

USER GUIDE

invitrogen™
by *life* technologies™

T-REx™ System

**A Tetracycline-Regulated Expression System
for Mammalian Cells**

Catalog numbers K1020-01, K1020-02, K1030-01, K1030-02

Revision date 8 November 2011

Publication Part number 25-0271

MAN0000105

For Research Use Only. Not for diagnostic dfcWXi fYg.

life
technologies™

Table of Contents

Important Information.....	iv
Introduction	1
The T-REx™ System	1
Methods	4
Using pcDNA™6/TR.....	4
Transfection	6
Creation of Stable Cell Lines	9
Appendix.....	13
Recipes.....	13
Blasticidin	14
pcDNA™6/TR Vector.....	15
Technical Support.....	17
Purchaser Notification	18
References.....	19

Important Information

Introduction

The T-REx™ System manual is supplied with the kits listed below. 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 following table for a detailed description of the contents of each T-REx™ Kit.

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

Shipping/Storage

The T-REx™ Core System is shipped at room temperature. Store at -20°C.

The T-REx™ Complete System is shipped in 2 boxes. Store as described below:

- Box 1 contains vectors, primers, and blasticidin and is shipped at room temperature. Upon receipt, remove the vectors and primers and store at -20°C. Blasticidin powder should be stored at +4°C.
- Box 2 contains tetracycline and Zeocin™ and is shipped on blue ice. Store at +4°C. For long-term storage (> 6 months), store at -20°C. Store the tetracycline and Zeocin™ protected from exposure to light.

Kit Contents

Both the T-REx™ Complete and the T-REx™ Core Systems include the regulatory vector and sequencing primers listed in the following table. Store at -20°C.

Reagent	Amount	Comments
pcDNA™6/TR	20 µg, lyophilized in TE, pH 8.0	Regulatory vector that expresses the tetracycline (Tet) repressor
CMV Forward Primer (21-mer)	2 µg (306 pmoles), lyophilized in TE, pH 8.0	5'-CGCAAATGGGCGGTAGGCGTG-3'
BGH Reverse Primer (18-mer)	2 µg (358 pmoles), lyophilized in TE, pH 8.0	5'-TAGAAGGCACAGTCGAGG-3'

Continued on next page

Important Information, continued

Inducible Expression Vector

Each T-REX™ Complete and Core System also includes one of the following inducible expression vectors and a corresponding positive control vector containing the *lacZ* gene. Please refer to the vector manual for specific information pertaining to each inducible expression vector. Store at -20°C.

Vector	Amount
pcDNA™4/TO plus pcDNA™4/TO/ <i>lacZ</i>	20 µg each, lyophilized in TE, pH 8.0
pcDNA™4/TO/ <i>myc</i> -His A, B, C plus pcDNA™4/TO/ <i>myc</i> -His/ <i>lacZ</i>	20 µg each, lyophilized in TE, pH 8.0

Inducing and Selection Agents

In addition to the vectors and primers provided in the T-REX™ Core System, the T-REX™ Complete System also includes the following inducing and selection agents. Store the tetracycline and Zeocin™ at -20°C protected from exposure to light. Store the blasticidin powder at +4°C.

Reagent	Amount Supplied	Comments
Tetracycline	5 g, powder	Inducing agent
Zeocin™	1 g	Selection agent for inducible expression plasmid
Blasticidin	50 mg, powder	Selection agent for pcDNA™6/TR plasmid

Additional Reagents

Many of the reagents in the T-REX™ kits are available separately from Invitrogen. Ordering information is provided below.

Item	Amount	Catalog no.
pcDNA™4/TO	20 µg, lyophilized	V1020-20
pcDNA™4/TO/ <i>myc</i> -His A, B, C	20 µg each, lyophilized	V1030-20
pcDNA™6/TR	20 µg, lyophilized	V1025-20
CMV Forward Primer	2 µg, lyophilized	N622-02
BGH Reverse Primer	2 µg, lyophilized	N575-02
Tetracycline	5 g, powder	Q100-19
Zeocin™	1 g	R250-01
	5 g	R250-05
Blasticidin	50 mg, powder	R210-01

*Also includes the corresponding positive control vector containing the *lacZ* gene

Continued on next page

Important Information, continued

T-REx™ Cell Lines

For your convenience, Invitrogen has available three mammalian cell lines that stably express the Tet repressor. T-REx™-293 cells and T-REx™-HeLa cells express the Tet repressor from pcDNA™6/TR and should be maintained in medium containing blasticidin. T-REx™-U2OS cells express the Tet repressor from pCEP4/*tetR* as described in Yao *et al.*, 1998, and should be maintained in medium containing hygromycin. Please note that the pCEP4/*tetR* plasmid in the T-REx™-U2OS cells is episomally-maintained, but is stable under hygromycin selection. For more information, please see our Web site (www.lifetechnologies.com) or call Technical Support (see page 17).

