

USER GUIDE

invitrogen™
by *life* technologies™

InsectSelect™ Glow System

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

Catalog numbers V8010-01; K810-01

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**For Research Use Only. Not intended for any animal or human
therapeutic or diagnostic use.**

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Kit Contents and Storage

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® II 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 |
|---------------------------------|--|--------|
| pIZT/V5-His | 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 | 40 µL |
| pIZT/V5-His/CAT | 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 | 40 µL |
| 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'-gacaatacaactaagatttagtcag-3' | 250 |

Zeocin™

Supplied with the InsectSelect™ Glow System Kit only. Zeocin™ is available separately, see page 30.

Store at -20°C, protected from light.

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

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

Continued on next page

Kit Contents and Storage, Continued

Cellfectin® II Reagent

Supplied with the InsectSelect™ Glow System kit only. Cellfectin® II Reagent is available separately, see page 30.

Store at 4°C.

Amount Supplied: 125 µL

Composition: Proprietary cationic 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 30.

Store the Sf9 cells in liquid nitrogen.

Amount supplied: 1 mL, 10⁷ 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

- 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)
-

Product Use

For research use only. Not intended for any human or animal therapeutic or diagnostic use.

Introduction

Product 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 (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 (OpMNPV). The virus' natural host is the Douglas fir tussock moth; however, the promoters allow protein expression in *Lymantria dispar* (LD652Y), *Spodoptera frugiperda* cells (Sf9) (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997), Sf21, *Trichoplusia ni* (High Five™), *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) to 8–10 µg/mL (melanotransferrin) (Hegedus *et al.*, 1999).

Continued on next page

Product 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). 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 |
| 3 | Ligate your gene into pIZT/V5-His and transform into a <i>recA, endA E. coli</i> strain (e.g. TOP10). Select on Low Salt LB plates containing 25–50 µg/mL Zeocin™. | Pages 6–7 |
| 4 | Isolate plasmid DNA and sequence your recombinant expression vector to confirm that your protein is in frame with the C-terminal peptide. | Page 7 |
| 5 | Transiently transfect Sf9 or High Five™ cells. | Page 9 |
| 6 | Assay for expression of your protein. | Page 12 |
| 7 | Create stable cell lines expressing the protein of interest by selecting with Zeocin™. | Page 17 |
| 8 | Scale-up expression for purification. | Page 20 |
| 9 | Purify your recombinant protein by chromatography on metal-chelating resin (i.e. ProBond™). | Page 20 |

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 (Cat. 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 www.lifetechnologies.com/support. Alternatively, you may request the manual from Technical Support (see page 32).

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).

Propagating and Maintaining pIZT/V5-His

To propagate and maintain pIZT/V5-His, use the supplied 0.5 µg/µL stock solution in TE buffer, pH 8.0 to transform a *recA*, *endA* *E. coli* strain like TOP10, DH5α™, or equivalent using your method of choice.

Select transformants on Low Salt LB plates containing 25–50 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**AT**GG

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.

Continued on next page

Cloning into pIZT/V5-His, Continued

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 nucleotide sequence of pIZT/V5-His is available for downloading from www.lifetechnologies.com or from Technical Support (see page 32). For a map and a description of the features of pIZT/V5-His, refer to pages 24–25.

```

491  CTTATCGCGC  CTATAATAC  AGCCCGCAAC  GATCTGGTAA  ACACAGTTGA  ACAGCATCTG  TTCGAATTTA
      TATA Box      OplE2 Forward priming site →

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

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

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

723  CAT CAC CAT TGA GTTTAT CTGACTAAAT CTTAGTTTGT ATTGTCATGT TTAATACAA TATGTTATGT
      OplE2 Reverse priming site
      His His His ***

791  TTAAATATGT TTTAAATAAA TTTTATAAAA TAATTTCAAC TTTTATTGTA ACAACATTGT CCATTTACAC
      OplE2 polyadenylation signal
      3' untranslated region of OplE2

861  ACTCCTTTCA AGCGCGTGGG ATCGATGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA
  
```

Continued on next page

Cloning into pLZT/V5-His, Continued

E. coli **Transformation**

Prepare competent *recA, endA 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 (*recA*) and endonuclease A deficient (*endA*). For your convenience, TOP10 is available as electrocompetent or chemically competent cells for purchase (see page 30).



