pcDNA6/TR

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

Catalog no. V1025-20

Version D
012502
25-0274

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General Information

Contents	20 µg of pcDNA6/TR, lyophilized in TE, pH 8.0
Shipping/Storage	Lyophilized plasmid is shipped at room temperature and should be stored at -20°C.

Methods

Using pcDNA6/TR

Introduction

pcDNA6/TR is a 6.7 kb vector designed for use with the T-REx™ System (Catalog nos. K1020-01, K1020-02, K1030-01, and K1030-02). 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. A map of pcDNA6/TR and a description of the features of the vector may be found in the **Appendix**, pages 7-8.

For more information about the T-REx™ System, please refer to the T-REx™ System manual. For information about T-REx™ inducible expression vectors, please refer to the manual for each specific vector. Manuals are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 9). To order components of the T-REx™ System separately, please see **Accessory Products** below.

A Note about the *TetR* Gene

The *TetR* gene used in pcDNA6/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 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, its interaction with Tet operator sequences, and tetracycline regulation, please refer to the T-REx™ System Manual or to published reviews (Hillen and Berens, 1994; Hillen *et al.*, 1983).

Accessory Products

Many of the reagents used in the T-REx™ System are available separately from Invitrogen. Ordering information is provided below.

Item	Amount	Purpose	Catalog no.
pcDNA4/TO	20 µg, lyophilized	Inducible expression vector	V1020-20
pcDNA4/TO/*myc*-His A, B, C	20 µg each, lyophilized	Inducible expression vector	V1030-20
Blasticidin	50 mg, powder	Selection agent for regulatory plasmid	R210-01
Tetracycline	5 g, powder	Inducing agent	Q100-19
Zeocin™	1 g	Selection agent for inducible expression vector	R250-01
	5 g		R250-05

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Using pcDNA6/TR, continued

Maintenance of pcDNA6/TR

The pcDNA6/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 pcDNA6/TR vector, we recommend resuspending the vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.

Use this stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10F´ (Catalog no. C615-00), DH5αF´, JM109, INVαF´, or equivalent. Select transformants on LB agar plates containing either 50 to 100 µg/ml ampicillin or 100 µg/ml blasticidin in Low Salt LB (see recipe below).

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 LB broth and plates using the recipe below.

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.

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 100 µg/ml final concentration.
4. Store plates at +4°C in the dark. Plates containing blasticidin are stable for up to 2 weeks.

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.

- Streak the original colony out on an LB plate containing 50-100 µg/ml ampicillin or 100 µg/ml blasticidin in Low Salt LB. Incubate the plate at 37°C overnight.
- Isolate a single colony and inoculate into 1-2 ml of LB containing 50-100 µg/ml ampicillin or 100 µg/ml blasticidin in Low Salt LB.
- Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
- Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
- Store at -80°C.

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Using pcDNA6/TR, continued

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01), or CsCl gradient centrifugation.

Methods of Transfection

To transfect established mammalian cell lines (e.g. HeLa, COS-1) with pcDNA6/TR, 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). Invitrogen offers the Calcium Phosphate Transfection Kit and Lipofectamine™ 2000 Reagent for mammalian cell transfection. For more information on transfection reagents available from Invitrogen, refer to our Web site (www.invitrogen.com) or contact Technical Service (see page 9).

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

Transient Transfection

You may use any of the methods above to transiently cotransfect pcDNA6/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 pcDNA6/TR DNA transfected into your host cell line relative to inducible expression plasmid DNA. Increasing the ratio of pcDNA6/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, please refer to the T-REx™ System manual.

Creation of Stable Cell Lines

Introduction

To generate a cell line that stably expresses the Tet repressor, you may transfect pcDNA6/TR into your mammalian host cell line and select with blasticidin. Cell lines expressing suitably high levels of the Tet repressor may then be used 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.

Determination of Antibiotic Sensitivity

To generate a stable cell line expressing pcDNA6/TR, you need to determine the minimum concentration of blasticidin required to kill your untransfected host cell line. Typically, concentrations between 2 and 10 µg/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, please refer to the **Appendix**, page 6.

- Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare 6 plates of cells.
- The next day, substitute culture medium with medium containing varying concentrations of blasticidin (e.g. 0, 1, 3, 5, 7.5, and 10 µg/ml).
- 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.
- Count the number of viable cells at regular intervals to determine the appropriate concentration of blasticidin that prevents growth within 1-2 weeks after addition of blasticidin.

