USER GUIDE

invitrogen™
by life technologies™

pShooter™ Vector (pCMV/*myc* vectors)

For the intracellular targeting of recombinant proteins and antibodies

Catalog numbers V820-20, V821-20, V822-20, V823-20

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For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

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Contents

Kit Contents and Storage

Shipping/Storage

All vectors are shipped at room temperature. Upon receipt, store at -20°C.

Kit Contents

The pShooter™ manual for vectors utilizing the CMV promoter and the *c-myc* epitope is included with the following vectors. All vectors are supplied at a concentration of 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL

Vector	Catalog no.
pCMV/*myc*/cyto pCMV/*myc*/cyto/GFP	V820-20
pCMV/*myc*/nuc pCMV/*myc*/nuc/GFP	V821-20
pCMV/*myc*/mito pCMV/*myc*/mito/GFP	V822-20
pCMV/*myc*/ER pCMV/*myc*/ER/GFP	V823-20

Product Use

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

Introduction

Product Overview

Background

The final location of a protein within a cell depends upon 'targeting sequences' encoded within the sequence of a protein. In the simplest case, the lack of a signal directs proteins to the default pathway which is the cytoplasm. The presence of a nuclear localization sequence within a protein or at the N- or C-terminus, directs the protein to the nucleus, while the mitochondrial leader sequence, which is removed upon translocation, directs proteins to the mitochondria. Lastly, proteins destined to be retained in the endoplasmic reticulum (ER) must have an N-terminal signal peptide to direct the protein into the secretory compartment and a C-terminal peptide (SEKDEL) to retain the protein in the ER.

Description

The pShooter™ vectors are a family of vectors designed to express and target your recombinant protein to the desired intracellular location in mammalian cells. They were originally designed to target single-chain antibodies (scFvs) to specific intracellular locations (Persic *et al.*, 1997a; Persic *et al.*, 1997b). These vectors can also be used to target other proteins to different intracellular compartments. The pShooter™ vectors described in this manual are 5.0 kb expression vectors that express your recombinant protein as a fusion to a targeting sequence (if necessary) and the *c-myc* epitope(Evans *et al.*, 1985). Proteins are targeted to the cytoplasm (no signal), mitochondria (Rizzuto *et al.*, 1992), nucleus (Fisher-Fantuzzi and Vesco, 1988), or endoplasmic reticulum (Munro and Pelham, 1987). Expression is driven by the strong, constitutive immediate-early cytomegalovirus (CMV) promoter (Stenberg et al., 1984; Thomsen et al., 1984). The table below summarizes the above features.

Vector	Desired Location	Targeting Signal
pCMV/*myc*/cyto	Cytoplasm	None
pCMV/*myc*/nuc	Nucleus	3X (DPKKKRKV)
pCMV/*myc*/mito	Mitochondria	MSVLTPLLLRGLTGSARRLPVPRAKIHSL
pCMV/*myc*/ER	ER	MGWSCIILFLVATATGAHS (N-terminus) + SEKDEL (C-terminus)

In addition, all vectors use the same backbone (pcDNA3) which includes the bovine growth hormone polyadenylation sequence, an f1 origin, the SV40 origin, the neomycin resistance gene, the SV40 late polyadenylation sequence, pUC origin, and the ampicillin resistance gene (Persic et al., 1997b). For more information on all of the above features, see page 16.

Uses of the pShooter™ Vectors

Targeting Recombinant Proteins

The vectors can be used to direct any recombinant protein to a particular intracellular location. However, success may be dependent on the specific protein used. To help analyze experiments, each vector is supplied with an optimized form of green fluorescent protein (SuperGFP) cloned into the vector as a control. See pages 22–25 for maps of the control vectors. Guidelines for assaying SuperGFP fluorescence are also provided (page 15).

Continued on next page

Uses of the pShooter™ Vectors, Continued

Targeting Antibodies

The pShooter™ vectors were originally designed for the targeting of scFvs to a specific intracellular location for intracellular immunization (Biocca and Cattaneo, 1995; Cattaneo and Biocca, 1997; Persic et al., 1997a). In this technique, an antibody which is inhibitory for a protein's function can be directed to the same compartment as the protein itself to inactivate the protein.

The pShooter™ vectors retain all of the features cited in Persic, et al., 1997a. Some of these features are summarized below.

- The restriction sites in the multiple cloning site were chosen because they are rare in both human and mouse antibody variable regions and have been removed from the rest of the vector.
- Vectors consist of a number of functional cassettes flanked by unique restriction sites, with junctional DNA reduced to a minimum.
- The nuclear localization signal is designed to be at the C-terminus of a scFv, positioned away from the antigen binding site, to reduce potential problems of steric hindrance.
- scFvs derived from phage antibody libraries can be easily cloned in from compatible vectors (e.g. pHEN; Hoogenboom *et al.*, 1991(Hoogenboom et al., 1991)) or amplified incorporating compatible ends.

For more information on cloning antibodies and antibody domains, refer to Persic, *et al.*, 1997a. For an example in which these vectors have been used in intracellular immunization to inhibit function within a cell, see Gargano and Cattaneo, 1997. (Gargano and Cattaneo, 1997)

Methods

General Guidelines

Introduction

This section contains general information on propagation and maintenance of the pShooter™ vectors and guidelines for *E. coli* transformation. Additional information is provided on the following pages:

- To develop a cloning strategy, refer to the multiple cloning sites on pages 5–9.
- Maps of the targeting vectors are on pages 18–21.
- Maps of the control vectors are on pages 22–25.
- Nucleotide sequences of any of the vectors described in this manual may be obtained by downloading them from www.lifetechnologies.com/support or by calling Technical Support (see page 27).

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 growth 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, TOP10F´ is available as chemically competent or electrocompetent cells (see page 26 for ordering).

E. coli Transformation

You may use any method you wish to prepare competent *E. coli* for transformation. Select transformants on LB plates containing 50–100 µg/mL ampicillin.

Continued on next page

Propagating and Maintaining Plasmids

To propagate and maintain any of the pShooter™ vectors, we recommend that you transform the plasmids into *E. coli* and prepare glycerol stocks for long-term storage. Transform plasmids into *E. coli* as follows:

1. Use the supplied stock solution in TE, pH 8.0 to transform a *rec*A, *end*A *E. coli* strain like TOP10F´, INVαF´, DH5αF´, or equivalent.
2. Select transformants on LB plates containing 50–100 µg/mL ampicillin.
3. Select a transformant and grow a log phase culture for a glycerol stock.
4. Prepare glycerol stocks by mixing 0.85 mL of the log phase culture with 0.15 mL of sterile glycerol.
5. Transfer the resulting solution to a cryovial and store at –80°C.

Cloning into the pShooter™ Vectors

Diagrams for each of the multiple cloning sites are provided on pages 5–9 to help you clone your gene of interest in frame with the desired targeting signal and/or the *c-myc* epitope for detection. For help with PCR, restriction digests, and ligations, refer to general molecular biology texts (Ausubel et al., 1994; Sambrook et al., 1989).

Transform ligation mixtures into competent *E. coli* as using the method of choice, and plate the cells on LB plates containing 50–100 µg/mL ampicillin.

