USER GUIDE

invitrogen™
by life technologies™

pIB/V5-His-DEST Gateway® Vector

A destination vector for stable expression of heterologous proteins in lepidopteran insect cell lines

Catalog number 12550-018

Revision date 10 January 2012
Publication Part number 25-0607

MAN0000348

life technologies™

Table of Contents

Important Information

Shipping and Storage

pIB/V5-His-DEST and pIB/V5-His-GW/*lac*Z are shipped at room temperature. Upon receipt, store at -20°C. Products are guaranteed for six months from date of shipment when stored properly.

Contents

The pIB/V5-His-DEST Gateway® Vector components are listed below.

Item	Concentration	Amount
pIB/V5-His-DEST	40 µl of 150 ng/µl vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	6 µg
pIB/V5-His-GW/*lac*Z Control Plasmid	20 µl of 0.5 µg/µl vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	10 µg

Product Use

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

Accessory Products

Additional Products

Additional products that may be used with the pIB/V5-His-DEST Gateway® Vector are available from Life Technologies. Ordering information is provided below.

Product	Amount	Catalog no.
Sf9 Cells, frozen	1×10^7 cells	B825-01
Sf21 Cells, frozen	1×10^7 cells	B821-01
High Five™ Cells, frozen	3×10^6 cells	B855-02
Grace's Insect Cell Culture Medium, Unsupplemented	500 ml	11595-030
Sf-900 II SFM	1 liter	10902-088
Express Five® SFM	1 liter	10486-025
Cellfectin® Reagent	1 ml	10362-010
Gateway® LR Clonase® Enzyme Mix	20 reactions	11791-019
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent Cells	10 reactions	C4040-50
	20 reactions	C4040-52
Blasticidin	50 mg	R210-01

Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using an antibody to the appropriate epitope. 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

continued on next page

Accessory Products, continued

Purification of Recombinant Fusion Protein

If your gene of interest is in frame with the C-terminal peptide containing the V5 epitope and the polyhistidine (6xHis) tag, you may use Immobilized Metal Affinity Chromatography (IMAC) to purify your recombinant fusion protein. The ProBond™ Purification System as well as the Ni-NTA Purification System are available separately from Life Technologies. See the table below for ordering information.

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

Methods

Overview

Description

pIB/V5-His-DEST is a 5.0 kb vector derived from pIB/V5-His and adapted for use with the Gateway® Technology. It is designed to allow transient or stable expression of the protein of interest in insect cell lines. For more information on the Gateway® Technology, see the next page.

Features

pIB/V5-His-DEST contains the following elements:

- *OpIE2* promoter for constitutive expression of the gene of interest
- Two recombination sites, *att*R1 and *att*R2, downstream of the *OpIE2* promoter for recombinational cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene located between the two *att*R sites for counterselection
- The *ccd*B gene located between the two *att*R sites for negative selection
- The C-terminal V5 epitope and 6xHis tag for detection and purification (optional)
- *OpIE2* polyadenylation sequence for proper termination and processing of the recombinant transcript
- The pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
- *GP64* promoter for expression of the blasticidin resistance gene (see page 3 for more information)
- EM7 promoter for expression of ampicillin (or blasticidin) resistance in *E. coli*
- Blasticidin resistance gene for selection of stable cell lines
- Ampicillin resistance gene for selection of transformants in *E. coli*

For a map of pIB/V5-His-DEST, see page 22.

continued on next page

Overview, continued

The Gateway® Technology

Gateway® is a universal cloning technology that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest using Gateway® Technology, simply:

1. Clone your gene of interest into a Gateway® entry vector to create an entry clone.
2. Generate an expression clone by performing an LR recombination reaction between the entry clone and a Gateway® destination vector (*e.g.* pIB/V5-His-DEST).
3. Introduce your expression clone into insect cells for transient or stable expression.

For more information on the Gateway® Technology, refer to the Gateway® Technology manual. This manual is available for downloading from our website (**www.lifetechnologies.com**) or by contacting Technical Support (page 27).

OpIE2 Promoter

Baculovirus immediate-early promoters utilize the host cell transcription machinery and do not require viral factors for activation. The *OpIE2* promoter is from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*Op*MNPV) and drives constitutive expression of the gene of interest in pIB/V5-His-DEST. The virus' natural host is the Douglas fir tussock moth; however, the promoter allows protein expression in *Lymantria dispar* (LD652Y), *Spodoptera frugiperda* cells (Sf9) (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997), Sf21 (Life Technologies), *Trichoplusia ni* (High Five™) (Life Technologies), *Drosophila* (Kc1, S2) (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997), and mosquito cell lines (unpublished data). The *OpIE2* promoter has been sequenced and analyzed. For more detailed information, see page 25.

continued on next page

Overview, continued

Expression Levels

Although the *OpIE2* promoter provides relatively high levels of constitutive expression, some proteins may not be expressed at levels seen with baculovirus late promoters such as polyhedrin or very late promoters such as p10 (Jarvis *et al.*, 1996). However, some researchers have found that the InsectSelect™ System expresses some proteins better than baculovirus systems. To date, reported expression levels range from 1-2 µg/ml (human IL-6; Life Technologies) to 8-10 µg/ml (human melanotransferrin) (Hegedus *et al.*, 1999).

