Version D
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pIB/His A, B, and C Vector Kit

For the selection of transfected cells and stable expression of N-terminal-tagged heterologous proteins in Lepidopteran insect cell lines

Catalog no. V8040-01

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Table of Contents

Important Information

Shipping/Storage

The pIB/His Vector Kit is shipped at room temperature. Upon receipt, store the kit at-20°C.

Kit Contents

The following items are supplied with each pIB/His Vector Kit. **Store the components at -20°C.**

Item	Composition	Amount Supplied
pIB/His A, B, and C	Lyophilized in TE, pH 8	20 µg each
pIB/His/*lacZ*	Lyophilized in TE, pH 8	20 µg
OpIE2 Forward Sequencing Primer	Lyophilized in TE, pH 8	2 µg
OpIE2 Reverse Sequencing Primer	Lyophilized in TE, pH 8	2 µg

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

Reagents Supplied by the User

Be sure to have the following reagents and equipment on hand before starting experiments:

- Sf9, Sf21, or High Five™ insect cells (see the next page for ordering information)
- Serum-Free Medium (SF-900 II SFM or Express Five® SFM 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 21)
- 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 22)
- PBS (see recipe on page 22)
- Cloning cylinders (optional)
- 96-well plates (optional)

Accessory Products

Introduction

The products listed in this section are intended for use with the pIB/His Vector kit. For more information, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 29).

Products Available Separately

The products listed below may be used with the pIB/His Vector Kit and are available separately from Invitrogen. For insect media products, other sizes may be available Call Technical Service for more information (see page 29).

Product	Amount	Catalog no.
Sf9 Cells, frozen	1 ml vial, 1 x 10^7 cells/ml	B825-01
Sf21 Cells, frozen	1 ml vial, 1 x 10^7 cells/ml	B821-01
High Five™ Cells, frozen	1 ml vial, 3 x 10^6 cells/ml	B855-02
Grace's Insect Cell Culture Medium, Unsupplemented	500 ml	11595-030
SF-900 II SFM	500 ml	10902-096
Express Five® SFM	500 ml	10486-017
Cellfectin® Reagent	1 ml	10362-010
Blasticidin S	50 mg	R210-01

Other InsectSelect™ Kits

Many 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 selection markers and epitope tags. 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 29). See the table below for ordering information.

Product	Catalog no.
pIB/V5-His TOPO TA Cloning® Kit	K890-01
pIB/V5-His Vector Kit	V8020-01
pMIB/V5-His Vector Kit	V8030-01
pIZ/V5-His Vector Kit	V8000-01
pIZT/V5-His Vector Kit	V8010-01

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Accessory Products, continued

InsectSelect™ System Kits

InsectSelect™ System kits are also available from Invitrogen. The InsectSelect™ System kits contain an InsectSelect™ expression vector plus transfection reagents, culture medium, selection agent, and frozen insect cells. For more details about the various InsectSelect™ System kits, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 29). See the table below for ordering information.

Kit	InsectSelect™ Vector	Catalog no.
InsectSelect™ BSD System Kit with Sf9 Cells	pIB/V5-His	K820-01
InsectSelect™ BSD System Kit with High Five™ Cells		K825-01
InsectSelect™ Kit with Sf9 Cells	pIZ/V5-His	K800-01
InsectSelect™ Kit with High Five™ Cells		K805-01
InsectSelect™ Glow Kit with Sf9 Cells	pIZT/V5-His	K810-01

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 N-terminal fusion proteins expressed using pIB/His. Horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-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-Xpress™ Antibody	Detects 8 amino acid Xpress™ epitope: DLYDDDDK	R910-25
Anti-Xpress™-HRP Antibody		R911-25
Anti-HisG Antibody	Detects the N-terminal polyhistidine (6xHis) tag followed by glycine: HHHHHHG	R940-25
Anti-HisG-HRP Antibody		R941-25
Anti-HisG-AP Antibody		R942-25

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Accessory Products, continued

Purification of Recombinant Protein

The metal binding domain encoded by the polyhistidine tag allows simple 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 (includes six 2 ml precharged ProBond™ resin columns and buffers for native and denaturing purification)	6 purifications	K850-01
ProBond™ Purification System with Anti-Xpress™-HRP Antibody	1 kit	K851-01
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. pIB/His A, B, and C are 3.6 kb vectors that use the InsectSelect™ technology to allow expression of your protein of interest in insect cell lines. The pIB/His vector contains the following features:

- *OpIE2* promoter for high-level, constitutive expression of the gene of interest (Theilmann and Stewart, 1992)
- N-terminal peptide containing the Xpress™ epitope and polyhistidine (6xHis) tag for detection and purification of your recombinant protein of interest
- *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*
- Three reading frames to facilitate in-frame cloning with the N-terminal peptide

The control plasmid, pIB/His/*lacZ*, is included for use as a positive control for expression.