Important

DO NOT USE any *E. coli* strain that contains the complete Tn5 transposon (i.e. DH5αFIQ, 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



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 (see page 30 for ordering). 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 Support, page 32.

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:

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

Transient Expression in Insect Cells

Introduction

Once you have cloned your gene of interest into 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 PureLink® HiPure MidiPrep Kit (see page 30 for ordering) or CsCl gradient centrifugation. The PureLink® HiPure MidiPrep Kit is a medium-scale plasmid isolation kit that isolates up to 150 µg of plasmid DNA from 25-100 mL of bacterial culture. Plasmid can be used directly for transfection of insect cells.

Method of Transfection

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

Expected Transfection Efficiency using Cellfectin® II 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

- ~1 µg of highly purified plasmid DNA (~1 µg/µL in TE buffer)
 - Either log phase Sf9 cells ($1.6\text{--}2.5 \times 10^6$ cells/mL, >95% viability) or log phase High Five™ cells ($1.8\text{--}2.3 \times 10^6$ cells/mL, >95% viability)
 - Serum-free medium (see page 10)
 - 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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Transient Expression in Insect Cells, Continued

Serum-Free Media

If you wish to transfect Sf9 cells in serum-free medium, Sf-900 II SFM (1X) is available for purchase (see page 30). 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 is available for purchase (see page 30).

Cellfectin® II Reagent

Cellfectin® II Reagent is a proprietary cationic liposome in membrane-filtered water. Cellfectin® II Reagent has been found to be superior for transfection of Sf9 and High Five™ insect cells.

Preparing Cells

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

1. For Sf9 cells or High Five™ cells, seed 1.8×10^6 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 at room temperature 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.

Note: Do not change media or wash the cells. The media carried over to the next step will enhance the transfection efficiency.

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® II Reagent, review the protocol on page 11 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

Guidelines for Transfection

- Cellfectin® II Reagent is a lipid suspension that may settle with time. Mix thoroughly by inverting the tube 5–10 times before use.
 - The DNA:lipid complex formation time is shorter (~15-30 minutes) when using Cellfectin® II Reagent as compared to Cellfectin® Reagent.
 - *Do not* add antibiotics to media during transfection; this causes cell death.
 - Transfection complexes *must* be formed in serum-free medium.
 - Test serum-free media for compatibility with Cellfectin® II Reagent since some serum-free formulations may inhibit cationic lipid-mediated transfection.
-

Transfection Procedure

Plasmid DNA and Cellfectin® II 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 interfere with the liposomes, causing the transfection efficiency to decrease.

1. Dilute 2.2 µg of pIZT/V5-His plasmid or construct (~1 µg/µL in TE, pH 8) in 250 µL Grace's Insect Media, Unsupplemented. Vortex briefly to mix. Incubate at room temperature.
Optional: If you are using SFM, add:
 2. Mix the Cellfectin® II Reagent before use (mix well and always add last).
 - a. Add 18 µL Cellfectin® II Reagent in 250 µL Grace's Insect Media.
 - b. Vortex briefly to mix.
 - c. *Incubate at room temperature for 15–30 minutes.*
 3. Combine the diluted DNA with the diluted Cellfectin® II Reagent. Mix gently and incubate for 5–15 minutes.
 4. Add ~520 µL of DNA-lipid mixture (from Step 3) *dropwise* onto the prepared cells (See **Preparing Cells**, page 10)
 5. **For Sf-900™ II SFM:** Incubate cells at 27°C for 24–48 hours and test for expression. There is no need to replace the medium.
For Grace's medium: Incubate the cells at 27°C for 3–5 hours. Remove the transfection mixture and replace with 5.5 mL of complete growth medium (e.g., Grace's Insect Medium, Supplemented, and 10% FBS). Adding antibiotics to the growth medium is optional. Incubate cells at 27°C.
 6. Harvest the cells 2, 3, and 4 days posttransfection and assay for expression of your protein (see page 12).
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Transient Expression in Insect Cells, Continued

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 for purchase (see page 30).