GP64 Promoter

The *GP64* promoter regulates expression of the baculovirus major envelope glycoprotein gene (*GP64*) of the budded virion. Studies have shown that while the *GP64* promoter is stimulated by the transcriptional transactivator IE-1, low levels of activity still occur without transactivation (Blissard *et al.*, 1992; Blissard and Rohrmann, 1991). Furthermore, deletion analysis has identified the specific region required for transcriptional initiation in the absence of IE-1 (Blissard *et al.*, 1992; Blissard and Rohrmann, 1991).

Increased Expression Levels

pIB/V5-His-DEST contains a 100 bp region of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) *GP64* promoter which is sufficient for activation of the blasticidin resistance gene (*bsd*) in the absence of any baculovirus proteins. Using standard blasticidin concentrations (50-80 µg/ml), stable transfectants will only be selected if the *bsd* gene is expressed at suitable levels. Because of the minimal activity of the *GP64* promoter, we reason that only stable transfectants containing pIB/V5-His-DEST integrated into the most transcriptionally active genomic loci will be selected. This allows generation of stable cell lines which will express higher levels of the protein of interest compared to cell lines expressing the *bsd* gene product from the *OpIE1* promoter, as in the parent pIB/V5-His vector.

Using pIB/V5-His-DEST

Culturing Cells

Before you start your cloning experiments, be sure to have cell cultures of Sf9, Sf21, or High Five™ cells growing and have frozen master stocks available. For detailed guidelines on culturing 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

This manual is available for downloading from our website (**www.lifetechnologies.com**) or by contacting Technical Support (page 27).

Important

The pIB/V5-His-DEST vector is supplied as a supercoiled plasmid. Although Life Technologies has previously recommended using a linearized destination vector for more efficient recombination, further testing has found that linearization of this vector is ***not*** required to obtain optimal results for any downstream application.

pIB/V5-His-DEST Concentration

The vector is supplied in solution at final concentration of 150 ng/µl in TE, pH 8.0.

Propagating pIB/V5-His-DEST

If you wish to propagate and maintain pIB/V5-His-DEST, we recommend using Library Efficiency® DB3.1™ Competent Cells (Catalog no. 11782-018) from Life Technologies for transformation. The DB3.1™ *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain integrity of the vector, select for transformants in media containing 50-100 µg/ml ampicillin and 15 µg/ml chloramphenicol.

Note: ***do not*** use general *E. coli* cloning strains including TOP10 or DH5α™ for propagation and maintenance as these strains are sensitive to CcdB effects.

continued on next page

Using pIB/V5-His-DEST, continued

Entry Clone

To recombine your gene of interest into pIB/V5-His-DEST, you should have an entry clone containing your gene of interest. For your convenience, Life Technologies offers the pENTR™ Directional TOPO® Cloning Kit (Catalog no. K2400-20) for 5 minute cloning of your gene of interest into an entry vector. For more information on entry vectors available from Life Technologies, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 27).

For detailed information on constructing an entry clone, refer to the specific entry vector manual. For detailed information on performing the LR recombination reaction, refer to the Gateway® Technology manual.

Points to Consider Before Recombining

- Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position -3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is shown underlined.

  (G/A)NNATG**G**

- If you wish to include the V5 epitope and 6xHis tag, your gene in the entry clone **should not** contain a stop codon. The gene should also be designed to be in frame with the C-terminal epitope tag after recombination. Refer to the **Recombination Region** on page 7.
- If you do NOT wish to include the V5 epitope and 6xHis tag, your gene should contain a stop codon in the entry clone.

Using pIB/V5-His-DEST, continued

Recombining Your Gene of Interest

Each entry clone contains *att*L sites flanking the gene of interest. Genes in an entry clone are transferred to the destination vector backbone by mixing the DNAs with the Gateway® LR Clonase® enzyme mix (see page v for ordering information). The resulting LR recombination reaction is then transformed into *E. coli* and the expression clone selected. Recombination between the *att*R sites on the destination vector and the *att*L sites on the entry clone replaces the *ccd*B gene and the chloramphenicol (Cm^R) gene with the gene of interest and results in the formation of *att*B sites in the expression clone.

Follow the instructions in the Gateway® Technology manual to set up the LR Clonase® reaction, transform *E. coli*, and select for the expression clone.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccd*B gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/ml chloramphenicol. A true expression clone will not grow in the presence of chloramphenicol.

