invitrogen™

pEF1/*myc*-His A, B, and C

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User Manual

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Kit Contents and Storage

Shipping and Storage

pEF1/*myc*-His vectors are shipped on wet ice. Upon receipt, store vectors at -20°C.

Kit Contents

All vectors are supplied as detailed below. **Store the vectors at –20°C.**

Vector	Composition	Amount
pEF1/*myc*-His A, B, and C	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg
pEF1/*myc*-His/*lacZ*	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg

Intended Use

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

Introduction

Product Overview

Description of the System

pEF1/*myc*-His A, B, and C are 6.2 kb vectors derived from pcDNA™3.1/*myc*-His and designed for overproduction of recombinant proteins in mammalian cell lines. Features of the vectors allow purification and detection of expressed proteins (see pages 11–12 for more information). High-level stable and transient expression can be carried out in most mammalian cells. The vectors contain the following elements:

- Human elongation factor 1α-subunit (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 10 for more information).
- Three reading frames to facilitate in-frame cloning with a C-terminal tag encoding the *myc* (*c-myc*) epitope and a polyhistidine metal-binding peptide.
- Neomycin resistance gene 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, pEF1/*myc*-His/*lacZ*, is included for use as a positive control for transfection, expression, and detection in the cell line of choice.

Experimental Outline

Use the following outline to clone and express your gene of interest using pEF1/*myc*-His.

1. Consult the multiple cloning sites described on pages 3–5 to determine which vector (A, B, or C) should be used to clone your gene in-frame with the C-terminal *myc* epitope and the polyhistidine tag.
2. Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants on 50 to 100 µg/mL ampicillin.
3. Analyze your transformants for the presence of insert by restriction digestion.
4. Select a transformant with the correct restriction pattern and use sequencing to confirm that your gene is cloned in-frame with the C-terminal peptide.
5. Transfect your construct into the cell line of choice using your own method of transfection.
6. Test for expression of your recombinant gene by western blot analysis or functional assay (for antibodies to the *myc* epitope or the polyhistidine, C-terminal tag, see page 15).
7. To purify your recombinant protein, you may use metal-chelating resin such as ProBond™. ProBond™ resin is available separately (see page 15 for ordering information).

Methods

Cloning into pEF1/*myc*-His A, B, and C

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).

E. coli Host

Many *E. coli* strains are suitable for the growth of this vector including TOP10F´, DH5α, JM109 and INVαF´. 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.

Transformation Method

You may use any method of 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.

Maintaining pEF1/*myc*-His

To propagate and maintain the pEF1/*myc*-His vectors, use a small amount of the supplied 0.5 µg/µL stock solution in TE, pH 8.0 to transform a *rec*A, *end*A *E. coli* strain like TOP10F´, DH5α, JM109, or equivalent. Select transformants on LB plates containing 50–100 µg/mL ampicillin. Be sure to prepare a glycerol stock of each plasmid for long term storage (see page 5)

Cloning Considerations

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; 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 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

