invitrogen™

InsectSelect™ System

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

Catalog nos. K800-01, K805-01, V8000-01

Version H
21 October 2010
25-0282

User Manual

Table of Contents

Kit Contents

Types of Kits

This manual covers the kits listed below.

Kit	Catalog no.
InsectSelect™ System with Sf9 Cells	K800-01
InsectSelect™ System with High Five™ Cells	K805-01
pIZ/V5-His Vector Kit	V8000-01

Shipping/Storage

See the table below for shipping and storage information.

Kit	Shipping	Storage
pIZ/V5-His Vector Kit	Wet Ice	–20°C
InsectSelect™ System with Sf9 Cells	Dry Ice	Vectors, primers: –20°C Zeocin™: –20°C, protected from light
InsectSelect™ System with High Five™ Cells	Dry Ice	Cells: Liquid nitrogen Cellfectin® Reagent: +4°C Medium: +4°C, protected from light

Vectors and Primer

Supplied with all the kits listed above.

Store at –20°C.

Item	Composition	Volume
pIZ/V5-His	20 µg at 0.5 µg/µl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	40 µl
pIZ/V5-His/CAT	20 µg at 0.5 µg/µl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	40 µl
OpIE2 Reverse Sequencing Primer	Lyophilized in TE, pH 8	2 µg

Primer Sequence

The sequence of the primer is provided below:

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

Zeocin™

Supplied with the InsectSelect™ System kits only. Zeocin™ is available separately, see page viii.

Store at –20°C, protected from light.

Amount Supplied: 1 g (8 tubes x 125 mg)

Composition: 100 mg/ml in autoclaved, deionized water (1.25 ml aliquots)

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Kit Contents, continued

Cellfectin® Reagent

Supplied with the InsectSelect™ System kits only. Cellfectin® Reagent is available separately, see page viii.

Store at +4°C.

Amount Supplied: 125 µl

Composition: 1 mg/ml lipid in membrane-filtered water

Cells and Medium

Supplied with the InsectSelect™ System kits only. Additional cells and other cell lines are available separately, see page viii.

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™ System kit you ordered. Refer to the table below. To culture Sf9 and High Five™ cells, refer to the Insect Cell Lines manual included with each kit.

Kit	Cells	Medium
InsectSelect™ System with Sf9 Cells	Sf9	Grace's Insect Cell Culture Medium, Unsupplemented (contains L-glutamine) 2 x 500 ml
InsectSelect™ System with High Five™ Cells	High Five™	Express Five® Serum-Free Medium 1 liter

Manuals

The following manuals are supplied with each kit.

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

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Kit Contents, continued

Reagents Supplied by the User

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

- Fetal Bovine Serum (FBS)
- 5, 10, and 25 ml sterile pipettes
- Cryovials
- Hemacytometer and Trypan Blue (see 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 page 21)
- PBS (see page 22)
- Cloning cylinders (optional)
- 96-well plates (optional)

Accessory Products

Products Available Separately

The following products are available separately from Invitrogen.

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	1 liter	10902-088
Express Five® SFM	1 liter	10486-025
Cellfectin® Reagent	1 ml	10362-010
Zeocin	1 gram	R250-01
	5 gram	R250-05
pIZ/V5-His Vector Kit	20 µg pIZ/V5-His 20 µg pIZ/V5-His/CAT 2 µg OpIE2 Reverse primer	V8000-01

Other InsectSelect™ Kits

Several other kits that allow you to clone and stably express your gene of interest using the InsectSelect™ technology are available from Invitrogen. These kits include InsectSelect™ vectors with different antibiotic resistance genes. In addition, the pIZT/V5-His Vector Kit enables expression of a gene of interest and a cycle 3-GFP/Zeocin™ fusion gene. This allows both visual monitoring of transfection efficiency and generation of a stable cell line. For more information about the various InsectSelect™ vector kits available from Invitrogen, visit our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 28). See the table below for ordering information.

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

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Accessory Products, continued

Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using an antibody to the appropriate epitope. The table below describes the antibodies available for detection of C-terminal fusion proteins expressed using the pIZ/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

Purification of Recombinant Protein

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

Product	Quantity	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Metal-Binding Resin	50 ml	R801-01
	150 ml	R801-15
Purification Columns (10 ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

The InsectSelect™ System allows constitutive stable or transient expression of your protein of interest in insect cell lines. The system utilizes a single expression vector, pIZ/V5-His to express your gene of interest. This 2.9 kb vector has the following features:

- *OpIE2* promoter for high-level, constitutive expression of the gene of interest (Theilmann and Stewart, 1992)
- Zeocin™ resistance gene for selection of stable cell lines (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997)
- Optional C-terminal peptide containing the V5 epitope and 6xHis tag for detection and purification of your protein of interest

Description of System

The gene of interest is cloned into pIZ/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 Zeocin™ 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 Promoter

Baculovirus immediate-early promoters utilize the host cell transcription machinery and do not require viral factors for activation. The OpIE2 promoter is from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*Op*MNPV). The virus' natural host is the Douglas fir tussock moth; however, the promoter allows 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, SL2) (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997), and mosquito cell lines (unpublished data). The OpIE2 promoter has been sequenced and analyzed. For more detailed information on this promoter, see page 26.

