



pMIB/V5-His A, B, and C Vector Kit

**For the Selection of Transfected Cells and
Stable Expression of Secreted Heterologous
Proteins in Lepidopteran Insect Cell Lines**

Catalog no. V8030-01

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User Manual

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Important Information

Shipping/Storage The pMIB/V5-His Vector Kit is shipped on wet ice. Upon receipt, store the kit at -20°C .

Kit Contents The following items are supplied with each pMIB/V5-His Vector Kit. Store at -20°C .

Item	Composition	Volume
pMIB/V5-His A, B, and C	20 μg each at 0.5 $\mu\text{g}/\mu\text{l}$, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	40 μl
pMIB/V5-His/CAT	20 μg at 0.5 $\mu\text{g}/\mu\text{l}$, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	40 μl
OpIE2 Forward Sequencing Primer	Lyophilized in TE, pH 8.0	2 μg
OpIE2 Reverse Sequencing Primer	Lyophilized in TE, pH 8.0	2 μg

Product Qualification The Certificate of Analysis provides detailed quality control information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Primer Sequences The sequence of each primer is provided below:

Primer	Sequence	pMoles Supplied
OpIE2 Forward	5'-CGCAACGATCTGGTAAACAC-3'	329
OpIE2 Reverse	5'-GACAATACAACTAAGATTTAGTCAG-3'	250

Reagents Supplied by the User Be sure to have the following reagents and equipment on hand before starting experiments:

- Express Five[®] Serum-Free Medium (recommended)
 - Grace's medium (optional)
 - Fetal Bovine Serum (FBS) (optional)
 - 1, 5, 10, and 25 ml sterile pipettes
 - Cryovials
 - Hemacytometer and Trypan Blue (see recipe on page 22)
 - Table-top centrifuge
 - 60 mm tissue culture plates (other flasks and plates may be used)
 - Sterile microcentrifuge tubes (1.5 ml)
 - Cell Lysis Buffer (see recipe on page 23)
 - PBS (see recipe on page 23)
 - Cloning cylinders (optional)
 - 96-well plates (optional)
-

Accessory Products

Introduction

The products listed in this section are intended for use with the pMIB/V5-His Vector kit. For more information, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 30).

Products Available Separately

The products listed below may be used with the pMIB/V5-His Vector Kit and are available separately from Invitrogen.

Product	Amount	Catalog no.
Sf9 Cells, frozen	1 ml vial, 1×10^7 cells/ml	B825-01
Sf21 Cells, frozen	1 ml vial, 1×10^7 cells/ml	B821-01
High Five™ Cells, frozen	1 ml vial, 3×10^6 cells/ml	B855-02
Grace's Insect Cell Culture Medium, Unsupplemented	500 ml	11595-030
Sf-900 II SFM	1 liter	10902-088
Express Five® SFM	1 liter	10486-025
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
Cellfectin® Reagent	1 ml	10362-010
Blasticidin S	50 mg	R210-01

Other InsectSelect™ Kits

Several other kits that allow you to clone and stably express your gene of interest using the InsectSelect™ technology are available from Invitrogen. These kits include InsectSelect™ vectors with different antibiotic resistance genes. In addition, the pIZT/V5-His Vector Kit enables expression of a gene of interest and a cycle 3-GFP/Zeocin™ fusion gene. This allows both visual monitoring of transfection efficiency and generation of a stable cell line. For more information about the various InsectSelect™ vector kits available from Invitrogen, visit our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 30). See the table below for ordering information.

Product	Catalog no.
pIB/V5-His TOPO® TA Expression Kit	K890-01
pIB/V5-His Vector Kit	V8020-01
pIZ/V5-His Vector Kit	V8000-01
pIZT/V5-His Vector Kit	V8010-01

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Accessory Products, continued

Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using an antibody to the appropriate epitope. The table below describes the antibodies available for detection of

C-terminal fusion proteins expressed using pMIB/V5-His. Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods.

Fifty microliters of each antibody is supplied which is sufficient for 25 Westerns.

Product	Epitope	Catalog no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern <i>et al.</i> , 1991) GKPIP NPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner <i>et al.</i> , 1997) HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His (C-term)-AP Antibody		R932-25

Purification of Recombinant Protein

The metal binding domain encoded by the polyhistidine tag allows simple, easy purification of your recombinant protein by Immobilized Metal Affinity Chromatography (IMAC) using Invitrogen's ProBond™ Resin (see below). To purify proteins expressed using the InsectSelect™ System, the ProBond™ Purification System or the ProBond™ resin in bulk are available separately. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Metal-Binding Resin (precharged resin provided as a 50% slurry in 20% ethanol)	50 ml	R801-01
	150 ml	R801-15
ProBond™ Purification System	6 purifications	K850-01
Purification Columns (10 ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

The InsectSelect™ technology facilitates constitutive stable or transient expression of recombinant proteins in insect cell lines. pMIB/V5-His A, B, and C are 3.6 kb vectors that use the InsectSelect™ technology to allow expression and secretion of your protein of interest in insect cell lines. The pMIB/V5-His vector contains the following features:

- *OpIE2* promoter for high-level, constitutive expression of the gene of interest (Theilmann and Stewart, 1992)
- Honeybee melittin secretion signal (HBM) for directing secreted expression of the gene of interest (Tessier *et al.*, 1991)
- *OpIE1* promoter for expression of the blasticidin resistance gene (see next bullet) (Theilmann and Stewart, 1991)
- Blasticidin resistance gene for selection of stable cell lines (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965)
- EM7 promoter for expression of ampicillin and blasticidin resistance in *E. coli*
- Ampicillin resistance gene for selection of transformants in *E. coli*
- C-terminal peptide containing the V5 epitope and 6xHis tag for detection and purification of your protein of interest (if desired)
- Three reading frames to facilitate in-frame cloning with the C-terminal peptide

The control plasmid, pMIB/V5-His/CAT, is included for use as a positive control for expression and secretion.

Description of System

The gene of interest is cloned into pMIB/V5-His and transfected into Sf9 or High Five™ cells using lipid-mediated transfection. After transfection, cells can be assayed for secreted expression of the gene of interest. Once you have confirmed that your gene expresses, you can select for a stable polyclonal population or stable clonal cell lines using blasticidin as a selection agent. Stable cell lines can be used to express the protein of interest in either adherent culture or suspension culture.

Description of Promoters

Baculovirus immediate-early promoters utilize the host cell transcription machinery and do not require viral factors for activation. Both the *OpIE2* and *OpIE1* promoters are from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*OpMNPV*). The virus' natural host is the Douglas fir tussock moth; however, the promoters allow protein expression in *Lymantria dispar* (LD652Y), *Spodoptera frugiperda* cells (Sf9) (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997), Sf21 (Invitrogen), *Trichoplusia ni* (High Five™) (Invitrogen), *Drosophila* (Kc1, S2) (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997), and mosquito cell lines (unpublished data). The *OpIE2* promoter has been shown to be about 5- to 10-fold stronger than the *OpIE1* promoter (Pfeifer *et al.*, 1997). Both promoters have been sequenced and analyzed. For more detailed information on the *OpIE2* and *OpIE1* promoters, see page 27 and page 28, respectively.

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Overview, continued

Expression Levels The *OpIE2* promoter provides relatively high levels of constitutive expression, although not all proteins will be expressed at levels equivalent to those obtained from baculovirus very late promoters (e.g. polyhedrin or p10). However, other proteins may be expressed more efficiently in the InsectSelect™ System than in baculovirus systems (Jarvis *et al.*, 1996). To date, reported expression levels range from 1–2 g/ml (human IL-6; Invitrogen) to 8–10 g/ml (human melanotransferrin) (Hegedus *et al.*, 1999).

Blasticidin Resistance Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two blasticidin S deaminase genes: *BSD* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a non-toxic deaminohydroxy (Kimura and Yamaguchi, 1996; Yamaguchi *et al.*, 1975).