Cell Line	Catalog no.
T-REx™-293	R710-07
T-REx™-HeLa	R714-07
T-REx™-U2OS	R712-07

Product Use

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

Introduction

The T-REx™ System

Overview

The T-REx™ System is a tetracycline-regulated mammalian expression system that uses regulatory elements from the *E. coli* Tn10-encoded tetracycline (Tet) resistance operon (Hillen and Berens, 1994; Hillen *et al.*, 1983). Tetracycline regulation in the T-REx™ 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). The major components of the T-REx™ System include:

- An inducible expression plasmid for expression of your gene of interest under the control of the strong human cytomegalovirus immediate-early (CMV) promoter and two tetracycline operator 2 (TetO₂) sites
- A regulatory plasmid, pcDNA™6/TR, which encodes the Tet repressor (*TetR*) under the control of the human CMV promoter (see pages 15-16 for further information)
- Tetracycline for inducing expression
- A control expression plasmid containing the *lacZ* gene, which when cotransfected with pcDNA™6/TR, expresses β-galactosidase upon induction with tetracycline.

For specific information on the inducible expression vector and the corresponding positive control vector containing the *lacZ* gene, please refer to the manual for the inducible expression vector you are using.

Description of the T-REx™ System

In the T-REx™ 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). Unlike other tetracycline-regulated systems which use hybrid regulatory molecules and viral transactivation domains (Gossen and Bujard, 1992), the T-REx™ System uses only regulatory elements from the native Tet operon (Yao *et al.*, 1998). Tetracycline-regulated gene expression in the T-REx™ System more closely resembles the regulation of the native bacterial *tet* operon (Hillen and Berens, 1994; Hillen *et al.*, 1983) and avoids the potentially toxic effects of viral transactivation domains observed in some mammalian cell lines.

The major component of the T-REx™ System is the inducible expression plasmid. Expression of your gene of interest from the inducible expression vector is controlled by the strong CMV promoter (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987) into which 2 copies of the *tet* operator 2 (TetO₂) sequence have been inserted in tandem. The TetO₂ 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 TetO₂ sequence serves as the binding site for 2 molecules of the Tet repressor. For more information about the Tet operator sequences and the specific features of each inducible expression vector, please refer to the manual for the vector you are using.

The second major component of the T-REx™ System is the pcDNA™6/TR regulatory vector which expresses high levels of the *TetR* gene (Postle *et al.*, 1984) under the control of the human CMV promoter (see **Appendix**, pages 15-16 for more information about pcDNA™6/TR and the *TetR* gene). Both T-REx™ vectors can be introduced into mammalian host cells by standard transfection methods.

Continued on next page

The T-REx™ System, continued

Mechanism of Repression

In the absence of tetracycline, the Tet repressor forms a homodimer that binds with extremely high affinity to each TetO₂ sequence in the promoter of the inducible expression vector (Hillen and Berens, 1994). The 2 TetO₂ sites in the promoter of the inducible expression vector serve as binding sites for 4 molecules (or 2 homodimers) of the Tet repressor. The affinity of the Tet repressor for the *tet* operator is $K_B = 2 \times 10^{11} \text{ M}^{-1}$ (as measured under physiological conditions), where K_B is the binding constant (Hillen and Berens, 1994). Binding of the Tet repressor homodimers to the TetO₂ 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 $3 \times 10^9 \text{ M}^{-1}$ (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.

