invitrogen™

pBudCE4.1

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User Manual

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Kit Contents and Storage

Shipping and Storage

pBudCE4.1 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
pBudCE4.1	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg
pBudCE4.1/*lacZ*/CAT	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg

Introduction

Product Overview

pBudCE4.1

pBudCE4.1 is a 4.6 kb vector designed for simultaneous expression of two genes in mammalian cell lines. The vector contains the human cytomegalovirus (CMV) immediate-early promoter and the human elongation factor 1α-subunit (EF-1α) promoter for high-level, constitutive, independent expression of two recombinant proteins (see page 13 for more information on the EF-1α promoter). Features of the vector allow detection and purification of expressed proteins (see pages 15–16) for more information). High-level stable and transient expression studies can be carried out in most mammalian cell types. In addition to the two promoters, the vector contains the following elements:

- C-terminal peptides encoding the *myc* (*c-myc*) epitope or the V5 epitope and a polyhistidine (6xHis) metal-binding tag for detection and purification of recombinant proteins
- Zeocin™ resistance gene for selection in *E. coli* and creation of stable, mammalian cell lines (Mulsant et al., 1988) (see pages 18–19 for more information)
- SV40 origin for episomal replication in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g., COS7)

pBudCE4.1/*lac*Z/CAT is included for use as a positive control for transfection, expression, and detection in the cell line of choice.

Note

pBudCE4.1 is an improved version of pBudCE4. During construction of the original vector, an ATG was inadvertently created in the multiple cloning site (672–674 bp) 3′ to the CMV promoter. Since it may interfere with proper translation of the cloned gene, this ATG was changed to ATT to create pBudCE4.1.

Continued on next page

Experimental Outline

Experimental Outline

Use the following outline to clone and express your genes of interest in pBudCE4.1.

Step	Action	Page
1	Determine a cloning strategy.	3–5
2	Ligate your inserts into the vector and transform into *E. coli*. Select transformants on Low Salt LB containing 25–50 µg/mL Zeocin™.	6, 20
3	Analyze your transformants for the presence of both inserts by restriction digestion.	6
4	Select a transformant with the correct restriction pattern and sequence to confirm that both genes are cloned in frame with the C-terminal peptide (if desired).	6
5	Transfect your construct into the cell line of choice.	7
6	Test for expression of your recombinant proteins by western blot analysis or functional assay. For antibodies to the *myc* epitope, the V5 epitope, or the C-terminal polyhistidine tag, see page 22.	8–9
7	Purify your recombinant proteins using a metal-chelating resin such as ProBond™ (see page 21 for ordering information).	9
8	Generate a stable cell line, if desired.	10–11

Methods

Cloning into pBudCE4.1

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 Strain

Most *E. coli* strains are suitable for the growth of this vector including TOP10 and DH5α™ T1^R. We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A). See below for an important note about *E. coli* strains.

For your convenience, TOP10 and DH5α™ T1^R are available from Invitrogen as chemically competent or electrocompetent cells (TOP10 only) in One Shot® format (see page 21).

Important

Any *E. coli* strain that contains the complete Tn*5* transposable element (i.e. DH5 F´IQ, SURE, SURE2) encodes the *ble* (bleomycin resistance gene). These strains will confer resistance to Zeocin™. We recommend that you choose an *E. coli* strain that does not contain the Tn*5* gene (i.e. TOP10).

Transformation Method

You may use your 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 pBudCE4.1

To propagate and maintain the pBudCE4.1 vector, 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 TOP10 or equivalent. Select transformants on Low Salt LB plates containing 25–50 µg/mL Zeocin™ (see page 20). Be sure to prepare a glycerol stock of each plasmid for long-term storage (see page 6).

