USER GUIDE

invitrogen™
by life technologies™

pcDNA™5/FRT Vector

Expression vector designed for use with the Flp-In™ System

Catalog Number V6010-20

Revision Date 12 September 2012

Publication Part Number 25-0307

MAN0000131

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

life technologies™

Contents

Kit Contents and Storage

Shipping/Storage

The pcDNA™5/FRT Vectors are shipped on wet ice. Upon receipt, **store at –20°C.**

Kit Contents

The following vectors are provided with pcDNA™5/FRT:

Vector	Quantity	Contents
pcDNA™5/FRT	20 µg	40 µL of 0.5 µg/µL pcDNA™5/FRT in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
pcDNA™5/FRT/CAT	20 µg	40 µL of 0.5 µg/µL pcDNA™5/ FRT/CAT in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.

Product Use

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

Accessory Products

Accessory Products

Additional products available from Life Technologies are listed below. For more information, visit our website at **www.lifetechnologies.com** or contact **Technical Support** (page 10).

Product	Amount	Catalog No.
T7 Promoter Primer	2 µg, lyophilized	N560–02
Zeocin®	1 g 5 g	R250–01 R250–05
Hygromycin	1 g	R220–05
pFRT/*lac*Zeo	20 µg, suspended as 40 µL of 0.5 µg/µL pFRT/*lac*Zeo in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.	V6015–20
pFRT/*lac*Zeo2	20 µg, suspended as 40 µL of 0.5 µg/µL pFRT/*lac*Zeo2 in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.	V6022–20
pOG44	20 µg, suspended as 40 µL of 0.5 µg/µL pOG44 in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.	V6005–20
One Shot® Kit (TOP10 **Chemically Competent** Cells)	10 reactions	C4040–10
	20 reactions	C4040–03
	40 reactions	C4040–06
One Shot® Kit (TOP10 **Electrocompetent** Cells)	10 reactions	C4040–50
	20 reactions	C4040–52

Flp-In™ Expression Vectors

Additional Flp-In™ expression vectors are available from Life Technologies. For more information about the features of each vector, visit our website at **www.lifetechnologies.com** or contact **Technical Support** (page 10).

Product	Amount	Catalog No.
pcDNA™5/FRT/V5-His TOPO® TA Expression Kit	1 kit	K6020–01
pSecTag/FRT/V5-His TOPO® TA Expression Kit	1 kit	K6025–01
pEF5/FRT/V5 Directional TOPO® Expression Kit	1 kit	K6035–01
pEF5/FRT/V5-DEST Gateway® Vector Pack	6 µg, supplied as 40 µL of 150ng/µL vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0	V6020–20

Continued on next page

Flp-In™ Host Cell Lines

For your convenience, Life Technologies has available several mammalian Flp-In™ host cell lines that stably express the *lacZ-Zeocin*® fusion gene from pFRT/*lac*Zeo or pFRT/*lac*Zeo2. Each cell line contains a single integrated FRT site as confirmed by Southern blot analysis. The cell lines should be maintained in medium containing Zeocin®. For more information, visit our website at **www.lifetechnologies.com** or contact **Technical Support** (page 10).

Cell Line	Amount	Catalog No.
Flp-In™-293	3×10^6 cells, frozen	R750–07
Flp-In™-CV-1	3×10^6 cells, frozen	R752–07
Flp-In™-CHO	3×10^6 cells, frozen	R758–07
Flp-In™-BHK	3×10^6 cells, frozen	R760–07
Flp-In™-3T3	3×10^6 cells, frozen	R761–07
Flp-In™-Jurkat	3×10^6 cells, frozen	R762–07

Introduction

Overview

Introduction

pcDNA™5/FRT is a 5.1 kb expression vector designed for use with the Flp-In™ System (Catalog nos. K6010-01 and K6010-02) available from Life Technologies. When cotransfected with the pOG44 Flp recombinase expression plasmid into a Flp-In™ mammalian host cell line, the pcDNA™5/FRT vector containing the gene of interest is integrated in a Flp recombinase-dependent manner into the genome. The vector contains the following elements:

- The human cytomegalovirus (CMV) immediate-early enhancer/promoter for high-level constitutive expression of the gene of interest in a wide range of mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
- Multiple cloning site with 10 unique restriction sites to facilitate cloning the gene of interest
- FLP Recombination Target (FRT) site for Flp recombinase-mediated integration of the vector into the Flp-In™ host cell line (see next page for more information)
- Hygromycin resistance gene for selection of stable cell lines (Gritz & Davies, 1983)

The control plasmid, pcDNA™5/FRT/CAT, is included for use as a positive control for transfection and expression in the Flp-In™ host cell line of choice.

