User Manual

Tet-Off® and Tet-On® Gene Expression Systems User Manual

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Table of Contents

Table of Contents *continued*

List of Figures

List of Tables

I. Introduction

A. Summary

The **Tet-Off** and **Tet-On Gene Expression Systems** and the premade **Tet-Off** and **Tet-On Cell Lines** give researchers ready access to the regulated, high-level gene expression systems described by Gossen & Bujard (1992; Tet-Off) and Gossen *et al.* (1995; Tet-On). In the Tet-Off system, gene expression is turned on when tetracycline (Tc) or doxycycline (Dox; aTc derivative) is removed from the culture medium. In contrast, expression is turned on in the Tet-On system by the addition of Dox (Figure 1A). The Tet-On system is responsive only to Dox, not to Tc. Both systems permit gene expression to be tightly regulated in response to varying concentrations of Tc or Dox (Figure 1B).

Maximal expression levels in Tet systems are very high and compare favorably with the maximal levels obtainable from strong, constitutive mammalian promoters such as CMV (Yin *et al.* , 1996). Unlike other inducible mammalian expression systems, gene regulation in the Tet Systems is highly specific, so interpretation of results is not complicated by pleiotropic effects or nonspecific induction.

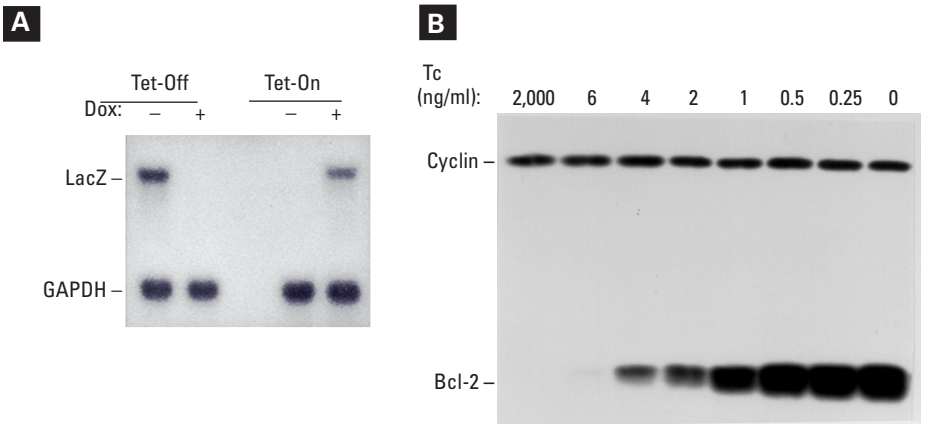


Figure 1. Inducible on/off control of gene expression in the Tet Systems. Panel A. Double-stable cell lines were developed by stably transfecting HeLa Tet-Off or HeLa Tet-On cells with a plasmid containing *E. coli* lacZ under control of the Tet response element (TRE). Cells were cultured +/– 1 µg/ml Dox. For Northern analysis, 10 µg of total RNA was loaded per lane, and the blot was hybridized simultaneously with probes to lacZ and the GAPDH housekeeping gene (Gossen *et al.*, 1995; reprinted with permission of the author). **Panel B.** HeLa S3 Tet-Off cells were stably transfected with a plasmid expressing Bcl-2 under control of the TRE and grown in the presence of the indicated amounts of Tc. A Western blot containing 100 µg of total protein from each condition was probed with human Bcl-2-specific and human cyclin-B1-specific mouse monoclonal antibodies. Based on scanning densitometry, removal of Tc gave ~100-fold induction of Bcl-2. For details, see Yin & Schimke (1995).

I. Introduction *continued*

See Appendix A or the Vector Information Packets provided for maps and detailed information on the Tet System Vectors. For a complete list of Tet Systems references, visit our web site at **www.clontech.com.**

B. The Tet-Off and Tet-On Systems

In *E. coli*, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon on the Tn *10* transposon. TetR blocks transcription of these genes by binding to the tet operator sequences (*tetO*) in the absence of Tc. TetR and *tetO* provide the basis of regulation and induction for use in mammalian experimental systems.

The first critical component of the Tet Systems is the **regulatory protein,** based on TetR. In the Tet-Off System, this 37-kDa protein is a fusion of amino acids 1–207 of TetR and the C-terminal 127 a.a. of the Herpes simplex virus VP16 activation domain (AD; Triezenberg *et al.* , 1988). Addition of the VP16 domain converts the TetR from a transcriptional repressor to a transcriptional activator, and the resulting hybrid protein is known as the tetracycline-controlled transactivator (tTA). tTA is encoded by the pTet-Off regulator plasmid, which also includes a neomycin-resistance gene to permit selection of stably transfected cells.

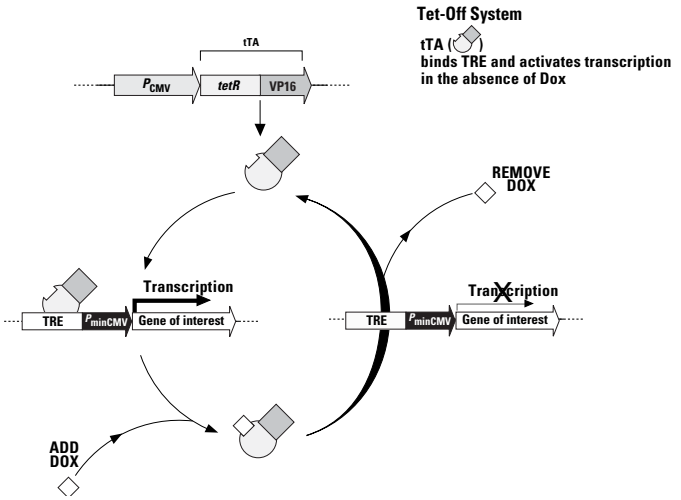
The Tet-On system is similar to the Tet-Off system, but the regulatory protein is based on a "reverse" Tet repressor (rTetR) which was created by four amino acid changes in TetR (Hillen & Berens, 1994; Gossen *et al.* , 1995). The resulting protein, rtTA (reverse tTA), is encoded by the pTet-On regulator plasmid, which also contains a neomycin-resistance gene.

The second critical component is the **response plasmid** which expresses a **gene of interest (Gene X)** under control of the tetracycline-response element, or TRE. We provide two response vector series for the Tet Systems. Our original vector series—pTRE or its variants—contain the TRE, which consists of seven direct repeats of a 42-bp sequence containing the *tetO*, located just upstream of the minimal CMV promoter (P_{minCMV}). P_{minCMV} lacks the strong enhancer elements normally associated with the CMV immediate early promoter. Because these enhancer elements are missing, there is extremely low background expression of Gene X from the TRE in the absence of binding by the TetR domain of tTA or the rTetR domain of rtTA. Our second response vector series—pTRE-Tight—contain a modified TRE (TRE_{mod}) upstream of an altered minimal CMV promoter ($P_{minCMV\Delta}$), resulting in further reduced basal expression of Gene X. pTRE-Tight can fully minimize background expression in certain cell lines, and is especially useful in cases where background expression is unacceptable, such as the expression of proteins that are extremely potent or toxic to the host cell.

The ultimate goal in setting up a functional Tet System is creating a double-stable **Tet cell line** which contains both the regulatory and

I. Introduction *continued*

Tet-Off



Tet-On

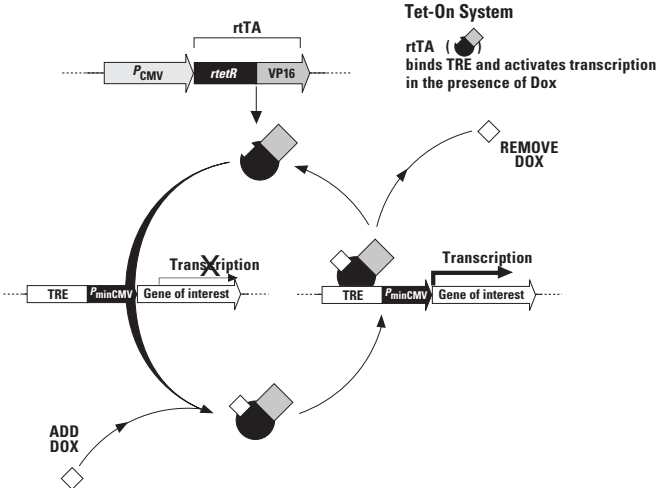


Figure 2. Schematic of gene regulation in the Tet-Off and Tet-On Systems. Tet-Off: The TRE is located upstream of the minimal immediate early promoter of cytomegalovirus (P_{minCMV}), which is silent in the absence of activation. tTA binds the TRE—and thereby activates transcription of Gene X—in the absence of Tc or Dox. **Tet-On:** The "reverse" Tet repressor (rTetR) was created by four amino acid changes that reverse the protein's response to Dox. As a result of these changes, the rTetR domain of rtTA binds the TRE and activates transcription in the presence of Dox. Please see Appendix A for maps and detailed vector information.