Description of System

The gene of interest is cloned into pIB/His and transfected into Sf9, Sf21, 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 26 and page 27, 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 µg/ml (human IL-6; Invitrogen) to 8-10 µg/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 pIB/His A, B, or C in frame with the N-terminal peptide encoding the Xpress™ epitope and the polyhistidine (6xHis) tag.	4-7
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 or 100-150 µg/ml blasticidin in Low Salt LB.	8
4	Use sequencing to confirm that your protein is cloned in frame with the N-terminal peptide.	8
5	Transfect Sf9, Sf21, or High Five™ cells.	10-12
6	Assay for transient expression of your protein.	12-13
7	Create stable cell lines expressing the protein of interest by selecting with the appropriate concentration of blasticidin.	15-18
8	Scale-up expression for purification.	19
9	Purify your recombinant protein by chromatography on metal-chelating resin (i.e. ProBond™).	19-20

Methods

Culturing Insect Cells

Introduction

Before you start your cloning experiments, be sure to have 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 may be viewed and printed from our web site (www.invitrogen.com) as a PDF (portable document format) file if you have Adobe® Reader (available **free** from www.adobe.com). Alternatively, you may request the manual from Technical Service (see page 29).

Cloning into pIB/His A, B, and C

Introduction

The pIB/His vector is supplied with the multiple cloning site in three reading frames (A, B, and C) to facilitate cloning your gene of interest in frame with the N-terminal peptide containing the Xpress™ epitope and a polyhistidine (6xHis) tag. Use the diagrams provided on pages 5-7 to help you design a strategy to clone your gene of interest in frame with the N-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 pIB/His A, B, C, and pIB/His/*lacZ* 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 pIB/His and pIB/His/*lacZ* plasmids, we recommend using the following procedure:

1. Resuspend each vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.
2. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain such as TOP10, DH5α, JM109, or equivalent (see page 8 for more information).
3. Select transformants on LB agar plates containing 50-100 µg/ml ampicillin or Low Salt LB agar plates containing 100-150 µg/ml blasticidin (see page 21 for a recipe).
4. Prepare a glycerol stock from each transformant containing plasmid for long-term storage (see page 9).

Cloning Considerations

The pIB/His vectors are fusion vectors. To ensure proper expression of your recombinant protein, you must clone your gene in frame with the ATG at base pairs 566-568. This will create a fusion with the N-terminal polyhistidine (6xHis) tag, Xpress™ epitope, and the enterokinase cleavage site. The vector is supplied in three reading frames to facilitate cloning. See pages 5-7 to develop a cloning strategy.

If you wish to clone as closely as possible to the enterokinase cleavage site, follow the guidelines below:

- Digest pIB/His A, B, or C with *Kpn* I.
- Create blunt ends with T4 DNA polymerase and dNTPs.
- Clone your blunt-ended insert in frame with the lysine codon (AAG) of the enterokinase recognition site.

If you wish to separate your recombinant protein of interest from the N-terminal peptide tag, you may use any suitable enterokinase including EnterokinaseMax™ (EKMax™, Catalog no. E180-01) from Invitrogen. Using the strategy outlined above, no vector-encoded amino acid residues will be present in your protein following enterokinase cleavage.

