pEF4/V5-His A, B, and C

Catalog no. V941-20

Version C
102810
25-0228

www.invitrogen.com
tech_service@invitrogen.com

Table of Contents

Table of Contents iii
General Information 1
Methods 2
Overview 2
Accessory Products 3
Cloning into pEF4/V5-His A, B, and C 4
Transfection and Analysis 9
Creation of Stable Cell Lines 12
Appendix 16
Human EF-1α Promoter 16
pEF4/V5-His Vector 17
pEF4/V5-His/*lac*Z 19
Technical Service 20
Purchaser Notification 22
References 23

General Information

Contents

20 µg each pEF4/V5-His A, B, and C, lyophilized
20 µg pEF4/V5-His/*lacZ*, lyophilized

Shipping/Storage

Lyophilized plasmids are shipped at room temperature and stored at -20°C.

Product Qualification

Invitrogen qualifies the vectors by restriction digest. Restriction digestion must demonstrate the correct banding patterns when electrophoresed on an agarose gel.

Restriction Enzyme	pEF4/V5-His A	pEF4/V5-His B	pEF4/V5-His C	pEF4/V5-His A/*lacZ*
*Bst*E II	No cut	No cut	5765 bp	8816 bp
*Hin*d III	4447 bp, 1322 bp	4451 bp, 1322 bp	4443 bp, 1322 bp	7494 bp, 1322 bp
Mlu I	4273 bp, 1496 bp	4273 bp, 1500 bp	4273 bp, 1492 bp	4273 bp, 2721 bp, 780 bp, 617 bp, 425 bp
Sma I	5573 bp, 196 bp	5577 bp, 196 bp	5569 bp, 196 bp	8620 bp, 196 bp
Sac II	5422 bp, 347 bp	4700 bp, 726 bp, 347 bp	5418 bp, 347 bp	8469 bp, 347 bp

Methods

Overview

Introduction

pEF4/V5-His A, B, and C are 5.7 kb vectors derived from pEF1/V5-His and designed for overproduction of recombinant proteins in mammalian cell lines. Features of the vectors allow purification and detection of expressed proteins. High-level stable and transient expression can be carried out in most mammalian cells. The vectors contain the following elements:

- Human elongation factor 1a-subunit promoter (hEF-1α) promoter for high-level expression across a broad range of species and cell types (Goldman et al., 1996) (Mizushima and Nagata, 1990) (see page 16 for more information).
- Three reading frames to facilitate in-frame cloning with a C-terminal tag encoding the V5 epitope and a polyhistidine metal-binding peptide
- Zeocin™ resistance gene for selection of stable cell lines (Mulsant et al., 1988) (see page 12 for more information).
- Episomal replication in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS7)

The control plasmid, pEF4/V5-His/*lac*Z is included for use as a positive control for transfection, expression, purification, and detection in the cell line of choice (see page 19).

Experimental Outline

Use the following outline to clone and express your gene of interest in pEF4/V5-His.

- Consult the multiple cloning sites described on pages 5-7 to determine which vector (A, B, or C) should be used to clone your gene in-frame with the C-terminal V5 epitope and the polyhistidine tag.
- Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants on 50 to 100 µg/ml ampicillin or 25 to 50 µg/ml Zeocin™ (prepare in Low Salt LB). For more information, see page 8.
- Analyze your transformants for the presence of insert by restriction digestion.
- Select a transformant with the correct restriction pattern and confirm that your gene is in-frame with the C-terminal peptide by sequencing.
- Transfect your construct into the cell line of choice using your own method of transfection. For information about transfection kits from Invitrogen, see page 9.
- Test for expression of your recombinant gene by western blot analysis or functional assay. For antibodies to the V5 epitope or the C-terminal polyhistidine tag, see the next page.
- To purify your recombinant protein, you may use metal-chelating resin such as ProBond™. ProBond™ resin is available separately (see next page for ordering information).

Accessory Products

Introduction

The products listed below are designed to help you detect and purify your recombinant fusion protein expressed from pEF4/V5-His. In addition, Invitrogen has a wide variety of mammalian expression vectors, many of which can be utilized with pEF4/V5-His to express multiple proteins in the same cell.

Antibodies for Detection

If you do not have an antibody to your protein, Invitrogen offers the Anti-V5 Antibodies or the Anti-His(C-term) Antibodies to detect your recombinant fusion protein. Horse-radish peroxidase (HRP)-conjugated antibodies are available for convenient one-step detection.

Antibody	Epitope	Catalog no.
Anti-V5	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	Same as above	R961-25
Anti-His(C-term)	Detects the C-terminal polyhistidine tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP	Same as above	R931-25

ProBond™ Resin

Ordering information for ProBond™ resin is provided below.

Item	Amount	Catalog no.
ProBond™ Protein Purification System	6 purifications	K850-01
ProBond™ Protein Purification Kit with Anti-V5-HRP Antibody	1 Kit	K854-01
ProBond™ Resin	50 ml	R801-01
	150 ml	R801-15

Other Mammalian Expression Vectors

We have a wide variety of mammalian expression vectors utilizing the CMV or EF-1α promoters. Vectors are available with the Xpress™ (N-terminal), *c-myc* (C-terminal), V5 (C-terminal), or C-terminal polyhistidine epitopes for detection and either the neomycin, blasticidin, or Zeocin™ resistance genes. All vectors utilize the polyhistidine tag for purification using ProBond™ resin. For more information on the mammalian expression vectors available, see our web site (www.invitrogen.com) or call Technical Service (see page 20).

