pEF4/His A, B, and C

Catalog no. V943-20

Version D
102810
25-0229

Invitrogen™
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General Information

Contents

20 µg each pEF4/His A, B, and C, lyophilized
20 µg pEF4/His/*lac*Z, 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/His A	pEF4/His B	pEF4/His C	pEF4/His/*lacZ*
Avr II	4652 bp, 1122 bp	5775 bp	5773 bp	8837 bp
*Bsu*36 I	No cut	5775 bp	No cut	8600 bp, 237 bp
Hind III	4452 bp, 1322 bp	4453 bp, 1322 bp	4451 bp, 1322 bp	7515 bp, 1322 bp
Mlu I	5774 bp	5775 bp	5773 bp	4852 bp, 2780 bp, 780 bp, 425 bp
Nsi I	5702 bp, 72 bp	5701 bp, 72 bp	5701 bp, 72 bp	8765 bp, 72 bp
Sma I	5578 bp, 196 bp	5579 bp, 196 bp	5577 bp, 196 bp	8641 bp, 196 bp

Methods

Overview

Introduction

pEF4/His A, B, and C are 5.8 kb vectors derived from pEF1/V5-His and designed for high-level expression and purification of recombinant proteins in mammalian hosts. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. The vectors contain the following elements:

- The human elongation factor 1 α subunit promoter provides high-level expression in a wide range of mammalian cells(Kim *et al.*, 1990; Mizushima and Nagata, 1990; Uetsuki *et al.*, 1989).
- Three reading frames to facilitate in-frame cloning with a N-terminal tag encoding the Xpress™ epitope and a polyhistidine metal-binding peptide.
- Zeocin™ resistance gene (*Sh ble*) for selection of stable cell lines.
- 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/His/*lac*Z, is included for use as a positive control for transfection, expression, and purification in the cell line of choice.

Experimental Outline

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

1. Consult the multiple cloning sites described on pages 4-6 to determine which vector (A, B, or C) should be used to clone your gene in frame with the N-terminal Xpress™ epitope and polyhistidine tag.
2. Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants on 50 to 100 µg/ml ampicillin (or 25 µg/ml Zeocin™).
3. Analyze your transformants for the presence of insert by restriction digestion.
4. Select a transformant with the correct restriction pattern and confirm that your gene is in frame with the N-terminal peptide by sequencing.
5. Transfect your construct into the cell line of choice.
6. Test for expression of your recombinant gene by western blot analysis or other functional assay. For antibody to the Xpress™ epitope, see the next page.
7. 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/His. In addition, we have a wide variety of mammalian expression vectors, many of which can be utilized with pEF4/His to express multiple proteins in the same cell.

Antibody for Detection

If you do not have an antibody to your protein, Invitrogen offers the Anti-Xpress™ Antibody (Catalog no. R910-25) to detect your recombinant protein. This antibody detects an 8 amino acid epitope (Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys).

ProBond™ Resin

Ordering information for ProBond™ resin is provided below.

Item	Amount	Catalog no.
ProBond Protein™ Purification System	6 purifications	K850-01
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. For more information on the mammalian expression vectors available, see our Web site (www.invitrogen.com) or call Technical Service (page 19).

Cloning into pEF4/His A, B, and C

Introduction

Diagrams are provided on pages 4-6 to help you ligate your gene of interest in frame with the N-terminal peptide. General considerations for ligation 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, see *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994)(See **References**, page 12).

E. coli Strain

Note that 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 be resistant to Zeocin™.

For the most efficient selection it is highly recommended that you choose an *E. coli* strain that does not contain the full Tn*5* transposon. We recommend that you propagate pEF4/His in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A-deficient (*end*A) such as TOP10F´ (Catalog no. C615-00), DH5αF´, JM109, and INVαF´ (Catalog no. C658-00).

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/His

In order to propagate and maintain the pEF4/His vectors, we recommend that you take each vector and resuspend the lyophilized material in 20 µl sterile water to make a 1 µg/µl stock solution. Store at -20°C.

Use this stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, TOP10F´, DH5α, JM109, or equivalent. Transformants are selected on LB plates containing 50-100 µg/ml ampicillin or Low Salt LB plates containing 25 µg/ml Zeocin™ (see page 18 for recipe).

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

Note

The pEF4/His vectors are fusion vectors. To ensure proper expression of your recombinant protein, you must clone your gene in frame with the ATG at base pairs 1718-1720. This will create a fusion with the N-terminal polyhistidine tag, Xpress™ epitope, and the enterokinase cleavage site . The vector is supplied in three reading frames to facilitate cloning. See pages 5-7 to develop a cloning strategy.

