USER GUIDE

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pcDNA™6/TR

A regulatory vector designed for use with the T-REx™ System

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For Research Use Only. Not for diagnostic procedures.

life technologies™

Contents

Kit Contents and Storage

Kit Contents	20 µg (40 µL at 0.5 µg/µL) of pcDNA™6/TR vector in TE Buffer, pH 8.0. **TE Buffer:** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
Shipping and Storage	The plasmid is shipped on wet ice. Upon receipt, store the plasmid at –20°C.
Product Use	**For research use only.** Not intended for any human or animal diagnostic or therapeutic uses.

Additional Products

T-REx™ System

The pcDNA™6/TR regulatory plasmid is designed for use with the T-REx™ System for tetracycline-regulated expression of your gene of interest in mammalian cells. The Core System includes the inducible expression vector of choice, the regulatory vector, and primers for sequencing. The Complete System includes the Core System plus inducing and selection agents. See the table below for a detailed description of the contents of each T-REx™ Kit.
For more information on the T-REx™ System, refer to our website (www.lifetechnologies.com) or contact Technical Support (see page 10).

T-REx™ Kit	Inducible Expression Vector	Catalog no.
Complete System	pcDNA™4/TO	K1020-01
	pcDNA™4/TO/*myc*-His	K1030-01
Core System	pcDNA™4/TO	K1020-02
	pcDNA™4/TO/*myc*-His	K1030-02

T-REx™ Cell Lines

For your convenience, Life Technologies has available several mammalian cells lines that stably express the Tet repressor. For more information, refer to our website (www.lifetechnologies.com) or contact Technical Support (see page 10).

Cell Line	Source	Catalog no.
T-REx™-293	Human embryonic kidney	R710-07
T-REx™-HeLa	Human cervical adenocarcinoma	R714-07
T-REx™-CHO	Chinese hamster ovary	R718-07
T-REx™-Jurkat	Human lymphocyte	R714-07

Additional Products, continued

Accessory Products

Many of the reagents used with the T-REx™ System are available separately from Life Technologies. See the table below for ordering information.

Item	Amount	Catalog no.
pcDNA™4/TO	20 µg	V1020-20
pcDNA™4/TO/*myc*-His A, B, C	20 µg each	V1030-20
Blasticidin S HCl	50 mg	R210-01
Blasticidin S HCl, liquid	20 mL	A11139-02
	10 × 1 mL	A11139-03
Zeocin™	1 g	R250-01
	5 g	R250-05
Lipofectamine® 2000 Reagent	0.75 mL	11668-027
	1.5 mL	11668-019
	15 mL	11668-500
PureLink® Quick Plasmid Miniprep Kit	50 preps	K2100-10
	250 preps	K2100-11

Methods

Using pcDNA™6/TR

Introduction

pcDNA6™/TR is a 6.7 kb vector designed for use with the T-REx™ System (see page v). The vector expresses high levels of the tetracycline (Tet) repressor under the control of the human cytomegalovirus immediate-early (CMV) promoter. High-level stable and transient expression of the Tet repressor can be carried out in most mammalian cells. Tetracycline-regulated expression of a gene of interest may then be tested by transfecting the inducible expression plasmid into host cells expressing the Tet repressor. Refer to the **Appendix**, pages 8–9, for a map and description of the features of the pcDNA6/TR vector.

For more information about the T-REx™ System, refer to the T-REx™ System manual. For information about T-REx™ inducible expression vectors, refer to the manual for each specific vector. Manuals are available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 10). To order components of the T-REx™ System separately, see page vi.

A Note about the *TetR* Gene

The *TetR* gene used in pcDNA™6/TR was originally isolated from the Tn*10* transposon which confers resistance to tetracycline in *E. coli* and other enteric bacteria (Postle *et al.*, 1984). The *TetR* gene from Tn*10* encodes a class B Tet repressor and is often referred to as *TetR(B)* in the literature (Hillen & Berens, 1994).