Cloning into pEF4/V5-His A, B, and C

Introduction

Diagrams are provided on pages 5-7 to help you ligate your gene of interest in frame with the C-terminal peptide. General considerations for cloning and transformation are listed below.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, 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) (See **References**, page 22).

E. coli Strain

Many *E. coli* strains are suitable for the growth of this vector including TOP10F´ (Catalog no. C615-00), DH5αF´, JM109, and INVαF´ (Catalog no. C658-00). We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10F´ is available as chemically competent or electrocompetent cells from Invitrogen.

Item	Quantity	Catalog no.
Electrocomp™ TOP10F´	5 x 80 µl	C665-55
Ultracomp™ TOP10F´ (chemically competent cells)	5 x 300 µl	C665-03
One Shot™ TOP10F´ (chemically competent cells)	21 x 50 µl	C3030-03

Transformation Method

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of pEF4/V5-His

To propagate and maintain the pEF4/V5-His vectors, we recommend resuspending each vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.

Use this stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10F´, DH5α, JM109, or equivalent. Select transformants on LB plates containing 50 to 100 µg/ml ampicillin or 25 to 50 µg/ml Zeocin™. Be sure to prepare a glycerol stock of each plasmid for long-term storage.

Cloning Considerations

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

ANN<u>ATG</u>**G**

To express your gene as a recombinant fusion protein, you must clone your gene in frame with the C-terminal peptide. The vector is supplied in three reading frames to facilitate cloning. See pages 5-7 to develop a cloning strategy.

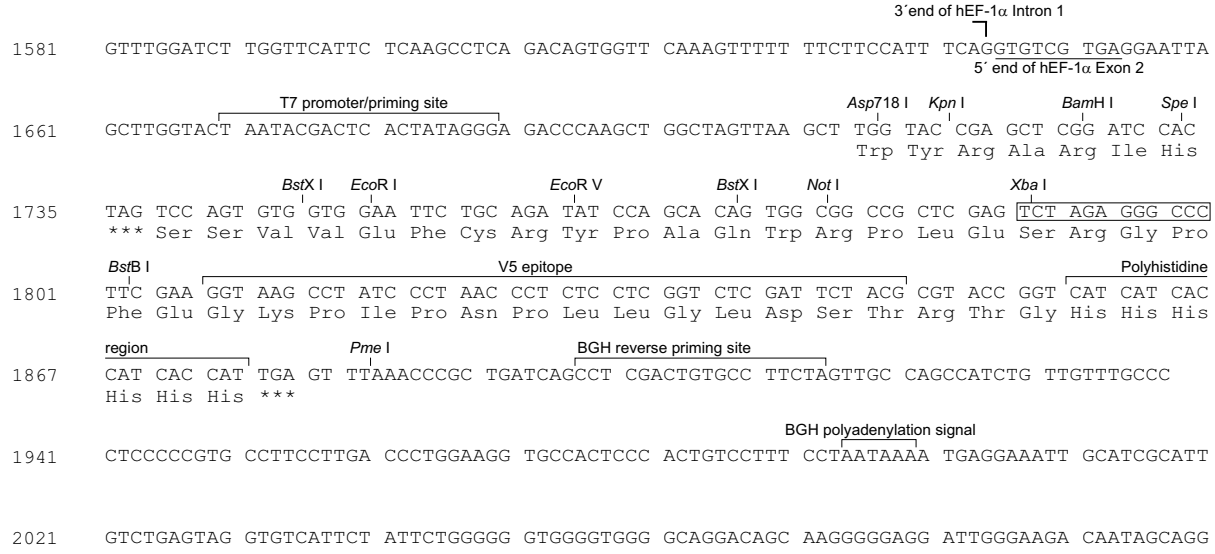
If you wish to express your protein WITHOUT the C-terminal peptide, be sure to include a stop codon.

continued on next page

Cloning into pEF4/V5-His A, B, and C, continued

Multiple Cloning Site of Version A

Below is the multiple cloning site for pEF4/V5-His A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. **Note that there is a stop codon between the *Spe* I site and the *Bst*X I site.** The multiple cloning site has been confirmed by sequencing and functional testing. For more information on the hEF-1α promoter, see page 16. **The complete sequence of pEF4/V5-His A is available for downloading from our web site (www.invitrogen.com) or from Technical Service.** See page 20 for more information.