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, expression levels range from 1 μg/ml (human IL-6; Invitrogen) to 8–10 μg/ml (human melanotransferrin) (Hegedus *et al.*, 1999).

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Overview, continued

Zeocin™ Resistance

Zeocin™, a member of the phleomycin family of antibiotics, exhibits toxicity towards a broad range of prokaryotic and eukaryotic organisms. Recently it has been demonstrated that Zeocin™ can be used to select resistant insect cell lines (i.e. Sf9 and *Drosophila* Kcl and SL2) (Pfeifer *et al.*, 1997). Shuttle vectors were developed that utilized a second copy of the OpIE2 promoter to express the *Streptoalloteichus hindustanus ble* gene (*Sh ble;* Zeocin™ resistance gene) (Hegedus *et al.*, 1998). Insect cells transfected with these plasmids can be selected for stable integration of the plasmid. Analysis of stable cell lines reveals that vector integration into chromosomal DNA is multi-copy in nature (Pfeifer *et al.*, 1997). For more information on Zeocin™, see page 27.

Experimental Outline

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

Step	Action	Source
1	Establish culture of Sf9 or High Five™ cells from supplied frozen stock. **Note**: Other cell lines (i.e. Sf21) may be used.	Refer to the Insect Cell Lines manual included with the System Kits or use your own laboratory protocols.
2	Develop a cloning strategy to ligate your gene of interest into pIZ/V5-His.	Page 4, this manual
3	Ligate your gene into pIZ/V5-His and transform into a *rec*A, *end*A *E. coli* strain (e.g. TOP10). Select on Low Salt LB plates containing 25–50 μg/ml Zeocin™.	Pages 6-7, 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	Transiently transfect Sf9 or High Five™ cells.	Page 9, this manual
6	Assay for expression of your protein.	Page 12, this manual
7	Create stable cell lines expressing the protein of interest by selecting with Zeocin™.	Page 15, this manual
8	Scale-up expression for purification.	Page 19, this manual Insect Cell Lines manual
9	Purify your recombinant protein by chromatography on metal-chelating resin (i.e. ProBond™).	Page 19, 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™ System kits (Catalog nos. K800-01 or K805-01), you will receive either Sf9 or High Five™ cells and the Insect Cell Lines manual. Use this manual as a guide to initiate cell culture.

Insect Cell Lines Manual

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 28).

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 complete TNM-FH (TNM-FH containing 10% FBS).

Sf21 Cells

You may also use Sf21 cells as a host for pIZ/V5-His. Sf21 cells are larger and 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 9-14 to determine how many cells you will need for transfection.

Cloning into pIZ/V5-His

Introduction

This chapter provides information to help you clone your gene of interest into pIZ/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 pages 7-8.
- For information on transfection into Sf9 or High Five™ cells see pages 9-14.

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 pIZ/V5-His

To propagate and maintain pIZ/V5-His, use 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 Low Salt LB plates containing 25 to 50 μg/ml Zeocin™ (see page 7).

Translation Initiation

Your insert should contain a Kozak translation initiation sequence and an ATG start codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Note that 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 consensus sequence. The ATG start 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 or utilize one of the stop codons available in the multiple cloning site. For example, the *Xba* I site contains a stop codon. Be sure to clone in frame with the stop codon.

