supernatant by vacuum aspiration and resuspend the cell pellet in 0.3 ml of Solution I. Gently vortex or pipet up and down to resuspend.
 5. Add 0.3 ml of Solution II and gently mix. Incubate at room temperature for 5 minutes. **Note:** The appearance of the suspension should change from turbid to almost translucent.
 6. Slowly add 0.3 ml of 3 M potassium acetate, pH 5.5, mixing gently during addition. A thick white precipitate of protein and *E. coli* genomic DNA will form. Place the sample on ice for 5 to 10 minutes.
 7. Centrifuge for 10 minutes at 14,000 x g.
 8. Gently transfer the supernatant to a microcentrifuge tube containing 0.8 ml of isopropanol. Do not transfer any white precipitate. Invert the tube a few times to mix and place on ice for 5 to 10 minutes. Proceed directly to Step 9 or store sample at -20°C overnight.
 9. Centrifuge the sample for 15 minutes at 14,000 x g at room temperature.
 10. Carefully remove the supernatant, taking care not to disturb the pellet. Add 0.5 ml of 70% ethanol. Invert the tube several times to wash the pellet.
 11. Centrifuge for 5 minutes at 14,000 x g at room temperature. Repeat Steps 10 and 11, if desired.
-

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Isolating Recombinant Bacmid DNA, continued

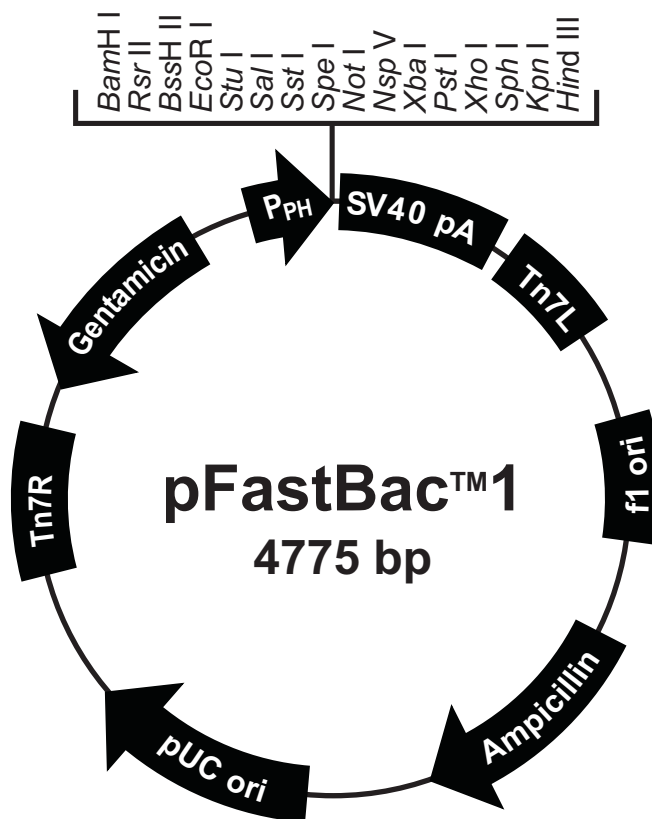
DNA Isolation Procedure, continued

12. Remove as much of the supernatant as possible, taking care not to disturb the pellet. Air dry the pellet for 5 to 10 minutes at room temperature. Do not overdry the pellet. **Important Note:** Do not use a SpeedVac to dry the DNA pellet as this may shear the DNA.
 13. Dissolve the DNA pellet in 40 μ l of 1X TE Buffer, pH 8.0. To avoid shearing, do not mechanically resuspend the DNA. Allow the solution to sit in the tube with occasional gentle tapping of the bottom of the tube.
 14. Store the DNA at +4°C. **Note:** We do not recommend storing the purified bacmid DNA at -20°C as repeated freezing and thawing may shear the DNA.
 15. Proceed to analyze the recombinant bacmid DNA (see **Analyzing Recombinant Bacmid DNA by PCR**, page 23) or to transfect the DNA into insect cells (see **Transfecting Insect Cells**, page 26).
-

Map and Features of pFastBac™ 1

pFastBac™ 1 Map

The map below shows the elements of pFastBac™ 1. The complete sequence of pFastBac™ 1 is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62).



