InsectSelect™ Glow System
with pIZT/V5-His

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InsectSelect™ Glow System

For Detection of Transfected Cells and Stable Expression of Heterologous Proteins in Lepidopteran Insect Cell Lines using pIZT/V5-His

Catalog no. V8010-01; K810-01

Invitrogen™
life technologies

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Table of Contents

Kit Contents

Types of Kits

This manual covers the kits listed below.

Kit	Catalog no.
InsectSelect™ Glow System with Sf9 Cells	K810-01
pIZT/V5-His Vector Kit	V8010-01

Shipping/Storage

See the table below for shipping and storage information.

Kit	Shipping	Storage
pIZT/V5-His Vector Kit	Room Temperature	-20°C
InsectSelect™ Glow System with Sf9 Cells	Dry Ice	Vectors, primers: -20°C Zeocin™: -20°C, protected from light Cells: Liquid nitrogen Cellfectin® Reagent: +4°C Medium: +4°C, protected from light

Vectors and Primers

Supplied with all kits listed above.

Store at -20°C.

Item	Composition	Amount Supplied
pIZT/V5-His	Lyophilized in TE, pH 8	20 µg
pIZT/V5-His/CAT	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 sequences of the primers are provided below:

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

Zeocin™

Supplied with the InsectSelect™ Glow System Kit only. Zeocin™ is available separately, see page vii.

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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Cellfectin® Reagent

Supplied with the InsectSelect™ Glow System kit only. Cellfectin® Reagent is available separately, see page vii.

Store at +4°C.

Amount Supplied: 125 µl

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

Cells and Medium

Supplied with the InsectSelect™ Glow System Kit only. Additional cells, other cell lines, and media are available separately, see page vii.

Store the Sf9 cells in liquid nitrogen.

Amount supplied: 1 ml, 10^7 cells/ml in 60% complete TNM-FH, 30% FBS, 10% DMSO.

Store the Grace's Insect Cell Culture Medium, Unsupplemented (contains L-glutamine) at +4°C, protected from light.

Manuals

The following manuals are supplied with each kit.

Kit	Manual
InsectSelect™ Glow System with Sf9 Cells	InsectSelect™ Glow System manual Insect Cell Lines manual
pIZT/V5-His Vector Kit	InsectSelect™ Glow System manual only

Reagents Supplied by the User

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

- Fetal Bovine Serum
- 5, 10, and 25 ml sterile pipettes
- Cryovials
- Hemacytometer and Trypan Blue (see page 22)
- Table-top centrifuge
- 60 mm tissue culture plates (other flasks and plates may be used)
- Sterile microcentrifuge tubes (1.5 ml)
- Cell Lysis Buffer (see page 22)
- PBS (see page 23)
- Cloning cylinders (optional)
- 96-well plates (optional)

Accessory Products

Products Available Separately

The following products are available separately from Invitrogen.

Product	Amount	Catalog no.
pIB/V5-His TOPO® TA Expression Kit (see information below)	1 kit	K890-01
pIB/V5-His Vector Kit (see information below)	20 µg pIB/V5-His 20 µg pIB/V5-His/CAT 2 µg OpIE2 Forward primer 2 µg OpIE2 Reverse primer	V8020-01
pIZ/V5-His Vector Kit (see information on the next page)	20 µg pIZ/V5-His 20 µg pIZ/V5-His/CAT 2 µg OpIE2 Reverse primer	V8000-01
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
Cellfectin® Reagent	1 ml	10362-010
Zeocin	1 gram	R250-01
	5 gram	R250-05
Blasticidin S	50 mg	R210-01

pIB/V5-His Vector Kit

pIB/V5-His utilizes the *bsd* gene from *Aspergillus terreus* to confer resistance to blasticidin and select stable cell lines. For more information, contact Technical Service (page 30).

pIB/V5-His TOPO® TA Expression Kit

The pIB/V5-His TOPO® TA Expression Kit allows you to rapidly clone and express *Taq* polymerase-amplified PCR products in insect cell lines using the InsectSelect™ technology. For more information, contact Technical Service (page 30).