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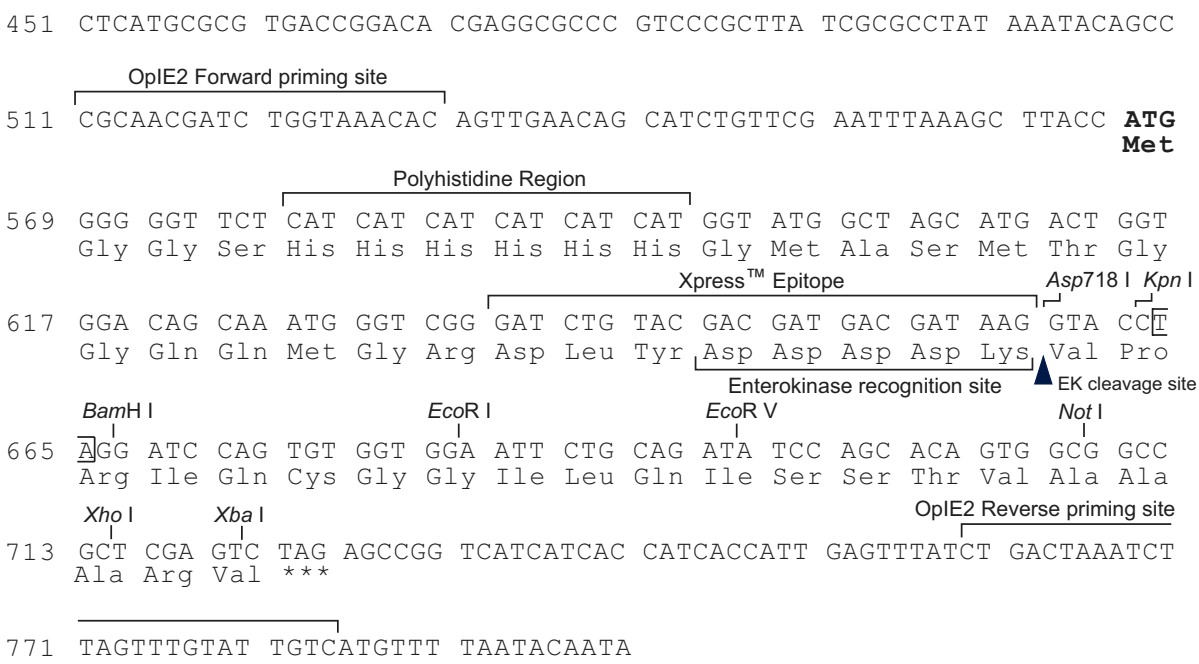
Cloning into pIB/His A, B, and C, continued

Multiple Cloning Site of pIB/His A

Below is the multiple cloning site for pIB/His A. 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 pIB/His A is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 29).** For a map and a description of the features of pIB/His A, refer to pages 23-24.



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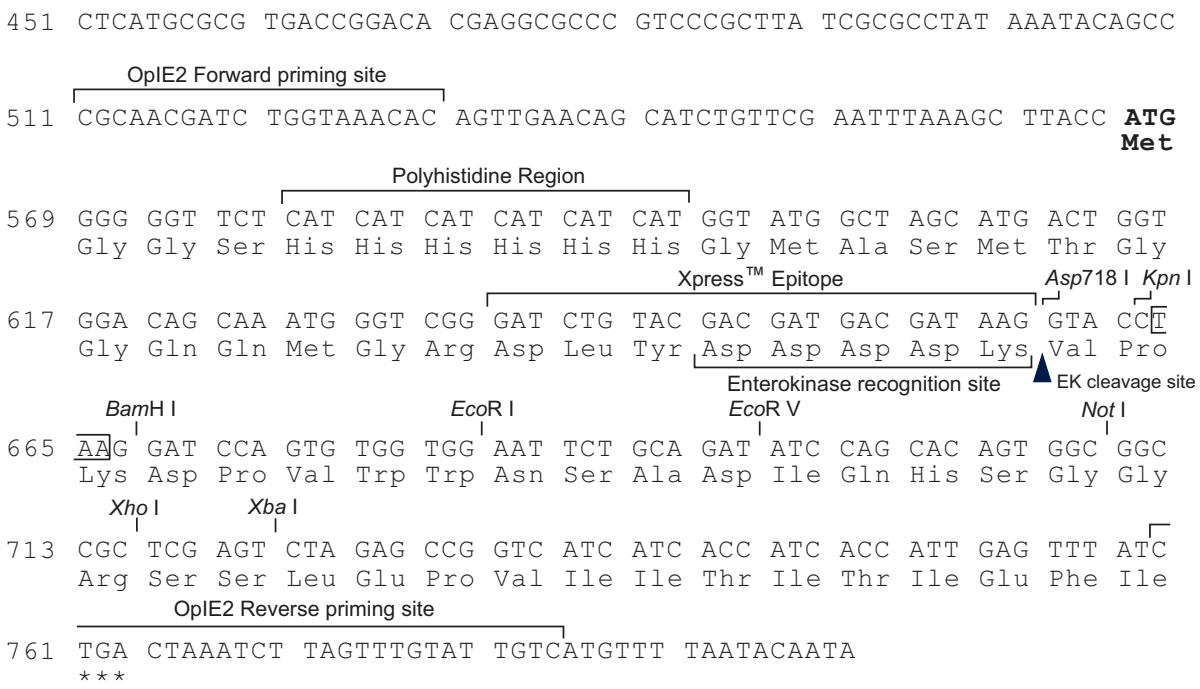
Cloning into pIB/His A, B, and C, continued

Multiple Cloning Site of pIB/His B

Below is the multiple cloning site for pIB/His B. 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 pIB/His B is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 29).** For a map and a description of the features of pIB/His B, refer to pages 23-24.



