invitrogen™

InsectSelect™ BSD System

For Stable Expression of Heterologous Proteins in Lepidopteran Insect Cell Lines using pIB/V5-His

Catalog nos. K820-01, K825-01, V8020-01

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User Manual

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Kit Contents and Storage

Types of Kits

This manual is supplied with the products listed below.

Product	Catalog no.
InsectSelect™ BSD System with Sf9 Cells	K820–01
InsectSelect™ BSD System with High Five™ Cells	K825–01
pIB/V5-His Vector Kit	V8020–01

Shipping/Storage

See the table below for shipping and storage information.

Product	Shipping	Storage
InsectSelect™ BSD System with Sf9 Cells	Dry ice	Vectors, primers, blasticidin: **–20°C** Cellfectin® Reagent: **4°C** Cells: **Liquid nitrogen** Medium: **4°C**, protected from light
InsectSelect™ BSD System with High Five™ Cells		
pIB/V5-His Vector Kit	Wet ice	**–20°C**

Vectors and Primer

The vectors and primers are supplied with all of the kits listed above. Store at –20°C.

Product	Amount Supplied	Composition
pIB/V5-His	20 µg	40 µl of 0.5 µg/µl pIB/V5-His in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
pIB/V5-His/CAT	20 µg	40 µl of 0.5 µg/µl pIB/V5-His/CAT control vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
OpIE2 Forward Sequencing Primer	2 µg	Lyophilized in TE, pH 8.0
OpIE2 Reverse Sequencing Primer	2 µg	Lyophilized in TE, pH 8.0

Primer Sequences

The sequence of each primer is provided below:

Primer	Sequence	pMoles Supplied
OpIE2 Forward	5´-CGCAACGATCTGGTAAACAC-3´	329
OpIE2 Reverse	5´-GACAATACAAACTAAGATTTAGTCAG-3´	250

Continued on next page

Kit Contents and Storage, Continued

Blasticidin

Blasticidin is supplied **with the InsectSelect™ BSD System kits only.**

Blasticidin is shipped as 50 mg (powder). Upon receipt, **store Blasticidin at room temperature**.

Blasticidin is available to order separately (see page vi).

Cellfectin® Reagent

Cellfectin® Reagent is supplied **with the InsectSelect™ BSD System kits only.**

Cellfectin® Reagent is shipped as 1 ml (composition: 1 mg/ml lipid in membrane-filtered water).

Upon receipt, **store Cellfectin® Reagent at 4°C**.

Cellfectin® Reagent is available to order separately (see page vi).

Cells and Medium

Supplied with the InsectSelect™ BSD System kits only. Additional cells and other cell lines are available to order separately (see page vii).

Store the cells in liquid nitrogen.

Store the medium at 4°C, protected from light.

Different cells and media are included, depending on which InsectSelect™ BSD System kit you ordered. Refer to the table below. For guidelines and instructions to culture Sf9 and High Five™ cells, refer to the Insect Cell Lines manual included with each kit.

Kit	Cells	Medium
InsectSelect™ BSD System with Sf9 Cells	Sf9	• Sf-900 II SFM (1X) or • Grace's Insect Cell Culture Medium, Unsupplemented (contains L-glutamine)
InsectSelect™ BSD System with High Five™ Cells	High Five™	Express Five® Serum-Free Medium

Manuals

The following manuals are supplied with each kit.

Kit	Manual
InsectSelect™ BSD System with Sf9 Cells	InsectSelect™ BSD System manual Insect Cell Lines manual
InsectSelect™ BSD System with High Five™ Cells	InsectSelect™ BSD System manual Insect Cell Lines manual
pIB/V5-His Vector Kit	InsectSelect™ BSD System manual

Accessory Products

Introduction

The products listed in this section are intended for use with the InsectSelect™ BSD System and the pIB/V5-His Vector Kit. For more information, refer to our web site at www.invitrogen.com or contact **Technical Support** (page 27).

Additional Products

The following products are available separately from Invitrogen.

Product	Quantity	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
TOP10 Electrocomp™ Cells	5 × 80 µl	C664–55
	10 × 80 µl	C664–11
	30 × 80 µl	C664–24
One Shot™ TOP10 (chemically competent cells)	21 × 50 µl	C4040–03
Electrocomp™ TOP10	5 × 80 µl	C664–55
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
Cellfectin® Reagent	1 ml	10362–010
Blasticidin S	50 mg	R210–01
S.N.A.P. Miniprep Kit	100 reactions	K1900–01
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100–03
Positope™ Control Protein	5 µg	R900–50

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Additional 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 and those that allow secreted protein expression. 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 web site at www.invitrogen.com or contact **Technical Support** (page 27). See the table below for ordering information.

Product	Catalog no.
pIB/V5-His TOPO® TA Expression Kit	K890–01
pIZ/V5-His Vector Kit	V8000–01
InsectSelect™ System with Sf9 Cells	K800–01
InsectSelect™ System with High Five™ Cells	K805–01
pIZT/V5-His Vector Kit	V8010–01
pMIB/V5-His Vector Kit	V8030–01

Polyacrylamide Gel Electrophoresis

To facilitate separation of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® 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 at www.invitrogen.com or contact **Technical Support** (page 27).