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 or obtain 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 μ L 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 μ L lysate with 10 μ L **4X SDS-PAGE** sample buffer.
 - Cell Pellet: Resuspend pellet in 100 μ L **1X SDS-PAGE** sample buffer.
 - Medium: 30 μ L medium with 10 μ L **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 μ g 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 31).
 11. Visualize proteins using your desired method. We recommend using chemiluminescent or colorimetric methods for detection.
-



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

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 30) or an antibody against CAT (see page 30). 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. PureLink[®] HiPure 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:

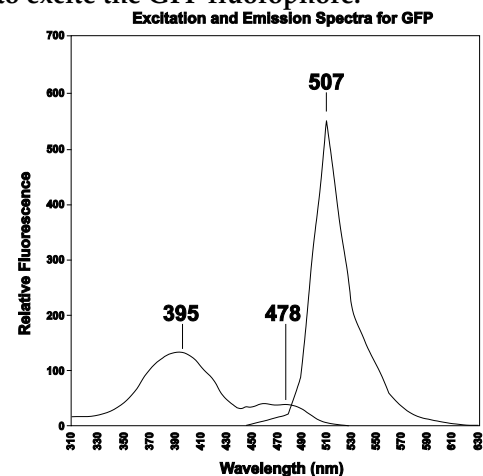
- Excitation and emission maxima that are the same as wild-type GFP (395 nm and 478 nm for primary and secondary excitation, respectively, and 507 nm for emission)
- High solubility in *E. coli* for visual detection of transformed cells
- >40-fold increase in fluorescent yield over wild-type GFP

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 5–10-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).



Continued on next page

Detection of Fluorescence, Continued

Detecting 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 page 17).

| 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

Selecting Stable Transformants, 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 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 (page 18) 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.**
-

Continued on next page

Selecting Stable Transformants, Continued

Isolating 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, Cat. no. 378747-00 or Belco, Cat. 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.

Continued on next page

Selecting Stable Transformants, Continued

Isolation of Clonal Cell Lines Using a Dilution Method

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

1. Forty-eight hours after transfection, dilute the cells to 1×10^4 cells/mL in medium **without** 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 μ L of the cell solution from Step 1 to 32 wells of a 96-well microtiter plate (8 rows by 4 columns).
3. Dilute the remaining cells 1:1 with medium **without** Zeocin™ and add 100 μ L of this solution to the next group of 32 wells (8×4).
4. Once again, dilute the remaining cells 1:1 with medium **without** Zeocin™ and add 100 μ L of this solution to the last group of 32 wells.
Note: Although the cells can be diluted to low numbers, cell density is critical for viability. If the density drops below a certain level, the cells will not grow.
5. Let the cells attach overnight, then remove the medium and replace with medium containing 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 μ g/mL. Human melanotransferrin has been secreted to levels of 8–10 μ g/mL (Hegedus *et al.*, 1999).



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 ~50 µg/mL. We have grown stably transformed Sf9 and High Five™ cell lines under non-selecting 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.



Important

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.

Purifying 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

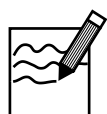
Conditions for successful ion exchange chromatography will vary depending on the protein. For more information, refer to *Current Protocols in Protein Science* (Coligan *et al.*, 1998), *Current Protocols in Molecular Biology*, Unit 10 (Ausubel *et al.*, 1994) or the *Guide to Protein Purification* (Deutscher,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

Continued on next page

Scale-Up and Purification, Continued

Metal-chelating Resin

You may use the ProBond™ Purification System (see page 30 for ordering) 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.

Purifying Intracellularly Expressed Proteins

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

1. Seed cells at 2×10^6 cells in two or three 25 cm² flasks.
 2. Grow the cells in selective medium until they reach confluence (4×10^6 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 $1000 \times g$ 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 (see page 30 for ordering) may be purchased separately for purchase. 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 Na₂HPO₄
1.8 mM KH₂PO₄

