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pCEP4

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User Manual

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Kit Contents and Storage

Shipping and Storage

pCEP4 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
pCEP4	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg
pCEP4/CAT	20 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	10 µg

Introduction

Product Overview

pCEP4

pCEP4 is an episomal mammalian expression vector that uses the cytomegalovirus (CMV) immediate early enhancer/promoter for high level transcription of recombinant genes inserted into the multiple cloning site. The Epstein-Barr Virus replication origin (oriP) and nuclear antigen (encoded by the EBNA-1 gene) is carried by this plasmid to permit extrachromosomal replication in human, primate, and canine cells. pCEP4 also carries the hygromycin B resistance gene for stable selection in transfected cells.

pCEP4/CAT is provided as a positive control for the relative level of expression of recombinant proteins in a cell line of interest. It expresses the chloramphenicol acetyl transferase (CAT) protein from the CMV enhancer/promoter. Like its parent vector pCEP4, pCEP4/CAT contains the hygromycin B resistance gene for selection.

Experimental Outline

Use the following outline to clone and express your gene of interest in pCEP4.

1. Consult the multiple cloning site described on page 3 to design a strategy to clone your gene into pCEP4.
2. Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants on LB plates containing 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 the proper orientation.
5. Transfect your construct into the mammalian cell line of interest using your own method of choice. Generate a stable cell line, if desired.
6. Test for expression of your recombinant gene by western blot analysis or functional assay.

Methods

Cloning into pCEP4

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

Many *E. coli* strains are suitable for the propagation of this vector. 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, TOP10 is available as chemically competent or electrocompetent cells from Invitrogen (see page 11).

Maintaining pCEP4

To propagate and maintain pCEP4, 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 LB plates containing 50 to 100 µg/mL ampicillin. Be sure to prepare a glycerol stock of your plasmid-containing *E. coli* strain for long-term storage (see page 4).

Important

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

Your insert should also contain a stop codon for proper termination of your gene.