The *TetR* gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa. For more information about the Tet repressor, its interaction with Tet operator sequences, and tetracycline regulation, refer to the T-REx™ System Manual or to published reviews (Hillen & Berens, 1994; Hillen *et al.*, 1983).

Maintaining pcDNA™6/TR

The pcDNA™6/TR vector contains the ampicillin resistance gene and the blasticidin resistance gene, either of which allows selection of the plasmid in *E. coli*.

To propagate and maintain the pcDNA™6/TR vector, use 10 ng of each vector to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™-T1^R, JM109, or equivalent. Select transformants on LB agar plates containing 50–100 µg/ml ampicillin or on Low Salt LB plates containing 100 mg/ml blasticidin (see the **Appendix**, page 7, for recipe). Be sure to prepare a glycerol stock of each plasmid for long-term storage (see page 2).

Selection in *E. coli*

To facilitate selection of blasticidin-resistant *E. coli*, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.0. Prepare Low Salt LB broth and plates using the recipe in the Appendix (page 7).

Failure to lower the salt content of your LB medium will result in non-selection due to inhibition of the drug unless a higher concentration of blasticidin is used.

Continued on next page

Using pcDNA™6/TR, continued

Preparing a Glycerol Stock

Once you have identified the correct clone, 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 50–100 mg/mL ampicillin or 100 mg/mL blasticidin in Low Salt LB. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 ml of LB containing 50–100 mg/ml ampicillin or 100 mg/mL blasticidin in Low Salt LB.
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.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be clean and free of phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink® HQ Mini Plasmid Purification Kit (page vi). Other methods of obtaining high quality plasmid DNA may be suitable.

Method of Transfection

To transfect established mammalian cell lines (*e.g.*, HeLa, COS-1) with pcDNA™6/TR, 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.*, 1987; Felgner & Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa & Dower, 1988). Life Technologies offers the Lipofectamine® 2000 Reagent for mammalian cell transfection (see page vi). For more information on transfection reagents available from Life Technologies, refer to our website (www.lifetechnologies.com) or contact Technical Support (see page 10).

Continued on next page

Using pcDNA™6/TR, continued

Transient Transfection

You may use any of the methods on page 2 to transiently cotransfect pcDNA™6/TR and your inducible expression construct into the mammalian host cell line.

Because the amount of Tet repressor expressed in the cell will determine the level of transcriptional repression of the hybrid CMV/$TetO_2$ promoter in the inducible expression plasmid, we recommend that you increase the amount of pcDNA™6/TR DNA transfected into your host cell line relative to inducible expression plasmid DNA. Increasing the ratio of pcDNA™6/TR:inducible expression plasmid DNA from 1:1 to **at least 6:1** should ensure that a sufficient amount of Tet repressor is expressed to suitably repress basal transcription of your gene of interest.

For more information about transfection and induction of expression using tetracycline, refer to the T-REx™ System manual.

Creating Stable Cell Lines

Introduction

To generate a cell line that stably expresses the Tet repressor, you may transfect pcDNA™6/TR into your mammalian host cell line and select with blasticidin. You may then use the cell lines expressing suitably high levels of the Tet repressor as hosts to stably or transiently express your gene of interest from the inducible expression vector. Before transfection, we recommend that you first test the sensitivity of your mammalian host cell to blasticidin as natural resistance varies among cell lines.

Determining Antibiotic Sensitivity

To generate a stable cell line expressing pcDNA™6/TR, you need to determine the minimum concentration of blasticidin required to kill your untransfected host cell line. Typically, concentrations between 2 and 10 mg/mL blasticidin are sufficient to kill the untransfected host cell line. Test a range of concentrations (see below) to ensure that you determine the minimum concentration necessary for your cell line. For more information about blasticidin and instructions for use, refer to the **Appendix**, page 6.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare 6 plates of cells.
2. The next day, substitute culture medium with medium containing varying concentrations of blasticidin (*e.g.*, 0, 1, 3, 5, 7.5, and 10 mg/mL).
3. Replenish the selective medium every 3–4 days. Cells sensitive to blasticidin will round up and detach from the plate. Dead cells will accumulate in the medium.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of blasticidin that prevents growth within 1–2 weeks after its addition.