If you wish to clone as close as possible to the enterokinase cleavage site, follow the guidelines below:

- Digest pEF4/His A, B, or C with *Kpn* I
- Create blunt ends with T4 DNA polymerase and dNTPs
- Clone your blunt-ended insert in frame with the lysine codon (AAG) of the enterokinase recognition sequence.

Following enterokinase cleavage, no vector-encoded amino acid residues will be present in your protein.

Multiple Cloning Site of pEF4/His A

Below is the multiple cloning site for pEF4/His A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotide indicates the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **Note that there is a stop codon after the *Xba* I site.** The complete sequence may be downloaded from our web site (www.invitrogen.com) or requested from Technical Service (see page 19).

```
                                                                    3´ end of hEF-1α Intron 1
1581  GTTTGGATCT TGGTTCATTC TCAAGCCTCA GACAGTGGTT CAAAGTTTTT TTCTTCCATT TCAGGTGTCG TGAGGAATTA
                                                                                5´ end of hEF-1α Exon 2

                          T7 promoter/priming site
1661  GCTTGGTACT AATACGACTC ACTATAGGGA GACCCAAGCT GGCTAGTTAA GCTTACC ATG GGG GGT TCT CAT CAT
                                                                      Met Gly Gly Ser His His

      Polyhistidine Region                                                        Xpress™ epitope
1736  CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT
      His His His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp

                  Asp718 I  Kpn I   BamH I          BstX I  EcoR I            EcoR V          BstX I    Not I
1802  GAC GAT AAG GTA CCT AGG ATC CAG TGT GGT GGA ATT CTG CAG ATA TCC AGC ACA GTG GCG GCC GCT
      Asp Asp Lys Val Pro Arg Ile Gln Cys Gly Gly Ile Leu Gln Ile Ser Ser Thr Val Ala Ala Ala
      EK Recognition site
                  EK Cleavage site
          Xba I                 Pme I                          BGH Reverse priming site
1868  CGA GTC TAG AGG GCC CGT TTA AAC CCG CTG ATC AGC CTC GAC TGT GCC TTC TAG TTGCCAGCC
      Arg Val *** Arg Ala Arg Leu Asn Pro Leu Ile Ser Leu Asp Cys Ala Phe ***

                                                                          BGH polyadenylation signal
1931  ATCTGTTGTT TGCCCCTCCC CCGTGCCTTC CTTGACCCTG GAAGGTGCCA CTCCCACTGT CCTTTCCTAA TAAAATGAGG

2011  AAATTGCATC GCATTGTCTG AGTAGGTGTC ATTCTATTCT GGGGGGTGGG GTGGGGCAGG ACAGCAAGGG GGAGGATTGG
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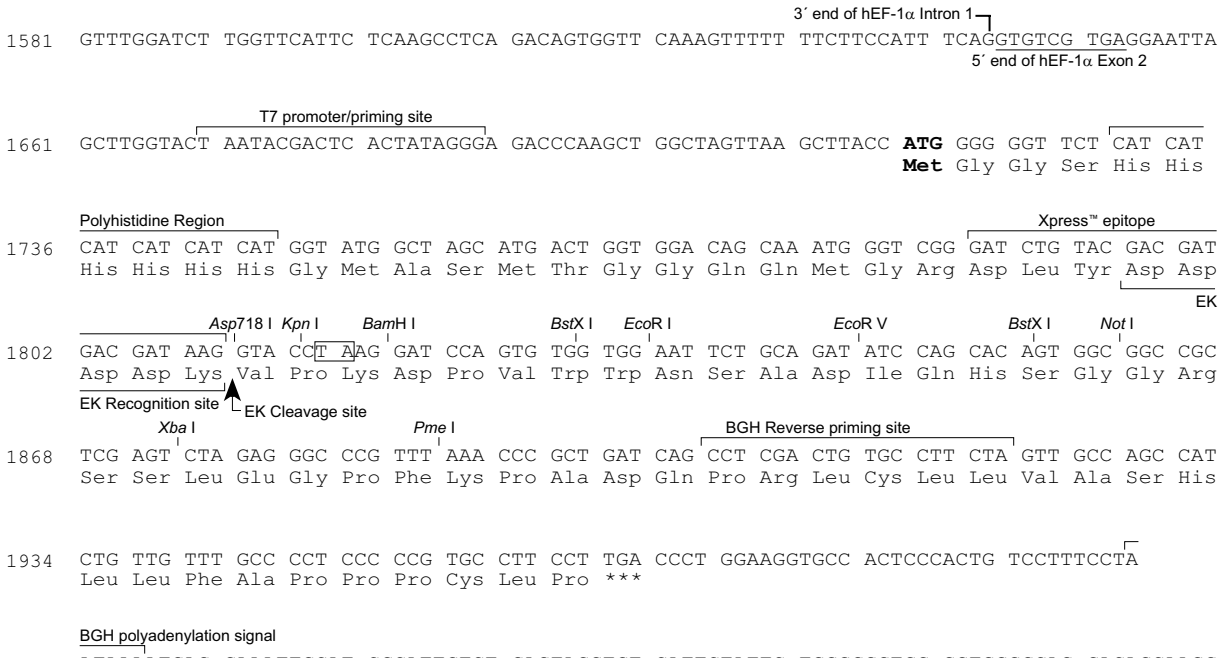
Cloning into pEF4/His A, B, and C, continued

Multiple Cloning Site of pEF4/His B

Below is the multiple cloning site for pEF4/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. The complete sequence may be downloaded from our web site (www.invitrogen.com) or requested from Technical Service (see page 19).