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Detecting 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 pIB/V5-His. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods.

The amount of antibody supplied 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 *et al.*, 1991) GKPIPNPLLGLDST	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 *et al.*, 1997) HHHHHH-COOH	R930–25
Anti-His(C-term)-HRP Antibody		R931–25
Anti-His(C-term)-AP Antibody		R932–25

Purifying 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 ProBond™ Resin available from Invitrogen. The ProBond™ Purification System and Ni-NTA Purification System are also available from Invitrogen. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Purification System	6 purifications	K850–01
ProBond™ Purification Kit with Anti-V5-HRP Antibody	1 kit	K854–01
ProBond™ Purification Kit with Anti-His(C-term)-HRP Antibody	1 kit	K853–01
ProBond™ Nickel-Binding Resin	50 ml	R801–01
	150 ml	R801–15
Purification Columns (10 ml polypropylene columns)	50	R640–50
Ni-NTA Purification System	6 purifications	K950–01
Ni-NTA Purification System with Anti-V5-HRP Antibody	1 kit	K954–01
Ni-NTA Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K953–01
Ni-NTA Agarose	10 ml	R901–01
	25 ml	R901–15

Introduction

Overview

Introduction

The InsectSelect™ System allows you to express your protein of interest in insect cell lines either transiently or stably. The system utilizes a single expression vector, pIB/V5-His to express your gene of interest. This 3.5 kb vector has the following features:

- *OpIE2* promoter for constitutive expression of the gene of interest (Theilmann & Stewart, 1992)
- *OpIE1* promoter for expression of the blasticidin resistance gene (see next bullet) (Theilmann & 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 (or blasticidin) resistance in *E. coli*
- Ampicillin resistance gene for selection of transformants in *E. coli*
- Optional C-terminal peptide containing the V5 epitope and 6xHis tag for detection and purification of your protein of interest

For more information and a map of the vector, see pages 22–23.

Description of System

The gene of interest is cloned into pIB/V5-His and transfected into Sf9 or High Five™ cells using lipid-mediated transfection. After transfection, cells can be assayed for 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 (*Op*MNPV). 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 25 and page 26, respectively.

Expression Levels

The *OpIE2* promoter provides relatively high levels of constitutive expression, although not all proteins will express as high as might be expected from baculovirus late promoters such as polyhedrin or very late promoters such as p10 (Jarvis *et al.*, 1996). However, some researchers have found that the InsectSelect™ System expresses some proteins better than baculovirus systems. To date, reported expression levels range from 1–2 µg/ml (human IL-6; Invitrogen) to 8–10 µg/ml (human melanotransferrin) (Hegedus *et al.*, 1999).

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Blasticidin Resistance

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromo-genes* 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 & Yamaguchi, 1996; Yamaguchi *et al.*, 1975).

Experimental Outline

The table below describes the general steps needed to clone and express your gene of interest using the InsectSelect™ BSD kit of your choice. For more details, refer to the manual and the pages indicated.

Step	Action	Source
1	Establish culture of Sf9 or High Five™ cells **Note:** Other cell lines (*e.g.* Sf21) may be used.	Refer to the Insect Cell Lines manual or use your own protocols.
2	Develop a cloning strategy to ligate your gene of interest into pIB/V5-His.	Pages 4–6, this manual
3	Transform your ligation reactions into a *rec*A, *end*A *E. coli* strain (*e.g.* TOP10). Select on LB plates containing 50–100 µg/ml ampicillin.	Page 6, this manual
4	Isolate plasmid DNA and sequence your recombinant expression vector to confirm that your protein is in frame with the C-terminal peptide.	Page 6, this manual
5	Transfect Sf9 or High Five™ cells.	Pages 7–9, this manual
6	Assay for transient expression of your protein.	Pages 10–11, this manual
7	Create stable cell lines expressing the protein of interest by selecting with blasticidin.	Pages 12–15, this manual
8	Scale-up expression for purification.	Page 16, this manual Insect Cell Lines manual
9	Purify your recombinant protein by chromatography on metal-chelating resin (*e.g.* ProBond™).	Pages 16–17, this manual

Methods

Culturing Insect Cells

Introduction

Before you start your cloning experiments, be sure to have cell cultures of either Sf9 or High Five™ cells growing and have frozen master stocks available. If you purchased one of the InsectSelect™ BSD System kits, you will receive either Sf9 cells or High Five™ cells along with the Insect Cell Lines manual. Use this manual as a guide to initiate cell culture. This manual is available for downloading from our web site at www.invitrogen.com or by contacting **Technical Support** (page 27).

Culturing Sf9 and High Five™ Cells

To culture Sf9 or High Five™ cells, refer to the Insect Cell Lines manual. This manual covers the following topics:

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

Note

For the best recovery and viability, thaw High Five™ cells into Express Five® Serum-Free medium and thaw Sf9 cells into Sf-900 II SFM (1X) (see page 8)

Sf21 Cells

You may also use Sf21 cells as a host for pIB/V5-His. Sf21 cells are larger and we have found that they may produce more protein than Sf9 cells. Refer to the Insect Cell Lines manual for more information.