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Accessory Products, continued

pIZ/V5-His Vector Kit

pIZ/V5-His utilizes a second OpIE2 promoter to express the Zeocin™ resistance gene. In addition, the Zeocin™ resistance gene is not fused to Cycle 3 GFP. For more information, contact Technical Service (page 30).

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 pIZT/V5-His vector. 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™ Glow 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™ Purification System with Anti-V5-HRP Antibody	1 kit 25 Westerns	K854-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit 25 Westerns	K853-01
Purification Columns (10 ml polypropylene columns)	50	R640-50
ProBond™ Metal-Binding Resin	50 ml	R801-01
	150 ml	R801-15

Introduction

Overview

Introduction

The InsectSelect™ Glow System allows you to express your protein of interest in insect cell lines either transiently or stably. The system utilizes a single expression vector to express your gene of interest and the Zeocin™ resistance marker to select for stable cell lines. The InsectSelect™ Glow expression vector, pIZT/V5-His, contained in this kit has the following features:

- OpIE2 promoter for constitutive expression of the gene of interest (Theilmann and Stewart, 1992)
- OpIE1 promoter for expression of the Zeocin™ resistance gene fusion (see next bullet) (Theilmann and Stewart, 1991)
- Zeocin™ resistance gene fused to Cycle 3 GFP for detection of transfected cells and 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 pIZT/V5-His and transfected into Sf9 or High Five™ cells using lipid-mediated transfection. After transfection, cells can be assayed for transfection efficiency using fluorescence and 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 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, SL2) (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997), and mosquito cell lines (unpublished data). The OpIE2 promoter has been shown to be about 5-10-fold stronger than the OpIE1 promoter (Pfeifer *et al.*, 1997). Both promoters have been sequenced and analyzed. For more detailed information on the OpIE2 and OpIE1 promoters, see page 27 and page 28, respectively.

Expression Levels

The OpIE2 promoter provides relatively high levels of constitutive expression, although not as high as might be expected from baculovirus late promoters such as polyhedrin or very late promoters such as p10 (Jarvis *et al.*, 1996). To date, expression levels range from 1 µg/ml (IL-6; Invitrogen) to 8-10 µg/ml (melanotransferrin) (Hegedus *et al.*, 1999).

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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). Insect cells transfected with plasmids expressing the *Streptoalloteichus hindustanus ble* gene (*Sh ble;* Zeocin™ resistance gene) (Gatignol *et al.*, 1988) can be selected for stable integration of the plasmid. Analysis of stable cell lines reveals that integration is primarily multi-copy. For more information on Zeocin™, see page 29.

Experimental Outline

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

Step	Action	Source
1	Establish culture of Sf9 cells from supplied frozen stock (InsectSelect™ Glow System Kit only) or culture the insect cell line of choice using your own methods. **Note**: Other cell lines (Sf21 or High Five™) can be used	Refer to the Insect Cell Lines manual included with the System Kit or use your own laboratory protocols.
2	Develop a cloning strategy to ligate your gene of interest into pIZT/V5-His.	Page 4, this manual
3	Ligate your gene into pIZT/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 16, this manual
8	Scale-up expression for purification.	Page 20, this manual
9	Purify your recombinant protein by chromatography on metal-chelating resin (i.e. ProBond™).	Page 20, this manual

Methods

Culturing Insect Cells

Introduction

Before you start your cloning experiments, be sure to have cell cultures of Sf9 cells growing and have frozen master stocks available. If you purchased the InsectSelect™ Glow System Kit (Catalog no. K810-01), you will receive Sf9 cells and the Insect Cell Lines manual. Use this manual to help you 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 30).

