pcDNA5/FRT/TO-E© Echo™-Adapted Expression Vector

Version G
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25-0372

pcDNA5/FRT/TO-E© Echo™-Adapted Expression Vector

For cloning of the gene of interest using the Echo™ Cloning System and targeted, inducible expression in mammalian cells using the Flp-In™ T-REx™ System

Catalog nos. ET470-XXX

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Important Information

Types of Kits

This manual is supplied with the following pcDNA5/FRT/TO-E© Echo™-Adapted Expression Vector Kits listed below.

Kit	Reagents Supplied	Catalog nos.
pcDNA5/FRT/TO-E© Echo™-Adapted Expression Vector Kit	pcDNA5/FRT/TO-E© vector Expression Control vector Cre Recombinase and 10X buffer CMV Forward Sequencing primer	ET470-01
pcDNA5/FRT/TO-E© Echo™-Adapted Expression Vector Kit with a choice of Donor Vector Kit and One Shot® TOP10 Chemically Competent *E. coli* (see page vi for more information on donor vectors)	pUni/V5-His TOPO® TA Cloning Kit	ET470-10C
	pUniBlunt/V5-His TOPO® Cloning Kit	ET470-20C
	pUni/V5-His A, B, and C	ET470-30C
	pUniD/V5-His TOPO® Cloning Kit	ET470-40C

Shipping/Storage

The pcDNA5/FRT/TO-E© Echo™-Adapted Expression Vector Kit is shipped on dry ice. Upon receipt, store the pcDNA5/FRT/TO-E© reagents at -20°C.

pcDNA5/FRT/TO-E© Reagents

The pcDNA5/FRT/TO-E© reagents are listed below. **Store at -20°C.**

Item	Concentration	Amount
pcDNA5/FRT/TO-E©	Supercoiled, lyophilized in TE, pH 8.0	20 µg
Cre Recombinase	Please check the label on the tube for exact concentration of the enzyme. Enzyme is supplied in: 50 mM Tris-HCl, pH 8.0 5 mM EDTA 1 mM EGTA 10 mM β-mercaptoethanol 20% Glycerol	15 µl
10X Recombinase Buffer	500 mM Tris-HCl, pH 7.5 100 mM $MgCl_2$ 300 mM NaCl 1.0 mg/ml BSA	25 µl

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pcDNA5/FRT/TO-E© Reagents, continued

Item	Concentration	Amount
CMV Forward Sequencing Primer (21- mer)	Lyophilized in TE Buffer, pH 8.0	2 µg
Expression Control (pcDNA5/FRT/TO-E/Uni-CAT©)	Supercoiled, lyophilized in TE, pH 8.0	20 µg

Sequence of the Primer

The CMV Forward sequencing primer is supplied in a total amount of 306 pmoles and its sequence is 5′-CGCAAATGGGCGGTAGGCGTG-3′.

One Shot® TOP10 Reagents (Optional)

The table below describes the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit.

Store at -80°C.

Item	Concentration	Amount
SOC Medium (may be stored at room temperature or at +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 *E. coli*	--	11 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH8	50 µl

Genotype of TOP10

TOP10: Use this strain for general cloning of your gene of interest. **Note**: This strain cannot be used for transformation and growth of donor vectors.

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lacX*74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Accessory Products

Introduction

The products listed in this section are intended for use with the pcDNA5/FRT/TO-E© Echo™-Adapted Expression Vector Kit. For more information, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 23).

Additional Products

Many of the reagents in the pcDNA5/FRT/TO-E© Echo™-Adapted Expression Vector Kit, as well as additional reagents that may be used with this kit, are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Catalog no.
One Shot® PIR1 Chemically Competent *E. coli*	11 x 50 µl	C1010-10
One Shot® PIR2 Chemically Competent *E. coli*	11 x 50 µl	C1111-10
One Shot® TOP10 Chemically Competent *E. coli* Kit	11 x 50 µl	C4040-10
Cre Recombinase	10 reactions	R100-10
Calcium Phosphate Transfection Kit	75 reactions	K2780-01
Anti-CAT Antiserum	50 µl	R902-25

Donor Vectors

The table below lists a variety of donor vectors currently available from Invitrogen to facilitate cloning of your gene of interest for use with the Echo™ Cloning System.

Product	Application	Quantity	Catalog no.
pUniD/V5-His-TOPO® Cloning Kit	Directional cloning of blunt PCR products	10 reactions	ET004-10
pUni/V5-His-TOPO® TA Cloning Kit	Cloning A-tailed PCR products	10 reactions	ET001-10
pUniBlunt/V5-His-TOPO® Cloning Kit	Cloning blunt PCR products	10 reactions	ET002-10
pUni/V5-His A, B, and C	Cloning DNA fragments using restriction enzymes	10 reactions	ET003-10

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Accessory Products, continued

Flp-In™ T-REx™ Products

The pcDNA5/FRT/TO-E© vector is designed to allow tetracycline-regulated expression of your PCR product in the Flp-In™ T-REx™ System. The plasmids required for use of the Flp-In™ T-REx™ System are available separately or in a core kit from Invitrogen. Other reagents including the selection agents that may be used with the Flp-In™ T-REx™ System are also available. Ordering information is provided below.

Product	Amount	Catalog no.
Flp-In™ T-REx™ Core Kit	1 kit	K6500-01
pFRT/*lac*Zeo	20 µg	V6015-20
pFRT/*lac*Zeo2	20 µg	V6022-20
pcDNA6/TR©	20 µg	V1025-20
pOG44	20 µg	V6005-20
Tetracycline	5 g	Q100-19
Hygromycin B	1 g	R220-05
Zeocin™	1 g	R250-01
	5 g	R250-05
Blasticidin	50 mg	R210-01
Flp-In™ T-REx™-293 Cell Line	3 x 10^6 cells	R780-07

Flp-In™ Host Cell Lines

For your convenience, Invitrogen has available several mammalian Flp-In™ host cell lines that stably express the lacZ-Zeocin™ fusion gene from pFRT/lacZeo or pFRT/lacZeo2. Each cell line contains a single integrated FRT site as confirmed by Southern blot analysis. By transfecting the pcDNA6/TR plasmid into these cell lines, you can easily generate Flp-In™ T-REx™ host cell lines. For more information, see our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 23).

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

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Accessory Products, continued

Detection of Fusion Protein

If you choose to clone your PCR product in frame with the C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag (provided by the donor vector), a number of antibodies and immunodetection kits are available from Invitrogen to detect expression of your fusion protein from the pcDNA5/FRT/TO-E© vector after recombination. Enzyme-conjugated antibodies allow one-step detection in Western blots using colorimetric or chemiluminescent detection methods. Sufficient antibody is provided for 25 Westerns. The WesternBreeze® kits contains sufficient reagents to allow detection of 20 blots.