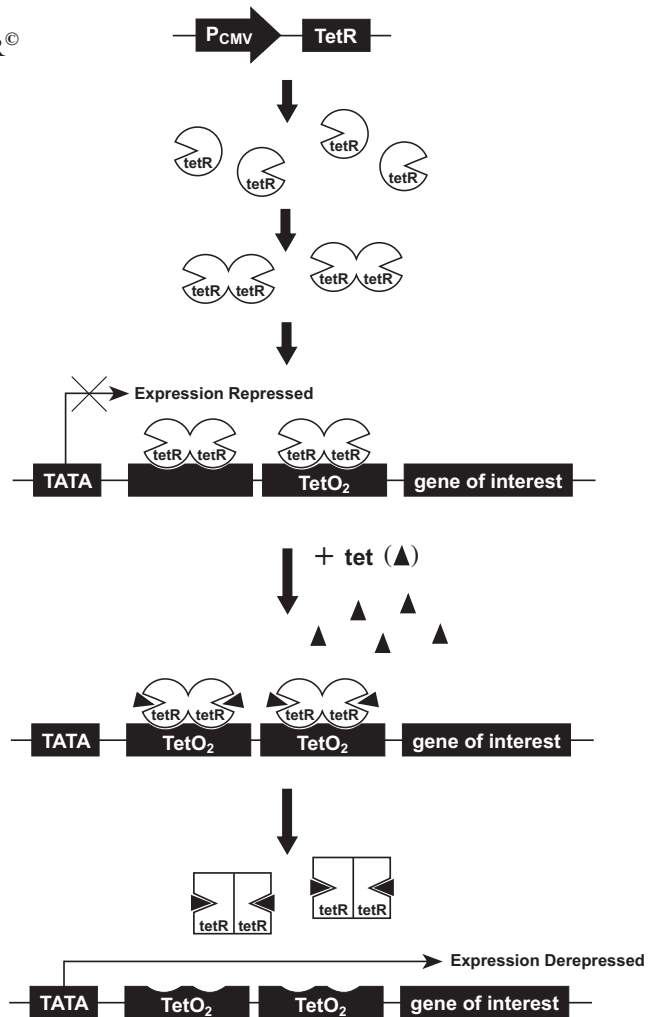
Note: The figure below illustrates the components of the T-REx™ System.

1. Tet repressor (*tetR*) protein is expressed from pcDNA6/TR[©] in cultured cells.

2. TetR homodimers bind to Tet operator 2 (TetO₂) sequences in the inducible expression vector, repressing transcription of the gene of interest.

3. Upon addition, tetracycline (*tet*) binds to *tetR* homodimers.

4. Binding of *tet* to *tetR* homodimers causes a conformational change in *tetR*, release from the Tet operator sequences, and induction of transcription from the gene of interest.



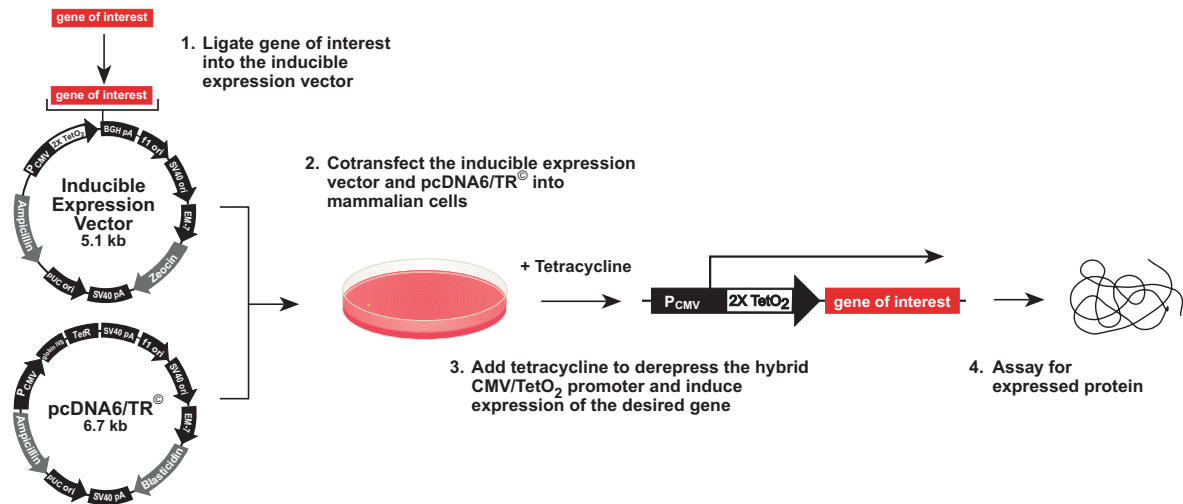
Continued on next page

The T-REx™ System, continued

Experimental Outline

The gene of interest is cloned into the multiple cloning site of the inducible expression vector, and the resulting construct cotransfected with the regulatory plasmid, pcDNA™6/TR into mammalian cells. After transfection, cells are treated with tetracycline to derepress the hybrid CMV/TetO₂ promoter in the inducible expression vector and induce transcription of your gene of interest.

The positive control vector containing the *lacZ* gene can be transiently cotransfected into mammalian cells with pcDNA™6/TR to demonstrate that the system is working properly in your cell line. Stable cell lines expressing Tet repressor from pcDNA™6/TR can be established to serve as hosts for inducible expression vector-based constructs.