Select 10 to 20 transformants and analyze your construct by restriction enzyme digestion or sequencing to ensure that your insert is cloned in the correct orientation. If you wish to sequence your insert, use the pCMV Forward and BGH Reverse primers (see page 26 for ordering) to confirm that your gene is correctly fused to the targeting signal and/or the *c-myc* epitope.

Cloning into pCMV/*myc*/cyto

Special Considerations

Since the cytoplasm is the default location for translated proteins, this vector contains no targeting signals. One thing to note:

The ATG in the *Nco* I site is part of a Kozak consensus sequence (ANNATGG)(Kozak, 1987; Kozak, 1990). If you can clone in frame or flush with this ATG, it will facilitate expression of your protein.

Note that you may have to use PCR to clone your gene in frame or flush with the ATG and/or the *c-myc* epitope. Note that the *c-myc* epitope will add ~1.5 kDa to your protein. If you do not wish to fuse your protein to the *c-myc* epitope, remember to include a stop codon.

pCMV/*myc*/cyto MCS

Restriction sites are labeled to indicate the cleavage site. For more information on the CMV promoter, see page 17. The multiple cloning site has been confirmed by sequencing and functional testing.

```
480  AGTTTGTTTT GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA CTCCGCCCCA

                CAAT                                    TATA          Start of Transcription
540  TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG AGCTTTCTGG
           pCMV Forward priming site

                                  Nco I                          Pst I  Sal I      Xho I
600  CTAACTAGAG AACCCGTGGC CACC ATG GCC CAG GTG CAG CTG CAG GTC GAC CTC GAG
                                Met Ala Gln Val Gln Leu Gln Val Asp Leu Glu

                    Not I                    myc epitope
657  ATC AAA CGG GCG GCC GCA GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG
     Ile Lys Arg Ala Ala Ala Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly

                                                BGH Reverse priming site
711  GCC GCA TAG TCTAGAAGCT CGCTGATCAG CCTCGACTGT GCCTTCTAGT TGCCAGCCAT
     Ala Ala ***


770  CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT TGACCCTGGA AGGTGCCACT CCCACTGTCC

          BGH polyadenylation signal
830  TTTCCTAATA AAATGAGGAA ATTGCATCGC ATTGTCTGAG TAGGTGTCAT TCTATTCTGG
```

Cloning into pCMV/*myc*/nuc

Special Considerations

The ATG in the *Nco* I site is part of a Kozak consensus sequence (ANNATGG) (Kozak, 1987; Kozak, 1990). If you can clone in frame or flush with this ATG, it will facilitate expression of your protein.

To efficiently target your protein to the nucleus, the nuclear localization signal (NLS) from SV40 large T antigen has been triplicated and placed downstream of the multiple cloning site for C-terminal fusion to your protein (Fisher-Fantuzzi and Vesco, 1988). Note that this signal will not be removed from your protein upon entry to the nucleus. If you clone in-frame with the NLS you will also be in frame with the *c-myc* epitope. The NLS and the *c-myc* epitope will add ~5 kDa to your protein.

Note that you may have to use PCR to facilitate in-frame cloning with the ATG (if desired) and the NLS.

pCMV/*myc*/nuc MCS

Restriction sites are labeled to indicate the cleavage site. For more information on the CMV promoter, see page 17. The multiple cloning site has been confirmed by sequencing and functional testing.

```
480  AGTTTGTTTT GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA CTCCGCCCCA

                CAAT                                        TATA          Start of Transcription
540  TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG AGCTTTCTGG
          pCMV Forward priming site

                                                                    Nco I
600  CTAACTAGAG AACCCACTGC TTACTGGCAC GTGGAAATTA ATACGACGTG GCCACC ATG GCC
                                                                      Met Ala

                                                                          NLS #1
                         Pst I  Sal I      Xho I                  Not I
662  CAG GTG CAG CTG CAG GTC GAC CTC GAG ATC AAA CGG GCG GCC GCA GAT CCA AAA
     Gln Val Gln Leu Gln Val Asp Leu Glu Ile Lys Arg Ala Ala Ala Asp Pro Lys

                                        NLS #2                          NLS #3
716  AAG AAG AGA AAG GTA GAT CCA AAA AAG AAG AGA AAG GTA GAT CCA AAA AAG AAG
     Lys Lys Arg Lys Val Asp Pro Lys Lys Lys Arg Lys Val Asp Pro Lys Lys Lys

                                                  myc epitope
770  AGA AAG GTA GAT ACG GCC GCA GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT
     Arg Lys Val Asp Thr Ala Ala Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn

                                                BGH Reverse priming site
824  GGG GCC GCA TAG TCTAGAAGCT CGCTGATCAG CCTCGACTGT GCCTTCTAGT TGCCAGCCAT
     Gly Ala Ala ***

886  CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT TGACCCTGGA AGGTGCCACT CCCACTGTCC

       BGH polyadenylation signal
946  TTTCCTAATA AAATGAGGAA ATTGCATCGC ATTGTCTGAG TAGGTGTCAT TCTATTCTGG
```

Cloning into pCMV/*myc*/mito

Special Considerations

To direct your protein to the mitochondria, clone in frame with the targeting sequence. The mitochondrial targeting sequence is removed upon translocation into the mitochondrial matrix; however, at least four additional amino acids (Ile-His-Ser-Leu) will be left at the N-terminus of your protein. Not enough is known about mitochondrial targeting sequences to provide a consensus sequence that will produce a native protein upon translocation. To clone your gene flush with the last leucine codon, use PCR and design the 5´ end of your primer to include sequence from the unique *Bss*H II site to the end of the targeting sequence.

This vector does not have an *Nco* I site in the multiple cloning site. Since the *Nco* I site contains an ATG, removal of this site insures that translation reinitiation does not occur downstream of the mitochondrial targeting sequence.

If you wish to include the *c-myc* epitope, remember to clone in-frame with the epitope. Note that the *c-myc* epitope will add ~1.5 kDa to your protein. If you wish to express your protein without the *c-myc* epitope, remember to include a stop codon.

pCMV/*myc*/mito MCS

Restriction sites are labeled to indicate the cleavage site. For more information on the CMV promoter, see page 17. The multiple cloning site has been confirmed by sequencing and functional testing.

```
480  AGTTTGTTTT GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA CTCCGCCCCA

                CAAT                                          TATA          Start of Transcription
540  TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG AGCTTTCTGG
          pCMV Forward priming site

                                                                  ┌
600  CTAACTAGAG AACCCACTGC TTACTGGCAC GTGACC ATG TCC GTC CTG ACG CCG CTG CTG
                                             Met Ser Val Leu Thr Pro Leu Leu

                                  Mitochondrial targeting signal          BssH II
     ──────────────────────────────────────────────────────────────────────┐
660  CTG CGG GGC TTG ACA GGC TCG GCC CGG CGG CTC CCA GTG CCG CGC GCC AAG ATC
     Leu Arg Gly Leu Thr Gly Ser Ala Arg Arg Leu Pro Val Pro Arg Ala Lys▲Ile
                                                                Putative Cleavage Site
     ┌──────────────   Pst I  Sal I    Xho I              Not I          myc epitope
714  CAT TCG TTG CTGCAGGTCG ACCTCGAGAT CAAACGGGCG GCCGCA GAA CAA AAA CTC ATC
     His Ser Leu                                         Glu Gln Lys Leu Ile

                                                                      BGH Reverse
     ──────────────────────┐                                         ┌
774  TCA GAA GAG GAT CTG AAT GGG GCC GCA TAG TCTAGAAGCT CGCTGATCAG CCTCGACTGT
     Ser Glu Glu Asp Leu Asn Gly Ala Ala ***
     priming site
     ┌
834  GCCTTCTAGT TGCCAGCCAT CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT TGACCCTGGA

                                  BGH polyadenylation signal
                                        ┌
894  AGGTGCCACT CCCACTGTCC TTTCCTAATA AAATGAGGAA ATTGCATCGC ATTGTCTGAG
```