Possible Sites for Linearization

To obtain stable transfectants, you may choose to linearize the pcDNA™6/TR plasmid before transfection. While linearizing your vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts the *TetR* gene or other elements required for mammalian expression. The table below lists the unique sites that you may use to linearize pcDNA™6/TR prior to transfection. **Other restriction sites are possible.**

Enzyme	Restriction Site (bp)	Location
*Bst*1107 I	4470	Backbone
Sap I	4733	Backbone
*Bsp*LU11 I	4849	Backbone
*Eam*1105 I	5739	Ampicillin gene
Fsp I	5961	Ampicillin gene

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Creating Stable Cell Lines, continued

Selecting Stable Integrants

Once you have determined the appropriate blasticidin concentration to use for selection, you can generate a stable cell line expressing pcDNA™6/TR.

1. Transfect your cell line of choice with pcDNA™6/TR using the desired protocol. Include a sample 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 containing blasticidin at the appropriate concentration for your cell line. Split the cells such that they are no more than 25% confluent. If the cells are too dense, the blasticidin will not kill the untransfected cells.
4. Replenish selective medium every 3–4 days until blasticidin-resistant colonies are detected. Typically, blasticidin selection takes 7–10 days.
5. Pick and expand at least 20 colonies. To screen the clones for those expressing the highest levels of Tet repressor, transiently transfect the postive control plasmid containing the *lacZ* gene into the cells and assay for β-galactosidase expression after induction with tetracycline. Select for those clones exhibiting the lowest basal levels and highest inducible levels of β-galactosidase expression. For more information about the positive control plasmid and how to assay for β-galactosidase expression, refer to the manual for the inducible expression vector that you have obtained. For more information about induction of gene expression with tetracycline, refer to the T-REx™ System manual.
6. Once you have obtained cell lines that stably express the Tet repressor from pcDNA™6/TR, you may use these cell lines to assay for tetracycline-regulated expression of your gene of interest from the pcDNA™4/TO-based expression vector.

Appendix

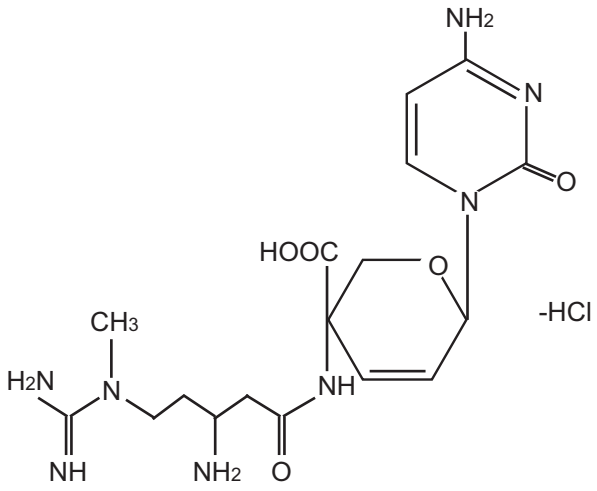
Blasticidin

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of one of two blasticidin S deaminase genes: *bsd from Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Molecular Weight, Formula, and Structure

The formula for blasticidin S is $C_{17}H_{36}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (*e.g.*, a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

1. Dissolve blasticidin in sterile water to make up the desired concentration, and filter-sterilize the solution.
2. Aliquot in small volumes suitable for one time use, and store at –20°C for long-term (6–8 weeks), or at 4°C for short-term (1–2 weeks). Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**). You may store media containing blasticidin at 4°C for up to 2 weeks.

   Note: The pH of the aqueous solution should be 7.0 to prevent inactivation of blasticidin.

Recipes

LB (Luria-Bertani) Medium

10 g Tryptone
5 g Yeast Extract
10 g NaCl

1. Dissolve tryptone, yeast extract, and 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. Allow solution to cool to ~55°C and add antibiotic, if desired.
4. Store at 4°C.