Comments for pFastBac™ 1 4775 nucleotides

f1 origin: bases 2-457
 Ampicillin resistance gene: bases 589-1449
 pUC origin: bases 1594-2267
 Tn7R: bases 2511-2735
 Gentamicin resistance gene: bases 2802-3335 (complementary strand)
 Polyhedrin promoter (P_{PH}): bases 3904-4032
 Multiple cloning site: bases 4037-4142
 SV40 polyadenylation signal: bases 4160-4400
 Tn7L: bases 4429-4594

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Map and Features of pFastBac™ 1, continued

Features of the Vector

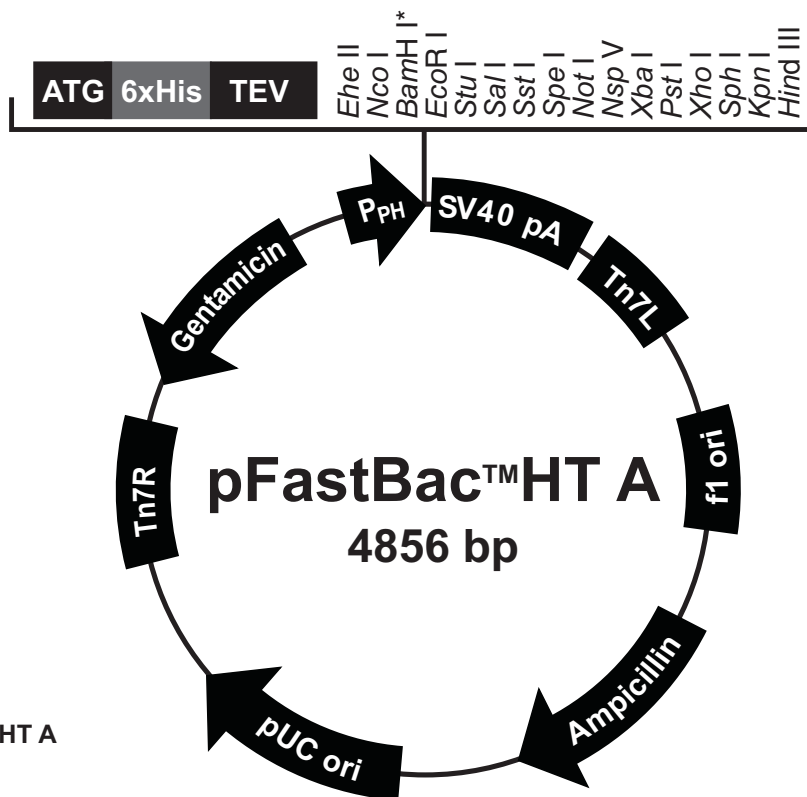
pFastBac™1 (4775 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Polyhedrin promoter (P _{PH})	Allows efficient, high-level expression of your recombinant protein in insect cells (O'Reilly <i>et al.</i> , 1992).
Multiple cloning site	Allows restriction enzyme-mediated cloning of your gene of interest.
SV40 polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA (Westwood <i>et al.</i> , 1993).
Tn7L and Tn7R	Mini Tn7 elements that permit site-specific transposition of the gene of interest into the baculovirus genome (<i>i.e.</i> bmon14272 bacmid) (Luckow <i>et al.</i> , 1993).
f1 origin	Allows rescue of single-stranded DNA.
Ampicillin resistance gene	Allows selection of the plasmid in <i>E. coli</i> .
pUC origin	Permits high-copy replication and maintenance in <i>E. coli</i> .
Gentamicin resistance gene	Permits selection of the recombinant bacmid in DH10Bac™ <i>E. coli</i> .

Map and Features of pFastBac™ HT

pFastBac™ HT A Map

The map below shows the elements of pFastBac™ HT A. The complete sequences of the pFastBac™ HT A, B, and C vectors are available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62).