Cells for Transfection

You will need log-phase cells with >95% viability to perform a successful transfection. Review pages 7–10 to determine how many cells you will need for transfection.

Cloning into pIB/V5-His

Introduction

This chapter provides information to help you clone your gene of interest into pIB/V5-His. A diagram is provided on page 5 to help you ligate your gene of interest in frame with the C-terminal peptide sequence.

- For information on transformation into *E. coli*, see page 6.
- For information on transfection into Sf9 or High Five™ cells see pages 7–9.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, 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 pIB/V5-His

20 µg of pIB/V5-His vector is supplied in suspension at a concentration of 40 µl of 0.5 µg/µl pIB/V5-His in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0. If you wish to propagate and maintain the pIB/V5-His, we recommend using 10 ng of the vector to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5 , or equivalent using your method of choice.

Select transformants on LB plates containing 50–100 µg/ml ampicillin (see page 6).

Translation Initiation

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position +4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

(G/A)NN<u>ATG</u>**G**

Fusion to the C-terminal Peptide

If you wish to include the C-terminal peptide for detection with either the V5 or His(C-term) antibodies or purification using the 6xHis tag, you must clone your gene in frame with the peptide. **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.

Secretion of Recombinant Protein

If your protein of interest is normally secreted, try expressing the protein using the native secretion signal. To date, all mammalian secretion signals tested have functioned properly in insect cells. We have successfully expressed human interleukin-6 (IL6) using the native secretion signal to levels of 1–2 µg/ml.

In addition, we recommend that you create a construct to express your protein intracellularly in the event that your protein is not secreted.

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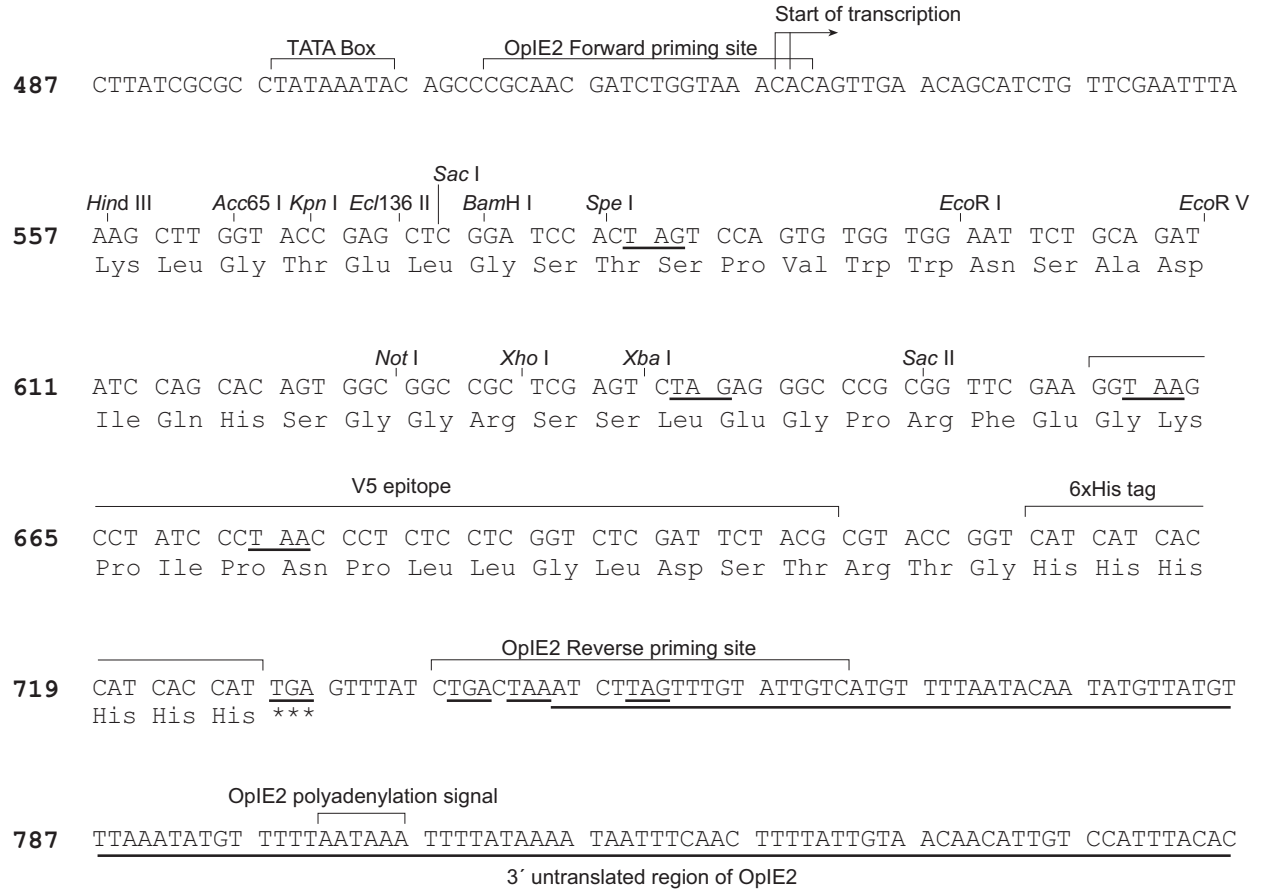
Cloning into pIB/V5-His, Continued

MCS of pIB/V5-His

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. Potential stop codons are shown underlined. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pIB/V5-His is available for downloading from our web site at www.invitrogen.com or from Technical Support (page 27).** For a map and a description of the features of pIB/V5-His, refer to pages 22–23.