(**G/A**)NN<u>ATG</u>**G**

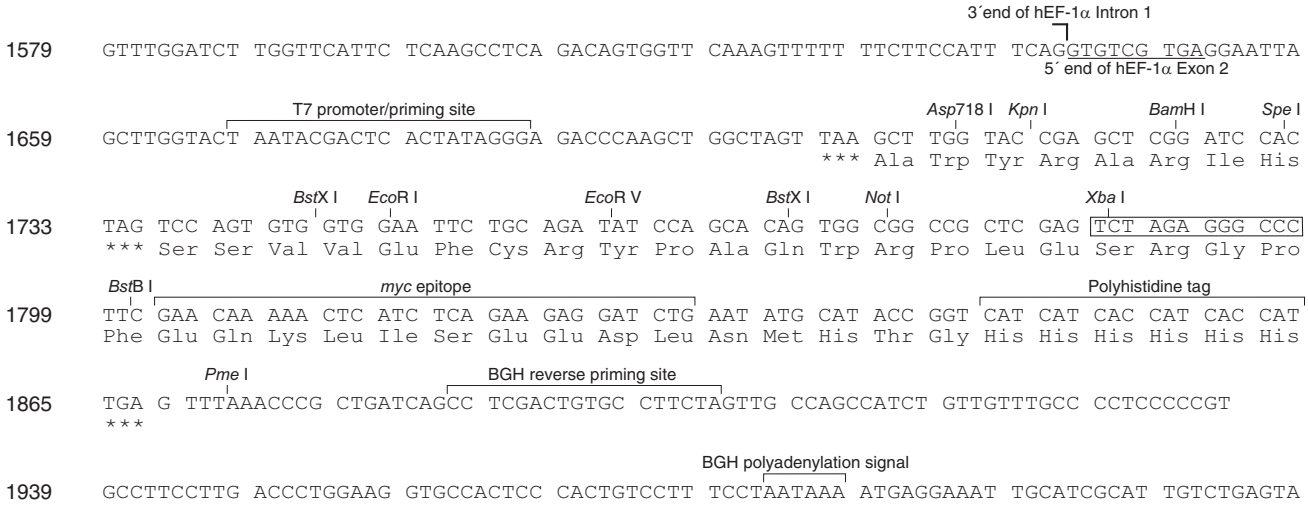
If you wish to express your protein WITHOUT the C-terminal peptide, be sure to include a stop codon.

Continued on next page

Cloning into pEF1/*myc*-His A, B, and C, Continued

Multiple Cloning Site of Version A

Below is the multiple cloning site for pEF1/*myc*-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 *BstX* I site.** The multiple cloning site has been confirmed by sequencing and functional testing. For more information on the hEF-1α promoter, see page 10. The vector sequence of pEF1/*myc*-His A is available for downloading from www.invitrogen.com or from **Technical Support** (see page 16).



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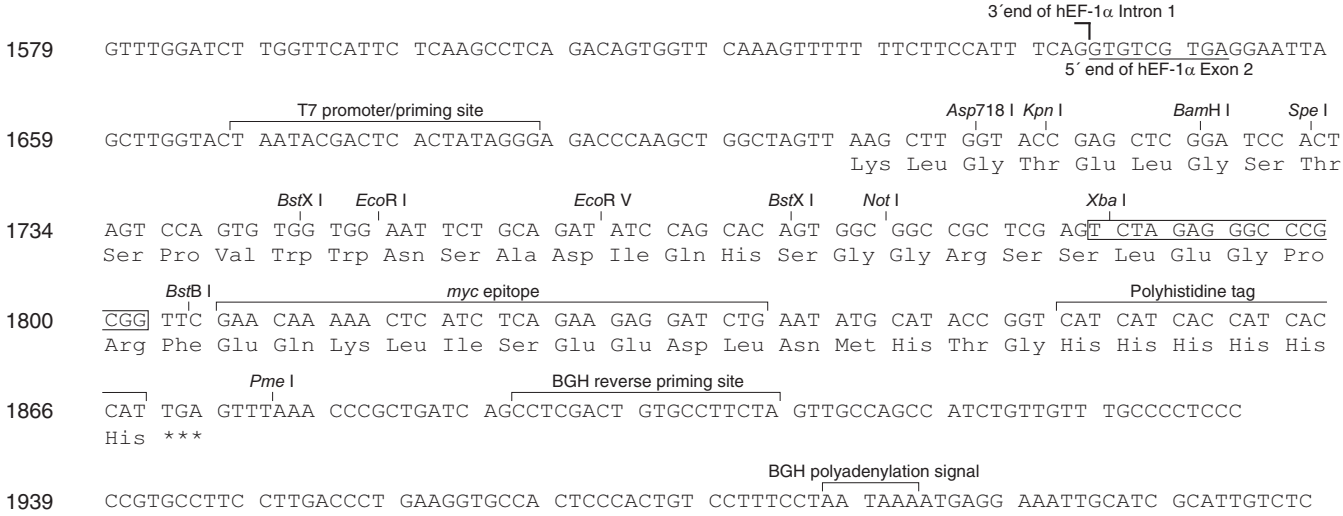
Cloning into pEF1/*myc*-His A, B, and C, Continued

Multiple Cloning Site of Version B

Below is the multiple cloning site for pEF1/*myc*-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 10. The vector sequence of pEF1/*myc*-His B is available for downloading from www.invitrogen.com or from **Technical Support** (see page 16).