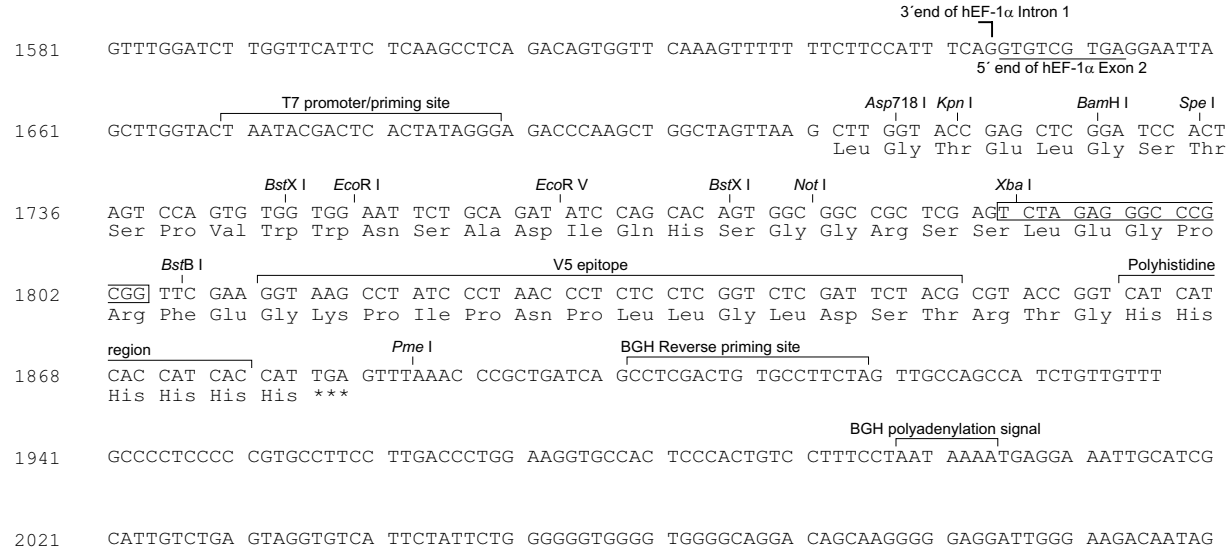


continued on next page

Cloning into pEF4/V5-His A, B, and C, continued

Multiple Cloning Site of Version B

Below is the multiple cloning site for pEF4/V5-His B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. For more information on the hEF-1α promoter, see page 16. **The complete sequence of pEF4/V5-His B is available for downloading from our web site (www.invitrogen.com) or from Technical Service.** See page 20 for more information.

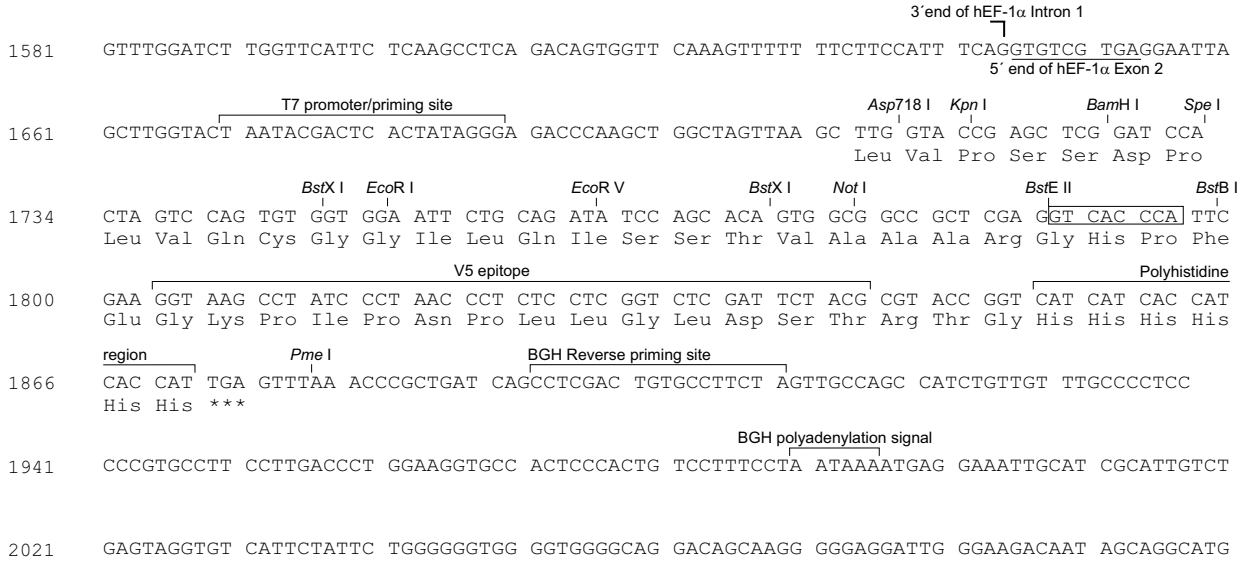


continued on next page

Cloning into pEF4/V5-His A, B, and C, continued

Multiple Cloning Site of Version C

Below is the multiple cloning site for pEF4/V5-His C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. For more information on the hEF-1α promoter, see page 16. **The complete sequence of pEF4/V5-His C is available for downloading from our web site (www.invitrogen.com) or from Technical Service.** See page 20 for more information.



continued on next page

Cloning into pEF4/V5-His A, B, and C, continued

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10F´, DH5α) and select on LB plates containing 50-100 µg/ml ampicillin or 25-50 µg/ml Zeocin™ in Low Salt LB medium (see below). Select 10-20 clones and analyze for the presence and orientation of your insert.

Low Salt LB Medium with Zeocin™

For Zeocin™ to be active, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.5. For selection in *E. coli*, it is **imperative** that you prepare LB broth and plates using the following recipe. Note the lower salt content of this medium. Failure to lower the salt content of your LB medium will result in non-selection because of inactivation of the drug.

Low Salt LB Medium:

10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.5 with 5M NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
3. Thaw Zeocin™ on ice and vortex before removing an aliquot.
4. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 µg/ml final concentration.
5. Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

Important

Any *E. coli* strain that contains the complete Tn*5* transposable element (i.e. DH5αF´IQ, SURE, SURE2) encodes the *ble* (bleomycin resistance gene). These strains will confer resistance to Zeocin™. For the most efficient selection, we highly recommend that you choose an *E. coli* strain that does not contain the Tn*5* gene (i.e. TOP10, DH5α, etc.).