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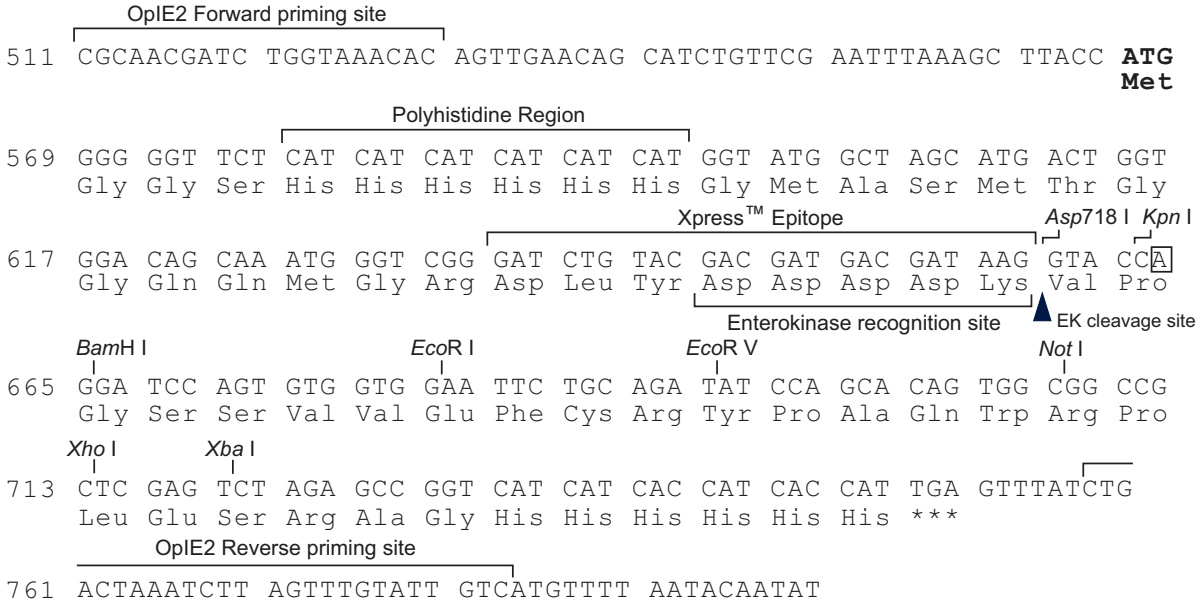
Cloning into pIB/His A, B, and C, continued

Multiple Cloning Site of pIB/His C

Below is the multiple cloning site for pIB/His C. 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 pIB/His C is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 29).** For a map and a description of the features of pIB/His C, refer to pages 23-24.



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/His including TOP10 (Catalog no. C610-00) or DH5α. 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.

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 100-150 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 21.

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 peptide containing the Xpress™ 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 pIB/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 S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) or other resin-based DNA purification systems. The S.N.A.P.™ MiniPrep Kit is a small-scale plasmid isolation kit that isolates 10-15 µg of plasmid DNA from 10-15 ml of bacterial culture. 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 or Sf21 cells**
- **40-60% for High Five™ cells**

Note: Other transfection methods (i.e. calcium phosphate and electroporation (Mann and King, 1989)) have 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 (1.6-2.5 x 10^6 cells/ml, >95% viability) or log-phase High Five™ cells (1.8-2.3 x 10^6 cells/ml, >95% viability), growing in serum-free medium. Note: You may transfect Sf9 or Sf21 cells in Grace's Medium without supplements.
- 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

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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/His. Express Five® SFM is recommended for use with High Five™ cells while Sf-900 II SFM is optimized for use with Sf9 and Sf21 cells (see page vi for ordering information). Other serum-free media may be used, although you may have to optimize conditions for transfection and selection. Note that if you are culturing Sf9 or Sf21 cells in Grace's Medium and wish to switch to serum-free medium, you will need to adapt the cells to serum-free medium before transfection (see the 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, or High Five™ cells**, seed 2 x 10^6 cells in the 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:

- pIB/His/*lacZ* 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 Medium (Sf9) **OR** Appropriate serum-free medium	1 ml
pIB/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. If the medium contained serum, wash the cells by carefully adding 2 ml of fresh Grace's Medium without supplements or FBS. This will remove trace amounts of serum that will decrease the efficiency of liposome transfection.
5. Again, carefully remove the medium from the monolayer and add the entire transfection mix dropwise 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. Following the 4-hour incubation period, add 1-2 ml of complete TNM-FH medium (Sf9 or Sf21 cells) or the appropriate serum-free medium (Sf9, Sf21, or High Five™ cells) 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 is no need to add fresh medium if the cells are sealed in an airtight plastic bag with moist paper towels.