For more information about the Flp-In™ System, the pOG44 plasmid, and generation of the Flp-In™ host cell line, refer to the Flp-In™ System manual. The Flp-In™ System manual is supplied with the Flp-In™ Complete or Core Systems, but is also available for downloading from our Website (**www.lifetechnologies.com**) or by contacting Technical Support (see page 10).

A Note About pcDNA™5/FRT

The pcDNA™5/FRT vector contains a single FRT site immediately upstream of the hygromycin resistance gene for Flp recombinase-mediated integration and selection of the pcDNA™5/FRT plasmid following cotransfection of the vector (with pOG44) into Flp-In™ mammalian host cells. The FRT site serves as both the recognition and cleavage site for the Flp recombinase and allows recombination to occur immediately adjacent to the hygromycin resistance gene. The Flp recombinase is expressed from the pOG44 plasmid. For more information about the FRT site and recombination, see the next page. For more information about pOG44, refer to the Flp-In™ System manual.

Important

The hygromycin resistance gene in pcDNA™5/FRT lacks a promoter and an ATG initiation codon; therefore, transfection of the pcDNA™5/FRT plasmid alone into mammalian host cells will **not** confer hygromycin resistance to the cells. The SV40 promoter and ATG initiation codon required for expression of the hygromycin resistance gene are integrated into the genome (in the Flp-In™ host cell line) and are only brought into the correct proximity and frame with the hygromycin resistance gene through Flp recombinase-mediated integration of pcDNA™5/FRT at the FRT site. For more information about the generation of the Flp-In™ host cell line and details of the Flp-In™ System, refer to the Flp-In™ System manual.

Continued on next page

Overview, Continued

Flp Recombinase-Mediated DNA Recombination

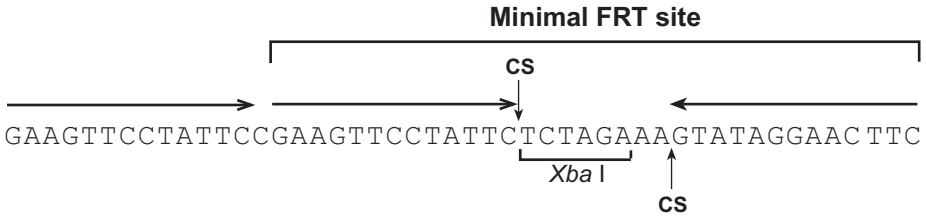
In the Flp-In™ System, integration of your pcDNA™5/FRT expression construct into the genome occurs via Flp recombinase-mediated intermolecular DNA recombination. The hallmarks of Flp-mediated recombination are listed below.

- Recombination occurs between specific FRT sites (see below) on the interacting DNA molecules.
- Recombination is conservative and requires no DNA synthesis; the FRT sites are preserved following recombination and there is minimal opportunity for introduction of mutations at the recombination site.
- Strand exchange requires only the small 34 bp minimal FRT site (see below).

For more information about the Flp recombinase and conservative site-specific recombination, refer to published reviews (Craig, 1988; Sauer, 1994).

FRT Site

The FRT site, originally isolated from *Saccharomyces cerevisiae*, serves as a binding site for Flp recombinase and has been well-characterized (Gronostajski & Sadowski, 1985; Jayaram, 1985; Sauer, 1994; Senecoff *et al.*, 1985). The minimal FRT site consists of a 34 bp sequence containing two 13 bp imperfect inverted repeats separated by an 8 bp spacer that includes an *Xba* I restriction site (see figure below). An additional 13 bp repeat is found in most FRT sites, but is not required for cleavage (Andrews *et al.*, 1985). While Flp recombinase binds to all three of the 13 bp repeats, strand cleavage actually occurs at the boundaries of the 8 bp spacer region (see figure below) (Andrews *et al.*, 1985; Senecoff *et al.*, 1985).