Cloning into pCMV/*myc*/ER

Retaining in the ER

To direct and retain your protein to the ER, clone in frame with the second exon of signal peptide and the *c-myc* epitope (see page 9). The signal peptide contains an intron which when spliced out, puts the peptide in frame with your protein. The signal peptide is removed after the serine codon upon translocation into the ER and the protein is retained because of the SEKDEL peptide which is in frame with, and C-terminal to, the *c-myc* epitope. To clone your gene flush with the serine codon in the signal peptide, use PCR and design the 5´ end of your primer to include sequence from the unique *Bss*H II site to the end of the signal peptide (GGC GCG CAC TCC ....). refer to the diagram on page 9.

Note: The signal peptide is from a mouse V_h chain (Kabat et al., 1987) and contains an intron. The presence of introns in signal peptides is reputed to increase expression levels.

Secretion

If you wish to secrete your protein, include the native stop codon of your gene of interest. This will prevent fusion with the *c-myc* epitope and the SEKDEL ER retention signal. Note that if a protein is normally secreted, then fusing the protein to the ER signal peptide (and omitting the ER retention signal) should allow secretion. However, proteins that are not normally secreted may be nonspecifically retained in the ER. This is very much protein-dependent.

Note: You will not be able to detect your protein with antibody to the *c-myc* epitope.

Other Considerations

This vector does not have an *Nco* I site in the multiple cloning site. Since the *Nco* I site contains an ATG, removal of this site insures that translation reinitiation does not occur downstream of the ER signal peptide.

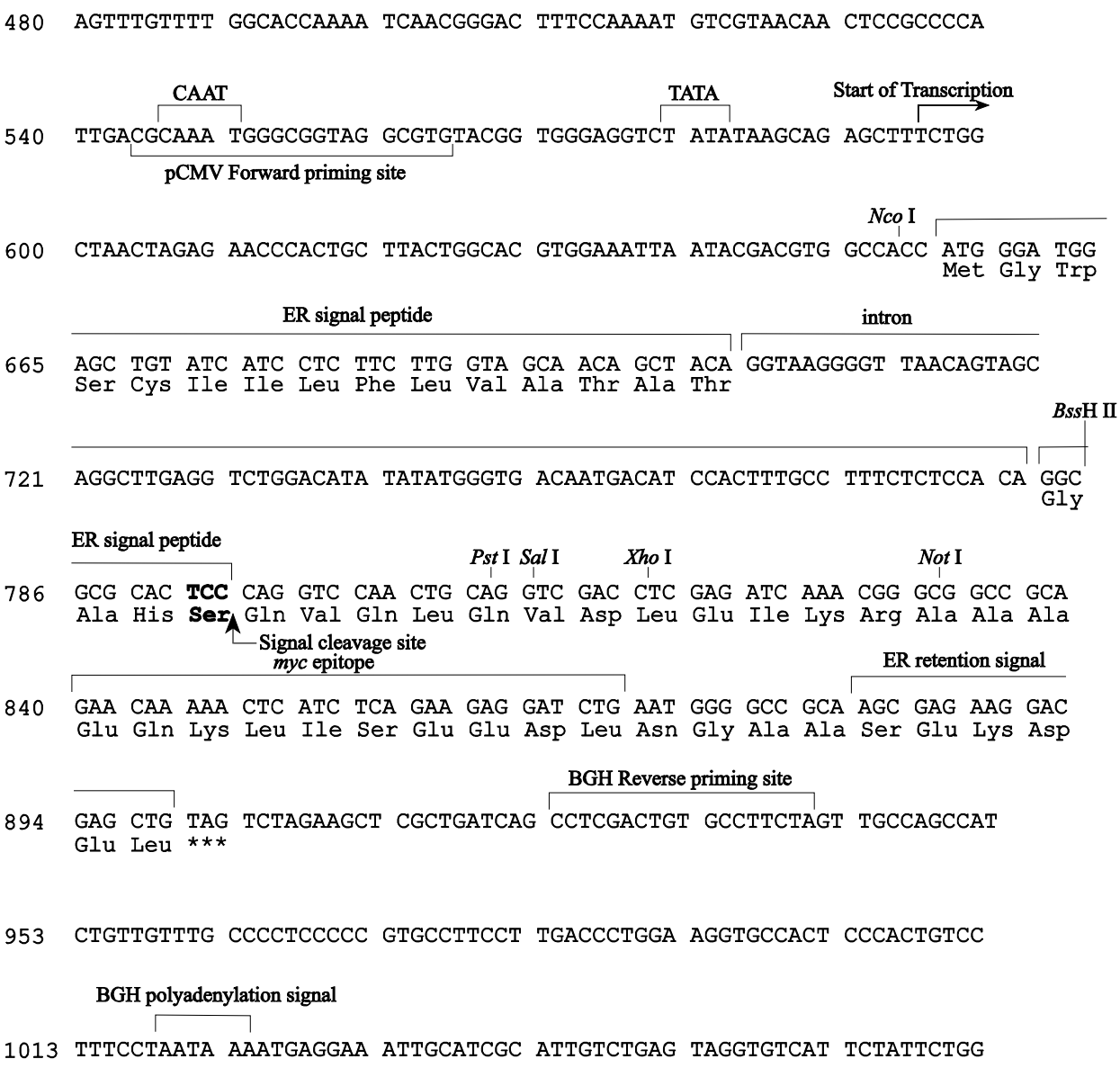
Note that the C-terminal peptide containing the *c-myc* epitope and the SEKDEL peptide will add ~2 kDa to your protein.

Continued on next page

Cloning into pCMV/*myc*/ER, Continued

pCMV/*myc*/ER MCS

Restriction sites are labeled to indicate the cleavage site. For more information on the CMV promoter, see page 17. The multiple cloning site has been confirmed by sequencing and functional testing.



Transfecting Mammalian Cells

Introduction

General information is provided below for transfection of mammalian cells with the pShooter™ vectors. Positive control vectors are supplied with each vector to optimize transfection conditions for your cell line. pShooter™ vectors have been tested in CHO and COS cells. A sample transfection is provided on page 11 for CHO cells.