1. Dissolve: 8 g NaCl
0.2 g KCl
1.44 g Na₂HPO₄
0.24 g KH₂PO₄
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 |

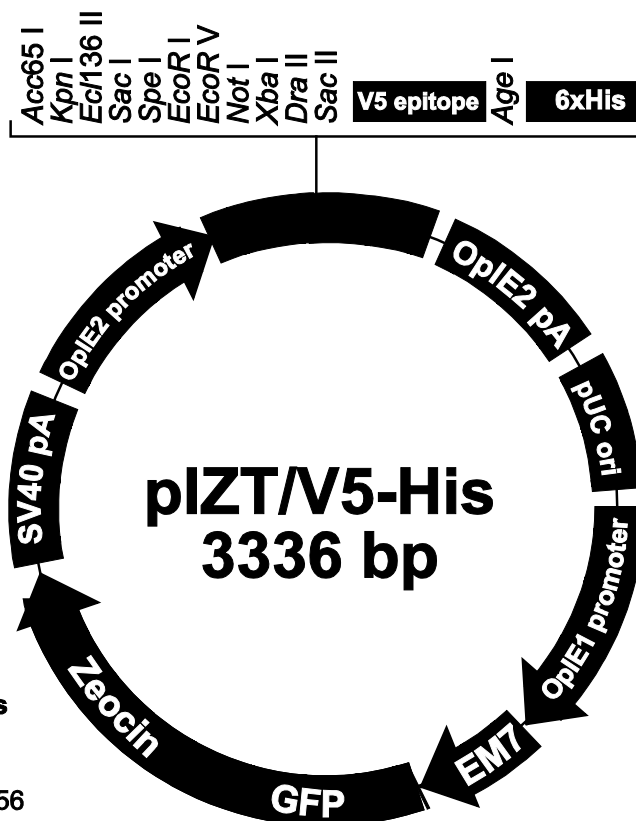
Yield is ~10 mL.

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 page 25. The nucleotide sequence of pIZT/V5-His is available from www.lifetechnologies.com or from Technical Support (see page 32).



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

pIZT/V5-His Map and Features, Continued

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 <i>et al.</i> , 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 <i>E. coli</i> . |
| 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 <i>E. coli</i> . |
| GFP-Zeocin™ resistance gene fusion | Detection of transfected insect cells. Selection of transformants in <i>E. coli</i> and stable insect cell lines. |
| SV40 early polyadenylation sequence | Efficient transcription termination and mRNA stability. |

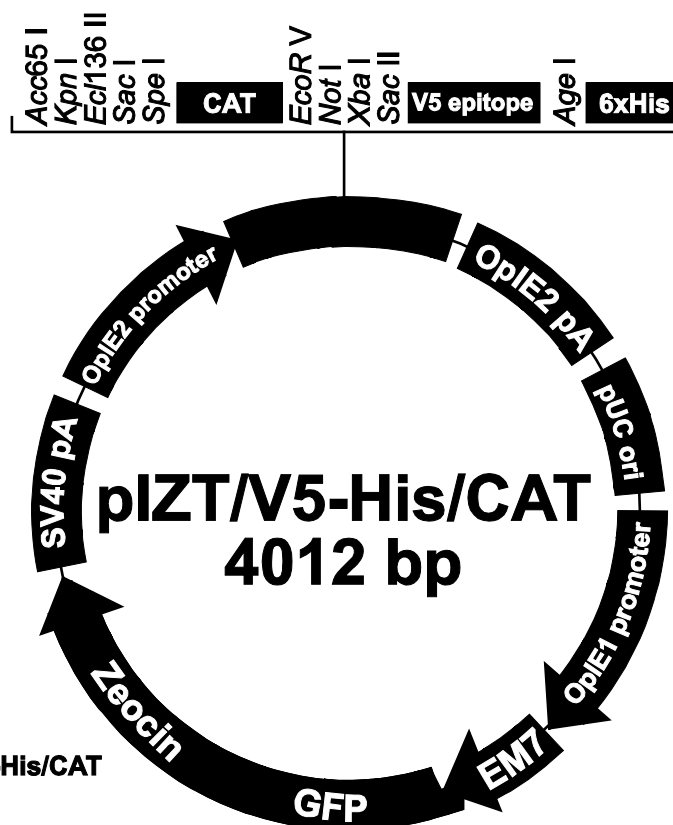
pIZT/V5-His/CAT Map

Description

pIZT/V5-His/CAT is a 4012 bp control vector expressing chloramphenicol 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 pIZT/V5-His/CAT vector. The nucleotide sequence for pIZT/V5-His/CAT is available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 32).