CS = cleavage site

Experimental Outline

The following table outlines the steps required to clone and express your gene of interest in pcDNA™5/FRT.

Step	Action
1	Consult the multiple cloning site diagrammed on page 4 to design your cloning strategy.
2	Ligate your insert into pcDNA™5/FRT and transform into *E. coli*. Select transformants on 50–100 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 sequence to confirm that your gene is cloned in the correct orientation.
5	Cotransfect your pcDNA™5/FRT construct and pOG44 into the Flp-In™ host cell line using your own method of choice and select for hygromycin resistant clones (see the Flp-In™ System manual for more information).
6	Assay for expression of the gene of interest.

Methods

Cloning into pcDNA™5/FRT

Introduction

A diagram is provided on the next page to help you clone your gene of interest into pcDNA™5/FRT. General considerations for cloning and transformation are listed below.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, 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 and maintenance 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 Life Technologies (page v).

Transformation Method

You may use any method of your choice for transformation. Chemical transformation is the most convenient method for many researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of Plasmids

To propagate and maintain the pcDNA™5/FRT and pcDNA™5/FRT/CAT vectors, we recommend using 10 ng of the vector to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™, JM109, or equivalent. Select transformants on LB agar plates containing 50–100 µg/mL ampicillin. Be sure to prepare a glycerol stock of each plasmid for long-term storage (see page 4).

Cloning Considerations

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). 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**

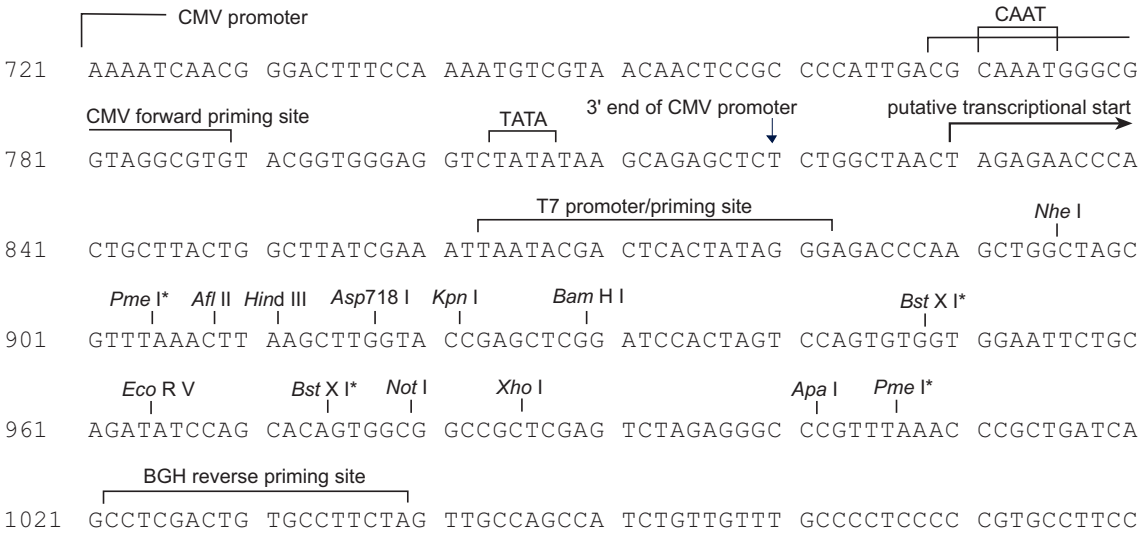
Your insert must also contain a stop codon for proper termination of your gene.

Continued on next page

Cloning into pcDNA™5/FRT, Continued

Multiple Cloning Site of pcDNA™5/FRT

Below is the multiple cloning site for pcDNA™5/FRT. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pcDNA™5/FRT is available for downloading from our website at www.lifetechnologies.com or from Technical Support (page 10).** For a map and a description of the features of pcDNA™5/FRT, refer to the **Appendix**, pages 7–8.



