invitrogen™

Flp-In™ System

For Generating Stable Mammalian Expression Cell Lines by Flp Recombinase-Mediated Integration

Catalog nos. K6010–01, K6010–02

Version E
9 November 2010
25–0306

User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

The Flp-In™ System manual is supplied with the kits listed below. The Core System includes vectors and primers for sequencing. The Complete System includes the Core System plus selection agents. See below for a detailed description of the contents of each Flp-In™ System.

Product	Catalog no.
Flp-In™ Complete System	K6010–01
Flp-In™ Core System	K6010–02

System Components

The following table shows the components associated with each Flp-In™ system listed above.

Components	Catalog no.	
	K6010–01	K6010–02
pcDNA™5/FRT Expression Vector	✓	✓
pcDNA™5/FRT/CAT Positive Control Vector	✓	✓
pOG44 Plasmid Vector	✓	✓
pFRT/*lac*Zeo Target-site Vector	✓	✓
CMV Forward Primer (21-mer)	✓	✓
BGH Reverse Primer (18-mer)	✓	✓
Hygromycin B	✓	
Zeocin™ Selection Reagent	✓	

Shipping/Storage

The components supplied with Catalog nos. K6010–01 and K6010–02 are shipped as described in the table below. Upon receipt, store each component as listed below.
Note: The components of K6010–01 are shipped in 2 boxes. Box 1 contains vectors, primers, and hygromycin. Box 2 contains Zeocin™.

Item	Shipping	Storage
Vectors: • pcDNA™5/FRT Expression Vector • pcDNA™5/FRT/CAT Positive Control Vector • pOG44 Plasmid Vector • pFRT/*lac*Zeo Target-site Vector	Wet ice	Store all vectors at **–20°C**
Primers • CMV Forward Primer (21-mer) • BGH Reverse Primer (18-mer)	Wet ice	Store all primers at **–20°C**
Hygromycin B (K6010–01 only)	Wet ice	Store at **4°C**, protected from light
Zeocin™ (K6040–01 only)	Wet ice	Store at **–20°**C, protected from light

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Kit Contents

Both the Flp-In™ Complete and the Flp-In™ Core Systems include the following components. Note that the vectors are supplied in suspension.

Product	Quantity	Composition
pcDNA™5/FRT Expression Vector	20 ug	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
pcDNA™5/FRT/CAT Positive Control	20 µg	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
pOG44 Expression Vector	20 µg	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
pFRT/*lac*Zeo Vector	20 µg	40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
CMV Forward Primer (21-mer)	2 µg	lyophilized in TE, pH 8.0
BGH Reverse Primer (18-mer)	2 µg	lyophilized in TE, pH 8.0
Zeocin™ (K6010–01 only)	1 g	100 mg/ml
Hygromycin B (K6010–01 only)	1 g	100 mg/ml

Primer Sequences

The sequence of each primer is provided below.

Primer	Sequence	pMoles Supplied
CMV Forward Primer (21-mer)	5´-CGCAAATGGGCGGTAGGCGTG-3´	306
BGH Reverse Primer (18-mer)	5´-TAGAAGGCACAGTCGAGG-3´	358

Accessory Products

Additional Products

The products listed in this section are intended for use with he Flp-In™ Systems For more information, refer to our web site at www.invitrogen.com or contact **Technical Support** (page 26).

Product	Amount	Catalog no.
pFRT/*lac*Zeo	20 µg, (supplied as 40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0)	V6015–20
pFRT/*lac*Zeo2	20 µg, (supplied as 40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0)	V6022–20
pOG44	20 µg, (supplied as 40 µl of 0.5 µg/µl vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0)	V6005–20
T7 Promoter Primer	2 µg, lyophilized	N560–02
One Shot® Kit	10 reactions	C4040–10
(TOP10 **Chemically Competent** Cells)	20 reactions	C4040–03
	40 reactions	C4040–06
One Shot® Kit	10 reactions	C4040–50
(TOP10 **Electrocompetent** Cells)	20 reactions	C4040–52
S.N.A.P. Miniprep Kit	100 reactions	K1900–01
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100–03
PureLink™ HiPure Plasmid Midiprep Kit	15–200 reactions	K2100–04
Easy-DNA™ Kit		K1800–01
Zeocin™	1 g	R250–01
	5 g	R250–05
Hygromycin B	1 g	R220–05
β-Gal Assay Kit	100 reactions	K1455–01
β-Gal Staining Kit	1 kit	K1465–01
CAT Antiserum*	50 µl	R902–25
Calcium Phosphate Transfection Kit	75 reactions	K2780–01
Lipofectamine™ 2000 Transfection Reagent	15 ml	11668–500
	1.5 ml	11668–019
imMedia™ Amp Agar.	20 pouches	Q601–20

*The amount supplied is sufficient to perform 25 Western blots using 10 ml working solution per reaction.

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Accessory Products, Continued

Flp-In™ Expression Vectors

Additional Flp-In™ expression vectors are available from Invitrogen. For more information about the features of each vector, refer to our web site at www.invitrogen.com or contact **Technical Support** (page 26).

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/ul vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0	V6020–20

Flp-In™ Host Cell Lines

For your convenience, Invitrogen has available several mammalian Flp-In™ host cell lines that stably express the *lac*Z-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 web site at www.invitrogen.com or contact **Technical Support** (see page 26).

Cell Line	Source	Amount	Catalog no.
Flp-In™-293	Human embryonic kidney	1×10^7 cells, frozen	R750–07
Flp-In™-CV-1	African Green Monkey kidney	1×10^7 cells, frozen	R752–07
Flp-In™-CHO	Chinese Hamster ovary	1×10^7 cells, frozen	R758–07
Flp-In™-BHK	Baby hamster kidney	1×10^7 cells, frozen	R760–07
Flp-In™-3T3	Mouse (NIH Swiss) embryonic fibroblast	1×10^7 cells, frozen	R761–07
Flp-In™-Jurkat	Human T-cell leukemia	1×10^7 cells, frozen	R762–07

Introduction

Overview

Introduction

The Flp-In™ System allows integration and expression of your gene of interest in mammalian cells at a specific genomic location. The Flp-In™ System involves introduction of a Flp Recombination Target (FRT) site into the genome of the mammalian cell line of choice. An expression vector containing your gene of interest is then integrated into the genome via Flp recombinase-mediated DNA recombination at the FRT site (O'Gorman *et al.*, 1991).

System Components

The major components of the Flp-In™ System include:

- A Flp-In™ target site vector, pFRT/*lac*Zeo, for generation of a host cell line containing an integrated FRT site (see pages 22–23 for more information).
- An expression plasmid containing a FRT site linked to the hygromycin resistance gene for Flp recombinase-mediated integration and selection of a stable cell line expressing your gene of interest under the control of the human cytomegalovirus (CMV) immediate-early enhancer/promoter.
- A Flp recombinase expression plasmid, pOG44, for expression of the Flp recombinase under the control of the human CMV promoter (see pages 24–25 for further information).
- A control expression plasmid containing the chloramphenicol acetyl transferase (*CAT)* gene, which when cotransfected with pOG44 into your Flp-In™ host cell line, expresses CAT.

