USER GUIDE

invitrogen™
by life technologies™

pcDNA™4/TO

Expression vector designed for use with the T-REx™ System

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Contents

Contents iii
Important Information iv
Methods 1
Overview 1
Cloning into pcDNA™4/TO 4
Transfection 7
Creation of Stable Cell Lines 9
Appendix 12
Zeocin™ 12
Detection of β-Galactosidase Fusion Proteins 14
pcDNA™4/TO Vector 15
pcDNA™4/TO/*lac*Z Vector 17
Accessory Products 18
Technical Support 19
Purchaser Notification 20
References 21

Important Information

pcDNA™ Vectors

This manual is supplied with the following products.

Product	Catalog no.
pcDNA™4/TO Vector	V1020-20

Shipping/Storage

Plasmids are shipped on wet ice and should be stored at –20°C.

Contents

20 µg pcDNA™4/TO at 0.5 µg/µL, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Volume: 40 µL.

20 µg pcDNA™4/TO/*lac*Z at 0.5 µg/µL, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Volume: 40 µL.

Product Use

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

Methods

Overview

Introduction

pcDNA™4/TO is a 5.1 kb expression vector designed for use with the T-REx™ System (Catalog nos. K1020-01 and K1020-02) available from Life Technologies. The vector allows tetracycline-regulated expression of the gene of interest in mammalian host cells cotransfected with the pcDNA™6/TR vector (Catalog no. V1025-20). The vector contains the following elements:

- Hybrid promoter consisting of the human cytomegalovirus immediate-early (CMV) promoter and tetracycline operator 2 ($TetO_2$) sites for high-level tetracycline-regulated expression in a wide range of mammalian cells (see below)
- Zeocin™ resistance gene for selection of stable cell lines (Mulsant et al., 1988) (see pages 10 for more information)

The control plasmid, pcDNA™4/TO/*lacZ*, is included for use as a positive control for transfection and tetracycline-regulated expression in the cell line of choice.

For more information about pcDNA™6/TR and the T-REx™ System, please refer to the T-REx™ System manual, our website (**www.lifetechnologies.com**), or call Technical Support (see page 19).

A Note About pcDNA™4/TO

The pcDNA™4/TO vector contains two tetracycline operator 2 ($TetO_2$) sites within the human cytomegalovirus immediate-early (CMV) promoter for tetracycline-regulated expression of your gene of interest (Yao et al., 1998). The $TetO_2$ sequences serve as binding sites for four Tet repressor molecules (comprising two Tet repressor homodimers) and confer tetracycline-responsiveness to your gene of interest. The Tet repressor is expressed from the pcDNA™6/TR plasmid. For more information about the $TetO_2$ sequences, please see page 2. For more information about the pcDNA™6/TR plasmid and the Tet repressor, please refer to the T-REx™ System manual. The T-REx™ System manual is available for downloading from our website (**www.lifetechnologies.com**) or from Technical Support (see page 18).

In the absence of tetracycline, expression of your gene of interest is repressed by the binding of Tet repressor homodimers to the $TetO_2$ sequences. Addition of tetracycline to the cells derepresses the hybrid CMV/$TetO_2$ promoter in pcDNA™4/TO and allows expression of your gene of interest.

Continued on next page

Tet Operator Sequences

The promoters of bacterial *tet* genes contain two types of operator sequences, O_1 and O_2, that serve as high affinity binding sites for the Tet repressor (Hillen and Berens, 1994; Hillen et al., 1983). Each O_1 and O_2 site binds to one Tet repressor homodimer. While Tet repressor homodimers bind to both *tet* operators with high affinity, studies have shown that the affinity of the Tet repressor homodimer for O_2 is three- to five-fold higher than it is for O_1 (Hillen and Berens, 1994).

Tet operators have been incorporated into heterologous eukaryotic promoters to allow tetracycline-regulated gene expression in mammalian cells (Gossen and Bujard, 1992; Yao et al., 1998). In the T-REx™ System, two copies of the O_2 operator sequence ($TetO_2$) were inserted into the strong CMV promoter of pcDNA™4/TO to allow regulated expression of your gene of interest by tetracycline. We use the $TetO_2$ operator sequence in pcDNA™4/TO to maximize repression of basal gene expression. For more detailed information about *tet* operators, please refer to Hillen and Berens (1994).