LB Plates Containing Ampicillin

Follow the instructions below to prepare LB agar plates containing ampicillin.

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add ampicillin to a final concentration of 100 µg/mL and pour into 10 cm plates.
4. Invert and store at 4°C.

Low Salt LB Medium with Blasticidin

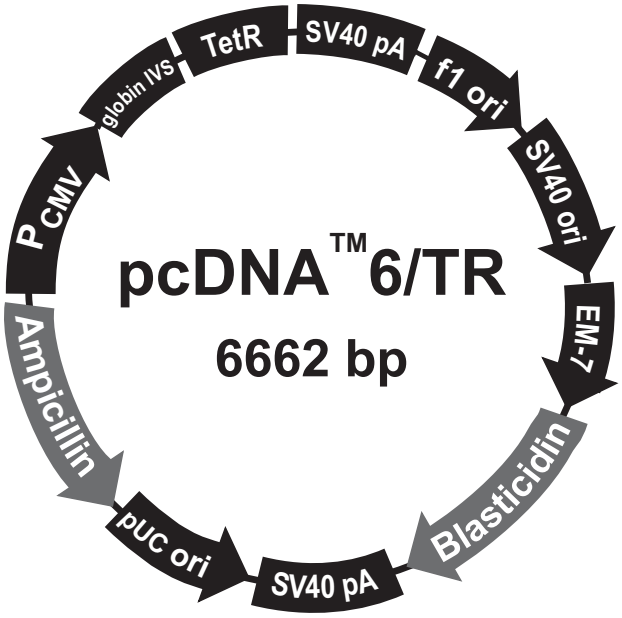
10 g Tryptone
5 g Yeast Extract
5 g NaCl

1. Dissolve tryptone, yeast extract, and NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.5 with 5 M NaOH and bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
3. Autoclave on liquid cycle for 20 minutes. Allow the medium to cool to at least 55°C before adding the blasticidin to 100 µg/ml final concentration.
4. Store plates at 4°C in the dark. Plates containing blasticidin are stable for up to 2 weeks..

pcDNA™6/TR

Map of pcDNA™6/TR

The figure below summarizes the features of the pcDNA™6/TRvector. **The sequence of pcDNA™6/TR is available for downloading from our website site (www.lifetechnologies.com) or from Technical Support (see page 10).** See the next page for a description of the features of the vector.



Comments for pcDNA™6/TR
6662 nucleotides

CMV promoter: bases 232-819
Rabbit β-globin intron II (IVS): bases 1028-1600
TetR gene: bases 1684-2340
SV40 early polyadenylation sequence: bases 2346-2477
f1 origin: bases 2897-3325
SV40 promoter and origin: bases 3335-3675
EM-7 promoter: bases 3715-3781
Blasticidin resistance gene: bases 3782-4180
SV40 early polyadenylation sequence: bases 4338-4468
pUC origin: bases 4851-5521
bla promoter: bases 6521-6625 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 5666-6526 (complementary strand)

Features of pcDNA™6/TR

Features

The table below describes the relevant features of pcDNA™6/TR. The vector includes the rabbit β-globin intron II to enhance expression of the *TetR* gene. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate early promoter	Allows high-level expression of the *TetR* gene (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
Rabbit β-globin intron II (IVS)	Enhances expression of the *TetR* gene (van Ooyen *et al.*, 1979)
TetR gene	Encodes the Tet repressor that binds to *tet* operator sequences to repress transcription of the gene of interest in the absence of tetracycline (Postle *et al.*, 1984; Yao *et al.*, 1998)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the blasticidin resistance gene in mammalian cells and episomal replication in cells expressing SV40 large T antigen
EM-7 promoter	Synthetic prokaryotic promoter for expression of the blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene	Allows selection of stable transfectants in mammalian cells (Kimura *et al.*, 1994) and transformants in *E. coli*
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

Technical Support

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