For specific information on the expression vector and the corresponding positive control vector containing the *CAT* gene, refer to the pcDNA™5/FRT vector manual.

Advantages of the Flp-In™ System

Use of the Flp-In™ System to generate stable expression cell lines provides a number of advantages as described below:

- Once the Flp-In™ host cell line containing an integrated FRT site has been created, subsequent generation of Flp-In™ cell lines expressing the gene(s) of interest is rapid and efficient.
- The Flp-In™ System allows the generation of isogenic stable cell lines.
- The Flp-In™ System permits polyclonal selection of stable expression cell lines.

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Description of the Flp-In™ System

The Flp-In™ System streamlines the generation of stable mammalian expression cell lines by taking advantage of a *Saccharomyces cerevisiae*-derived DNA recombination system. This DNA recombination system uses a recombinase (Flp) and site-specific recombination (Craig, 1988; Sauer, 1994) to facilitate integration of the gene(s) of interest into a specific site in the genome of mammalian cells.

In the Flp-In™ System, three different vectors are used to generate isogenic stable mammalian cells lines expressing your gene(s) of interest. The first major component of the Flp-In™ System is the pFRT/*lac*Zeo target site vector that is used to generate a Flp-In™ host cell line. The vector contains a *lac*Z-Zeocin™ fusion gene whose expression is controlled by the SV40 early promoter (see the **Appendix**, pages 22–23 for more information). A FRT site has been inserted just downstream of the ATG initiation codon of the *lac*Z-Zeocin™ fusion gene. The FRT site (see page 4 for more information) serves as the binding and cleavage site for the Flp recombinase. The pFRT/*lac*Zeo plasmid is transfected into the mammalian cell line of interest and cells are selected for Zeocin™ resistance. Zeocin™-resistant clones are screened to identify those containing a single integrated FRT site. The resulting Flp-In™ host cell line contains an integrated FRT site and expresses the *lac*Z-Zeocin™ fusion gene (see the diagram, next page).

Note: Integration of the pFRT/lacZeo plasmid into the genome is random.

The second major component of the Flp-In™ System is the pcDNA™5/FRT expression vector into which the gene of interest will be cloned. Expression of the gene of interest is controlled by the human CMV promoter. The vector also contains the hygromycin resistance gene with a FRT site embedded in the 5′ coding region. The hygromycin resistance gene lacks a promoter and the ATG initiation codon. For more information about the pcDNA™5/FRT vector, refer to the vector manual.

The third major component of the Flp-In™ System is the pOG44 plasmid which constitutively expresses the Flp recombinase (Broach *et al.*, 1982; Broach & Hicks, 1980; Buchholz *et al.*, 1996) under the control of the human CMV promoter. For more information about pOG44 and the *FLP* gene, see the **Appendix**, pages 24–25.

The pOG44 plasmid and the pcDNA™5/FRT vector containing your gene of interest are cotransfected into the Flp-In™ host cell line. Upon cotransfection, the Flp recombinase expressed from pOG44 mediates a homologous recombination event between the FRT sites (integrated into the genome and on pcDNA™5/FRT) such that the pcDNA™5/FRT construct is inserted into the genome at the integrated FRT site (see diagram, next page). Insertion of pcDNA™5/FRT into the genome at the FRT site brings the SV40 promoter and the ATG initiation codon (from pFRT/*lac*Zeo) into proximity and frame with the hygromycin resistance gene, and inactivates the *lac*Z-Zeocin™ fusion gene. Thus, stable Flp-In™ expression cell lines can be selected for hygromycin resistance, Zeocin™ sensitivity, lack of β-galactosidase activity, and expression of the recombinant protein of interest (see diagram, next page).

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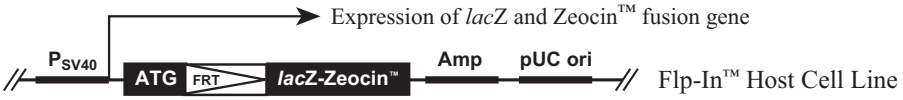
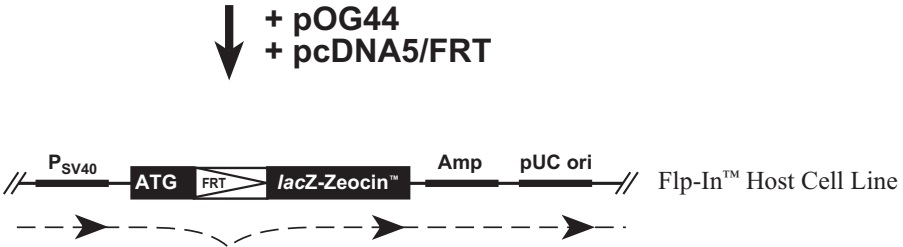
Diagram of the Flp-In™ System

The figure below illustrates the major features of the Flp-In™ System as described on the previous page. For a brief description about FRT sites and the mechanism of Flp-mediated recombination, see the next page and published reviews (Craig, 1988; Sauer, 1994).

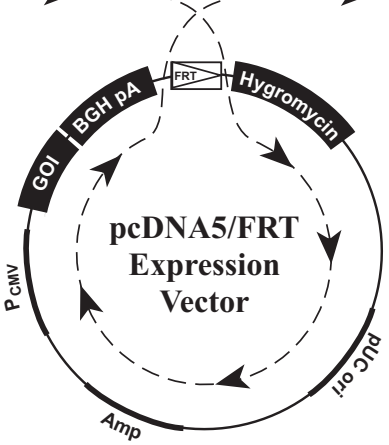
1. pFRT/*lac*Zeo is stably transfected into the mammalian cells of interest to generate the Zeocin™-resistant Flp-In™ Host Cell Line(s)



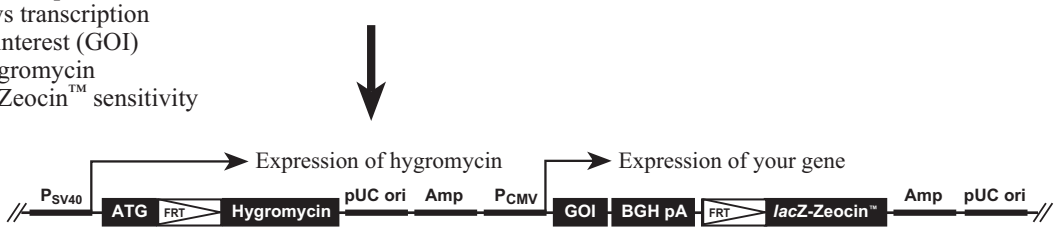
2. The pcDNA5/FRT expression vector containing your gene of interest (GOI) is cotransfected with pOG44 into the Flp-In™ Host Cell Line.