I. Introduction *continued*

response plasmids. When cells contain both the regulatory (pTet-Off or pTet-On) and the response (e.g. pTRE-Gene X) Vectors, Gene X is only expressed upon binding of the tTA or rtTA protein to the TRE (Figure 2). In the Tet-Off System, tTA binds the TRE and activates transcription in the *absence* of Tc or Dox. In the Tet-On System, rtTA binds the TRE and activates transcription in the *presence* of Dox. In both Tet-On and Tet-Off Systems, transcription is turned on or off in response to Dox in a precise and dose-dependent manner.

You can greatly reduce the time needed to establish a Tet cell line by purchasing one of our premade Tet Cell Lines, which already stably express the appropriate regulatory protein. A list of available Tet-Off and Tet-On Cell Lines is available from our Tet Systems product page at **www.clontech.com**.

Note that addition of a nuclear localization sequence [*nls*] to tTA or rtTA alters the protein's regulatory function (M. Gossen & H. Bujard, pers. comm.). Addition of an *nls* to tTA or rtTA increases maximum expression but also increases background expression due to altered binding affinity to *tetO* sequences (unpublished observations). Therefore, we recommend that you do not add a *nls* to either tTA or rtTA for creating stable Tet cell lines.

C. Advantages of the Tet Systems

The Tet-Off and Tet-On systems have several advantages over other regulated gene expression systems that function in mammalian cells:

- Extremely tight on/off regulation. Background, or leaky, expression of Gene X in the absence of induction is extremely low with pTRE or its variants (Figure 1). For the lowest background expression, use Tet-On 3G vectors.
- No pleiotropic effects. When introduced into mammalian cells, the prokaryotic regulatory proteins (TetR or rTetR, the prokaryotic precursors to tTA and rtTA) act very specifically on their target sequences, presumably because these regulatory DNA sequences are nonexistent in eukaryotic genomes (Harkin *et al.*, 1999).
- High inducibility and fast response times. With the Tet Systems, induction can be detected within 30 minutes (Figure 3) using nontoxic levels of inducer. Induction levels up to 10,000-fold have been observed (results not shown). In contrast, other systems for mammalian expression exhibit slow induction (up to several days), incomplete induction (compared to repressor-free controls), low overall induction (often no more than 100-fold), and high (nearly cytotoxic) levels of inducer (reviewed by Gossen *et al.*, 1993; Yarronton, 1992).

I. Introduction *continued*

- High absolute expression levels. Maximal expression levels in the Tet systems can be higher than expression levels obtained from the CMV promoter or other constitutive promoters. For example, Yin *et al. ,* (1996) reported that the maximal level of luciferase expression in HeLa Tet-Off cells transiently transfected with pTRE-Luc is 35-fold higher than that obtained with HeLa cells transiently transfected with a plasmid expressing luciferase from the wild-type CMV promoter.
- Well-characterized inducer. In contrast to the inducer used in other systems, such as in the ecdysone system, Tc and Dox are inexpensive, well characterized, and yield highly reproducible results.
- *Activation* of a promoter, rather than repression, to control expression. To completely shut off transcription, *repression*-based systems require very high—and difficult to attain—levels of repressor to ensure 100% occupancy of the regulatory sites. Even if suitably high levels of repressor can be obtained, the presence of high repressor levels makes it difficult to achieve rapid, high-level induction (Yao *et al.*, 1998). For a more complete discussion of the advantages of activation versus repression, see Gossen *et al.,* (1993).

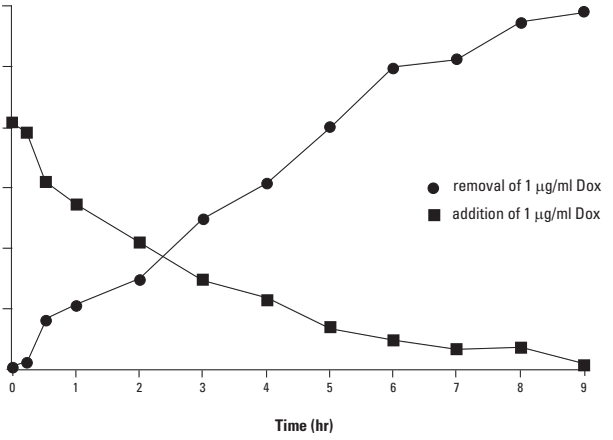


Figure 3. Luciferase expression is rapidly induced in a Tet-Off cell line in response to removal of Dox. The CHO-K1-EGFP-Luc Tet-Off control cell line expresses the tTA and contains a stably integrated copy of the firefly luciferase gene under control of the TRE. Luciferase activity was continuously monitored with a fluorescent imaging plate reader (FLIPR, Molecular Devices Corp.) after addition or removal of 1 μg/ml Dox from the culture medium (Cunningham et al., 1997).

I. Introduction *continued*

In contrast to the heterologous Tet Systems, homologous systems based on eukaryotic regulatory elements are subject to one or more of the following problems:

- Inducing stimulus is pleiotropic, i.e., the gene of interest is not the only gene affected by the inducing stimulus.
- It can be very difficult to distinguish specific from nonspecific events in an expression system based on homologous regulatory elements. This is largely due to the modular nature of eukaryotic promoters which interact with a variety of transcription factors that are, in turn, involved in the regulation of many promoters and/or enhancers.
- Most of the commonly used eukaryotic promoters are too "leaky" to maintain the gene of interest in the fully repressed ("off") state, limiting their usefulness for expressing toxic proteins.
- Maximal level of induction is usually not very high.

Thus, of the systems described to date, only the Tet Systems exhibit tight on/off regulation, absence of pleiotropic effects, high induction levels, high absolute expression, and rapid induction times (Gossen *et al.*, 1993; 1994).

D. Tet-Off vs. Tet-On Systems

Although the Tet-Off system has been studied more extensively than the Tet-On system, the two systems are truly complementary. When properly optimized, both systems give tight on/off control of gene expression, regulated dose-dependent induction with similar kinetics of induction, and high absolute levels of gene expression. Thus, for most purposes, there is no inherent advantage of using one system over the other.

With the Tet-Off system, it is necessary to keep Tc or Dox in the medium to maintain the native (off) state. Because Tc and Dox have relatively short half-lives (see below), you must add Tc or Dox to the medium at least every 48 hours to suppress expression of Gene X. Conversely, in the Tet-On system, the native (off) state is maintained until induction. For this reason, Tet-On may be more convenient in transgenic applications, because you need only add Dox to the animals' diet when induction is desired.

E. Tetracycline vs. Doxycycline

The Tet-On System is only responsive to Dox, not Tc (Gossen & Bujard, 1995). In contrast, Tet-Off systems respond equally well to either Tc or Dox. We recommend that you use Dox for all Tet System experiments, in part because a significantly lower concentration of Dox is required for complete activation or inactivation (0.01–1 µg/ml Dox vs. 1–2 µg/ml Tc). In both systems, the antibiotics are used at concentrations far below cytotoxic levels for either cell culture or transgenic studies. In addition,

I. Introduction *continued*

Dox has a longer half-life (24 hours) than Tc (12 hours). Thus, for the Tet-Off System, you may prefer to use Dox for long-term maintenance of antibiotic levels and switch to Tc in preparation for induction.