***Note**: there are two *Pme* I sites and two *Bst*X I sites in the polylinker.

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g., TOP10, DH5α™) and select on LB agar plates containing 50–100 µg/mL ampicillin. Select 10–20 clones and analyze for the presence and orientation of your insert.

We recommend that you sequence your construct to confirm that your gene is in the correct orientation for expression and contains an ATG initiation codon and a stop codon. To sequence your construct, we suggest using the T7 Promoter and BGH Reverse primer sequences. See page 4 for sequences and location of primer binding sites.
For your convenience, Life Technologies offers the T7 Promoter Primer (page v) as well as custom primer services. For more information on custom primer services, visit **www.lifetechnologies.com** or contact **Technical Support** (page 10).

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.

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

Transfection

Introduction

Once you have cloned your gene of interest into pcDNA™5/FRT and have prepared clean plasmid preparations of your pcDNA™5/FRT construct and pOG44, you are ready to cotransfect the plasmids into your mammalian Flp-In™ host cell line to generate your stable Flp-In™ expression cell line. We recommend that you include the pcDNA™5/FRT/CAT positive control vector and a mock transfection (negative control) to evaluate your results. General information about transfection and selection is provided below. Specific guidelines and protocols for generation of the Flp-In™ expression cell line can be found in the Flp-In™ System manual.

For detailed information about pOG44 and generation of the Flp-In™ host cell line, refer to the Flp-In™ System manual.

Several Flp-In™ host cell lines which stably express the *lacZ-Zeocin*® fusion gene from pFRT/*lac*Zeo or pFRT/*lac*Zeo2 and which contain a single integrated FRT site are available from Life Technologies (see page vi for ordering information). If you wish to express your gene of interest in 293, CV-1, CHO, 3T3, BHK, or Jurkat cells, may want to use one of Flp-In™ cell lines as the host to establish your stable expression cell line. For more information, visit our website **www.lifetechnologies.com** or contact **Technical Support** (see page 10).

Important

We have observed down-regulation of the viral CMV promoter and subsequent loss of gene expression when pcDNA™5/FRT-based expression constructs are introduced into 3T3 or BHK cells. This behavior is not observed with pEF5/FRT-based expression constructs. If you are generationg Flp-In™ expression cell lines using a 3T3 or BHK host cell line, we recommend that you clone your gene of interest into a pEF5/FRT-based expression plasmid (e.g., pEF5/FRT/V5-D-TOPO® or pEF5/FRT/V5-DEST). For more information, visit our website **www.lifetechnologies.com** or contact **Technical Support** (see page 10).

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 S.N.A.P. ™ MiniPrep Kit (10–15 µg DNA, Catalog No. K1900-01), the S.N.A.P. ™ MidiPrep Kit (10–200 µg DNA, Catalog No. K1910-01), or CsCl gradient centrifugation.

Positive Control

pcDNA™5/FRT/CAT is provided as a positive control vector for mammalian cell transfection and expression (see page 9) and may be used to assay for recombinant protein expression levels in your Flp-In™ expression cell line. Cotransfection of the positive control vector and pOG44 into your Flp-In™ host cell line allows you to generate a stable cell line expressing chloramphenicol acetyl transferase (CAT) at the same genomic locus as your gene of interest. If you have several different Flp-In™ host cell lines, you may use the pcDNA™5/FRT/CAT control vector to compare protein expression levels between the various cell lines.

Continued on next page

Assay for CAT Protein

The CAT protein expressed from the pcDNA™5/FRT/CAT control plasmid is approximately 32 kDa in size. 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). For Western blot analysis, you may use CAT Antiserum available from Life Technologies for detection. Other commercial kits to assay for CAT protein are available.

Hygromycin B

The pcDNA™5/FRT vector contains the hygromycin resistance gene (Gritz & Davies, 1983) for selection of transfectants with the antibiotic, hygromycin B (Palmer *et al.*, 1987). When added to cultured mammalian cells, hygromycin B acts as an aminocyclitol to inhibit protein synthesis. Hygromycin B liquid is supplied with the Flp-In™ Complete System and is also available separately from Life Technologies. For instructions to handle and store hygromycin B, refer to the Flp-In™ System manual.