3. The Flp recombinase expressed from pOG44 catalyzes a homologous recombination event between the FRT sites in the host cells and the pcDNA5/FRT expression vector.



4. Integration of the expression construct allows transcription of the gene of interest (GOI) and confers hygromycin resistance and Zeocin™ sensitivity to the cells.



Flp-In™ Expression Cell Line

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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).

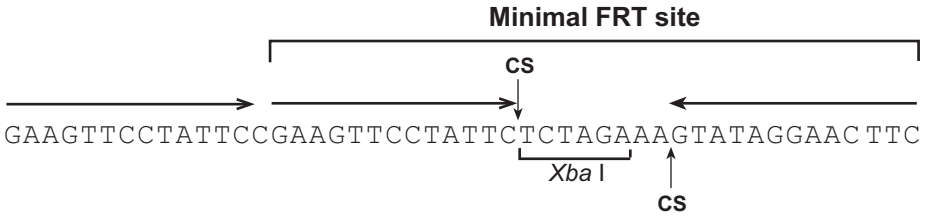
For more information about the Flp recombinase and conservative site-specific recombination, refer to published reviews (Craig, 1988; Sauer, 1994).

Note: If your cell line contains multiple integrated FRT sites, Flp-mediated intramolecular recombination may also occur. Intramolecular recombination may result in:

- Excision of the intervening DNA if the FRT sites are directly repeated (*i.e*. integration of multiple FRT sites on the same DNA strand).
- DNA inversion if the sites are in opposing orientations.
- Deletion of genomic sequences.

FRT Sites

As described above, Flp recombinase-mediated recombination occurs between specific FRT sites. 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 for cleavage sites (CS) (Andrews *et al.*, 1985; Senecoff *et al.*, 1985).



CS = cleavage site

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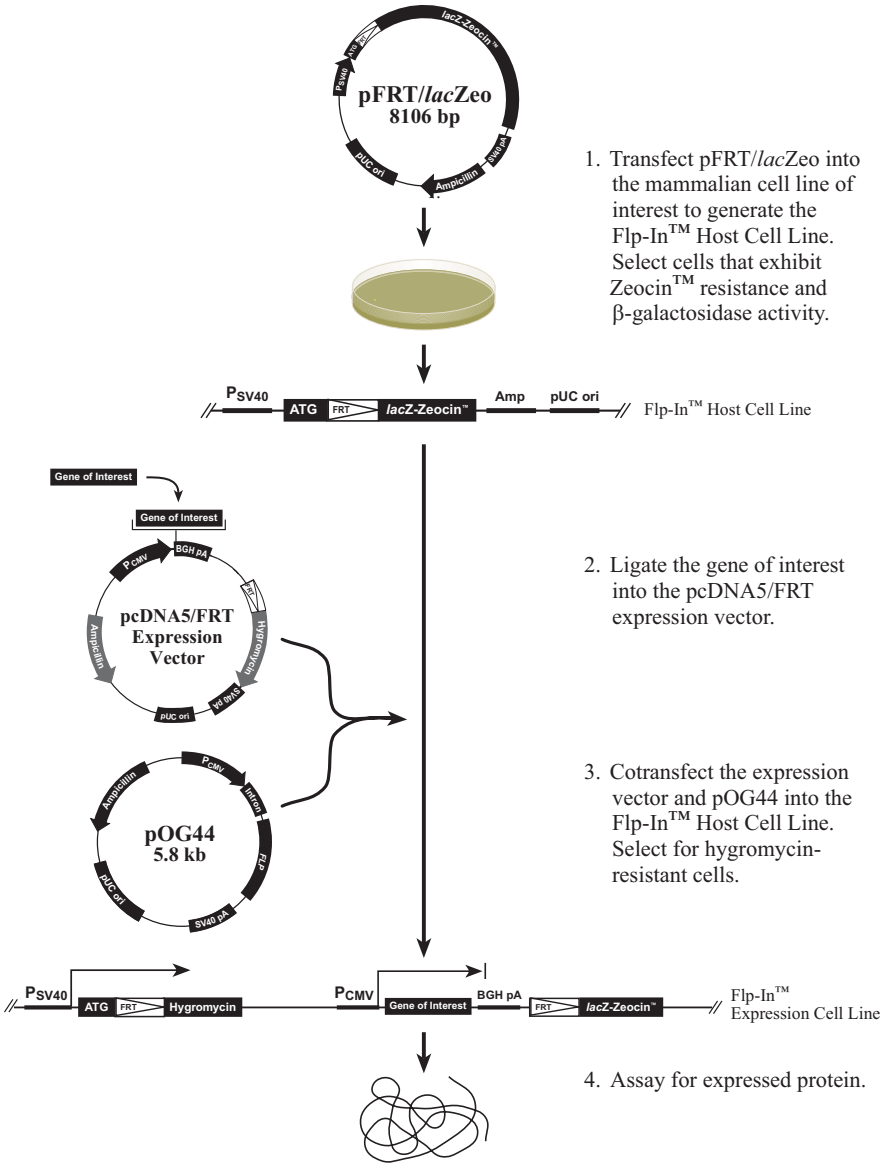
Experimental Outline

To create a stable Flp-In™ cell line expressing your gene of interest at a site-specific genomic locus, you will perform the following steps:

1. Transfect the Flp-In™ target site vector, pFRT/*lac*Zeo, into the mammalian cell line of choice to generate your Flp-In™ host cell line(s) (see figure below).
2. Clone your gene of interest into the pcDNA™5/FRT expression vector.
3. Co-transfect your pcDNA™5/FRT construct and the Flp recombinase expression vector, pOG44, into your Flp-In™ host cell line to generate your Flp-In™ expression cell line (see figure below).
4. Assay for expression of your recombinant protein of interest.

Note: The positive control vector containing the *CAT* gene can be cotransfected into your Flp-In™ host cell line with pOG44 to demonstrate that the system is working properly.



Methods

Propagation and Maintenance of Plasmids

Introduction

The following section contains guidelines for maintaining and propagating the pFRT/*lac*Zeo and pOG44 vectors. For information about maintaining and propagating the pcDNA™5/FRT expression vector, refer to the vector manual.

General Molecular Biology Techniques

For assistance with *E. coli* transformations, restriction enzyme analysis, DNA biochemistry, and plasmid preparation, 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 the pFRT/*lac*Zeo and pOG44 vectors. We recommend that you propagate the pFRT/*lac*Zeo and pOG44 vectors in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10 *E. coli* are available as chemically competent or electrocompetent cells from Invitrogen (page vii).

Transformation Method

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

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Propagation and Maintenance of Plasmids, Continued

Maintenance of Plasmids

The pFRT/*lac*Zeo and pOG44 vectors contain the ampicillin gene to allow selection of the plasmid using ampicillin (see pages 22–25 for more information about each vector).