Experimental Outline The table below describes the general steps needed to clone and express your gene of interest. For more details, refer to the pages indicated. Information on how to culture insect cell lines may be found in our Insect Cell Lines manual. This manual may be downloaded from our Web site (www.invitrogen.com).

Step	Action	Page
1	Establish culture of Sf9, Sf21, or High Five™ cells.	3
2	Develop a cloning strategy to ligate your gene of interest into pMIB/V5-His A, B, or C in frame with the honeybee melittin secretion signal and the C-terminal peptide encoding the V5 epitope and the polyhistidine (6xHis) tag (if desired).	4-7
3	Transform your ligation reactions into a <i>recA</i> , <i>endA</i> <i>E. coli</i> strain (e.g. TOP10). Select on LB plates containing 50–100 µg/ml ampicillin or 100 µg/ml blasticidin in Low Salt LB.	8
4	Use sequencing to confirm that your protein is cloned in frame with the honeybee melittin secretion signal and the C-terminal peptide (if desired).	8
5	Transfect Sf9, Sf21, or High Five™ cells.	10-12
6	Assay for transient expression of your protein.	12-14
7	Create stable cell lines expressing the protein of interest by selecting with the appropriate concentration of blasticidin.	16-19
8	Scale-up expression for purification.	20
9	Purify your recombinant protein by chromatography on metal-chelating resin (e.g. ProBond™).	20-21

Methods

Culturing Insect Cells

Introduction

Before you start your cloning experiments, be sure to have cell cultures of Sf9, Sf21, or High Five™ cells growing and have frozen master stocks available.

Cells for Transfection

You will need log-phase cells with >95% viability to perform a successful transfection. Review pages 10-12 to determine how many cells you will need for transfection.

Insect Cell Lines Manual

For additional information on insect cell culture, refer to the Insect Cell Lines manual. This manual contains information on:

- Thawing frozen cells
- Maintaining and passaging cells
- Freezing cells
- Using serum-free medium
- Growing cells in suspension
- Scaling up cell culture

This manual is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 30).

Cloning into pMIB/V5-His A, B, and C

Introduction

The pMIB/V5-His kit supplies vectors with multiple cloning sites in three reading frames (A, B, and C) to facilitate cloning your gene of interest in frame with the C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag. Use the diagrams provided on pages 5-7 to design a strategy to clone your gene of interest in frame with the HBM secretion signal and the C-terminal peptide.

General Molecular Biology Techniques

For help with *E. coli* transformations, DNA ligations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, 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 pMIB/V5-His A, B, C, and pMIB/V5-His/CAT vectors contain the ampicillin and blasticidin resistance genes to allow selection of the plasmid in *E. coli* using ampicillin or blasticidin, respectively. To propagate and maintain the pMIB/V5-His and pMIB/V5-His/CAT plasmids, we recommend using the following procedure:

1. Use 10 ng of each vector to transform a *recA*, *endA* *E. coli* strain like TOP10, DH5, JM109, or equivalent (see page 8 for more information).
 2. Select transformants on LB agar plates containing 50–100 µg/ml ampicillin or Low Salt LB agar plates containing 100 µg/ml blasticidin (see recipe page 22).
 3. Prepare a glycerol stock from each transformant containing plasmid for long-term storage (see page 9).
-

Cloning Considerations

pMIB/V5-His is a terminal fusion vector. To express your gene as a recombinant fusion protein, you **must** clone your gene in frame with the N-terminal HBM secretion signal. If you wish to include the C-terminal peptide for detection with either the V5 or His(C-term) antibodies or purification using the polyhistidine (6xHis) tag, you must also clone your gene in frame with the C-terminal peptide. The vector is supplied in three reading frames to facilitate cloning. Refer to the diagrams on pages 5-7 to develop a cloning strategy. **Be sure that your gene does not contain a stop codon upstream of the C-terminal peptide.**

If you do not wish to include the C-terminal peptide, include the native stop codon for your gene of interest.

Signal Sequence Processing

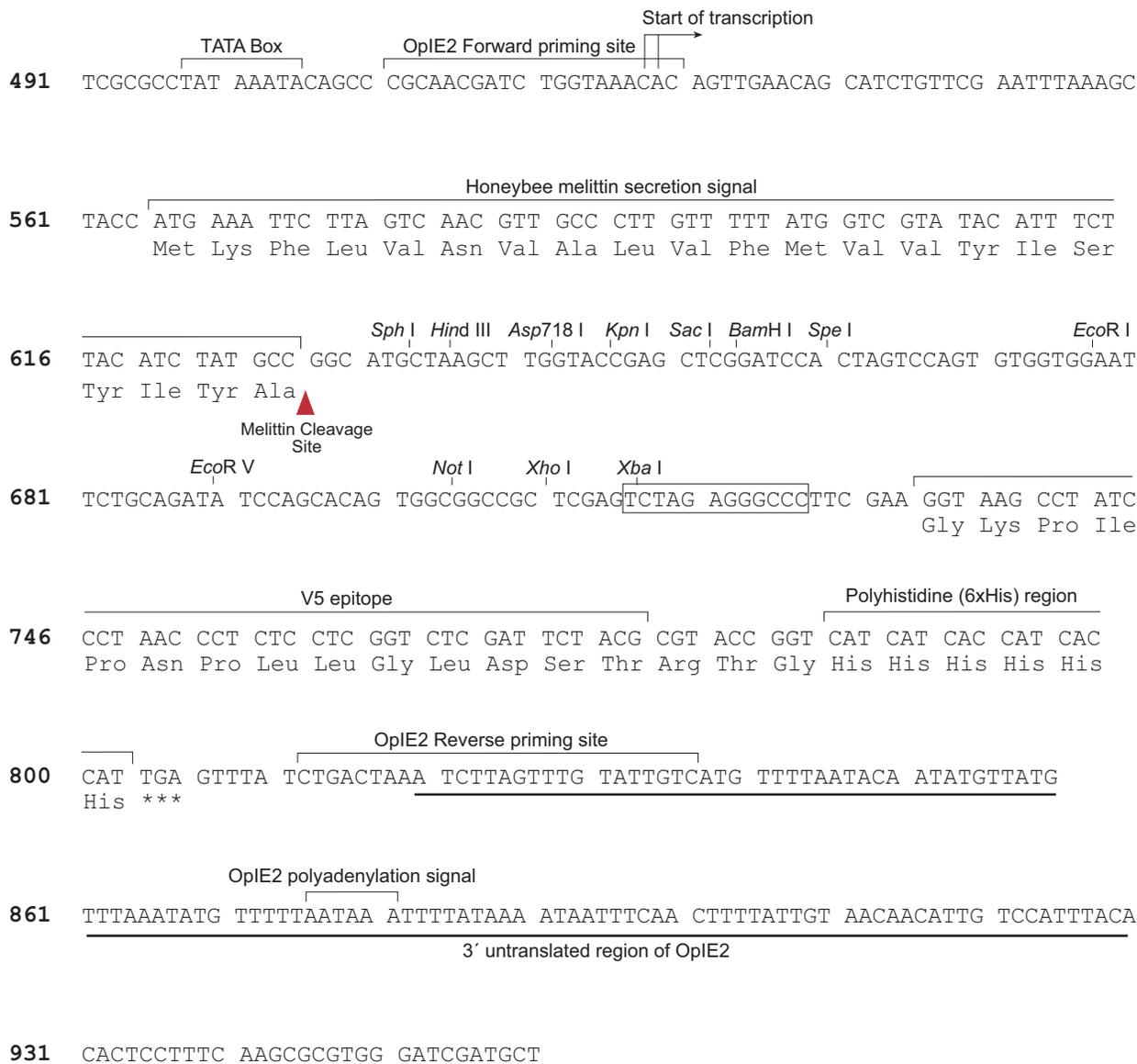
The HBM secretion signal is processed from your recombinant protein by a signal peptidase-directed cleavage after alanine²¹ in the signal sequence (Tessier *et al.*, 1991). For the location of the melittin cleavage site, refer to the diagrams on pages 5-7. Note that you will not obtain native protein following cleavage of the signal sequence because of the intervening sequences between the melittin cleavage site and the restriction site of interest in the multiple cloning site (see diagrams on pages 5-7). For example, your recombinant protein will contain at least two extra amino acids following cleavage of the secretion signal if you clone your gene into the *Sph* I site.