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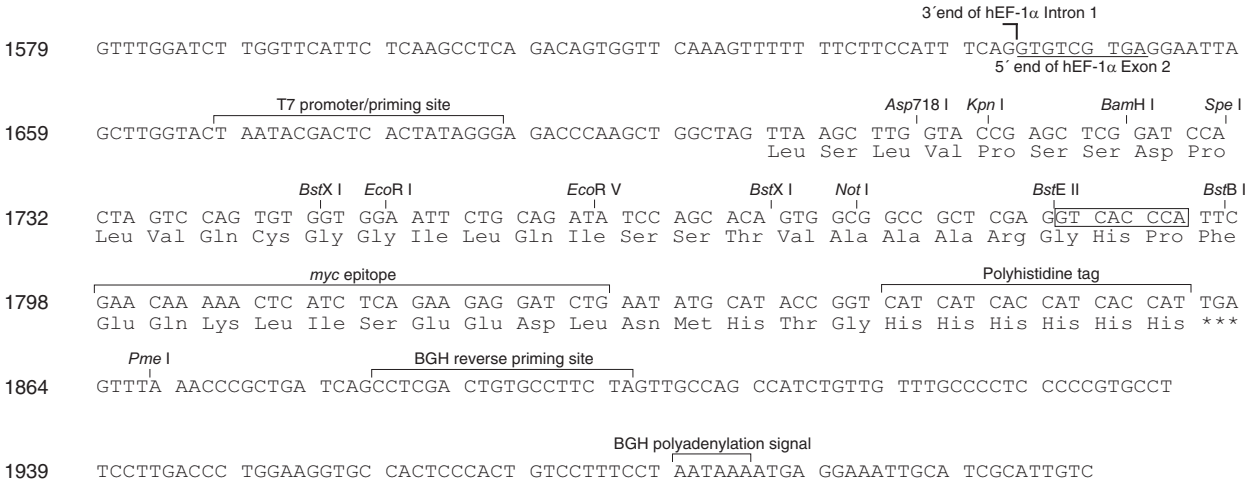
Cloning into pEF1/*myc*-His A, B, and C, Continued

Multiple Cloning Site of Version C

Below is the multiple cloning site for pEF1/*myc*-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 10. The vector sequence of pEF1/*myc*-His C is available for downloading from www.invitrogen.com or from **Technical Support** (see page 16).



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. 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 Primer and BGH Reverse primer sequences to confirm that your gene is fused in frame with the *myc* 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 ordering primers, see page 15.

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. Maintain DNA stock of your plasmid at –20°C.

1. Streak the original colony out on an LB plate containing 50 µg/mL ampicillin. 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.
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 and Analysis

Introduction

Once you have confirmed that your construct is in the correct orientation and fused in frame with 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 PureLink™ HiPure Miniprep Kit or the PureLink™ HiPure Midiprep Kit (see page 15 for ordering information).

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*.

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 Lipofectamine™ 2000 Reagent for mammalian transfection For details, contact **Technical Support** (see page 16) or visit www.invitrogen.com.

Positive Control

pEF1/*myc*-His/*lac*Z is provided as a positive control vector for mammalian transfection and expression (see page 13), 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 below).

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 and the β-Gal Staining Kit for fast and easy detection of β-galactosidase expression (see page 15).

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Detecting Fusion Proteins

Several antibodies are available from Invitrogen to detect expression of your fusion protein from pEF1/*myc*-His (see page 15).

To detect fusion protein by western blot, you 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 1,500 × *g* for 5 minutes.
3. Resuspend in 50 µL Cell Lysis Buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.8, see Recipe on page 14).
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.
5. Centrifuge the cell lysate at 10,000 × *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 C-terminal peptide containing the *myc* epitope and the polyhistidine region will add approximately 3 kDa to your protein.

Purification

You will need 5 × 10^6 to 1 × 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 9.