Methods

Using pcDNA™6/TR

Introduction

The following section contains guidelines for maintaining and propagating the pcDNA™6/TR regulatory vector.

General Molecular Biology Techniques

For assistance with *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, please 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 pcDNA™6/TR vector including TOP10F' (Catalog no. C615-00), DH5 F', and INV F' (Catalog no. C658-00). We recommend that you propagate the pcDNA™6/TR vector in *E. coli* strains that are recombination deficient (*recA*) and endonuclease A deficient (*endA*).

For your convenience, TOP10F' *E. coli* are available as chemically competent or electrocompetent cells from Invitrogen.

Item	Quantity	Catalog no.
One Shot® TOP10F' (chemically competent cells)	21 x 50 µl	C3030-03
Electrocomp™ TOP10F' (electrocompetent cells)	5 x 80 µl	C665-55
Max Efficiency™ DH10B™ Chemically Competent Cells	5 x 0.2 ml	18297-010

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.

Maintenance of Plasmids

The pcDNA™6/TR vector contains the ampicillin and blasticidin resistance genes to allow selection of the plasmid using ampicillin or blasticidin (see pages 15-16 for more information about pcDNA™6/TR). For more information about preparation and handling of blasticidin, please refer to the **Appendix**, page 14.

To propagate and maintain the pcDNA™6/TR plasmid, we recommend using the following procedure:

1. Resuspend the vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.
 2. Use the stock solution to transform a *recA*, *endA* *E. coli* strain like TOP10F', INV F', or equivalent.
 3. Select transformants on LB agar plates containing 50 to 100 µg/ml ampicillin or 50 µg/ml blasticidin in Low Salt LB (see the **Appendix**, page 13 for a recipe)
 4. Prepare a glycerol stock of each plasmid for long-term storage (see page 5).
-

Continued on next page

Using pcDNA™ 6/TR, continued

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 13.

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.

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 or 100 µg/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 µg/ml ampicillin or 50 µg/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 very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipids decreasing transfection efficiency. We recommend isolating DNA using the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01) or CsCl gradient centrifugation.

Transfection

Introduction

Once you have obtained clean plasmid preparations of your inducible expression plasmid and pcDNA™6/TR, you are ready to cotransfect the two plasmids into the mammalian cell line of choice. We recommend that you include the positive control vector and a mock transfection (negative control) to evaluate your results. For more information about the positive control vector, please refer to the manual for the inducible expression vector you are using.

Methods of Transfection

For established cell lines (e.g. HeLa, COS-1), please 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 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). Lifetechnologies offers the Calcium Phosphate Transfection Kit for mammalian cell transfection and Lipofectamine® 2000 Reagent to perform lipid-mediated transfection.

Catalog No.	Description	Quantity
K2780-01	Calcium Phosphate Transfection Kit	75 reactions
11668-019	Lipofectamine® 2000 Reagent	1.5 ml

Tetracycline-Reduced Serum

When culturing cells in medium containing fetal bovine serum (FBS), please note that many lots of FBS contain tetracycline as FBS is generally isolated from cows that have been fed a diet containing tetracycline. If you culture your cells in medium containing FBS that is not reduced in tetracycline, you may observe low basal expression of your gene of interest in the absence of tetracycline. We have cultured our mammalian cells in medium containing FBS that may not be reduced in tetracycline, and have observed undetectable to very low basal expression of β -galactosidase from the positive control vector in the absence of additional tetracycline. If your gene of interest produces a toxic protein, you may wish to culture your cells in tetracycline-reduced FBS. For more information, please consult the supplier of your serum.

Tetracycline

Tetracycline (MW = 444.4) is commonly used as a broad spectrum antibiotic and acts to inhibit translation by blocking polypeptide chain elongation in bacteria. In the T-REx™ System, tetracycline is used as an inducing agent to induce transcription of the gene of interest from the inducible expression vector. Tetracycline induces transcription by binding to the Tet repressor homodimer and causing the repressor to undergo a conformational change that renders it unable to bind to the Tet operator. The association constant of tetracycline to the Tet repressor is $3 \times 10^9 \text{ M}^{-1}$ (Takahashi *et al.*, 1991). Please note that the concentrations of tetracycline used to induce gene expression in the T-REx™ System are generally not high enough to be toxic to mammalian cells.