RECOMMENDATION

We recommend that you sequence your construct with the T7 Promoter and BGH Reverse primer sequences to confirm that your gene is fused in frame with the V5 epitope and the C-terminal polyhistidine tag. Refer to the diagram on the previous pages for the sequence and location of the primer binding sites. For your convenience, Invitrogen offers the T7 Promoter Primer (Catalog no. N560-02) as well as a custom primer synthesis service. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 20).

Preparing a Glycerol Stock

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at -20°C.

- Streak the original colony out on an LB plate containing 50 µg/ml ampicillin or 25 µg/ml Zeocin™ (in low salt LB). Incubate the plate at 37°C overnight.
- Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml ampicillin or 25 µg/ml Zeocin™.
- Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
- Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
- Store at -80°C.

Transfection and Analysis

Introduction

Once you have confirmed that your construct is in the correct orientation and fused in frame to the C-terminal peptide, you are ready to transfect your cell line of choice. We recommend that you include the positive control vector and a mock transfection to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells and salt will interfere with lipids decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 µg, Catalog no. K1910-01), or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (e.g. HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (**Reference** section, page 22).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988).

Invitrogen offers the Calcium Phosphate Transfection Kit (K2780-01) and a large variety of reagents for mammalian transfection. For more details on the transfection products, contact Technical Service (see page 20) or visit our Web site at www.invitrogen.com..

Positive Control

pEF4/V5-His/*lacZ* is provided as a positive control vector for mammalian transfection and expression (see page 19), and may be used to optimize transfection conditions for your cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the control of the hEF-1α promoter. A successful transfection will result in β-galactosidase expression that can be easily assayed (see next page).

continued on next page

Transfection and Analysis, continued

Assay for β-galactosidase Activity

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

Detection of Fusion Proteins

Several antibodies are available from Invitrogen to detect expression of your fusion protein from pEF4/V5-His (see page 3).

To detect fusion protein by Western blot, you will need to prepare a cell lysate from transfected cells. We recommend that you perform a time course to optimize expression of the fusion protein (*e.g.* 24, 48, 72 hours, etc. after transfection). To lyse cells:

1. Wash cell monolayers (~10^6 cells) once with phosphate-buffered saline (PBS).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 50 µl Cell Lysis Buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, see recipe below). **Note:** Other lysis buffers may be suitable.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.
5. Centrifuge the cell lysate at 10,000 x g for 10 minutes to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration. **Note:** Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer to a final concentration of 1X and boil the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

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.

Note: Protease inhibitors may be added at the following concentrations:

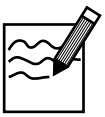
1 mM PMSF

1 µg/ml pepstatin

1 µg/ml leupeptin

continued on next page

Transfection and Analysis, continued



Note

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

Purification

You will need lysate from 5 x 10^6 to 1 x 10^7 **transfected** cells for purification of your protein on a 2 ml ProBond™ column (or other metal-chelating column). Refer to the manufacturer's instructions before attempting to purify your fusion protein. To prepare cells for lysis, refer to the protocol on page 15.

Creation of Stable Cell Lines

Introduction

The pEF4/V5-His vectors contain the Zeocin™ resistance gene to allow for selection of stable cell lines using Zeocin™. We recommend that you test the sensitivity of your mammalian host cell to Zeocin™ as natural resistance varies among cell lines. General information and guidelines are provided below for your convenience.

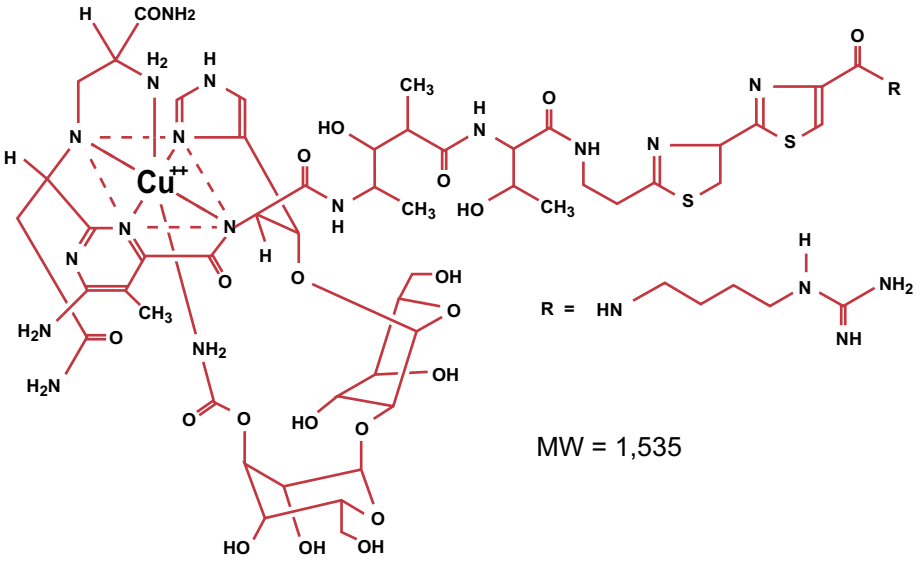
Zeocin™

Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells.

The Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ in a stoichiometric manner to inhibit its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.



continued on next page

Creation of Stable Cell Lines, continued

Applications of Zeocin™

Zeocin™ is used for selection in mammalian cells (Mulsant *et al.*, 1988); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection in mammalian cell lines and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25-50 µg/ml in l**ow salt LB** medium* (see page 8 for recipe)
Mammalian Cells	50-1000 µg/ml (varies with cell line)

*Efficient selection requires that the concentration of NaCl be no more than 5 g/liter (< 90 mM).

Handling Zeocin™

- High salt and acidity or basicity 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 (see page 8).
- 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 Zeocin™-containing solutions.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Ordering Information

Zeocin™ can be purchased from Invitrogen. For your convenience, the drug is prepared in autoclaved, deionized water and available in 1.25 ml aliquots at a concentration of 100 mg/ml. The stability of Zeocin™ is guaranteed for six months, if stored at -20°C.

Amount	Catalog no.
1 gram	R250-01
5 grams	R250-05

Effect of Zeocin™ on Sensitive and Resistant Cells

Zeocin™'s method of killing is quite different from neomycin and hygromycin. Cells do not round up and detach from the plate. Sensitive cells will exhibit the following morphological changes upon exposure to Zeocin™:

- Vast increase in size (similar to the effects of cytomegalovirus infecting permissive cells)
- Abnormal cell shape
- 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)

Eventually, these "cells" will completely break down and only "strings" of protein 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™.

continued on next page

Creation of Stable Cell Lines, continued

Selection in Mammalian Cell Lines

To generate a stable cell line expressing your protein, you need to determine the minimum concentration of Zeocin™ required to kill your untransfected host cell line. Typically, concentrations between 50 and 1000 µg/ml Zeocin™ are sufficient to kill the untransfected host cell line. Test a range of concentrations (see below) to ensure that you determine the minimum concentration necessary for your cell line.

- Seed cells (2 x 10^5 cells/60 mm plate) for each time point and allow cells to adhere overnight.
- The next day, substitute culture medium with medium containing varying concentrations of Zeocin™ (e.g. 0, 50, 125, 250, 500, 750, and 1000 µg/ml).
- Replenish the selective medium every 3-4 days, and observe the percentage of surviving cells.
- Count the number of viable cells at regular intervals to determine the appropriate concentration of Zeocin™ that prevents growth.

Possible Sites for Linearization

To obtain stable transfectants, you may choose to linearize your vector before transfection. While linearizing your vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts the gene of interest. The table below lists unique sites that may be used to linearize your construct prior to transformation. **Other restriction sites are possible. Note that for the enzymes listed below, the cleavage site is indicated for versions A, B, and C of pEF4/V5-His.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)(A,B,C)	Location	Supplier
Ssp I	4	Upstream of EF-1α promoter	Invitrogen (Catalog no. 15458-011)
Nru I	331	Upstream of EF-1α promoter	Invitrogen (Catalog no. 15423-015)
*Bst*1107 I	3697 (A), 3701 (B), 3693 (C)	End of SV40 poly A	AGS*, Fermentas, Takara, Boehringer-Mannhiem
*Eam*1105 I	4969 (A), 4973 (B), 4965 (C)	Ampicillin gene	AGS*, Fermentas, Takara
Fsp I	5191 (A), 5195 (B), 5187 (C)	Ampicillin gene	Many
Pvu I	5339 (A), 5343 (B), 5335 (C)	Ampicillin gene	Invitrogen (Catalog no. 25420-068)
Sca I	5449 (A), 5453 (B), 5445 (C)	Ampicillin gene	Invitrogen (Catalog no. 15436-017)

*Angewandte Gentechnologie Systeme

continued on next page

Creation of Stable Cell Lines, continued

Selection of Stable Integrants

Once the appropriate Zeocin™ concentration is determined, you can generate a stable cell line with your construct.

- Transfect your cells using the appropriate protocol for your cell line. Include a sample of untransfected cells as a negative control.
- After transfection, wash the cells once with 1X PBS and add fresh medium to the cells.
- 48 hours after transfection, split the cells into fresh medium containing Zeocin™ at the appropriate concentration for your cell line. Split the cells such that they are no more than 25% confluent.
- Change selective medium every 3-4 days until Zeocin™-resistant colonies are detected.
- Pick and expand colonies.

Preparation of Cells for Lysis

Use the procedure below to prepare cells for lysis prior to purification of your protein on ProBond™. You will need 5 x 10^6 to 1 x 10^7 cells for purification of your protein on a 2 ml ProBond™ column (see Xpress™ Protein Purification manual).

1. Seed cells in either five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are 80-90% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with fresh medium (if necessary) and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 1500 rpm for 5 minutes. Resuspend the cells in PBS.
6. Centrifuge the cells at 1500 rpm for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -70°C until needed.

Lysis of Cells

If you are using ProBond™ resin, refer to the ProBond™ Protein Purification manual for details about sample preparation for chromatography.