Other Tc derivatives have been used successfully as the inducer in Tet systems (Gossen & Bujard, 1993). Affinity for TetR and antibiotic potency are apparently mediated by different chemical moieties; some derivatives, such as anhydrotetracycline, have an increased affinity for TetR and decreased antibiotic activity (Gossen *et al.* , 1993).

F. Additional Tet Response Vectors

The complete Tet-Off and Tet-On Gene Expression Systems are provided with pTRE2hyg as the response vector. In addition to the TRE regulatory element and a multiple cloning site, this vector also expresses the hygromycin resistance gene, permitting easy selection of stable transfectants. We also offer pTRE2pur (Cat. No. 631013) for an alternative selection scheme using puromycin.

Another response vector, the pTRE-Tight Vector (Cat. No. 631059), is available separately. pTRE-Tight contains a modified TRE element (TRE_{mod}) that can minimize basal expression in certain cell lines.

Additionally, response vectors are available that express your protein with a tag to aid in detection and protein purification. These vectors provide a way to screen colonies directly for protein expression by Western analysis using readily available antibodies. These Vectors are available with or without a mammalian selection marker.

See Appendix A for additional information on these vectors.

G. Beyond the Basics: pBI, VP16, and pTet-tTS Vectors

The Bidirectional (pBI) Tet Vectors are specially designed response vectors that allow coregulated expression of two genes under control of a single TRE. They are ideal response vectors to use if you do not have a functional assay for your gene of interest, because you can select for expression of the coregulated marker gene, either β-galactosidase or luciferase. These vectors do not contain a selectable gene and should be cotransfected with one of the Linear Selection Markers, pTK-Hyg (Cat. No. 631625) or pPUR (Cat. No. 631626).

The pTRE-Tight response plasmid (Cat. No. 631059), contains a modified TRE element that can minimize basal expression in certain cell lines).

See Appendix A for additional information on these vectors.

H. Viral Tet Expression

Tet-On 3G Systems come in adenoviral, lentiviral, and retroviral formats. For more information, visit the Tet-On 3G Systems product pages at **www.clontech.com**.

II. Protocol Overview

Figure 4 provides an overview for creating double-stable Tet-Off or Tet-On cell lines which contain integrated copies of the regulatory and response vector—the ultimate goal in establishing the Tet System. For more detailed flow charts of each of the transfection procedures see Figure 7 (Section VIII) and Figure 8 (Section IX). If you have purchased a premade Tet-Off or Tet-On Cell Line from Clontech, you need only perform the second transfection with your pTRE-Gene X construct.

Important note on simultaneous versus consecutive transfections

In general, we recommend that you do not attempt to save time by cotransfecting the regulator and response plasmids. Cotransfected plasmids tend to cointegrate into the chromosome, and enhancer elements from the CMV promoter on the regulator plasmid (pTet-Off or pTet-On) can induce basal expression of Gene X. Furthermore, cotransfection prevents comparison of multiple clones, since differences in induction or absolute expression could be due to clone-to-clone variation in tTA or rtTA expression. In contrast, consecutive transfections have several advantages. Most importantly, the response plasmid generally will not cointegrate with the regulator, and you can select a double-stable cell line that gives very low to no background expression of Gene X. Furthermore, once you have developed a suitable Tet-Off or Tet-On cell line, it provides a proven genetic background into which you can introduce many different response plasmids.

II. Protocol Overview *continued*

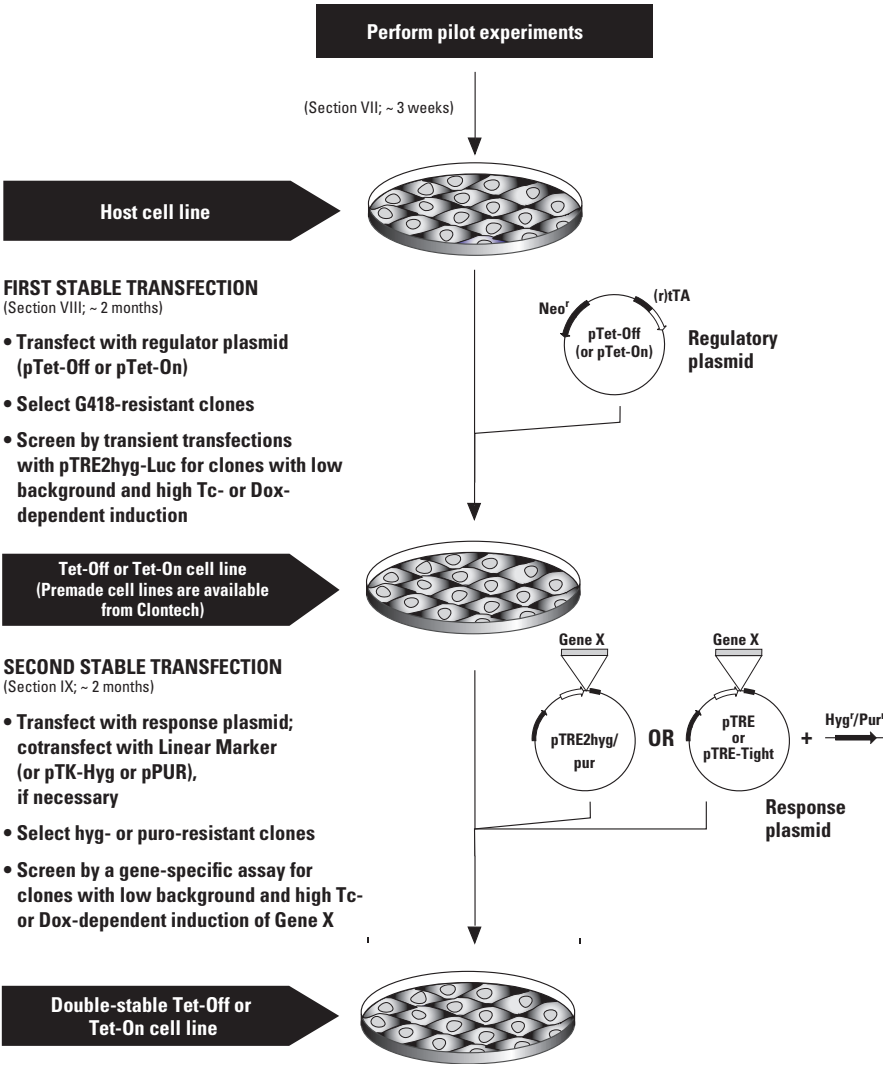


Figure 4. Overview of developing Tet-Off and Tet-On and double-stable Tet-Off and Tet-On cell lines. To use the Tet Gene Expression Systems, you will need to make a "double-stable" Tet cell line, as outlined above. If you are starting with your own cell line, you will need to perform the entire procedure outlined above. If you are starting with one of our premade Tet-Off or Tet-On Cell Lines, only perform the second stable transfection.

III. List of Components

Store all mammalian cell lines in liquid nitrogen (–196°C).
Store all plasmids and Fetal Bovine Serum at –20°C.
Visit our Tet Systems product pages **www.clontech.com** for a current list of cell lines and products available for the Tet Systems.

Tet-Off Cell Lines

- 1.0 ml Tet-Off Cell Line (2 x 10^6 cells/ml)
- 1.0 ml CHO-AA8-Luc Tet-Off Control Cell Line (2 x 10^6 cells)
- 50 ml Tet System Approved Fetal Bovine Serum
- Tet Cell Lines Protocol-at-a-Glance (PT3001-2)

Tet-On Cell Lines

- 1.0 ml Tet-On Cell Line (2 x 10^6 cells/ml)
- 1.0 ml U2-OS-Luc Tet-On Control Cell Line (2 x 10^6 cells)
- 50 ml Tet System Approved Fetal Bovine Serum
- Tet Cell Lines Protocol-at-a-Glance (PT3001-2)

IV. Additional Materials Required

For cell culture

- **Dulbecco's Modified Eagle's Medium** (DMEM, Sigma, Cat. No. D5796), **Alpha Minimal Essential Medium Eagle** (alpha-MEM), **RPMI-1640**, or other specified medium. The appropriate medium for growing Clontech premade Tet-Off and Tet-On cell lines is described on the Product Analysis Certificate provided with each cell line.