Culturing Sf9 Cells

To culture Sf9 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 Sf9 cells into complete TNM-FH (TNM-FH containing 10% FBS).

Other Cell Lines

You may also use Sf21 or High Five™ cells as a host for pIZT/V5-His. Sf21 cells are larger and may produce more protein than Sf9 cells. High Five™ cells may be better for secretion of proteins. 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 9-13 to determine how many cells you will need for transfection.

Cloning into pIZT/V5-His

Introduction

This chapter provides information to help you clone your gene of interest into pIZT/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 9-13.

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 pIZT/V5-His

To use pIZT/V5-His, resuspend the vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.

To propagate and maintain pIZT/V5-His, use this stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, or equivalent using your method of choice.

Select transformants on Low Salt LB plates containing 25 to 50 µg/ml Zeocin™ (see page 29).

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 are the most critical for function (shown in bold). 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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MCS of pIZT/V5-His

The TATA box, start of transcription, and polyadenylation site 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 pIZT/V5-His is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 30).** For a map and a description of the features of pIZT/V5-His, refer to pages 24-25.

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                TATA Box             OpIE2 Forward priming site
491  CTTATCGCGC CTATAAATAC AGCCCGCAAC GATCTGGTAA ACACAGTTGA ACAGCATCTG TTCGAATTTA

                           Sac I
              Acc65 I Kpn I Ecl136 II      Spe I                     EcoR I          EcoR V
561  AAGCTT GGT ACC GAG CTC GGA TCC ACT AGT CCA GTG TGG TGG AAT TCT GCA GAT
            Gly Thr Glu Leu Gly Ser Thr Ser Pro Val Trp Trp Asn Ser Ala Asp

                         Not I               Xba I   Dra II      Sac II
615  ATC CAG CAC AGT GGC GGC CGC TCG AGT CTA GAG GGC CCG CGG TTC GAA GGT AAG
     Ile Gln His Ser Gly Gly Arg Ser Ser Leu Glu Gly Pro Arg Phe Glu Gly Lys

                        V5 epitope                                    6xHis tag
669  CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC
     Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His

                                   OpIE2 Reverse priming site
723  CAT CAC CAT TGA GTTTAT CTGACTAAAT CTTAGTTTGT ATTGTCATGT TTTAATACAA TATGTTATGT
     His His His ***

                OpIE2 polyadenylation signal
791  TTAAATATGT TTTTAATAAA TTTTATAAAA TAATTTCAAC TTTTATTGTA ACAACATTGT CCATTTACAC
                           3´ untranslated region of OpIE2