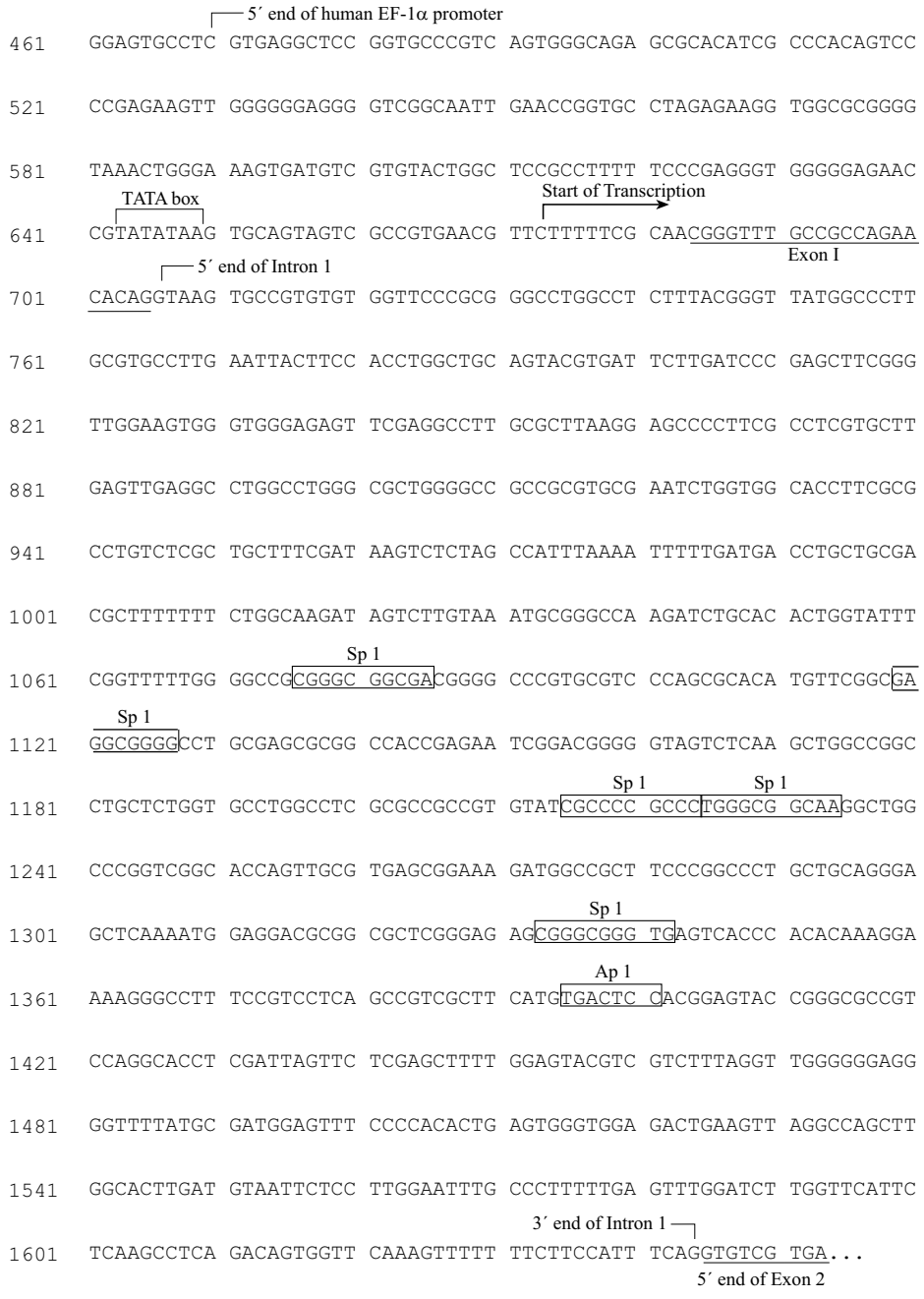
If you are using other metal-chelating resin, refer to the manufacturer's instruction for recommendations on sample preparation.

Appendix

Human EF-1α Promoter

Description

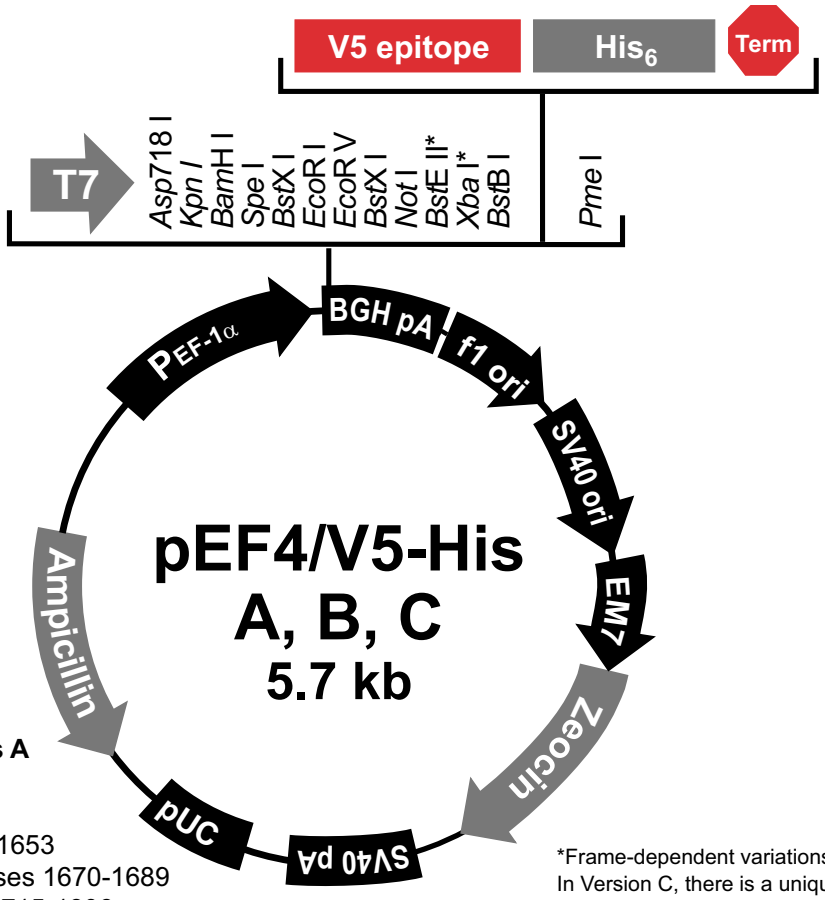
The diagram below shows all the features of the EF-1α promoter used in pEF4/V5-His vectors (Mizushima and Nagata, 1990). Features are marked as per Uetsuki *et al.*, 1989.