Possible Sites for Linearization

To obtain stable transfectants, you may choose to linearize the pcDNA6/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 either the *TetR* gene or other elements required for mammalian expression. The table below lists unique sites that may be used to linearize pcDNA6/TR prior to transfection. **Other restriction sites are possible.**

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

*Angewandte Gentechnologie Systeme

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Creation of Stable Cell Lines, continued

Selection of Stable Integrants

Once you have determined the appropriate blasticidin concentration to use for selection, you can generate a stable cell line expressing pcDNA6/TR.

1. Transfect your cell line of choice with pcDNA6/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. You will want to 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, please refer to the manual for the inducible expression vector that you have obtained. For more information about induction of gene expression with tetracycline, please refer to the T-REx™ System manual.
6. Once you have obtained cell lines that stably express the Tet repressor from pcDNA6/TR, you may use these cell lines to assay for tetracycline-regulated expression of your gene of interest from the pcDNA4/TO-based expression vector.

Appendix

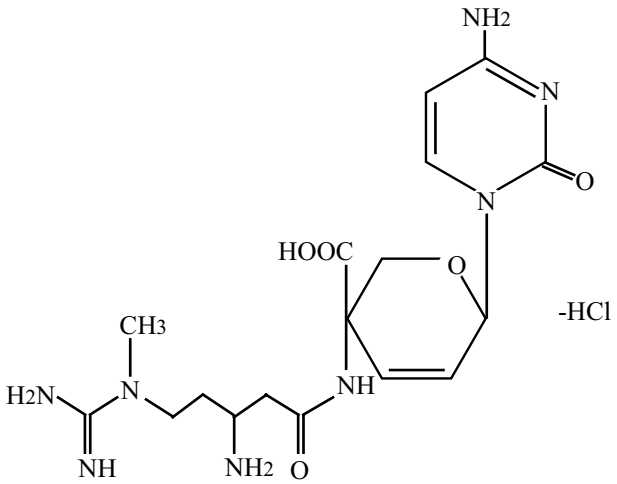
Blasticidin

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 $C_{17}H_{26}N_8O_5$-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

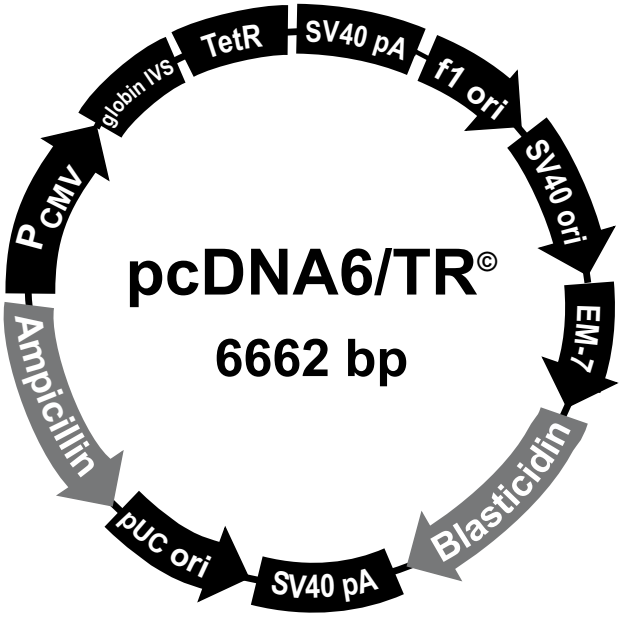
Blasticidin is available from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 10 mg/ml.

- 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°C for long-term storage or store at +4°C for short-term storage.
- Aqueous stock solutions are stable for 1-2 weeks at +4°C and 6-8 weeks at -20°C.
- pH of the aqueous solution should be 7.0 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 stock solution at +4°C for up to 2 weeks.
- Medium containing blasticidin may be stored at +4°C for up to 2 weeks.

pcDNA6/TR Vector

Map of pcDNA6/TR

The figure below summarizes the features of the pcDNA6/TR vector. **The complete sequence for pcDNA6/TR is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 9).** Please see the next page for a description of the features of the vector.



Comments for pcDNA6/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)

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pcDNA6/TR Vector, continued

Features of pcDNA6/TR

The table below describes the relevant features of pcDNA6/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	Permits 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	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 *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	Permits high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

Technical Service

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