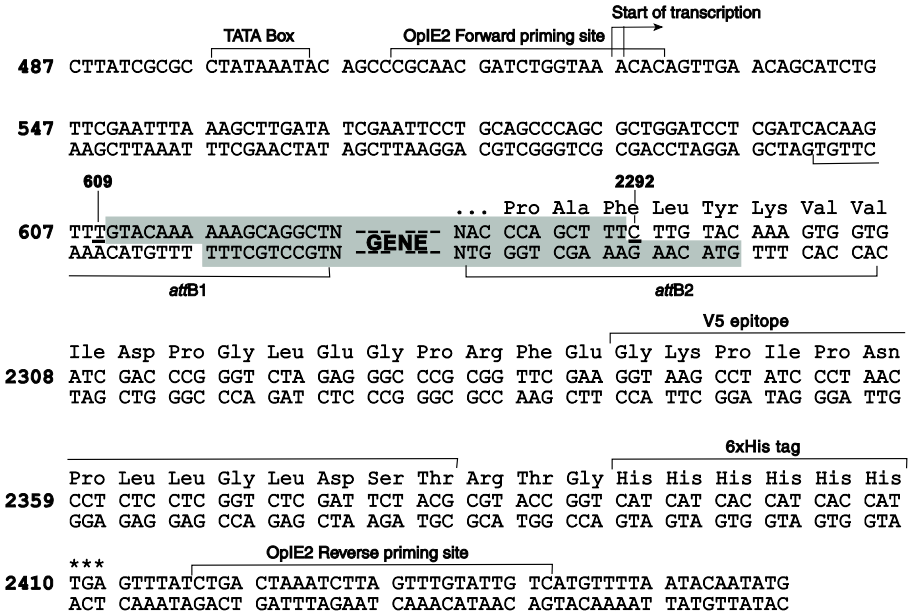
Using pIB/V5-His-DEST, continued

Recombination Region

The recombination region of the expression clone resulting from pIB/V5-His-DEST × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into pIB/V5-His-DEST by recombination. Non-shaded regions are derived from the pIB/V5-His-DEST vector.
- The underlined nucleotides flanking the shaded region correspond to bases 609 and 2292, respectively, of the pIB/V5-His-DEST vector sequence.




```
                                                    Start of transcription
                          TATA Box                  OpIE2 Forward priming site
487 CTTATCGCGC CTATAAATAC AGCCCGCAAC GATCTGGTAA ACACAGTTGA ACAGCATCTG

547 TTCGAATTTA AAGCTTGATA TCGAATTCCT GCAGCCCAGC GCTGGATCCT CGATCACAAG
    AAGCTTAAAT TTCGAACTAT AGCTTAAGGA CGTCGGGTCG CGACCTAGGA GCTAGTGTTC

        609                                                 2292
                                        ... Pro Ala Phe Leu Tyr Lys Val Val
607 TTTGTACAAA AAAGCAGGCTN ---  ---  NAC CCA GCT TTC TTG TAC AAA GTG GTG
    AAACATGTTT TTTCGTCCGTN -GENE- NTG GGT CGA AAG AAC ATG TTT CAC CAC
                attB1                                   attB2
                                                                V5 epitope
    Ile Asp Pro Gly Leu Glu Gly Pro Arg Phe Glu Gly Lys Pro Ile Pro Asn
2308 ATC GAC CCG GGT CTA GAG GGC CCG CGG TTC GAA GGT AAG CCT ATC CCT AAC
    TAG CTG GGC CCA GAT CTC CCG GGC GCC AAG CTT CCA TTC GGA TAG GGA TTG

                                                                6xHis tag
    Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His His His
2359 CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT
    GGA GAG GAG CCA GAG CTA AGA TGC GCA TGG CCA GTA GTA GTG GTA GTG GTA

    ***                 OpIE2 Reverse priming site
2410 TGA GTTTATCTGA CTAAATCTTA GTTTGTATTG TCATGTTTTA ATACAATATG
    ACT CAAATAGACT GATTTAGAAT CAAACATAAC AGTACAAAAT TATGTTATAC
```

Sequencing

To confirm that your gene of interest is in frame with the C-terminal V5 epitope and polyhistidine tag, you may sequence your expression construct. We suggest using the OpIE2 Forward and Reverse primer sequences. Refer to the diagram above for the sequence and location of the primer binding sites. Life Technologies also offers a custom primer synthesis service. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 27).

Transient Transfection

Introduction

Follow the guidelines below to perform transient transfection of your expression clone into an insect cell line of choice.

Plasmid Preparation

Once you have generated your expression clone, you must isolate plasmid DNA for transfection. 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 using the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) for isolation of 10-15 µg of plasmid DNA from 10-15 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, although transfection conditions may have to be optimized.

Expected Transfection Efficiency using Cellfectin® Reagent:

- **40-60% for Sf9 or Sf21 cells**
- **40-60% for High Five™ cells**

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

Positive and Negative Controls

We recommend that you include the following controls:

- pIB/V5-His-GW/*lac*Z vector as a positive control for transfection and expression (see page 9)
- Lipid only as a negative control
- DNA only to check for DNA contamination

continued on next page

Transient Transfection, continued

Positive Control

pIB/V5-His-GW/*lac*Z is provided as a positive control vector for transfection and expression (see page 24 for a map). The vector allows expression of a C-terminally tagged β-galactosidase fusion protein that may be detected by Western blot or functional assay.

To propagate and maintain the plasmid:

1. pIB/V5-His-GW/*lac*Z is provided at a concentration of 0.5 µg/µl in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Use the supplied stock to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™, JM109, or equivalent.
2. Select transformants on LB agar plates containing 50-100 µg/ml ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

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 35 mm plate for each time point.

1. For Sf9, Sf21, or High Five™ cells, seed 9×10^5 cells in appropriate serum-free medium in a 35 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.

Note

- If you use another lipid besides Cellfectin® Reagent, consult the manufacturer's instructions to adapt the protocol on the next page 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.

continued on next page

Transient Transfection, continued

Before Starting

You will need the following for each transfection experiment:

- Either log-phase Sf9, Sf21, or High Five™ cells (9×10^5 cells/ml, >95% viability) growing in serum-free medium. **Note:** You may transfect Sf9 or Sf21 cells in Grace's Medium without supplements or FBS.
- 1-2 µg of purified pIB/V5-His-DEST expression construct (~1 µg/µl in TE buffer)
- Serum-free medium (Sf-900 II SFM for Sf9 or Sf 21 cells, Express Five® SFM for High Five™ cells)
- Cellfectin® Reagent
- 35 mm tissue-culture dishes
- 1.5 ml sterile microcentrifuge tubes
- 27°C incubator
- Paper towels and air-tight bags or containers

Transfection Procedure

Plasmid DNA and Cellfectin® Reagent are mixed together in the appropriate medium and incubated with freshly seeded insect cells. The amount of cells, liposomes, and plasmid DNA has been optimized for 35 mm culture plates. It is important that you optimize transfection conditions if you use plates or flasks other than 30 mm plates.