```
                                                                3´ end of hEF-1α Intron 1
1581  GTTTGGATCT TGGTTCATTC TCAAGCCTCA GACAGTGGTT CAAAGTTTTT TTCTTCCATT TCAGGTGTCG TGAGGAATTA
                                                                          5´ end of hEF-1α Exon 2

                    T7 promoter/priming site
1661  GCTTGGTACT AATACGACTC ACTATAGGGA GACCCAAGCT GGCTAGTTAA GCTTACC ATG GGG GGT TCT CAT CAT
                                                                    Met Gly Gly Ser His His

      Polyhistidine Region                                                        Xpress™ epitope
1736  CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT
      His His His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp
                                                                                        EK
                  Asp718 I  Kpn I     BamH I          BstX I  EcoR I          EcoR V          BstX I  Not I
1802  GAC GAT AAG GTA CCT AAG GAT CCA GTG TGG TGG AAT TCT GCA GAT ATC CAG CAC AGT GGC GGC CGC
      Asp Asp Lys Val Pro Lys Asp Pro Val Trp Trp Asn Ser Ala Asp Ile Gln His Ser Gly Gly Arg
      EK Recognition site
                 EK Cleavage site
          Xba I                Pme I                          BGH Reverse priming site
1868  TCG AGT CTA GAG GGC CCG TTT AAA CCC GCT GAT CAG CCT CGA CTG TGC CTT CTA GTT GCC AGC CAT
      Ser Ser Leu Glu Gly Pro Phe Lys Pro Ala Asp Gln Pro Arg Leu Cys Leu Leu Val Ala Ser His

1934  CTG TTG TTT GCC CCT CCC CCG TGC CTT CCT TGA CCCT GGAAGGTGCC ACTCCCACTG TCCTTTCCTA
      Leu Leu Phe Ala Pro Pro Pro Cys Leu Pro ***

      BGH polyadenylation signal
2001  ATAAAATGAG GAAATTGCAT CGCATTGTCT GAGTAGGTGT CATTCTATTC TGGGGGGTGG GGTGGGGCAG GACAGCAAGG