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 pIB/V5-His including TOP10 or DH5 . We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A). For your convenience, TOP10 is available as electrocompetent or chemically competent cells from Invitrogen (see page vi).

Transformation Method

You may use your method of choice to transform *E. coli*. To select transformants, use LB plates containing 50–100 µg/ml ampicillin.

Note: It is possible to select *E. coli* transformants on 100–150 µg/ml blasticidin. You will need to prepare Low Salt LB (5 g NaCl per liter) and adjust the pH to below 7 before autoclaving. After autoclaving and cooling, add blasticidin. Plates are stable for 1–2 weeks. Since blasticidin is sensitive to salt and pH, we recommend that you use ampicillin for selection of *E. coli* transformants.

We recommend that you sequence your construct to confirm that your gene is fused in frame with the 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.

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 pIB/V5-His, you are ready to transfect your construct into Sf9 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 clean 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 S.N.A.P.™ Miniprep Kit or the PureLink™ HiPure Plasmid Miniprep Kit (page vi). The purified 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 cells**
- **40–60% for High Five™ cells**

Note: Other transfection methods (*e.g.* calcium phosphate and electroporation (Mann & 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 in all transfection experiments. At about 24–48 hours post–transfection, 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.

Materials Needed

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 cells (1.6–2.5 × 10^6 cells/ml, >95% viability) or log-phase High Five™ cells (1.8–2.3 × 10^6 cells/ml, >95% viability), growing in serum-free medium

  Note: You may transfect Sf9 cells in Grace's Medium without supplements or FBS. The proteins in the FBS and supplements will interfere with the liposomes, causing the transfection efficiency to decrease.
- Serum-free medium (see 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 8.0

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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 pIB/V5-His. Express Five® SFM (page vi) is recommended for use with High Five™ cells while Sf-900 II SFM (1X) (page vi) is optimized for use with Sf9 cells and Sf21 cells. Other serum-free media may be used, although you may have to optimize conditions for transfection and selection. You will need to adapt the cells to serum-free medium before transfection (see the Insect Cell Lines manual for a protocol).

Note: You may transfect Sf9 cells in Grace's Medium without supplements or FBS. The proteins in the FBS and supplements will interfere with the liposomes, causing the transfection efficiency to decrease.

Preparing 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 post–transfection. You will need at least one 60 mm plate for each time point.

1. For Sf9 cells or High Five™ cells, seed 1×10^6 cells in appropriate serum-free medium in a 60 mm dish.

   Note: You may transfect Sf9 cells in Grace's Medium without supplements or FBS. The proteins in the FBS and supplements will interfere with the liposomes, causing the transfection efficiency to decrease.
2. Rock gently from side to side for 2–3 minutes to evenly distribute the cells. Cells should be 50–60% confluent.
3. 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.
4. 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:

- pIB/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.

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Transfection Procedure

Plasmid DNA and Cellfectin® Reagent are mixed together in the appropriate medium (see **Serum-Free Media**, previous page) 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.

1. To prepare each transfection mixture, add the following reagents to a 1.5-ml microcentrifuge tube:
   a. 1 ml Appropriate serum-free medium (see previous page)
   b. 1–10 µl pIB/V5-His plasmid or construct (~1 µg/µl in TE, pH 8.0)
   c. 6 µl Cellfectin® Reagent (**mix well before use and always add last**)
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. If the medium contained serum, wash the cells by carefully adding 2 ml of fresh serum-free medium (see previous page for appropriate media to use).

   Note: This will remove trace amounts of serum that will decrease the efficiency of liposome transfection).
5. Again, carefully remove all of the medium from the monolayer and add the entire transfection mix drop-wise into the 60 mm dish. **Repeat** for all transfections.

   Note: 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. After incubation, add 1–2 ml of appropriate serum-free medium (see previous page) to each 60 mm dish.
8. 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 twice with medium, and replace with 1–2 ml of fresh serum-free medium.
9. Harvest the cells 2*, 3, and 4 days post–transfection 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.

   ***Note:** To create stable cell lines, proceed to **Stable Transfection Procedure** (page 12) at 48-hours post-transfection. Otherwise, continue to harvest cells at days 3 and 4 post-transfection.

Continued on next page

Detecting Recombinant Proteins

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 viii for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (page vi) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope or a 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 contact **Technical Support** (see page 27).

Testing for Expression

Use the cells from one 60 mm plate for each expression experiment.

Before starting prepare Cell Lysis Buffer and SDS-PAGE sample buffer. Recipes are provided on page 19 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. Remove the medium from the cells. **If your protein is predicted to be secreted, be sure to save and assay both the medium and the cell pellet.**
3. Add 100 µl Cell Lysis Buffer to the plate 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.