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**

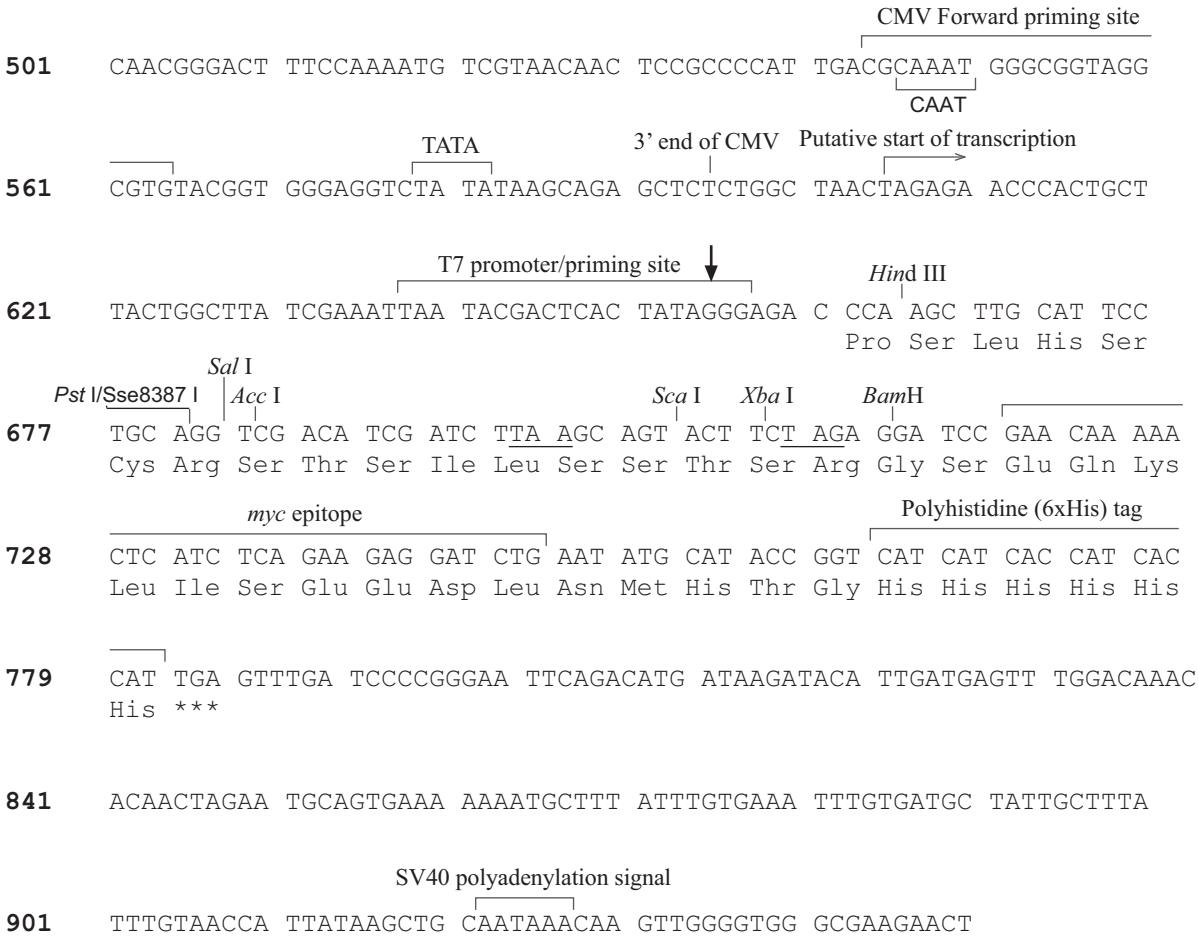
If you wish to express your protein WITHOUT the C-terminal peptide, be sure to include a stop codon.

Continued on next page

Cloning into pBudCE4.1, Continued

CMV Multiple Cloning Site

Below is the multiple cloning site of pBUDCE4.1 located downstream of the CMV promoter. Restriction sites are labeled to indicate the cleavage site. Potential stop codons are underlined. The arrow indicates the predicted start of transcription using T7 RNA polymerase. Sequencing primers are available separately (see page 22).