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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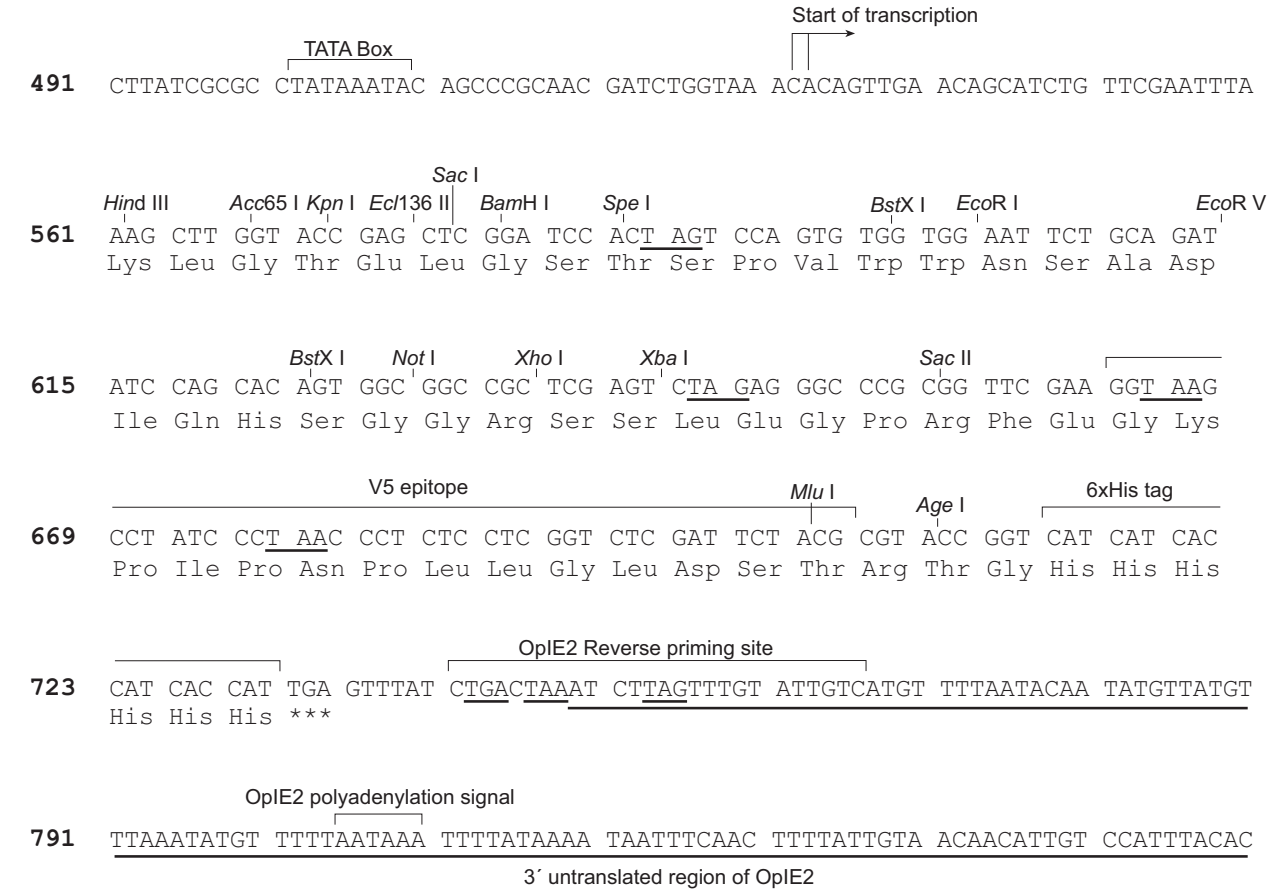
Cloning into pIZ/V5-His, continued

MCS of pIZ/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 cleavage site. Potential stop codons are shown underlined. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pIZ/V5-His is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 28).** For a map and a description of the features of pIZ/V5-His, refer to pages 23-24.



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Cloning into pIZ/V5-His, continued

E. coli Transformation

Prepare competent *rec*A, *end*A *E. coli* cells (e.g. TOP10) using your method of choice. Transform your ligation mixtures and select on Low Salt LB plates containing 25–50 μg/ml Zeocin™ (see page 7 for more information). Select 10–20 clones and analyze for the presence and orientation of your insert.

Important

Note that pIZ/V5-His contains two copies of the OpIE2 promoter (see map on page 23). We have tested the stability of this plasmid with and without insert by serially passaging transformed *E. coli* TOP10 cells (*recA*) over 3 or 4 days. We have detected some rearrangement by day 3. We have not observed rearrangement after overnight growth.

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 Reverse primer included in your kit and a primer to your gene of interest to sequence your insert (see previous page).

Important: Do not use a primer that is homologous to sequences in the OpIE2 promoter. A second OpIE2 promoter drives expression of the Zeocin™ resistance gene. The primer will bind to both locations and give you unreadable sequence.

Transforming *E. coli*

Introduction

The pIZ/V5-His vector contains the Zeocin™ resistance gene for selection of transformants in *E. coli* and selection of stable cell lines in insect cells (Drocourt *et al.*, 1990; Pfeifer *et al.*, 1997). The Zeocin™ antibiotic can be inactivated by high salt concentrations and extremes in pH. Special considerations are listed below to help you successfully isolate transformants in *E. coli*.

E. coli Host

Many *E. coli* strains are suitable for transformation of pIZ/V5-His including TOP10 (Catalog no. C610-00) or DH5 . We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*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
One Shot® TOP10 (chemically competent cells)	21 x 50 µl	C4040-03

Important

DO NOT USE any *E. coli* strain that contains the complete Tn*5* transposon (i.e. DH5 F´IQ, SURE, SURE2). This transposon encodes a *ble* (bleomycin) resistance gene which will confer resistance to Zeocin™, preventing selection of colonies containing your pIZ/V5-His construct.