pEF4/V5-His Vector

Map of pEF4/V5-His

The figure below summarizes the features of the pEF4/V5-His vectors. **The sequences for pEF4/V5-His A, B, and C are available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 20).**



*Frame-dependent variations. In Version C, there is a unique *Bst*E II site, but no *Xba* I site.

Comments for pEF4/V5-His A
5769 nucleotides

EF-1α promoter: bases 468-1653
T7 promoter/priming site: bases 1670-1689
Multiple cloning site: bases 1715-1806
V5 epitope: bases 1807-1848
Polyhistidine tag: bases 1858-1875
BGH reverse priming site: bases 1898-1915
BGH polyadenylation signal: bases 1901-2128
f1 origin: bases 2174-2602
SV40 promoter and origin: bases 2630-2938
EM7 promoter: bases 2986-3041
Zeocin resistance gene: bases 3060-3434
SV40 polyadenylation signal: bases 3564-3694
pUC origin: bases 4077-4750
Ampicilllin resistance gene: bases 4895-5755

continued on next page

pEF4/V5-His Vector, continued

Features of pEF4/V5-His

pEF4/V5-His A (5769 bp), pEF4/V5-His B (5773 bp), and pEF4/ V5-His C (5765 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
Human elongation factor 1α (hEF-1α) promoter	Permits overexpression of your recombinant protein in a broad range of mammalian cell types (Goldman *et al.*, 1996; Mizushima and Nagata, 1990)
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
Multiple cloning site in three reading frames	Allows insertion of your gene and facilitates cloning in frame with the V5 epitope and polyhistidine C-terminal tag
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 Antibody (Catalog no. R960-25) or Anti-V5-HRP Antibody (Catalog no. R961-25) (Southern *et al.*, 1991)
C-terminal polyhistidine tag	Permits purification of your recombinant protein on metal-chelating resin such as ProBond™ In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody (Catalog no. R930-25) (Lindner *et al.*, 1997) and the Anti-His (C-term)-HRP Antibody (Catalog no. R931-25)
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the Zeocin™ resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM7 promoter	Synthetic promoter based on the bacteriophage T7 promoter for expression of the Zeocin™ resistance gene in *E. coli*
Zeocin™ resistance gene	Selection of transformants in *E. coli* and stable transfectants in mammalian cells (Drocourt *et al.*, 1990; Mulsant *et al.*, 1988)
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Selection of transformants in *E. coli*

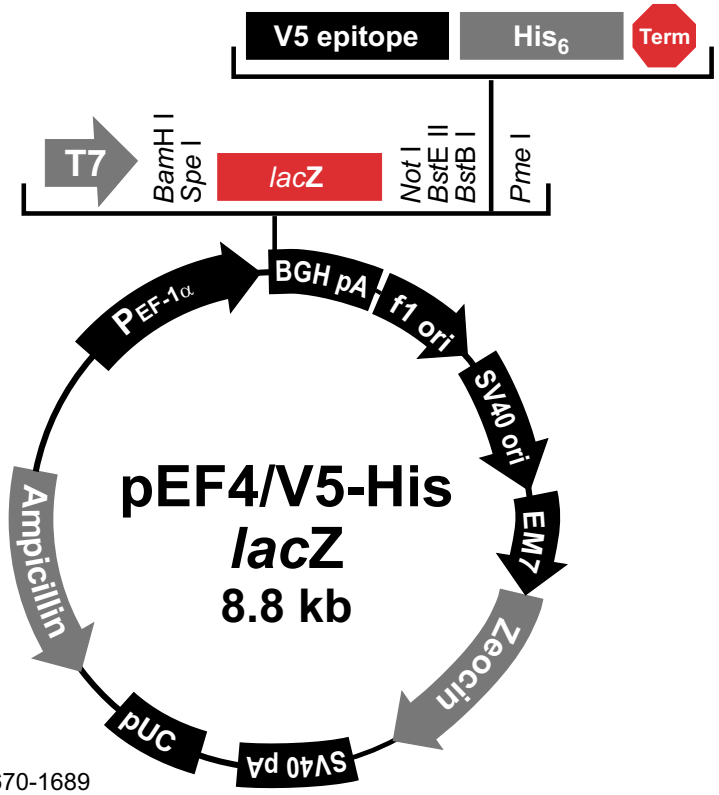
pEF4/V5-His/*lacZ*

Description

pEF4/V5-His/*lacZ* is a 8816 bp control vector containing the 3.0 kb gene for β-galactosidase. This vector was constructed by ligating a 3852 bp *Bam*H I-*Bsm* I fragment containing the EF-1α promoter from pEF1/V5-His to a 4964 bp *Bam*H I-*Bsm* I fragment containing the *lacZ* gene, V5 epitope, polyhistidine tag and Zeocin™ resistance gene from pcDNA4/V5-His/*lacZ*.