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Cloning into pBudCE4.1, Continued

EF-1α Multiple Cloning Site

Below is the multiple cloning site of pBUDCE4.1 located downstream of the EF-1α promoter. Restriction sites are labeled to indicate the cleavage site. The promoter is marked using the convention of Uetsuki *et al.*, 1989. For more information see page 13. Sequencing primers are available separately (see page 22).

```
2940   GCACTTGATG TAATTCTCGT TGGAATTTGC CCTTTTTGAG TTTGGATCTT GGTTCATTCT

       EF-1α Forward priming site                    3´end of hEF-1α Intron 1
3000   CAAGCCTCAG ACAGTGGTTC AAAGTTTTTT TCTTCCATTT CAGGTGTCGT GAACACGTGG
                                                          5´ end of hEF-1α Exon 2

          Not I          BstB I*          Kpn I        BstX I     Xho I       Bgl II
3060   T CGC GGC CGC TTC GAA GGT ACC AGC ACA GTG GAC TCG AGA GAT CTG GCC
         Arg Gly Arg Phe Glu Gly Thr Ser Thr Val Asp Ser Arg Asp Leu Ala

          Sfi I          BstB I*                                V5 epitope
3109   GGC TGG GCC CGT TTC GAA GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT
       Gly Trp Ala Arg Phe Glu Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly

                                                  Polyhistidine (6xHis) tag
3157   CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA G
       Leu Asp Ser Thr Arg Thr Gly His His His His His His ***

                                  BGH Reverse priming site
3200   TTTAAACCCG CTGATCAGCC TCGACTGTGC CTTCTAGTTG CCAGCCATCT GTTGTTTGCC

                                                        BGH polyadenylation signal
3260   CCTCCCCCGT GCCTTCCTTG ACCCTGGAAG GTGCCACTCC CACTGTCCTT TCCTAATAAA

3320   ATGAGGAAAT TGCATCGCAT TGTCTGAGTA GGTGTCATTC TATTCTGGGG GGTGGGGTGG

3380   GGCAGGACAG CAAGGGGGAG GATTGGGAAG ACAATAGCAG GCATGCTGGG GATGCGGTGG
```

*Note that there are two *Bst*B I sites in the polylinker.

Continued on next page

Cloning into pBudCE4.1, Continued

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g., TOP10) and select on Low Salt LB plates containing 25–50 µg/mL Zeocin™ (see page 20). Select 10–20 clones and analyze for the presence and orientation of your insert.

We recommend that you sequence your construct to confirm that each of your genes is fused in frame with the C-terminal peptide. Several primers are available separately that you may use to sequence your construct. These are marked in the multiple cloning site diagrams on pages 4–5. For ordering information, see page 22. Alternatively, you may design your own primers for sequencing.

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.

1. Streak the original colony out on a Low Salt LB plate containing 25 µg/mL Zeocin™.
2. Incubate the plate at 37°C overnight.
3. Isolate a single colony and inoculate into 1–2 mL of Low Salt LB containing 25 µg/mL Zeocin™.
4. Grow the culture to stationary phase (OD_{600} = 1–2).
5. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
6. Store at –80°C.

Transfection and Analysis

Introduction

Once you have confirmed that your inserts are in the correct orientation and fused in frame with the C-terminal peptide (if desired), 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 lipid complexing decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HiPure Miniprep Kit or the PureLink™ HiPure Midiprep Kit (see page 21 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. 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* (Ausubel et al., 1994).

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 lipid-mediated transfection.

Positive Control

pBudCE4.1/*lac*Z/CAT is provided as a positive control vector for mammalian cell transfection and expression and may be used to optimize transfection conditions for your cell line (see page 17). The gene encoding β-galactosidase is expressed from the CMV promoter as a fusion to the *myc* epitope in mammalian cells. The gene encoding chloramphenicol acetyltransferase (CAT) is expressed as a fusion to the V5 epitope from the EF-1α promoter. A successful transfection results in β-galactosidase and CAT expression that can be easily assayed (see page 9.