Comments for pFastBac™ HT A 4856 nucleotides

f1 origin: bases 2-457
 Ampicillin resistance gene: bases 589-1449
 pUC origin: bases 1594-2267
 Tn7R: bases 2511-2735
 Gentamicin resistance gene: bases 2802-3335 (complementary strand)
 Polyhedrin promoter (P_{PH}): bases 3904-4032
 Initiation ATG: bases 4050-4052
 6xHis tag: bases 4062-4079
 TEV recognition site: bases 4101-4121
 Multiple cloning site: bases 4119-4222
 SV40 polyadenylation signal: bases 4240-4480
 Tn7L: bases 4509-4674

*Frameshift occurs at the *Bam*H I site in each vector

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Map and Features of pFastBac™ HT, continued

Features of the Vector

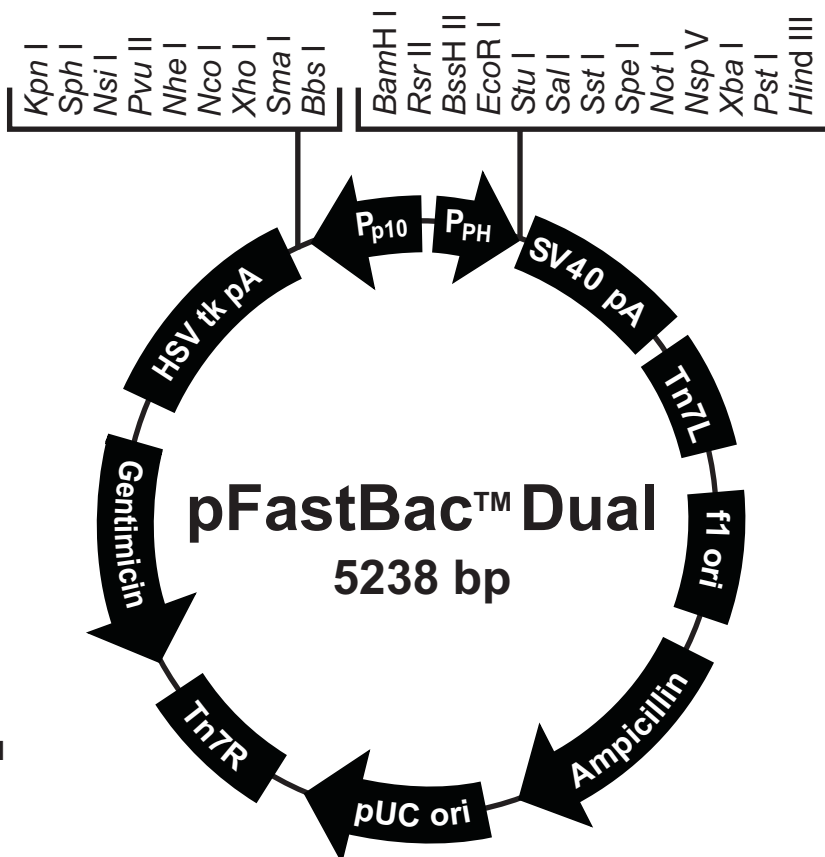
The pFastBac™ HT A (4856 bp), B (4857 bp), and C (4858 bp) vectors contain the following elements. All features have been functionally tested.

Feature	Benefit
Polyhedrin promoter (P _{PH})	Allows efficient, high-level expression of your recombinant protein in insect cells (O'Reilly <i>et al.</i> , 1992).
6xHis tag	Allows purification of your recombinant protein using a metal-chelating resin such as ProBond™ (Catalog no. K850-01) or Ni-NTA (Catalog no. K950-01).
TEV recognition site	Permits removal of the N-terminal tag from your recombinant protein using TEV protease (Carrington and Dougherty, 1988; Dougherty <i>et al.</i> , 1988).
Multiple cloning site	Allows restriction enzyme-mediated cloning of your gene of interest.
SV40 polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA (Westwood <i>et al.</i> , 1993).
Tn7L and Tn7R	Mini Tn7 elements that permit site-specific transposition of the gene of interest into the baculovirus genome (<i>i.e.</i> bmon14272 bacmid) (Luckow <i>et al.</i> , 1993).
f1 origin	Allows rescue of single-stranded DNA.
Ampicillin resistance gene	Allows selection of the plasmid in <i>E. coli</i> .
pUC origin	Permits high-copy replication and maintenance in <i>E. coli</i> .
Gentamicin resistance gene	Permits selection of the recombinant bacmid in DH10Bac™ <i>E. coli</i> .