To propagate and maintain the pFRT/*lac*Zeo and pOG44 plasmids, we recommend using the following procedure:

1. Use 10 ng of the vector to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, JM109, or equivalent.
2. Select transformants on LB agar plates containing 50–100 µg/ml ampicillin. For fast and easy microwaveable preparation of Low Salt LB agar containing ampicillin, imMedia™ Amp Agar is available from Invitrogen (page vii). For more information, visit our web site at www.invitrogen.com or contact **Technical Support** (see page 26).
3. Prepare a glycerol stock of each plasmid for long-term storage (below).

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 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.

Generating Stable Flp-In™ Host Cell Lines

Introduction

Before you can create a stable Flp-In™ cell line(s) expressing your gene of interest, you will first need to generate a stable mammalian cell line containing an integrated FRT site (Flp-In™ host cell line). The following section provides guidelines and instructions to generate stable Flp-In™ host cell lines by transfection using the pFRT/*lac*Zeo plasmid. For a map and a description of the features of pFRT/*lac*Zeo, refer to the **Appendix**, pages 22–23.

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 Invitrogen (see table below). If you wish to express your gene of interest in one of the cell lines listed below, you may want to use one of Invitrogen's Flp-In™ cell lines as the host to establish your stable expression cell line. For more information, refer to our web site at www.invitrogen.com or contact **Technical Support** (page 26).

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 Flp-In™-3T3 or Flp-In™-BHK cells. If you will be cloning your gene of interest into a pcDNA™5/-FRT-based expression construct, we recommend that you **do not** use 3T3 or BHK cells to create your Flp-In™ host cell line.

Alternatively, if you prefer to use 3T3 or BHK cells to create your Flp-In™ 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). Loss of gene expression due to down-regulation of the promoter is not observed in these cell lines when using pEF5/FRT-based expression constructs. For more information about the pEF5/FRT/V5-D-TOPO® or pEF5/FRT/V5-DEST vectors, visit our web site at www.invitrogen.com or contact **Technical Support** (page 26).

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 DNA using the PureLink™ or S.N.A.P.™ Miniprep or Midiprep Kit (page vii) or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (*e.g.* HeLa, COS-1), 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 & Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner & Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa & Dower, 1988). Invitrogen offers the Calcium Phosphate Transfection Kit and Lipofectamine™ 2000 Reagent (page vii) for mammalian cell transfection. For more information, refer to our web site at www.invitrogen.com or contact **Technical Support** (page 26).

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Zeocin™

The pFRT/*lac*Zeo plasmid contains a *lac*Z-Zeocin™ fusion gene under the control of the SV40 early promoter. Expression of the *lac*Z-Zeocin™ fusion gene allows selection of stable integrants using Zeocin™ antibiotic. The resulting stable integrants can then be screened by assaying for expression of β-galactosidase. For more information about preparing and handling Zeocin™, refer to the **Appendix**, page 21.

Note

The pFRT/*lac*Zeo2 plasmid contains a *lac*Z-Zeocin™ fusion gene under the control of a truncated SV40 promoter and is available separately from Invitrogen (page vii). The minimal activity of the promoter allows for isolation of clones that have FRT sites integrated in the most transcriptionally active genomic loci. For details, visit our web site at www.invitrogen.com or contact **Technical Support** (page 26).

Determination of Zeocin™ Sensitivity

To successfully generate a stable cell line containing an integrated FRT site and expressing the *lac*Z-Zeocin™ fusion protein, you need to determine the minimum concentration of Zeocin™ required to kill your untransfected mammalian cell line. Typically, concentrations ranging from 50–1000 µg/ml Zeocin™ are sufficient to kill most untransfected mammalian cell lines, with the average being 100–400 µg/ml. We recommend that you test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line. Refer to the **Appendix**, page 21 for instructions on how to prepare and store Zeocin™.

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 Zeocin™ (0, 50, 100, 250, 500, 750, and 1000 µg/ml Zeocin™).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of Zeocin™ that kills the cells within 1–2 weeks after addition of Zeocin™.

Effect of Zeocin™ on Sensitive and Resistant Cells

Zeocin™'s method of killing is quite different from other antibiotics including hygromycin, G418, and blasticidin. **Cells do not round up and detach from the plate.** Sensitive cells may exhibit the following morphological changes to Zeocin™ exposure:

- 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 other scaffolding proteins)
- Breakdown of plasma and nuclear membrane (appearance of many holes)

Eventually, these "cells" completely break down and only "strings" of protein 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™. For more information about Zeocin™ and its mechanism of action, see **Appendix,** page 21.

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Transfection Considerations

Once you have determined the appropriate Zeocin™ concentration to use, you are ready to transfect the pFRT/*lac*Zeo plasmid into your mammalian cell line of choice to generate the Flp-In™ host cell line. You will need to consider the following factors:

- **Insertion of the FRT site into the genome:** Integration of the pFRT/*lac*Zeo plasmid containing the FRT site into the genome will occur randomly. Subsequent integration of the pcDNA™5/FRT expression plasmid containing your gene of interest will occur through Flp recombinase-mediated recombination at the genomic FRT site.
- **Transfection efficiency of your cell line:** The aim of most users will be to create stable cell lines containing a single integrated FRT site ("single integrants"; see **Note** on the next page). The probability of obtaining stable integrants containing a single FRT site or multiple FRT sites depends on the transfection efficiency of your cell line and the amount of DNA transfected. To increase the likelihood of obtaining single integrants, lower the transfection efficiency by limiting the amount of plasmid DNA that you transfect (see **Recommendation** next page).
- **Selection of foci:** You will select for stable transfectants by plating cells in medium containing Zeocin™. Zeocin™-resistant foci can then be screened by Southern blot analysis to identify single integrants. To increase the chances of obtaining single integrants, we recommend you pick foci from plates that have been transfected with the least amount of plasmid DNA.
- **Chromosomal position effects:** Because integration of the pFRT/*lac*Zeo plasmid into the genome occurs randomly, expression levels of the *lac*Z-Zeocin™ fusion gene will be dependent on the transcriptional activity of the surrounding sequences at the integration site (*i.e.* chromosomal position effect). Once you have obtained single integrants, you may want to screen the Zeocin™-resistant clones for those expressing the highest β-galactosidase levels. Those clones expressing the highest levels of β-galactosidase should contain single FRT sites which have integrated into the most transcriptionally active regions.
- **Antibiotic concentration:** Single integrants will express only a single copy of the *lac*Z-Zeocin™ fusion gene and therefore, may be more sensitive to Zeocin™ selection than multiple integrants. If you have previously used your mammalian cell line for transfection and Zeocin™ selection, you may need to use lower concentrations of Zeocin™ to obtain single integrants.

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Note

If you want to increase the expression levels of your gene of interest in the cell line of choice, you may wish to generate a Flp-In™ host cell line containing multiple integrated FRT sites. In theory, cotransfection of your pcDNA™5/FRT construct and pOG44 into these cells will allow integration of your gene of interest into multiple genomic loci. **Note that the presence of multiple integrated FRT sites in the genome may increase the occurrence of chromosomal rearrangements or unexpected recombination events in your host cell line**.