Transformation Method

You may use your method of choice to transform *E. coli*. To select transformants, use Low Salt LB plates containing 25–50 µg/ml Zeocin™ (see recipe below). **Zeocin™ can be inactivated by high salt and extremes in pH.**

Low Salt LB Medium and Agar Plates

Composition: 1.0% Tryptone; 0.5% Yeast Extract; **0.5% NaCl;** pH 7.5

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and **5 g NaCl** in 950 ml deionized water.
2. Adjust pH of solution to 7.5 with NaOH and bring volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add Zeocin™ to a final concentration of 25–50 µg/ml.
4. Store at room temperature or at +4°C, protected from light. Medium is stable for ~2 weeks.

Low Salt LB agar plates

1. Prepare Low Salt LB medium as above, but add 15 g/L agar.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. Let cool to ~55°C, add Zeocin™ (25–50 µg/ml), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark. Plates are stable for ~2 weeks to 1 month.

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Transforming *E. coli*, continued

Note

For convenient preparation of Low Salt LB medium or plates containing Zeocin™, we offer imMedia™. imMedia™ is premixed, pre-sterilized *E. coli* growth medium that contains everything you need in a convenient pouch. You can easily prepare either Low Salt LB liquid medium (200 ml) or agar plates (8–10 plates). Simply mix the pouch contents with distilled water, microwave the solution, and pour plates or cool the liquid medium before inoculating *E. coli*. Ordering information is provided below. For more information, contact Technical Service, page 28.

Item	Amount	Catalog no.
imMedia™ Zeo Liquid	20 pouches	Q620-20
imMedia™ Zeo Agar	20 pouches	Q621-20

Long Term Storage

For long-term storage, prepare a glycerol stock of each strain containing plasmid. It is also a good idea to keep a stock of the DNA at –20°C.

To prepare a glycerol stock:

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

Important

Note that pIZ/V5-His contains two copies of the OpIE2 promoter (see map on page 23). We have tested the stability of this plasmid with and without insert by serially passaging transformed *E. coli* TOP10 cells (*recA*) over 3 or 4 days. We have detected some rearrangement by day 3. We have not observed rearrangement after overnight growth.

Transient Expression in Insect Cells

Introduction

Once you have cloned your gene of interest into pIZ/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 PureLink™ HiPure Plasmid Midiprep Kit (Catalog no. K2100-04) or CsCl gradient centrifugation. The PureLink™ HiPure Plasmid MidiPrep Kit is a medium-scale plasmid isolation kit that isolates 10–200 μg of plasmid DNA from 10–100 ml of bacterial culture. Plasmid can be used directly for transfection of insect cells.

Method of Transfection

We recommend lipid-mediated transfection with Cellfectin® Reagent. Note that other lipids may be substituted.

Expected Transfection Efficiency using Cellfectin® Reagent:

- **40–60% for Sf9 cells**
- **40–60% for High Five™ cells**

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

Control of Plasmid Quality

To test the quality of a plasmid DNA preparation, include a mock transfection (DNA only; no lipids) in all transfection experiments. At about 24 to 48 hours posttransfection, compare the DNA only 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 1–10 μg of highly purified plasmid DNA (~1 μg/μl in TE buffer) for each transfection experiment and the following materials.

- Either log phase Sf9 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)
- Serum-free medium (see the next page)
- 60 mm tissue-culture dishes
- 1.5 ml sterile microcentrifuge tubes
- Rocking platform only (NOT orbital)
- 27°C incubator
- Inverted Microscope
- Paper towels and air-tight bags or containers
- 5 mM EDTA, pH 8

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Transient Expression in Insect Cells, continued

Serum-Free Media

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

Cellfectin® Reagent

Cellfectin® Reagent is a 1:1.5 (M/M) liposome formulation of the cationic lipid N, N^{I}, N^{II}, N^{III}-Tetramethyl- N, N^{I}, N^{II}, N^{III}-tetrapalmitylspermine (TM-TPS) and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water. Cellfectin® Reagent has been found to be superior for transfection of Sf9 and High Five™ insect cells.

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

   Rock gently from side to side for 2 to 3 minutes to evenly distribute the cells. Do not swirl the plates in a circular motion. Cells should be 50 to 60% confluent.
2. Incubate the cells for at least 15 minutes without rocking to allow the cells to fully attach to the bottom of the dish to form a monolayer of cells.
3. Verify that the cells have attached by inspecting them under an inverted microscope.

Positive and Negative Controls

We recommend that you include the following controls:

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

Note

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

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Transient Expression in Insect Cells, continued

Transfection Procedure

Mix plasmid DNA and Cellfectin® Reagent together in the appropriate medium (see below) and incubate 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 or FBS. The proteins in the FBS and supplements will interfere with the liposomes, causing the transfection efficiency to decrease.