Preparing the Plasmid

Once you have confirmed that your gene is in the correct reading frame, prepare plasmid DNA for transfection. 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 DNA using the PureLink® MidiPrep Kit (up to 150 µg, see page 26 for ordering) or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (e.g. COS, CHO), 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). We offer a wide variety of transfection reagents including Lipofectamine® 2000 for mammalian transfection (see page 26 for ordering). For more information, call Technical Support (see page 27) or visit www.lifetechnologies.com.

Expressing Your Fusion Protein

No matter which method of transfection you elect to use, it is very important to perform a time course to optimize expression and targeting of your particular protein. Be sure to transfect enough cells to collect time points, particularly if you are using immunofluorescence or a functional assay.

Methods of Detection

There are a variety of methods for detection, depending on what protein you are expressing and targeting.

- **Visual Method**. If you want to be sure that your protein is targeting to the correct location, use immunofluorescence (see page 12).
- **Functional Assay**. If you are targeting a protein that inhibits or alters the function of another protein, you may have a visual assay (e.g. changes in cell morphology) or an enzymatic assay.

Continued on next page

Stable Transfection

For stable transfection, the pShooter™ vectors contain the resistance factor to G418. G418 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 G418 (Southern and Berg, 1982).

G418 (Neomycin) Selection Guidelines

G418 is available for purchase (contact Technical Support for ordering information). Use as follows:

- Prepare G418 in a buffered solution (e.g. 100 mM HEPES, pH 7.3).
- Test varying concentrations of G418 on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to G418.
- Use 100 to 1000 µg/mL of G418 in complete medium.
- Calculate concentration based on the amount of active drug (check the lot label).

Cells will divide once or twice in the presence of lethal doses of G418, so the effects of the drug take several days to become apparent. Complete selection can take from 3 to 6 weeks of growth in selective medium.

Linearizing Vectors for Stable Transfection

While linearizing a plasmid is not necessary to obtain stable transfectants, it will ensure that the vector does not integrate in a way that disrupts the gene of interest. The table below lists possible restriction enzymes you could use to linearize your particular construct.

Vector	Sites	Location
All vectors	*Pvu* I, *Sca* I	Ampicillin resistance gene
	Kpn I, *Eco*R I	5´ end of CMV promoter

Detecting Fusion Proteins

Introduction

To ensure that your protein is targeted correctly, it is important to visualize its cellular location. Inclusion of the *c-myc* epitope allows detection by immunofluorescence although you can use antibody to your own protein. A basic protocol is included for your convenience. Other protocols may be appropriate.

Detecting Fusion Proteins

Antibodies to the *c-myc* epitope are available for purchase and can be used to detect expression of your fusion protein by immunofluorescence (see below) or western blot. **Note that the *c-myc* epitope will add an additional 1.5 kDa to your protein.** The table below describes the antibodies available and ordering information. The amount supplied is sufficient for 25 westerns and 2–3 immunofluorescence experiments.

Antibody	Purpose	Catalog no.
Anti-*myc*	Detects 10 amino acid epitope derived from the *c-myc* protein (Evans *et al.*, 1985)	R950-25
Anti-*myc*-HRP	See above. Provided as an HRP conjugate for time-saving detection.	R951-25

Basic Immunofluorescent Labeling of Cells

Antibodies can be used for immunofluorescence using standard techniques (Ausubel et al., 1994). A basic protocol is supplied below for adherent cells. For more information, refer to Chapter 14.6 in *Current Protocols in Molecular Biology*.

1. Cool the cells on ice. (Culture cells in a 3- to 5-cm dish. Cells should be confluent or as close to confluent as possible).
2. Aspirate off the culture medium and wash the cells with 4°C PBS.
3. Remove PBS and fix cells for either 30 minutes in 2% paraformaldehyde/0.1% Triton X-100 or 15 minutes in 100% methanol at -20°C. **Note**: Be sure to wash the cells thoroughly with methanol or they will freeze.
4. Remove fixative and wash the cells twice with cold PBS (~5 minutes/wash).
5. Dilute primary antibody in PBS to a final concentration of 5 to 10 µg/mL. Prepare enough antibody to cover cells.
6. Centrifuge antibody for 2 minutes at 13,500 × *g* (4°C) to precipitate any particulate matter.
7. Carefully layer primary antibody onto the cells until they are just covered and incubate for 1 hour at 4°C.
8. Remove antibody and wash four times with cold PBS (~5 minutes/wash).
9. Dilute labeled secondary antibody in PBS to a final concentration of 5-10 µg/mL. Prepare enough antibody to cover cells.
10. Centrifuge antibody for 2 minutes at 13,500 × *g* (4°C) to precipitate any particulate matter.
11. Layer secondary antibody over cells and incubate for 1 hour at 4°C.
12. Remove antibody and wash four times with cold PBS (~5 minutes/wash). Store cells in PBS. Analyze cells by fluorescence immediately; or, cover dishes, wrap in aluminum foil, and refrigerate. Be sure to examine preparations within 24 hours or the fluorescence will fade.

Continued on next page

Detection of Fusion Proteins, Continued

Patterns of Expression

Transformation of the vector expressing your gene of interest or the control vectors should give the following expression patterns using immunofluorescence or fluorescence (SuperGFP).

Cytoplasmic Expression: A number of different distributions may be observed. Some cells will show a typical diffuse pattern throughout the cytoplasm while others will show a puntiform distribution. In cases where greater accumulation of intracellular protein is seen, a "donut-like" pattern may also be seen. Examples of these cytoplasmic distributions are found in Persic, et al, 1997a. We have only observed the typical diffuse pattern with the GFP control.

Nuclear expression: Recombinant proteins should be primarily localized to the nucleus.

Mitochondrial expression: A punctate pattern will be apparent indicating proper targeting to the mitochondria.

ER expression: The ER is a reticular network found throughout the cell and normally appears as a vesicular structure in immunofluorescence. In some cases brighter areas will be visible indicating movement into the Golgi apparatus, located near the nucleus. This can be confirmed by staining with rhodamine-conjugated wheat germ lectin (Virtanen et al., 1980). The ER retention signal allows rescue from the Golgi apparatus so in most cases, ER-targeted proteins should only appear minimally in the Golgi.

If you have trouble expressing and targeting your protein, read the section on the positive control vectors below and the **Troubleshooting** section on page 14.

Using the Positive Controls

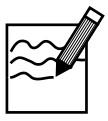
Each of the pShooter™ vectors described in this manual is also provided with a control vector expressing SuperGFP. These vectors may be used to:

- Optimize transfection conditions for your cell line
- Confirm that the targeting signals function properly in your cell line

For more information on the control vectors, see pages 22–25. For information on detection of SuperGFP, see page 15.