Yao et al. (1998) have recently demonstrated that the location of *tet* operator sequences in relation to the TATA box of a heterologous promoter is critical to the function of the *tet* operator. Regulation by tetracycline is only conferred upon a heterologous promoter by proper spacing of the $TetO_2$ sequences from the TATA box (Yao et al., 1998). For this reason, the first nucleotide of the $TetO_2$ operator sequence has been placed 10 nucleotides after the last nucleotide of the TATA element in the CMV promoter in pcDNA™4/TO. Please refer to the diagram on page 5 for the sequence and placement of the $TetO_2$ sequences in relation to the TATA box.

In other tetracycline-regulated systems, the $TetO_2$ sequences are located upstream of the TATA element in the promoter of the inducible expression vector (Gossen and Bujard, 1992). These systems differ substantially from the T-REx™ System in that they use regulatory molecules composed of the Tet repressor fused to a viral transactivation domain. The presence of viral transactivation domains appears to overcome the requirement for specific positioning of the $TetO_2$ sequences in relation to the TATA box of the heterologous promoter. However, the presence of viral transactivation domains has been found to have deleterious effects in some mammalian cell lines.

Continued on next page

Experimental Outline

Use the following outline to clone and express your gene of interest in pcDNA™4/TO.

Step	Action
1	Consult the multiple cloning site diagrammed on page 5 to design your cloning strategy.
2	Ligate your insert into pcDNA™4/TO and transform into *E. coli*. Select transformants on 50 to 100 µg/mL ampicillin or 25 to 50 mg/mL Zeocin™ in Low Salt LB. For more information, see page 6.
3	Analyze your transformants for the presence of insert by restriction digestion.
4	Select a transformant with the correct restriction pattern and use sequencing to confirm that your gene is cloned in the correct orientation.
5	Cotransfect your pcDNA™4/TO construct and pcDNA™6/TR into the cell line of choice using your own method of choice, and induce expression of your gene of interest with tetracycline. Generate a double stable cell line, if desired. For more information about pcDNA™6/TR, please refer to the T-REx™ System manual.

Cloning into pcDNA™4/TO

Introduction

A diagram is provided on page 5 to help you clone your gene of interest into pcDNA™4/TO. General considerations for cloning and transformation are listed below.

General Molecular Biology Techniques

For help with DNA ligations, *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 and maintenance of this vector including TOP10F´, DH5αF´™, JM109, and INVαF´. We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10F´ is available as chemically competent or electrocompetent cells from Life Technologies.

Item	Quantity	Catalog no.
One Shot® TOP10F´ (chemically competent cells)	20 × 50 µL	C3030-03
Electrocomp™ TOP10F´	5 × 80 µL	C665-55
Max Efficiency DH5αF´IQ™ Competent Cells	1 mL	18288-019
One Shot® INVαF´ (chemically competent cells)	20 × 50 µL	C2020-03

Transformation Method

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

Maintenance of pcDNA™4/TO

To propagate and maintain the pcDNA™4/TO vector, transform 10 ng of the vector into a *rec*A, *end*A *E. coli* strain like TOP10F´, DH5α™, JM109, or equivalent. Select transformants on LB agar plates containing 50-100 µg/mL ampicillin or 25-50 µg/mL Zeocin™ in Low Salt LB (see page 6 for a recipe). Be sure to prepare a glycerol stock of each plasmid for long-term storage (see page 6).

Cloning Considerations

Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position +4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

(G/A)NNATG**G**

Continued on next page

Cloning into pcDNA™4/TO, continued

Multiple Cloning Site of pcDNA™4/TO

Below is the multiple cloning site for pcDNA™4/TO. Restriction sites are labeled to indicate the cleavage site. Potential stop codons are shown underlined. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pcDNA™4/TO is available for downloading from our website (www.lifetechnologies.com) or from Technical Support (see page 19).** For a map and a description of the features of pcDNA™4/TO, please refer to the **Appendix**, page 15–16.