1. To prepare each transfection mixture, use a 1.5 ml microcentrifuge tube. Add the following reagents:

Grace's Insect Media (Sf9) **OR** Appropriate serum-free medium	1 ml
pIZ/V5-His plasmid or construct (~1 µg/µl in TE, pH 8)	1–10 µl
Cellfectin® Reagent (**mix well before use and always add last**)	20 µl

2. Gently mix the transfection mixture for 10 seconds.
3. Incubate the transfection mixture at room temperature for 15 minutes. While the transfection mixture is incubating, proceed to Step 4.
4. Carefully remove the medium from the cells without disrupting the monolayer. **Note:** If you are using medium containing serum, wash the cells by carefully adding 2 ml of fresh Grace's Insect Media **without supplements or FBS**. This will remove trace amounts of serum that will decrease the efficiency of liposome transfection. Remove all of the medium from the monolayer.
5. Add the entire transfection mix dropwise into the 60 mm dish. Repeat for all transfections.

   (Distribute the drops evenly over the monolayer. This method reduces the chances of disturbing the monolayer.)
6. Incubate the dishes at room temperature for 4 hours on a side-to-side, rocking platform. Adjust speed to ~2 side to side motions per minute. **Note**: If you do not have a rocker, manually rock the dishes periodically.
7. Following the 4-hour incubation period, add 1–2 ml of complete TNM-FH medium (Sf9 cells) or the appropriate serum-free medium to each 60 mm dish, place the dishes in a sealed plastic bag with moist paper towels to prevent evaporation and incubate at 27°C. **Note**: It is not necessary to remove the transfection solution as Cellfectin® Reagent is not toxic to the cells. If you are using a different lipid and observe loss of viability, then remove the transfection solution after 4 hours, rinse two times with medium, and replace with 1–2 ml of fresh medium.
8. Harvest the cells 2, 3, and 4 days posttransfection and assay for expression of your gene (see next page). There's no need to add fresh medium if the cells are sealed in an airtight plastic bag with moist paper towels.

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Transient Expression in Insect Cells, continued

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 pages 21-22 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 secreted, be sure to save and assay the medium.**
3. Optional. You may wash the cells with PBS prior to adding the Cell Lysis Buffer if you are concerned about the presence of serum.
4. 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.
5. Centrifuge a 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).
6. Assay the lysate for protein concentration. You may use the Bradford method, Lowry assay, or BCA assay (Pierce).
7. 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.
   - Cell 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.
8. Boil the samples for 5 minutes. Centrifuge briefly.
9. Load approximately 3 to 30 µg protein per lane. For the cell pellet sample, load the same volume as the lysate. Amount loaded depends on the amount of your protein produced.
10. Electrophorese your samples, blot, and probe with antibody to your protein, antibody to the V5 epitope, or antibody to the C-terminal polyhistidine tag (see page ix).
11. Visualize proteins using your desired method. We recommend using chemiluminescence or alkaline phosphatase for detection.

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

Transient Expression in Insect Cells, continued

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 (www.invitrogen.com) or call Technical Service (see page 28).

Western Analysis

To detect expression of your recombinant fusion protein by Western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page ix for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope or a polyhistidine (6xHis) tag. WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 28).

Assay for CAT

If you use pIZ/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 (see page ix) or an antibody against CAT (Catalog no. R902-25). The CAT/V5-His protein fusion migrates around 34 kDa on an SDS-PAGE gel.

continued on next page

Troubleshooting

Cells Growing Too Slowly (Or Not At All).

For troubleshooting guidelines regarding cell culture, refer to the Insect Cell Lines manual included with the kit.

Low Transfection Efficiency.

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

- **Impure DNA.** Cells will appear unhealthy when compared to the negative control (DNA only, no lipids). Use clean, pure DNA isolated by resin based DNA isolation kits (i.e. PureLink™ HiPure Plasmid Midiprep Kit) or CsCl gradient ultracentrifugation.
- **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 or try a different method.

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 (i.e. High Five™).

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.

Effect of Zeocin™ on Sensitive and Resistant Cells

Cells may round up and detach from the plate. Sensitive cells may exhibit the following morphological changes upon exposure to Zeocin™.

- Cells stop growing
- Vast increase in size (similar to the effects of cytomegalovirus infecting permissive cells)
- Abnormal cell shape
- Granular appearance
- Presence of large empty vesicles in the cytoplasm (breakdown of the endoplasmic reticulum and Golgi apparatus or other scaffolding proteins)
- Breakdown of plasma and nuclear membrane (appearance of many holes in these membranes)
- Cellular debris in the medium

Eventually, cells sensitive to Zeocin™ will completely break down and only cellular debris will remain.

Zeocin™-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes in Zeocin™-resistant cells when compared to cells not under selection with Zeocin™. For more information on Zeocin™, see page 27.

Suggested Zeocin™ Concentrations

The table below provides recommended concentrations of Zeocin™ to use with Sf9, Sf21, and High Five™ cells. Effective concentrations are media-dependent. If you have trouble selecting cells using the concentrations below, we recommend that you perform a kill curve (see next page).