Creating Stable Cell Lines

Introduction

The pEF1/*myc*-His vectors contain the neomycin resistance gene to allow for selection of stable cell lines using Geneticin®. General information and guidelines are provided below for your convenience.

Geneticin® Activity

Geneticin® blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression of the bacterial aminoglycoside phosphotransferase gene (APH), derived from Tn*5*, in mammalian cells results in detoxification of Geneticin® (Southern and Berg, 1982).

Geneticin® Selection Guidelines

Geneticin® is available from Invitrogen (see page 15). Use as follows:

- Prepare Geneticin® in a buffered solution (e.g., 100 mM HEPES, pH 7.3). Filter-sterilize the solution.
- Freeze at –20°C for long-term storage or store at 4°C for short-term storage.
- Use 100 to 1,000 µg/mL of Geneticin® in complete medium.
- Test varying concentrations of Geneticin® on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to Geneticin®. Typically, concentrations between 100 and 1,000 µg/mL Geneticin® are sufficient to kill the untransfected host cell line.

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 ensures 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.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector. **Note that the cleavage site is indicated for versions A, B, and C of pEF1/*myc*-His.**

Enzyme	Restriction Site (bp) (A,B,C)	Location	Supplier
Ssp I	4	Upstream of EF-1α promoter	Many
Aat II	122	Upstream of EF-1α promoter	Many
Mlu I	351	Upstream of EF-1α promoter	Many
*Eam*1105 I	5379 (A), 5383 (B), 5375 (C)	Ampicillin gene	AGS*, Fermentas, Takara
Pvu I	5749 (A), 5753 (B), 5745 (C)	Ampicillin gene	Many
Sca I	5859 (A), 5863 (B), 5855 (C)	Ampicillin gene	Many

*Angewandte Gentechnologie Systeme

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Selecting Stable Integrants

Cells divide once or twice in the presence of lethal doses of Geneticin®, so the effects of the drug take several days to become apparent. Geneticin® resistant colonies can generally be identified in 7 to 10 days with complete selection and expansion in 2 to 4 weeks.

1. Transfect your cells using the appropriate protocol for your cell line. Include a sample of untransfected cells as a negative control.
2. After transfection, wash the cells once with 1X PBS and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing Geneticin® at the appropriate concentration for your cell line. Split the cells such that they are no more than 25% confluent.
4. Change selective medium every 3–4 days until neomycin-resistant colonies are detected (7 to 10 days).
5. Pick and expand colonies (1–2 weeks).

Preparing Cells for Lysis

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

1. Seed cells in 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 $240 \times g$ for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at –80°C until needed.