Troubleshooting

Problem	Reason	Solution
No targeting observed	Low expression levels	Could be a variety of reasons. Check for expression by western blot. You may have to optimize transfection conditions (use the SuperGFP control vector to evaluate transfection). Many of the other solutions below may help.
	No expression of your protein	Check for expression by western blot. If your protein is not expressed, sequence your construct to confirm that it is in frame with the targeting sequence.
	Cell line may not recognize targeting signal	Check for targeting using the appropriate GFP control vector.
Non-specific labeling	The *c-myc* tag is derived from an endogenous protein (*c-myc*)	Transfect with the empty vector (negative control) and assay for immunofluorescence. You may need to use a different tag or use antibody to your protein.
Pattern of cytoplasmic expression is not diffuse	Protein is not very soluble or is normally expressed in another compartment	Assay earlier after transfection. Targeting proteins to a compartment other than the normal compartment may change disulfide bond formation and solubility characteristics.
Difficulty expressing protein in stable clones	Protein is toxic when redirected to another compartment	Selection of stable clones may lead to down regulation of the protein. Try a different promoter for expression.
	Continuous culture may lead to loss of protein expression	Remember to prepare an early set of back-up stocks.



Note

Some proteins (e.g. antibodies expressed intracellularly) may give a very good immunofluorescent signal, but may not be detectable in a western blot. This may be due to aggregation and/or precipitation of the antibody, so be sure your SDS-PAGE samples are well solubilized.

Detection of GFP

Introduction

SuperGFP has been optimized for expression in *E. coli* and mammalian cells. Fluorescent yield is >40-fold over wild-type GFP, yet it has the same excitation maxima (395 nm and 478 nm for primary and secondary excitation) and emission maxima (507 nm). Guidelines for detection and optimization of expression are described below.

Construct of the Control Vectors

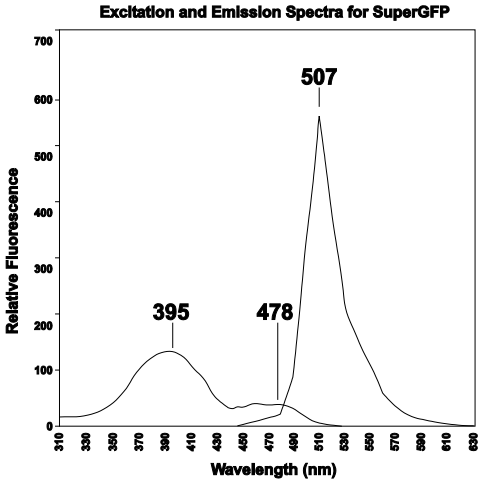
The control vectors were synthesized by amplifying a 716 bp fragment from pαGFP (Crameri et al., 1996) using oligomers that introduced a *Pst* I site at the 5´ end and a *Not* I site at the 3´ end of SuperGFP. In addition each of the oligomers was specifically designed to clone in frame with the targeting sequence and/or the *c-myc* epitope.

Detecting Fluorescence

To detect fluorescent cells, it is important to pick the best filter set to optimize detection. The primary excitation peak of SuperGFP is at 395 nm. There is a secondary excitation peak at 478 nm. Excitation at these wavelengths yields a fluorescent emission peak with a maximum at 507 nm (see below).

Use of the best filter set will ensure that the optimal regions of the SuperGFP spectra are excited and passed (emitted). For example, the FITC filter set that we use excites SuperGFP with light from 460 to 490 nm, which covers the secondary excitation peak. The filter set passes light from 515 to 550, allowing detection of most of the GFP fluorescence. Standard FITC filters easily suit most purposes; however, it is important to keep in mind that fluorescence will be affected by the sample assayed and the filter you choose.

For general information about GFP fluorescence and detection, refer to Current Protocols in Molecular Biology.



Detecting Transfected Cells

After transfection, allow the cells to recover for 24 to 48 hours before assaying for fluorescence. **Note**: Most media fluoresce because of the presence of riboflavin (Zylka and Schnapp, 1996) and may interfere with detection of SuperGFP fluorescence. Medium can be removed and replaced with PBS to alleviate this problem.

Estimate the total number of cells before assaying for fluorescence. Then check your plate for fluorescent cells. You can use fluorescence to estimate transfection efficiency and normalize any subsequent assay for your gene of interest.

Optimizing Expression

It is recommended that a time course be performed to determine the optimal time to assay for transient expression of GFP. **Optimal times may vary from 12 to 96 hours from the time of transfection depending on cell line.**

Appendix

Features of pCMV/*myc* Plasmids

Table

The table below summarizes the features of the pShooter™ vectors. These vectors were derived from pcDNA3. Features that are unique to one vector are noted.

Feature	Benefit
Immediate-early CMV promoter	Permits efficient, high-level expression of your recombinant protein (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987). For more detailed information on this promoter, see page 17.
Mitochondrial targeting sequence pCMV/*myc*/mito only	Allows efficient targeting to the mitochondria. Isolated from subunit VIII of human cytochrome c oxidase (Rizzuto et al., 1992).
ER signal peptide pCMV/*myc*/ER only	Directs the protein of interest to the ER for retention in the ER or secretion. This is the signal peptide from a mouse V_h chain (Kabat et al., 1987).
Multiple cloning site	Allows insertion of your gene.
Nuclear targeting sequence pCMV/*myc*/nuc only	Permits efficient targeting of your protein to the nucleus. Sequence is triplicated to ensure proper localization. Isolated from SV40 large T antigen (Fisher-Fantuzzi and Vesco, 1988).
c-myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of your recombinant protein by immunofluorescence with the Anti-*myc* Antibody (see page 26) (Evans *et al.*, 1985)
ER retention signal pCMV/*myc*/ER only	Permits retention of your protein in the ER (Munro and Pelham, 1987).
TAG termination codon	For efficient termination of translation.
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 (i.e. COS). *Nco* I site removed by site-directed mutagenesis.
Neomycin (G418) resistance gene	Selection of stable transfectants in mammalian cells (Southern and Berg, 1982). Tn*5* sequence removed and the Kozak sequence improved by PCR at the 5´ end of the ORF. *Nco* I, *Pst* I, and *Bss*H II sites removed by site-directed mutagenesis.
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA.
pUC origin	High-copy number replication and growth in *E. coli. Apa*L I site removed by site-directed mutagenesis.
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli. Apa*L I site removed by site-directed mutagenesis.

CMV Promoter

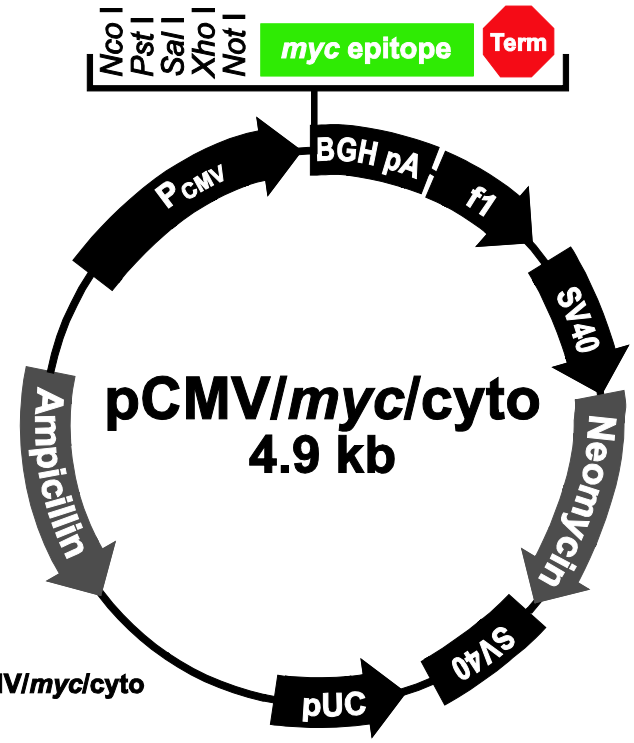
Description

The diagram below shows all the features of the CMV promoter used in the pShooter™ vectors (Persic et al., 1997a). The original sequence has been changed to remove the *Mlu* I, *Spe* I, *Sac* I, *Sna*B I and *Nco* I restriction sites. In addition, *Eco*R I and *Pml* I were introduced by PCR. The CMV promoter can be excised using *Kpn* I or *Eco*R I and *Pml* I or *Nco* I.

pCMV/*myc*/cyto Map

Map

The figure below summarizes the features of pCMV/*myc*/cyto. The nucleotide sequence for pCMV/*myc*/cyto is available for downloading from www.lifetechnologies.com or from Technical Support (see page 27).