Cells	Media	Concentration of Zeocin™ (μg/ml)
Sf9	TNM-FH	300–400
	Express Five® Serum-Free	200–300
Sf21	TNM-FH	300–500
	Express Five® Serum-Free	300–400
High Five™	TNM-FH	400–600
	Express Five® Serum-Free	400–500

continued on next page

Selecting Stable Cell Lines, continued

Zeocin™ Selection Guidelines

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

- Prepare complete TNM-FH (Sf9) or Express Five® Serum-Free Medium (High Five™) supplemented with 100 to 1000 µg/ml Zeocin™. Generally, concentrations that kill lepidopteran insect cells are in the 200 to 600 µg/ml range.
- Test varying concentrations of Zeocin™ on the cell line to determine the concentration that kills your cells within a week (kill curve).
- Use the concentration of drug which kills your cells within a week.

Zeocin™ Selection in High Five™ Cells

If you are using High Five™ cells to generate stable cell lines, note that the state of confluency of the cells is important for effective Zeocin™ selection. Zeocin™ selection is less effective when cells are overly confluent, therefore, make sure that your cells are not greater than 20% confluent when adding Zeocin™ (see below).

Important: High Five™ cells do not form an even monolayer on the tissue culture dish when confluent. As the density increases, cells will pile up on one another and form patches on the plate.

Stable Transfection

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

1. Follow the transfection procedure on page 11, Steps 1 to 7.
2. Forty-eight hours posttransfection, remove the transfection solution and add fresh medium (**no Zeocin™**).
3. Split cells 1:5 (25% confluent) and let cells attach for 15 minutes before adding selective medium.
4. Remove medium and replace with medium containing Zeocin™ 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). **Note**: When the cells in the mock transfection are dead, you can drop the concentration of Zeocin™ by half.
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** Zeocin 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 Zeocin™ when maintaining stable lepidopteran cell lines. You may lower the concentration of Zeocin™ to 50 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 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 foci you can choose at a time. To select more foci, increase the number of plates or wells, not the size.

To select foci:

1. Examine the closed plate under a microscope and mark the location of each foci on the top of the plate. Transfer the markings to the bottom of the plate. Be sure to include orientation marks. **Note**: Each foci 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. **Note**: Silicon grease can be sterilized by placing a small amount in a glass petri dish and autoclaving it.
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 Zeocin™) to slough the cells. Try to hold the pipette tip away from the sides of the cloning cylinder (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** Zeocin 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.

1. Forty-eight hours after transfection, dilute the cells to 1 x 10^4 cells/ml in medium **without** Zeocin™. **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** Zeocin™ 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** Zeocin™ 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 Zeocin™. **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's not necessary to change the medium.
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 Zeocin™.
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 medium.** You may wish to compare the yield of protein in the cells and supernatant.

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 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 reduce the concentration of Zeocin™ to about 50 µg/ml. We have grown stably transformed Sf9 and High Five™ cell lines under nonselecting conditions for 60 passages without loss of protein expression.

Serum-Free Medium

If your protein is secreted, use a serum-free medium to facilitate expression and purification.

Adapting Cells to Different Medium

Sf9 cells can be switched from complete TNM-FH to Sf-900 II SFM during passage. Refer to the Insect Cell Lines manual for more information on how to adapt cells to different medium.

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

Purifying Proteins from Medium

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

Purification of 6xHis-tagged Proteins from Medium

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

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

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

continued on next page

Scale-Up and Purification, continued

Metal-chelating Resin

You may use the ProBond™ 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.

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 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 the cells.
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

TNM-FH Medium, Complete TNM-FH Medium

Grace's Insect Cell Culture Medium with additional supplements (TC yeastolate and lactalbumin hydrolysate) is referred to as **TNM-FH** (*Trichoplusia ni* Medium-Formulation Hink).

TNM-FH is not considered to be a complete medium until fetal bovine serum is added to a final concentration of 10%. The serum does not have to be heat inactivated; however, the quality of the serum is important for optimal cell growth.

Penicillin-Streptomycin may be added to a final concentration of 50 units/ml of penicillin G and 50 µg/ml of streptomycin sulfate. Many scientists prefer to leave out penicillin and streptomycin to avoid propagating low-level contamination.

Grace's Insect Cell Culture Medium, Unsupplemented (Catalog no. 11595-030) may be purchased separately from Invitrogen. Shelf life of the medium after opening is approximately 2 weeks at 27°C. Shelf life is increased to about a month if the medium is stored at +4°C. The color of the medium may vary from clear to yellow. This is not harmful to the cells.

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.

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 µM leupeptin and 0.1 µM aprotinin.

continued on next page

Recipes, continued

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 autoclave the solution to increase shelf life.