Lysis of Cells

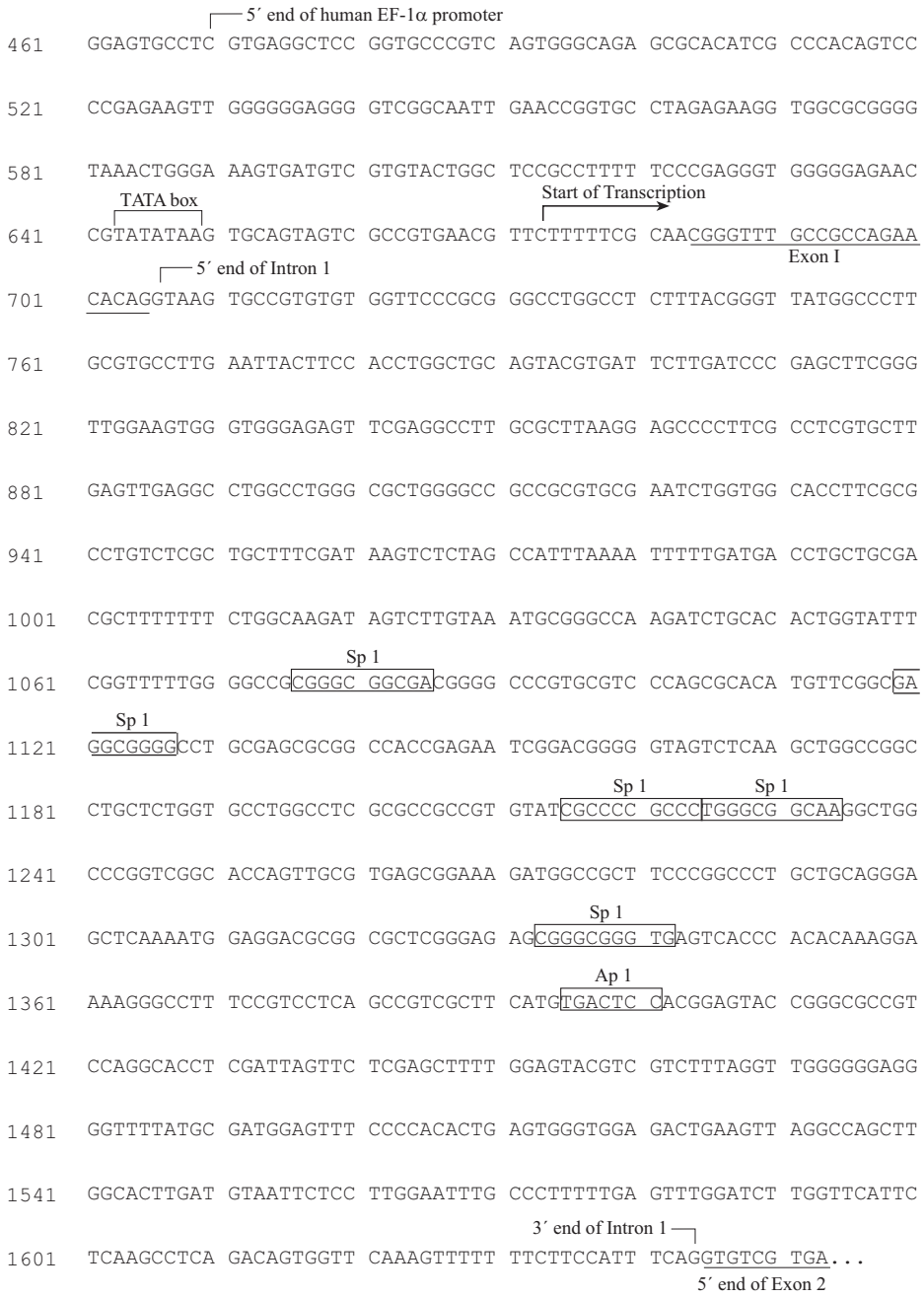
If you are using ProBond™ resin, refer to the ProBond™ Purification manual for details about sample preparation for chromatography. If you are using another metal-chelating resin, refer to the manufacturer's instruction for recommendations on sample preparation.

Appendix

Human EF-1α Promoter

Description

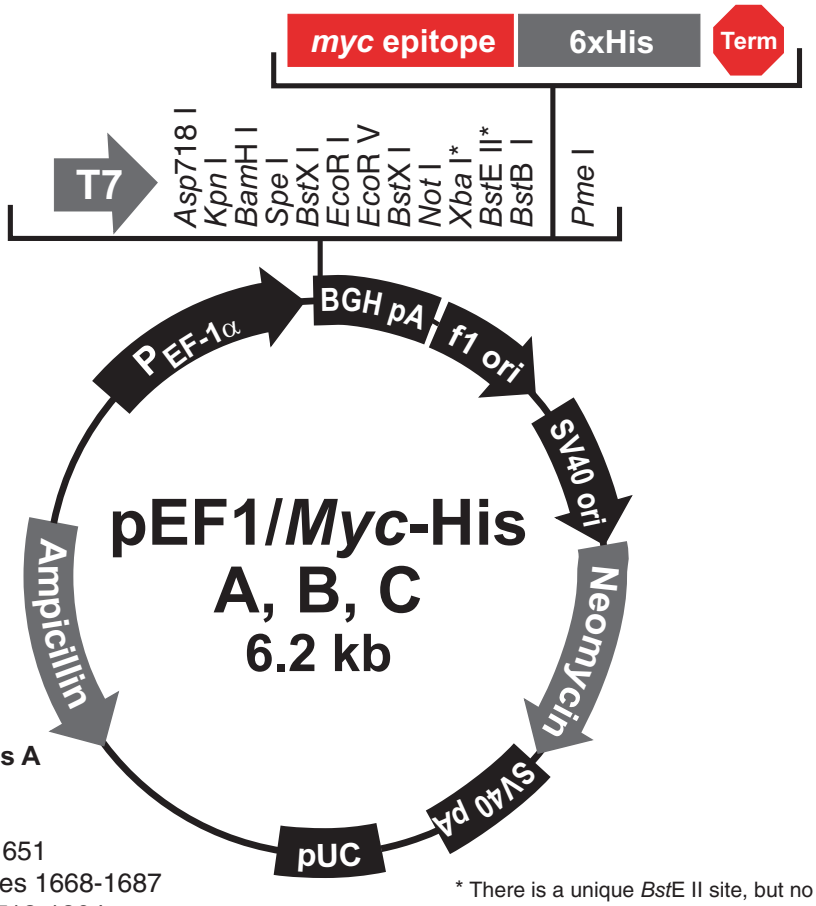
The diagram below shows all the features of the EF-1α promoter used in pEF1/*myc*-His vectors (Mizushima and Nagata, 1990). Features are marked as per Uetsuki *et al.*, 1989.