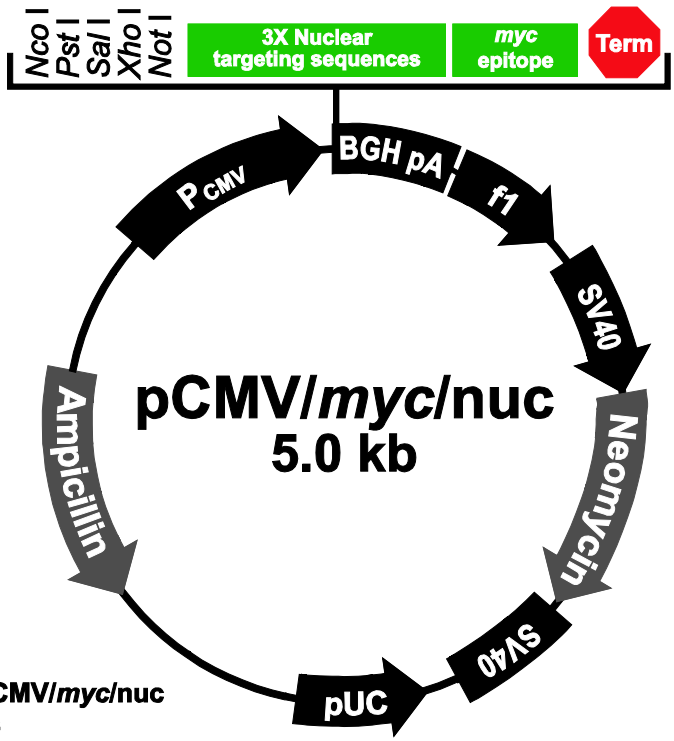
Comments for pCMV/*myc*/cyto
4883 nucleotides

CMV immediate-early promoter: bases 12-627
CMV priming site: bases 544-564
Multiple cloning site: bases 622-673
myc epitope: bases 675-704
BGH Reverse priming site: bases 740-757
BGH polyadenylation sequence: bases 739-953
f1 origin: bases 1016-1429
SV40 promoter/origin: bases 1494-1801
Neomycin (G418) resistance gene (ORF): bases 1813-2607
SV40 polyadenylation sequence: bases 2623-2862
pUC origin: bases 3047-3720
Ampicillin resistance gene (ORF): 3865-4725

pCMV/*myc*/nuc Map

Map

The figure below summarizes the features of pCMV/*myc*/nuc. The nucleotide sequence for pCMV/*myc*/nuc is available for downloading from www.lifetechnologies.com or from Technical Support (see page 27).



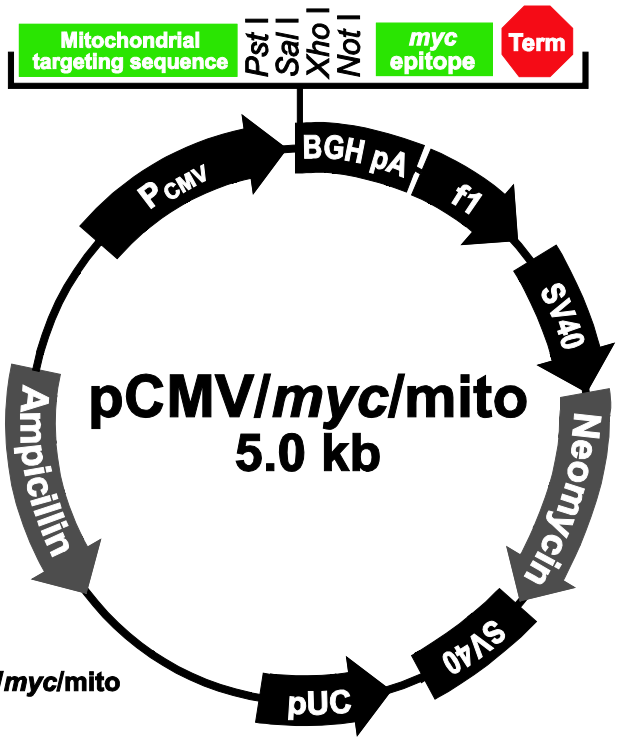
Comments for pCMV/*myc*/nuc
4999 nucleotides

CMV immediate-early promoter: bases 12-627
CMV priming site: bases 544-564
Multiple cloning site: bases 654-705
Nuclear localization signal: bases 707-730; 731-754; 755-778
myc epitope: bases 791-820
BGH Reverse priming site: bases 856-873
BGH polyadenylation sequence: bases 855-1069
f1 origin: bases 1132-1545
SV40 promoter/origin: bases 1610-1921
Neomycin (G418) resistance gene (ORF): bases 1929-2723
SV40 polyadenylation sequence: bases 2739-2978
pUC origin: bases 3163-3836
Ampicillin resistance gene (ORF): 3981-4841

pCMV/*myc*/mito Map

Map

The figure below summarizes the features of pCMV/*myc*/mito. The nucleotide sequence for pCMV/*myc*/mito is available for downloading from www.lifetechnologies.com or from Technical Support (see page 27).



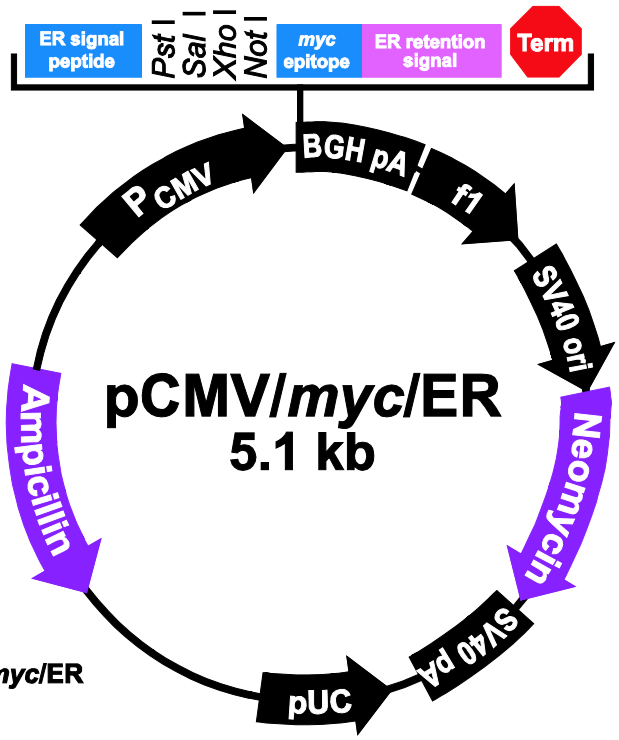
Comments for pCMV/*myc*/mito
4967 nucleotides