Continued on next page

Transfection, continued



- Tetracycline is light sensitive. Store the powdered drug at +4°C in the dark. Prepare medium containing tetracycline immediately before use.
 - Tetracycline is toxic. Do not ingest or inhale the powder or solutions containing the drug.
 - Wear gloves, a laboratory coat, and safety glasses or goggles when handling tetracycline and tetracycline-containing solutions.
-

Preparation of Tetracycline

To prepare a stock solution from the tetracycline salt supplied with the Complete Kit:

1. Weigh out 10 mg of tetracycline and transfer to a sterile 15 ml conical polypropylene tube.
 2. Resuspend 10 mg of tetracycline in 10 ml of water to produce a 1 mg/ml stock solution that is yellow in color. **Note:** If you are using a different form of tetracycline (i.e. free base form), resuspend in 100% ethanol rather than water.
 3. Store the stock solution at -20°C protected from exposure to light.
-

Important

Because tetracycline-regulated expression in the T-REx™ System is based on a repression/derepression mechanism, the amount of Tet repressor that is expressed in the host cell line from pcDNA™6/TR will determine the level of transcriptional repression of the Tet operator sequences in your inducible expression construct. Tet repressor levels should be sufficiently high to suitably repress basal level transcription. We have varied the ratio of pcDNA™6/TR and inducible expression plasmid that we transiently cotransfect into mammalian cells to optimize repression and inducibility of the hybrid CMV/TetO₂ promoter in the inducible expression plasmid. **We recommend that you cotransfect your mammalian host cell line with a ratio of at least 6:1 (w/w) pcDNA™6/TR:inducible expression plasmid DNA.**

Transfection, continued

Cotransfection and Induction with Tetracycline

Guidelines are provided below to cotransfect your inducible expression construct (or the control plasmid) and pcDNA™6/TR into your mammalian cell line and to induce expression of your protein of interest with tetracycline. Since every cell line is different and may require a different method of transfection, some empirical experimentation may be needed to determine the optimal conditions for inducible expression.

- Use cells that are approximately 60% confluent for transfection.
- Cotransfect the pcDNA™6/TR plasmid and your inducible expression construct at a ratio of 6:1 (w:w) into the cell line of choice using your preferred method. Absolute amounts of plasmid will vary depending on the method of transfection and the cell line used.
- After transfection, add fresh medium and allow the cells to recover for 24 hours before induction.
- Remove medium and add fresh medium containing the appropriate concentration of tetracycline to the cells. In general, we recommend that you 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.
- Harvest the cells and assay for expression of your gene.

Optimization of Expression

You may want to vary the concentration of tetracycline (0.1 to 1 µg/ml) and time of exposure to tetracycline (8 to 24 hours) to optimize or modulate expression for your cell line.

Other Inducers

You may use doxycycline as an alternative inducing agent in the T-REx™ System. Doxycycline is similar to tetracycline in its mechanism of action, and exhibits similar dose response and induction characteristics as tetracycline in the T-REx™ System. Doxycycline has been shown to have a longer half-life than tetracycline (48 hours vs. 24 hours, respectively). Doxycycline may be obtained from Sigma-Aldrich (Catalog no. D9891).

Continued on next page

Creation of Stable Cell Lines

Introduction

Once you have established that your construct can be inducibly expressed, you may wish to establish a stable cell line that constitutively expresses the Tet repressor and inducibly expresses your gene of interest. We recommend that you first create a stable cell line that expresses only the Tet repressor, then use that cell line to create a second cell line that will express your gene of interest from the inducible expression plasmid (see **Note** below). Alternatively, you can transfect with both plasmids (pcDNA™6/TR and inducible expression vector) and dual-select with blasticidin and Zeocin™ to isolate a single stable cell line expressing both the Tet repressor and your gene of interest.



Three T-REx™ cell lines that stably express the Tet repressor are available from Invitrogen (see page vi for ordering information). If you wish to assay for tetracycline-inducible expression of your gene of interest in 293, HeLa, or U2OS cells, you may want to use one of the T-REx™ cell lines as the host to establish your double stable cell line. For more information, please refer to our Web site (www.lifetechnologies.com) or call Technical Support (see page 17).

Note

Reminder: When generating a stable cell line expressing the Tet repressor, you will want to select for clones that express the highest levels of Tet repressor to use as hosts for your inducible expression construct. Those clones that express the highest levels of Tet repressor should exhibit the most complete repression of basal transcription of your gene of interest.