Continued on next page

Detecting Fusion Proteins

Antibodies are available from Invitrogen to detect expression of fusion proteins from pBudCE4.1 (see page 22). In pBudCE4.1/*lacZ*/CAT, β-galactosidase and CAT are expressed as fusion proteins to the *myc* epitope or the V5 epitope, respectively. In addition you may assay for activity of either control protein using one of the assays described on the next page.

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 1,500 × g for 5 minutes.
3. Resuspend in 50 µL Cell Lysis Buffer (see recipe on page 20). Other lysis buffers may be suitable.
4. Incubate cell suspension at 37°C for 10 minutes to completely lyse the cells. **Note:** You may prefer to lyse the cells at room temperature or on ice if degradation of your proteins are a potential problem.
5. Centrifuge the cell lysate at 10,000 × g for 10 minutes at room temperature 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 (see below) and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Polyacrylamide Gel Electrophoresis

To facilitate separation of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast Novex® NuPAGE® and Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The patented Novex® NuPAGE® Gel System prevents the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits for visualization of recombinant proteins. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our website (www.invitrogen.com) or call Technical Support (see page 23).

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Western Analysis

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-*myc*, Anti-V5, or the Anti-His(C-term) antibodies available from Invitrogen (see page 22 for ordering information) or an antibody to your protein of interest. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods (see page 21 for ordering). For more information, refer to our website (www.invitrogen.com) or call Technical Support (see page 23).

Note

The C-terminal peptide containing the *myc* epitope and the polyhistidine tag or the V5 epitope and polyhistidine tag will add approximately 3 kDa to the size of your protein.

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 21).

Assay for CAT Activity

You may assay for CAT expression by ELISA assay, western blot analysis, fluorometric assay, or radioactive assay (Ausubel *et al.*, 1994; Neumann *et al.*, 1987). The CAT assay kit is available from Invitrogen for detection of CAT protein (see page 21).

Purifying Cells

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 20.

Creating Stable Cell Lines

Introduction

pBudCE4.1 contains the Zeocin™ resistance gene 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. For more information about Zeocin™, refer to page 18.

Effect of Zeocin™ on Sensitive and Resistant Cells

The method of killing with Zeocin™ is quite different from neomycin (G418) 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 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 will remain.
- Zeocin™-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes in Zeocin™-resistant cells when compared to cells not under selection with Zeocin™.

Selection in Mammalian Cell Lines

To generate a stable cell line expressing your protein, you need to determine the minimum concentration of Zeocin™ required for killing your untransfected host cell line. Typically, concentrations between 50 and 1,000 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.

1. Seed cells (2×10^5 cells/60 mm plate) for each time point and allow cells to adhere overnight.
2. 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).
3. Replenish the selective medium every 3–4 days, and observe the percentage of surviving cells.
4. Observe the cells at regular intervals to determine the appropriate concentration of Zeocin™ that prevents growth.
5. Select the concentration that kills cells in 7–10 days.

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Creating Stable Cell Lines, Continued

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 or elements necessary for expression of the gene.

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.**

Enzyme	Restriction Site (bp)	Location	Supplier
Nhe I	1877	Upstream of EF-1α promoter	Many
BspH I	4240	Backbone	New England Biolabs
Fsp I	4547	Backbone	Many
Pvu I	4568	Backbone	Many

Selection of Stable Integrants

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

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 (no Zeocin™) and allow cells to attach.
4. Remove medium and add medium containing Zeocin™ at the appropriate concentration for your cell line. Split the cells such that they are no more than 25% confluent.
5. Replenish selective medium every 3–4 days until Zeocin™-resistant colonies are detected.
6. Pick and expand colonies.