CMV immediate-early promoter: bases 12-627
CMV priming site: bases 544-564
Mitochondrial targeting sequence: bases 636-722
Multiple cloning site: bases 723-757
myc epitope: bases 759-788
BGH Reverse priming site: bases 824-841
BGH polyadenylation sequence: bases 823-1037
f1 origin: bases 1100-1513
SV40 promoter/origin: bases 1578-1889
Neomycin (G418) resistance gene (ORF): bases 1897-2691
SV40 polyadenylation sequence: bases 2707-2946
pUC origin: bases 3131-3804
Ampicillin resistance gene (ORF): 3949-4809

pCMV/*myc*/ER Map

Map

The figure below summarizes the features of pCMV/*myc*/ER. The nucleotide sequence for pCMV/*myc*/ER is available for downloading from www.lifetechnologies.com or from Technical Support (see page 27).



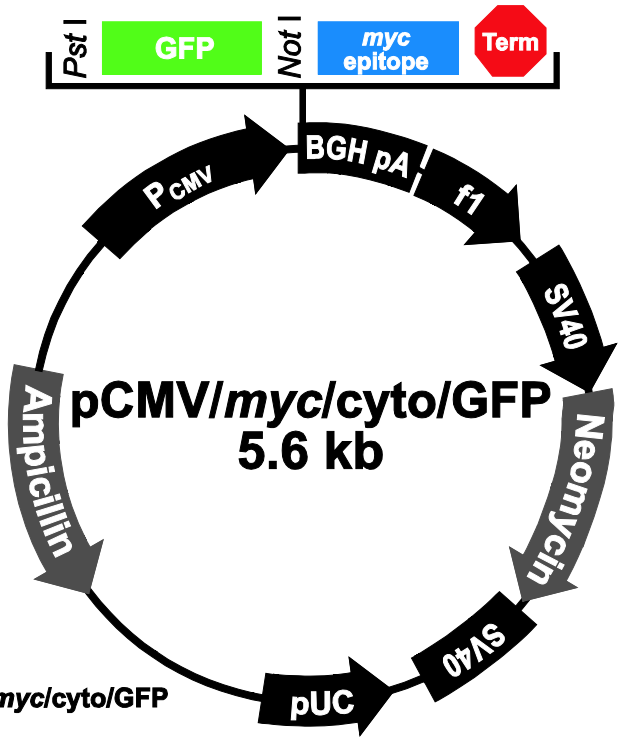
Comments for pCMV/*myc*/ER
5066 nucleotides

CMV immediate-early promoter: bases 12-627
pCMV priming site: bases 544-564
ER Signal sequence (exon 1): bases 656-700
Intron: bases 701-782
ER Signal sequence (exon 2): bases 783-794
Multiple cloning site: bases 804-838
myc epitope: bases 840-869
ER retention signal sequence: bases 882-899
BGH Reverse priming site: bases 923-940
BGH polyadenylation sequence: bases 922-1136
f1 origin: bases 1199-1612
SV40 promoter/origin: bases 1677-1988
Neomycin (G418) resistance gene (ORF): bases 1996-2790
SV40 polyadenylation sequence: bases 2806-3045
pUC origin: bases 3230-3903 (opposite strand)
Ampicillin resistance gene (ORF): 4048-4908 (opposite strand)

pCMV/*myc*/cyto/GFP Map

Map

The figure below summarizes the features of pCMV/*myc*/cyto/GFP. The nucleotide sequence for pCMV/*myc*/cyto/GFP is available for downloading from www.lifetechnologies.com or from Technical Support (see page 27).



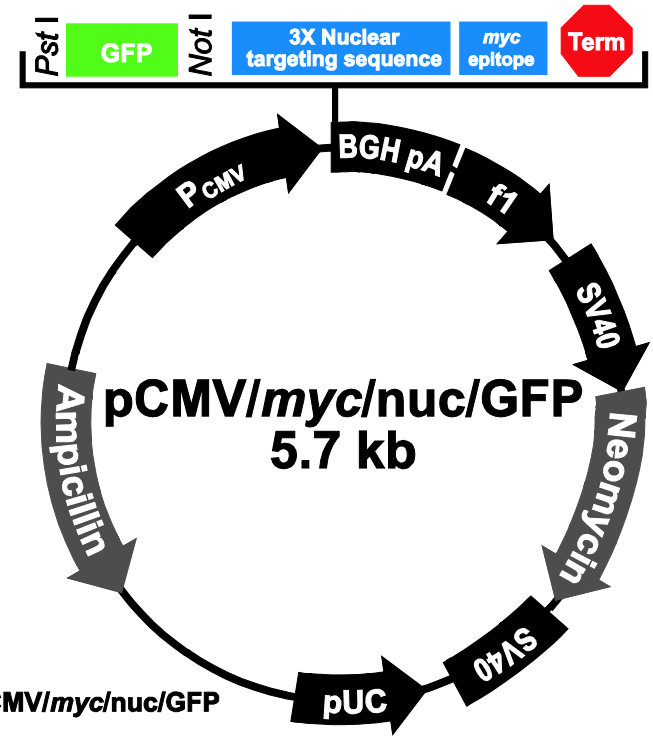
Comments for pCMV/*myc*/cyto/GFP
5611 nucleotides

CMV immediate-early promoter: bases 12-627
CMV priming site: bases 544-564
GFP ORF: bases 667-1393
myc epitope: bases 1403-1432
BGH Reverse priming site: bases 1468-1485
BGH polyadenylation sequence: bases 1467-1681
f1 origin: bases 1744-2157
SV40 promoter/origin: bases 2222-2533
Neomycin (G418) resistance gene (ORF): bases 2541-3335
SV40 polyadenylation sequence: bases 3351-3590
pUC origin: bases 3775-4448
Ampicillin resistance gene (ORF): 4593-5453

pCMV/*myc*/nuc/GFP Map

Map

The figure below summarizes the features of pCMV/*myc*/nuc/GFP. The nucleotide sequence for pCMV/*myc*/nuc/GFP is available for downloading from www.lifetechnologies.com or from Technical Support (see page 27).