Map and Features of pFastBac™ Dual

pFastBac™ Dual Map

The map below shows the elements of pFastBac™ Dual. The complete sequence of pFastBac™ Dual is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62).



Comments for pFastBac™ Dual 5238 nucleotides

f1 origin: bases 102-557

Ampicillin resistance gene: bases 689-1549

pUC origin: bases 1694-2367

Tn7R: bases 2611-2835

Gentamicin resistance gene: bases 2902-3435 (complementary strand)

HSV tk polyadenylation signal: bases 3992-4274 (complementary strand)

Multiple cloning site: bases 4274-4337 (complementary strand)

p10 promoter (P_{p10}): bases 4338-4459 (complementary strand)

Polyhedrin promoter (P_{PH}): bases 4478-4606

Multiple cloning site: bases 4606-4704

SV40 polyadenylation signal: bases 4722-4962

Tn7L: bases 4991-5156

continued on next page

Map and Features of pFastBac™ Dual, continued

Features of the Vector

pFastBac™ Dual (5238 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Polyhedrin promoter (P _{PH})	Allows efficient, high-level expression of your recombinant protein in insect cells (O'Reilly <i>et al.</i> , 1992).
Multiple cloning site (P _{PH})	Allows restriction enzyme-mediated cloning of your gene of interest.
SV40 polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA (Westwood <i>et al.</i> , 1993).
Tn7L and Tn7R	Mini Tn7 elements that permit site-specific transposition of the gene of interest into the baculovirus genome (<i>i.e.</i> bmon14272 bacmid) (Luckow <i>et al.</i> , 1993).
f1 origin	Allows rescue of single-stranded DNA.
Ampicillin resistance gene	Allows selection of the plasmid in <i>E. coli</i> .
pUC origin	Permits high-copy replication and maintenance in <i>E. coli</i> .
Gentamicin resistance gene	Permits selection of the recombinant bacmid in DH10Bac™ <i>E. coli</i> .
Herpes Simplex Virus (HSV) thymidine kinase (tk) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985).
Multiple cloning site (P _{p10})	Allows restriction enzyme-mediated cloning of your gene of interest.
p10 promoter (P _{p10})	Allows efficient, high-level expression of your recombinant protein in insect cells (O'Reilly <i>et al.</i> , 1992).