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Cloning into pMIB/V5-His A, B, and C, continued

Multiple Cloning Site of pMIB/V5-His A

Below is the multiple cloning site for pMIB/V5-His A. The TATA box, start of transcription, and the polyadenylation signal are marked as described in Theilmann and Stewart, 1992. Restriction sites are labeled to indicate the actual cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pMIB/V5-His A is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 30).** For a map and a description of the features of pMIB/V5-His A, refer to pages 24-25.



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Cloning into pMIB/V5-His A, B, and C, continued

Multiple Cloning Site of pMIB/V5-His B

Below is the multiple cloning site for pMIB/V5-His B. The TATA box, start of transcription, and the polyadenylation signal are marked as described in Theilmann and Stewart, 1992. Restriction sites are labeled to indicate the actual cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pMIB/V5-His B is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 30).** For a map and a description of the features of pMIB/V5-His B, refer to pages 24-25.

Start of transcription

491 TCGCGCCTAT AAATACAGCC CGCAACGATC TGGTAAACAC AGTTGAACAG CATCTGTTTCG AATTTAAAGC

Honeybee melittin secretion signal

561 TACC ATG AAA TTC TTA GTC AAC GTT GCC CTT GTT TTT ATG GTC GTA TAC ATT TCT
Met Lys Phe Leu Val Asn Val Ala Leu Val Phe Met Val Val Tyr Ile Ser

616 TAC ATC TAT GCC GGC ATGCTAAGCT TGGTACCGAG CTCGGATCCA CTAGTCCAGT GTGGTGGAA
Tyr Ile Tyr Ala

Melittin Cleavage Site

681 TCTGCAGATA TCCAGCACAG TGGCGGCCGC TCGAGTCTAG AGGGCCCGCG GTTCGAA GGT AAG CCT
Gly Lys Pro

V5 epitope

747 ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT
Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His

OpIE2 Reverse priming site

801 CAC CAT TGA G TTTATCTGAC TAAATCTTAG TTTGTATTGT CATGTTTTAA TACAATATGT
His His ***

OpIE2 polyadenylation signal

861 TATGTTTAAA TATGTTTTTA ATAAATTTTA TAAAATAATT TCAACTTTTA TTGTAACAAC ATTGTCCATT

3' untranslated region of OpIE2

931 TACACACTCC TTTCAAGCGC GTGGGATCGA TGCTCACTCA

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Cloning into pMIB/V5-His A, B, and C, continued

Multiple Cloning Site of pMIB/V5-His C

Below is the multiple cloning site for pMIB/V5-His C. The TATA box, start of transcription, and the polyadenylation signal are marked as described in Theilmann and Stewart, 1992. Restriction sites are labeled to indicate the actual cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pMIB/V5-His C is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 30).** For a map and a description of the features of pMIB/V5-His C, refer to pages 24-25.

491 TCGCGCCTAT AAATACAGCC CGCAACGATC TGGTAAACAC AGTTGAACAG CATCTGTTTCG AATTTAAAGC

TATA Box OplE2 Forward priming site Start of transcription

561 TACC ATG AAA TTC TTA GTC AAC GTT GCC CTT GTT TTT ATG GTC GTA TAC ATT TCT
Met Lys Phe Leu Val Asn Val Ala Leu Val Phe Met Val Val Tyr Ile Ser

Honeybee melittin secretion signal

616 TAC ATC TAT GCC GGC ATGCTAAGCT TGGTACCGAG CTCGGATCCA CTAGTCCAGT GTGGTGGAAAT
Tyr Ile Tyr Ala

Sph I Hind III Asp718 I Kpn I Sac I BamHI Spe I EcoRI

681 TCTGCAGATA TCCAGCACAG TGGCGGCCGC TCGAG GTCAC CCA TTTCGAA GGT AAG CCT ATC CCT
Gly Lys Pro Ile Pro

EcoR V Not I Xho I BstE II

745 AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT
Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His His His

V5 epitope Polyhistidine (6xHis) region

799 TGA GTTTATCTG ACTAAATCTT AGTTTGTATT GTCATGTTTT AATACAATAT GTTATGTTTA

OplE2 Reverse priming site

861 AATATGTTTT TAATAAATTT TATAAAATAA TTTCAACTTT TATTGTAACA ACATTGTCCA TTTACACACT

OplE2 polyadenylation signal

3' untranslated region of OplE2

931 CCTTTCAAGC GCGTGGGATC GATGCTCACT

Transforming *E. coli*

Introduction

Once you have completed your ligation reactions, you are ready to transform into *E. coli*. Many strains and transformation protocols are suitable. General recommendations are provided below.

E. coli Host

Many *E. coli* strains are suitable for transformation of pMIB/V5-His including TOP10 (Catalog no. C610-00) or DH5. We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*recA*) and endonuclease A deficient (*endA*). For your convenience, TOP10 is available as electrocompetent or chemically competent cells from Invitrogen.

Item	Quantity	Catalog no.
Electrocomp™ TOP10	5 x 80 µl	C664-55
	10 x 80 µl	C664-11
	30 x 80 µl	C664-24
One Shot™ TOP10 (chemically competent cells)	21 x 50 µl	C4040-03

Transformation Method

You may use any method of choice to transform *E. coli*. Chemical transformation is the most convenient for many researchers. Electroporation is the most efficient and the method of choice for large plasmids. To select transformants, use LB agar plates containing 50–100 µg/ml ampicillin or Low Salt LB agar plates containing 100 µg/ml blasticidin (see below). Once you have obtained ampicillin (or blasticidin) resistant colonies, pick 10 transformants and screen for the presence and orientation of your insert.



Important

To facilitate selection of blasticidin-resistant *E. coli*, the salt concentration of the medium must remain low (<90 mM) and the pH must be 7.0. Prepare Low Salt LB broth and plates using the recipe in the **Appendix**, page 22.

Failure to lower the salt content of your LB medium will result in non-selection due to inhibition of the drug.



We recommend that you sequence your construct to confirm that your gene is fused in frame with the N-terminal HBM secretion signal and the C-terminal V5 epitope and the polyhistidine tag. Use the OpIE2 Forward and Reverse sequencing primers included in your kit or a primer to your gene of interest to sequence your insert.

Note: Resuspend each primer in 20 µl sterile water to prepare a 0.1 µg/µl stock solution.

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Transforming *E. coli*, continued

Long-Term Storage

Once you have confirmed that you have the correct clone, prepare a glycerol stock for long-term storage. It is also a good idea to keep a stock of plasmid DNA at -20°C .

To prepare a glycerol stock:

1. Grow the *E. coli* strain containing the plasmid overnight.
 2. Combine 0.85 ml of the overnight culture with 0.15 ml of sterile glycerol.
 3. Vortex and transfer to a labeled cryovial.
 4. Freeze the tube in liquid nitrogen or dry ice/ethanol bath and store at -80°C .
-

Transient Expression in Insect Cells

Introduction

Once you have cloned your gene of interest into pMIB/V5-His, you are ready to transfect your construct into Sf9, Sf21, or High Five™ cells using lipid-mediated transfection and test for expression of your protein.