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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-Xpress™ antibodies or the Anti-HisG antibodies available from Invitrogen (see page vii 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 an Xpress™ 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 call Technical Service (see page 29).

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 22 for your convenience, but other recipes are suitable. If you are using pre-cast polyacrylamide gels (see the next page), 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.
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 (Pierce).
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.
7. Boil the samples for 5 minutes. Centrifuge briefly.
8. Load approximately 3 to 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 method of choice.

Note

The N-terminal tag containing the Xpress™ epitope and 6xHis tag will increase the size of your protein by ~3.1 kDa. Note that any additional amino acids between your protein and the tags are not included in this molecular weight calculation.

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Transient Expression in Insect Cells, continued

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The patented NuPAGE® Gel System avoids the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. 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 World Wide Web site (www.invitrogen.com) or call Technical Service (see page 29).

Assay for β-galactosidase Activity

You may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. Invitrogen offers the β-Gal Assay Kit (Catalog no. K1455-01) and the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression.

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 (lipids only; no DNA). Use clean, pure DNA isolated by resin based DNA isolation kits (i.e. 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 N-terminal peptide containing the Xpress™ epitope and the polyhistidine (6xHis) tag.** If your gene is not cloned in frame with the N-terminal peptide, the recombinant protein may not be expressed at all. Re-design your cloning strategy to make sure that you clone your gene in frame with the N-terminal peptide.
- **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.

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 S2 cells using the *Drosophila* Expression System is generally not observed. For more information about the *Drosophila* Expression System, see our Web site or call Technical Service (see page 29).

Before Starting

Review the information on blasticidin S on page 28. 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 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 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 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 (i.e. 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).

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 done in 24-well tissue culture plates.

- Prepare TNM-FH medium or the serum-free medium of choice supplemented with concentrations ranging from 0 to 100 µg/ml blasticidin. Generally, concentrations that effectively kill lepidopteran insect cells within a week are in the 50 to 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.
- Test varying concentrations of blasticidin on the cell line to determine the concentration that kills your cells within a week (kill curve).

Use the concentration of drug that kills your cells within a week.

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 (pIB/His/*lacZ*).

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 µl 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 x 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 x 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.

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.

Metal-chelating Resin

You may use the ProBond™ Purification System (Catalog no. K850-01) or a similar product to purify your 6xHis-tagged protein. The ProBond™ Purification System contains ProBond™, a metal-chelating resin specifically designed to purify 6xHis-tagged proteins. Before starting, be sure to consult the ProBond™ 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.

continued on next page

Scale-Up and Purification, continued

Purification of Recombinant Protein

You may lyse the cells and add the lysate directly to the ProBond™ column. You will need 5 x 10^6 to 1 x 10^7 cells for purification of your protein on a 2 ml ProBond™ column (see ProBond™ Purification System manual).

1. Seed 2 x 10^6 cells in two or three 25 cm^2 flasks.
2. Grow the cells in selective medium until they reach confluence (4 x 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 x 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.

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 100-150 μg/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 μM leupeptin, and 0.1 μM aprotinin before use.

1X PBS

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

1. Dissolve: 8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_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:

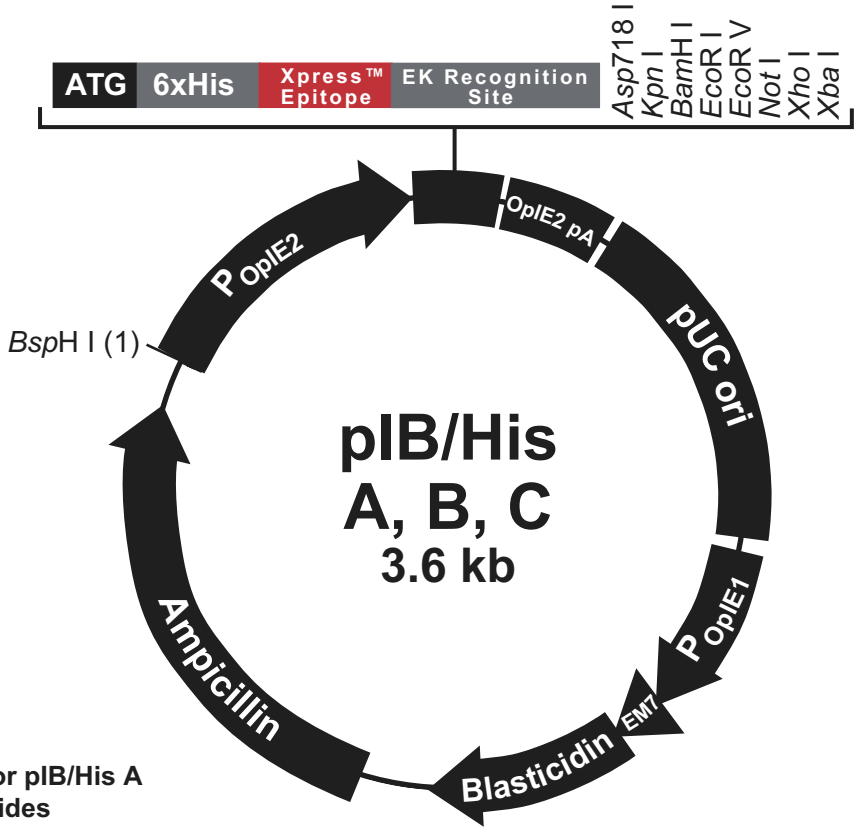
0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol (100%)	2 ml
β-mercaptoethanol	0.4 ml
Bromophenol Blue	0.02 g
SDS	0.4 g