Product	Application	Catalog no.
Anti-V5 Antibody	Detects the 14 amino acid epitope derived from the P and V proteins of the para-myxovirus, SV5 (Southern *et al.*, 1991): GKPINPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-V5-FITC Antibody		R963-25
Anti-His(C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25
Anti-His(C-term)-FITC Antibody		R933-25
WesternBreeze® Chromogenic Kit-αMouse	Chromogenic detection of proteins on blots using alkaline phosphatase substrate	WB7103
WesternBreeze® Chemiluminescent Kit-αMouse	Chemiluminescent detection of proteins on blots using alkaline phosphatase substrate	WB7104

Purification of Fusion Protein

The polyhistidine (6xHis) tag allows purification of the recombinant fusion protein using metal-chelating resins such as ProBond™. Ordering information for ProBond™ resin is provided below.

Product	Quantity	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti-V5-HRP Antibody	1 kit	K854-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K853-01
ProBond™ Resin	50 ml	R801-01
	150 ml	R801-15
Purification Columns	50 polypropylene columns	R640-50

Introduction

Overview

Introduction

The Echo™ Cloning System allows direct recombination of your gene of interest downstream of an appropriate promoter for expression in the host system of choice. pcDNA5/FRT/TO-E© is a member of the Echo™ Cloning System family of expression vectors and is specifically designed to allow targeted, inducible expression of the gene of interest in mammalian cells using the Flp-In™ T-REx™ System. The hybrid human cytomegalovirus (CMV)/TetO_2 promoter provides high-level, tetracycline-regulated recombinant protein expression in the mammalian cell line of choice when cotransfected with the pOG44 Flp recombinase expression plasmid into a Flp-In™ T-REx™ host cell line.

The Echo™ Cloning System

The Echo™ Cloning System is based on the univector plasmid-fusion system (UPS) described by Elledge and coworkers to quickly and easily recombine a gene of interest into a series of recipient (acceptor) vectors (Liu *et al.*, 1998; Liu *et al.*, 1999). The system consists of the univector (donor) vector containing the gene of interest and recipient (acceptor) vectors containing various regulatory sequences for expression in the host of choice. The Echo™ System utilizes the *cre-lox* site-specific recombination system of bacteriophage P1 (Abremski *et al.*, 1983; Sternberg and Hamilton, 1981) The product of the *cre* gene is a site-specific recombinase that catalyzes conservative recombination between two 34 bp *loxP* or *loxH* sequences to resolve P1 dimers generated by replication of circular lysogens.

Plasmid Fusion

The donor vector (pUni) and the acceptor vector (*i.e.* pcDNA5/FRT/TO-E©) each contain a single *lox* site. The donor vector contains a *loxP* site, while the acceptor vector contains either a *loxP* or a *loxH* (see the next page for more information about *loxP* and *loxH* sites). You may insert your gene of interest into the donor vector via the TOPO® Cloning method or traditional restriction enzyme-mediated cloning (see the manual for the specific donor vector that you are using). pcDNA5/FRT/TO-E© allows tetracycline-regulated expression of the gene of interest at a targeted genomic locus in mammalian cells using the Flp-In™ T-REx™ System. The unique *loxP* site is located downstream of the regulatory sequences. By mixing the donor vector containing the gene of interest with pcDNA5/FRT/TO-E© in the presence of Cre recombinase, a plasmid fusion is created that inducibly expresses the gene of interest in mammalian cells. A generic diagram is shown on the next page.

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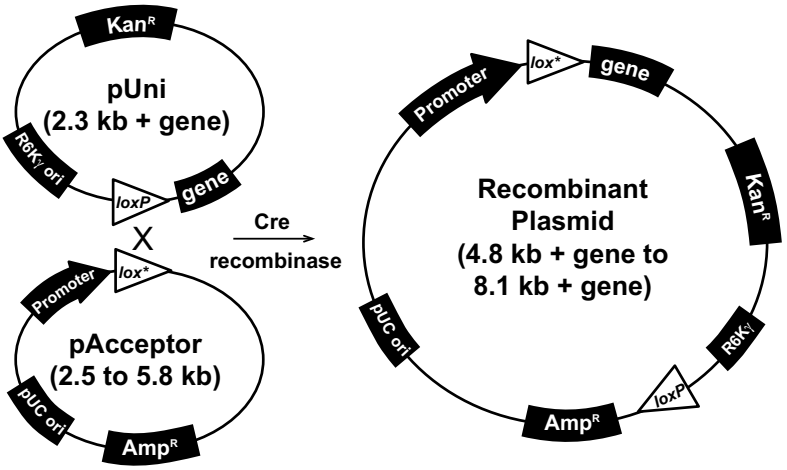
Overview, continued

Plasmid Fusion, continued



*lox** = *loxP* or *loxH* depending on acceptor vector

loxP or *loxH* Sites

The sequence of the *loxP* site is shown below. The *loxP* site consists of a 34 bp sequence containing two 13 bp inverted repeats (see underlined bases) separated by an 8 bp spacer (Hoess *et al.*, 1982). The inverted repeats may form a stem and loop structure that may reduce expression of the gene of interest in some cases. A variation of the *loxP* site (*loxH*, see below) may eliminate the formation of this structure and improve expression. We have not observed any differences in expression levels in constructs containing a *loxP* or a *loxH* site. Mutated bases are shown in boldface. **Note:** Some acceptor vectors contain a *loxH* site. Cre-mediated recombination can still occur between a *loxP* and a *loxH* site although the efficiency may be slightly reduced.

- *loxP*: ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T
- *loxH*: AT**T** AC**C** TC**A** TAT AGC ATA CAT TAT ACG AAG TTA T

Cre Recombinase

Cre recombinase (MW = 35 kDa) is a site-specific recombinase that binds to specific sequences (*loxP* and *loxH* sites), brings together the target sites, cleaves them, and covalently attaches to the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel (recombinant) form. A nucleophilic hydroxylated tyrosine initiates the DNA cleavage event by attack on a specific phosphodiester bond followed by the covalent attachment of the recombinase to the target sequence through a phosphoamino acid bond (Abremski and Hoess, 1992; Argos *et al.*, 1986). The reaction does not require any host factors or ATP, but does require Mg^{2+} or spermidine for activity (Abremski *et al.*, 1983). Recombination between two supercoiled substrates, each containing a *loxP* or *loxH* site, results in a supercoiled dimer. The extent of the reaction is 10-20% and appears to be stoichiometric (Abremski and Hoess, 1984; Abremski *et al.*, 1983).

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Selection of Recombinants

By fusing the two plasmids, kanamycin resistance is now linked to the pUC origin of replication. The recombination reaction is transformed into TOP10 *E. coli* and recombinants selected by plating the transformation reaction onto plates containing kanamycin. Because the donor plasmid carries the R6Kγ origin of replication, it will not propagate in *E. coli* strains such as TOP10, which do not carry the *pir* gene. In addition, the acceptor vector, which carries the ampicillin resistance gene will not be selected. Therefore every colony that is selected on kanamycin will represent a recombined fusion plasmid.

pcDNA5/FRT/TO-E©

pcDNA5/FRT/TO-E© is a 4.9 kb vector derived from pcDNA5/FRT/TO© and designed for targeted, high-level, tetracycline-inducible expression in most mammalian hosts. The vector contains the following elements:

- Human cytomegalovirus (CMV) immediate-early promoter containing two tetracycline operator 2 ($TetO_2$) sites for tetracycline-regulated expression of your gene of interest in mammalian cells (Yao *et al.*, 1998)
- A *loxP* site for plasmid fusion
- FLP Recombination Target (FRT) site for Flp recombinase-mediated integration of the vector into the Flp-In™ T-REx™ host cell line (see next page)
- Hygromycin resistance gene for selection of stable mammalian cell lines
- The pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*
- The ampicillin (*bla*) resistance gene for selection in *E. coli*

For a map and a description of the features of pcDNA5/FRT/TO-E©, see pages 20-21.