Determination of Antibiotic Sensitivity

To successfully generate a stable cell line expressing the Tet repressor and your protein of interest, you need to determine the minimum concentration of each antibiotic (blasticidin and Zeocin™) required to kill your untransfected host cell line. For each antibiotic, test a range of concentrations (see step 1 below) to ensure that you determine the minimum concentration necessary for your cell line. Use the protocol below to determine the minimal concentrations of Zeocin™ and blasticidin required to prevent growth of the parental cell line. Please refer to the **Appendix**, page 14 for instructions on how to prepare and store blasticidin. Instructions on how to prepare and store Zeocin™ can be found in the inducible expression vector manual.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. For each antibiotic, prepare a set of 6-7 plates. Add the following concentrations of antibiotic to each plate in a set:
 - For blasticidin selection, test 0, 1, 3, 5, 7.5, and 10 µg/ml blasticidin
 - For Zeocin™ selection, test 0, 50, 125, 250, 500, 750, and 1000 µg/ml Zeocin™
 2. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
 3. Count the number of viable cells at regular intervals to determine the appropriate concentration of antibiotic that prevents growth within 1-2 weeks after addition of the antibiotic.
-

Continued on next page

Creation of Stable Cell Lines, continued

Effect of Zeocin™ on Sensitive and Resistant Cells

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

- 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 in these membranes)

Eventually, these "cells" will 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, please refer to the inducible expression vector manual.

Possible Sites for Linearization of pcDNA™ 6/TR

To obtain stable transfectants, you may choose to linearize pcDNA™ 6/TR 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 Tet repressor gene 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.

Enzyme	Restriction Site (bp)	Location	Supplier
<i>Bst</i> 1107 I	4470	Backbone	AGS*, Fermentas, Takara
<i>Sap</i> I	4733	Backbone	New England Biolabs
<i>Bsp</i> LU11 I	4849	Backbone	Boehringer-Mannheim
<i>Eam</i> 1105 I	5739	Ampicillin gene	AGS*, Fermentas, Takara
<i>Fsp</i> I	5961	Ampicillin gene	Many

*Angewandte Gentechnologie Systeme

Continued on next page

Creation of Stable Cell Lines, continued

Selection of Stable Integrants

Once you have determined the appropriate antibiotic concentrations to use for selection, you can generate a stable cell line expressing pcDNATM6/TR and your inducible expression construct.

1. Transfect mammalian cells with pcDNATM6/TR 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 containing blasticidin at the pre-determined concentration required for your cell line. Split the cells such that the cells 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. Feed the cells with selective medium every 3-4 days until foci can be identified.
5. Pick at least 20 foci and expand them to test for tetracycline-inducible gene expression by transiently transfecting with the positive control plasmid expressing β -galactosidase. Screen for those clones that exhibit the lowest levels of basal transcription and the highest levels of β -galactosidase expression after addition of tetracycline (see below).
6. Once you have obtained a stable cell line expressing the Tet repressor, you can use this cell line to isolate a stable cell line expressing your gene of interest from the inducible expression plasmid. Repeat Steps 1 through 4, above, using your inducible expression vector construct and ZeocinTM to select foci. Remember to maintain your cells in medium containing blasticidin as well.
7. Pick and expand at least 20 foci to test for tetracycline-regulated gene expression.

Continued on next page

Creation of Stable Cell Lines, continued

Dual Selection of Stable Integrants

We recommend using the protocol above to generate double stable cell lines. However, if you wish to perform dual selection, you may cotransfect both pcDNATM6/TR and your inducible expression construct into the cell line of choice. **When cotransfecting your cell line, we recommend using a ratio of at least 6:1 (w/w) pcDNATM6/TR DNA:inducible expression plasmid DNA (see page 8) to increase the chances that your double stable cell lines will express sufficient levels of Tet repressor.** Following the steps listed above, add selective medium containing blasticidin and ZeocinTM at the concentrations determined previously. Screen at least 40 foci for tetracycline-regulated expression of your gene of interest using an appropriate assay for your protein (e.g. western blot, enzymatic assay).



Once you have isolated stable cell lines expressing both pcDNATM6/TR and your inducible expression construct and have tested for tetracycline-inducible expression of your gene of interest, we recommend that you perform a time course of tetracycline induction to optimize expression of your protein of interest (e.g. 0, 8, 16, 24, 32 hours, etc.). Use the appropriate concentration of tetracycline for your cell line as previously determined (see page 9). We have observed as much as 120-fold induction of β -galactosidase in a double stable cell line (T-RExTM-293 cells transfected with the positive control plasmid containing the *lacZ* gene) after 24 hours of treatment with 1 μ g/ml tetracycline. Please note that your induction levels may vary depending on the nature of your gene of interest and the particular clone that you choose.