As mentioned previously, we recommend that you transfect your mammalian cell line with a limiting amount of pFRT/*lac*Zeo plasmid. We generally use 250 ng to 2 µg of plasmid DNA per 4×10^6 cells for transfection, but the amount of plasmid DNA may vary due to the nature of the cell line, the transfection efficiency of your cells, and the method of transfection used. When transfecting your mammalian cell line of choice, we suggest that you try a range of plasmid DNA concentrations (*e.g.* 0.25, 0.5, 1, 2, 5 µg/ml DNA) to optimize transfection conditions for your cell line.

We generally use electroporation to transfect cells, but other methods of transfection are suitable. For a protocol to electroporate cells, refer to *Current Protocols in Molecular Biology*, Unit 9.3 (Ausubel *et al.*, 1994). Note that if you use calcium phosphate or lipid-mediated transfection methods, the amount of **total** DNA required for transfection is typically higher than for electroporation (usually between 10 and 20 µg DNA). Depending on the amount of pFRT/*lac*Zeo plasmid that you use for transfection, you may need to supplement your plasmid DNA with carrier DNA (*e.g.* salmon sperm DNA).

Possible Sites for Linearization of pFRT/*lac*Zeo

To obtain stable transfectants, we recommend that you linearize the pFRT/*lac*Zeo plasmid before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts the ATG-FRT-*lac*Z-*Zeocin*™ cassette or other elements necessary for expression in mammalian cells. The table below lists unique sites that may be used to linearize your construct prior to transfection. Other restriction sites are possible.

Note: We generally use *Sca* I to linearize pFRT/*lac*Zeo.

Enzyme	Restriction Site (bp)	Location	Supplier
*Tth*111 I	125	Backbone	Many
Apa I	5617	Backbone	Invitrogen (Catalog no. 15440–019)
Swa I	6075	Backbone	New England Biolabs, Sigma, Takara
Xmn I	6487	Ampicillin gene	Many
Sca I	6606	Ampicillin gene	Invitrogen (Catalog no. 15436–017)
Bsa I	7021	Ampicillin gene	New England Biolabs
*Eam*1105 I	7087	Ampicillin gene	AGS*, Fermentas, Takara
Sap I	8092	Backbone	New England Biolabs

*Angewandte Gentechnologie Systeme

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Selection of Stable Integrants

Once you have determined the appropriate Zeocin™ concentration to use for selection, you can generate a stable cell line with pFRT/*lac*Zeo.

1. Transfect mammalian cells with pFRT/*lac*Zeo using the desired protocol. Remember to include a plate of untransfected cells as a negative 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 such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Incubate the cells at 37°C for 2–3 hours until they have attached to the culture dish.
5. Remove the medium and add fresh medium containing Zeocin™ at the pre-determined concentration required for your cell line.
6. Feed the cells with selective medium every 3–4 days until foci can be identified.
7. Pick at least 20 Zeocin™-resistant foci and expand each clone to test for the number of integrated FRT sites. Isolate genomic DNA and use Southern blot analysis to distinguish between single and multiple integrants (see below and the next page). Select the single integrants and proceed to the next step.
8. Screen the single integrants for β-galactosidase activity (see the next page). Select those clones which exhibit the highest levels of β-galactosidase expression (if desired) to use as your Flp-In™ host cell line(s).
9. Once you have obtained a stable Flp-In™ host cell line, you can use this cell line to isolate a stable cell line expressing your gene of interest from the pcDNA™5/FRT plasmid (see the next section).

Note: The Flp-In™ host cell line should be maintained in medium containing the appropriate amount of Zeocin™ until generation of your Flp-In™ expression cell line.

Isolation of Genomic DNA

Once you have obtained Zeocin™-resistant foci, you will need to expand the cells and isolate genomic DNA. You may use any standard protocol to isolate genomic DNA from your cells. Protocols may be found in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989). For easy isolation of genomic DNA, the Easy-DNA™ Kit (page vii) is available from Invitrogen. Contact **Technical Support** for more information (page 26).

Continued on next page

Screening Clones by Southern Blot Analysis

You can use Southern blot analysis to determine the number of integrated FRT sites present in each of your Zeocin™-resistant clones. When performing Southern blot analysis, you should consider the following factors:

- **Probe:** We recommend that you use a fragment of the *lacZ* gene (100 to 500 bp) as the probe to screen your samples. Mammalian cells do not contain an endogenous *lacZ* gene, therefore, a *lacZ* probe should allow you to identify those clones which contain pFRT/*lac*Zeo DNA. To label the probe, we generally use a standard random priming kit (*e.g.* Ambion, DECAprime II™ Kit, Catalog no. 1455). Other random priming kits are suitable.
- **Restriction digest:** When choosing a restriction enzyme to digest the genomic DNA, we recommend choosing an enzyme that cuts at a single known site outside of the *lacZ* gene in the pFRT/*lac*Zeo vector. Hybridization of the *lacZ* probe to digested DNA should then allow you to detect a single band containing the *lacZ* gene from pFRT/*lac*Zeo. We generally use *Hin*d III to digest genomic DNA from the Zeocin™-resistant clones. pFRT/*lac*Zeo contains a single *Hin*d III site within the FRT site.
- **Protocol:** You may use any Southern blotting protocol of your choice. Refer to *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) for detailed protocols.

What You Should See

If you digest genomic DNA from your transfectants with *Hin*d III and use a *lacZ* fragment as a probe in your Southern analysis, you should be able to easily distinguish between single and multiple FRT integrants.

- DNA from single integrants should contain only one hybridizing band corresponding to a single copy of the integrated pFRT/*lac*Zeo plasmid.
- DNA from multiple integrants should contain more than one hybridizing band. If the pFRT/*lac*Zeo plasmid integrates into multiple chromosomal locations, the bands may be of varying sizes.

Assay for β-Galactosidase Activity

Once you have identified single integrants, proceed to screen the clones for β-galactosidase expression. 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 (page vii) for fast and easy detection of β-galactosidase expression. Select those clones expressing the highest levels of β-galactosidase (if desired) to use as the host cell lines for your pcDNA™5/FRT expression construct.

Generating Stable Flp-In™ Expression Cell Lines

Introduction

Once you have established your Flp-In™ host cell line, you may cotransfect your pcDNA™5/FRT construct and the pOG44 expression plasmid into the host cell line to generate a stable Flp-In™ expression cell line. Integration of the pcDNA™5/FRT construct into the genome will occur at the FRT site in the Flp-In™ host cells. The pcDNA™5/FRT plasmid contains the hygromycin resistance gene to allow selection of stable cell lines (see **Important,** below). For more information about the pcDNA™5/FRT plasmid and generating the pcDNA™5/FRT expression construct, refer to the vector manual. For more information about the pOG44 plasmid, see below.