   Note: If you are expressing a membrane protein, it may be located in the pellet. Be sure to assay the pellet (see below).
5. Assay the lysate for protein concentration. You may use the Bradford, Lowry, or BCA assays.
6. To assay your samples, mix them with SDS-PAGE sample buffer as follows:
   - Lysate: 30 µl lysate with 10 µl **4X SDS-PAGE** sample buffer.
   - Pellet: Resuspend pellet in 100 µl **1X SDS-PAGE** sample buffer.
   - Medium: 30 µl medium with 10 µl **4X SDS-PAGE** sample buffer.

   Note: Because of the volume of medium, it is difficult to normalize the amount loaded on an SDS-PAGE gel. If you are concerned about normalization, concentrate the medium.
7. Boil the samples for 5 minutes. Centrifuge briefly.
8. Load approximately 3–30 µg protein per lane. For the cell pellet sample, load the same volume as the lysate. Amount to load depends on the amount of your protein produced.
9. Electrophorese your samples, blot, and probe with a suitable antibody (see above).
10. 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.

Continued on next page

Polyacrylamide Gel Electrophoresis

To facilitate separation of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® 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 at www.invitrogen.com or contact **Technical Support** (page 27).

Assay for CAT

If you use pIB/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 (page viii or an antibody against CAT. The CAT/V5-His protein fusion migrates around 34 kDa on an SDS-PAGE gel.

Selecting Stable Cell Lines

Introduction

Once you have demonstrated that your protein is expressed in Sf9 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 in 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 21. Prepare a stock solution of blasticidin S as described in the protocol provided.

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 not be any distinct morphological changes between blasticidin-resistant cells compared to cells not under selection with blasticidin.

Suggested Blasticidin Concentrations

In general, concentrations around 10 µg/ml will kill Sf9 (in complete Sf-900 II SFM medium) and concentrations around 20 µg/ml will kill High Five™ cells (in Express Five® SFM) within one week, although a few cells may remain that exclude trypan blue. To obtain faster and more thorough killing, we recommend using 50–80 µg/ml blasticidin. Once blasticidin-resistant clones have been obtained, cells may be maintained in lower concentrations of blasticidin (*e.g.* 10–20 µg/ml). If you are using other media or have trouble selecting cells using the concentrations above, we recommend that you perform a kill curve (see below).

Blasticidin Selection Guidelines

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

1. Prepare Sf-900 II SFM medium (1X) or the serum-free medium of choice, supplemented with concentrations ranging from 0–100 µg/ml blasticidin. Generally, concentrations that effectively kill lepidopteran insect cells within a week are in the 50–80 µg/ml range.

   Note: While 10–20 µg/ml blasticidin will kill cells within a week, higher concentrations will result in faster and more thorough killing. In addition, using higher concentrations of blasticidin may result in enrichment of clones containing multiple integrations of your gene of interest.

2. Test varying concentrations of blasticidin on the cell line to determine the concentration that kills your cells within a week (kill curve).
3. Use the concentration of drug that kills your cells within a week.

Continued on next page

Selecting Stable Cell Lines, Continued

Note

Do not linearize the plasmid prior to transfection. Linearizing the plasmid appears to decrease protein expression.

Stable Transfection Procedure

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

1. Complete the **Transfection Procedure** (page 9), through Step 9 until 2-days post-transfection.
2. At 2-days, post-transfection, remove the transfection solution and add fresh medium (**without 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 (colonies) 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.

   Important: 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

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.

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 the 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 20–100 µl of medium (**without** 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

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: 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 µl 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 µl of this solution to the next group of 32 wells (8 × 4).
4. Once again, dilute the remaining cells 1:1 with medium **without** blasticidin and add 100 µl 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. **If your protein is secreted, remember to assay the cell pellet as well as the medium.** You may wish to compare the yield of protein in the cells and medium.

Yield of Expressed Protein

In general, the level of secreted protein is comparable to that obtained with viral expression systems in insect cells. We have obtained stable cell lines that express and secrete human interleukin-6 to levels of 1–2 µg/ml. Human melanotransferrin has been expressed to levels of 8–10 µg/ml (Hegedus *et al.*, 1999).

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. If your protein is secreted, you may 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.

Serum-Free Medium

If your protein is secreted, use serum-free medium to facilitate expression and purification (see page 8).

Adapting Cells to Different Medium

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

CAUTION

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

Metal-chelating Resin

You may use the ProBond™ Purification System or Ni-NTA Purification System (page viii), or a similar product to purify your 6xHis-tagged protein. Both purification systems contain a metal-chelating resin specifically designed to purify 6xHis-tagged proteins. Before starting, be sure to consult the ProBond™ Purification System manual or Ni-NTA Purification System 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. When using the ProBond™ Purification System 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 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.

Purification of Intracellularly Expressed Proteins

If you are expressing your 6xHis-tagged protein intracellularly, you may lyse the cells and add the lysate directly to the ProBond™ column. You will need 5×10^6 to 1×10^7 cells for purification of your protein on a 2 ml ProBond™ column (see ProBond™ Purification System manual).