861  ACTCCTTTCA AGCGCGTGGG ATCGATGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA
```

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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.

We recommend that you sequence your construct to confirm that your gene is fused in frame with the V5 epitope and the polyhistidine tag. Use the OpIE2 Forward and Reverse primers included in the kit

Transforming *E. coli*

Introduction

The pIZT/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). High salt concentrations and extremes in pH can inactivate the Zeocin™ antibiotic. 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 pIZT/V5-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

Important

DO NOT USE any *E. coli* strain that contains the complete Tn*5* transposon (*i.e.* DH5αF′IQ, SURE, SURE2). This transposon encodes a *ble* (bleomycin) resistance gene which will confer resistance to Zeocin™, preventing selection of colonies containing your pIZT/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). **High salt and extremes in pH can inactivate Zeocin™.**

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 the pH of the solution to 7.5 with NaOH and bring the 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 before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, 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 30.

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

Transient Expression in Insect Cells

Introduction

Once you have cloned your gene of interest into pIZT/V5-His, you are ready to transfect your construct into Sf9 cells using lipid-mediated transfection and test for expression of your protein. **Note**: Instructions for use with High Five™ cells are also provided if you wish to use those cells.

Plasmid Preparation

Plasmid DNA for transfection into insect cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) or CsCl gradient centrifugation. The S.N.A.P.™ 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 (lipid only) in all transfection experiments. At about 24 to 48 hours posttransfection, compare the 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 µg of highly purified plasmid DNA (~1 µg/µl in TE buffer)
- 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 next page)
- 60 mm tissue-culture dishes
- 1.5 ml sterile microcentrifuge tubes
- Rocking platform only (NOT orbital)
- 27°C incubator and Inverted Microscope
- Paper towels moistened with 5 mM EDTA, pH 8 and air-tight bags or containers

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Serum-Free Media

If you wish to transfect Sf9 cells in serum-free medium, Sf-900 II SFM (1X) (Catalog no. 10902-088) is available from Invitrogen. Note that you will need to adapt cells to serum-free medium before transfection (see the Insect Cell Lines manual for a protocol). Other serum-free media may be used, although you may have to optimize conditions for transfection and selection.

If you are using High Five™ cells, Express Five® SFM (Catalog no. 10486-025) is available from Invitrogen.

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. 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:

- pIZT/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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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 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
pIZT/V5-His plasmid or construct (~1 µg/µl in TE, pH 8)	1 µl (1 µg)
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. Again, carefully remove all of the medium from the monolayer and 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 serum-free medium (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 protein (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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Monitoring Transfection