pEF1/*myc*-His Vector

Map of pEF1/*myc*-His

The figure below summarizes the features of the pEF1/*myc*-His vectors. The sequences for pEF1/*myc*-His A, B, and C are available for downloading from www.invitrogen.com or from **Technical Support** (see page 16).



* There is a unique *Bst*E II site, but no *Xba* I site in version C.

Comments for pEF1/*Myc*-His A
6165 nucleotides

EF-1α promoter: bases 473-1651
T7 promoter/priming site: bases 1668-1687
Multiple cloning site: bases 1713-1804
myc epitope: bases 1802-1831
Polyhistidine tag: bases 1847-1864
BGH reverse priming site: bases 1887-1904
BGH polyadenylation signal: bases 1890-2179
f1 origin: bases 2163-2591
SV40 promoter and origin: bases 2618-2926
Neomycin resistance gene: bases 3001-3795
SV40 polyadenylation signal: bases 3970-4100
pUC origin: bases 4484-5146
Ampicillin resistance gene: 5291-6151

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Features of pEF1/*myc*-His

pEF1/*myc*-His A (6165 bp), pEF1/*myc*-His B (6169 bp), and pEF1/ *myc*-His C (6161 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 *myc* epitope and polyhistidine C-terminal tag.
myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of your recombinant protein with the Anti-*myc* Antibody or Anti-*myc*-HRP Antibody (Evan *et al.*, 1985) (see page 15 for ordering).
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 (Lindner *et al.*, 1997) and the Anti-His (C-term)-HRP Antibody (see page 15 for ordering).
BGH reverse priming site	Permits sequencing through the insert.
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 the SV40 large T antigen.
Neomycin (Geneticin®) resistance gene	Selection of stable transfectants in mammalian cells (Southern and Berg, 1982).
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.*