- **Fetal bovine serum (FBS)**
  It is critical that the FBS not inhibit Tet-responsive expression. You can eliminate Tc contamination problems by using Clontech Tet System Approved FBS. This serum has been functionally tested in the Tet Systems to ensure against possible Tc contamination. Alternatively, use the CHO-AA8-Luc Control Cell Line to test for Tc contamination in other sera, as described in Section VII.A.

  Note: The PC-12 Tet-Off and Tet-On Cell Lines require horse serum (Sigma Cat. No. 0146) for growth, which does not normally contain Tc.

- **200 mM L-Glutamine** (Sigma, Cat. No. G7513)

- Solution of 10,000 units/ml **Penicillin G sodium** and 10,000 µg/ml **Streptomycin sulfate** (Sigma, Cat. No. P0781)

- **Antibiotics for clonal selection**
  Prior to use, determine the optimal concentration of each antibiotic for selection as described in Section VII.B.

  G418 (for selection of Tet-Off and Tet-On Cell Lines)
  G418 is available in powdered form from Clontech (Cat. No. 631307). Note that the effective weight is about 0.7 g per gram of powder. Make a 10 mg/ml stock solution by dissolving 1 g of powder in approximately 70 ml of DMEM or alpha-MEM (without supplements). Filter sterilize and store at 4°C.
  Recommended working concentration:
  Maintenance: 100 µg/ml Selection (HeLa or CHO cells): 400–500 µg/ml
  (acceptable range): 50–800 µg/ml

  Hygromycin (for selection of double-stable Tet-Off and Tet-On Cell Lines)
  Hygromycin B is available from Clontech (Cat. No. 631309).
  Recommended working concentration:
  Maintenance: 100 µg/ml Selection (HeLa or CHO cells): 200 µg/ml
  (acceptable range): 50–800 µg/ml

  Puromycin (for maintenance of the MDCK Tet-Off Cell Line and for selection of double-stable Tet-On and Tet-Off cells) available from Clontech (Cat. No. 631305 & 631306)
  Recommended working concentration:
  Maintenance: 0.5 µg/ml Selection (acceptable range): 0.5–5 µg/ml

- **Trypsin-EDTA** (Trypsin; Sigma, Cat. No. T3924)

IV. Additional Materials Required *continued*

- **Dulbecco's phosphate buffered saline**
  (DPBS; Sigma, Cat. No. D8662)
- **Cell Freezing Medium,** with or without DMSO
  (Sigma, Cat. No. C6164 or Cat. No. C6039)
- **Tissue culture plates and flasks,** available from BD Discovery Labware
  (www.bdbiosciences.com/discovery_labware)
- **Cloning cylinders or discs**
  (PGC Scientific, Cat. No. 62-6150-40, -45 or Cat. No. 62-6151-12, -16)

For transient and stable transfections

The transient and stable transfections in this protocol can be performed by various methods. Reagents will depend on which transfection method you use. Although we generally use electroporation for both transient and stable transfections with the Tet-Off and Tet-On System, other methods work well and may be preferable, depending on cell type.

The CalPhos™ Mammalian Transfection Kit (Cat. No. 631312) and Xfect™ Transfection Reagent (Cat. Nos. 631317 & 631318), are available for high-efficiency calcium-phosphate or nanoparticle-mediated transfections.

The efficiency of transfection for different cell lines may vary greatly. A method that works well for one host cell line may be inferior for another. Therefore, when working with a cell line for the first time, you may want to compare the efficiencies of several transfection protocols. You can transfect the host cell line with a noninducible reporter/expression vector, such as pCMV-LacZ (Cat. No. 631719) or pAcGFP1-N1 (Cat. Nos. 632469 & 632426) and assay for reporter gene activity.

After a method of transfection is chosen, it may be necessary to optimize parameters such as cell density, the amount and purity of the DNA, media conditions, and transfection time. Once optimized, these parameters should be kept constant to obtain reproducible results.

If cotransfection is required to create a stable cell line with your pTRE vector, we recommend cotransfection with Linear Hygromycin Marker (Cat. No. 631625) or Linear Puromycin Marker (Cat. No. 631626). These markers are short, purified linear DNA fragments comprised of the marker gene, an SV40 promoter, and the SV40 polyadenylation signal. Because of their small size, these markers are highly effective at generating stable transfectants. Alternatively, you can use pTK-Hyg Vector (Cat. No. 631750) or pPUR Vector (Cat. No. 631601).

Note: If you are using a selection vector other than a Linear Selection Marker, pTK-Hyg, or pPUR, the promoter should not contain an enhancer element. If it does, cointegration of the response and selection plasmids may lead to high background expression of Gene X in the uninduced state.

IV. Additional Materials Required *continued*

For regulation of gene expression

- **Doxycycline** (Cat. No. 631311). Dilute to 1–2 mg/ml in H_2O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.
- **Tetracycline hydrochloride** (Sigma Cat. No. T3383) Dilute to 1 mg/ml in 70% ethanol. Filter sterilize, aliquot, and store at –20°C in the dark. Use within two months.

For luciferase assays

- Use any standard luciferase assay system.

For PCR confirmation of integrated plasmids (optional)

- If you wish to confirm the presence of integrated plasmids in clonal hygromycin-, puromycin-, or neomycin-resistant cell lines, you will need to design PCR primers that amplify a portion of the appropriate regulator or response plasmid.

V. Plasmid Manipulations

A. Propagation of Vector Plasmids

1. Transform each of the plasmids provided in this kit into a suitable *E. coli* host strain (e.g., DH5α) to ensure that you have a renewable source of DNA. Tet vectors are low copy-number, so use chloramphenicol amplification to increase plasmid yields.
2. You will need to perform large-scale plasmid preparations of any plasmid that will be introduced into mammalian cells. To ensure the purity of the DNA, prepare transfection-grade plasmid by purification on a NucleoBond column. Visit **www.clontech.com** for complete product information.

B. Generating Your Gene-Specific Expression Vector

Generate your pTRE-Gene X construct using standard molecular biology techniques, as described below. For more detailed information, see Sambrook *et al.* (2001).

1. Purify the Gene X fragment by any standard method, such as the NucleoTrap Gel Extraction Kit (Cat. No. 740584) or NucleoTrap PCR Purification Kit (Cat. No. 740587). The cDNA or gene fragment must contain an ATG initiation codon. In some cases, addition of a Kozak consensus ribosome binding site (Kozak, 1987) may improve expression levels; however, many genes have been efficiently expressed in Tet systems without the addition of a Kozak sequence. The fragment can be generated using compatible restriction sites that are present on either side of the gene and in the cloning vector. If no such sites are present, the gene fragment can be generated by PCR with suitable restriction sites incorporated into the primers.
2. Digest the response vector (pTRE or its variant) with the appropriate restriction enzyme(s), treat with phosphatase, and purify.
3. Ligate the response vector and the Gene X fragment.
4. Transform ligation mixtures into *E. coli*.
5. Identify the desired recombinant plasmid by restriction analysis, and confirm orientation and junctions by sequencing.

VI. Cell Culture Guidelines

A. General Information

The protocols in this User Manual provide only general guidelines for mammalian cell culture techniques. Perform all steps involving cell culture using sterile technique in a suitable hood. For those requiring more information on mammalian cell culture, we recommend the following general references:

- *Culture of Animal Cells*, Fourth Edition, ed. by R. I. Freshney (2000, Wiley-Liss, NY)
- *Current Protocols in Molecular Biology*, ed. by F. M. Ausubel *et al.* (1995, Wiley & Sons)

B. Characteristics of Tet-Off and Tet-On Cell Lines

See the Certificate of Analysis for information on each Tet-Off and Tet-On Cell Line. Additional information for all the currently available Tet-Off and Tet-On Cell Lines, including propagation information, is provided in the Tet Cell Lines Protocol-At-A-Glance (PT3001-2) which is available from our website at **www.clontech.com/manuals**.