To monitor transfection, you may assay for expression of Cycle 3 GFP by fluorescence microscopy (see page 14). After transfection, allow the cells to recover for 24 hours before assaying for expression of GFP.

Expected transfection efficiency is about 40-60% for Sf9 cells and High Five™ cells.

You can also assay for Cycle 3 GFP by Western blotting using antisera to GFP, although you will not be able to estimate transfection efficiency. GFP Antiserum is available from Invitrogen (Catalog no. R970-01).

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 22-23 for your convenience, but other recipes are suitable.

1. Prepare an SDS-PAGE gel that will resolve your expected recombinant protein.
2. Remove the medium from 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, be sure to assay the cell 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 histidine tag (see page viii).
11. Visualize proteins using your desired method. We recommend using chemiluminescent or colorimetric methods 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

Assay for CAT

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

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.** Use clean, pure DNA isolated by resin based DNA isolation kits (i.e. S.N.A.P. 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™).

Detection of Fluorescence

Introduction

After transfecting your cells, you can monitor for fluorescence of Cycle 3 GFP using fluorescence microscopy. Only transfected cells will emit a green fluorescent signal upon illumination, and the fluorescence can be used to estimate the transfection efficiency.

Description of Cycle 3 GFP

The GFP gene used in this vector is described by Crameri *et al.*, 1996. In this paper, the codon usage of GFP was optimized for expression in *E. coli*, followed by three cycles of DNA shuffling. A mutant form of GFP was selected that gave the greatest fluorescence signal in mammalian cells. "Cycle 3 GFP" has the following characteristics:

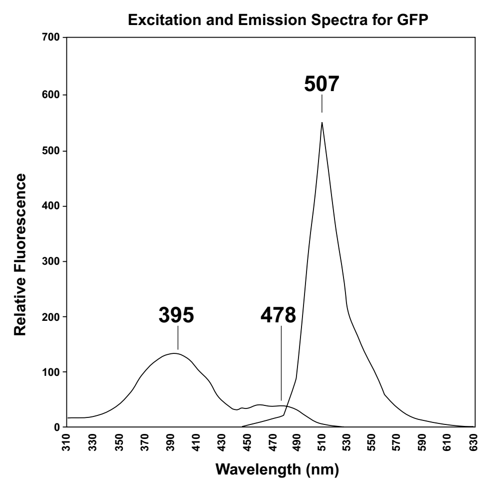
- Excitation and emission maxima that are the same as wild-type GFP (395 nm and 478 nm for primary and secondary excitation, respectively, and 507 nm for emission)
- High solubility in *E. coli* for visual detection of transformed cells
- >40-fold increase in fluorescent yield over wild-type GFP

The Cycle 3 GFP gene is fused to the Zeocin™ resistance gene to correlate GFP fluorescence with resistance to Zeocin™.

Detection of Fluorescence

To detect fluorescent cells, it is important to pick the best filter set to optimize detection. The primary excitation peak of Cycle 3 GFP is at 395 nm. There is a secondary excitation peak at 478 nm. Excitation at these wavelengths yield a fluorescent emission peak with a maximum at 507 nm, as shown in the figure below. **Note that the quantum yield can vary as much as 5- to 10-fold depending on the wavelength of light that is used to excite the GFP fluorophore.**

Use of the best filter set will ensure that the optimal regions of the Cycle 3 GFP spectra are excited and passed (emitted). For best results, use a filter set designed to detect fluorescence from wild-type GFP (e.g. XF76 filter from Omega Optical, www. omegafilters.com). FITC filter sets can also be used to detect Cycle 3 GFP fluorescence. For example, the FITC filter set that we use excites Cycle 3 GFP with light from 460 to 490 nm, which covers the secondary excitation peak. The filter set passes light from 515 to 550, allowing detection of most of the Cycle 3 GFP fluorescence.



For general information about GFP fluorescence and detection, refer to Current Protocols in Molecular Biology, pages 9.7.22 to 9.7.28 (Ausubel et al., 1994).