CMV Forward priming site

```
721  AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
```

TATA box; Tetracycline operator ($TetO_2$)

```
781  GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CCCTATCAGT GATAGAGATC
```

Tetracycline operator ($TetO_2$)

```
841  TCCCTATCAG TGATAGAGAT CGTCGACGAG CTCGTTTAGT GAACCGTCAG ATCGCCTGGA

901  GACGCCATCC ACGCTGTTTT GACCTCCATA GAAGACACCG GGACCGATCC AGCCTCCGGA
```

Pme I* *Afl* II *Hind* III *Asp*718 I *Kpn* I *Bam*H I *Bst*X I* *Eco*R I

```
961  CTCTAGCGTT TAAACTTAAG CTTGGTACCG AGCTCGGATC CACTAGTCCA GTGTGGTGGA
```

Pst I *Eco*R V *Bst*X I* *Not* I *Xho* I *Xba* I *Eco*0109 I *Apa* I *Pme* I*

```
1021 ATTCTGCAGA TATCCAGCAC AGTGGCGGCC GCTCGAGTCT AGAGGGCCCG TTTAAACCCG
```

BGH Reverse priming site

```
1081 CTGATCAGCC TCGACTGTGC CTTCTAGTTG CCAGCCATCT
```

*Please note that there are two *Pme* I sites and two *Bst*X I sites in the polylinker.

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E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10F´, DH5α™) and select on LB agar plates containing 50–100 µg/mL ampicillin or 25–50 µg/mL Zeocin™ in Low Salt LB (see the following section). Select 10–20 clones and analyze for the presence and orientation of your insert.

Low Salt LB Medium with Zeocin™

Zeocin™ activity, requires the salt concentration of the bacterial medium to remain low (<90 mM) and the pH must be 7.5. For selection in *E.* coli, it is **imperative** that you prepare LB broth and plates using the following recipe. Please note the lower salt content of this medium.

Failure to use lower the salt content of your LB medium will result in non-selection due to inactivation of the drug.

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 7.5 with 5 M NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
3. Thaw Zeocin™ on ice and vortex before removing an aliquot.
4. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 µg/mL final concentration.
5. Store plates at 2°C to 8°C in the dark. Plates containing Zeocin™ are stable for 1–2 weeks.

Important

Any *E. coli* strain that contains the complete Tn*5* transposon (i.e. DH5αF´IQ™, SURE, SURE2) encodes the *ble* (bleomycin resistance gene). These strains will confer resistance to Zeocin™. For the most efficient selection, we recommend that you choose an *E. coli* strain that does not contain the Tn*5* gene (i.e. TOP10F´).

We recommend that you sequence your construct with the CMV Forward and BGH Reverse primers to confirm that your gene is in the correct orientation for expression and contains an initiation ATG and a stop codon. Please refer to the diagram on the previous page for the sequences and location of the priming sites.

Preparing a Glycerol Stock

Once you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. You should keep a DNA stock of your plasmid at –20°C.

- Streak the original colony out on an LB plate containing 50 µg/mL ampicillin or 25 µg/mL Zeocin™ 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 µg/mL ampicillin or 25 µg/mL Zeocin™ 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.

Transfection

Introduction

Once you have cloned your gene of interest into pcDNA™4/TO and have prepared clean plasmid preparations of your pcDNA™4/TO construct and pcDNA™6/TR, you are ready to cotransfect the 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. Please refer to the T-REx™ System manual for information on pcDNA™6/TR, transfection, and induction of expression using tetracycline.

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 plasmid DNA using the PureLink® HQ Mini Plasmid Purification Kit (Catalog no. K2100-01), the PureLink® HiPure Plasmid Midiprep Kit (Catalog no. K2100-04), or CsCl gradient centrifugation.

Positive Control

pcDNA™4/TO/*lac*Z is provided as a positive control vector for mammalian cell transfection and expression (see page 17) and may be used to optimize transfection conditions for your cell line. Cotransfection of the positive control vector and pcDNA™6/TR results in the induction of β-galactosidase expression upon addition of tetracycline. A successful cotransfection will result in β-galactosidase expression that can be easily assayed by staining with X-gal (see the following section).

Assay for β-galactosidase Activity

You may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. Life Technologies offers the β-Gal Assay Kit (Catalog no. K1455-01) and the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression.

Note

Please note that the *lacZ* gene in pcDNA™4/TO/*lacZ* is fused to an N-terminal peptide containing an ATG initiation codon, a polyhistidine (6xHis) tag and the Xpress™ epitope. The presence of the Xpress™ epitope allows detection of the β-galactosidase fusion protein on a western blot using the Anti-Xpress™ Antibody available from Life Technologies (Catalog no. R910-25). The Anti-Xpress™ Antibody recognizes an 8 amino acid epitope (Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys). The N-terminal peptide adds approximately 4.3 kDa to the size of the β-galactosidase fusion protein (total size of the fusion protein is approximately 120 kDa). For more information about detection of the β-galactosidase fusion protein by western blot, please see the **Appendix**, page 14.