Plasmid Preparation

Plasmid DNA for transfection into insect cells must be very pure and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or other resin-based DNA purification systems. The PureLink™ HQ Mini Plasmid Purification Kit is a small-scale plasmid isolation kit that isolates 10–15 µg of plasmid DNA from 10–15 ml of bacterial culture. Plasmid can be used directly for transfection of insect cells.

Method of Transfection

We recommend lipid-mediated transfection with Cellfectin® Reagent. Note that other lipids may be substituted, although transfection conditions may have to be optimized.

Expected Transfection Efficiency using Cellfectin® Reagent:

- 40–60% for Sf9 or Sf21 cells
- 40–60% for High Five™ cells

Note: Other transfection methods (*e.g.* calcium phosphate and electroporation (Mann and King, 1989)) have also been tested with High Five™ cells.

Control of Plasmid Quality

To test the quality of a plasmid DNA preparation, include a mock transfection using DNA only (no lipids) in all transfection experiments. At about 24 to 48 hours posttransfection, compare the DNA only mock transfection with cells transfected with plasmid. If the plasmid preparation contains contaminants, then the cells will appear unhealthy and start to lyse.

Before Starting

You will need the following for each transfection experiment:

- 1–10 µg of highly purified plasmid DNA (~1 µg/µl in TE buffer)
 - Either log-phase Sf9 or Sf21 cells ($1.6\text{--}2.5 \times 10^6$ cells/ml, >95% viability) or log-phase High Five™ cells ($1.8\text{--}2.3 \times 10^6$ cells/ml, >95% viability)
 - Serum-free medium (see the next page)
 - 60 mm tissue-culture dishes
 - 1.5 ml sterile microcentrifuge tubes
 - Rocking platform only (NOT orbital)
 - 27°C incubator
 - Inverted Microscope
 - Paper towels and air-tight bags or containers
 - 5 mM EDTA, pH 8
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Transient Expression in Insect Cells, continued

Serum-Free Media Several serum-free media are available from Invitrogen for use in transfection experiments with pMIB/V5-His. Express Five® SFM (Catalog no. 10486-025) is recommended for use with High Five™ cells while Sf-900 II SFM (1X) (Catalog no. 10902-088) is optimized for use with Sf9 and Sf21 cells. Other serum-free media may be used, although you may have to optimize conditions for transfection and selection. Note that if you wish to transfect Sf9 or Sf21 cells in serum-free medium, you will need to adapt the cells to serum-free medium before transfection (see Insect Cell Lines manual for a protocol).

Prepare Cells For each transfection, use log-phase cells with greater than 95% viability. We recommend that you set up enough plates to perform a time course for expression of your gene of interest. Test for expression 2, 3, and 4 days posttransfection. You will need at least one 60 mm plate for each time point.

1. **For Sf9, Sf21 cells, or High Five™ cells**, seed 2×10^6 cells in appropriate serum-free medium in a 60 mm dish.
Rock gently from side to side for 2 to 3 minutes to evenly distribute the cells. Do not swirl the plates in a circular motion. Cells should be 50 to 60% confluent.
2. Incubate the cells for at least 15 minutes without rocking to allow the cells to fully attach to the bottom of the dish to form a monolayer of cells.
3. Verify that the cells have attached by inspecting them under an inverted microscope.

Positive and Negative Controls We recommend that you include the following controls:

- pMIB/V5-His/CAT vector as a positive control for transfection and expression
- Lipid only as a negative control
- DNA only to check for DNA contamination



Note

- If you use another lipid besides Cellfectin® Reagent, review the protocol on the next page and consult the manufacturer's instructions to adapt the protocol for your use. You may have to empirically determine the optimal conditions for transfection.
 - **Do not linearize** the plasmid prior to transfection. Linearizing the plasmid appears to decrease protein expression. The reason for this is not known.
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Transient Expression in Insect Cells, continued

Transfection Procedure

Plasmid DNA and Cellfectin[®] Reagent are mixed together in the appropriate medium (see below) and incubated with freshly seeded insect cells. The amount of cells, liposomes, and plasmid DNA has been optimized for 60 mm culture plates. It is important that you optimize transfection conditions if you use plates or flasks other than 60 mm plates.

Note: If you are using serum-free medium, we recommend using Sf-900 II SFM to transfect Sf9 cells and Express Five[®] SFM to transfect High Five[™] cells. If you are using Grace's Medium, be sure to use Grace's Medium without supplements. The proteins in the FBS and supplements will interfere with the liposomes, causing the transfection efficiency to decrease.

1. To prepare each transfection mixture, use a 1.5 ml microcentrifuge tube. Add the following reagents:
Grace's Insect Media (Sf9) **OR**
Appropriate serum-free medium 1 ml
pMIB/V5-His plasmid or construct (~1 µg/µl in TE, pH 8) 1–10 µl
Cellfectin[®] Reagent (**mix well before use and always add last**) 20 µl
2. Gently mix the transfection mixture for 10 seconds.
3. Incubate the transfection mixture at room temperature for 15 minutes. While the transfection mixture is incubating, proceed to Step 4.
4. Carefully remove the medium from the cells without disrupting the monolayer. **Note:** If you are using medium containing serum, wash the cells by carefully adding 2 ml of fresh Grace's Insect Media **without supplements or FBS**. This will remove trace amounts of serum that will decrease the efficiency of liposome transfection. Remove all of the medium from the monolayer.
5. Add the entire transfection mix dropwise into the 60 mm dish. Repeat for all transfections.
(Distribute the drops evenly over the monolayer. This method reduces the chances of disturbing the monolayer.)
6. Incubate the dishes at room temperature for 4 hours on a side-to-side, rocking platform. Adjust speed to ~2 side to side motions per minute. **Note:** If you do not have a rocker, manually rock the dishes periodically.
7. Following the 4-hour incubation period, add 1–2 ml of complete TNM-FH medium (Sf9 cells) or the appropriate serum-free medium to each 60 mm dish, place the dishes in a sealed plastic bag with moist paper towels to prevent evaporation and incubate at 27°C. **Note:** It is not necessary to remove the transfection solution as Cellfectin[®] Reagent is not toxic to the cells. If you are using a different lipid and observe loss of viability, then remove the transfection solution after 4 hours, rinse two times with medium, and replace with 1–2 ml of fresh medium.
8. Harvest the cells 2, 3, and 4 days posttransfection and assay for expression of your gene (see next page). There's no need to add fresh medium if the cells are sealed in an airtight plastic bag with moist paper towels.

continued on next page

Transient Expression in Insect Cells, continued

Testing for Secreted Protein Expression

Use the medium from one 60 mm plate for each expression experiment. If you are also harvesting cells, see **Preparing Cell Lysates** below. Before starting, prepare 2X SDS-PAGE sample buffer. A recipe is provided on page 23 for your convenience, but other recipes are suitable. If you are using pre-cast polyacrylamide gels (see below), refer to the manufacturer's instructions to prepare the appropriate sample buffer.

1. Prepare an SDS-PAGE gel that will resolve your expected recombinant protein.
2. Harvest the medium from the cells.

Note: Depending on the sensitivity of your antibody, media samples can be concentrated to approximately 10-fold prior to Western blot analysis. You may use any method to concentrate the media samples. We suggest using commercially available ultrafiltration devices (*e.g.* Centricon) or a Speed-Vac.

3. Mix 20 μ l of the media samples with 20 μ l of 2X SDS-PAGE sample buffer.
 4. Boil the samples for 5 minutes. Centrifuge briefly.
 5. Load samples, electrophorese, blot, and probe with a suitable antibody (see the next page).
 6. Visualize proteins using your desired method.
-



Note

The C-terminal tag containing the V5 epitope and 6xHis tag will increase the size of your protein by ~3 kDa. Note that any additional amino acids between your protein and the tags are not included in this molecular weight calculation.

Preparing Cell Lysates

Before starting, prepare Cell Lysis Buffer. A recipe is provided on page 23 for your convenience, but other recipes are suitable.