Note: If you are using serum-free medium, we recommend using Sf-900 II SFM to transfect Sf9 or Sf21 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 transfection mixture A, use a 1.5 ml microcentrifuge tube. Add the following reagents:

Grace's Medium **OR** Appropriate serum-free medium	100 µl
pIB/V5-His-DEST expression construct (~1 µg/µl in TE, pH 8)	1-2 µl

continued on next page

Transient Transfection, continued

Transfection Procedure, continued

2. To prepare transfection mixture B, use a fresh 1.5 ml microcentrifuge tube. Add the following reagents:

Appropriate serum-free medium	100 µl
Cellfectin® Reagent	1.5-9 µl

   (mix well before use and always add last)
3. Combine transfection mixture A and transfection mixture B. Gently mix for 10 seconds. Incubate the transfection mixture at room temperature for 15-45 minutes.
4. To the combined transfection mixture, add 0.8 ml of the appropriate serum-free medium or Grace's Medium without supplements or FBS. Mix gently.
5. Carefully remove the medium from the cells without disrupting the monolayer. Wash the cells by adding 2 ml of the appropriate serum-free medium or Grace's Medium without supplements or FBS.
6. Again, carefully remove the medium from the monolayer and add the entire transfection mix from step 4 dropwise into the 35 mm dish. Distribute the drops evenly over the monolayer. This method reduces the chances of disturbing the monolayer. Repeat for all transfections.
7. Incubate the dishes at room temperature for 5 hours in a 27°C incubator.
8. Following the 5-hour incubation period, add 1-2 ml of complete TNM-FH medium (Sf9 or Sf21 cells) or the appropriate serum-free medium (Sf9, Sf21, or High Five™ cells) to each 35 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 twice with medium, and replace with 1-2 ml of fresh medium.
9. Harvest the cells 2, 3, and 4 days posttransfection and assay for expression of your gene. There's no need to add fresh medium if the cells are sealed in an airtight plastic bag with moist paper towels.

Expression and Analysis

Introduction

Expression of your gene of interest from the expression clone can be performed in transiently transfected cells or stable cell lines (see page 14 for guidelines to create stable cell lines). A sample protocol to detect your fusion protein by Western blot is provided below.

Testing for Expression

Use the cells from one 35 mm plate for each expression experiment. Before starting, prepare Cell Lysis Buffer and SDS-PAGE sample buffer (see pages 20-21 for recipes). If you are using pre-cast polyacrylamide gels (see page 13), refer to the manufacturer's instructions to prepare the appropriate sample buffer.

1. Prepare an SDS-PAGE gel that will resolve your expected recombinant protein.
2. Remove the medium from the cells. **If your protein is predicted to be secreted, be sure to save and assay both the medium and the cell pellet.**
3. Add 100 µl Cell Lysis Buffer to the plate and slough (or scrape) the cells into a microcentrifuge tube. Vortex the cells to ensure they are completely lysed.
4. Centrifuge at maximum speed for 1-2 minutes to pellet nuclei and cell membranes. Transfer the supernatant to a new tube. **Note**: If you are expressing a membrane protein, it may be located in the pellet. Be sure to assay the pellet (see step 6).
5. Assay the lysate for protein concentration. You may use the Bradford, Lowry, or BCA assays (Pierce).
6. To assay your samples, mix them with SDS-PAGE sample buffer as follows:
   - Lysate: 30 µl lysate with 10 µl **4X SDS-PAGE** sample buffer.
   - Pellet: Resuspend pellet in 100 µl **1X SDS-PAGE** sample buffer.
   - 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.

continued on next page

Testing for Expression, continued

7. Boil the samples for 5 minutes. Centrifuge briefly.
8. Load approximately 3 to 30 µg protein per lane. For the cell pellet sample, load the same volume as the lysate. Amount to load depends on the amount of your protein produced.
9. Electrophorese your samples, blot, and probe with a suitable antibody (see Western Analysis).
10. Visualize proteins using your desired method.

Polyacrylamide Gel Electrophoresis

To facilitate separation of your recombinant protein, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels are available from Life Technologies. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 27).

Western Analysis

To detect expression of your recombinant fusion protein by Western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Life Technologies (see page v for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available for use as a positive control for detection of fusion proteins containing a V5 epitope or a 6xHis tag. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 27).

Assay for β-galactosidase

If you use the pIB/V5-His-GW/*lac*Z plasmid as a positive control vector, you may assay for β-galactosidase expression by Western blot analysis or activity assay (Miller, 1972). Life Technologies offers β-Gal Antiserum (Catalog no. R901-25), the β-Gal Assay Kit (Catalog no. K1455-01), and the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression.

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine tag will add approximately 5 kDa to your protein.

Selecting Stable Cell Lines

Introduction

Once you have demonstrated that your protein is expressed, you may wish to create stable expression cell lines for long-term storage and large-scale production of the desired protein.

Nature of Stable Cell Lines

Note that stable cell lines are created by multiple copy integration of the vector. Amplification as in the case with calcium phosphate transfection and hygromycin resistance in *Drosophila* is generally not observed.

Before Starting

Review the information on blasticidin on page 26. Prepare a stock solution of blasticidin as described.