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 GATAACAAT GTATGGTGCT AATGTTGCTT CAACAACAAT TCTGTTGAAC

61  TGTGTTTTCA TGTTTGCCAA CAAGCACCTT TATACTCGGT GGCCTCCCCA CCACCAACTT

121 TTTTGCAC TG CAAAAAACA CGCTTTTGCA CGCGGGCCCA TACATAGTAC AACTCTACG

181 TTTCGTAGAC TATTTTACAT AAATAGTCTA CACCGTTGTA TACGCTCCAA ATACACTACC

241 ACACATTGAA CCTTTTTGCA GTGCAAAAAA GTACGTGTCG GCAGTCACGT AGGCCGGCCT

301 TATCGGGTCG CGTCCTGTCA CGTACGAATC ACATTATCGG ACCGACGAG TGTGTCTTA

361 TCGTGACAGG ACGCCAGCTT CCTGTGTTGC TAACCGCAGC CGGACGCAAC TCCTTATCGG

421 AACAGGACGC GCCTCCATAT CAGCCGCGCG TTATCTCATG CGCGTGACCG GACACGAGGC

481 GCCCGTCCCG CTTATCGCGC TATAAATAC AGCCCGCAAC GATCTGGTAA ACACAGTTGA
      TATA                                     Start of Transcription
      |-----|-----|-----|-----|-----|-----|-----|-----|-----|
      |-----|-----|-----|-----|-----|-----|-----|-----|-----|