Continued on next page

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 pcDNA™4/TO construct. Tet repressor levels should be sufficiently high to suitably repress basal level transcription. We have varied the ratio of pcDNA™6/TR and pcDNA™4/TO plasmid that we transiently cotransfect into mammalian cells to optimize repression and inducibility of the hybrid CMV/TetO_2 promoter in pcDNA™4/TO. We recommend that you cotransfect your mammalian host cell line with a ratio of **at least 6:1** (w/w) pcDNA™6/TR:pcDNA™4/TO plasmid DNA, but you may want to try varying ratios of pcDNA™6/TR:pcDNA™4/TO plasmid to optimize repression and expression for your particular cell line and your gene of interest.

Cotransfection and Induction with Tetracycline

General guidelines are provided below to cotransfect your pcDNA™4/TO construct (or the control plasmid) and pcDNA™6/TR into your cell line of interest and to induce expression of your protein of interest with tetracycline. Please refer to the T-REx™ System manual for more information on transfection and the preparation and handling of tetracycline.

- Use cells that are approximately 60% confluent for transfection.
- Cotransfect your pcDNA™4/TO construct and pcDNA™6/TR at a ratio of 6:1 (w:w) into the cell line of choice using your preferred method. Absolute amounts of plasmid used for transfection 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 solution 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 of interest.

Creation of Stable Cell Lines

Introduction

Once you have established that your construct can be inducibly expressed, you may create a stable cell line that inducibly expresses your gene of interest. pcDNA™4/TO contains the Zeocin™ resistance gene to allow selection of stable lines using Zeocin™.

Note

Please note that your gene of interest will be constitutively expressed if you transfect your pcDNA™4/TO construct into mammalian host cells prior to transfecting the pcDNA™6/TR plasmid. For more information on selection of stable cell lines using pcDNA™6/TR and blasticidin, please refer to the T-REx™ System manual.

Reminder: When generating a stable cell line expressing the Tet repressor (from pcDNA™6/TR), you will want to select for clones that express the highest levels of Tet repressor to use as hosts for your pcDNA™4/TO expression plasmid. Those clones which 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 generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of Zeocin™ required to kill your untransfected host cell line. Typically, concentrations between 50 and 1000 µg/mL Zeocin™ 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 instructions on how to prepare and store Zeocin™, please refer to the **Appendix**, pages 13.

Note: Before transfecting your host cell line with pcDNA™6/TR, you will need to perform a similar experiment to determine the minimum concentration of blasticidin required to kill the untransfected cell line. Please refer to the T-REx™ System manual for information about blasticidin.

- Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates.
- The next day, substitute culture medium with medium containing varying concentrations of Zeocin™ (e.g. 0, 50, 125, 250, 500, 750, and 1000 µg/mL).
- Replenish the selective medium every 3–4 days, and observe the percentage of surviving cells.
- Count the number of viable cells at regular intervals to determine the appropriate concentration of Zeocin™ that prevents growth within 1–2 weeks after addition of Zeocin™.

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Effect of Zeocin™ on Sensitive and Resistant Cells

The Zeocin™ method of killing is different from 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 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 will 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™, please see the **Appendix**, page 12.

Possible Sites for Linearization

To obtain stable transfectants, you may choose to linearize your pcDNA™4/TO construct 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 gene of interest or other elements important for mammalian expression. The table below lists unique sites that may be used to linearize your construct prior to transfection. Other restriction sites are possible. **Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.**

Enzyme	Restriction Site (bp)	Location	Supplier
Mun I	161	Upstream of CMV promoter	Many
Nru I	208	Upstream of CMV promoter	Many
Sap I	3149	Backbone	New England Biolabs
*Eam*1105 I	4155	Ampicillin gene	AGS*, Fermentas, Takara
Fsp I	4377	Ampicillin gene	Many
Pvu I	4525	Ampicillin gene	Many
Sca I	4635	Ampicillin gene	Many
Ssp I	4959	Backbone	Many

*Angewandte Gentechnologie Systeme

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Selection of Stable Integrants

Once you have determined the appropriate Zeocin™ concentration to use for selection, you can generate a stable cell line expressing pcDNA™6/TR and your pcDNA™4/TO construct. We recommend that you first generate a stable cell line expressing pcDNA™6/TR and then use this cell line as the host for your pcDNA™4/TO construct.