Other Echo™-adapted acceptor vectors are available separately. For more information on other available acceptor vectors, visit our Web site (www.invitrogen.com) or call Technical Service (see page 23).

Flp-In™ T-REx™ System

The Flp-In™ T-REx™ System allows the generation of stable mammalian cell lines exhibiting tetracycline-regulated expression of a gene of interest from a specific genomic location. The Flp-In™ T-REx™ System is based in part on a *Saccharomyces cerevisiae* derived DNA recombination system which uses Flp recombinase and site-specific recombination to facilitate integration of the gene of interest into a specific genomic locus. Tetracycline regulation is imparted to the system by incorporating regulatory elements from the *E. coli* tetracycline (Tet) resistance operon into the promoter used to control expression of the gene of interest. For more information about the mechanism of Flp recombinase-mediated DNA recombination and the Flp-In™ T-REx™ System, refer to the Flp-In™ T-REx™ Core Kit manual. The Flp-In™ T-REx™ Core Kit manual is available for downloading from our World Wide Web site (www.invitrogen.com) or by calling Technical Service (see page 23). For more information about the mechanism of tetracycline regulation, see the next page.

Generation of stable Flp-In™ T-REx™ expression cell lines which inducibly express the gene of interest requires the following major steps:

1. Generation of a Flp-In™ T-REx™ host cell line (see the next page)
2. Cotransfection of your pcDNA5/FRT/TO-E© expression construct and the pOG44 Flp recombinase plasmid into the Flp-In™ T-REx™ host cell line
3. Induction of the gene of interest by the addition of tetracycline

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Overview, continued

Flp-In™ T-REx™ Host Cell Line

To use the pcDNA5/FRT/TO-E© construct in the Flp-In™ T-REx™ System, you will need to have a Flp-In™ T-REx™ host cell line on hand. The Flp-In™ T-REx™ host cell line exhibits the following key features:

- Contains an integrated Flp Recombination Target (FRT) site (from the pFRT/*lac*Zeo plasmid)
- Expresses the tetracycline (Tet) repressor (from the pcDNA6/TR plasmid)

For more information about the pFRT/*lac*Zeo and pcDNA6/TR plasmids and detailed instructions to generate a Flp-In™ T-REx™ host cell line, refer to the Flp-In™ T-REx™ Core Kit manual. The pFRT/*lac*Zeo and the pcDNA6/TR© plasmids are available separately from Invitrogen (see page vii for ordering information). Alternatively, the Flp-In™ T-REx™-293 cell line is available from Invitrogen (see page vii for ordering information). This human embryonic kidney cell line contains a single integrated FRT site and expresses the Tet repressor and may be used as a host for your pcDNA5/FRT/TO-E© construct to generate a Flp-In™ T-REx™ expression cell line.

Tetracycline Regulation in the Flp-In™ T-REx™ System

The Flp-In™ T-REx™ System uses regulatory elements from the *E. coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen and Berens, 1994; Hillen *et al.*, 1983) to allow tetracycline-regulated expression of your gene of interest from pcDNA5/FRT/TO-E©. The mechanism of tetracycline regulation in the system is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest (Yao *et al.*, 1998). In the system, expression of your gene of interest is repressed in the absence of tetracycline and induced in the presence of tetracycline (Yao *et al.*, 1998).

Expression of your gene of interest from the pcDNA5/FRT/TO-E© vector is controlled by the human cytomegalovius (CMV) promoter into which 2 tandem copies of the *tet* operator 2 ($TetO_2$) sequence have been inserted. The $TetO_2$ sequences consist of 2 copies of the 19 nucleotide sequence:

5′-TCCCTATCAGTGATAGAGA-3′

separated by a 2 base pair spacer (Hillen and Berens, 1994; Hillen *et al.*, 1983). Each 19 nucleotide $TetO_2$ sequence serves as the binding site for 2 molecules of the Tet repressor.

Mechanism of Repression/ Derepression

In the absence of tetracycline, the Tet repressor (expressed from the pcDNA6/TR© plasmid) forms a homodimer that binds with extremely high affinity to each $TetO_2$ sequence in the promoter of the pcDNA5/FRT/TO-E© vector (Hillen and Berens, 1994). The 2 $TetO_2$ sites in the promoter of pcDNA5/FRT/TO-E© serve as binding sites for 4 molecules (or 2 homo-dimers) of the Tet repressor. The affinity of the Tet repressor for the *tet* operator is $K_B = 2 \times 10^{11}\ M^{-1}$ (as measured under physiological conditions), where K_B is the binding constant (Hillen and Berens, 1994). Binding of the Tet repressor homodimers to the $TetO_2$ sequences represses transcription of your gene of interest. Upon addition, tetracycline binds with high affinity to each Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The association constant, K_A, of tetracycline for the Tet repressor is $3 \times 10^9\ M^{-1}$ (Hillen and Berens, 1994). The Tet repressor:tetracycline complex then dissociates from the Tet operator and allows induction of transcription from the gene of interest. For more information about the pcDNA6/TR© plasmid, refer to the Flp-In™ T-REx™ Core Kit manual.

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Overview, continued

Experimental Outline

The table below describes the general steps needed to recombine, transform, and express your protein of interest.

Step	Action	Page
1	Perform the recombination reaction using your donor vector and pcDNA5/FRT/TO-E©.	6
2	Transform the recombination reaction into chemically competent TOP10 *E. coli* and select transformants on LB plates containing 50 µg/ml kanamycin.	7
3	Analyze transformants by restriction digestion and sequencing, if desired.	8-10
4	Select the correct clone and transfect your construct and pOG44 into the Flp-In™ T-REx™ host cell line of interest using your method of choice. Select for stable transfectants using hygromycin.	11-14
5	Induce expression of your recombinant protein with tetracycline and analyze by Western blot or functional assay.	14-15
6	Purify your protein (if desired).	16

Methods

Recombining Your Gene into pcDNA5/FRT/TO-E©

Introduction

At this point, you should have a plasmid preparation of your donor vector construct in addition to pcDNA5/FRT/TO-E©. Review the information below and on the next page before performing the recombination reaction.

Preparation and Maintenance of pcDNA5/FRT/TO-E©

To prepare pcDNA5/FRT/TO-E© for use, add 20 µl sterile water to prepare a 1 µg/µl stock solution. Use the stock solution as is or a diluted aliquot of the plasmid. Store the stock solution at -20°C when you are finished.

If you wish to propagate the pcDNA5/FRT/TO-E© plasmid or prepare plasmid DNA, transform the plasmid into One Shot® TOP10 *E. coli* as described on page 7. Use 10-100 ng of plasmid DNA for transformation and select transformants on LB plates containing 50-100 µg/ml ampicillin. Prepare a glycerol stock of your plasmid-containing TOP10 strain for long-term storage (see page 10).