4X SDS-PAGE Sample Buffer

1. Combine the following reagents:

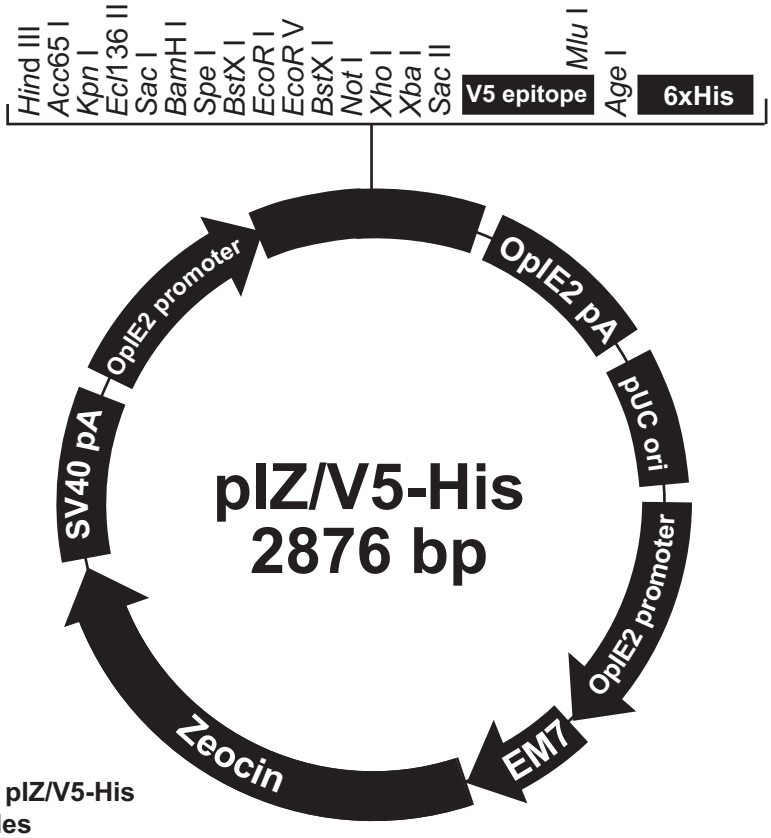
0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

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

pIZ/V5-His Map and Features

Map

The figure below summarizes the features of the pIZ/V5-His vector. For a more detailed explanation of each feature, see the next page. **The complete sequence of pIZ/V5-His is available from our Web site (www.invitrogen.com) or from Technical Service (see page 28).**



Comments for pIZ/V5-His 2876 nucleotides

OpIE2 promoter: bases 4-552
Multiple cloning site: bases 561-656
V5 epitope: bases 663-704
6xHis tag: bases 714-731
OpIE2 Reverse priming site: bases 741-766
OpIE2 polyadenylation sequence: bases 749-878
pUC origin: bases 947-1620
OpIE2 promoter: bases 1665-2213
EM7 promoter: bases 2231-2308
Zeocin™ resistance marker (ORF): bases 2309-2683
SV40 early polyadenylation sequence: bases 2747-2876

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pIZ/V5-His Map and Features, continued

Features

The features of pIZ/V5-His (2876 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 high-level, constitutive expression of the gene of interest in lepidopteran insect cells (Theilmann and Stewart, 1992).
Multiple cloning site (14 unique sites)	Permits 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	Permits purification of your recombinant protein on metal-chelating resin such as ProBond™. In addition, the C-terminal 6xHis tag is the epitope for the Anti-His(C-term) 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 and Stewart, 1992).
pUC origin	Replication, maintenance, and high copy number in *E. coli*.
OpIE2 promoter	Provides high-level, constitutive expression of the Zeocin™ resistance gene in lepidopteran insect cells (Theilmann and Stewart, 1992).
EM7 promoter	Allows efficient expression of the Zeocin™ resistance gene fusion in *E. coli*.
Zeocin™ resistance gene	Selection of transformants in *E. coli* and stable insect cell lines.
SV40 early polyadenylation sequence	Efficient transcription termination and mRNA stability.