1. Seed 2×10^6 cells in two or three 25 cm^2 flasks.
2. Grow the cells in selective medium until they reach confluence (4×10^6 cells).
3. Wash cells once with PBS.
4. Harvest the cells by sloughing.
5. Transfer the cells to a sterile centrifuge tube.
6. Centrifuge the cells at 1000 × g for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at –80°C until needed.

Scale-Up

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

Troubleshooting

Problem	Cause	Solution
Cells Growing Too Slowly (Or not at all)	Refer to the Insect Cell Lines manual	For troubleshooting guidelines regarding cell culture, refer to the Insect Cell Lines manual included with the InsectSelect™ BSD kits.
Low Transfection Efficiency	Impure DNA	Transfected cells will appear unhealthy when compared to the negative control (DNA only). 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 Protein Expression	Gene not cloned in frame with the C-terminal sequence	If it is not in frame with the C-terminal peptide sequence, expression will not be detected using the antibody to the V5 epitope or the C-terminal histidine tag.
	No Kozak sequence for proper initiation of translation	Translation will be inefficient and the protein will not be expressed at its optimal level.
	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 Cell Lysis buffer to prevent degradation of recombinant protein.
	Poor secretion	Check the cell pellet as well as the medium when analyzing secreted expression. Protein may be trapped in the cell and not secreted. To improve secretion, try a different cell line (*e.g.* High Five™).

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
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 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.

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

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.

Add 1 mM PMSF, 1 M leupeptin, and 0.1 µM aprotinin to prevent proteolysis, before use.

4X SDS-PAGE Sample Buffer

Combine the following reagents to obtain 10 ml:

0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

Aliquot and freeze at –20°C until needed.

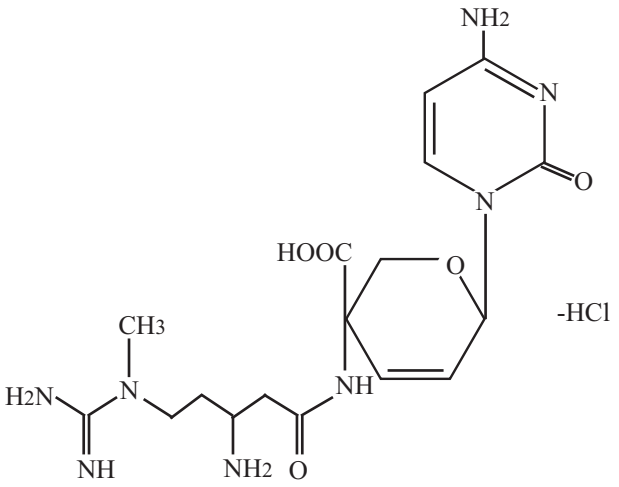
Blasticidin S

Molecular Weight, Formula, and Structure

Merck Index: 12: 1350

MW: 458.9

Formula: $C_{17}H_{26}N_8O_5$-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.

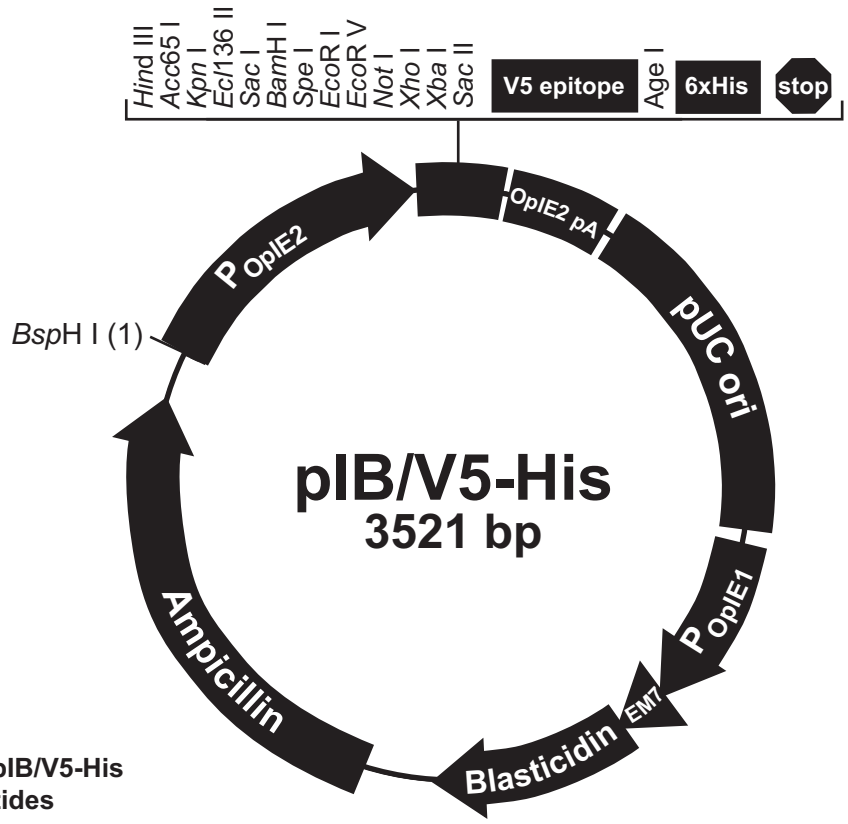
Preparing and Storing Stock Solutions

- 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 8.0. Be sure the pH of the solution is 7.0.
- Aliquot in small volumes (see below) and freeze at –20°C for long-term storage or store at 4°C for short term storage.
- Aqueous stock solutions are stable for 1–2 weeks at 4°C and 6–8 weeks at –20°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°C. Discard after 1–2 weeks.