Before Starting

You will need the following reagents and equipment.

- 100 ng of your donor vector construct
- 100 ng of pcDNA5/FRT/TO-E© (included in the kit)
- Microcentrifuge tubes
- Heat blocks set at 37°C and 65°C
- Ice bucket with ice
- Cre Recombinase and 10X Recombinase Buffer (included in the kit)

Recombination Reaction

1. Set up each 20 µl recombination reaction **on ice** as follows:

Donor vector (100 ng)		x µl
pcDNA5/FRT/TO-E© (100 ng)		y µl
10X Recombinase Buffer		2 µl
Deionized water	add to a total volume of	17 µl
Cre Recombinase		1 µl
Final Volume		20 µl

2. Incubate at 37°C for 20 minutes.
3. Incubate at 65°C for 5 minutes to inactivate the recombinase.
4. Place the tube on ice and proceed to **Transformation**, next page. If you run out of time, you may store the recombination reaction at +4°C or -20°C overnight. Longer storage times have not been tested.

Transforming the Recombination Reaction

Introduction

Once you have performed the recombination reaction, you are ready to transform your *E. coli* host. We recommend using One Shot® TOP10 *E. coli* (available with some kits; see page iv) for transformation, but other strains are suitable. *E. coli* strains should be endonuclease A deficient (*endA*) to ensure quality plasmid preparations and recombination deficient (*recA*) to reduce non-specific recombination.

Materials Supplied by the User

In addition to general microbiological supplies (*i.e.* plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath
- LB plates containing 50 µg/ml kanamycin (see **Important**, below)
- 37°C shaking and non-shaking incubator

Important

It is important to select the fusion plasmid using kanamycin. Remember that the donor vector contains the R6Kγ origin. This origin can only be maintained in *E. coli* strains containing the *pir* gene. After the donor vector and pcDNA5/FRT/TO-E© have recombined to form the fusion plasmid, the kanamycin resistance gene (from the donor vector) is linked to the pUC origin (from pcDNA5/FRT/TO-E©). The fusion plasmid can be maintained in *E. coli* strains that do not contain the *pir* gene (*i.e.* TOP10). By selecting for kanamycin resistance, you ensure that only colonies containing the fusion plasmid are selected.

Preparation for Transformation

The following transformation protocol is for use with the One Shot® TOP10 Chemically Competent *E. coli* available with the kit. Please follow the manufacturer's protocol, if you are using other competent cells.

For each transformation, you will need one vial of One Shot® TOP10 Competent *E. coli* and two selective plates. Perform the following steps before beginning.

1. Equilibrate a water bath to 42°C.
2. Bring the vial of SOC medium from the kit to room temperature.
3. Warm LB plates containing 50 µg/ml kanamycin at 37°C for 30 minutes.
4. Thaw **on ice** 1 vial of One Shot® cells for each transformation.

One Shot® Transformation Reaction

1. Add 5 µl of the recombination reaction to a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Heat-shock the cells for 30 seconds at 42°C without shaking.
3. Immediately transfer the tubes to ice.
4. Add 450 µl of room temperature SOC medium.
5. Cap the tube tightly and shake the tube horizontally at 37°C for 45 minutes.
6. Spread 50 µl from each transformation onto a prewarmed LB plate containing 50 µg/ml kanamycin. The remaining cells are pelleted, resuspended in 50 µl SOC and plated. Incubate plates overnight at 37°C.
7. An efficient recombination reaction will produce hundreds of colonies.

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Transforming the Recombination Reaction, continued

Analysis of Positive Clones

1. Pick 5 colonies (from step 7, see previous page) and culture them overnight in 2-5 ml LB or SOB medium containing 50 µg/ml kanamycin. See page 17 for recipes for LB and SOB media.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01) or the S.N.A.P.™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01).
3. Analyze the plasmids by restriction analysis. Use an enzyme or enzymes that cut once in the donor vector and once in the acceptor vector to yield two fragments that are distinguishable from one another. Note that other strategies are possible.
4. (Optional) To sequence the fusion plasmid to confirm the fusion junctions, use the CMV Forward (supplied with the kit) and the Uni1 Forward primers. Refer to the diagram on the next page for the sequence around the pcDNA5/FRT/TO-E© *loxP* site. Refer to the donor vector manual for the sequence around the donor vector *loxP s*ite.
5. If you need help with setting up restriction enzyme digests or DNA sequencing, refer to general molecular biology texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

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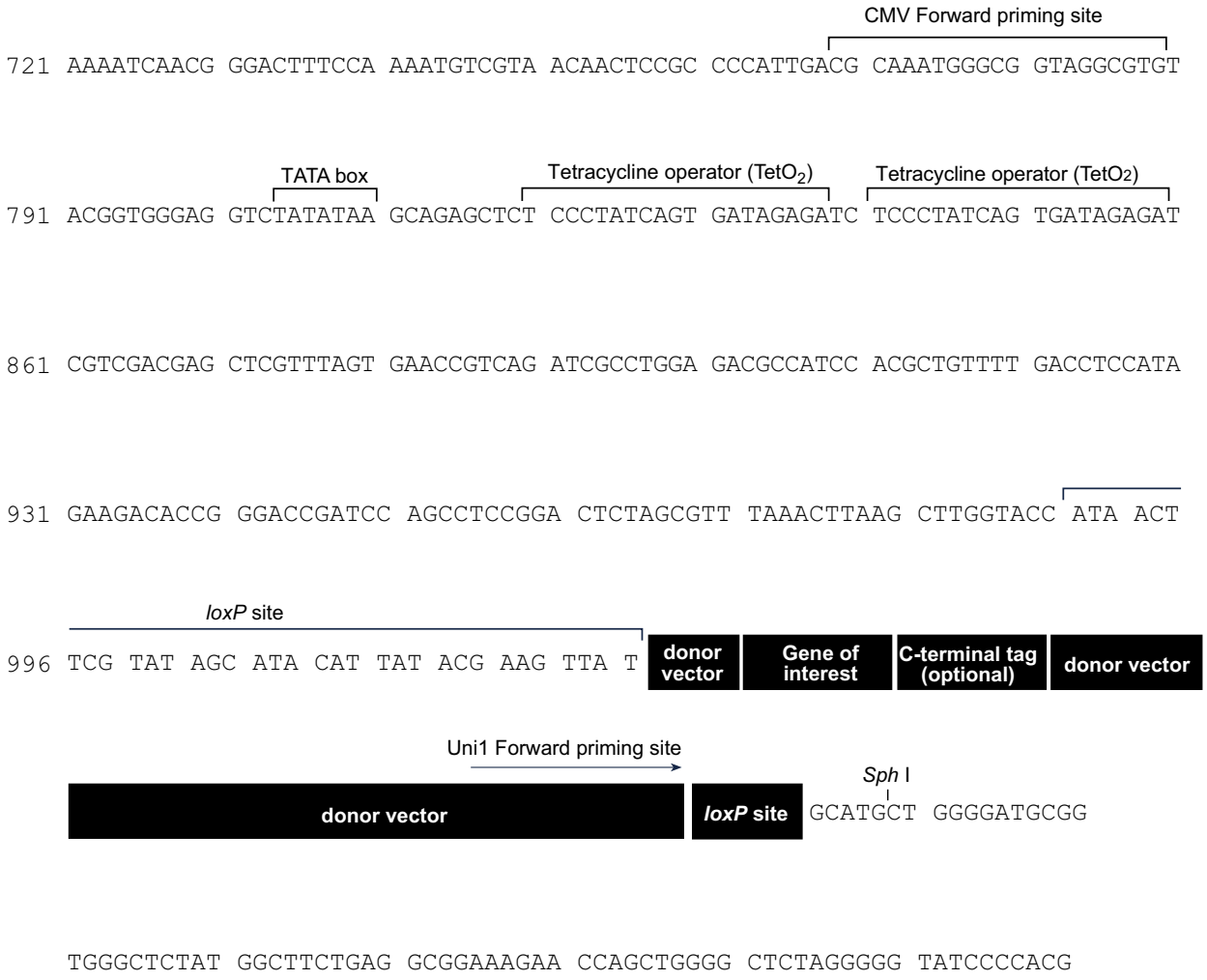
Transforming the Recombination Reaction, continued