Effect of Blasticidin on Sensitive and Resistant Cells

Cytopathic effects should be visible within 3-5 days depending on the concentration of blasticidin in the medium. Sensitive cells will enlarge and become filled with vesicles. The outer membrane will show signs of blebbing, and cells will eventually detach from the plate.

Blasticidin-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes between blasticidin-resistant cells compared to cells not under selection with blasticidin.

Suggested Blasticidin Concentrations

In general, concentrations around 10 µg/ml will kill Sf9 or Sf21 cells (in complete TNM-FH medium) and concentrations around 20 µg/ml will kill High Five™ cells (in Express Five® SFM) within one week, although a few cells may remain that exclude trypan blue. To obtain faster and more thorough killing, we recommend using 50-80 µg/ml blasticidin. Once blasticidin-resistant clones have been obtained, cells may be maintained in lower concentrations of blasticidin (e.g. 10-20 µg/ml). If you are using other media or have trouble selecting cells using the concentrations above, we recommend that you perform a kill curve (see page 15).

continued on next page

Selecting Stable Cell Lines, continued

Blasticidin Selection Guidelines

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

- Prepare TNM-FH medium or the serum-free medium of choice supplemented with concentrations ranging from 0 to 100 μg/ml blasticidin. Generally, concentrations that effectively kill lepidopteran insect cells within a week are in the 50 to 80 μg/ml range. **Note**: While 10-20 μg/ml blasticidin will kill cells within a week, higher concentrations will result in faster and more thorough killing. In addition, using higher concentrations of blasticidin may result in enrichment of clones containing multiple integrations of your gene of interest.
- Test varying concentrations of blasticidin on the cell line to determine the concentration that kills your cells within a week (kill curve).
- Use the concentration of drug that kills your cells within a week.

Note

Do not linearize the plasmid prior to transfection. Linearization of the plasmid appears to decrease protein expression. The reason for this is not known.

Stable Transfection

We recommend including a mock transfection and a positive control (pIB/V5-His-GW/*lac*Z) in your experiments.

1. Follow steps 1-7 of the transfection procedure on pages 10-11.
2. Forty-eight hours posttransfection, remove the transfection solution and add fresh medium (**no blasticidin**).
3. Split cells 1:5 (20% confluent) and let cells attach overnight before adding selective medium.
4. Remove medium and replace with medium containing blasticidin at the appropriate concentration. Incubate cells at 27°C.

continued on next page

Selecting Stable Cell Lines, continued

Stable Transfection, continued

5. Replace selective medium every 3 to 4 days until you observe foci forming. At this point you may use cloning cylinders or limiting dilution to isolate clonal cell lines or you can let resistant cells grow out to confluence for a polyclonal cell line (2 to 3 weeks).
6. To isolate a polyclonal cell line, let the resistant cells grow to confluence and split the cells 1:5 and test for expression. **Important**: Always use medium **without** blasticidin when splitting cells. Let the cells attach before adding selective medium.
7. Expand resistant cells into flasks to prepare frozen stocks. **Always use medium containing blasticidin when maintaining stable lepidopteran cell lines. You may lower the concentration of blasticidin to 10 µg/ml for maintenance.**

Isolation of Clonal Cell Lines

If you select to isolate clonal cell lines, you may use clonal cylinders or limiting dilution. Protocols for both methods are provided in the InsectSelect™ BSD System manual. This manual is available from our website (**www.lifetechnologies.com**) or by contacting Technical Support (page 27).

Assay for Expression

Assay each of your cell lines for yield of the desired protein and select the one with the highest yield for scale-up and purification of recombinant protein. **If your protein is secreted, remember to assay the cell pellet as well as the medium.** You may wish to compare the yield of protein in the cells and medium.

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. Refer to the next section for guidelines on purifying your protein of interest.

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.

Metal-Chelating Resin

You may use the ProBond™ Purification System, the Ni-NTA Purification System, or a similar product to purify your 6xHis-tagged protein (see page vi for ordering information). Both purification systems contain a metal-chelating resin specifically designed to purify 6xHis-tagged proteins. Before starting, be sure to consult the ProBond™ or Ni-NTA Purification System manual to familiarize yourself with the buffers and the binding and elution conditions. If you are using another resin, follow the manufacturer's instructions.

Important

As you expand your stable cell line, you can maintain the concentration of blasticidin at 10 µg/ml.

Adapting Cells to Different Medium

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

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.** Refer to **Purification of 6xHis-tagged Proteins from Medium** (see page 18) for a general recommendation to address this issue.

continued on next page

Purification, continued

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.

Purification of 6xHis-Tagged Proteins from Medium

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

Dialysis allows:

- Removal of media components that strip Ni^{+2} from metal-chelating resins

Ion exchange chromatography allows:

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

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

Note

Many insect cell proteins are naturally rich in histidines, with some containing stretches of six histidines. When using the ProBond™ Purification System or other similar products to purify 6xHis-tagged proteins, these histidine-rich proteins may co-purify with your protein of interest. The contamination can be significant if your protein is expressed at low levels. We recommend that you add 5 mM imidazole to the binding buffer prior to addition of the protein mixture to the column. Addition of imidazole may help to reduce background contamination by preventing proteins with low specificity from binding to the metal-chelating resin.

continued on next page

Purification of Intracellularly Expressed Proteins

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

1. Seed 2×10^6 cells in two or three 25 cm^2 flasks.
2. Grow the cells in selective medium until they reach confluence (4×10^6 cells).
3. Wash cells once with PBS (Phosphate Buffered Saline, pH 7.4; Catalog no. 10010-023).
4. Harvest the cells by sloughing.
5. Transfer the cells to a sterile centrifuge tube.
6. Centrifuge the cells at 1000 × g for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -80°C until needed.