Map of pIB/V5-His

Map of pIB/V5-His

The figure below summarizes the features of the pIB/V5-His vector (3521 bp). For a more detailed explanation of each feature, see the next page. **The complete sequence of pIB/V5-His is available from our web site at www.invitrogen.com or from Technical Support (page 27).**



Features of pIB/V5-His
3521 nucleotides

OpIE2 promoter: bases 1-548
OpIE2 Forward priming site: bases 511-530
Multiple cloning site: bases 557-652
V5 epitope: bases 659-700
6xHis tag: bases 710-727
OpIE2 Reverse priming site: bases 737-762
OpIE2 polyadenylation sequence: bases 745-874
pUC origin: bases 943-1616 (c)
OpIE1 promoter: bases 1665-1956
EM7 promoter: bases 1971-2029
Blasticidin resistance gene (*bsd*): bases 2048-2447
Ampicillin resistance gene (*bla*): 2566-3426

Continued on next page

Features of pIB/V5-His

Features of pIB/V5-His

The features of pIB/V5-His (3521 bp) are described below. All features have been functionally tested. The multiple cloning site has been tested by restriction analysis.

Features	Function
OpIE2 promoter	Provides constitutive expression of the gene of interest in lepidopteran insect cells (Theilmann & Stewart, 1992).
OpIE2 Forward priming site	Allows sequencing of the insert from the 5´ end.
Multiple cloning site (13 unique sites)	Allows insertion of the gene of interest for 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 Antibodies (Southern *et al.*, 1991).
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 Anti-His(C-term) Antibodies (Lindner *et al.*, 1997).
OpIE2 Reverse priming site	Allows sequencing of the insert from the 3´ end.
OpIE2 polyadenylation sequence	Efficient transcription termination and polyadenylation of mRNA (Theilmann & Stewart, 1992).
pUC origin	Replication, maintenance, and high copy number in *E. coli*.
OpIE1 promoter	Provides constitutive expression of the blasticidin resistance gene in lepidopteran insect cells (Theilmann & Stewart, 1991).
EM7 promoter	Allows efficient expression of the blasticidin and ampicillin resistance genes in *E. coli*.
Blasticidin resistance gene (*bsd*)	Allows generation of stable insect cell lines (Kimura *et al.*, 1994).
Ampicillin resistance gene (*bla*)	Selection of transformants in *E. coli*. **Note**: The native promoter has been removed. Transcription is assumed to start from the EM7 promoter.

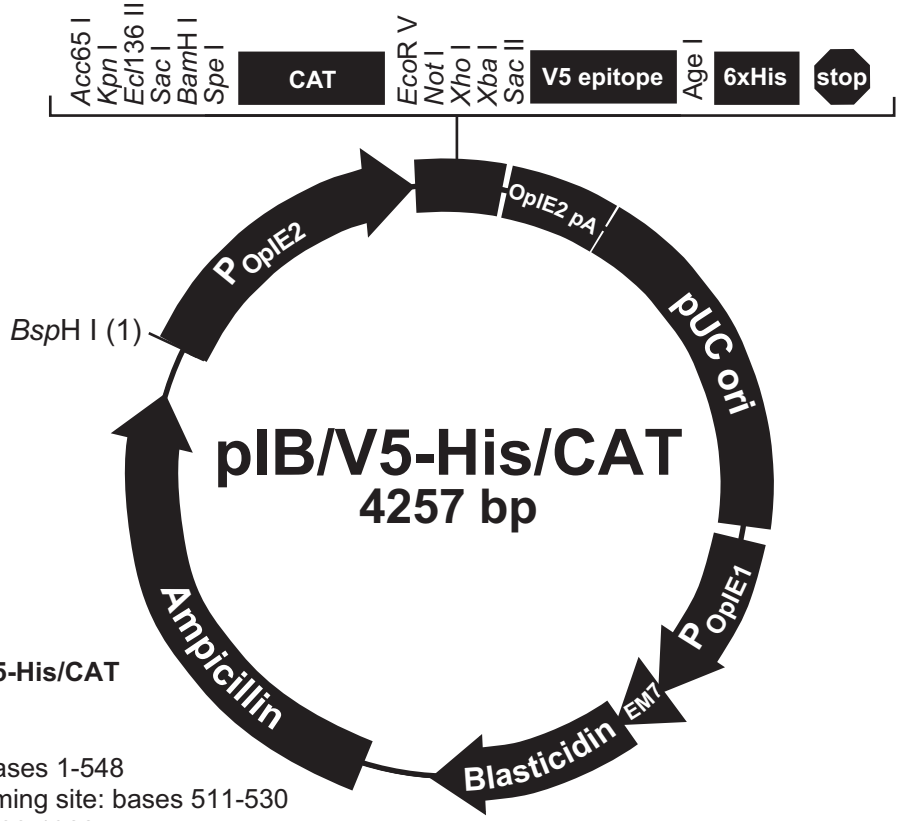
Map of pIB/V5-His/CAT

Description

pIB/V5-His/CAT is a 4257 bp control vector expressing chloramphenicol acetyltransferase (CAT). The CAT gene was amplified using PCR and TOPO® Cloned into pIB/V5-His-TOPO®. CAT is expressed as a fusion to the V5 epitope and 6xHis tag. The molecular weight of the protein is 34 kDa.