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

pIB/His Map and Features

Map of pIB/His

The figure below summarizes the features of the pIB/His A, B, and C vectors. For a more detailed explanation of each feature, see the next page. **The complete sequences of pIB/His A, B, and C are available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 29).**



Comments for pIB/His A
3543 nucleotides

OpIE2 promoter: bases 1-548
OpIE2 Forward priming site: bases 511-530
Initiation ATG: bases 566-568
Polyhistidine (6xHis) region: bases 578-595
Xpress™ epitope: bases 635-658
EK recognition site: bases 644-658
Multiple cloning site: bases 658-725
OpIE2 Reverse priming site: bases 759-784
OpIE2 polyadenylation sequence: bases 767-896
pUC origin: bases 965-1638 (complementary strand)
OpIE1 promoter: bases 1687-1978
EM7 promoter: bases 2003-2069
Blasticidin resistance gene (*bsd*): bases 2070-2468
Ampicillin resistance gene (*bla*): 2588-3448

continued on next page

pIB/His Map and Features, continued

Features of pIB/His

The features of pIB/His A (3543 bp), pIB/His B (3544 bp), and pIB/His C (3542 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 and Stewart, 1992).
OpIE2 Forward priming site	Allows sequencing of the insert from the 5′ end.
N-terminal polyhistidine (6xHis) tag	Allows purification of your recombinant protein on metal-chelating resin such as ProBond™. In addition, the N-terminal 6xHis tag is the epitope for the Anti-HisG Antibody (Catalog no. R940-25) and the Anti-HisG-HRP Antibody (Catalog no. R941-25).
Xpress™ epitope	Allows detection of your recombinant protein with the Anti-Xpress™ Antibody (Catalog no. R910-25).
Enterokinase cleavage site	Allows removal of the N-terminal tag from your recombinant protein using an enterokinase such as EnterokinaseMax™ (Catalog no. E180-01).
Multiple cloning site	Allows insertion of the gene of interest.
OpIE2 Reverse priming site	Allows sequencing of the insert from the 3′ end.
OpIE2 polyadenylation sequence	Allows efficient transcription termination and polyadenylation of mRNA (Theilmann and Stewart, 1992).
pUC origin	Allows high-copy replication and maintenance in *E. coli*.
OpIE1 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 *E. coli*.
Blasticidin resistance gene (*bsd*)	Allows generation of stable insect cell lines (Kimura *et al.*, 1994).
Ampicillin resistance gene (*bla*)	Allows selection of transformants in *E. coli*. **Note**: The native promoter has been removed. Transcription is assumed to start from the EM7 promoter.