Sequencing Your Construct

The sequence surrounding your insert is shown below. The unique restriction site is labeled to indicate the cleavage site. The complete sequence of pcDNA5/FRT/TO-E© is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 23).



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Transforming the Recombination Reaction, continued

Fusion Plasmid Analysis

It should be clear from restriction analysis that you have a dimer plasmid consisting of the donor vector and pcDNA5/FRT/TO-E©. Occasionally, trimers will result. Trimers usually consist of two donor vector molecules and one acceptor molecule, but they express as well as the dimer product.

In theory, trimers may result from two sequential fusion events or a single fusion event between a pre-existing monomeric substrate and a dimeric substrate. The production of trimers can be eliminated if gel-purified monomeric supercoiled DNA is used in the recombination reaction.

Preparing a Glycerol Stock for Long-Term Storage

Once you have identified the correct clone, prepare a glycerol stock for long term storage.

1. Streak out the original colony on LB plates containing 50 µg/ml kanamycin to isolate single colonies.
2. Select a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow overnight until culture is saturated.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Note: You may also want to store a stock of plasmid DNA at -20°C.

Creation of Stable Flp-In™ T-REx™ Expression Cell Lines

Introduction

Once you have created the pcDNA5/FRT/TO-E© fusion plasmid, have verified its integrity, and have prepared clean plasmid preparations of both the fusion plasmid and the pOG44 Flp recombinase plasmid, you are ready to cotransfect the plasmids into the Flp-In™ T-REx™ host cell line of choice to generate a stable Flp-In™ T-REx™ expression cell line. You will use hygromycin to select for stable transfectants which have properly undergone Flp-mediated recombination via the FRT sites (see below). General guidelines are provided in this section to transfect cells and induce expression of your gene of interest with tetracycline.

For more information about the characteristics of the Flp-In™ T-REx™ host cell line, see page 4. For instructions to generate a Flp-In™ T-REx™ host cell line, refer to the Flp-In™ T-REx™ Core Kit manual. The Flp-In™ T-REx™ Core Kit manual also includes detailed information on the pOG44 plasmid, Flp recombinase, transfection, and induction of expression.

Important

The hygromycin resistance gene in the pcDNA5/FRT/TO-E© vector lacks an ATG initiation codon and a promoter to drive expression of the gene. Transfection of pcDNA5/FRT/TO© expression construct alone into a Flp-In™ T-REx™ 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 pcDNA5/FRT/TO-E© plasmid and the Flp-In™ T-REx™ host cell line.

pOG44 Plasmid

You will cotransfect the pOG44 plasmid and your pcDNA5/FRT/TO-E© construct into your Flp-In™ T-REx™ host cell line to generate stable cell lines that inducibly express your protein of interest. The pOG44 plasmid allows expression of a weakened Flp recombinase, which then facilitates integration of the pcDNA5/FRT-TO-E© plasmid into the genome via the FRT sites. For more information about the pOG44 plasmid and the Flp recombinase, refer to the Flp-In™ T-REx™ Core Kit manual

Note: 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.

If you wish to express your gene of interest in 293 cells, you may want to use the Flp-In™ T-REx™-293 host cell line available from Invitrogen to establish your expression cell line (see page vii for ordering information). For more information, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 23).

Important

We have observed down-regulation of the viral CMV promoter and subsequent loss of gene expression when pcDNA5/FRT-based expression constructs are introduced into Flp-In™-3T3 or Flp-In™-BHK cells. We recommend that you **DO NOT** use 3T3 or BHK cells when generating your Flp-In™ T-REx™ host cell line.

continued on next page

Creation of Stable Flp-In™ T-REx™ Expression Cell Lines, continued

Note

Integration of the pcDNA5/FRT/TO-E© construct into the genome via the FRT sites will result in the occurrence of the following events:

- 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/$TetO_2$ promoter, your gene of interest, and the BGH polyadenylation signal upstream of the *lacZ-Zeocin*™ fusion gene
- Disruption of the functional *lacZ-Zeocin*™ transcriptional unit caused by loss of the SV40 early promoter and the ATG initiation codon and insertion of the cassette containing the CMV/$TetO_2$ promoter, gene of interest, and the BGH polyadenylation signal

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

- Hygromycin resistance
- Zeocin™ sensitivity
- Lack of β-galactosidase activity
- Blasticidin resistance
- Tetracycline-regulated expression of the gene of interest

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 the transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) or any other resin-based method.

Methods of Transfection

For established cell lines (*e.g.* HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to passage the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Transfection methods include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Invitrogen offers the Calcium Phosphate Transfection Kit (Catalog no. K2780-01) and a large selection of reagents for transfection. For more information about the reagents available, visit our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 23).

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Creation of Stable Flp-In™ T-REx™ Expression Cell Lines, continued

Positive Expression Control

pcDNA5/FRT/TO-E/Uni-CAT© is provided as a positive control vector for mammalian transfection and expression (see page 22) and may be used to optimize transfection and expression conditions for your cell line. The gene encoding chloramphenicol acetyltransferase (CAT) is expressed in mammalian cells under the control of the CMV/TetO_2 promoter. A successful transfection will result in CAT expression that can be easily assayed (see below).

Detection of CAT

The CAT gene in pcDNA5/FRT/TO-E/Uni-CAT© is expressed with a V5 epitope and a polyhistidine (6xHis) tag as a C-terminal fusion. CAT can be detected by Western blot as a 32 kDa polypeptide using antibodies against the tag or an antibody against CAT (available from Invitrogen, see pages vi and viii). Alternatively, you may assay for CAT protein by ELISA assay, fluorometric assay, or radioactive assay (Ausubel *et al.*, 1994; Neumann *et al.*, 1987).