1. Prepare an SDS-PAGE gel that will resolve your expected recombinant protein.
 2. Remove the medium from each plate and prepare samples as detailed above.
 3. Wash the cells once with 1X PBS (see page 23 for a recipe). Add 100 μ l Cell Lysis Buffer and slough (or scrape) the cells into a microcentrifuge tube. Vortex the cells to ensure they are completely lysed.
 4. Centrifuge at maximum speed for 1–2 minutes to pellet nuclei and cell membranes. Transfer the supernatant to a new tube.
 5. Assay the lysate for protein concentration. You may use the Bradford, Lowry, or BCA assays (Pierce).
 6. Mix 30 μ l of lysate with 30 μ l of 2X SDS-PAGE sample buffer. Proceed with Steps 5 and 6 as detailed above.
-

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Tris-Glycine polyacrylamide gels are available from Invitrogen. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 30).

continued on next page

Transient Expression in Insect Cells, continued

Western Analysis

To detect expression of your recombinant fusion protein by Western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page vi for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope or a polyhistidine (6xHis) tag. WesternBreeze™ Chromogenic Kits and WesternBreeze™ Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 30).

Assay for CAT

If you use pMIB/V5-His/CAT as a positive control vector, you may assay for CAT expression using your method of choice. Commercial kits to assay for CAT protein are available. There is also a novel, rapid radioactive assay (Neumann *et al.*, 1987).

CAT can be detected by Western blot using antibodies against the C-terminal fusion tag or an antibody against CAT (Catalog no. R902-25). The CAT/V5-His protein fusion migrates around 34 kDa on an SDS-PAGE gel.

continued on next page

Transient Expression in Insect Cells, continued

Troubleshooting

Cells Growing Too Slowly (Or Not At All).

For troubleshooting guidelines regarding cell culture, refer to the Insect Cell Lines manual. This manual may be downloaded from our Web site (www.invitrogen.com).

Low Transfection Efficiency.

If the transfection efficiencies are too low, check the following:

- **Impure DNA.** Transfected cells will appear unhealthy when compared to the negative control (DNA only; no lipids). Use clean, pure DNA isolated by resin based DNA isolation kits (*e.g.* S.N.A.P. MidiPrep Kit).
- **Poor Cell Viability.** Be sure to test cells for viability and make sure you use log-phase cells. Refer to the Insect Cell Lines manual to troubleshoot cell culture.
- **Method of Transfection.** Optimize transfection.

Low or No Secreted Protein Expression

- **Gene not cloned in frame with the N-terminal HBM secretion signal.** If it is not in frame with the N-terminal secretion signal, the recombinant protein may be poorly expressed or not expressed at all. Re-design your cloning strategy to make sure that you clone your gene in frame with the HBM secretion signal.
 - **Optimize expression.** If you've tried a time course to optimize expression, try switching cell lines. Proteins may express better in a different cell line.
 - **Proteins are degraded.** Include protease inhibitors in the medium when harvesting to prevent degradation of recombinant protein.
 - **Poor secretion.** If the protein is normally localized to the nucleus, addition of the secretion signal to force the protein into the secretory pathway may result in incorrect folding and retention in the cell. You may detect expression of your protein in the cellular fraction using the Anti-V5 antibodies or the Anti-His(C-term) antibodies (see page vi for ordering information).
-

Selecting Stable Cell Lines

Introduction

Once you have demonstrated that your protein is expressed in Sf9, Sf21, or High Five™ cells, you may wish to create stable expression cell lines for long-term storage and large-scale production of the desired protein.

Nature of Stable Cell Lines

Note that stable cell lines are created by multiple copy integration of the vector. Amplification as is the case with calcium phosphate transfection and hygromycin resistance in *Drosophila* is generally not observed.

Before Starting

Review the information on blasticidin S on page 29. Prepare a stock solution of blasticidin S as described.

Effect of Blasticidin on Sensitive and Resistant Cells

Cytopathic effects should be visible within 3–5 days depending on the concentration of blasticidin in the medium. Sensitive cells will enlarge and become filled with vesicles. The outer membrane will show signs of blebbing, and cells will eventually detach from the plate.

Blasticidin-resistant cells should continue to divide at regular intervals to form distinct colonies. There should be no distinct morphological changes between blasticidin-resistant cells and cells not under selection with blasticidin.

Suggested Blasticidin Concentrations

In general, concentrations around 10 µg/ml will kill Sf9 cells (in complete TNM-FH medium) and concentrations around 20 µg/ml will kill High Five™ cells (in Express Five® SFM) within one week, although a few cells will remain that exclude trypan blue. To obtain faster and more thorough killing (in 3–4 days), we recommend using 50–80 µg/ml blasticidin. Using higher concentrations of blasticidin may result in enrichment of clones containing multiple integrations of your gene of interest. Once you have obtained your stable cell line(s), the concentration of blasticidin can be lowered and cells maintained at 10 µg/ml blasticidin. If you use other media or have trouble selecting cells using the concentrations above, we recommend that you perform a kill curve (see below).

Determining Blasticidin Sensitivity

If you wish to test your cell line for sensitivity to blasticidin, perform a kill curve as described below. Assays can be performed in 24-well tissue culture plates.

1. Seed insect cells in TNM-FH or serum-free medium of choice.
 2. The next day, substitute culture medium with medium containing varying concentrations of blasticidin (0–100 µg/ml blasticidin).
 3. Replenish the selective medium every 3–4 days and observe the percentage of surviving cells.
 4. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of blasticidin that kills the cells within 1 week after addition of blasticidin.
-

continued on next page

Selecting Stable Cell Lines, continued



Note

Reminder: Do not linearize the plasmid prior to transfection. Linearizing the plasmid appears to decrease protein expression. The reason for this is not known.

Stable Transfection

For stable transfections, follow the steps below. Include a mock transfection and a positive control (pMIB/V5-His/CAT).

1. Follow the transfection procedure on page 12, Steps 1 to 6.
2. Forty-eight hours posttransfection, remove the transfection solution and add fresh medium (**no blasticidin**).
3. Split cells 1:5 (20% confluent) and let cells attach overnight before adding selective medium.
4. Remove medium and replace with medium containing blasticidin at the appropriate concentration. Incubate cells at 27°C.
5. Replace selective medium every 3 to 4 days until you observe foci forming. At this point you may use cloning cylinders or dilution to isolate clonal cell lines (next page) or you can let resistant cells grow out to confluence for a polyclonal cell line (2 to 3 weeks).
6. To isolate a polyclonal cell line, let the resistant cells grow to confluence and split the cells 1:5 and test for expression. **Important:** Always use medium **without** blasticidin when splitting cells. Let the cells attach before adding selective medium.
7. Expand resistant cells into flasks to prepare frozen stocks. **Always use medium containing blasticidin when maintaining stable lepidopteran cell lines. You may lower the concentration of blasticidin to 10 µg/ml for maintenance.**

continued on next page

Selecting Stable Cell Lines, continued

Isolation of Clonal Cell Lines Using Cloning Cylinders

If you elect to select clonal cell lines, try to isolate as many foci (colonies) as possible for expression testing. As in mammalian cell culture, the location of integration may affect expression of your gene.

Tip: Perform selections in small plates or wells. When you remove the medium, you must work quickly to prevent the cells from drying out. Using smaller plates or wells limits the number of colonies you can choose at a time. To select more colonies, increase the number of plates or wells, not the size.