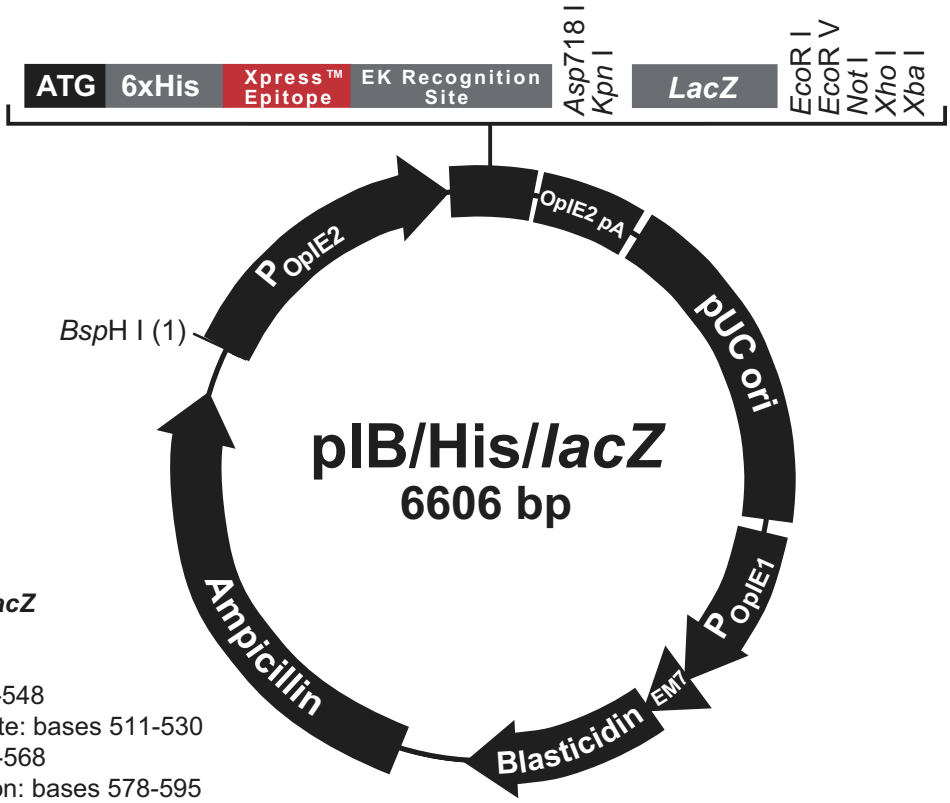
pIB/His/*lacZ* Map

Description

pIB/His/*lacZ* is a 6606 bp control vector expressing β-galactosidase. The plasmid was constructed by cloning a *Hin*d III/*Xho* I fragment containing the *lacZ* gene into pIB/His B. In pIB/His/*lacZ*, β-galactosidase is expressed as a fusion to the Xpress™ epitope and 6xHis tag. The molecular weight of the protein is 120 kDa.

Map

The figure below summarizes the features of the pIB/His/*lacZ* vector. **The complete nucleotide sequence for pIB/His/*lacZ* is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (see page 29).**



Comments for pIB/His/*lacZ*
6606 nucleotides

OpIE2 promoter: bases 1-548
OpIE2 Forward priming site: bases 511-530
Initiation ATG: bases 566-568
Polyhistidine (6xHis) region: bases 578-595
Xpress™ epitope: bases 635-658
EK recognition site: bases 644-658
LacZ ORF (no ATG): bases 683-3733
OpIE2 Reverse priming site: bases 3822-3847
OpIE2 polyadenylation sequence: bases 3830-3959
pUC origin: bases 4028-4701 (complementary strand)
OpIE1 promoter: bases 4750-5041
EM7 promoter: bases 5066-5132
Blasticidin resistance gene (*bsd*): bases 5133-5531
Ampicillin resistance gene (*bla*): 5651-6511