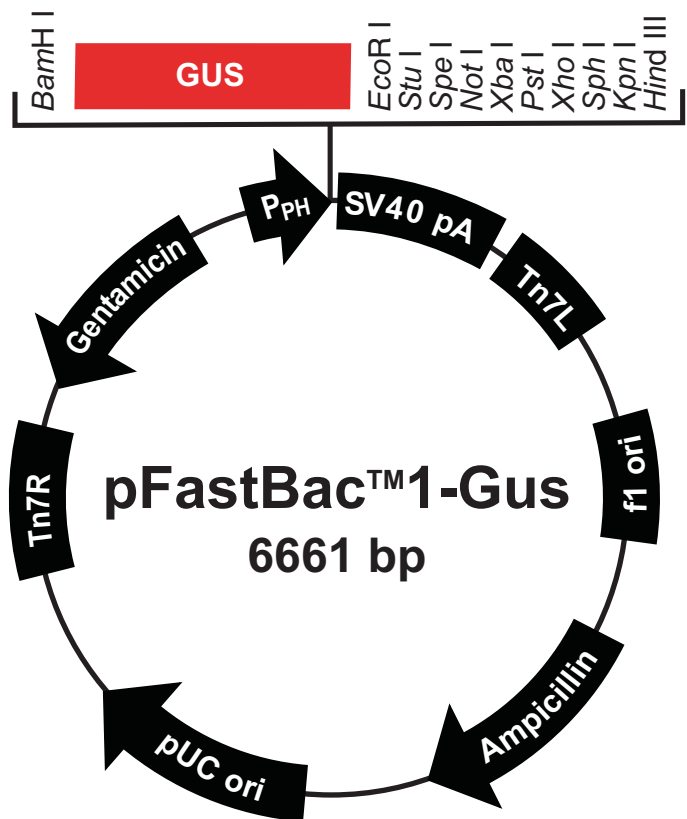
Map of pFastBac™ 1-Gus

Description

pFastBac™1-Gus is a 6661 bp control vector containing the *Arabidopsis thaliana* gene for β -glucuronidase (Gus) (Kertbundit *et al.*, 1991), and was generated by restriction cloning of the Gus gene into pFastBac™1. The molecular weight of β -glucuronidase is 68.5 kDa.

pFastBac™ 1-Gus Map

The figure below summarizes the features of the pFastBac™1-Gus vector. The complete sequence of pFastBac™1-Gus is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62).



Comments for pFastBac™1-Gus 6661 nucleotides

f1 origin: bases 2-457

Ampicillin resistance gene: bases 589-1449

pUC origin: bases 1594-2267

Tn7R: bases 2511-2735

Gentamicin resistance gene: bases 2802-3335 (complementary strand)

Polyhedrin promoter (P_{PH}): bases 3904-4032

GUS ORF: bases 4081-5892

SV40 polyadenylation signal: bases 6047-6287

Tn7L: bases 6315-6480

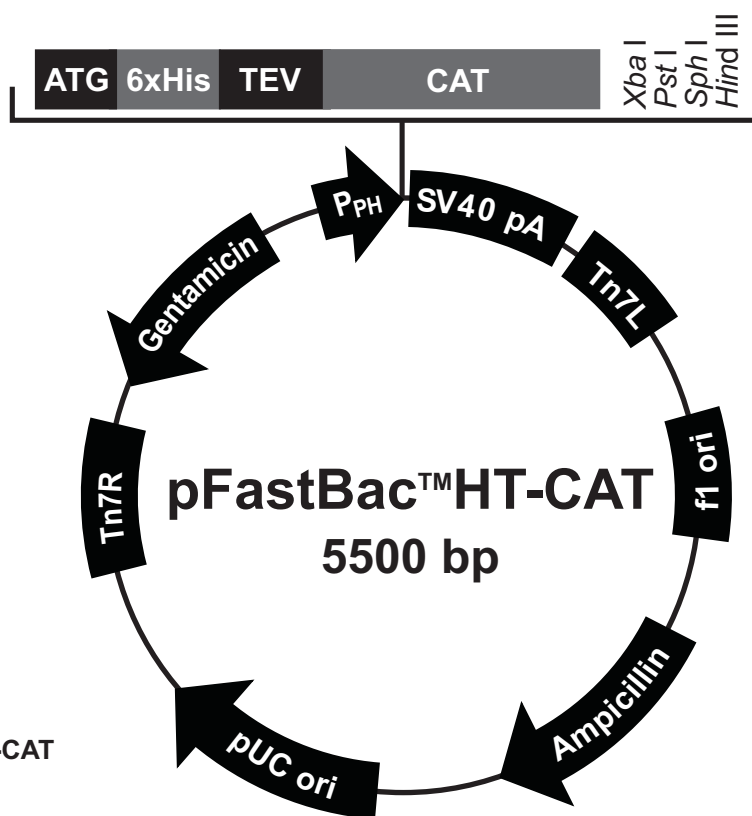
Map of pFastBac™ HT-CAT

Description

pFastBac™ HT-CAT is a 5500 bp control vector containing the gene for chloramphenicol acetyltransferase (CAT), and was generated by restriction cloning of the CAT gene into pFastBac™ HT. The CAT gene is expressed as a fusion to the N-terminal 6xHis tag. The molecular weight of the fusion protein is 28 kDa.

pFastBac™ HT-CAT Map

The figure below summarizes the features of the pFastBac™ HT-CAT vector. The complete sequence of pFastBac™ HT-CAT is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62).