Before beginning, have sterile cloning cylinders on hand. To select colonies:

1. Examine the closed plate under a microscope and mark the location of each colony on the top of the plate. Transfer the markings to the bottom of the plate. Be sure to include orientation marks. **Note:** Each colony will contain 50 to 200 cells. Sf9 cells tend to spread more than High Five™ cells.
2. Move the culture dish to the sterile cabinet and remove the lid.
3. Apply a thin layer of sterile silicon grease to the bottom of a cloning cylinder (Scienceware, Catalog no. 378747-00 or Belco, Catalog no. 2090-00608), using a sterile cotton-tipped wooden applicator. The layer should be thick enough to retard the flow of liquid from the cylinder, without obscuring the opening on the inside. **Tip:** Cloning cylinders and silicon grease can be sterilized together by placing a small amount of grease in a glass petri dish and placing the cloning cylinders upright in the grease. After autoclaving, the grease will have spread out in a thin layer to coat the bottom of the cylinders.
4. Aspirate the culture medium and place the cylinder firmly and directly over the marked area. Use a microscope if it is available to help you direct placement of the cylinder.
5. Use 20 to 100 μ l of medium (no blasticidin) to slough the cells. Try to hold the pipette tip away from the sides of the cloning cylinder to avoid the grease (this will take a little practice).
6. Remove the cells and medium and transfer to a microtiter plate and let the cells attach. Remove medium and replace with selective medium for culturing. Expand the cell line and test for expression of your gene of interest. **Important:** Always use medium **without** blasticidin when splitting cells. Let the cells attach before adding selective medium.

continued on next page

Selecting Stable Cell Lines, continued

Isolation of Clonal Cell Lines Using a Dilution Method

You may also select clonal cell lines using a quick dilution method. The objective of this method is to dilute the cells so that under selective pressure only one stable viable cell per well is achieved. **Note that the higher your transfection efficiency, the more you should dilute out your cells. The protocol below works well with cells transfected at 5–10% efficiency.**

1. Forty-eight hours after transfection, dilute the cells to 1×10^4 cells/ml in medium **without** blasticidin. **Note:** Other dilutions of the culture should also be used as transfection efficiency will determine how many transformed cells there will be per well.
2. Add 100 μ l of the cell solution from Step 1 to 32 wells of a 96-well microtiter plate (8 rows by 4 columns).
3. Dilute the remaining cells 1:1 with medium **without** blasticidin and add 100 μ l of this solution to the next group of 32 wells (8 x 4).
4. Once again, dilute the remaining cells 1:1 with medium **without** blasticidin and add 100 μ l of this solution to the last group of 32 wells. **Note:** Although the cells can be diluted to low numbers, cell density is critical for viability. If the density drops below a certain level, the cells will not grow.
5. Let the cells attach overnight, then remove the medium and replace with medium containing blasticidin. **Note:** Removing and replacing medium may be tedious. If you slough the cells gently, it is possible to dilute the cells directly into selective medium.
6. Wrap the plate and incubate at 27°C for 1 week. It is not necessary to change the medium or place in a humid environment.
7. Check the plate after a week and mark the wells that have only one colony.
8. Continue to incubate the plate until the colony fills most of the well.
9. Harvest the cells and transfer to a 24-well plate with 0.5 ml of fresh medium containing blasticidin.
10. Continue to expand the clone to 12- and 6-well plates, and finally to a T-25 flask.

Assay for Expression

Assay each of your cell lines for yield of the desired protein and select the one with the highest yield for scale-up and purification of recombinant protein.



Important

Remember to prepare master stocks and working stocks of your stable cell lines prior to scale-up and purification. Refer to the Insect Cell Lines manual for information on freezing your cells and scaling up for purification.

Scale-Up and Purification

Introduction

Once you have obtained stable cell lines expressing the protein of interest and prepared frozen stocks of your cell lines, you are ready to purify your protein. General information for protein purification is provided below. Eventually, you may expand your stable cell line into larger flasks, spinners, shake flasks, or bioreactors to obtain the desired yield of protein. We recommend that you culture cells in serum-free medium to simplify purification.



Important

As you expand your stable cell line, you can maintain the concentration of blasticidin at 10 µg/ml.

Adapting Cells to Different Medium

Cells can be switched from complete TNM-FH to serum-free medium during passage. Refer to the Insect Cell Lines manual for more information on how to adapt cells to different medium.



If you plan to use a metal-chelating resin such as ProBond™ to purify your secreted protein from serum-free medium, **note that adding serum-free medium directly to the column will strip the nickel ions from the resin.** See the information below in **Purification of 6xHis-tagged Proteins from Medium** for a general recommendation to address this issue.

Purifying Proteins from Medium

Many protocols are suitable for purifying proteins from the medium. The choice of protocol depends on the nature of the protein being purified. Note that the culture volume needed to purify sufficient quantities of protein is dependent on the expression level of your protein and the method of detection. To purify 6xHis-tagged proteins from the medium, see below.

Purification of 6xHis-tagged Proteins from Medium

To purify 6xHis-tagged recombinant proteins from the culture medium, we recommend that you perform dialysis or ion exchange chromatography prior to affinity chromatography on metal-chelating resins.

Dialysis allows:

- Removal of media components that strip Ni⁺² from metal-chelating resins

Ion exchange chromatography allows:

- Removal of media components that strip Ni⁺² from metal-chelating resins
- Concentration of your sample for easier manipulation in subsequent purification steps

Conditions for successful ion exchange chromatography will vary depending on the protein. For more information, refer to *Current Protocols in Protein Science* (Coligan *et al.*, 1998), *Current Protocols in Molecular Biology*, Unit 10 (Ausubel *et al.*, 1994) or the *Guide to Protein Purification* (Deutscher, 1990).

continued on next page

Scale-Up and Purification, continued

Metal-chelating Resin

You may use the ProBond™ Protein Purification Kit (Catalog no. K850-01) or a similar product to purify your 6xHis-tagged protein. The ProBond™ Protein Purification Kit contains ProBond™, a metal-chelating resin specifically designed to purify 6xHis-tagged proteins. Before starting, be sure to consult the ProBond™ Protein Purification manual to familiarize yourself with the buffers and the binding and elution conditions. If you are using another resin, consult the manufacturer's instructions.



Note

Many insect cell proteins are naturally rich in histidines, with some containing stretches of six histidines. Some of these proteins may be secreted. When using the ProBond™ Protein Purification Kit or other similar products to purify 6xHis-tagged proteins, these histidine-rich proteins may co-purify with your protein of interest. The contamination can be significant if your protein is expressed and secreted at low levels. We recommend that you add 5 mM imidazole to the binding buffer prior to addition of the protein mixture to the column. Addition of imidazole may help to reduce background contamination by preventing proteins with low specificity from binding to the metal-chelating resin.

Scale-Up

To scale up insect cell culture, refer to the Insect Cell Lines manual.

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

10 g Tryptone
5 g Yeast Extract
10 g NaCl
pH 7.0

1. Combine the dry reagents above and add deionized, distilled water to 950 ml.
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 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 and pour into 10 cm plates.
 4. Let harden, then invert and store at +4°C, in the dark.
-

Low Salt LB Medium with Blasticidin

10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 ml.
 2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
 3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C before adding the blasticidin to 100 g/ml final concentration.
 4. Store plates at +4°C in the dark. Plates containing blasticidin are stable for up to 2 weeks.
-

Trypan Blue Exclusion Assay

1. Prepare a 0.4% stock solution of trypan blue in phosphate buffered saline, pH 7.4
 2. Mix 0.1 ml of trypan blue solution with 1 ml of cells and examine under a microscope at low magnification.
 3. Dead cells will take up trypan blue while live cells will exclude it. Count live cells versus dead cells. Cell viability should be at least 95–99% for healthy log-phase cultures.
-

continued on next page

Recipes, continued

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions.
For 100 ml, combine

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml
2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

To prevent proteolysis, you may add 1 mM PMSF, 1 M leupeptin, and 0.1 μ M aprotinin before use.