Continued on next page

Cloning into pCEP4, Continued

Multiple Cloning Site of pCEP4

Below is the multiple cloning site for pCEP4. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. **The sequence of pCEP4 is available for downloading from our website (www.invitrogen.com) or from Technical Support (see page 12).** For a map and a description of the features, refer to pages 8–9.

```
1    GTTGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA
                                ┌ enhancer region (5' end)
61   GCCCATATAT GGAGTTCCGC GTTACATAAC TTACGGTAAA TGGCCCGCCT GGCTGACCGC

121  CCAACGACCC CCGCCCATTG ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG

181  GGACTTTCCA TTGACGTCAA TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC

241  ATCAAGTGTA TCATATGCCA AGTCCGCCCC CTATTGACGT CAATGACGGT AAATGGCCCG

301  CCTGGCATTA TGCCCAGTAC ATGACCTTAC GGGACTTTCC TACTTGGCAG TACATCTACG

361  TATTAGTCAT CGCTATTACC ATGGTGATGC GGTTTTGGCA GTACACCAAT GGGCGTGGAT
                    AP1
421  AGCGGTTTGA CTCACGGGGA TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT
     enhancer region (3' end)
481  TTTGGCACCA AAATCAACGG GACTTTCCAA AATGTCGTAA TAACCCCGCC CCGTTGACGC
     CAAT                                    TATA    pCEP Forward primer
541  AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCGT TTAGTGAACC
     ┐┌──► Transcriptional start   Kpn I Pvu II Nhe I*   Hind III  Nhe I*  Not I   Xho I
601  GTCAGATCTC TAGAAGCTGG GTACCAGCTG CTAGCAAGCT TGCTAGCGGC CGCTCGAGGC
       Sfi I     BamH I                          EBV Reverse primer
661  CGGCAAGGCC GGATCCAGAC ATGATAAGAT ACATTGATGA GTTTGGACAA ACCACAACTA
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Cloning into pCEP4, Continued

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (*e.g.* TOP10) and select transformants on LB plates containing 50 to 100 μg/mL ampicillin. Select 10–20 clones and analyze for the presence and orientation of your insert.

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.

Sequencing Your Construct

Several primers are available separately that you may use to sequence your construct. These are marked in the multiple cloning site diagram on page 3. For ordering information, see page 11. Alternatively, you may design your own primer for sequencing.

Preparing a Glycerol Stock

Once you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. You should keep a 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

Introduction

Once you have verified that your gene is cloned in the correct orientation and contains an initiation ATG and a stop codon, you are ready to transfect your cell line of choice. We recommend that you include the positive control vector and a mock transfection (negative control) to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be 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™ HQ Mini Plasmid Purification Kit or PureLink™ HiPure Miniprep or Midiprep Kits available from Invitrogen (see page 11).

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 mammalian cell transfection (see page 11).

Positive Control

pCEP4/CAT is provided as a positive control vector for mammalian transfection and expression (see page 10) and may be used to optimize transfection conditions for your cell line. The gene encoding chloramphenicol acetyl transferase (CAT) is expressed in mammalian cells under the control of the CMV promoter. A successful transfection will result in CAT expression that can be easily assayed (see below).

Assay for CAT Protein

You may assay for CAT expression using your method of choice. Invitrogen offers a CAT assay kit for detection of the protein (page 11).

Creating Stable Cell Lines

Introduction

pCEP4 contains the hygromycin resistance gene for selection of stable cell lines using hygromycin B. We recommend that you test the sensitivity of your mammalian host cell to hygromycin B, as natural resistance varies among cell lines. General information and guidelines are provided in this section for your convenience.

Hygromycin B Activity

Hygromycin B (527.5 MW) is an aminocyclitol that inhibits protein synthesis by disrupting translocation and promoting mistranslation. Hygromycin B-phosphotransferase detoxifies hygromycin-B by phosphorylation.

- Hygromycin is light sensitive. Store the liquid stock solution at +4°C protected from exposure to light.
- Hygromycin is toxic. Do not ingest solutions containing the drug.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling hygromycin and hygromycin-containing solutions.

Determining Antibiotic Sensitivity

To successfully generate a stable cell line expressing your gene of interest from pCEP4, you need to determine the minimum concentration of hygromycin B required to kill your untransfected host cell line. Typically, concentrations ranging from 10 to 400 μg/mL hygromycin are sufficient to kill most untransfected mammalian cell lines. We recommend that you test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your host cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of hygromycin (0, 10, 25, 50, 100, 200, 400 μg/mL hygromycin).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of hygromycin that prevents growth within 2–3 weeks after addition of hygromycin.