Determination of Hygromycin Sensitivity

To successfully generate a stable cell line expressing your gene of interest from pcDNA5/FRT/TO-E©, you need to determine the minimum concentration of hygromycin B required to kill your untransfected Flp-In™ T-REx™ 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 to ensure that you determine the minimum concentration necessary for your cell line. Hygromycin B is available separately from Invitrogen (see page vii for ordering information). Refer to the **Appendix**, page 19 for instructions to prepare and store hygromycin.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of hygromycin (0, 10, 50, 100, 200, 400 , 600 µg/ml hygromycin).
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 pcDNA5/FRT/TO-E© 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 the Flp-In™ T-REx™ Core Kit manual for details). We have varied the ratio of pOG44 and pcDNA5/FRT/TO-E© expression plasmid that we cotransfect into mammalian Flp-In™ T-REx™ host cells to optimize the recombination efficiency. **We recommend that you cotransfect your Flp-In™ T-REx™ host cell line with a ratio of <u>at least</u> 9:1 (w/w) pOG44:pcDNA5/FRT/TO-E© 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.

continued on next page

Creation of Stable Flp-In™ T-REx™ Expression Cell Lines, continued

Important

When transfecting your Flp-In™ T-REx™ host cell line, do not use linearized pOG44 and pcDNA5/FRT/TO-E© plasmid DNA. Flp-mediated recombination between the FRT site on pcDNA5/FRT/TO-E© and the integrated FRT site in the Flp-In™ T-REx™ host cell line will only occur if the pcDNA5/FRT/TO-E© plasmid is circularized. The pOG44 plasmid should be circularized to minimize the possiblity of the plasmid integrating into the genome.

Selection of Stable Integrants

Once you have determined the appropriate hygromycin concentration to use for selection in your Flp-In™ T-REx™ host cell line, you are ready to generate a stable cell line which inducibly expresses your gene of interest. Tetracycline may be obtained separately from Invitrogen (see page vii for ordering information). Refer to the Flp-In™ T-REx™ Core Kit manual for instructions to prepare and handle tetracycline.

Reminder: Following cotransfection, your Flp-In™ T-REx™ expression clones should become sensitive to Zeocin™ (see Note on page 12); therefore, your selection medium should not contain Zeocin™. Remember that your selection medium should contain blasticidin to select for the pcDNA6/TR© plasmid.

1. Cotransfect your mammalian Flp-In™ T-REx™ host cell line with a 9:1 ratio of pOG44:pcDNA5/FRT/TO-E© fusion plasmid DNA using the desired protocol. Remember to include a plate of untransfected cells as a negative control and the pcDNA5/FRT/TO-E/Uni-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. Split the cells such that they are no more than 25% confluent.
4. Incubate the cells at 37ºC for 2-3 hours until they have attached to the culture dish.
5. Remove the medium and add fresh medium containing hygromycin (and blasticidin) at the pre-determined concentration required for your cell line.
6. Feed the cells with selective medium every 3-4 days until hygromycin-resistant foci can be identified.
7. Pick 5-20 hygromycin-resistant foci and expand the cells. Verify that the pcDNA5/FRT/TO-E© construct has integrated into the FRT site by testing each clone for Zeocin™ sensitivity and lack of β-galactosidase activity.
8. Select those clones that are hygromycin-resistant, Zeocin™-sensitive, and LacZ⁻, and assay for tetracycline-regulated expression of the gene of interest. To induce expression of the gene of interest, add tetracycline to a final concentration of 1 µg/ml (5 µl of a 1 mg/ml stock per 5 ml of medium) to the cells and incubate the cells for 24 hours at 37°C.
9. Harvest the cells and assay for expression of your gene.

continued on next page

Creation of Stable Flp-In™ T-REx™ Expression Cell Lines, continued

Polyclonal Selection

If you use a single integrant as your Flp-In™ T-REx™ host cell line (*i.e.* the cells contain only a single integrated FRT site), all of the hygromycin-resistant foci that you obtain after cotransfection of pcDNA5/FRT/TO-E© and pOG44 and selection with hygromycin should be isogenic. In this case, pcDNA5/FRT/TO-E© 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 tetracycline-regulated expression of your protein of interest.

Detection of Recombinant Fusion Proteins

If you have expressed your protein as a fusion to the C-terminal V5 epitope and polyhistidine (6xHis) tag, you can detect expression using the Anti-V5 or Anti-His(C-term) antibodies (see page viii for ordering information). You may also use an antibody to your protein of interest. **Note:** The C-terminal peptide containing the V5 epitope and the polyhistidine (6xHis) tag will add approximately 5 kDa to the size of your protein.

To detect your fusion protein by Western blot, you will need to prepare a cell lysate from transfected cells. Use the protocol below to lyse cells. Other protocols and lysis buffers are also suitable.

1. Wash cell monolayers (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (PBS, see recipe on page 18).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend pellet in 50 µl Cell Lysis Buffer (see page 18 for recipe) and vortex.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells. **Note:** You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
5. Centrifuge the cell lysate at 10,000 x g for 10 minutes to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration. **Note:** Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer to a final concentration of 1X and heat the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Polyacrylamide Gel Electrophoresis

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

Purification

Introduction

Once you have created stable cell lines expressing your protein of interest, you may purify your recombinant fusion protein from cells, if desired. You may harvest the cells and store them at -80°C until you are ready to purify the fusion protein, or you may proceed directly with protein purification.

Purification

If you have expressed your protein as a fusion to the C-terminal polyhistidine (6xHis) tag, you can purify it using metal-chelating resins such as ProBond™.

If the protein is not expressed as a fusion to the C-terminal polyhistidine (6xHis) tag, then you may use any other chromatographic method of choice.

If you need to perform large-scale purification of your fusion protein (>1 x 10^7 transfected cells), you may need to prepare a column containing the desired amount of resin from bulk precharged ProBond™ resin available separately (see page viii for ordering information).

Binding Capacity of ProBond™

One milliliter of ProBond™ resin binds at least 1 mg of recombinant protein. This amount can vary depending on the nature of the recombinant protein.

Preparation of Cells for Lysis

Use the procedure below to prepare cells for lysis if you will be purifying your protein on ProBond™ Resin. You will need 5 x 10^6 to 1 x 10^7 stably transfected cells for purification of your protein on a 2 ml ProBond™ column (see ProBond™ Purification System manual).

1. Seed cells in either five T-75 flasks or two to three T-175 flasks.
2. Grow the cells in selective medium until they are approximately 80% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with fresh medium (if necessary) and transfer the cells to a microcentrifuge tube.
5. Centrifuge the cells at 1500 rpm for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 1500 rpm for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -70°C until needed.

Lysis of Cells and ProBond™ Column Purification

If you are using ProBond™ resin, refer to the ProBond™ Purification System manual for details about sample preparation and column purification. The ProBond™ Purification System manual is available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (see page 23).

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

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
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 (50 µg/ml of kanamycin), and pour into 10 cm plates.
4. Let harden, then invert the plates and store at +4°C, in the dark.

SOB Medium (with Kanamycin)

2% Tryptone
0.5% Yeast Extract
0.05% NaCl
2.5 mM KCl
10 mM $MgCl_2$

1. Dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 ml deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.5 with 5 N NaOH and add deionized water to 1 liter.
5. Autoclave this solution, cool to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$. You may also add kanamycin to 50 µg/ml.
6. Store at +4°C. Medium is stable for only ~1 month.

continued on next page

Recipes, continued

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. Prepare the buffer from the following stock solutions. For 100 ml, combine:

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

Note: Just before use, add protease inhibitors to a small amount of buffer at the following final concentrations:

1 mM PMSF

1 µg/ml pepstatin

1 µg/ml leupeptin

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 up to 1 liter and autoclave for 20 minutes on liquid cycle.
4. Store at +4°C or room temperature.