2081  GGGAGGATTG GGAAGACAAT AGCAGGCATG CTGGGGATGC GGTGGGCTCT ATGGCTTCTG AGGCGGAAAG AACCAGCTGG
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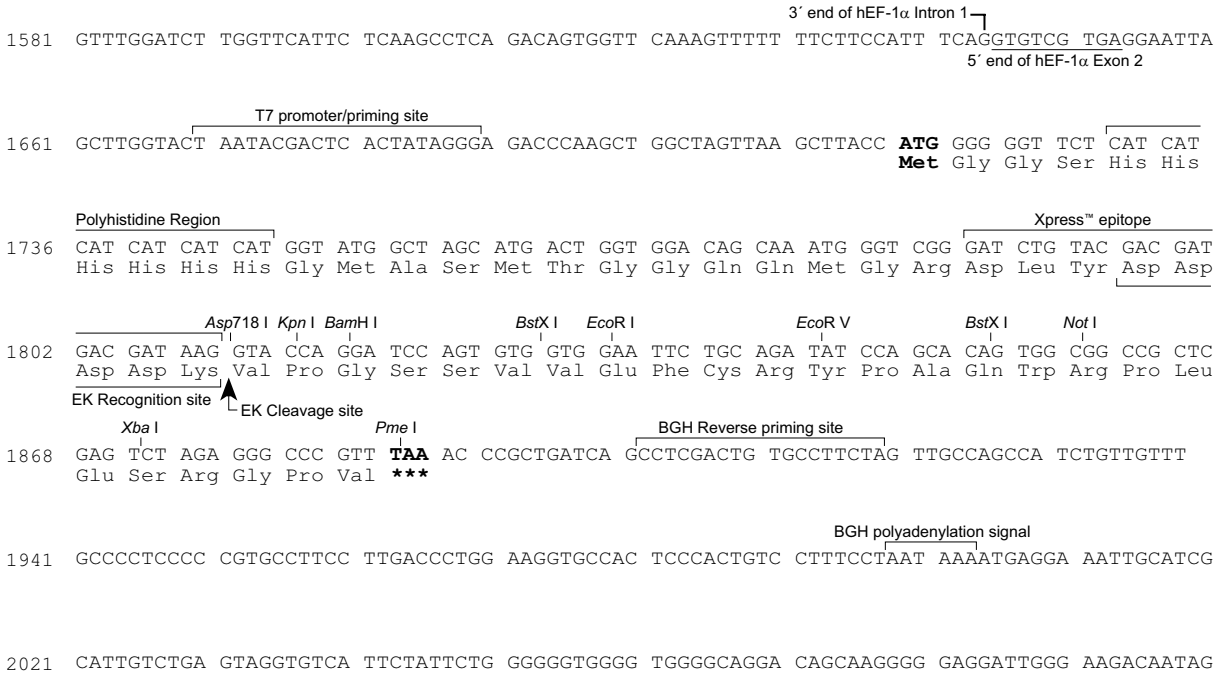
Cloning into pEF4/His A, B, and C, continued

Multiple Cloning Site of pEF4/His C

Below is the multiple cloning site for pEF4/His C. Restriction sites are labeled to indicate the cleavage site. **Note that there is a stop codon within the *Pme* I site.** The multiple cloning site has been confirmed by sequencing and functional testing. The complete sequence may be downloaded from our web site (www.invitrogen.com) or requested from Technical Service (see page 19).



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

Transformation of Ligation Mixtures

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 Low Salt LB plates containing 25 µg/ml Zeocin™ (see page 18 for recipe). Select 10-20 clones and analyze for the presence and orientation of your insert.

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 Xpress™ epitope and the N-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 19).

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 in case the glycerol stock dies.

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

Transfection

Introduction

Once you have confirmed that your construct is in the correct orientation and fused to the N-terminal tag, then 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. It is recommended 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).

Invitrogen offers a wide variety of transfection reagents including the Calcium Phosphate Transfection Kit for mammalian transfection. For more information, call Technical Service (page 19) or visit our Web site at www.invitrogen.com.

Positive Control

pEF4/His/*lac*Z is provided as a positive control vector for mammalian transfection and expression (see page 10). It may be used to optimize transfection conditions for your cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the EF-1α promoter. A successful transfection will result in β-galactosidase expression and can be easily assayed (see next page).

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Transfection, 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, easy detection of β-galactosidase expression.

Detection of Fusion Proteins

The Anti-Xpress™ Antibody is available from Invitrogen and can be used to detect expression of your fusion protein from pEF4/His (see page 2).

To detect the 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 NP-40 Cell Lysis Buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.8, see page 18) or other suitable lysis buffer.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.
5. Centrifuge resulting cell lysate at 10,000 x g for 10 minutes to pellet nuclei and transfer the post-nuclear lysate 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.

Note

The N-terminal peptide containing the Xpress™ epitope and the polyhistidine region will add approximately 3.4 kDa to your protein.

Purification

You will need 5 x 10^6 to 1 x 10^7 of **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 13.

Creation of Stable Cell Lines

Introduction

The pEF4/His vectors contain the Zeocin™ resistance gene (*Sh ble*) to allow for selection of stable cell lines using Zeocin™. It is always important to test the sensitivity of your mammalian host cell to Zeocin™ as natural resistance varies among cell lines.