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Detection of Transfected Cells

After transfection, allow the cells to recover for at least 24 hours before assaying for fluorescence. If fluorescence seems low, assay the cells again at 48 hours. **Note**: Media that contain riboflavin (e.g. Grace's Insect Medium) will fluoresce and may interfere with detection of GFP fluorescence (Zylka and Schnapp, 1996). Medium can be removed and replaced with PBS to alleviate this problem. Be sure to replace PBS with fresh medium if you wish to continue growing the cells.

You can use fluorescence to estimate transfection efficiency and normalize any subsequent assay for your gene of interest. Estimate the total number of cells before assaying for fluorescence. Then check your plate for fluorescent cells. Cells can be incubated further in order to optimize expression of your gene of interest.

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

Sensitive cells exhibit the following morphological changes upon exposure to Zeocin™.

- Cessation of growth
- 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

Cells do not necessarily round up and detach from the plate. 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 29.

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	
Sf21	TNM-FH	300-500
	Express Five® Serum-Free	
High Five™	TNM-FH	400-600
	Express Five® Serum-Free	

continued on next page

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 that 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 (pIZT/V5-His/CAT). You can use fluorescence to monitor selection of resistant colonies.

Note that GFP fluorescence will decrease as stable integrants form and the plasmid is diluted out of the cells.

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 (20% 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 colonies forming. At this point you may use cloning cylinders to isolate clonal cell lines (next page) or you can let resistant cells grow out to confluence for a polyclonal cell line (3 to 4 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 drop the concentration of Zeocin™ to 50 µg/ml for maintenance.**

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Isolation of Clonal Cell Lines Using Cloning Cylinders

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

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

To select colonies:

1. Examine the closed plate under a microscope and mark the location of each colony on the top of the plate. Transfer the markings to the bottom of the plate. Be sure to include orientation marks. **Note**: Each colony will contain 50 to 200 cells. Sf9 cells tend to spread more than High Five™ cells.
2. Move the culture dish to the sterile cabinet and remove the lid.
3. Apply a thin layer of sterile silicon grease to the bottom of the cloning cylinder (Scienceware, Catalog no. 378747-00 or Belco, Catalog no. 2090-00608), using a sterile cotton-tipped wooden applicator. The layer should be thick enough to retard the flow of liquid from the cylinder, without obscuring the opening on the inside. **Tip**: Cloning cylinders and silicon grease can be sterilized together by placing a small amount of grease in a glass petri dish and placing the cloning cylinders upright in the grease. After autoclaving, the grease will have spread out in a thin layer to coat the bottom of the cylinders.
4. Aspirate the culture medium and place the cylinder firmly and directly over the marked area. Use a microscope if it is available to help you direct placement of the cylinder.
5. Use 20 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.