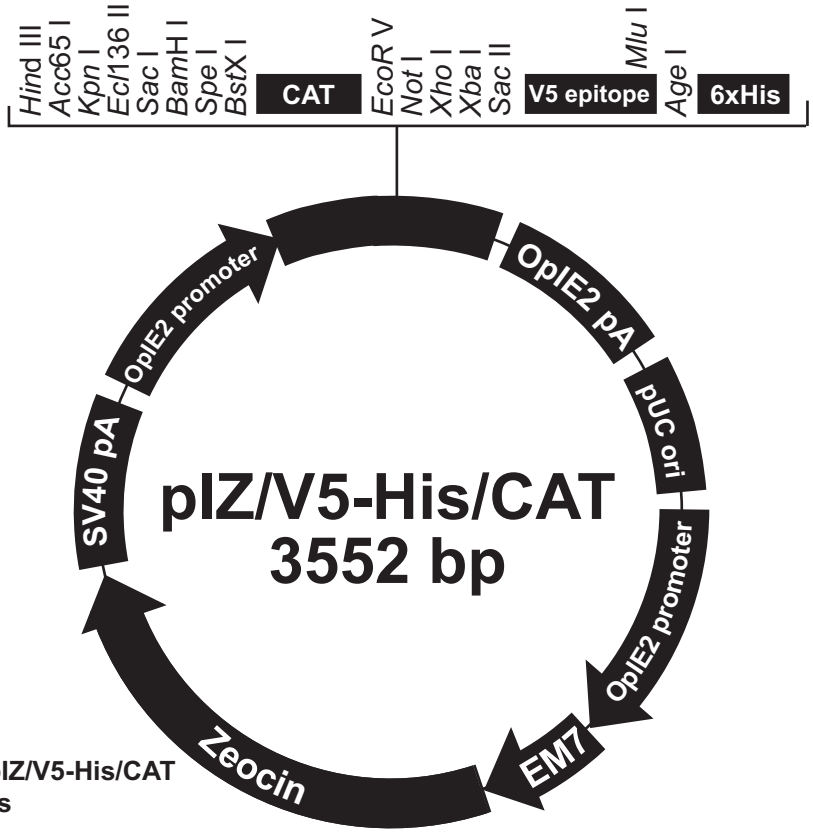
pIZ/V5-His/CAT Map

Description

pIZ/V5-His/CAT is a 3552 bp control vector expressing chloramphenicol acetyltransferase (CAT). CAT is expressed as a fusion to the V5 epitope and 6xHis tag. The molecular weight of the protein is 34 kDa.

Map

The figure below summarizes the features of the pIZ/V5-His/CAT vector. **The complete nucleotide sequence for pIZ/V5-His/CAT is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 28).**



Comments for pIZ/V5-His/CAT 3552 nucleotides

OpIE2 promoter: bases 4-552
CAT ORF: bases 616-1272
V5 epitope: bases 1339-1380
6xHis tag: bases 1390-1407
OpIE2 Reverse priming site: bases 1417-1442
OpIE2 polyadenylation sequence: bases 1425-1554
pUC origin: bases 1623-2296
OpIE2 promoter: bases 2341-2889
EM7 promoter: bases 2907-2984
Zeocin™ resistance marker (ORF): bases 2985-3359
SV40 early polyadenylation sequence: bases 3423-3552

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 are 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
```

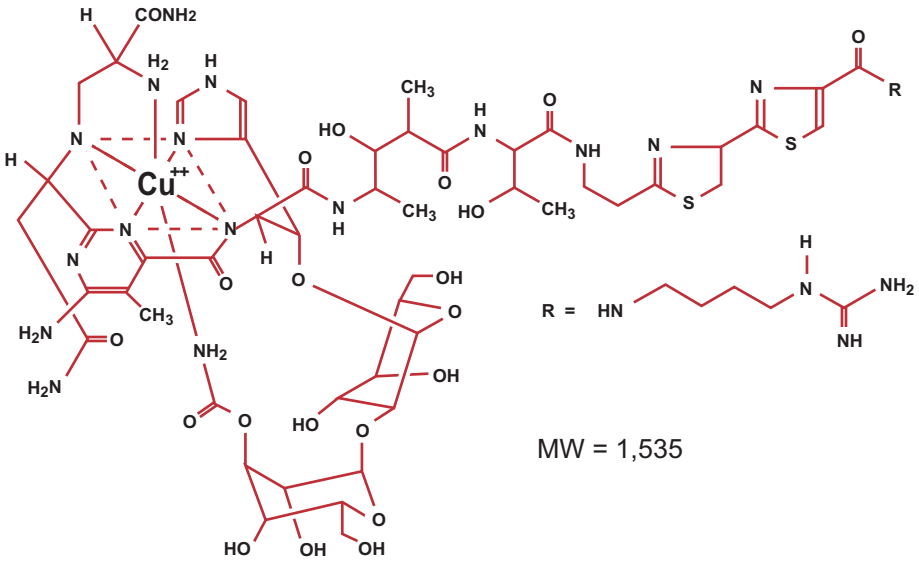
Zeocin™

Introduction

Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces* (Berdy, 1980). Zeocin™ and the resistance gene (*Sh ble*) can be used for selection in mammalian cells (Mulsant *et al.*, 1988); insect cells (Pfeifer *et al.*, 1997); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). It is particularly well-suited for selection of mammalian and insect stable cell lines.

Chemical Structure of Zeocin™

Zeocin™ is a formulation of phleomycin D1, a basic, water-soluble, copper-chelated glycopeptide isolated from *Streptomyces verticillus*. The presence of copper gives the solution its blue color. This copper-chelated form is inactive. When the antibiotic enters the cell, the copper cation is reduced from Cu^{2+} to Cu^{1+} and removed by sulfhydryl compounds in the cell. Upon removal of the copper, Zeocin™ is activated and will bind DNA and cleave it, causing cell death. The structure of Zeocin™ is shown below (Berdy, 1980).



Handling Zeocin™

- High salt and extremes in pH inactivate Zeocin™. Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active.
- Store Zeocin™ at –20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store drug, plates and medium containing drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling solutions containing Zeocin™.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Technical Service

World Wide Web

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog

Additional product information and special offers

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E-mail: eurotech@invitrogen.com

MSDS

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