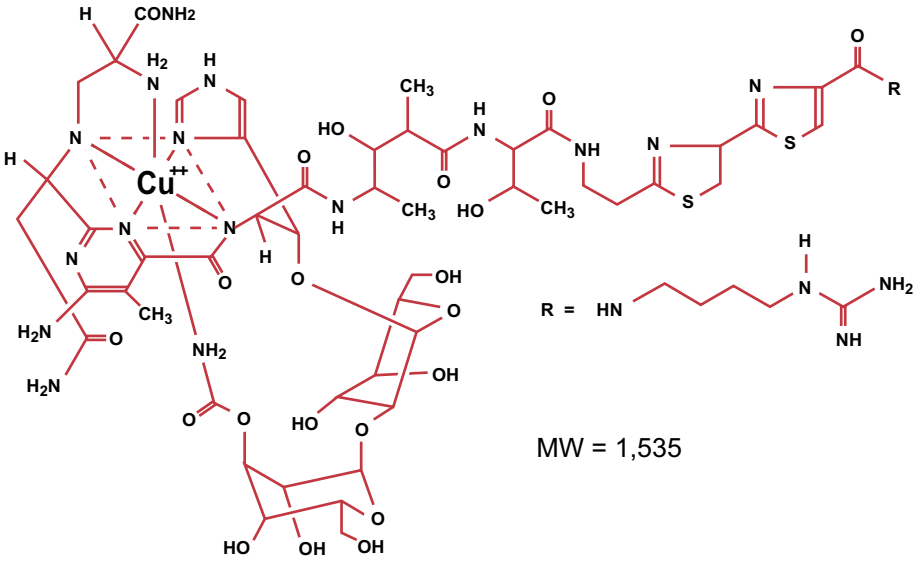
General information and guidelines are provided below for your convenience.

Zeocin™

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong anti-bacterial and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells. Zeocin™ is not as toxic as bleomycin on fungi. As a broad-spectrum antibiotic Zeocin™ is particularly useful, allowing selection in a number of cell types containing vectors with a Zeocin™ resistance gene.

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™.



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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 tissue culture cells and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25-50 µg/ml in **low salt** LB medium*
Mammalian cells	50-1000 µg/ml (depends on cell line)

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

Ordering Information

Additional Zeocin™ can be purchased from Invitrogen. For your convenience, the drug is prepared in autoclaved, deionized water and aliquoted into 1.25 ml aliquots at 100 mg/ml. The stability of Zeocin™ is guaranteed for six months, if stored at -20°C. It can be frozen and thawed several times without losing activity.

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

Handling Zeocin™

Always wear gloves, mask, goggles, and protective clothing (e.g. a laboratory coat) when handling Zeocin™. Use Zeocin™ solutions in a hood.

Effect of Zeocin™ on Sensitive and Resistant Cells

Zeocin™'s method of killing is quite different from neomycin and hygromycin. Cells do not necessarily round up and detach from the plate. Sensitive cells will exhibit the following changes in morphology 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 breakdown so that only "strings" of protein are left.

In contrast, Zeocin™ resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct changes in morphology, when compared to cells not under selection with Zeocin™.

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Creation of Stable Cell Lines, continued

Determination of Zeocin™ Sensitivity

To obtain a stable integrant, you must first determine if the cell line in question can grow as an isolated colony. You may already know this for your cell line. If you do not, seed ~100 cells in a 60 mm plate and feed every 4 days for 10-12 days. Count the number of colonies. Growing in soft agar can help cells to grow when they are diluted; however, some cell lines (e.g. NIH3T3) require plating at a certain density in order to grow properly (see Ausubel, *et al.*, 1990).

Next, determine the minimal concentration of Zeocin™ required to prevent growth of the parental cell line using the protocol below:

1. Plate or split a confluent plate so there are approximately 2.5 x 10^5 cells per 60-100 mm dish. Prepare 7 plates and add varying concentrations of Zeocin™ (0, 50, 125, 250, 500, 750, and 1000 µg/ml) to each plate.
2. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
3. Count the number of viable cells at regular intervals to determine the appropriate concentration of Zeocin™ that prevents growth.

Possible Linearization Sites

To obtain stable transfectants, you may choose to linearize your vector before transfection. While linearizing your vector may not improve your chances of obtaining stable transfectants, 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. For the complete sequence and a restriction list of any of the pEF4/His vectors, refer to our web site (www.invitrogen.com) or contact Technical Service.**

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 promoter	Invitrogen (Catalog no. 15458-011)
Mlu I	351	Upstream of promoter	Invitrogen (Catalog no. 15432-016)
*Bst*1107 I	3702, 3703, 3701	End of SV40 pA	AGS*, Fermentas, Takara
Sap I	3965, 3966, 3964	Backbone	New England Biolabs
*Eam*1105 I	4974, 4975, 4973	Ampicillin gene	AGS*, Fermentas, Takara
Fsp I	5196, 5197, 5195	Ampicillin gene	Many
Sca I	5498, 5502, 5494	Ampicillin gene	Invitrogen (Catalog no. 15436-017)
Pvu I	5344, 5345, 5343	Ampicillin gene	Invitrogen (Catalog no. 25420-068)
Sca I	5454, 5455, 5453	Ampicillin gene	Invitrogen (Catalog no. 15436-017)