541 ACAGCATCTG TTCGAATTTA
```

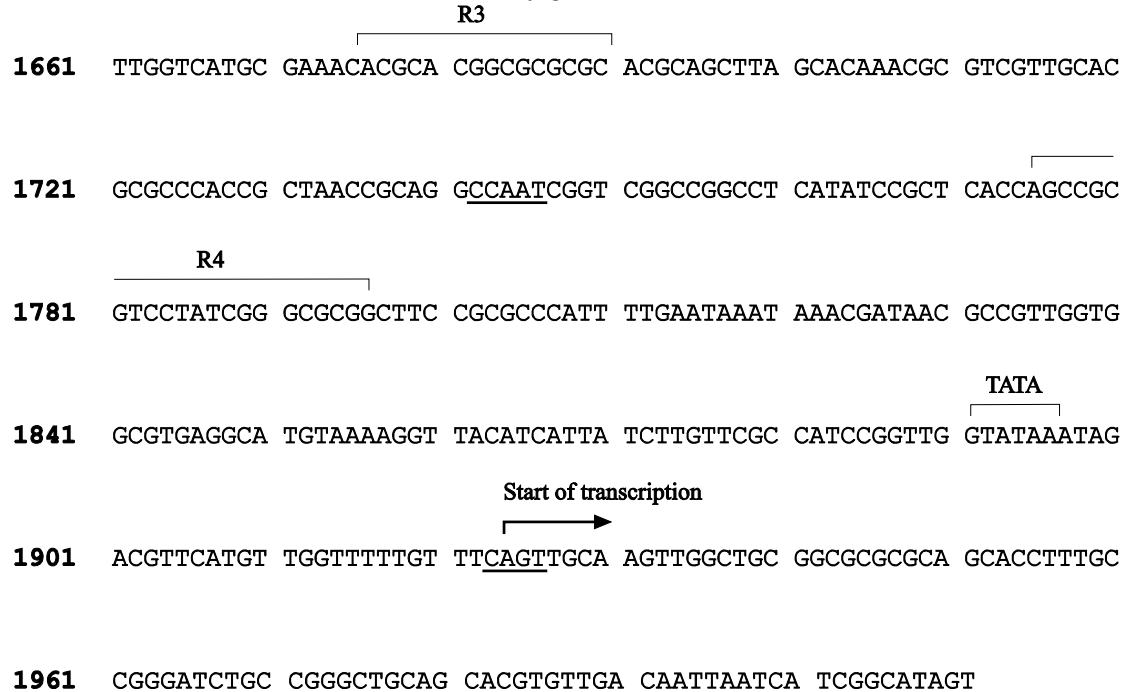
OpIE1 Promoter

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).



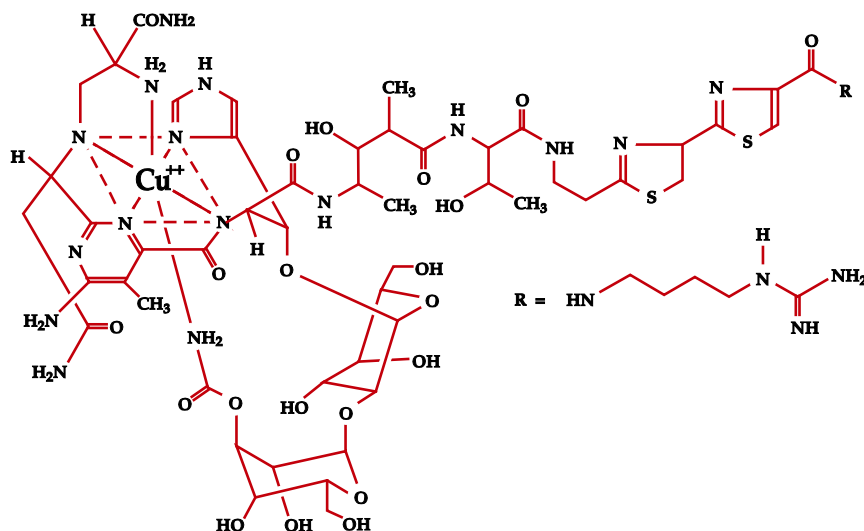
Zeocin™

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 chemical formula for Zeocin™ is $\text{C}_{55}\text{H}_{86}\text{N}_{20}\text{O}_{21}\text{S}_2\text{Cu}\cdot\text{HCl}$ and its molecular weight is 1,527.5 Da. 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.

Accessory Products

Additional Products

Many of the reagents supplied with the InsectSelect™ Glow System and other reagents suitable for use with it are available separately. Ordering information for these reagents is provided below. For more information, refer to www.lifetechnologies.com or call Technical Support (see page 32).

| Product | Amount | Catalog no. |
|---|-------------------------------------|-------------|
| pIB/V5-His Vector Kit | 1 Kit | V8020-01 |
| pIZ/V5-His Vector Kit | 1 Kit | V8000-01 |
| Sf9 Cells, frozen | 1 mL vial, 1×10^7 cells/mL | B825-01 |
| Sf21 Cells, frozen | 1 mL vial, 1×10^7 cells/mL | B821-01 |
| High Five™ Cells, frozen | 1 mL vial, 3×10^6 cells/mL | B855-02 |
| Grace's Insect Cell Culture Medium, Unsupplemented | 500 mL | 11595-030 |
| Sf-900 II SFM | 1 liter | 10902-088 |
| Express Five® SFM | 1 liter | 10486-025 |
| Grace's Insect Cell Culture Medium, Unsupplemented | 500 mL | 11595-030 |
| Cellfectin® II Reagent | 1 mL | 10362-100 |
| Zeocin™ | 1 gram | R250-01 |
| | 5 gram | R250-05 |
| Blasticidin S | 50 mg | R210-01 |
| One Shot™ TOP10 Chemically Competent <i>E. coli</i> | 20 reactions | C4040-03 |
| Electrocomp™ TOP10 | 20 reactions | C664-55 |
| | 2 × 20 reactions | C664-11 |
| | 6 × 20 reactions | C664-24 |
| imMedia™ Zeo Liquid | 20 pouches | Q620-20 |
| imMedia™ Zeo Agar | 20 pouches | Q621-20 |
| PureLink® HiPure Midiprep Kit | 25 preps | K2100-04 |

Continued on next page

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 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 <i>et al.</i> , 1991) GKPIP NPLLGLDST | R960-25 |
| Anti-V5-HRP Antibody | | R961-25 |
| Anti-V5-AP Antibody | | R962-25 |
| Anti-His (C-term) Antibody | Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner <i>et al.</i> , 1997) HHHHHH-COOH | R930-25 |
| Anti-His (C-term)-HRP Antibody | | R931-25 |
| Anti-His (C-term)-AP Antibody | | R932-25 |

Purification of Recombinant Protein

The metal binding domain encoded by the polyhistidine tag allows simple, easy purification of your recombinant protein by Immobilized Metal Affinity Chromatography (IMAC) using the 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 |
| Purification Columns (10 mL polypropylene columns) | 50 | R640-50 |
| ProBond™ Metal-Binding Resin | 50 mL | R801-01 |
| | 150 mL | R801-15 |

Technical Support

Obtaining support For the latest services and support information for all locations, go to www.lifetechnologies.com/support.

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- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
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The (cycle 3) mutant GFP gene was produced by Maxygen, Inc. using the DNA shuffling technology. Crameri, A., Whitehorn, E.A., and Stemmer, W.P.C. (1996) Improved Green Fluorescent Protein by Molecular Evolution Using DNA Shuffling. *Nature Biotechnology*, 14: 315-319.

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Accessory Products, Continued

**Limited Use Label
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Technology**