Comments for pFastBac™ HT-CAT 5500 nucleotides

f1 origin: bases 2-457

Ampicillin resistance gene: bases 589-1449

pUC origin: bases 1594-2267

Tn7R: bases 2511-2735

Gentamicin resistance gene: bases 2802-3335 (complementary strand)

Polyhedrin promoter (P_{PH}): bases 3904-4032

Initiation ATG: bases 4050-4052

6xHis tag: bases 4062-4079

TEV recognition site: bases 4101-4121

CAT ORF: bases 4131-4790

SV40 polyadenylation signal: bases 4884-5124

Tn7L: bases 5153-5318

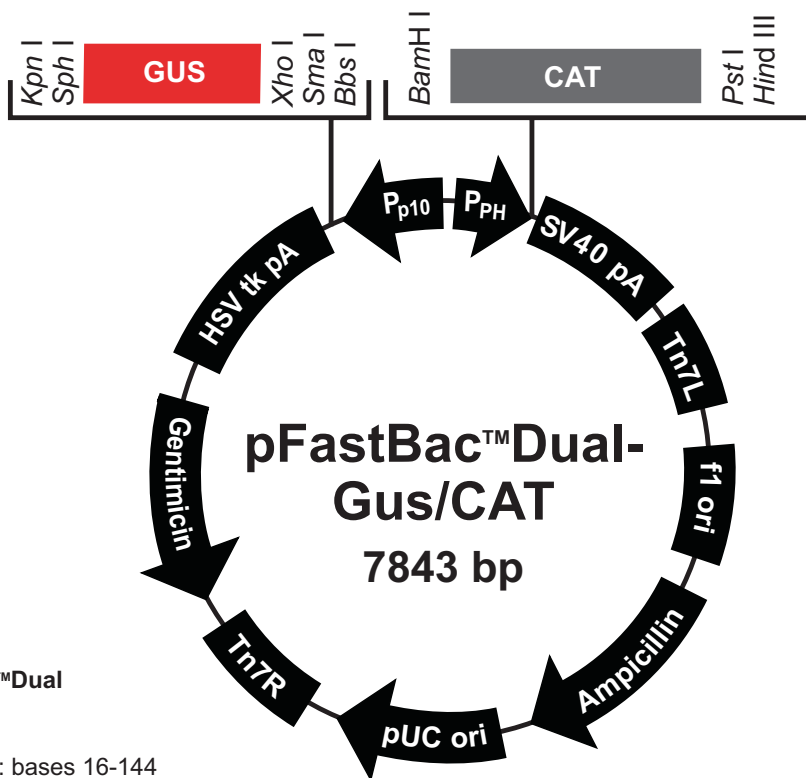
Map of pFastBac™ Dual-Gus/CAT

Description

pFastBac™ Dual-Gus/CAT is a 7843 bp control vector containing the *Arabidopsis thaliana* gene for β-glucuronidase (Gus) (Kertbundit *et al.*, 1991) and the chloramphenicol acetyltransferase (CAT) gene. The vector was generated by restriction cloning of the Gus and CAT genes into pFastBac™ Dual. Expression of CAT and Gus are controlled by the polyhedrin (PH) and p10 promoters, respectively. The molecular weight of β-glucuronidase and CAT are 68.5 kDa and 26 kDa, respectively.

pFastBac™ Dual-Gus/CAT Map

The figure below summarizes the features of the pFastBac™ Dual-Gus/CAT vector. **The complete sequence of pFastBac™ Dual-Gus/CAT is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 62).**



Comments for pFastBac™Dual 5238 nucleotides

- Polyhedrin promoter (P_{PH}): bases 16-144
- CAT ORF: bases 181-840
- SV40 polyadenylation signal: bases 964-1204
- Tn7L: bases 4991-5156
- f1 origin: bases 1582-2037
- Ampicillin resistance gene: bases 2169-3029
- pUC origin: bases 3174-3847
- Tn7R: bases 4091-4315
- Gentamicin resistance gene: bases 4382-4915 (complementary strand)
- HSV tk polyadenylation signal: bases 5472-5754 (complementary strand)
- GUS ORF: bases 5878-7689 (complementary strand)
- p10 promoter (P_{p10}): bases 7719-7840 (complementary strand)

Technical Service

World Wide Web



Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

<http://www.invitrogen.com>

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

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MSDS Requests

To request an MSDS, visit our Web site at www.invitrogen.com. On the home page, go to 'Technical Resources', select 'MSDS', and follow instructions on the page.