Note: Cells will divide once or twice in the presence of lethal doses of hygromycin, so the effects of the drug may take several days to become apparent. Complete inhibition of cell growth can take 2–3 weeks of growth in selective medium.

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Selecting Stable Integrants

Once you have determined the appropriate hygromycin concentration to use for selection in your host cell line, you can generate a stable cell line expressing your gene of interest.

1. Transfect your mammalian host cell line with your pCEP4 construct using the desired protocol. Remember to include a plate of untransfected cells as a negative control and the pCEP4/CAT plasmid as a positive 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 hygromycin at the pre-determined concentration required for your cell line. Split the cells such that they are no more than 25% confluent.
4. Feed the cells with selective medium every 3–4 days until hygromycin-resistant foci can be identified.
5. Pick and expand colonies in 96- or 48-well plates.

Maintaining Stable Transfectants

pCEP4 is an episomally-maintained plasmid (Reisman and Sugden, 1986; Yates *et al.*, 1985). Transfected cells may lose the pCEP4 plasmid if they are not maintained under selection or are continuously cultured for long periods of time (over six months). To prevent loss of pCEP4 from transfected cells, we recommend that you follow these guidelines when working with the cells:

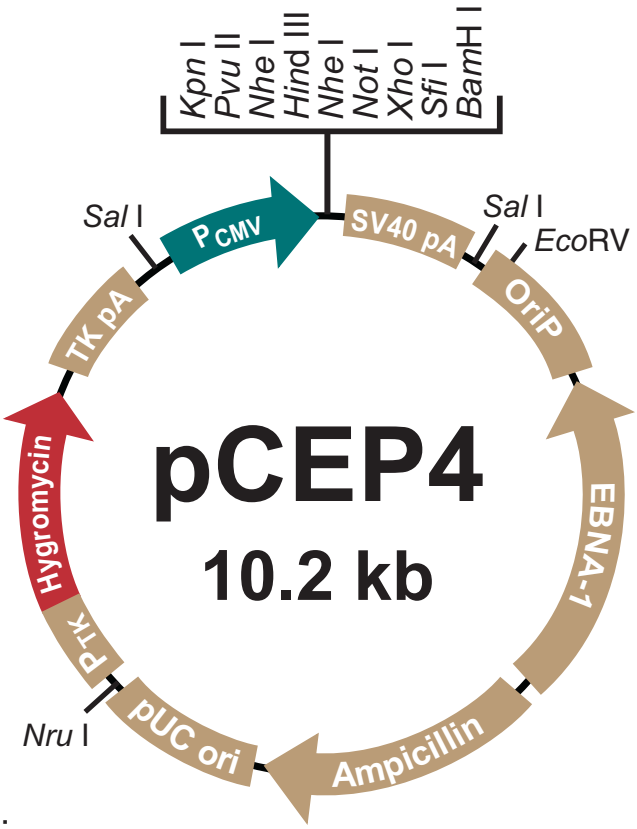
- Always use early-passage cells. Grow and freeze multiple vials of transfected cells to ensure that you have an adequate supply of early-passage cells.
- Always maintain cells in medium containing 50 μg/mL hygromycin.
- Do not maintain cells in continuous culture for longer than 6 months.

Appendix

pCEP4 Vector

Map of pCEP4

The figure below summarizes the features of the pCEP4 vector. **The sequence for pCEP4 is available for downloading from our website (www.invitrogen.com) or from Technical Support (see page 12).**



Comments for pCEP4:
10186 nucleotides

CMV promoter: bases 1-588
Multiple cloning site: bases 619-676
SV40 polyadenylation signal: bases 685-926
OriP: bases 1344-3319
EBNA-1 gene (complementary strand): bases 3620-5545
Ampicillin resistance gene: bases 6171-7031
pUC origin: bases 7040-7815
TK promoter: bases 8183-8345
Hygromycin resistance gene: bases 8409-9419
TK polyadenylation signal: bases 9431-9702

Continued on next page

Features of pCEP4

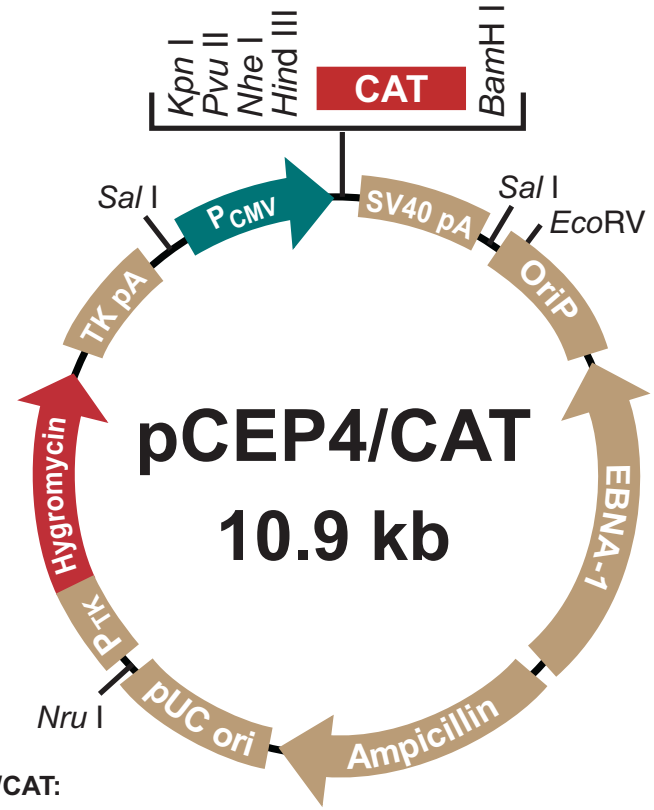
pCEP4 (10,186 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987)
Multiple cloning site	Allows insertion of your gene and facilitates cloning
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
EBV origin of replication (oriP) and nuclear antigen (EBNA-1)	High-copy episomal replication in primate and canine cell lines (Reisman and Sugden, 1986; Yates et al., 1985)
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli*
pUC origin	High-copy number replication and growth in *E. coli*
Herpes Simplex Virus thymidine kinase (TK) promoter	Allows efficient, high-level expression of the hygromycin resistance gene (McKnight, 1980)
Hygromycin resistance gene	Selection of stable transfectants in mammalian cells (Gritz and Davies, 1983; Palmer et al., 1987)
Herpes Simplex Virus thymidine kinase (TK) promoter polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA

pCEP4/CAT

Map of pCEP4/CAT

The figure below summarizes the features of the pCEP4/CAT vector. **The nucleotide sequence for pCEP4/CAT is available for downloading from our website (www.invitrogen.com) or by contacting Technical Support (page 12).**



Comments for pCEP4/CAT:
10891 nucleotides

CMV promoter: bases 1-588
Chloramphenicol acetyl transferase (CAT) gene: bases 675-1334
SV40 polyadenylation signal: bases 1390-1631
OriP: bases 2049-4024
EBNA-1 gene (complementary strand): bases 4325-6250
Ampicillin resistance gene: bases 6876-7736
pUC origin: bases 7745-8520
TK promoter: bases 8888-9050
Hygromycin resistance gene: bases 9114-10124
TK polyadenylation signal: bases 10136-10407

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 12).

Item	Quantity	Catalog no.
One Shot® TOP10 Chemically Competent Cells	21 × 50 µL	C4040-03
One Shot® TOP10 Electrocomp™ Cells	21 × 50 µL	C4040-52
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
Lipofectamine™ 2000 Reagent	1.5 mL	11668-019
Hygromycin B	20 mL	10687-010
Fast Cat® Chloramphenicol Acetyltransferase Assay Kit	1 kit	F2900

Primers

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

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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