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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 ProBond™ Purification System 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 1,500 rpm for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 1,500 rpm 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 System manual for details about sample preparation for chromatography. The ProBond™ Purification System manual is available for downloading at our website (www.invitrogen.com) or by contacting Technical Support (see page 23).

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

Appendix

Human EF-1α Promoter

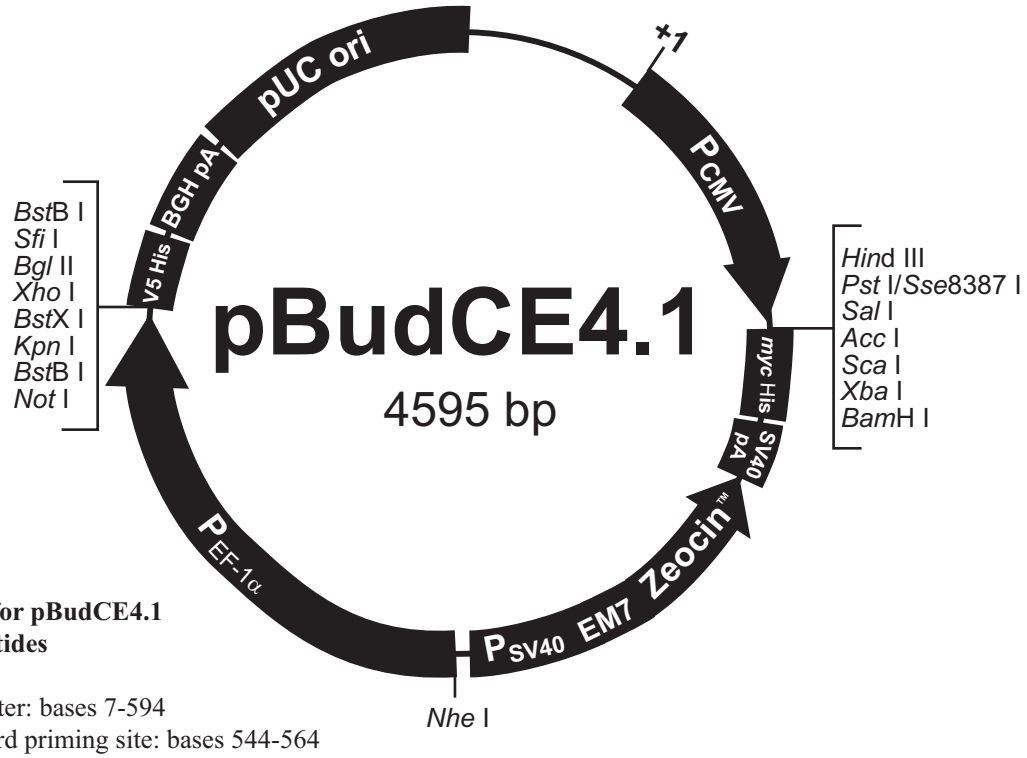
Description

The diagram below shows the features of the EF-1α promoter used in pBudCE4.1 (Mizushima and Nagata, 1990). Features are marked as per Uetsuki *et al.*, 1989(Uetsuki et al., 1989).

pBudCE4.1 Vector

Map of pBudCE4.1

The figure below summarizes the features of the pBudCE4.1 vector. The vector sequence is available for downloading from our website (www.invitrogen.com) or by contacting Technical Support (see page 23).