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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** 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 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 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 secreted 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 GFP fluorescence or protein expression.

Serum-Free Medium

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

Adapting Cells to Different Medium

Sf9 cells can be switched from complete TNM-FH to serum-free medium (*e.g.* Sf-900 II SFM) during passage. Refer to the Insect Cell Lines manual for more information.

CAUTION

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

Purifying Proteins from Medium

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

Purification of 6xHis-tagged Proteins from Medium

To purify 6xHis-tagged recombinant proteins from the culture medium, we recommend that you perform 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 cells at 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 the 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

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

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

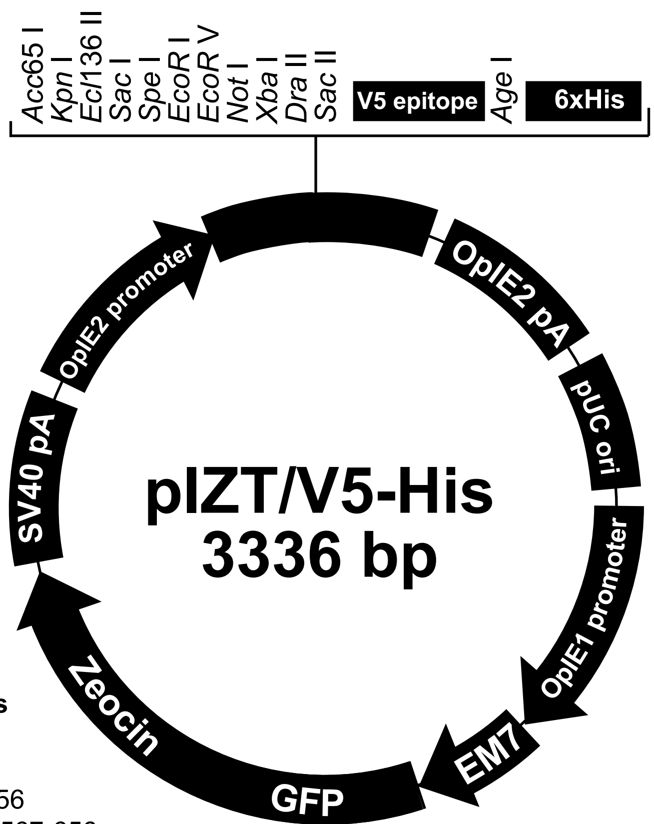
Yield is ~10 ml.

Aliquot and freeze at -20°C until needed.

pIZT/V5-His Map and Features

Map

The figure below summarizes the features of the pIZT/V5-His vector. For a more detailed explanation of each feature, see the next page. **The complete sequence of pIZT/V5-His is available from our Web site (www.invitrogen.com) or from Technical Service (see page 30).**



Comments for pIZT/V5-His
3336 nucleotides

OpIE2 promoter: bases 4-556
Multiple cloning site: bases 567-656
V5 epitope: bases 663-704
6xHis tag: bases 714-731
OpIE2 polyadenylation sequence: bases 749-878
pUC origin: bases 947-1620
OpIE1 promoter: bases 1669-1960
EM7 promoter: bases 1975-2033
GFP-Ble fusion: bases 2067-3140
  GFP ORF: bases 2067-2771
  Zeocin resistance (*ble*) ORF: bases 2772-3140
SV40 early polyadenylation sequence: bases 3210-3336

continued on next page

Features

The features of pIZT/V5-His (3336 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).
OpIE2 Forward priming site	Allows sequencing of the insert from the 5′ end.
Multiple cloning site (12 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 or Anti-V5-HRP Antibody (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) and the Anti-His(C-term)-HRP Antibodies.
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*.
OpIE1 promoter	Provides high-level, constitutive expression of the GFP-Zeocin™ resistance gene fusion in lepidopteran insect cells (Theilmann and Stewart, 1991).
EM7 promoter	Allows efficient expression of the Zeocin™ resistance gene fusion in *E. coli*.
GFP-Zeocin™ resistance gene fusion	Detection of transfected insect cells. Selection of transformants in *E. coli* and stable insect cell lines.
SV40 early polyadenylation sequence	Efficient transcription termination and mRNA stability.

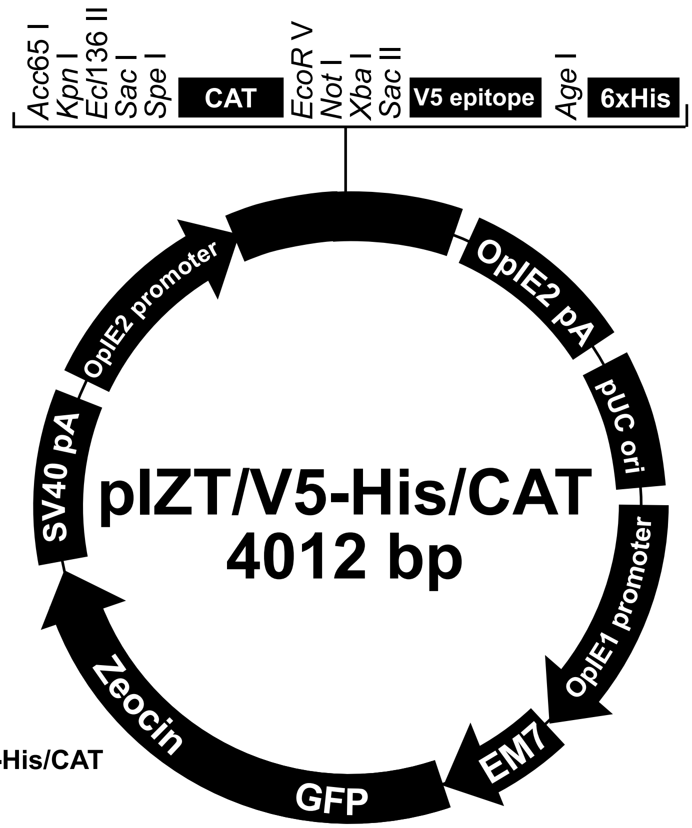
pIZT/V5-His/CAT Map

Description

pIZT/V5-His/CAT is a 4012 bp control vector expressing chloramphenicol acetyl-transferase (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 pIZT/V5-His/CAT vector. **The complete nucleotide sequence for pIZT/V5-His/CAT is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 30).**



Comments for pIZT/V5-His/CAT
4012 nucleotides

OpIE2 promoter: bases 4-556
CAT ORF: bases 616-1272
V5 epitope: bases 1339-1380
6xHis tag: bases 1390-1407
OpIE2 polyadenylation sequence: bases 1426-1555
pUC origin: bases 1623-2296
OpIE1 promoter: bases 2345-2636
EM7 promoter: bases 2651-2709
GFP-Ble fusion: bases 2743-3819
  GFP ORF: bases 2743-3447
  Zeocin resistance (*ble*) ORF: bases 3448-3819
SV40 early polyadenylation sequence: bases 3883-4012

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
```