1. Once you have obtained a stable cell line expressing the Tet repressor, follow the steps below to transfect your stable cell line with the pcDNA™4/TO construct. Use Zeocin™ to select for double stable clones. Remember to maintain your cells in medium containing blasticidin as well.
2. Transfect your cell line of choice with pcDNA™4/TO using the desired protocol. Include a sample of untransfected cells as a negative control.
3. 24 hours after transfection, wash the cells and add fresh medium to the cells.
4. 48 hours after transfection, split the cells into fresh medium containing Zeocin™ 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 antibiotic will not kill the untransfected cells.
5. Replenish selective medium every 3–4 days until Zeocin™-resistant colonies are detected.
6. Pick at least 20 foci and expand them to test for tetracycline-inducible gene expression.

Dual Selection of Stable Integrants

If you wish to select for stable cell lines by dual selection, you may cotransfect your pcDNA™4/TO expression plasmid and pcDNA™6/TR into your cell line of choice and select with Zeocin™ and blasticidin. Pick and expand at least 40 foci to screen for tetracycline-regulated expression of your gene of interest.

Appendix

Zeocin™

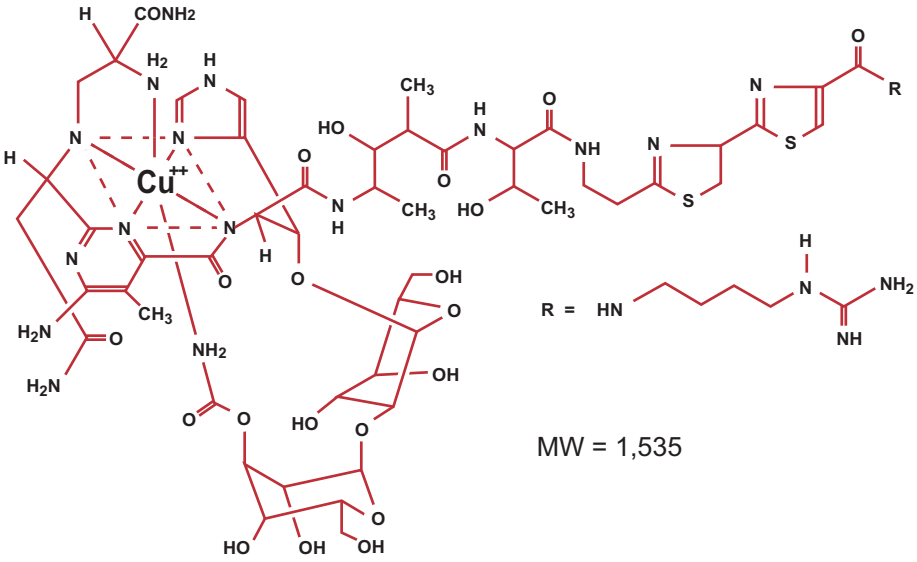
Zeocin™

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and antitumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron et al., 1992; Drocourt et al., 1990; Mulsant et al., 1988; Perez et al., 1989).

The Zeocin™ resistance protein has been isolated and characterized (Calmels et al., 1991; Drocourt et al., 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.



Applications of Zeocin™

Zeocin™ is used for selection in mammalian cells (Mulsant et al., 1988); plants (Perez et al., 1989); yeast (Baron et al., 1992); and prokaryotes (Drocourt et al., 1990). Suggested concentrations of Zeocin™ for selection in mammalian cell lines and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25–50 µg/mL in **low salt LB** medium* (see page 6 for recipe)
Mammalian Cells	50–1000 µg/mL (varies with cell line)

* Efficient selection requires that the concentration of NaCl be no more than 5 g/liter (<90 mM).

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Handling Zeocin™

- **High salt and acidity or basicity inactivate Zeocin™.** Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see **Low Salt LB Medium,** page 6). Please note that the pH and salt concentration do not need to be adjusted when preparing tissue culture medium containing Zeocin™.
- Store Zeocin™ at –20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store the drug, and plates or medium containing drug, in the dark at 2°C to 8°C. Culture medium containing Zeocin™ may be stored at 2°C to 8°C protected from exposure to light for up to 1 month.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling Zeocin™-containing solutions.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Preparing and Storing Zeocin™

Zeocin™ is available from Life Technologies (see page 18 for ordering information). For your convenience, Zeocin™ is prepared in autoclaved, deionized water in 1.25 mL aliquots at a concentration of 100 mg/mL. The stability of Zeocin™ is guaranteed for six months, if stored at –20°C protected from exposure to light.