General cell culture conditions: Premade Tet-Off and Tet-On Cell Lines should be grown at 37°C in a humidified chamber with 5–10% CO_2. See the PAC for details particular to each cell line.

Relative growth rates: The incubation times in this User Manual are for cells such as CHO or HeLa with relatively rapid doubling times. Other cell types will differ in their growth rates.

Selection in G418 and hygromycin: Maintain stable and double-stable Tet-Off and Tet-On Cell Lines in the appropriate selective medium; however, the concentration can be reduced (typically to 100 µg/ml for each drug) from the levels used to select stably transfected clones. You may wish to alternate between selecting and nonselecting conditions.

C. Starting Tet Cell Cultures From Frozen Stocks

Note: Frozen cells should be cultured immediately upon receipt or as soon thereafter as possible. Increased loss of viability may occur after shipping if culturing is delayed.

1. Thaw vial of cells rapidly in a 37°C water bath with constant agitation. Immediately upon thawing, wipe the outside of the vial with 70% EtOH. Transfer the contents of the vial to a 10-cm dish, or a T25 or T75 flask, containing 1 ml of medium (without antibiotics). Mix gently.
2. Add an additional 4 ml of medium to the flask/dish and mix gently.
3. Add additional medium to the culture as follows:

T25 flask or 10-cm dish	add 5 ml
T75 flask	add 10 ml

Note: For Jurkat and other suspension cultures, suspend cells at a density of no less than $2x10^5$ cells/ml in the appropriate medium.

VI. Cell Culture Guidelines *continued*

4. Mix the cell suspension thoroughly. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place it in a 37°C humidified incubator (5–10% CO_2 as appropriate).
5. [Alternative method]The cells can also be rinsed prior to incubation. If rinsing is desired, perform steps 1 and 2 in a 15-ml conical centrifuge tube. Centrifuge at 125 x g for 10 min, and resuspend in complete medium for culturing. This step removes the cryopreservative and can be beneficial when resuspending in small volumes. However, this step can damage fragile cell membranes.
6. The next day, examine the cells under a microscope. If the cells were not rinsed upon thawing (step 5), centrifuge cells (if suspension cultures), aspirate the medium, and replace with fresh, prewarmed, complete medium (without antibiotics).
7. Expand the culture as needed. Note: The appropriate selective antibiotic(s) may be added to the medium after 48–72 hr in culture.

D. Preparing Frozen Stocks of Tet Cell Lines

Once you have started growing a Tet-Off or Tet-On Cell Line from Clontech, prepare frozen aliquots to ensure a renewable source of cells. Similarly, prepare frozen aliquots of any double-stable Tet-Off or Tet-On cell line or of any Tet-Off or Tet-On cell line that you make.

1. Trypsinize the desired number of flasks.
2. Pool cell suspensions together, count cells, and calculate total viable cell number.
3. Centrifuge cells at 125 x g for 10 min. Aspirate the supernatant.
4. *Resuspend the pellet at a density of at least 1–2 x10^6 cells/ml* in freezing medium. Freezing medium can be purchased from Sigma (Cat. No. C6164), or freeze cells in 70–90% FBS, 0–20% medium (no additives), and 10% DMSO.
5. Dispense 1-ml aliquots into sterile cryovials.
6. Freeze slowly (1°C per min). Nalgene makes cryo-containers (Nalgene Cat. No. 5100) for this purpose if a specialized freezer is not available (freeze at –80°C overnight). Alternatively, place vials in a thick-walled styrofoam container at –20°C for 1–2 hr. Transfer to –80°C overnight. Remove vials from styrofoam container or cryo-containers the following day and place in liquid nitrogen storage or ultra low-temperature freezer (–150°C).
7. (Two or more weeks later) Plate a vial of frozen cells, as described in Section C, to confirm viability.

VII. Pilot Experiments

A. Pilot Experiment with the CHO-AA8-Luc Tet-Off or U2-OS-Luc Tet-On Control Cell Line

Before you perform any other experiments, we strongly recommend that you perform a dose-response curve with the CHO-AA8-Luc Tet-Off Control Cell Line provided with Tet-Off kits or the U2-OS-Luc Tet-On Cell Line provided with the Tet-On kits. These premade double-stable Tet Cell Lines can exhibit over 10^4-fold induction of luciferase upon removal (Tet-Off) or addition (Tet-On) of Tc or Dox from the culture medium (Figure 5). In addition to providing a "hands-on" introduction to the Tet Systems, this experiment serves two critical functions:

- **Determination of effective concentrations of Tc or Dox stocks**: The concentrations of Tc and Dox listed throughout this protocol are approximate. The optimal concentration may vary with different cell lines and with different lots of antibiotic. In general, full repression of gene expression in Tet-Off cell lines can be obtained with 1–2 µg/ml Tc or 10 ng–1 µg/ml Dox. Full activation of gene expression in Tet-On cell lines can be obtained with 100 ng–1 µg/ml Dox.
- **Testing of serum for Tc contamination**: As shown in Figure 5, different lots of FBS exhibit significant variation in their effect on Tet System expression, presumably due to the widespread use of tetracyclines in the diet of cattle. The ~10,000-fold induction of luciferase in CHO-AA8-Luc Tet-Off Control Cells in response to Tc or Dox is highly reproducible. If you see a significantly lower level of induction (e.g., 100–1,000-fold or less), this may suggest that your serum contains Tc. This test should be repeated with each different lot of serum. Alternatively, use Tet System Approved FBS (Cat. No. 631101 or Cat. No. 631106), which has been functionally tested and shown to allow the full range of induction possible with the Tet System cell lines.

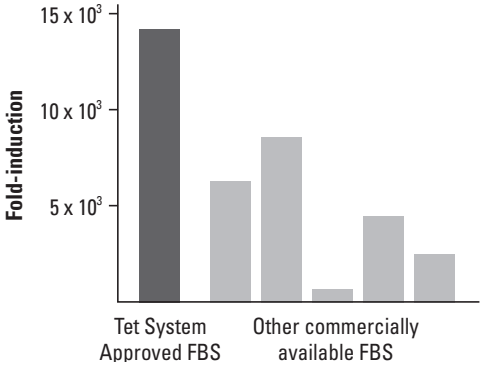


Figure 5. Fold induction of luciferase activity in different lots of FBS. The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different lots of FBS. Average uninduced expression level = 0.21 RLU (n=21, S.D.=0.07); maximum expression levels varied from 123 to 3,176 RLU.

VII. Pilot Experiments *continued*

Procedure:

1. Plate 6 aliquots of 0.5 x 10^5 CHO-AA8-Luc Tet-Off or U2-OS-Luc Tet-On cells each into 5 ml of complete culture medium in 6-well culture dishes.
2. To titrate Tc: add Tc to final concentrations of 0, 1 x 10^{-4}, 1 x 10^{-3}, 1 x 10^{-2}, 0.1, 1.0, and 10.0 µg/ml.

   To titrate Dox, add Dox to final concentrations of 0, 1 x 10^{-3}, 1 x 10^{-2}, 0.1, 1.0, 10, and 100 ng/ml.
3. Allow the cells to grow for 48 hr.
4. Assay each sample for luciferase activity using any standard luciferase assay. Plot your results logarithmically and compare to Figure 6.

B. Titrating G418, Hygromycin, and Puromycin (Kill Curves)

Prior to using G418, hygromycin, or puromycin to establish stable and double-stable cell lines, it is important to titrate your selection agent stocks to determine the optimal concentration for selection with the particular host cell line being tested. This is also important because of lot-to-lot variation in the potency of these drugs. Therefore, you should titrate each new lot of antibiotic to determine the optimal concentration. We recommend that you perform two experiments for each drug: (1) a titration to determine the optimal drug concentration, and (2) an experiment to determine the optimal plating density. This step is recommended even if you are using premade Tet Cell Lines.

1. Titrate at fixed cell density.
   a. Plate 2 x 10^5 cells in each of six 10-cm tissue culture dishes containing 10 ml of the appropriate complete medium plus varying amounts (0, 50, 100, 200, 400, 800 µg/ml) of hygromycin or G418. For puromycin, add the drug at 0, 1, 2.5, 5, 7.5, and 10 µg/ml.