Scale Up

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

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:
1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add antibiotic and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

Trypan Blue Exclusion Assay

1. Prepare a 0.4% stock solution of trypan blue in phosphate buffered saline, pH 7.4 (Catalog no. 10010-023).
2. Mix 0.1 ml of trypan blue solution with 1 ml of cells and examine under a microscope at low magnification.
3. Dead cells will take up trypan blue while live cells will exclude it. Count live cells versus dead cells. Cell viability should be at least 95-99% for healthy log-phase cultures.

continued on next page

Recipes, continued

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 ml, combine

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.
4. To prevent proteolysis, you may add 1 mM PMSF, 1 μM leupeptin, and 0.1 μM aprotinin before use.

4X SDS-PAGE Sample Buffer

1. Combine the following reagents:

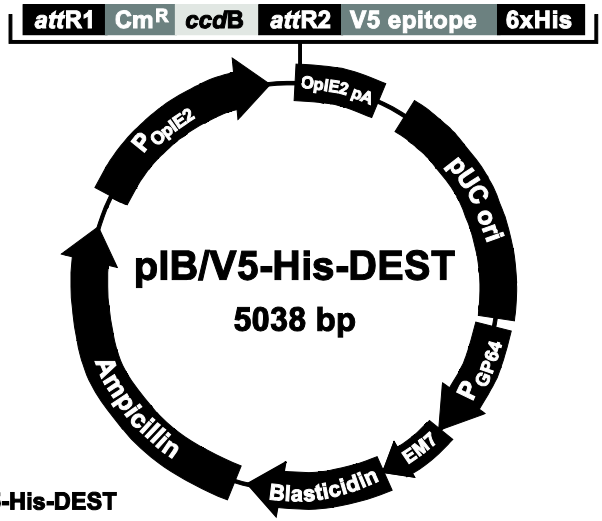
0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

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

Map and Features of pIB/V5-His-DEST

Map of pIB/V5-His-DEST

The map below shows the elements of pIB/V5-His-DEST. DNA from the entry clone replaces the region between bases 609 and 2292. **The complete sequence of pIB/V5-His-DEST is available from our website (www.lifetechnologies.com) or by contacting Technical Support (page 27).**



Features of pIB/V5-His-DEST
5038 nucleotides

OpIE2 promoter: bases 1-548
OpIE2 Forward priming site: 511-530
*att*R1 recombination site: bases 602-726
Chloramphenicol resistance gene: bases 835-1494
*ccd*B gene: bases 1836-2141
*att*R2 recombination site: bases 2182-2306 (c)
V5 epitope: bases 2341-2382
6xHis tag: bases 2392-2409
OpIE2 Reverse priming site: 2419-2444
OpIE2 polyadenylation sequence: bases 2427-2556
pUC origin: bases 2625-3298
GP64 promoter: bases 3364-3463
EM7 promoter: bases 3488-3546
Blasticidin (*bsd*) resistance ORF: bases 3565-3963
Ampicillin (*bla*) resistance ORF: 4083-4943
(c) = complementary strand

continued on next page

Map and Features of pIB/V5-His-DEST, continued

Features of pIB/V5-His-DEST pIB/V5-His-DEST (5038 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
OpIE2 promoter	Allows constitutive expression of the gene of interest in lepidopteran insect cells (Theilmann and Stewart, 1992).
*att*R1 and *att*R2 sites	Allows recombinational cloning of the gene of interest from an entry clone.
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of expression clones.
*ccd*B gene	Allows negative selection of expression clones.
V5 epitope	Allows detection of your recombinant protein with the Anti-V5 Antibodies (Southern *et al.*, 1991).
C-terminal polyhistidine tag	Allows purification of recombinant proteins on metal-chelating resin such as ProBond™ or Ni-NTA. Allows detection of the recombinant protein by the Anti-His (C-term) Antibodies (Lindner *et al.*, 1997).
OpIE2 polyadenylation sequence	Efficient transcription termination and polyadenylation of mRNA (Theilmann and Stewart, 1992).
pUC origin	Allows high-copy number replication and growth in *E. coli.*
GP64 promoter	Allows constitutive expression of the blasticidin resistance gene in lepidopteran insect cells (Blissard *et al.*, 1992; Blissard and Rohrmann, 1991).
EM7 promoter	Allows efficient expression of the blasticidin and ampicillin resistance genes in *E. coli.*
Blasticidin resistance gene (*bsd*)	Allows generation of stable insect cell lines (Kimura *et al.*, 1994).
Ampicillin resistance gene (*bla*)	Allows selection of transformants in *E. coli* **Note**: The native promoter has been removed. Transcription is assumed to start from the EM7 promoter.