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Technical Service, continued

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Information for European Customers

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Purchaser Notification, continued

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Vectors and
Reagents**

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Product Qualification

pFastBac™ Vectors

1. Each vector is tested for transposition efficiency into bacmid DNA upon transformation into MAX Efficiency® DH10Bac™ competent cells following the procedure on page 21. Transposition frequency should be greater than 8% (% white colonies).
2. Restriction analysis with the enzymes below is performed on each vector to confirm its identity. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

Vector	Restriction Enzyme	Expected Fragments (bp)
pFastBac™1	<i>Bam</i> H I	4776
	<i>Bgl</i> II	470, 4306
	<i>Eco</i> R I	4776
	<i>Hind</i> III	4776
	<i>Not</i> I	4776
	<i>Sal</i> I	4776
pFastBac™1-Gus	<i>Bam</i> H I	6661
	<i>Bgl</i> II	2508, 2886
	<i>Eco</i> R I	1267, 5394
	<i>Hind</i> III	6661
	<i>Not</i> I	6661
	<i>Sal</i> I	1886, 4775
pFastBac™HT A	<i>Bam</i> H I	4856
	<i>Bgl</i> II	1080, 1268, 2508
	<i>Eco</i> R I	4856
	<i>Eco</i> R V	1263, 3593
	<i>Hind</i> III	4856
	<i>Not</i> I	4856
	<i>Sal</i> I	4856
pFastBac™HT B	<i>Bam</i> H I	4857
	<i>Bgl</i> II	470, 4387
	<i>Eco</i> R I	4857
	<i>Eco</i> R V	404, 1263, 3190
	<i>Hind</i> III	4857
	<i>Not</i> I	4857
	<i>Sal</i> I	4857

continued on next page

Product Qualification, continued

pFastBac™ Vectors, continued

Vector	Restriction Enzyme	Expected Fragments (bp)
pFastBac™ HT C	<i>Bam</i> H I	4858
	<i>Bgl</i> II	470, 1474, 2914
	<i>Eco</i> R I	4858
	<i>Eco</i> R V	1263, 3595
	<i>Hind</i> III	4858
	<i>Not</i> I	4858
	<i>Sal</i> I	4858
pFastBac™ HT-CAT	<i>Bam</i> H I	707, 4793
	<i>Bgl</i> II	1268, 1724, 2508
	<i>Eco</i> R I	5500
	<i>Eco</i> R V	1263, 4237
	<i>Hind</i> III	5500
	<i>Sal</i> I	5500
	pFastBac™ Dual	<i>Bam</i> H I
<i>Bgl</i> II		470, 4768
<i>Eco</i> R I		5238
<i>Hind</i> III		5238
<i>Nco</i> I		5238
<i>Not</i> I		5238
<i>Sal</i> I		5238
pFastBac™ Dual-Gus/CAT	<i>Acc</i> I	479, 2028, 5336
	<i>Nco</i> I	852, 6991
	<i>Sal</i> I	7843

Cellfectin® Reagent

Cellfectin® Reagent is tested for the absence of microbial contamination using blood agar plates, Sabaraud dextrose agar plates, and fluid thioglycolate medium, and functionally by transfection of COS-7 cells with a luciferase reporter-containing plasmid.

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Product Qualification, continued

**MAX Efficiency[®]
DH10Bac[™]
Chemically
Competent *E. coli***

1. MAX Efficiency[®] DH10Bac[™] competent cells are tested for transformation efficiency using the pUC19 control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Transformation efficiency should be greater than 1×10^8 cfu/µg pUC19 with non-saturating amounts (50 µg) of DNA, and greater than 1×10^5 cfu/µg pUC19 with saturating amounts (25 ng) of DNA.
 2. DH10Bac[™] competent cells are tested for transposition efficiency using 1 ng of the pFastBac[™]1-Gus plasmid and following the procedure on page 21. Transposition frequency should be greater than 8% (% white colonies).
 3. Untransformed cells are tested for the appropriate antibiotic sensitivity and absence of phage contamination.
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