Map of pIB/V5-His/CAT

The figure below summarizes the features of the pIB/V5-His/CAT vector. **The complete nucleotide sequence for pIB/V5-His/CAT is available for downloading from our web site at www.invitrogen.com or by contacting Technical Support (see page 27).**



Features of pIB/V5-His/CAT
4257 nucleotides

OpIE2 promoter: bases 1-548
OpIE2 Forward priming site: bases 511-530
CAT ORF: bases 666-1322
V5 epitope: bases 1395-1436
6xHis tag: bases 1446-1463
OpIE2 Reverse priming site: 1473-1498
OpIE2 polyadenylation sequence: bases 1481-1610
pUC origin: bases 1679-2352 (c)
OpIE1 promoter: bases 2401-2692
EM7 promoter: bases 2707-2765
Blasticidin resistance gene (*bsd*): bases 2784-3183
Ampicillin resistance gene (*bla*): bases 3302-4162

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 & 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 & 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 & Rohrmann, 1989).

```
1    GGATCATGAT GATAAACAAT GTATGGTGCT AATGTTGCTT CAACAACAAT TCTGTTGAAC

61   TGTGTTTTCA TGTTTGCCAA CAAGCACCTT TATACTCGGT GGCCTCCCCA CCACCAACTT

121  TTTTGCACTG CAAAAAAACA 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

                          TATA                                  Start of Transcription
481  GCCCGTCCCG CTTATCGCGC CTATAAATAC AGCCCGCAAC GATCTGGTAA ACACAGTTGA

541  ACAGCATCTG TTCGAATTTA
```

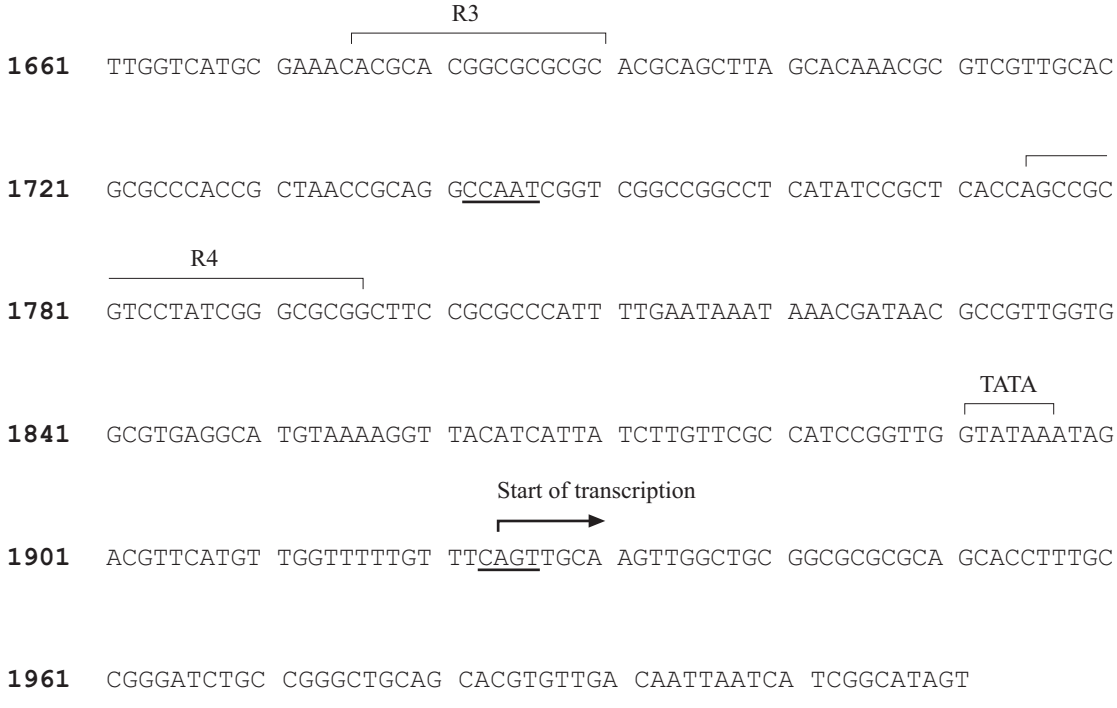
OpIE1 Promoter

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 & Stewart, 1991).

This region contains a canonical CCAAT site (underlined) (Johnson & 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 & Rohrmann, 1989).



Technical Support

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