Map of Control Vector

The figure below summarizes the features of the pEF4/V5-His/*lacZ* vector. **The complete nucleotide sequence for pEF4/V5-His/*lacZ* is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service. See the next page for more information.**



Comments for pEF4/V5-His/lacZ
8816 nucleotides

EF-1α promoter: bases 468-1653
T7 promoter/priming site: bases 1670-1689
LacZ gene: bases 1770-4826
V5 epitope: bases 4854-4895
Polyhistidine tag: bases 4905-4922
BGH reverse priming site: bases 4945-4962
BGH polyadenylation signal: bases 4948-5175
f1 origin: bases 5221-5649
SV40 promoter and origin: bases 5677-5985
EM7 promoter: bases 6033-6088
Zeocin resistance gene: bases 6107-6481
SV40 polyadenylation signal: bases 6611-6741
pUC origin: bases 7124-7797
Ampicilllin resistance gene: bases 7942-8802

Technical Service

World Wide Web

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

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

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

http://www.invitrogen.com

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

Contact Us

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

Corporate Headquarters:

Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
USA

Tel: 1 760 603 7200

Tel (Toll Free): 1 800 955 6288

Fax: 1 760 602 6500

E-mail:
tech_service@invitrogen.com

Japanese Headquarters:

Invitrogen Japan K.K.
Nihonbashi Hama-Cho Park
Bldg. 4F
2-35-4, Hama-Cho, Nihonbashi

Tel: 81 3 3663 7972

Fax: 81 3 3663 8242

E-mail: jpinfo@invitrogen.com

European Headquarters:

Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK

Tel: +44 (0) 141 814 6100

Tel (Toll Free): 0800 5345 5345

Fax: +44 (0) 141 814 6287

E-mail: eurotech@invitrogen.com

continued on next page

Technical Service, continued

Limited Warranty

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

Invitrogen warrants that all of its products will perform according to the specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Limited Use Label License No. 60: EF-1∝ Promoter	EF-1alpha promoter products are sold under license for research purposes only. The use of this product for any commercial purpose, including but not limited to, use in any study for the purpose of a filing of a new drug application, requires a license from: Mochida Pharmaceutical Co., Ltd., 7, Yotsuya 1-Chome, Shinjuku-Ku, Tokyo 160, Japan. Tel: 81-3-3225-5451; Fax: 81-3-3225-6091.
Limited Use Label License No. 22: Vectors and Clones containing sequences coding for Histidine Hexamer	This product is licensed under U.S. Patent Nos. 5,284,933 and 5,310,663 and foreign equivalents from Hoffmann-LaRoche, 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.

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 (New York: Greene Publishing Associates and Wiley-Interscience).

Baron, M., Reynes, J. P., Stassi, D., and Tiraby, G. (1992). A Selectable Bifunctional β-Galactosidase: Phleomycin-resistance Fusion Protein as a Potential Marker for Eukaryotic Cells. Gene *114*, 239-243.

Calmels, T., Parriche, M., Burand, H., and Tiraby, G. (1991). High Efficiency Transformation of *Tolypocladium geodes* Conidiospores to Phleomycin Resistance. Curr. Genet. *20*, 309-314.

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Molec. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326.

Drocourt, D., Calmels, T. P. G., Reynes, J. P., Baron, M., and Tiraby, G. (1990). Cassettes of the *Streptoalloteichus hindustanus ble* Gene for Transformation of Lower and Higher Eukaryotes to Phleomycin Resistance. Nucleic Acids Res. *18*, 4009.

Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121.

Felgner, P. L., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. Nature *337*, 387-388.

Goldman, L. A., Cutrone, E. C., Kotenko, S. V., Krause, C. D., and Langer, J. A. (1996). Modifications of Vectors pEF-BOS, pcDNA1, and pcDNA3 Result in Improved Convenience and Expression. BioTechniques *21*, 1013-1015.

Goodwin, E. C., and Rottman, F. M. (1992). The 3′-Flanking Sequence of the Bovine Growth Hormone Gene Contains Novel Elements Required for Efficient and Accurate Polyadenylation. J. Biol. Chem. *267*, 16330-16334.

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 Biol. *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.

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.

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

Mizushima, S., and Nagata, S. (1990). pEF-BOS, a Powerful Mammalian Expression Vector. Nucleic Acids Res. *18*, 5322.

Mulsant, P., Tiraby, G., Kallerhoff, J., and Perret, J. (1988). Phleomycin Resistance as a Dominant Selectable Marker in CHO Cells. Somat. Cell Mol. Genet. *14*, 243-252.

continued on next page

References, continued

Perez, P., Tiraby, G., Kallerhoff, J., and Perret, J. (1989). Phleomycin Resistance as a Dominant Selectable Marker for Plant Cell Transformation. Plant Mol. Biol. *13*, 365-373.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

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.

Uetsuki, T., Naito, A., Nagata, S., and Kaziro, Y. (1989). Isolation and Characterization of the Human Chromosomal Gene for Polypeptide Chain Elongation Factor-1α. J. Biol. Chem. *264*, 5791-5798.

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.