1X PBS

137 mM NaCl
2.7 mM KCl
10 mM Na₂HPO₄
1.8 mM KH₂PO₄

1. Dissolve:

8 g NaCl
0.2 g KCl
1.44 g Na ₂ HPO ₄
0.24 g KH ₂ PO ₄

in 800 ml deionized water.
 2. Adjust pH to 7.4 with concentrated HCl.
 3. Bring the volume to 1 liter. You may wish to filter-sterilize or autoclave the solution to increase shelf life.
-

2X SDS-PAGE Sample Buffer

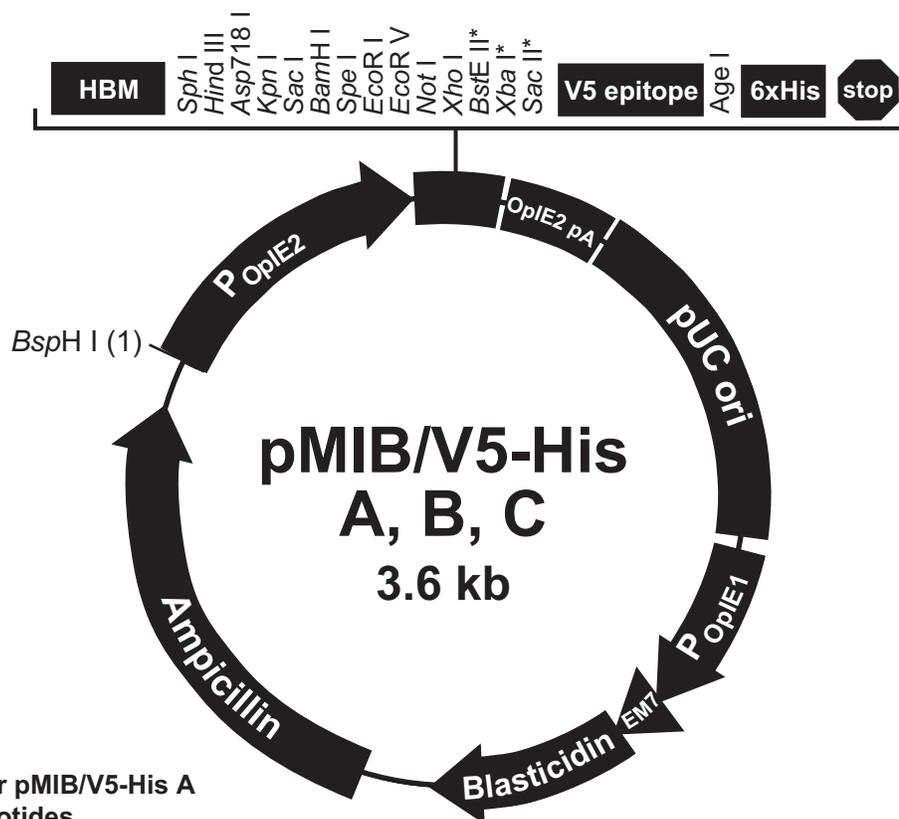
1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol (100%)	2 ml
β -mercaptoethanol	0.4 ml
Bromophenol Blue	0.02 g
SDS	0.4 g
 2. Bring the volume to 10 ml with sterile water.
 3. Aliquot and freeze at -20°C until needed.
-

pMIB/V5-His Map and Features

Map of pMIB/V5-His

The figure below summarizes the features of the pMIB/V5-His A, B, and C vectors. For a more detailed explanation of each feature, see the next page. **The complete sequences of pMIB/V5-His A, B, and C are available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 30).**



Comments for pMIB/V5-His A 3596 nucleotides

OpIE2 promoter: bases 1-549
 OpIE2 Forward priming site: bases 511-530
 Honeybee melittin secretion signal: bases 565-627
 Multiple cloning site: bases 629-721
 V5 epitope: bases 734-775
 Polyhistidine (6xHis) region: bases 785-802
 OpIE2 Reverse priming site: bases 812-837
 OpIE2 polyadenylation sequence: bases 820-949
 pUC origin: bases 1018-1691 (complementary strand)
 OpIE1 promoter: bases 1765-2056
 EM7 promoter: bases 2056-2122
 Blastidicin resistance gene (*bsd*): bases 2123-2521
 Ampicillin resistance gene (*bla*): bases 2641-3501

*Frame-dependent variations

continued on next page

pMIB/V5-His Map and Features, continued

Features of pMIB/V5-His

The features of pMIB/V5-His A, B, and C are described below. All features have been functionally tested. The multiple cloning site has been tested by restriction analysis.

Features	Function
<i>OpIE2</i> promoter	Provides constitutive expression of the gene of interest in lepidopteran insect cells (Theilmann and Stewart, 1992).
<i>OpIE2</i> Forward priming site	Allows sequencing of the insert from the 5' end.
Honeybee melittin secretion signal (HBM)	Directs secreted expression of the gene of interest (Tessier <i>et al.</i> , 1991).
Multiple cloning site	Allows insertion of the gene of interest for secreted expression.
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of your recombinant protein with the Anti-V5 Antibody (Catalog no. R960-25) or Anti V5-HRP Antibody (Catalog no. R961-25) (Southern <i>et al.</i> , 1991).
Polyhistidine (6xHis) tag	Allows purification of your recombinant protein on metal-chelating resin such as ProBond™. In addition, the C-terminal 6xHis tag is the epitope for the Anti-His(C-term) Antibody (Catalog no. R930-25) and the Anti-His(C-term)-HRP Antibody (Catalog no. R931-25) (Lindner <i>et al.</i> , 1997)
<i>OpIE2</i> Reverse priming site	Allows sequencing of the insert from the 3' end.
<i>OpIE2</i> polyadenylation sequence	Allows efficient transcription termination and polyadenylation of mRNA (Theilmann and Stewart, 1992).
pUC (pMB1-derived) origin	Allows high-copy replication and maintenance in <i>E. coli</i> .
<i>OpIE1</i> promoter	Provides constitutive expression of the blasticidin resistance gene in lepidopteran insect cells (Theilmann and Stewart, 1991).
EM7 promoter	Allows efficient expression of the blasticidin and ampicillin resistance genes in <i>E. coli</i> .
Blasticidin resistance gene (<i>bsd</i>)	Allows generation of stable insect cell lines (Kimura <i>et al.</i> , 1994).
Ampicillin resistance gene (<i>bla</i>)	Allows selection of transformants in <i>E. coli</i> . Note: The native promoter has been removed. Transcription is assumed to start from the EM7 promoter.