Important

The hygromycin resistance gene in the pcDNA™5/FRT vector lacks an ATG initiation codon and a promoter to drive expression of the gene. Transfection of pcDNA™5/FRT plasmid alone into a Flp-In™ host cell line will **not** confer hygromycin resistance to the cells containing the plasmid. The ATG initiation codon and the SV40 promoter required for expression of the hygromycin resistance gene are brought into proximity and frame with the gene only through Flp recombinase-mediated recombination between the FRT sites in the pcDNA™5/FRT plasmid and the Flp-In™ host cell line.

If you wish to express your gene of interest in one of the cell lines listed in the table below, you may want to use one of Invitrogen's Flp-In™ host cell lines. For more information, visit our web site at www.invitrogen.com or contact **Technical Support** (page 26).

Note

If you are generating Flp-In™ expression cell lines using the Flp-In™-3T3 or Flp-In™-BHK 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). 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 Flp-In™-3T3 or Flp-In™-BHK cells.

Continued on next page

pOG44 Plasmid

You will cotransfect the pOG44 plasmid and your pcDNA™5/FRT construct into your Flp-In™ host cell line to generate stable cell lines that express your protein of interest. Cotransfection of pOG44 and pcDNA™5/FRT allows expression of Flp recombinase and integration of the pcDNA™5/FRT plasmid into the genome via the FRT sites. Once the pcDNA™5/FRT construct has integrated into the genome, the Flp recombinase is no longer required. In fact, the continued presence of Flp recombinase would be detrimental to the cells because it could mediate excision of your pcDNA™5/FRT construct.

The pOG44 plasmid lacks an antibiotic resistance marker for selection in mammalian cells. Thus, the plasmid and therefore, Flp recombinase expression, will gradually be lost from transfected cells as they are cultured and selected in hygromycin.

Flp Recombinase

The *FLP* gene was originally isolated from the *Saccharomyces cerevisiae* 2 plasmid (Broach *et al.*, 1982; Broach & Hicks, 1980) (see the **Appendix**, page 25 for more information). When tested in mammalian cells, the Flp recombinase has been shown to possess optimum recombination activity near 30°C and relatively low activity at 37°C, a result consistent with its physiological role in yeast (Buchholz *et al.*, 1996).

The *FLP* gene in pOG44 is further limited in its activity because it contains a point mutation that encodes a Flp recombinase with a phenylalanine to leucine amino acid substitution at position 70 (Buchholz *et al.*, 1996). The resulting Flp recombinase (flp-F70L) exhibits increased thermolability at 37°C in mammalian cells when compared to the native Flp recombinase (Buchholz *et al.*, 1996). Studies have shown that the Flp recombinase expressed from pOG44 possesses only 10% of the activity at 37°C of the native Flp recombinase (Buchholz *et al.*, 1996).

Important

When generating Flp-In™ expression cell lines, it is important to remember that you are selecting for a relatively rare recombination event since you want recombination and integration of your pcDNA™5/FRT construct to occur only through the FRT site and for a limited time. In this case, using a highly inefficient Flp recombinase is beneficial and may decrease the occurrence of other undesirable recombination events.

Continued on next page

Note

Reminder: Integration of the pcDNA™5/FRT construct into the genome via the FRT sites will result in the following events (see page 3 for a diagram):

- Insertion of the hygromycin resistance gene downstream of the SV40 early promoter and the ATG initiation codon (provided by pFRT/*lac*Zeo)
- Insertion of the plasmid containing the CMV promoter, your gene of interest, and the BGH polyadenylation signal upstream of the *lac*Z-Zeocin™ fusion gene
- Disruption of the functional *lac*Z-Zeocin™ transcriptional unit caused by loss of the SV40 early promoter and the ATG initiation codon and insertion of the cassette containing the CMV promoter, gene of interest, and the BGH polyadenylation signal

As a result, your Flp-In™ expression cell lines should exhibit the following phenotype:

- Hygromycin resistance
- Zeocin™ sensitivity
- Lack of β-galactosidase activity
- Expression of the gene of interest

Positive Control

The pcDNA™5/FRT/CAT plasmid is provided as a positive control vector for mammalian cell transfection and expression and may be used to assay for expression levels in your Flp-In™ expression cell line. If you have several different Flp-In™ host cell lines (cell lines containing FRT sites integrated at different genomic loci), you may want to use the pcDNA™5/FRT/CAT control vector to compare protein expression levels from the various genomic loci. For more information about pcDNA™5/FRT/CAT, refer to the pcDNA™5/FRT vector manual.

Hygromycin B

The pcDNA™5/FRT vector contains the *E. coli* hygromycin resistance gene (*HPH*) (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 by disrupting translocation and promoting mistranslation. Hygromycin B liquid is supplied with the Flp-In™ Complete System and is also available separately from Invitrogen (see page vii).

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

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Preparing and Storing Hygromycin B

Hygromycin B (Flp-In™ Complete System, only) is supplied as a 100 mg/ml stock solution in autoclaved, deionized water and is filter-sterilized. The solution is brown in color. The stability of hygromycin B is guaranteed for six months, if stored at 4°C. Medium containing hygromycin is stable for up to six weeks.

Determination of Hygromycin Sensitivity

To successfully generate a stable cell line expressing your gene of interest from pcDNA™5/FRT, you need to determine the minimum concentration of hygromycin B required to kill your untransfected Flp-In™ host cell line. Typically, concentrations ranging from 10 to 400 µg/ml hygromycin B 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 Flp-In™ 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 B (0, 10, 50, 100, 200, 400, 600 µg/ml hygromycin B).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of hygromycin that kills the cells within 1–2 weeks after addition of hygromycin.

Because correct integration of your pcDNA™5/FRT construct into the genome is dependent on Flp recombinase, the expression levels of Flp recombinase in the cell will determine the efficiency of the recombination reaction. Flp recombinase levels must be sufficiently high to mediate recombination at the FRT sites (single recombination event) and overcome the low intrinsic activity of the enzyme (see previous page). We have varied the ratio of pOG44 and pcDNA™5/FRT expression plasmid that we cotransfect into mammalian Flp-In™ host cells to optimize the recombination efficiency. **We recommend that you cotransfect your Flp-In™ host cell line with a ratio of <u>at least</u> 9:1 (w/w) pOG44:pcDNA™5/FRT expression plasmid.** Note that this ratio may vary depending on the nature of the cell line. You may want to determine this ratio empirically for your cell line.

Important

When transfecting your Flp-In™ host cell line, be sure to use supercoiled pOG44 and pcDNA™5/FRT plasmid DNA. Flp-mediated recombination between the FRT site on pcDNA™5/FRT and the integrated FRT site in the Flp-In™ host cell line will only occur if the pcDNA™5/FRT plasmid is circularized. The pOG44 plasmid should be circularized to minimize the possibility of the plasmid integrating into the genome.