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 of ampicillin), and pour into 10 cm plates.
 4. Let harden, then invert and store at +4°C, in the dark.
-

Low Salt LB Medium with Blasticidin

Low Salt LB Medium:

10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.0 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
 2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
 3. Allow the medium to cool to at least 55°C before adding the blasticidin to 50 µg/ml final concentration.
 4. Store plates at +4°C in the dark. Plates containing blasticidin are stable for up to 2 weeks.
-

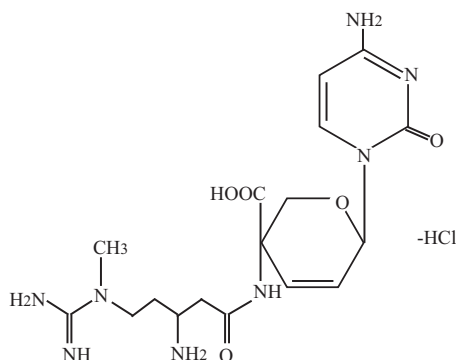
Blasticidin

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either 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 $C_{17}H_{26}N_8O_5 \cdot HCl$, 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

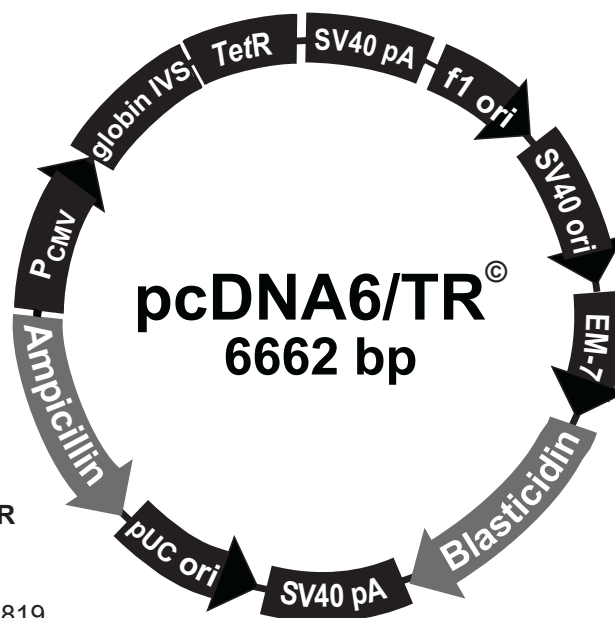
Blasticidin is supplied in the T-REx™ Complete Kit, but may also be obtained separately from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 10 mg/ml.

- Dissolve blasticidin in sterile water and filter-sterilize the solution.
 - Aliquot in small volumes suitable for one time use (see next to last point below) and freeze at $-20^{\circ}C$ for long-term storage or store at $+4^{\circ}C$ for short-term storage.
 - Aqueous stock solutions are stable for 1-2 weeks at $+4^{\circ}C$ and 6-8 weeks at $-20^{\circ}C$.
 - pH of the aqueous solution should be 7.5 to prevent inactivation of blasticidin.
 - Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
 - Upon thawing, use what you need and store the thawed stock solution at $+4^{\circ}C$ for up to 2 weeks.
 - Medium containing blasticidin may be stored at $+4^{\circ}C$ for up to 2 weeks.
-

pcDNA™ 6/TR Vector

Map of pcDNA™ 6/TR

pcDNA™ 6/TR is a 6662 bp vector that expresses the Tet repressor under the control of the human CMV promoter. The figure below summarizes the features of the pcDNA™ 6/TR vector. **The complete sequence for pcDNA™ 6/TR is available for downloading from our Web site (www.lifetechnologies.com) or by contacting Technical Support (see page 17).**



Comments for pcDNA6/TR 6662 nucleotides

CMV promoter: bases 232-819
Rabbit β -globin intron II: bases 1028-1600
TetR gene: bases 1684-2304
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-derived origin: bases 4851-5521
bla promoter: bases 6521-6625 (C)
Ampicillin (*bla*) resistance gene: bases 5666-6526 (C)

TetR Gene

The *TetR* gene used in pcDNA™ 6/TR was originally isolated from the Tn10 transposon which confers resistance to tetracycline in *E. coli* and other enteric bacteria (Postle *et al.*, 1984). The *TetR* gene from Tn10 encodes a class B Tet repressor and is often referred to as *TetR*(B) in the literature (Hillen and 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 and its interaction with the Tet operator, please refer to the review by Hillen and Berens (1994).