Determination of Hygromycin Sensitivity

Before generating a stable cell line expressing your protein of interest (Flp-In™ expression cell line), we recommend that you generate a kill curve to determine the minimum concentration of hygromycin required to kill your untransfected Flp-In™ host cell line. Generally, concentrations between 10 and 400 µg/mL hygromycin are required for selection of most mammalian cell lines. General guidelines for performing a kill curve are provided in the Flp-In™ System manual.

Generation of Flp-In™ Expression Cell Lines

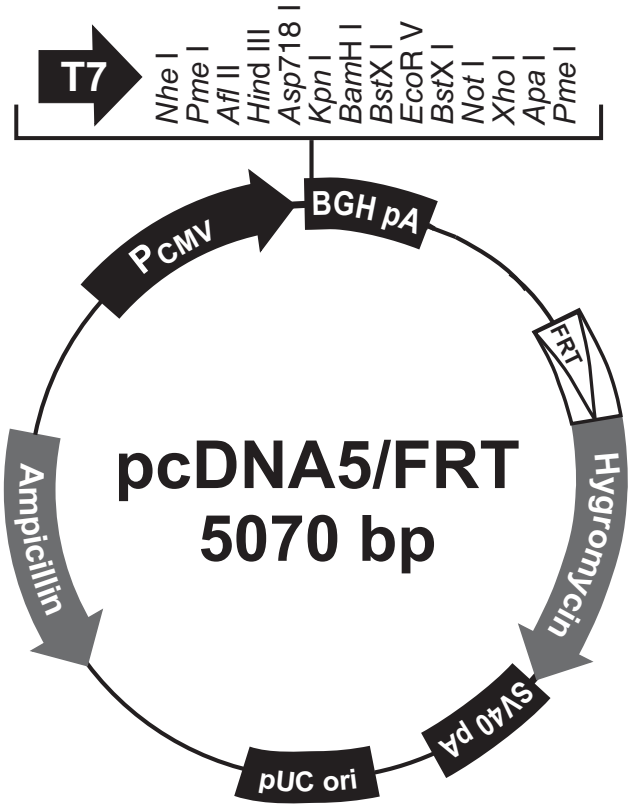
Refer to the Flp-In™ System manual for detailed guidelines and instructions to cotransfect your pcDNA™5/FRT construct and pOG44 into the Flp-In™ host cell line to generate stable Flp-In™ expression cell lines.

Appendix

Map of pcDNA™5/FRT Vector

Map of pcDNA™5/FRT

The figure below summarizes the features of the pcDNA™5/FRT vector. Note that the hygromycin resistance gene lacks a promoter and its native ATG start codon. Transfection of the pcDNA™5/FRT plasmid alone into mammalian cells will **not** confer hygromycin resistance to the cells. **The complete nucleotide sequence for pcDNA™5/FRT is available for downloading from our website at www.lifetechnologies.com or by contacting Technical Support (page 10).**



Comments for pcDNA5/FRT 5070 nucleotides

CMV promoter: bases 232-819
CMV forward priming site: bases 769-789
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1028-1252
FRT site: bases 1536-1583
Hygromycin resistance gene (no ATG): bases 1591-2611
SV40 early polyadenylation signal: bases 2743-2873
pUC origin: bases 3256-3929 (complementary strand)
bla promoter: bases 4935-5033 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4074-4934 (complementary strand)