Comments for pBudCE4.1
4595 nucleotides

CMV promoter: bases 7-594
CMV Forward priming site: bases 544-564
T7 promoter/priming site: bases 638-657
CMV multiple cloning site: bases 664-713
myc epitope: bases 719-748
6xHis tag: bases 764-782
SV40 polyadenylation sequence: bases 803-933
Zeocin™ resistance gene: bases 1063-1437 (complementary strand)
EM7 promoter: bases 1456-1510 (complementary strand)
SV40 early promoter: bases 1547-1869 (complementary strand)
EF-1α promoter: bases 1885-3051
EF-1α Forward priming site: bases 2999-3019
EF-1α multiple cloning site: bases 3062-3126
V5 epitope: bases 3127-3168
6xHis tag: bases 3178-3195
BGH Reverse priming site: bases 3218-3235 (complementary strand)
BGH polyadenylation sequence: bases 3224-3447
pUC origin: bases 3521-4194

Continued on next page

pBudCE4.1 Vector, Continued

Features of pBudCE4.1

pBudCE4.1 (4595 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of recombinant protein (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987).
CMV Forward priming site	Permits sequencing through the insert from the 5′ end.
T7 promoter/priming site	Permits sequencing through the insert from the 5′ end. Allows for *in vitro* transcription in the sense orientation.
CMV Multiple cloning site	Seven unique sites allow insertion of your gene.
myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of your recombinant protein with the Anti-*myc* Antibody, Anti-*myc*-HRP Antibody, or Anti-*myc*-AP Antibody (Evan *et al.*, 1985) (see page 22 for ordering).
C-terminal polyhistidine (6xHis) 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, the Anti-His (C-term)-HRP Antibody, or the Anti-His(C-term)-AP Antibody (Lindner et al., 1997) (see page 22 for ordering).
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA. **Note**: The SV40 late polyadenylation signal terminates transcription for the gene cloned into the CMV MCS while the SV40 early polyadenylation signal terminates transcription for the Zeocin™ resistance gene. The signals are encoded on opposite strands in the same fragment of DNA.
Zeocin™ resistance gene	Selection of transformants in *E. coli* and stable transfectants in mammalian cells (Drocourt et al., 1990; Mulsant et al., 1988).
EM7 promoter	Synthetic promoter based on the bacteriophage T7 promoter for expression of the Zeocin™ resistance gene in *E. coli.*

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Features of pBudCE4.1, Continued

Feature	Benefit
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.
Human elongation factor 1α (EF-1α) promoter	Permits efficient, high-level expression of recombinant protein (Goldman et al., 1996; Mizushima and Nagata, 1990).
EF-1α Forward priming site	Permits sequencing through the insert from the 5′ end.
EF-1α Multiple cloning site	Seven unique sites allow insertion of your gene.
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, Anti-V5-HRP Antibody or the Anti-V5-AP Antibody (Southern et al., 1991) (see page 22 for ordering).
6xHis tag	See previous page.
Bovine growth hormone (BGH) reverse priming site	Permits sequencing through the insert from the 3′ end.
BGH polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992).
pUC origin	High-copy number replication and growth in *E. coli.*