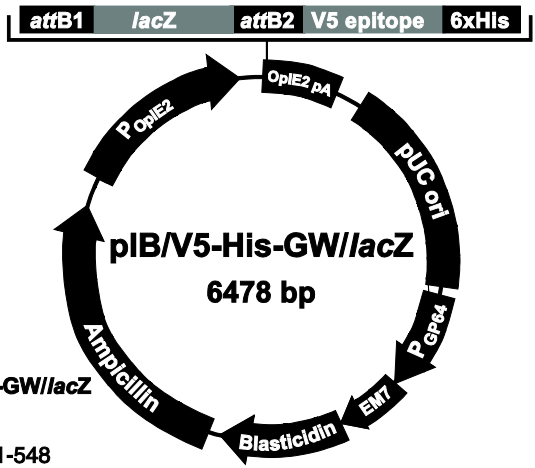
Map of pIB/V5-His-GW/lacZ

Description

pIB/V5-His-GW/*lac*Z is a 6478 bp control vector containing the gene for β-galactosidase. pIB/V5-His-GW/*lac*Z was constructed using the Gateway® LR recombination reaction between an entry clone containing the *lac*Z gene and pIB/V5-His-DEST. β-galactosidase is expressed as a fusion to the C-terminal tag. The molecular weight of the fusion protein is approximately 120 kDa.

Map of pIB/V5-His-GW/lacZ

The map below shows the elements of pIB/V5-His-GW/*lac*Z. **The complete sequence of pIB/V5-His-GW/lacZ is available from our website (www.lifetechnologies.com) or by contacting Technical Support (page 27).**



Features of pIB/V5-His-GW/*lac*Z
6478 nucleotides

OpIE2 promoter: bases 1-548
OpIE2 Forward priming site: 511-530
*att*B1 recombination site: bases 602-626
*lac*Z ORF: bases 646-3705
*att*B2 recombination site: bases 3722-3746 (c)
V5 epitope: bases 3781-3822
6xHis tag: bases 3832-3849
OpIE2 Reverse priming site: 3859-3884
OpIE2 polyadenylation sequence: bases 3867-3996
pUC origin: bases 4065-4738
GP64 promoter: bases 4804-4903
EM7 promoter: bases 4928-4986
Blasticidin (*bsd*) resistance ORF: bases 5005-5403
Ampicillin (*bla*) resistance ORF: 5523-6383

(c) = complementary strand

OpIE2 Promoter

Description

The *OpIE2* promoter has been analyzed by deletion analysis using a CAT reporter in both *Lymantria dispar* (LD652Y) and *Spodoptera frugiperda* (Sf9) cells. Expression in Sf9 cells was much higher than in LD652Y cells. Deletion analysis revealed that sequence up to -275 base pairs from the start of transcription is necessary for maximal expression (Theilmann and Stewart, 1992). Additional sequence beyond -275 may broaden the host range expression of this plasmid to other insect cell lines (Tom Pfeifer, personal communication).

In addition, an 18 bp element appears to be required for expression. This 18 bp element is repeated almost completely in three different locations and partially at six other locations. These are marked in the figure below. Elimination of the three major 18 bp elements reduces expression to basal levels (Theilmann and Stewart, 1992). The function of these elements is not known.

Primer extension experiments revealed that transcription initiates equally from either the C or the A indicated. These two transcriptional start sites are adjacent to a CAGT sequence motif that has been shown to be conserved in a number of early genes (Blissard and Rohrmann, 1989).

```
1    GGATCATGAT GATAAACAAT GTATGGTGCT AATGTTGCTT CAACAACAAT TCTGTTGAAC

61   TGTGTTTTCA TGTTTGCCAA CAAGCACCTT TATACTCGGT GGCCTCCCCA CCACCAACTT

121  TTTTGCACTG CAAAAAAACA CGCTTTTGCA CGCGGGCCCA TACATAGTAC AAACTCTACG

181  TTTCGTAGAC TATTTTACAT AAATAGTCTA CACCGTTGTA TACGCTCCAA ATACACTACC

241  ACACATTGAA CCTTTTTGCA GTGCAAAAAA GTACGTGTCG GCAGTCACGT AGGCCGGCCT

301  TATCGGGTCG CGTCCTGTCA CGTACGAATC ACATTATCGG ACCGGACGAG TGTTGTCTTA

361  TCGTGACAGG ACGCCAGCTT CCTGTGTTGC TAACCGCAGC CGGACGCAAC TCCTTATCGG

421  AACAGGACGC GCCTCCATAT CAGCCGCGCG TTATCTCATG CGCGTGACCG GACACGAGGC

                                TATA                          Start of Transcription
481  GCCCGTCCCG CTTATCGCGC CTATAAATAC AGCCCGCAAC GATCTGGTAA ACACAGTTGA

541  ACAGCATCTG TTCGAATTTA
```

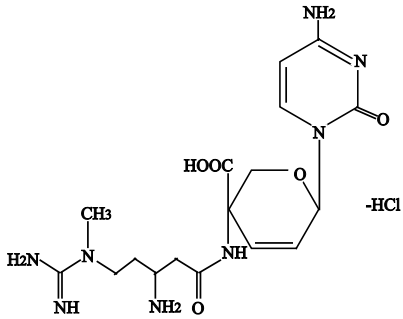
Blasticidin

Molecular Weight, Formula, and Structure

Merck Index: 12: 1350

MW: 458.9

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



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (*e.g.* a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

To inactivate blasticidin for disposal, add sodium bicarbonate.

Preparing and Storing Stock Solutions

- Blasticidin is soluble in water and acetic acid.
- Water is generally used to prepare stock solutions of 5 to 10 mg/ml.
- Dissolve blasticidin in sterile water and filter-sterilize the solution.
- Blasticidin is unstable in solutions with a pH greater than 8.0. Be sure the pH of the solution is 7.0.
- Aliquot in small volumes (see below) and freeze at -20°C for long-term storage or store at +4°C for short term storage.
- Aqueous stock solutions are stable for 1-2 weeks at +4°C and 6-8 weeks at -20°C.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store at +4°C. Discard after 1-2 weeks.