*Angewandte Gentechnologie Systeme

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Creation of Stable Cell Lines, continued

Selection of Stable Integrants

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

1. Transfect cells with your construct using the desired protocol and plate at the appropriate cell number or density. Remember to include a plate of untransformed cells as a negative control.
2. 24 hours after transfection, wash the cells and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing Zeocin™ at the pre-determined concentration required for your cell line. Split the cells such that the cells are no more than 25% confluent.
4. Feed the cells with selective medium every 3-4 days until foci can be identified. **Note**: The morphology of dead of dying cells is different with Zeocin™ than with neomycin, hygromycin, or blasticidin (see page 11).
5. Pick and expand the foci to test for expression of your recombinant protein.

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 ProBond™ Protein Purification manual).

1. Seed cells (from a stable cell line) 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. 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.

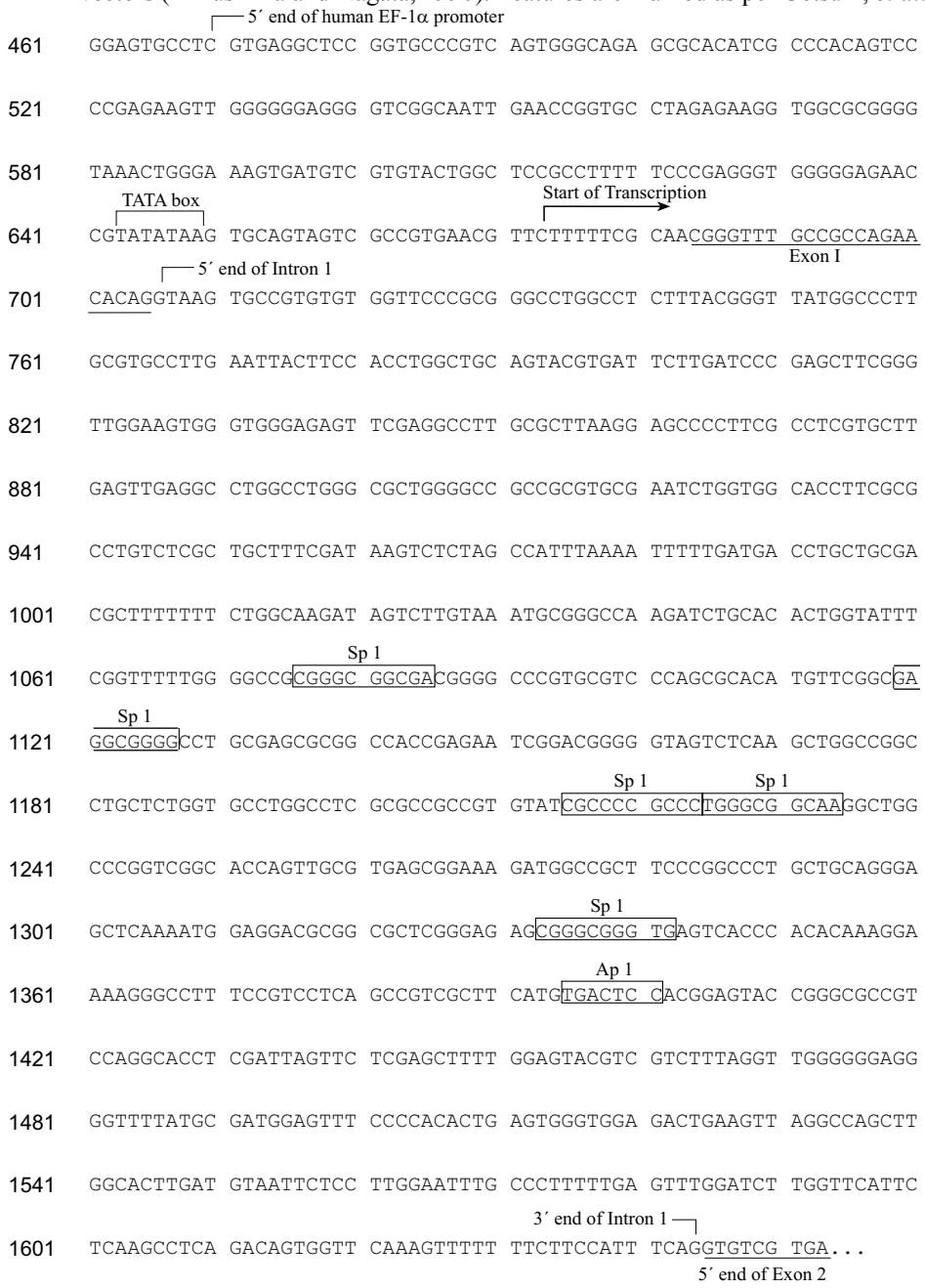
If you are using other metal-chelating resin, refer to the manufacturer's instruction for recommendations on sample preparation.