Detection of β-Galactosidase Fusion Proteins

Introduction

To detect expression of your β–galactosidase fusion protein from pcDNA™4/TO/*lacZ* by western blot, you may use the Anti-Xpress™ Antibody available from Life Technologies or an antibody to β-galactosidase. The antibody should detect a β-galactosidase fusion protein of approximately 120 kDa in size. To perform a western blot, you will need to prepare a cell lysate from transfected cells. A sample protocol is provided below. Other protocols are suitable. Please refer to *Current Protocols in Molecular Biology* (Ausubel et al., 1994) for more information.

Preparation of Cell Lysates

Follow the protocol below to prepare cell lysates.

1. Wash cell monolayers (~10^6 cells) once with phosphate-buffered saline (PBS).
2. Scrape cells into 1 mL PBS and pellet the cells at 1,500 × *g* for 5 minutes.
3. Resuspend in 50 µL Cell Lysis Buffer (see recipe below). Other cell lysis buffers are suitable.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells. **Note:** You may prefer to lyse the cells on ice or at room temperature.
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 boil 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.

Cell Lysis Buffer

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

1. This solution can be prepared from the following common 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.

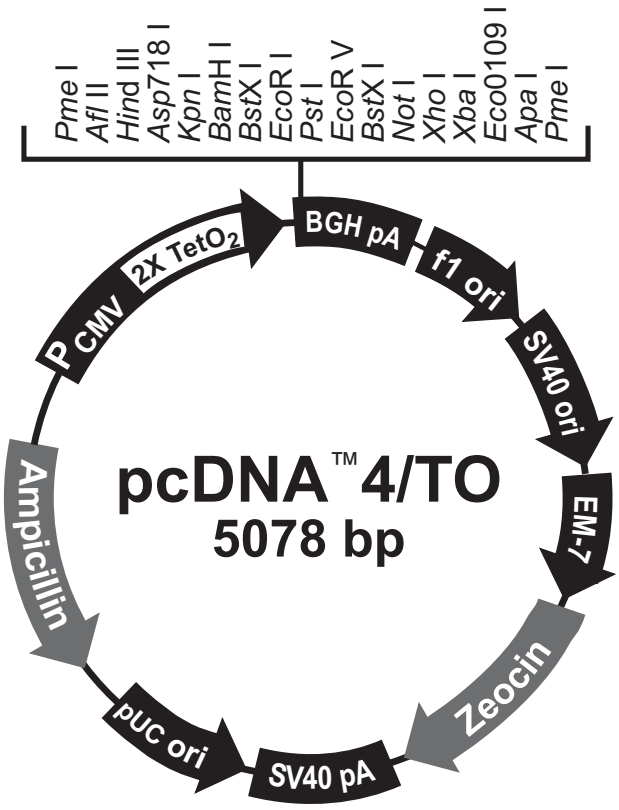
Note: Protease inhibitors may be added at the following concentrations:

1 mM PMSF
1 µg/mL pepstatin
1 µg/mL leupeptin

pcDNA™4/TO Vector

Map of pcDNA™4/TO

The figure below summarizes the features of the pcDNA™4/TO vector. **The complete nucleotide sequence for pcDNA™4/TO is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 19).**



Comments for pcDNA™4/TO
5078 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
Multiple cloning site: bases 967-1077
BGH reverse priming site: bases 1089-1106
BGH polyadenylation sequence: bases 1095-1319
f1 origin: bases 1365-1793
SV40 promoter and origin: bases 1803-2143
EM-7 promoter: bases 2183-2249
Zeocin™ resistance gene: bases 2250-2624
SV40 early polyadenylation sequence: bases 2754-2884
pUC origin: bases 3267-3937
bla promoter: bases 4937-5041 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4082-4942 (complementary strand)