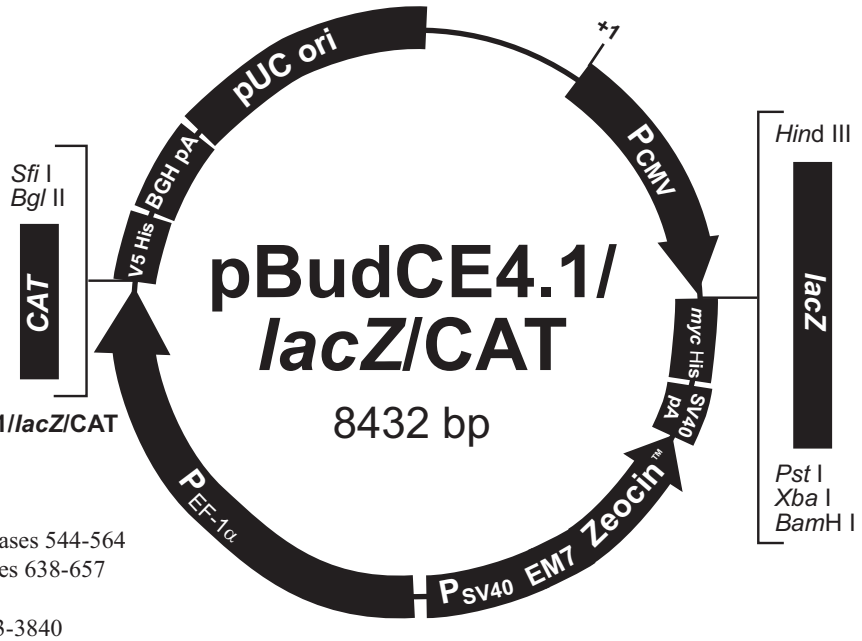
pBudCE4.1/*lacZ*/CAT

Description

pBudCE4.1/*lacZ*/CAT is an 8432 bp control vector containing the gene for β-galactosidase and the gene for chloramphenicol acetyltransferase (CAT). The *lacZ* gene was excised from pIND/*lacZ* using *Hin*d III and *Xba* I and cloned into *Hin*d III/*Xba* I digested pBudCE4.1. The CAT gene was cloned by digesting pBudCE4.1/*lacZ* and pBudCE4/*lacZ*/CAT with *Bgl* II and *Mun* I. A fragment containing the CAT gene and part of the EF-1α promoter from pBudCE4/*lacZ*/CAT was cloned into *Bgl* II/*Mun* I digested pBudCE4.1/*lacZ* to generate pBudCE4.1/*lacZ*/CAT.

Map of Control Vector

The figure below summarizes the features of the pBudCE4.1/*lacZ*/CAT vector. The nucleotide sequence for pBudCE4.1/*lacZ*/CAT is available for downloading from our website (www.invitrogen.com) or by contacting Technical Support (see page 23).



Comments for pBudCE4.1/*lacZ*/CAT
8432 nucleotides

CMV promoter: bases 7-594
CMV Forward priming site: bases 544-564
T7 promoter/priming site: bases 638-657
LacZ fusion: bases 673-3966
    LacZ ORF: bases 673-3840
    myc epitope: bases 3901-3963
    6xHis tag: bases 3946-3963
SV40 polyadenylation sequence: bases 3986-4116
Zeocin™ resistance gene: bases 4245-4619 (complementary strand)
EM7 promoter: bases 4638-4693 (complementary strand)
SV40 early promoter: bases 4728-5051 (complementary strand)
EF-1α promoter: bases 5067-6236
EF-1α Forward priming site: bases 6181-6201
CAT fusion: bases 6268-7032
    CAT ORF: bases 6268-6924
    V5 epitope: bases 6964-7005
    6xHis tag: bases 7015-7032
BGH Reverse priming site: bases 7055-7072 (complementary strand)
BGH polyadenylation sequence: bases 7061-7285
pUC origin: bases 7358-8031