Continued on next page

pcDNA™ 6/TR Vector, continued

Features of pcDNA™ 6/TR

The table below describes the relevant features of pcDNA™ 6/TR. The vector includes the rabbit γ -globin intron II to enhance expression of the *TetR* gene. For a more detailed description of the *TetR* gene and the Tet repressor, please refer to the previous page. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate early promoter	Permits high-level expression of the <i>TetR</i> gene (Andersson <i>et al.</i> , 1989; Boshart <i>et al.</i> , 1985; Nelson <i>et al.</i> , 1987)
Rabbit γ -globin intron II (IVS)	Enhances expression of the <i>TetR</i> gene (van Ooyen <i>et al.</i> , 1979) in cultured cells
<i>TetR</i> gene	Encodes the Tet repressor that binds to <i>tet</i> operator sequences to repress transcription of the gene of interest in the absence of tetracycline (Postle <i>et al.</i> , 1984; Yao <i>et al.</i> , 1998)
SV40 early polyadenylation signal	Permits 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 <i>E. coli</i>
Blasticidin (<i>bsd</i>) resistance gene	Allows selection of stable transfectants in mammalian cells (Kimura <i>et al.</i> , 1994)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Permits high-copy number replication and growth in <i>E. coli</i>
<i>bla</i> promoter	Allows expression of the ampicillin (<i>bla</i>) resistance gene
Ampicillin (<i>bla</i>) resistance gene (β -lactamase)	Allows selection of transformants in <i>E. coli</i>

Technical Support

Obtaining support For the latest services and support information for all locations, go to www.lifetechnologies.com.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited Warranty

Life Technologies and/or its affiliate(s) warrant their products as set forth in the Life Technologies General Terms and Conditions of Sale found on the Life Technologies web site at <http://www.lifetechnologies.com/termsandconditions>. If you have any questions, please contact Life Technologies.

Life Technologies and/or its affiliate(s) disclaim all warranties with respect to this document, expressed or implied, including but not limited to those of merchantability or fitness for a particular purpose. In no event shall Life Technologies and/or its affiliate(s) be liable, whether in contract, tort, warranty, or under any statute or on any other basis for special, incidental, indirect, punitive, multiple or consequential damages in connection with or arising from this document, including but not limited to the use thereof.

Purchaser Notification

**Limited Use Label
License No. 358:
Research Use
Only**

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

References

- 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.
- 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. *Mol. Cell. Biol.* 7, 2745-2752.
- Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. *Nuc. 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., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. *Nature* 337, 387-388.
- Gossen, M., and Bujard, H. (1992). Tight Control of Gene Expression in Mammalian Cells by Tetracycline-Responsive Promoters. *Proc. Natl. Acad. Sci. USA* 89, 5547-5551.
- Hillen, W., and Berens, C. (1994). Mechanisms Underlying Expression of Tn10 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 Tn10-encoded Tetracycline Resistance Genes: Equilibrium and Kinetic Investigations of the Regulatory Reactions. *J. Mol. Biol.* 169, 707-721.
- Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991). Blastocidin S-Resistance Gene (bsr): A Novel Selectable Marker for Mammalian Cells. *Exp. Cell Res.* 197, 229-233.
- Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994). Blastocidin S Deaminase Gene from *Aspergillus terreus* (BSD): A New Drug Resistance Gene for Transfection of Mammalian Cells. *Biochim. Biophys. Acta* 1219, 653-659.
- 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. *Mol. Cell. Biol.* 7, 4125-4129.
- Postle, K., Nguyen, T. T., and Bertrand, K. P. (1984). Nucleotide Sequence of the Repressor Gene of the Tn10 Tetracycline Resistance Determinant. *Nuc. Acids Res.* 12, 4849-4863.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Continued on next page

References, continued

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.

Takahashi, M., Degenkolb, J., and Hillen, W. (1991). Determination of the Equilibrium Association Constant Between Tet Repressor and Tetracycline at Limiting Mg^{2+} Concentrations: A Generally Applicable Method for Effector Dependent High Affinity Complexes. *Anal. Biochem.* 199, 197-202.

Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H., and Yonehara, H. (1958). Blasticidin S, A New Antibiotic. *The Journal of Antibiotics, Series A* 11, 1-5.

van Ooyen, A., van den Berg, J., Mantei, N., and Weissmann, C. (1979). Comparison of Total Sequence of a Cloned Rabbit Beta-globin gene and its Flanking Regions With a Homologous Mouse Sequence. *Science* 206, 337-344.

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.

Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965). Inhibition of Protein Synthesis by Blasticidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. *J. Biochem. (Tokyo)* 57, 667-677.

Yao, F., Svensjö, 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.

© 2011 Life Technologies Corporation. All rights reserved.

The trademarks mentioned herein are the property of Life Technologies Corporation or their respective owners. Zeocin is a trademark of CAYLA, SA.

Headquarters

5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288

For support visit www.lifetechnologies.com/support or email techsupport@lifetech.com

www.lifetechnologies.com