Continued on next page

Features of pcDNA™4/TO

pcDNA™4/TO is a 5078 bp vector that expresses your gene of interest under the control of a hybrid CMV/TetO_2 promoter. The table below describes the relevant features of pcDNA™4/TO. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate early promoter	Allows high-level expression of your gene of interest (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987)
CMV Forward priming site	Allows sequencing in the sense orientation
Tetracycline operator (O_2) sequences	Two tandem 19 nucleotide repeats which serve as binding sites for Tet repressor homodimers (Hillen and Berens, 1994)
Multiple cloning site	Allows insertion of your gene of interest
BGH Reverse priming site	Allows sequencing of the non-coding strand
Bovine growth hormone (BGH) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the Zeocin™ 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 Zeocin™ resistance gene in *E. coli*
Zeocin™ resistance (*Sh ble*) gene (expressed from the SV40 early promoter or the EM-7 promoter)	Allows selection of stable transfectants in mammalian cells (Drocourt et al., 1990; Mulsant et al., 1988) 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*

pcDNA™4/TO/*lacZ* Vector

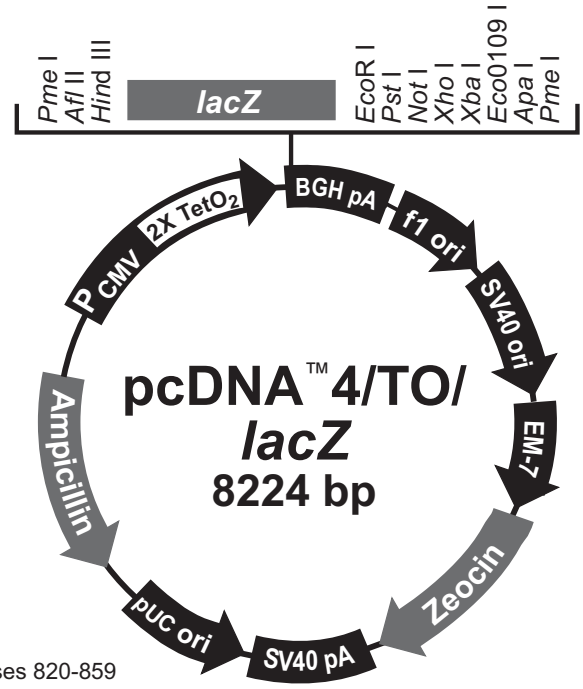
Description

pcDNA™4/TO/*lacZ* is a 8224 bp control vector containing the gene for β-galactosidase. This vector was constructed by ligating a 3.1 kb *Hin*d III-*Eco*R I fragment containing the *lacZ* gene from pcDNA™3/His/*lacZ* into the *Hin*d III-*Eco*R I site of pcDNA™4/TO.

Note: The *lacZ* gene is fused to an N-terminal peptide containing an ATG initiation codon, a polyhistidine (6xHis) tag, and the Xpress™ epitope. The size of the β-galactosidase fusion protein is approximately 120 kDa in size.

Map of pcDNA™4/TO/*lacZ*

The figure below summarizes the features of the pcDNA™4/TO/*lacZ* vector. **The complete nucleotide sequence for pcDNA™4/TO/*lacZ* is available for downloading from our website (www.lifetechnologies.com) or from Technical Support (see page 19).**



Comments for pcDNA™4/TO/*lacZ*
8224 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
LacZ fusion protein: bases 987-4157
  6xHis tag: bases 999-1016
  Xpress™ epitope: bases 1056-1079
  LacZ ORF: bases 1104-4154
BGH reverse priming site: bases 4235-4252
BGH polyadenylation sequence: bases 4241-4465
f1 origin: bases 4511-4939
SV40 promoter and origin: bases 4949-5289
EM-7 promoter: bases 5329-5395
Zeocin™ resistance gene: bases 5396-5770
SV40 early polyadenylation sequence: bases 5900-6030
pUC origin: bases 6413-7083
bla promoter: bases 8083-8187 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 7228-8088 (complementary strand)

Accessory Products

Introduction

The products listed in the tables below are designed for use with the T-REx™ System.

T-REx™ Cell Lines

For your convenience, Life Technologies has available several mammalian cell lines that stably express the Tet repressor. T-REx™-293 cells, T-REx™-HeLa cells, and T-REx™-CHO 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 is episomally-maintained in the T-REx™-U2OS cell line, but is stable under hygromycin selection. Expression of your gene of interest from pcDNA™4/TO may be assayed by transfection of your pcDNA™4/TO construct into any of the T-REx™ cell lines and induction with tetracycline. Ordering information is provided below.

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™ System Components

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

Item	Amount	Catalog no.
pcDNA™6/TR	20 µg	V1025-20
Zeocin™	1 g	R250-01
	5 g	R250-05
Blasticidin	50 mg, powder	R210-01

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.

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