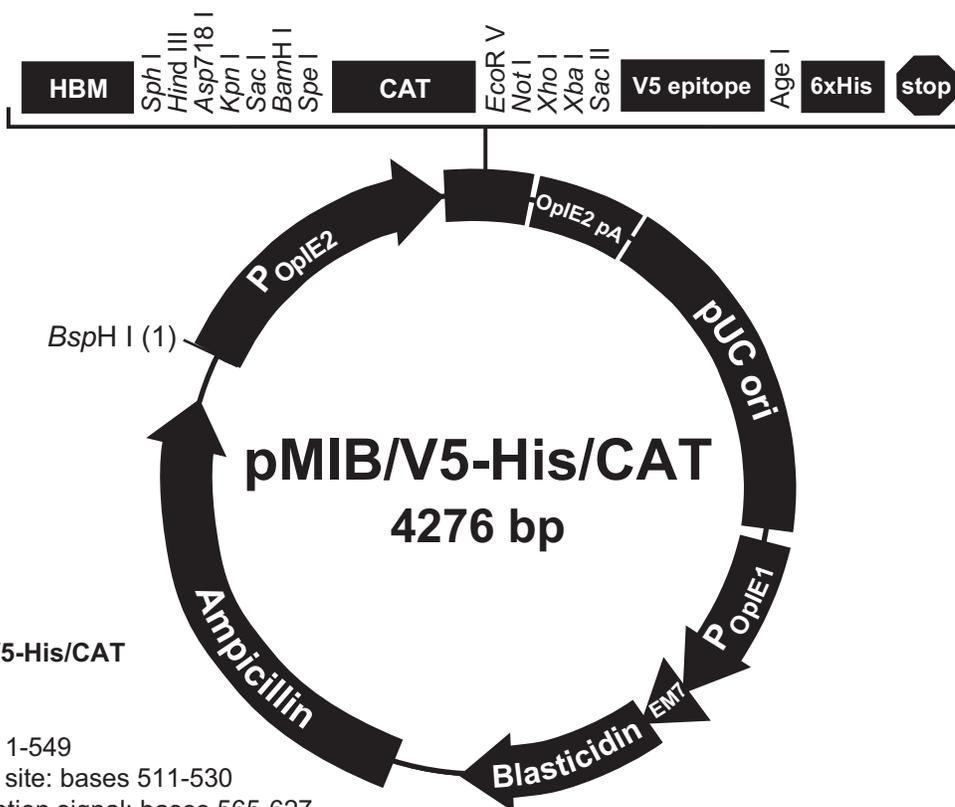
pMIB/V5-His/CAT Map

Description

pMIB/V5-His/CAT is a 4276 bp control vector expressing chloramphenicol acetyltransferase (CAT). The plasmid was constructed by cloning a *Hind* III/*Xho* I fragment containing the CAT gene into pMIB/V5-His B. In pMIB/V5-His/CAT, CAT is expressed as a fusion to the V5 epitope and 6xHis tag. The molecular weight of the protein is 34 kDa.

Map

The figure below summarizes the features of the pMIB/V5-His/CAT vector. The complete nucleotide sequence for pMIB/V5-His/CAT is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 30).



Comments for pMIB/V5-His/CAT 4276 nucleotides

OpIE2 promoter: bases 1-549
 OpIE2 Forward priming site: bases 511-530
 Honeybee melittin secretion signal: bases 565-627
 CAT ORF: bases 691-1347
 V5 epitope: bases 1414-1455
 Polyhistidine (6xHis) region: bases 1465-1482
 OpIE2 Reverse priming site: bases 1492-1517
 OpIE2 polyadenylation sequence: bases 1500-1629
 pUC origin: bases 1698-2371 (complementary strand)
 OpIE1 promoter: bases 2447-2735
 EM7 promoter: bases 2736-2802
 Blasticidin resistance gene (*bsd*): bases 2803-3201
 Ampicillin resistance gene (*bla*): bases 3321-4181

OpIE2 Promoter

Description

The *OpIE2* promoter has been analyzed by deletion analysis using a CAT reporter in both *Lymantria dispar* (LD652Y) and *Spodoptera frugiperda* (Sf9) cells. Expression in Sf9 cells was much higher than in LD652Y cells. Deletion analysis revealed that sequence up to -275 base pairs from the start of transcription is necessary for maximal expression (Theilmann and Stewart, 1992). Additional sequence beyond -275 may broaden the host range expression of this plasmid to other insect cell lines (Tom Pfeifer, personal communication).

In addition, an 18 bp element appears to be required for expression. This 18 bp element is repeated almost completely in three different locations and partially at six other locations. These are marked in the figure below. Elimination of the three major 18 bp elements reduces expression to basal levels (Theilmann and Stewart, 1992). The function of these elements is not known.

Primer extension experiments revealed that transcription initiates equally from either the C or the A indicated. These two transcriptional start sites are adjacent to a CAGT sequence motif that has been shown to be conserved in a number of early genes (Blissard and Rohrmann, 1989).

```
1   GGATCATGAT GATAACAAT GTATGGTGCT AATGTTGCTT CAACAACAAT TCTGTTGAAC

61  TGTGTTTTCA TGTTTGCCAA CAAGCACCTT TATACTCGGT GGCCTCCCCA CCACCAACTT

121 TTTTGCCTG CAAAAAACA CGCTTTTGCA CGCGGGCCCA TACATAGTAC AAACTCTACG

181 TTTCGTAGAC TATTTTACAT AAATAGTCTA CACCGTTGTA TACGCTCCAA ATACACTACC

241 ACACATTGAA CCTTTTTGCA GTGCAAAAAA GTACGTGTCG GCAGTCACGT AGGCCGGCCT

301 TATCGGGTCG CGTCCTGTCA CGTACGAATC ACATTATCGG ACCGGACGAG TGTTGTCTTA

361 TCGTGACAGG ACGCCAGCTT CCTGTGTTGC TAACCGCAGC CGGACGCAAC TCCTTATCGG

421 AACAGGACGC GCCTCCATAT CAGCCGCGCG TTATCTCATG CGCGTGACCG GACACGAGGC

481 GCCCGTCCCG CTTATCGCGC CTATAAATAC AGCCCGCAAC GATCTGGTAA ACACAGTGA

541 ACAGCATCTG TTCGAATTTA
```

Start of Transcription
→

OpIE1 Promoter

Description

The *OpIE1* promoter has been analyzed by deletion analysis using a CAT reporter in both *Lymantria dispar* (LD652Y) and *Spodoptera frugiperda* (Sf9) cells. Deletion analysis revealed that sequence between -186 and -106 is important for maximum transcription in Sf9 cells (Theilmann and Stewart, 1991).

This region contains a canonical CCAAT site (underlined) (Johnson and McKnight, 1989) and an element (R4) that is homologous to the proposed binding site of the *Drosophila* transcription factor Adf-1 (England *et al.*, 1990). Three other Adf-1-like elements are found at three other distal locations. These elements are referred to as R1, R2, R3, and R4. R3 and R4 are marked in the figure below. R1 and R2 are not present in pIB/V5-His but do not appear to be important for expression in Sf9 cells. The function of these elements has not been determined.

Primer extension experiments revealed that transcription initiates from the A in the CAGT sequence. This CAGT sequence motif has been shown to be conserved in a number of early genes (Blissard and Rohrmann, 1989).



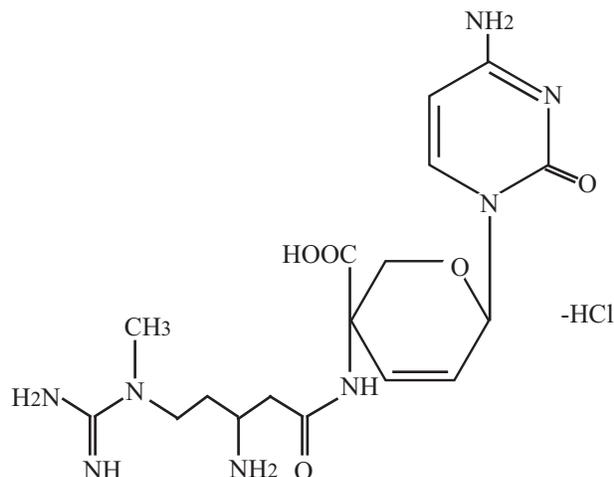
Blasticidin S

Molecular Weight, Formula, and Structure

Merck Index: 12: 1350

MW: 458.9

Formula: $C_{17}H_{26}N_8O_5 \cdot HCl$



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (*e.g.* a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

To inactivate blasticidin for disposal, add sodium bicarbonate.

Preparing and Storing Stock Solutions

- Blasticidin S is soluble in water and acetic acid. Water is generally used to prepare stock solutions of 5 to 10 mg/ml.
- Dissolve blasticidin S in sterile water and filter-sterilize the solution.
- Blasticidin S is unstable in solutions with a pH greater than 8. Be sure the pH of the solution is below 7.
- Aliquot in small volumes (see below) and freeze at $-20^{\circ}C$ for long-term storage or store at $+4^{\circ}C$ for short term storage.
- Aqueous stock solutions are stable for 1–2 weeks at $+4^{\circ}C$ and 6–8 weeks at $-20^{\circ}C$.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store at $+4^{\circ}C$. Discard after 1–2 weeks.

Technical Service

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Technical Service, continued

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