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 pIZT/V5-His but do not appear to be important for expression in Sf9 cells. The function of these elements has not been described.

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

```
                         R3
1661  TTGGTCATGC GAAACACGCA CGGCGCGCGC ACGCAGCTTA GCACAAACGC GTCGTTGCAC

1721  GCGCCCACCG CTAACCGCAG GCCAATCGGT CGGCCGGCCT CATATCCGCT CACCAGCCGC

          R4
1781  GTCCTATCGG GCGCGGCTTC CGCGCCCATT TTGAATAAAT AAACGATAAC GCCGTTGGTG

                                                             TATA
1841  GCGTGAGGCA TGTAAAAGGT TACATCATTA TCTTGTTCGC CATCCGGTTG GTATAAATAG

                            Start of transcription
1901  ACGTTCATGT TGGTTTTTGT TTCAGTTGCA AGTTGGCTGC GGCGCGCGCA GCACCTTTGC

1961  CGGGATCTGC CGGGCTGCAG CACGTGTTGA CAATTAATCA TCGGCATAGT
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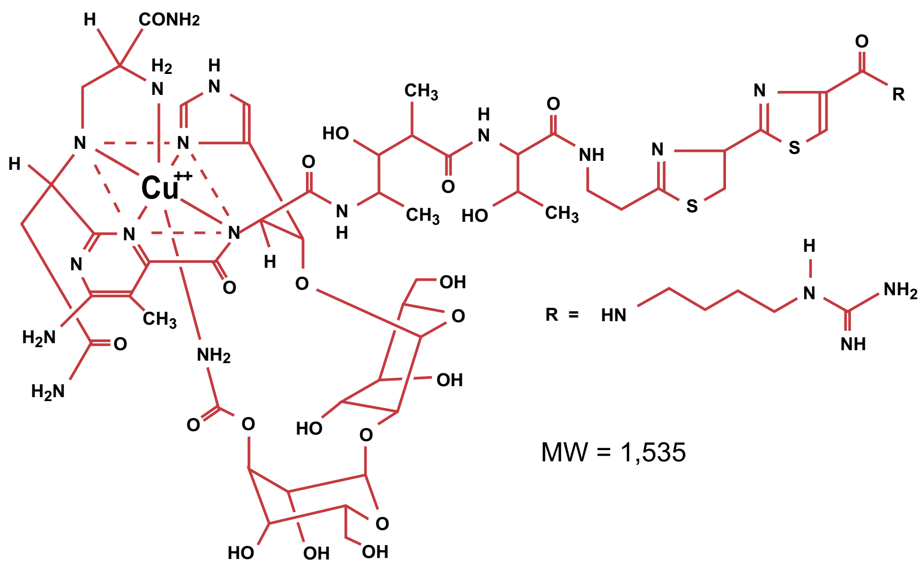
Zeocin™

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.

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Product Qualification

Introduction

This section describes the criteria used to qualify the components of the InsectSelect™ Glow System and the pIZT/V5-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)
pIZT/V5-His	*Bam*H I	1326, 2010
	Mlu I	684, 1005, 1647
	Xba I	3336
pIZT/V5-His/CAT	*Bam*H I	1326, 2868
	Mlu I	684, 1005, 2323
	Xba I	4012

Primers

The primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

Zeocin™

Zeocin™ is lot-qualified by demonstrating that Low Salt LB media containing 25 µg/ml Zeocin™ prevents growth of the *E. coli* strain, TOP10.

Cellfectin® Reagent

Cellfectin® Reagent is tested for the absence of bacterial and fungal contamination using blood agar plates and fluid thioglycolate medium. In addition, Cellfectin® Reagent is tested in a functional assay by transfecting subconfluent COS-7 cells with pCMV.SPORT.LUC DNA. Transfected cells are assayed for luciferase activity.

Insect Cells

Insect cells are in logarithmic growth with 98% viability before they are frozen. Frozen cells are tested to demonstrate that they can be recovered as healthy logarithmically growing cells within 2 to 3 days after thawing.

Grace's Medium

To assess the performance of each lot of medium, Grace's Medium is prepared as described in its manual and used to test the growth and viability of Sf9 cells. Sf9 cells are seeded at a density of 5×10^5 cells/ml. Cells are carried through three passages of suspension culture in the medium. In the third passage, cells are tested for doubling time and viability. Doubling time must be between 18 and 24 hours and viability must be greater than 98-99%.

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