      Note: 293 Tet-On and Tet-Off cells (Cat. No. 630903 and Cat. No. 630908, respecitively) are especially sensitive to hygromycin; test a concentration range with a midpoint of 25 µg/ml. Saos-2 Tet-Off cells (Cat. No. 630911) exhibit resistance to hygromycin; test a concentration range with a midpoint of 800 µg/ml.

   b. Incubate the cells for 10–14 days, replacing the selective medium every four days (or more often if necessary).
   c. Examine the dishes for viable cells every two days.

   For selecting stable transformants, use the lowest concentration that begins to give massive cell death in ~5 days and kills all the cells within two weeks. For HeLa and CHO cells, we have found 400 µg/ml G418 and 200 µg/ml hygromycin to be optimal. In mammalian cells the optimal level of puromycin is typically around 1 µg/ml.

VII. Pilot Experiments *continued*

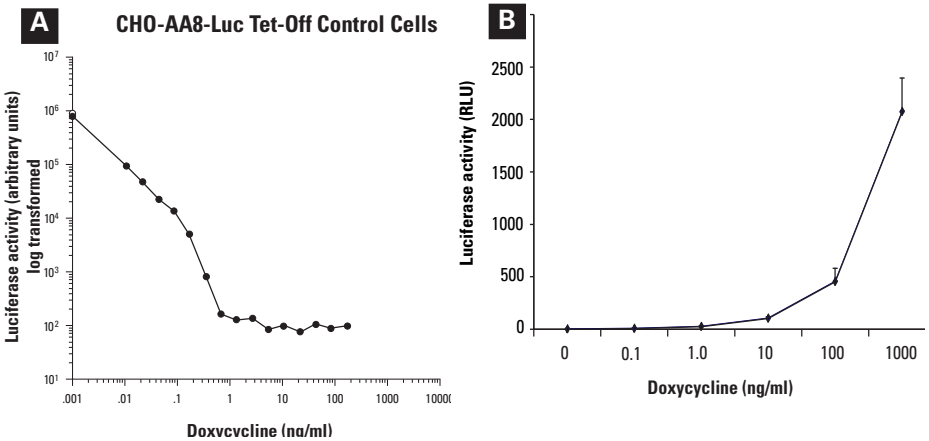


Figure 6. Dose-response curves for the Tet Control Cell Lines. Panel A. Dose-response curves for the CHO-AA8-Luc Control Cell Line. Experiments with another control cell line (CHO-K1-EGFP-Luc Tet-Off) have demonstrated that suppression can be maintained with Dox concentrations as low as 10 pg/ml (Cunningham *et al.* , 1997). Differences in background and induction levels can occur between multiple independent clonal lines when establishing double-stable Tet-Off Cell Lines (see Section IX.C). **Panel B.** Dose-response curve for the U2-OS-Luc Tet-On Control Cell Line.

2. Determine optimal plating density.
3. Once you have determined the optimal drug concentration, determine the optimal plating density by plating cells at several different densities in the presence of a constant amount of drug. If cells are plated at too high a density, they will reach confluency before the selection takes effect. Optimal plating density is dependent on population doubling time and cell surface area. For example, large cells that double rapidly have a lower optimal plating density than small cells that double slowly.
   a. Plate cells at several different densities in each of six 10-cm tissue culture dishes containing 10 ml of the appropriate selective medium. Suggested densities (cells/10-cm dish): 5 x 10^6, 1 x 10^6, 5 x 10^5, 2 x 10^5, 1 x 10^5, and 5 x 10^4.
   b. Incubate the cells for 5–14 days, replacing the selective medium every four days.
   c. Examine the dishes for viable cells every two days.

   For selecting stable transfectants, use a plating density that allows the cells to reach ~80% confluency before massive cell death begins (at about day 5).This is the cell density at which cells should be plated for selection of stable transfectants. For HeLa cells, we have found 2 x 10^5 cells/10-cm dish to be a good plating density.

VII. Pilot Experiments *continued*

C. [optional] Test Potential Host Cells by Transient Transfection with pTRE2hyg-Luc and pTet-Off or pTet-On

Tet expression systems have been established in numerous cell lines including HeLa, CHO, MCF7, HEK 293 and HepG2. However, the system may not be compatible with every cell type. Performing a transient expression assay with pTet-Off (or pTet-On) and pTRE2hyg-Luc may provide a quick indication of whether or not the Tet systems will work in a particular cell line. This test is not necessary if you have purchased a premade Tet-Off or Tet-On Cell Line.

You should transfect cells using varying ratios of pTet-Off/On to pTRE2hyg-Luc. For example, try:

pTet-Off/On	:	pTRE2hyg-Luc
1 µg	:	1 µg
1 µg	:	10 µg
10 µg	:	1 µg

Important Note: Fold-induction levels are almost always lower in transient assays than in properly screened stable and double-stable cell lines. For example, the Saos-2 Tet-Off Cell Line exhibits ~40-fold induction in transient expression assays, but stable clones can be isolated that exhibit 6,000-fold induction and background expression levels that are indistinguishable from control background expression. Therefore, an apparent lack of induction response in the transient assay should not be the sole reason for aborting your experiments in a particular cell line.

VIII. Development of Stable Tet Cell Lines

SKIP SECTION VIII IF YOU HAVE PURCHASED A PREMADE TET-OFF OR TET-ON CELL LINE

A. Transfection and Selection of Stable Cell Lines (Figure 7)

The following protocol describes the development of Tet-Off or Tet-On cell lines. You must optimize the protocol for each cell type. Some of the parameters most likely to need adjustment are: plating densities, transfection method, G418 concentrations for selection, and incubation and growing times.

Regardless of the cell type and transfection method, the goal is to generate a cell line that gives low background and high induction of luciferase activity when tested by transient transfection with pTRE2hyg-Luc in Section B. Because the level of expression of tTA or rtTA is profoundly affected by the site of integration, we recommend that you isolate and analyze as many clones as possible at Step 6. In general, test at least 30 clones. We have screened as many as 100 clones to obtain one that exhibits suitably high induction and low background.

1. Grow cells to ~80% confluency in complete medium or to a density appropriate for your transfection method.
2. Transfect the pTet-On or pTet-Off Vector by the desired method.

   Note: If desired, the regulator plasmid can be linearized by digestion with a restriction enzyme (Sca I for pTet-On/Off).
3. Plate transfected cells in ten 10-cm culture dishes, each containing 10 ml of the appropriate complete medium, at the optimal density determined in Section VII.
4. Allow cells to divide twice (24–48 hr), then add G418 to 400–500 µg/ml.

   Note: The exact concentration of G418 for selection and the optimal plating density may vary from cell type to cell type and with different lots of G418. See Section VII.B.
5. Replace medium with fresh complete medium plus G418 every four days, or more often if necessary.

   After about five days, cells that have not taken up the plasmid should start to die. Split the cells if they reach confluency before massive cell death begins.

   After 2–4 weeks, isolated colonies should begin to appear.
6. Isolate large, healthy colonies and transfer them to individual plates or wells. Suspension cultures must be cloned using the limiting dilution technique. When working with adherent cells at Clontech, we generally isolate clones using cloning cylinders or cloning discs.