Hygromycin

Hygromycin B

The pcDNA5/FRT/TO-E© vector contains the *E. coli* hygromycin resistance gene (*HPH*) (Gritz and 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.

Handling Hygromycin B

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

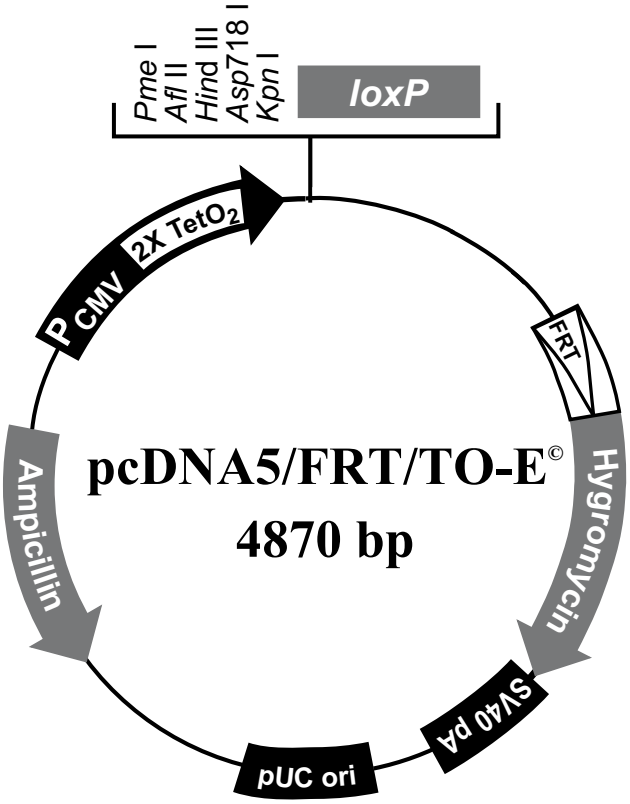
Preparing and Storing Hygromycin B

The hygromycin B (MW = 527.5 kDa) available from Invitrogen 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 B is stable for up to six weeks.

pcDNA5/FRT/TO-E© Vector

Map of pcDNA5/FRT/TO-E©

The figure below summarizes the features of the pcDNA5/FRT/TO-E© vector. The complete sequence for pcDNA5/FRT/TO-E© is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 23). Details of the sequences surrounding the *loxP* site in pcDNA5/FRT/TO-E© may be found on page 9.



Comments for pcDNA5/FRT/TO-E©
4870 nucleotides

CMV promoter: bases 232-958
TATA box: bases 804-810
Tetracycline operator (2X $TetO_2$) sequences: bases 820-859
CMV forward priming site: bases 769-789
loxP site: bases 990-1023
FRT site: bases 1336-1383
Hygromycin resistance gene (no ATG): bases 1391-2411
SV40 early polyadenylation signal: bases 2543-2673
pUC origin: bases 3056-3729 (complementary strand)
bla promoter: bases 4735-4833 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 3874-4734 (complementary strand)

continued on next page

pcDNA5/FRT/TO-E© Vector, continued

Features of pcDNA5/FRT/TO-E©

pcDNA5/FRT/TO-E© (4870 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
CMV Forward site	Allows sequencing through the insert
Tetracycline operator 2 ($TetO_2$) sequences	Two tandem 19 nucleotide repeats which serve as binding sites for tet repressor homodimers (Hillen and Berens, 1994; Hillen *et al.*, 1983)
loxP site	Allows recombination between the donor vector and pcDNA5/FRT/TO-E (Hoess, *et al*., 1982)
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 and Sadowski, 1985; Jayaram, 1985; Senecoff *et al.*, 1985)
Hygromycin resistance gene (no ATG)	Allows selection of stable transfectants in mammalian cells (Gritz and 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	Allows selection of transformants in *E. coli*

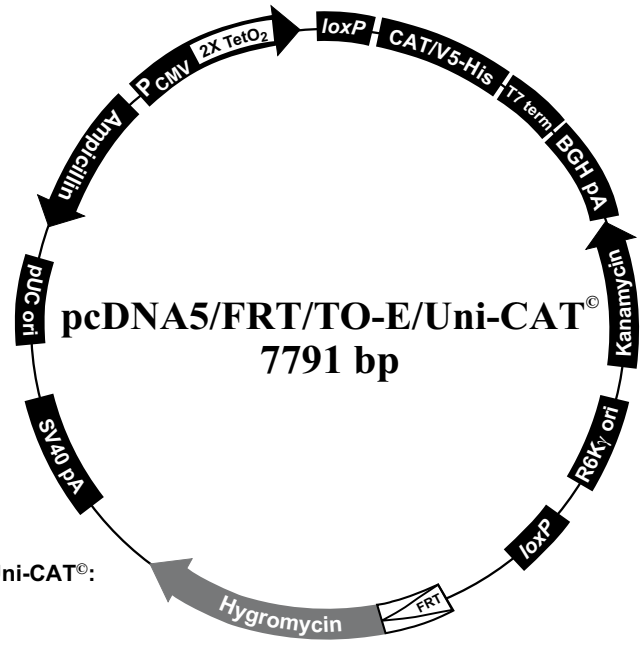
pcDNA5/FRT/TO-E/Uni-CAT©

Description

pcDNA5/FRT/TO-E/Uni-CAT© is a 7.8 kb expression control vector that contains the CAT gene. The CAT gene was amplified and TOPO® Cloned into pUni/V5-His/Gene-TOPO®. The resulting vector was recombined with pcDNA5/FRT/TO-E© using Cre recombinase to create pcDNA5/FRT/TO-E/Uni-CAT©. **Note**: pUni/V5-His/Gene-TOPO® is similar to pUni/V5-His-TOPO® TA except that it contains additional DNA between the TOPO® Cloning site and the V5 epitope.

Map of Expression Control Vector

The figure below summarizes the features of the pcDNA5/FRT/TO-E/Uni-CAT© vector. The complete nucleotide sequence for pcDNA5/FRT/TO-E/Uni-CAT© is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 23).



Comments for pcDNA5/FRT/TO-E/Uni-CAT©:
7791 nucleotides

CMV promoter: bases 232-958
TATA box: bases 804-810
Tetracycline operator 2 (2X $TetO_2$) sequences: bases 820-859
CMV Forward priming site: bases 769-789
loxP site: bases 990-1023
CAT ORF: bases 1056-1712
V5 epitope: bases 1731-1772
Polyhistidine (6xHis) region: bases 1773-1790
BGH polyadenylation sequence: bases 1814-2022
T7 transcription termination signal: bases 2037-2165
Kanamycin resistance gene: bases 2344-3138 (complementary strand)
R6Kγ origin: bases 3610-3890
loxP site: bases 3911-3944
FRT site: bases 4257-4304
Hygromycin resistance gene: bases 4312-5332
SV40 early polyadenylation sequence: bases 5464-5594
pUC origin: bases 5977-6650 (complementary strand)
bla promoter: bases 7656-7754 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 6795-7655 (complementary strand)

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact Us

For more information or technical assistance, please call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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MSDS Requests

To request an MSDS, please visit our Web site (www.invitrogen.com) and follow the instructions below.