Continued on next page

Note

Your gene of interest will be expressed from pcDNA™5/FRT under the control of the human CMV promoter. Once you have generated the Flp-In™ expression cell line, note that your recombinant protein should be expressed constitutively.

Selection of Stable Flp-In™ Expression Cell Lines

Once you have determined the appropriate hygromycin concentration to use for selection in your Flp-In™ host cell line, you can generate a stable cell line expressing your pcDNA™5/FRT construct. **Reminder: Following cotransfection, your Flp-In™ expression clones should become sensitive to Zeocin™ (see Note on page 14); therefore, your selection medium should not contain Zeocin™.**

1. Cotransfect your mammalian Flp-In™ host cells with a 9:1 ratio of pOG44:pcDNA™5/FRT plasmid DNA (see previous page) using the desired protocol. Remember to include a plate with no pOG44 as a Flp recombination control, a plate of untransfected cells as a negative control, and the pcDNA™5/FRT/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, such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Plate the trypsinized cells in the presence of hygromycin **immediately**, (at the predetermined concentration for your cell line) rather than waiting for the cells to attach and then adding antibiotic. This will ensure that ONLY the true transfectants survive and the untransfected cells die off very quickly.
5. Feed the cells with selective medium every 3–4 days until foci can be identified.
6. Pick 5–20 hygromycin-resistant foci and expand the cells. Verify that the pcDNA™5/FRT construct has integrated into the FRT site by testing each clone for Zeocin™ sensitivity and lack of β-galactosidase activity.
7. Select those clones that are hygromycin-resistant, Zeocin™-sensitive, and *lac*Z$^-$, then assay for expression of your gene of interest.

Note

We have observed that in cells where the FRT site has integrated into a very transcriptionally active locus in the host cell genome (seen more commonly in Flp-in CHO and Flp-in 293 cells but can happen in Flp-in 3T3 cells and any other Flp-in host cell line), there is some "read-through" transcription and translation of the *lac*Z-Zeocin ORF post Flp-in, even though the *lac*Z-Zeocin ORF does not have a bonafide promoter and ATG. In such cases, the hygromycin-resistant clones would also be *lac*Z positive and Zeocin-resistant. To make sure that the integration is FRT site-specific and not random, we recommend doing a parallel control transfection with no pOG44 present. This should yield no surviving clones upon hygromycin selection, indicating that all the hygromycin-resistant clones obtained in the presence of pOG44 are indeed Flp recombinase-dependent and hence have the gene of interest integrated at the FRT site. Also, a Southern blot analysis of these clones will help verify that they do indeed have proper FRT integration of the gene of interest despite the expression of *lac*Z (although this is usually not necessary). As long as you see hygromycin-resistant clones 9ost Flp-in, we recommend you select assay them for expression of your gene of interest.

Continued on next page

Polyclonal Selection

If you use a single integrant as your Flp-In™ host cell line, all of the hygromycin-resistant foci that you obtain after cotransfection of pcDNA™5/FRT and pOG44 and selection with hygromycin should, in theory, be isogenic (*i.e.* pcDNA™5/FRT should integrate into the same genomic locus in every clone, therefore, all clones should be identical). Having isogenic clones should allow you to perform "polyclonal" selection and screening of your hygromycin-resistant cells. If you wish, you do not need to pick and screen separate foci for expression of your protein of interest. After hygromycin selection, simply pool the foci and screen the entire population of cells for expression of your protein of interest.

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 using your method of choice. For Western blot analysis, you may use CAT Antiserum available from Invitrogen for detection (see page vii for ordering information). Other commercial kits are available for assaying CAT expression.

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

10 g Tryptone
5 g Yeast Extract
10 g NaCl
pH 7.0

1. Combine the dry reagents above and add deionized, distilled water to 950 ml.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed.
4. Store at room temperature or at 4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (*i.e.* 50–100 µg/ml ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at 4°C, in the dark.

Phosphate-Buffered Saline (PBS)

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

1. Dissolve the following in 800 ml of deionized water:

   8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4

2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter and autoclave for 20 minutes on liquid cycle.
4. Store at room temperature or at 4°C.

Zeocin™

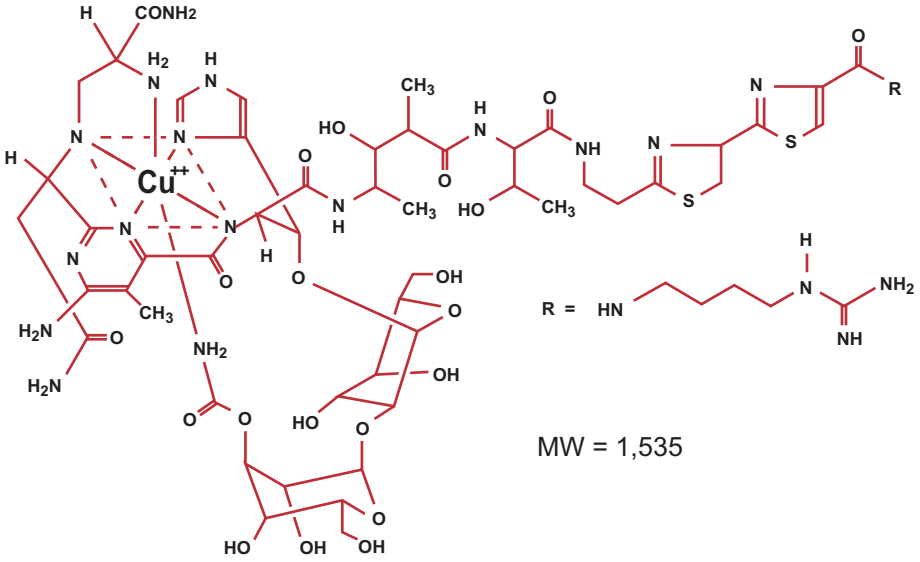
Zeocin™

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 (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989).

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™ and inhibits 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 $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. 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). Typically, Zeocin™ concentrations ranging from 50 to 1000 µg/ml are used for selection in mammalian cells. Before transfection, we recommend that you first test the sensitivity of your mammalian host cell to Zeocin™ as natural resistance varies among cell lines.

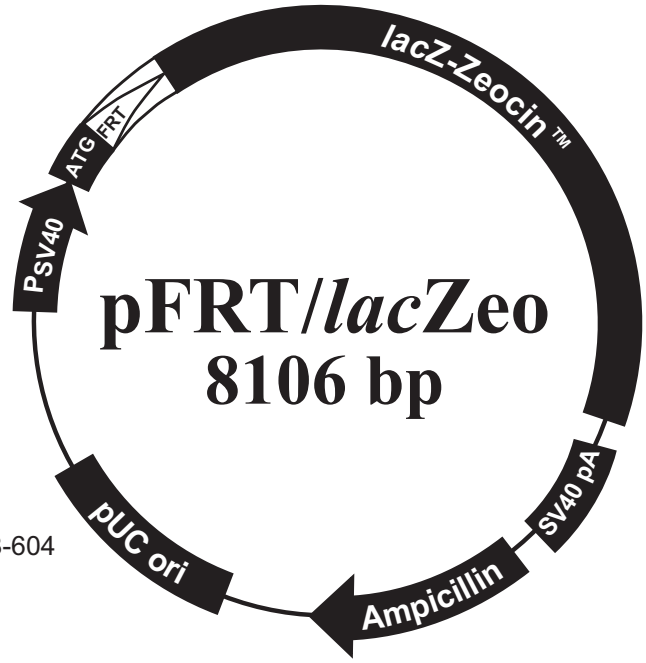
Handling Zeocin™

- 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.