VIII. Development of Cell Lines *continued*

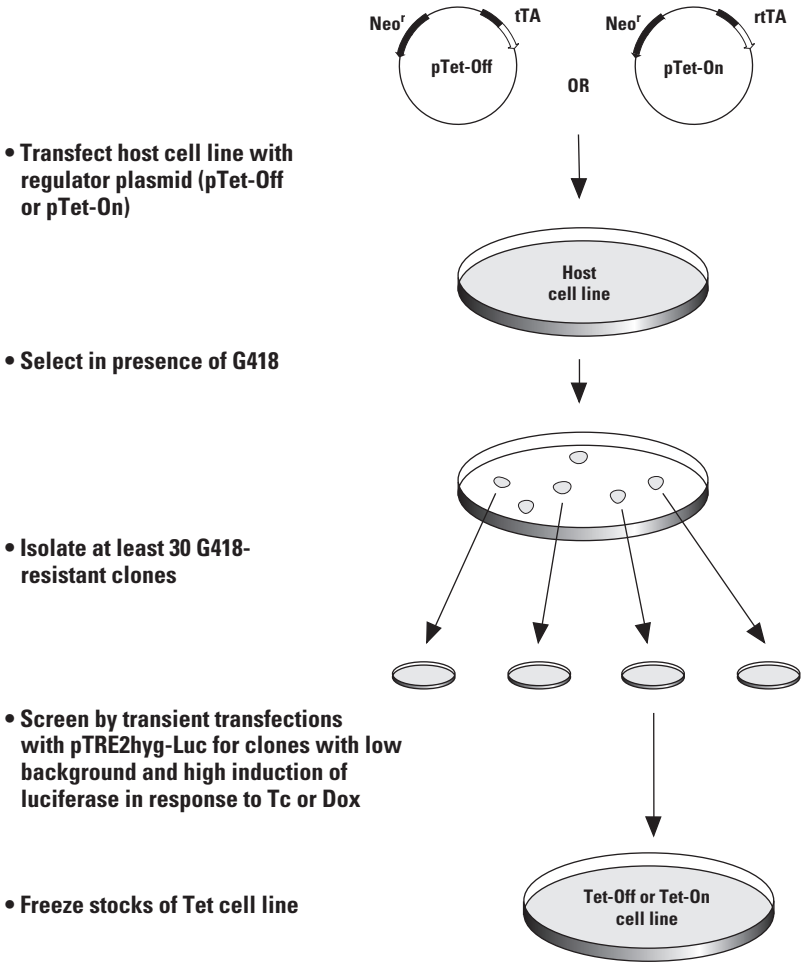


Figure 7. Flow chart for developing Tet cell lines.

VIII. Development of Cell Lines *continued*

B. Screening Stable Cell Lines

The next step is to perform transient transfection assays with a reporter vector, to identify G418-resistant clones that meet the criteria for stable Tet-Off or Tet-On cell lines. See Appendix A for maps and more information on these reporter vectors.

1. Pick clones and expand as needed for your particular cell line. Screen clones once they reach 50–80% confluency in a 6-well plate.
2. Trypsinize the cells and split about 1/3 into a single well of a 6-well plate. The cells in this "stock plate" will be propagated depending upon the results of the screening assay.
3. Transfect the remaining 2/3 of the cells with 1–2 µg of your reporter vector, using the desired transfection method. Decrease the amount of DNA if performing liposome-mediated transfection. Split into two wells of a six-well plate.
4. Add Dox (1–2 µg/ml) to one of the two wells from step 3.
5. Incubate the transfected cells for 48 hr.
6. Assay for induction:

   Luciferase Assay: calculate fold-induction
   For Tet-Off: Fold-induction = –Dox RLU/+Dox RLU
   For Tet-On: Fold-induction = +Dox RLU/–Dox RLU
7. Select clones with the highest fold-induction (highest expression with lowest background) for propagation and further testing. In general, only select clones that exhibit >20-fold induction.
8. Freeze stocks of each clone as soon as possible after expanding the culture.

   Note: Some researchers may desire to confirm the presence of the tTA and rtTA regulatory proteins in stable Tet cell lines by Western analysis. A Western blot only verifies the presence of tTA or rtTA; it does not reveal the functional inducibility of these cell lines. Furthermore, tTA and rtTA expression in stable cell lines may be below levels detectable by Western blotting. High levels of tTA or rtTA are not required for good induction, and in fact, overexpression of tTA can be toxic to cells. Therefore, Western analysis should NOT substitute for the functional screen.

IX. Development of Double-Stable Tet Cell Lines

A. Test pTRE-Gene X by Transient Transfection into a Tet-Off or Tet-On Cell Line

Prior to establishing your double-stable Tet-Off or Tet-On cell lines, you should test your pTRE-Gene X (or pBI Tet Vector) construct for functionality. Transiently transfect pTRE-Gene X into the cell line created in Section VIII, or the premade Clontech Tet Cell Line. If you are not using a pBI Vector, you will need to design a gene-specific assay to test for the induction of Gene X. Examples of gene-specific assays that can be used include:

- Western blot with an antibody to Protein X
- RT-PCR using Gene X primers. Be sure you can discriminate PCR products generated from genomic DNA from true RT-PCR products.
- Northern blot with Gene X probe
- Functional assay for Protein X

B. Stably Transfect and Select Double-Stable Cell Lines (Figure 8)

The next step is to stably transfect the stable (or premade) Tet cell line with your pTRE-Gene X construct. The goal is to generate a cell line that gives low background and high expression of Gene X when tested in Section IX.D. Both expression levels and induction of Gene X can be profoundly affected by the site of integration. Insertion near an enhancer may result in high basal expression of Gene X, whereas other insertion sites may result in suboptimal induction. To find the clone with the highest induction and lowest background, we recommend that you grow and analyze as many clones as possible. In general, test at least 30 clones. We have screened as many as 100 clones to obtain one that exhibits suitably high induction and low background.

IMPORTANT: If you are **not** using pTRE2hyg, pTRE2pur or another response vector bearing a mammalian selection marker, skip the steps below and use the cotransfection protocol in Section IX.C.

1. Grow cells to ~80% confluency in complete medium or to a density appropriate for your transfection method.
2. Transfect cells with pTRE2hyg-Gene X or pTRE2pur-Gene X.

   Note: If desired, the plasmids can be linearized by digestion with a restriction enzyme (check the Vector Information Packets provided with each vector for appropriate restriction sites).
3. Plate transfected cells in ten 10-cm culture dishes, each containing 10 ml of the appropriate complete medium, at the optimal density determined in Section VII.
4. Allow cells to divide twice (24–48 hr), then add the appropriate selection agent, hygromycin or puromycin, to the optimal concentration determined in Section VII. For hygromycin the range is generally

IX. Development of Double-Stable Tet Cell Lines *continued*

- **Insert Gene X into pTRE2hyg/pur, another pTRE variant, or a pBI vector**

- **Transfect Tet-Off or Tet-On cell line with pTRE2hyg/pur-Gene X response plasmid; or cotransfect pTRE-Gene X or pBI-Gene X with a Linear Marker**
- **Select in presence of hygromycin or puromycin**
  (Tc or Dox should be included in the medium when establishing double-stable Tet-Off cell lines.)

- **Isolate at least 30 hygromycin/puromycin-resistant clones**

- **(OPTIONAL) Confirm presence of integrated pTRE-Gene X in clones by PCR**

- **Screen by a gene-specific assay for clones with:**
  - **Low background of Gene X**
  - **High induction of Gene X**

  Possible assays:
  - **Western blot using an antibody to Protein X**
  - **RT-PCR using Gene X primers**
  - **Northern blot with Gene X probe**
  - **Functional assay for Protein X**
  - **Reporter activity (β-galactosidase, or luciferase on pBI vector)**

- **Freeze stocks of double-stable cell lines**

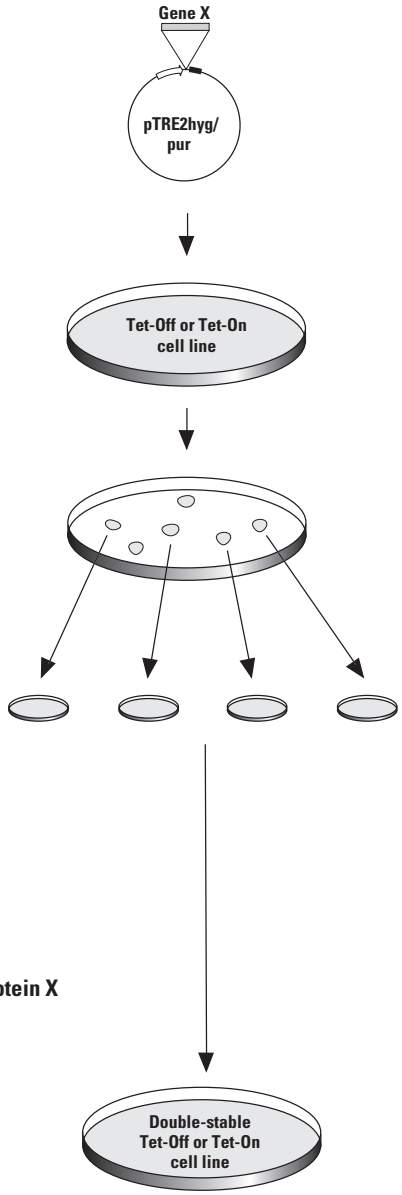


Figure 8. Flow chart for developing double-stable Tet cell lines.