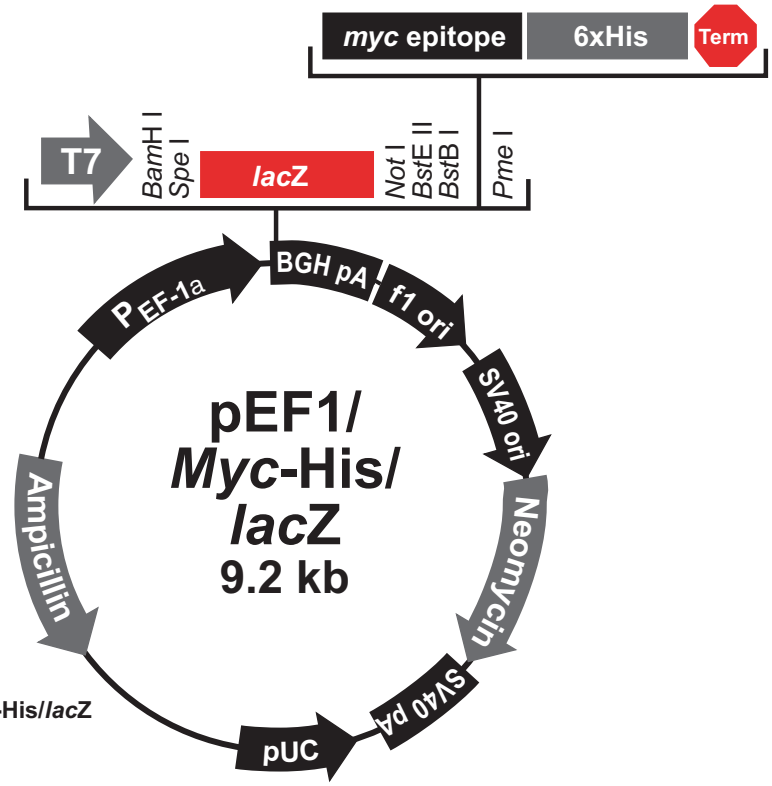
pEF1/*myc*-His/*lacZ*

Description

pEF1/*myc*-His/*lacZ* is a 9,226 bp control vector containing the gene for β-galactosidase. This vector was constructed by ligating a 3,852 bp *Bam*H I-*Bsm* I fragment containing the EF-1α promoter from pEF1/V5-His to a 5,374 bp *Bam*H I-*Bsm* I fragment containing the *lacZ* gene, *myc* epitope, polyhistidine tag, and neomycin resistance gene from pcDNA™3.1/*myc*-His/*lacZ*.

Map of Control Vector

The figure below summarizes the features of the pEF1/*myc*-His/*lacZ* vector. The nucleotide sequence for pEF1/*myc*-His/*lacZ* is available for downloading from www.invitrogen.com or by contacting **Technical Support** (see page 16).



Comments for pEF1/*Myc*-His/*lacZ*
9226 nucleotides

EF-1α promoter: bases 468-1653
T7 promoter/priming site: bases 1670-1689
*Lac*Z gene: bases 1770-4826
myc epitope: bases 4851-4880
Polyhistidine tag: bases 4896-4913
BGH reverse priming site: bases 4936-5403
BGH polyadenylation signal: bases 4939-5166
f1 origin: bases 5212-5640
SV40 promoter and origin: bases 5689-5976
Neomycin resistance gene: bases 6051-6845
SV40 polyadenylation signal: bases 7021-7151
pUC origin: bases 7534-8207
Ampicillin resistance gene: 8352-9212

Recipe

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

Accessory Products

Introduction

The following products may be used with the pEF1/*myc*-His vectors. For details, visit www.invitrogen.com or contact **Technical Support** (see page 16).

Item	Amount	Catalog no.
ProBond™ Purification System	6 × 2 mL precharged, prepacked ProBond™ resin columns and buffers for native and denaturing purification	K850-01
ProBond™ Resin	50 mL	R801-01
	150 mL	R801-15
Electrocomp™ TOP10F´	5 × 80 µL	C665-55
One Shot® TOP10F´ Chemically Competent *E. coli*	20 × 50 µL	C3030-03
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
β-Gal Assay Kit	80 mL	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
Geneticin®	1 gram	11811-023
	5 grams	11811-031
	25 grams	11811-098
Lipofectamine™ 2000 Reagent	0.75 mL	11668-027

Primers

For your convenience, Invitrogen offers a custom primer synthesis service. Visit www.invitrogen.com for more details.

Antibodies

If you do not have an antibody specific to your protein, Invitrogen offers the Anti-*myc*, or Anti-His(C-term) antibodies to detect your recombinant fusion protein. Horseradish peroxidase (HRP)- and alkaline phosphatase (AP)–conjugated antibodies are available for convenient one-step detection.

Antibody	Epitope	Catalog no.
Anti-*myc*	Detects a 10 amino acid epitope derived from *c-myc* (Evan *et al.*, 1985): EQKLISEEDL	R950-25
Anti-*myc*-HRP		R951-25
Anti-*myc*-AP		R952-25
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		R931-25
Anti-His(C-term)-AP		R932-25

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDS, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

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

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SDS

Safety Data Sheets (SDSs) are available on our website at www.invitrogen.com/sds.

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References

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