Features of pcDNA™5/FRT Vector

Features of pcDNA™5/FRT

pcDNA™5/FRT is a 5070 bp vector that expresses your gene of interest under the control of the human CMV promoter. The table below describes the relevant features of pcDNA™5/FRT. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate early promoter	Allows high-level expression of your gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
CMV Forward priming site	Allows sequencing in the sense orientation.
T7 promoter/priming site	Allows in vitro transcription in the sense orientation and sequencing through the insert.
Multiple cloning site	Allows insertion of your gene of interest.
pBGH Reverse priming site	Allows sequencing of the non-coding strand.
Bovine growth hormone (BGH) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Goodwin & Rottman, 1992).
Flp Recombination Target (FRT) site	Encodes a 34 bp (+14 bp of non-essential) sequence that serves as the binding and cleavage site for Flp recombinase (Gronostajski & Sadowski, 1985; Jayaram, 1985; Senecoff *et al.*, 1985).
Hygromycin resistance gene (no ATG)	Allows selection of stable transfectants in mammalian cells (Gritz & Davies, 1983) when brought in frame with a promoter and an ATG initiation codon through Flp recombinase-mediated recombination via the FRT site.
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin	Allows high-copy number replication and growth in *E. coli.*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene.
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli.*

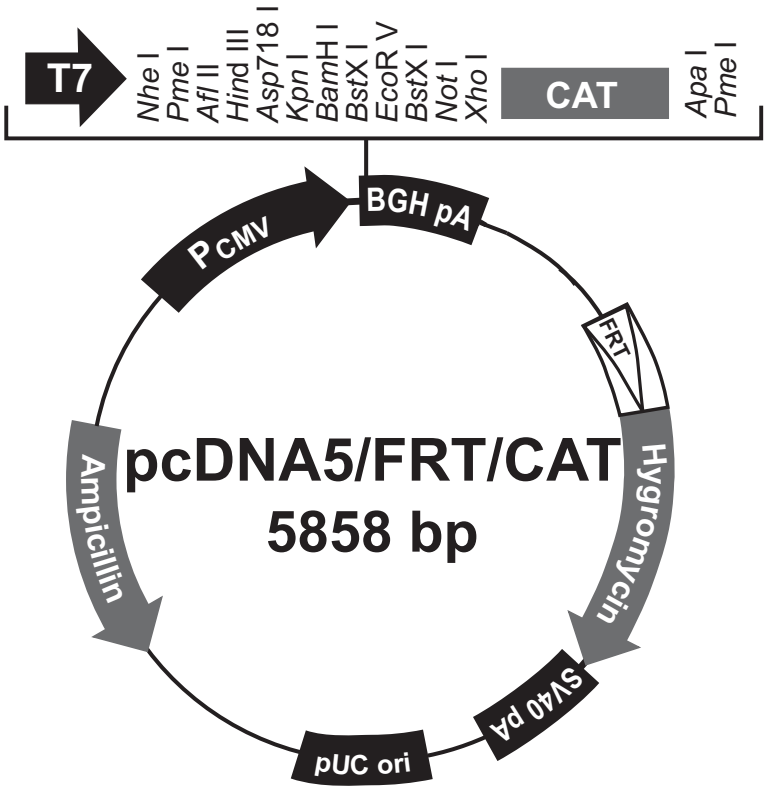
pcDNA™5/FRT/CAT Vector

Description

pcDNA™5/FRT/CAT is a 5858 bp control vector containing the gene for chloramphenicol acetyl transferase (CAT). This vector was constructed by ligating a 0.7 kb *Xho* I-*Apa* I fragment containing the CAT gene into the *Xho* I-*Apa* I site of pcDNA™5/FRT. The CAT protein expressed from pcDNA™5/FRT/CAT is approximately 32 kDa in size.

Map of pcDNA™5/FRT/CAT

The figure below summarizes the features of the pcDNA™5/FRT/CAT vector. **The complete nucleotide sequence for pcDNA™5/FRT/CAT is available for downloading from our website at www.lifetechnologies.com or from Technical Support (page 10).**



Comments for pcDNA5/FRT/CAT
5858 nucleotides

CMV promoter: bases 232-819
CMV forward priming site: bases 769-789
T7 promoter/priming site: bases 863-882
Chloramphenicol acetyl transferase (CAT) gene: bases 1026-1685
BGH reverse priming site: bases 1810-1827
BGH polyadenylation signal: bases 1816-2040
FRT site: bases 2324-2371
Hygromycin resistance gene (no ATG): bases 2379-3399
SV40 early polyadenylation signal: bases 3531-3661
pUC origin: bases 4044-4717 (complementary strand)
bla promoter: bases 5723-5821 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4862-5722 (complementary strand)

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- Obtain information about customer training
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