The InsectSelect™ System (the “Expression Kit”) was developed into an expression system by scientists at the University of British Columbia (UBC) for high-level expression of recombinant proteins. Components of the InsectSelect™ System are covered by one or more U.S. patents or patent applications and corresponding foreign patents or patent applications owned and/or licensed by UBC and others. Life Technologies Corporation (“Life Technologies”) has an exclusive license to sell the Expression Kit to scientists for academic research or one year commercial evaluation only, under the terms described below. Use of the Expression Kit for any Commercial Purpose (as defined below) other than evaluation requires the user to obtain a commercial license as detailed below. Before using the Expression Kit, please read the terms and conditions set forth below. Your use of the Expression Kit shall constitute acknowledgment and acceptance of these terms and conditions. If you do not wish to use the Expression Kit pursuant to these terms and conditions, please contact Life Technologies Technical Services to return the unused and unopened Expression Kit for a full refund. Otherwise, please complete the Product User Registration Card and return it to Life Technologies. Life Technologies grants you a non-exclusive license to use the enclosed Expression Kit for academic research or for commercial evaluation purposes only. The Expression Kit is being transferred to you in furtherance of, and reliance on, such license. You may not use the Expression Kit, or the materials contained therein, for any Commercial Purpose without a license for such purpose from Research Corporation Technologies (RCT). If you are a commercial entity, your right to use the Expression Kit expires after one year. Any commercial entity that wishes to use the Expression Kit beyond this one-year period, must obtain a commercial license from RCT. Commercial entities will be contacted by RCT during this one-year period regarding their desire to obtain a commercial license. You may terminate your use of the Expression Kit at any time by destroying all InsectSelect™ expression products in your control. Your right to use the Expression Kit will also terminate automatically if you fail to comply with the terms and conditions set forth herein. You shall, upon such termination of your rights, destroy all Expression Kits in your control, and notify Life Technologies of such in writing. Commercial Purpose include: any use of Expression Products in a Commercial Product; any use of Expression Products in the manufacture of a Commercial Product; any sale of Expression Products; any use (other than evaluation) of Expression products or the Expression Kit to facilitate or advance research or development of a Commercial Product; and any use (other than evaluation) of Expression Products or the Expression Kit to facilitate or advance any research or development program the results of which will be applied to the development of Commercial Products. “Expression Products” means products expressed with the Expression Kit, or with the use of any vectors or host strains in the Expression Kit. “Commercial Product” means any product intended for commercial use. Access to the Expression Kit must be limited solely to those officers, employees and students of your entity who need access to perform the aforementioned research or evaluation. Each such officer, employee and student must be informed of these terms and conditions and agree, in writing, to be bound by same. You may not distribute the Expression Kit or the vectors or host strains contained in it to others. You may not transfer modified, altered, or original material from the Expression Kit to a third party without written notification to, and written approval from Life Technologies. You may not assign, sub-license, rent, lease or otherwise transfer any of the rights or obligations set forth herein, except as expressly permitted by Life Technologies and RCT. Inquiries for commercial use should be directed to: Research Corporation Technologies, 101 North Wilmot Road, Suite 600, Tucson, AZ 85711-3335, Tel: 1-520-748-4400 Fax: 1-520-748-0025.

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