Map of pFRT/*lac*Zeo Vector

Map of pFRT/*lac*Zeo

pFRT/*lac*Zeo is a 8106 bp vector that expresses a fusion protein containing β-Galactosidase and the Zeocin™ resistance marker under the control of SV40 early promoter. Note that neither the *lac*Z gene nor the Zeocin™ resistance gene contains its native ATG initiation codon. The ATG initiation codon is placed directly upstream of a FRT site and allows expression of the *lac*Z-Zeocin™ fusion gene in cells. The figure below summarizes the features of the pFRT/*lac*Zeo. **The complete sequence for pFRT/*lac*Zeo is available for downloading from our web site at www.invitrogen.com or by contacting Technical Support (page 26).**



Comments for pFRT/*lac*Zeo
8106 nucleotides

SV40 early promoter and origin: bases 278-604
ATG initiation codon: bases 609-611
FRT site: bases 614-661
lacZ-Zeocin™ fusion gene
LacZ ORF (no ATG): bases 675-3722
Zeocin™ resistance gene (no ATG): bases 3810-4181
SV40 early polyadenylation signal: bases 5102-5425
bla promoter: bases 6201-6299
Ampicillin (*bla*) resistance gene: bases 6300-7160
pUC origin: bases 7305-7978

Continued on next page

Features of pFRT/lacZeo Vector

Features of pFRT/*lac*Zeo

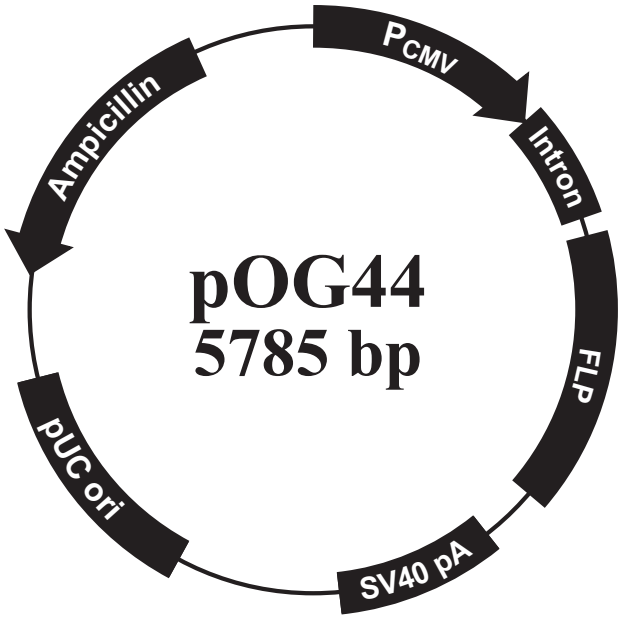
The table below describes the relevant features of pFRT/*lac*Zeo. All features have been functionally tested.

Feature	Benefit
SV40 early promoter and origin	Permits efficient, high-level expression of the *lac*Z-Zeocin™ fusion gene in mammalian cells and episomal replication in cells expressing the SV40 large T antigen.
ATG initiation codon	Allows translation initiation of the *lac*Z-Zeocin™ fusion protein.
Flp Recombination Target (FRT) site	Encodes a 34 bp (+14 bp non-essential) sequence that serves as the binding and cleavage site for Flp recombinase (Gronostajski & Sadowski, 1985; Jayaram, 1985; Senecoff *et al.*, 1985).
*lac*Z-Zeocin™ fusion gene	Encodes a fusion protein containing β-Galactosidase and the Zeocin™ resistance marker to permit selection of stable mammalian cell lines with Zeocin™ and screening by β-galactosidase activity assay.
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene.
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli.*
pUC origin	Permits high-copy number replication and growth in *E. coli.*

Map of pOG44 Vector

Map of pOG44

pOG44 is a 5785 bp vector that expresses the Flp recombinase under the control of the human CMV promoter as previously described (O'Gorman *et al.,* 1991). The vector contains a synthetic intron to enhance expression of the *FLP* gene. Note that the vector does not contain an antibiotic resistance marker to allow stable selection in mammalian cells. The figure below summarizes the features of the pOG44 vector. **The complete sequence for pOG44 is available for downloading from our web site at www.invitrogen.com or by contacting Technical Support (page 26).**



Comments for pOG44
5785 nucleotides

CMV promoter: bases 234-821
Synthetic intron: bases 871-1175
FLP ORF: bases 1202-2473
SV40 late polyadenylation signal: bases 2597-2732
pUC origin: bases 3327-3993 (complementary strand)
bla promoter: bases 4999-5097 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4138-4998 (complementary strand)

Features of pOG44 Vector

Features of pOG44

The table below describes the relevant features of pOG44. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate early promoter	Permits high-level expression of the *FLP* gene (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Synthetic intron	Hybrid fragment which contains sequences derived from the adenovirus major late region and an IgG variable region (Huang & Gorman, 1990; O'Gorman *et al.*, 1991) and functions to enhance expression of the *FLP* gene.
FLP ORF	Encodes a temperature-sensitive Flp recombinase (Buchholz *et al.*, 1996) that mediates conservative recombination via FRT sites (O'Gorman *et al.*, 1991).
SV40 late polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA.
pUC origin	Permits 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.*

FLP Gene

The *FLP* gene was originally isolated from the *Saccharomyces cerevisiae* 2 plasmid (Broach *et al.*, 1982; Broach & Hicks, 1980) and encodes a site-specific recombinase that is a member of the integrase family of recombinases (Argos *et al.*, 1986). The Flp recombinase mediates a site-specific recombination reaction between interacting DNA molecules via the pairing of interacting FRT sites. For more information about site-specific recombination, refer to page 4 and published reviews (Craig, 1988; Sauer, 1994).

The native *FLP* gene encodes a protein of 423 amino acids with a calculated molecular weight of 49 kDa. The *FLP* gene expressed from pOG44 encodes a temperature-sensitive Flp recombinase which carries a point mutation (flp-F70L) that results in a change in amino acid 70 from phenylalanine to leucine (Buchholz *et al.*, 1996). For more information about the properties of the flp-F70L protein, refer to page 15 and Buchholz *et al.*, 1996.

Technical Support

Web Resources

Visit the Invitrogen web site 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.
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