Technical Support

Obtaining Support

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

At the website, you can:

- 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
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

continued on next page

Technical Support, continued

Limited Warranty

Life Technologies and/or its affiliate(s) warrant their products as set forth in the Life Technologies General Terms and Conditions of Sale found on the Life Technologies website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies.

Life Technologies and/or its affiliate(s) disclaim all warranties with respect to this document, expressed or implied, including but not limited to those of merchantability or fitness for a particular purpose. In no event shall Life Technologies and/or its affiliate(s) be liable, whether in contract, tort, warranty, or under any statute or on any other basis for special, incidental, indirect, punitive, multiple or consequential damages in connection with or arising from this document, including but not limited to the use thereof.

Purchaser Notification

Limited Use Label License No. 358: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact **outlicensing@lifetech.com** or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License: ULB ccdB Selection Technology

ccdB selection technology is described in Bernard et al., "Positive Selection Vectors Using the F Plasmid ccdB Killer Gene" Gene 148 (1994) 71-74. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). For licensing information for use in other than research, please contact: **outlicensing@lifetech.com** or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License: 6X HisTag

This product is licensed from Hoffmann-La Roche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for use in research. Information about licenses for commercial use is available from QIAGEN GmbH, Max-Volmer-Str. 4, D-40724 Hilden, Germany.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 68: InsectSelect™ Technology

The InsectSelect™ System (the "Expression Kit") was developed into an expression system by scientists at the University of British Columbia (UBC) for high-level expression of recombinant proteins. 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.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 68: InsectSelect™ Technology, continued

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.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology.

Blissard, G. W., Kogan, P. H., Wei, R., and Rohrmann, G. F. (1992). A Synthetic Early Promoter from a Baculovirus: Roles of the TATA Box and Conserved Start Site CAGT Sequence in Basal Levels of Transcription. Virology *190*, 783-793.

Blissard, G. W., and Rohrmann, G. F. (1991). Baculovirus gp64 Gene Expression: Analysis of Sequences Modulating Early Transcription and Transactivation by IE1. J. Virology *65*, 5820-5827.

Blissard, G. W., and Rohrmann, G. F. (1989). Location, Sequence, Transcriptional Mapping, and Temporal Expression of the gp64 Envelope Glycoprotein Gene of the *Orgyia pseudotsugata* Multicapsid Nuclear Polyhedrosis Virus. Virology *170*, 537-555.

Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., and Wingfield, P. T. (1998). Current Protocols in Protein Science, V. B. Chanda, ed. (New York: John Wiley and Sons, Inc.).

Deutscher, M. P. (1990) Guide to Protein Purification. In *Methods in Enzymology*, Vol. 182. (M. I. Simon, ed. Academic Press, San Diego, CA.

Hegedus, D. D., Pfeifer, T. A., Hendry, J., Theilmann, D. A., and Grigliatti, T. A. (1998). A Series of Broad Host Range Shuttle Vectors for Constitutive and Inducible Expression of Heterologous Proteins in Insect Cell Lines. Gene *207*, 241-249.

Hegedus, D. D., Pfeifer, T. A., Theilmann, D. A., Kennard, M. L., Gabathuler, R., Jefferies, W. A., and Grigliatti, T. A. (1999). Differences in the Expression and Localization of Human Melanotransferrin in Lepidopteran and Dipteran Insect Cell Lines. Protein Expression and Purification *15*, 296-307.

Jarvis, D. L., Weinkauf, C., and Guarino, L. A. (1996). Immediate-Early Baculovirus Vectors for Foreign Gene Expression in Transformed or Infected Insect Cells. Protein Expression and Purification *8*, 191-203.

Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994). Blasticidin S Deaminase Gene from *Aspergillus terreus* (*BSD*): A New Drug Resistance Gene for Transfection of Mammalian Cells. Biochim. Biophys. ACTA *1219*, 653-659.

continued on next page

References, continued

Kozak, M. (1987). An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. *15*, 8125-8148.

Kozak, M. (1991). An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology *115*, 887-903.

Kozak, M. (1990). Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305.

Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Annu. Rev. Biochem. *58*, 913-949.

Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Pluckthun, A. (1997). Specific Detection of His-tagged Proteins With Recombinant Anti-His Tag scFv-Phosphatase or scFv-Phage Fusions. BioTechniques *22*, 140-149.

Mann, S. G., and King, L. A. (1989). Efficient Transfection of Insect Cells with Baculovirus DNA Using Electroporation. J. Gen. Virol. *70*, 3501-3505.

Miller, J. H. (1972). Experiments in Molecular Genetics (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Pfeifer, T. A., Hegedus, D. D., Grigliatti, T. A., and Theilmann, D. A. (1997). Baculovirus Immediate-Early Promoter-Mediated Expression of the Zeocin™ Resistance Gene for Use as a Dominant Selectable Marker in Dipteran and Lepidopteran Insect Cell Lines. Gene *188*, 183-190.

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991). Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557.

Theilmann, D. A., and Stewart, S. (1992). Molecular Analysis of the trans-Activating IE-2 Gene of *Orgyia pseudotsugata* Multicapsid Nuclear Polyhedrosis Virus. Virology *187*, 84-96.

Notes

Notes

Notes

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA
Phone +1 760 603 7200 | Toll Free in USA 800 955 6288

For support visit
www.lifetechnologies.com/support or email techsupport@lifetech.com

www.lifetechnologies.com

life technologies™