1. On the home page, go to the left-hand column under 'Technical Resources' and select 'MSDS Requests'.
2. Follow instructions on the page and fill out all the required fields.
3. To request additional MSDSs, click the 'Add Another' button.
4. All requests will be faxed unless another method is selected.
5. When you are finished entering information, click the 'Submit' button. Your MSDS will be sent within 24 hours.

continued on next page

Technical Service, continued

Emergency Information

In the event of an emergency, customers of Invitrogen can call the 3E Company, 24 hours a day, 7 days a week for disposal or spill information. The 3E Company can also connect the customer with poison control or with the University of California at San Diego Medical Center doctors.

3E Company
Voice: 1-760-602-8700

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, please contact our Technical Service Representatives.

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Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pcDNA5/FRT/TO-E© Echo™-Adapted Expression Vector Kit.

Vectors

pcDNA5/FRT/TO-E© and pcDNA5/FRT/TO-E/Uni-CAT© are qualified by restriction digest. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pcDNA5/FRT/TO-E©	pcDNA5/FRT/TO-E/Uni-CAT©
Xba I	4870 bp	2261, 5530 bp
*Hin*d III/*Sna*B I	388, 4482 bp	244, 388, 2632, 4527 bp

Primers

The CMV Forward sequencing primer has been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

Cre Recombinase

Purity: >95% homogeneity

Endonuclease activity: Negative

Exonuclease activity: Negative

Functional Assay: Cre recombinase is qualified using the assay on page 6 of this manual. The donor vector is pUni/*lacZ* and the acceptor vector is pcDNA3.1-E©. Five microliters of the recombination reaction is transformed into 50 µl One Shot® TOP10 chemically competent *E. coli* using the protocol on page 7. Twenty-five µl of the transformation reaction is plated on LB agar plates containing 50 µg/ml kanamycin (performed in duplicate). One microliter of Cre recombinase should yield >500 blue, kanamycin-resistant transformants.

One Shot® Competent *E. coli*

All competent cells are qualified as follows:

- Cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA for chemically competent cells and >1 x 10^9 for electrocompetent cells.
- To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
- Untransformed cells are plated on LB plates 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

References

Abremski, K., and Hoess, R. (1984). Bacteriophage P1 Site-Specific Recombination. Purification and Properties of the Cre Recombinase Protein. J. Biol. Chem. *259*, 1509-1514.

Abremski, K., Hoess, R., and Sternberg, N. (1983). Studies on the Properties of P1 Site-Specific Recombination: Evidence for Topologically Unlinked Products Following Recombination. Cell *32*, 1301-1311.

Abremski, K. E., and Hoess, R. H. (1992). Evidence for a Second Conserved Arginine Residue in the Integrase Family of Recombination Proteins. Protein Eng. *5*, 87-91.

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989). Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. J. Biol. Chem. *264*, 8222-8229.

Argos, P., Landy, A., Abremski, K., Egan, J. B., Ljungquist, E. H., Hoess, R. H., Kahn, M. L., Kalionis, B., Narayana, S. V. L., and Pierson, L. S. (1986). The Integrase Family of Site-Specific Recombinases: Regional Similarities and Global Diversity. EMBO J. *5*, 433-440.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell *41*, 521-530.

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Molec. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326.

Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121.

Felgner, P. L. a., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. Nature *337*, 387-388.

Gritz, L., and Davies, J. (1983). Plasmid-Encoded Hygromycin-B Resistance: The Sequence of Hygromycin-B-Phosphotransferase Gene and its Expression in *E. coli* and *S. Cerevisiae*. Gene *25*, 179-188.

Gronostajski, R. M., and Sadowski, P. D. (1985). Determination of DNA Sequences Essential for FLP-mediated Recombination by a Novel Method. J. Biol. Chem. *260*, 12320-12327.

Hillen, W., and Berens, C. (1994). Mechanisms Underlying Expression of Tn*10* Encoded Tetracycline Resistance. Annu. Rev. Microbiol. *48*, 345-369.

Hillen, W., Gatz, C., Altschmied, L., Schollmeier, K., and Meier, I. (1983). Control of Expression of the Tn*10*-encoded Tetracycline Resistance Genes: Equilibrium and Kinetic Investigations of the Regulatory Reactions. J. Mol. Biol. *169*, 707-721.

Hoess, R. H., Ziese, M., and Sternberg, N. (1982). P1 Site-Specific Recombination: Nucleotide Sequence of the Recombining Sites. Proc. Natl. Acad. Sci USA *79*, 3398-3402.

References, continued

Jayaram, M. (1985). Two-micrometer Circle Site-specific Recombination: The Minimal Substrate and the Possible Role of Flanking Sequences. Proc. Natl. Acad. Sci. USA *82*, 5875-5879.

Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Pluckthun, A. (1997). Specific Detection of His-tagged Proteins With Recombinant Anti-His Tag scFv-Phosphatase or scFv-Phage Fusions. BioTechniques *22*, 140-149.

Liu, Q., Li, M. Z., Leibham, D., Cortez, D., and Elledge, S. (1998). The Univector Plasmid-Fusion System, a Method for Rapid construction of Recombinant DNA Without Restriction Enzymes. Current Biology *8*, 1300-1309.

Liu, Q., Li, M. Z., Liu, D., and Elledge, S. J. (1999). Rapid Construction of Recombinant DNA by the Univector Plasmid-Fusion System. Methods in Enzymology, in press.

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987). Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129.

Neumann, J. R., Morency, C. A., and Russian, K. O. (1987). A Novel Rapid Assay for Chloramphenicol Acetyltransferase Gene Expression. BioTechniques *5*, 444-447.

Palmer, T. D., Hock, R. A., Osborne, W. R. A., and Miller, A. D. (1987). Efficient Retrovirus-Mediated Transfer and Expression of a Human Adenosine Deaminase Gene in Diploid Skin Fibroblasts from an Adenosine-Deficient Human. Proc. Natl. Acad. Sci. U.S.A. *84*, 1055-1059.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Senecoff, J. F., Bruckner, R. C., and Cox, M. M. (1985). The FLP Recombinase of the Yeast 2-micron Plasmid: Characterization of its Recombination Site. Proc. Natl. Acad. Sci. USA *82*, 7270-7274.

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991). Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557.

Sternberg, N., and Hamilton, D. (1981). Bacteriophage P1 Site-Specific Recombination. I. Recombination Between *loxP* Sites. J. Mol. Biol. *150*, 467-486.

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.

Yao, F., Svensjo, T., Winkler, T., Lu, M., Eriksson, C., and Eriksson, E. (1998). Tetracycline Repressor, tetR, Rather than the tetR-Mammalian Cell Transcription Factor Fusion Derivatives, Regulates Inducible Gene Expression in Mammalian Cells. Hum. Gene Ther. *9*, 1939-1950.