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 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
```

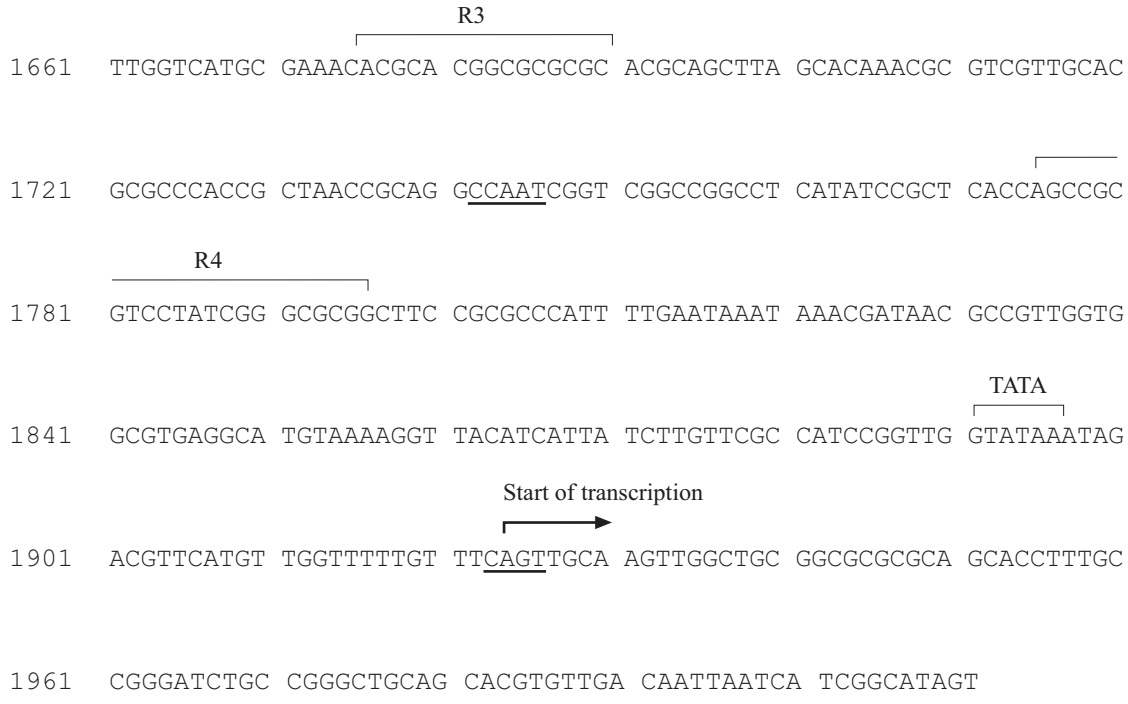
OpIE1 Promoter

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 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/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).



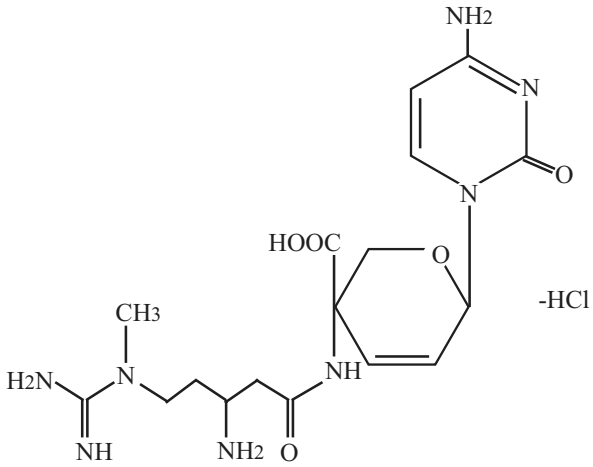
Blasticidin S

Molecular Weight, Formula, and Structure

Merck Index: 12: 1350

MW: 458.9

Formula: $C_{17}H_{26}N_8O_5$-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°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.

Technical Service

World Wide Web

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

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

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

www.invitrogen.com

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

Contact Us

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

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MSDS Requests

To request an MSDS, please visit our Web site (www.invitrogen.com) and follow the instructions below.

1. On the home page, go to the left-hand column under 'Technical Resources' and select 'MSDS Requests'.
2. Follow instructions on the page and fill out all the required fields.
3. To request additional MSDSs, click the 'Add Another' button.
4. All requests will be faxed unless another method is selected.
5. When you are finished entering information, click the 'Submit' button. Your MSDS will be sent within 24 hours.

continued on next page

Technical Service, continued

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, please contact our Technical Service Representatives.

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Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pIB/His Vector Kit.

Vectors

Each vector is qualified by restriction enzyme digestion with specific restriction enzymes as listed below. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel (see below).

Vector	Restriction Enzyme	Expected Results (bp)
pIB/His A	*Avr* II	3543
	*Bsa*H I	108, 1075, 2360
	*Bsu*36 I	No site
pIB/His B	*Avr* II	No site
	*Bsa*H I	108, 1075, 2361
	*Bsu*36 I	3544
pIB/His C	*Avr* II	No site
	*Bsa*H I	108, 1075, 2359
	*Bsu*36 I	No site
pIB/His/*lacZ*	*Avr* II	No site
	*Bsa*H I	108, 172, 821, 1075, 1528, 2902
	*Bsu*36 I	No site

Primers

Both primers have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

Purchaser Notification

Limited Use Label License No. 68: InsectSelect™ Technology

The InsectSelect™ System (the "Expression Kit") was developed into an expression system by scientists at the University of British Columbia (UBC) for high-level expression of recombinant proteins. The Expression Kit also incorporates use of the Zeocin™ selection marker that is licensed to Invitrogen.

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Purchaser Notification, continued

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Blasticidin and the blasticidin resistance gene (*bsd*) are sold under patent license and may be used for **research purposes only**. Inquiries for commercial use should be directed to: Kaken Pharmaceutical Company, Ltd., Bunkyo Green Court, Center Office Building, 19-20 Fl, 28-8 Honkomagome 2-chome, Bunkyo-ku, Tokyo 113-8650, Japan, Tel: 81 3-5977-5008; Fax: 81 3-5977-5008.

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