Appendix

Human EF-1α Promoter

Description

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



pEF4/His A, B, and C Vectors

Features of pEF4/His

pEF4/His A (5774 bp), pEF4/His B (5775 bp), and pEF4/His C (5773 bp) contain the following elements. All features have been functionally tested.

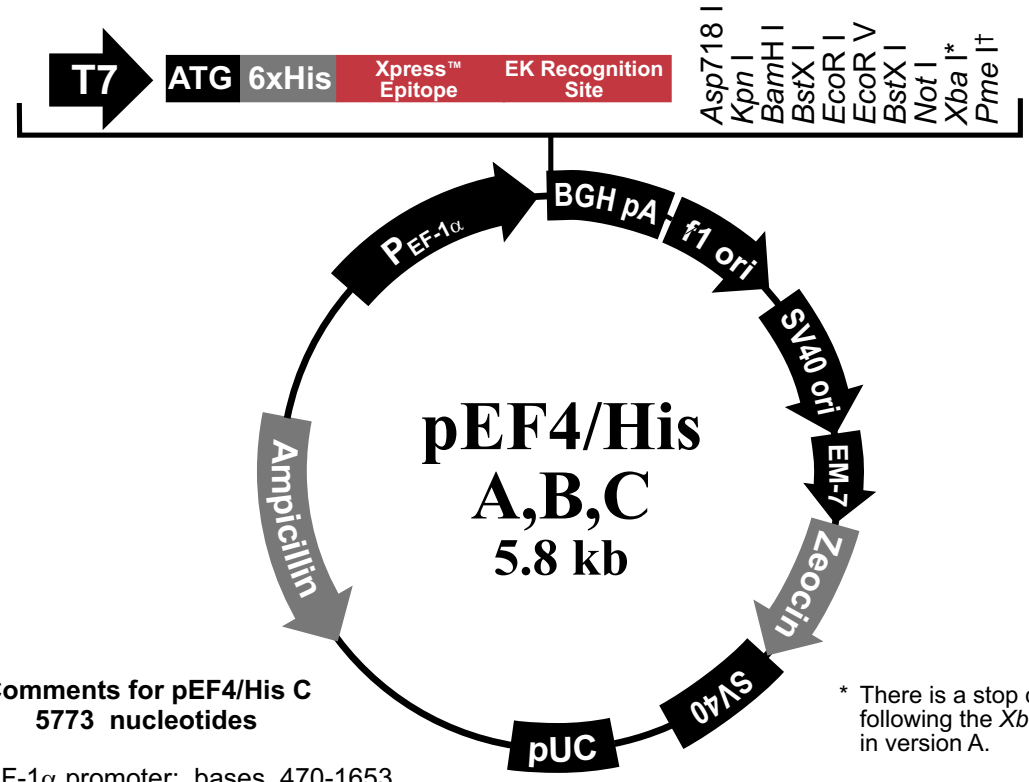
Feature	Benefit
Human elongation factor 1α (hEF-1α) promoter	Permits overexpression of your recombinant protein in a broad range of mammalian cell types (Goldman *et al.*, 1996; Mizushima and Nagata, 1990)
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
N-terminal polyhistidine tag	Permits purification of your recombinant protein on metal-chelating resin such as ProBond™
Xpress™ epitope tag	Allows detection of your recombinant protein with the Anti-Xpress™ Antibody (Catalog no. R910-25)
Enterokinase cleavage site	Allows removal of the N-terminal polyhistidine tag from your recombinant protein using an enterokinase such as EnterokinaseMax™ (Catalog no E180-01)
Multiple cloning site in three reading frames	Allows insertion of your gene and facilitates cloning in frame with the polyhistidine N-terminal tag
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 neomycin resistance gene and episomal replication in cells expressing SV40 large T antigen
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 vector in *E. coli*

continued on next page

pEF4/His A, B, and C Vectors, continued

Map of pEF4/His

The figure below summarizes the features of the pEF4/His vectors. The sequences for pEF4/His A, B, and C are available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 19).