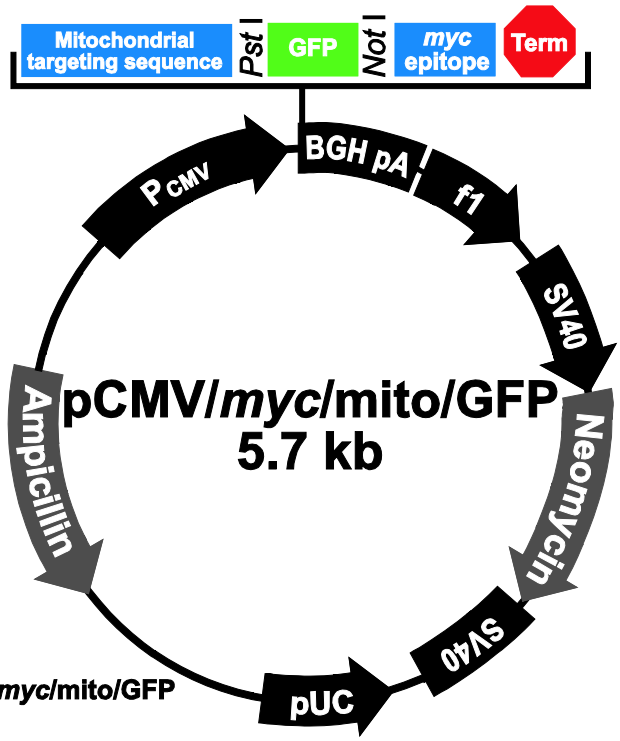
Comments for pCMV/*myc*/nuc/GFP
5695 nucleotides

CMV immediate-early promoter: bases 12-627
CMV priming site: bases 544-564
GFP ORF: bases 677-1393
Nuclear localization signal (3X): bases 1403-1426, 1427-1450, 1451-1474
myc epitope: bases 1487-1516
BGH Reverse priming site: bases 1552-1569
BGH polyadenylation sequence: bases 1551-1765
f1 origin: bases 1828-2241
SV40 promoter/origin: bases 2306-2617
Neomycin (G418) resistance gene (ORF): bases 2625-3419
SV40 polyadenylation sequence: bases 3435-3674
pUC origin: bases 3859-4532
Ampicillin resistance gene (ORF): 4677-5537

pCMV/*myc*/mito/GFP Map

Map

The figure below summarizes the features of pCMV/*myc*/mito/GFP. The nucleotide sequence for pCMV/*myc*/mito/GFP is available for downloading from www.lifetechnologies.com or from Technical Support (see page 27).



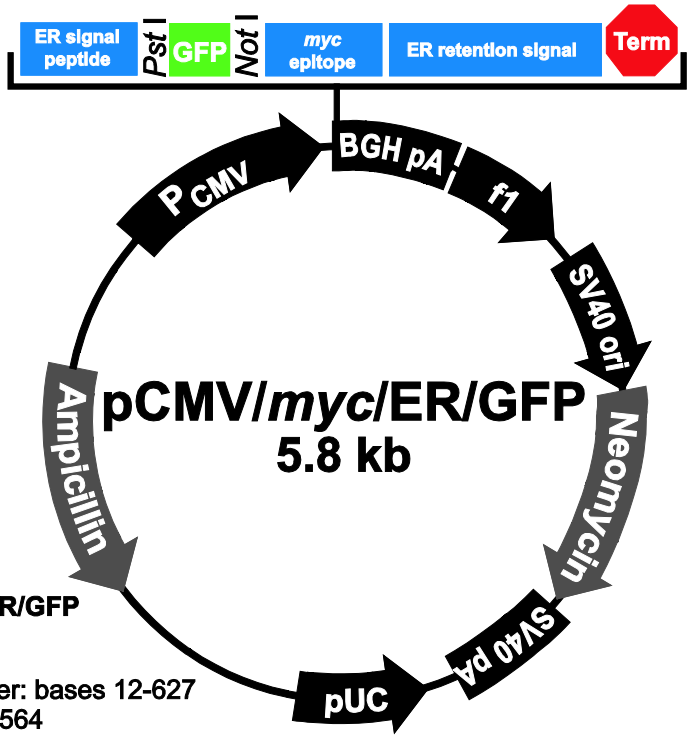
Comments for pCMV/*myc*/mito/GFP
5663 nucleotides

CMV immediate-early promoter: bases 12-627
CMV priming site: bases 544-564
Mitochondrial targeting sequence: bases 636-722
GFP ORF: bases 729-1445
myc epitope: bases 1455-1484
BGH Reverse priming site: bases 1520-1537
BGH polyadenylation sequence: bases 1519-1733
f1 origin: bases 1796-2209
SV40 promoter/origin: bases 2265-2585
Neomycin (G418) resistance gene (ORF): bases 2593-3387
SV40 polyadenylation sequence: bases 3403-3642
pUC origin: bases 3827-4500
Ampicillin resistance gene (ORF): 4645-5505

pCMV/myc/ER/GFP Map

Map

The figure below summarizes the features of pCMV/myc/ER/GFP. The nucleotide sequence for pCMV/myc/ER/GFP is available for downloading from www.lifetechnologies.com or from Technical Support (see page 27).



Comments for pCMV/*myc*/ER/GFP
5762 nucleotides

CMV immediate-early promoter: bases 12-627
CMV priming site: bases 544-564
Secretory leader (exon 1): bases 656-700
Intron: bases 701-782
Secretory leader (exon 2): bases 783-794
GFP portion of fusion: bases 799-1526
myc epitope: bases 1536-1565
Endoplasmic reticulum signal sequence: bases 1578-1595
BGH Reverse priming site: bases 1619-1636
BGH polyadenylation sequence: bases 1678-1832
f1 origin: bases 1895-2308
SV40 promoter/origin: bases 2373-2684
Neomycin (G418) resistance gene (ORF): bases 2692-3486
SV40 polyadenylation sequence: bases 3502-3741
pUC origin: bases 3926-4599 (opposite strand)
Ampicillin resistance gene (ORF): 4744-5604 (opposite strand)

Accessory Products

Introduction

The products listed in this section are intended for use with the pFLD vectors. For more information, refer to www.lifetechnologies.com or call Technical Support (see page 27).

Item	Quantity	Catalog no.
Electrocomp™ TOP10F´	5 × 80 µL	C665-55
One Shot® TOP10F´ (chemically competent cells)	21 × 50 µL	C3030-03
PureLink® HiPure Midiprep Kit	25 preps	K2100-04
Lipofectamine® 2000	1.5 mL	11668-019
	0.75 mL	11668-027

Products Available Separately

Primers to sequence your insert in the pCMV/*myc* vectors and antibodies to the *c-myc* epitope are available for purchase. pShooter™ vectors containing the EF1α promoter are also available. You may find that one promoter expresses your protein better than the other in your particular cell line. See the table below for ordering information.

Vector	Amount	Catalog no.
BGH Reverse Primer	2 µg	N575-02
Anti-*myc* Antibody	25 westerns	R950-25
Anti-*myc*-HRP Antibody	25 westerns	R951-25
pEF/*myc*/nuc pEF/*myc*/nuc/GFP	20 µg	V891-20

Technical Support

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- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

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Limited Use Label License: (Cycle 3) Mutant GFP

The (cycle 3) mutant GFP gene was produced by Maxygen, Inc. using the DNA shuffling technology. Crameri, A., Whitehorn,E.A., and Stemmer, W.P.C. (1996) Improved Green Fluorescent Protein by Molecular Evolution Using DNA Shuffling. *Nature Biotechnology*, 14: 315-319.

Limited Use Label License: GFP

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