Zeocin™

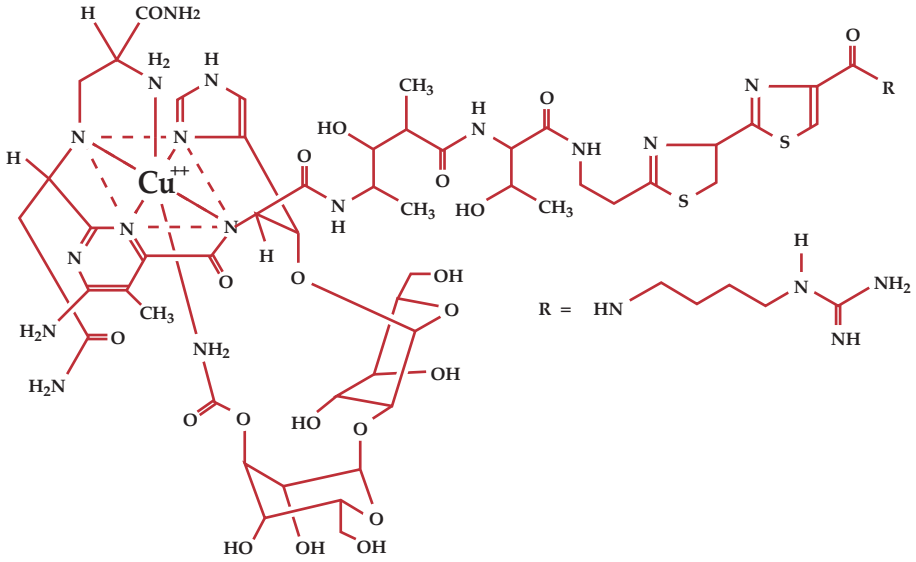
Zeocin™

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 anti-bacterial 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 $C_{55}H_{86}O_{21}N_{20}S_2Cu.HCl$ and the molecular weight is 1,527.5 daltons. Zeocin is an HCl salt. The diagram below shows the structure of Zeocin™.



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 **Low Salt LB** medium* (see page 20 for recipe)
Mammalian Cells	50-1,000 µg/mL (varies with cell line)

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

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Zeocin™, Continued

Handling Zeocin™

- High salt and acidity or basicity inactivates 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 next page). **Note**: The salt concentration should not be adjusted for mammalian cells. Changes to the salt concentration are detrimental to cells.
- Store Zeocin™ at –20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store drug, plates, and medium containing drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling solutions containing Zeocin™.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

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. 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 use low salt LB medium will result in non-selection due to inactivation of the drug. For more information about Zeocin™, refer to page 18.

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 5 M 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 25–50 µg/mL final concentration.
5. Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for 1–2 weeks.

For your convenience Low Salt LB medium containing 25 µg/ml Zeocin™ is available as premixed, pre-sterilized *E. coli* growth medium (imMedia™) that contains everything you need in a convenient pouch. Liquid and agar media are available, depending upon your application (see page 21).

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 **fresh** at the following concentrations: 1 mM PMSF; 1 µg/mL pepstatin; 1 µg/mL leupeptin.

Accessory Products

Introduction

The products listed below are designed for use with pBudCE4.1. For details, visit www.invitrogen.com or contact Technical Support (page 23).

Item	Quantity	Catalog no.
One Shot® TOP10 Chemically Competent *E. coli*	21 × 50 µL	C4040-03
One Shot® TOP10 Electrocomp™ Cells	21 × 50 µL	C4040-52
One Shot® Max Efficiency® DH5α™ T1R	20 × 50 µL	12297-016
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Lipofectamine™ 2000 Reagent	1.5 mL	11668-019
β-Gal Assay Kit	80 mL	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Resin	50 mL	R801-01
	150 mL	R801-15
Zeocin™	1 gram	R250-01
	5 grams	R250-05
imMedia™ Zeo Liquid	200 mL	Q620-20
imMedia™ Zeo Agar	8–10 agar plates	Q621-20
WesternBreeze® Chromogenic Kit, Anti-Mouse	1 kit	WB7103
WesternBreeze® Chromogenic Kit, Anti-Rabbit	1 kit	WB7105
WesternBreeze® Chromogenic Kit, Anti-Goat	1 kit	WB7107
WesternBreeze® Chemiluminescent Kit, Anti-Mouse	1 kit	WB7104
WesternBreeze® Chromogenic Kit, Anti-Rabbit	1 kit	WB7106
WesternBreeze® Chromogenic Kit, Anti-Goat	1 kit	WB7108
Fast Cat® Chloramphenicol Acetyltransferase Assay Kit	1 kit	F2900

Continued on next page

Accessory Products, Continued

Primers

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

Antibodies and Western Detection Kits

If you do not have an antibody specific to your protein, Invitrogen offers the Anti-*myc*, Anti-V5, 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-V5	Detects a 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		R961-25
Anti-V5-AP		R962-25
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, MSDSs, 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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MSDS

Material Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/msds.

Certificate of Analysis

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

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