Comments for pEF4/His C
5773 nucleotides

EF-1α promoter: bases 470-1653
T7 promoter/priming site: bases 1670-1689
ATG initiation codon: bases 1718-1720
Polyhistidine region: bases 1730-1747
Xpress™ epitope: bases 1787-1810
Enterokinase recognition site: bases 1797-1810
Multiple cloning site: bases 1810-1890
BGH reverse priming site: bases 1902-1919
BGH polyadenylation sequence: bases 1905-2132
f1 origin: bases 2178-2606
SV40 promoter and origin: bases 2660-2942
EM-7 promoter: bases 2990-3045
Zeocin resistance gene (ORF): bases 3064-3438
SV40 polyadenylation sequence: bases 3568-3698
pUC origin: bases 4081-4754
Ampicillin resistance gene (ORF): bases 4899-5759

* There is a stop codon following the *Xba* I site in version A.

† There is a stop codon in the *Pme* I site in version C.

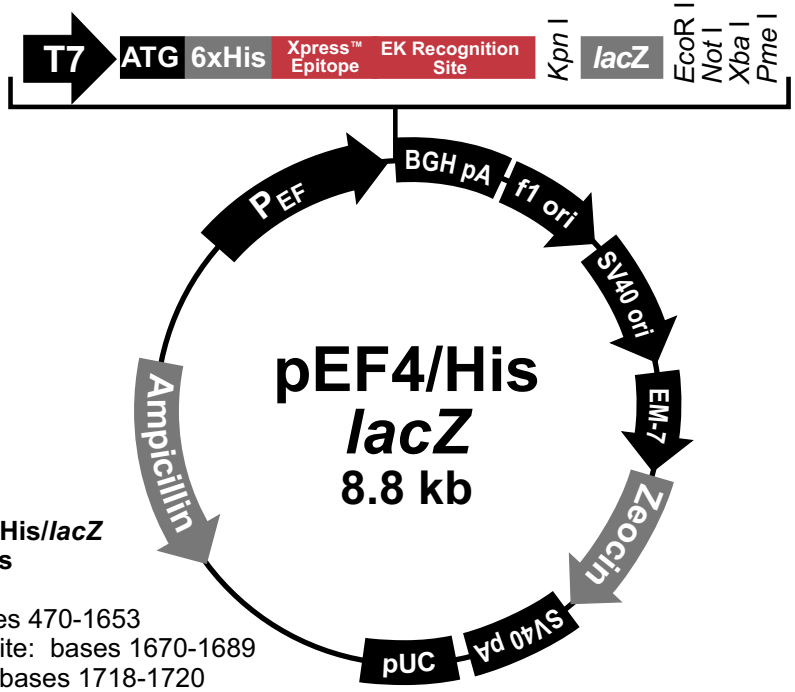
pEF4/His/*lacZ*

Description

pEF4/His/*lacZ* is a 8837 bp control vector containing the gene for β-galactosidase. pEF4/His B was digested with *Kpn* I and *Eco*R I. A 3.2 kb *Kpn* I-*Eco*R I fragment containing the β-galactosidase gene was then ligated into pEF4/His B in frame with the N-terminal peptide.

Map of Control Vector

The figure below summarizes the features of the pEF4/His/*lacZ* vector. The complete nucleotide sequence for pEF4/His/*lacZ* is available by downloading it from our Web site (www.invitrogen.com) or by contacting Technical Service. See page 19 for more information.



Comments for pEF4/His/*lacZ*
8837 nucleotides

EF-1α promoter: bases 470-1653
T7 promoter/priming site: bases 1670-1689
ATG initiation codon: bases 1718-1720
Polyhistidine region: bases 1730-1747
Xpress™ epitope: bases 1787-1810
Enterokinase recognition site: bases 1797-1810
LacZ ORF: bases 1839-4888
BGH reverse priming site: bases 4966-4983
BGH polyadenylation sequence: bases 4969-5196
f1 origin: bases 5242-5670
SV40 promoter and origin: bases 5724-6006
EM-7 promoter: bases 6054-6109
Zeocin resistance gene (ORF): bases 6128-6502
SV40 polyadenylation sequence: bases 6632-6762
pUC origin: bases 7145-7818
Ampicillin resistance gene (ORF): bases 7963-8823

Recipes

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. You must 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 due to 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 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 µg/ml final concentration.
4. Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

Note: Pre-mixed Low Salt LB Medium is available from Invitrogen in convenient pouches or in bulk. Contact Technical Service for more information (page 11).

Cell Lysis Buffer

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

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

Technical Service

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- 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):

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...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!

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Technical Service, continued

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