IX. Development of Double-Stable Cell Lines *continued*

200–400 µg/ml and for puromycin it is 1–5 µg/ml.

For Tet-Off cells only: When establishing a double-stable Tet-Off cell line, we recommend that you culture the cells in the presence of 2 µg/ml Tc or 1 µg/ml Dox in order to keep transcription of Gene X turned "off". This is essential if Protein X is toxic to the cell.

5. Replace medium with fresh complete medium containing the selection antibiotic (hyg or pur) every four days. Fresh Dox MUST be added every two days for Tet-Off cells.

   After about five days, cells should start to die. Split cells if they reach confluency before massive cell death begins.

   After 2–4 weeks, hyg-resistant or pur-resistant colonies will begin to appear.
6. Isolate large, healthy colonies and transfer them to individual plates or wells. Isolate as many clones as possible.
7. Proceed to Section IX.D.

C. Stably Transfect and Select Double-Stable Cell Lines—Cotransfection

pTRE2hyg-Gene X and pTRE2pur-Gene X response plasmids contain a selection marker in the backbone. Other pTRE response plasmids which do not contain a marker must be cotransfected with a selection vector such as a Linear Selection Marker, pTK-Hyg, or pPUR using the following protocol.

Note: If you are using a selection vector other than a Linear Selection Marker, pTK-Hyg, or pPUR, the promoter should not contain an enhancer element. If it does, cointegration of the response and selection plasmids may lead to high background expression of Gene X in the uninduced state.

1. Grow cells to ~80% confluency in complete medium or to a density appropriate for your transfection method.
2. Transfect pTRE-Gene X and a Linear Selection Marker, pTK-Hyg, or pPUR in a ratio of between 10:1 and 20:1 by the desired method. You may want to optimize ratios.

   Note: If desired, the plasmids can be linearized by digestion with a restriction enzyme (check the Vector Information Packets provided with each vector for appropriate restriction sites).
3. Plate transfected cells in ten 10-cm culture dishes, each containing 10 ml of the appropriate complete medium, at the optimal density determined in Section VII.
4. Allow cells to divide twice (24–48 hr; time may vary with cell line), then add hygromycin (or puromycin) to 200–400 µg/ml (or the optimal concentration determined in Section VII).

   For Tet-Off cells only: When establishing a double-stable Tet-Off cell line, you may wish to culture the cells in the presence of 2 µg/ml

IX. Development of Double-Stable Cell Lines *continued*

Tc or 1 µg/ml Dox in order to keep transcription of Gene X turned "off". This is essential if Protein X is toxic to the cell.

5. Replace medium with fresh complete medium containing hygromycin (or puromycin) every four days. Fresh Dox MUST be added every two days for Tet-Off cells.

   After about five days, cells should start to die. Split cells if they reach confluency before massive cell death begins.

   After 2–4 weeks, hyg- (or puro-) resistant colonies will begin to appear.
6. Using cloning cylinders or discs, isolate large, healthy colonies and transfer them to individual plates or wells. Isolate as many clones as possible.

D. Screening Double-Stable Cell Lines

1. Test isolated resistant clones for Dox-regulated gene expression by dividing a suitable number of cells in half and testing for Gene X expression (or pBI reporter expression) in the presence and absence of 1 µg/ml Dox.

   As with the development of Tet-Off or Tet-On cell lines, you should generally choose the cell line that gives you the highest overall induction and lowest background (i.e., uninduced expression level) of Gene X.
2. Allow the cells to grow for at least 48 hr, then assay each sample for Gene X expression using one of the methods described in Section A.
3. [Optional] Confirm the presence of integrated pTRE-Gene X by performing PCR on chromosomal DNA using primers that will amplify an internal portion of the plasmid.
4. Once you have developed a suitable double-stable Tet-Off or Tet-On cell line, prepare frozen aliquots to ensure a renewable source of the cells (Section VI.D).

E. Working with Double-Stable Tet Cell Lines

The Tet System has been established successfully in many cell types, as well as transgenic mice, rats, plants, and yeast. In general, failure to obtain a cell line with a low background level of Gene X expression is a result of the integration site in the tested lines, and can be overcome simply by screening more clones.

Perform a dose-response curve similar to the experiments described in Section VII.A. The kinetics of induction are dependent on the stability of the mRNA and protein. It may take some time before stably expressed proteins accumulate to equilibrium levels. Refer to the results seen in Figures 1B, 3, and 6.

IX. Development of Double-Stable Cell Lines *continued*

Loss of regulation: On occasion, well-characterized double-stable cell lines can lose their responsiveness to Tc or Dox. This can occur after changing lots of calf or fetal bovine serum and appears to be due to contamination of some lots of serum with Tc. If you observe a sudden loss of responsiveness, check your serum by performing a dose-response curve as described in Section VII.A. You can also try replating and washing the cells 3 hr later to remove any residual antibiotic that may be interfering with induction control (Rennel & Gerwins, 2002). Loss of regulation can also be due to switching off or methylation of the viral promoter. It is recommended that you subclone and freeze stocks of your cells at various stages.

Toxicity of the VP16 activation domain: Some researchers have inquired about the possible toxic effects of expressing the VP16 AD in mammalian cells. In our experience and that of the Bujard laboratory and the many other labs that have successfully used the Tet system, this has not been a problem in tissue culture. Like other transcription factors, the tTA regulator does not have to be expressed at high levels in order to give very high-level expression of the genes it regulates (i.e., genes encoded on the response plasmid). For example, Gossen and Bujard have characterized HeLa Tet-Off cell lines that contain 6,000–10,000 molecules of tTA per cell and give 10^5-fold induction of the Tet-regulated genes (pers. comm.). For *in vivo* applications, however, it may be preferable to use the VP16 Minimal Domain Vectors, which are tolerated at higher intracellular concentrations and allow activation over different ranges. See Appendix A for more information.

X. References

Clontech's Tet Systems were developed in cooperation with Dr. Bujard and his colleagues at the Center for Molecular Biology in Heidelberg (ZMBH). Additional background information on Tet-regulated gene expression systems is available at the site maintained by Dr. Bujard's laboratory:

http://www.zmbh.uni-heidelberg.de/bujard/homepage.html

Please note that Clontech is not responsible for the information on, or the maintenance of, this site.

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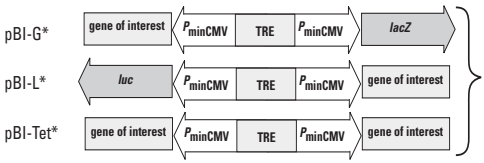
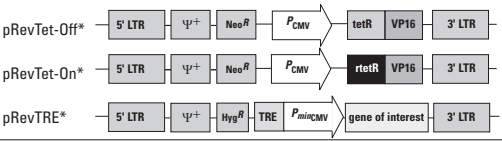
Appendix A: Vector Information

Table I. Tet-Off and Tet-On Vector alignment.

Name		Applications
Basic Vectors		
pTet-Off		Regulator vector for use in Tet-Off system
pTet-On		Regulator vector for use in Tet-On system
pTRE2		Response plasmids encoding the Tet Responsive Element (TRE) for use in either Tet-Off or Tet-On
pTRE2hyg/pur		
pTRE-Tight		Response plasmid encoding a modified Tet Responsive Element (TRE_{mod}) for use in either Tet-Off or Tet-On
Accessory Vectors		
pTet-tTS		For tighter control of gene expression in Tet-On Systems
ptTA-2, 3, 4*		Minimal domain vectors used in Tet-Off System; minimizes VP16 toxicity
pTRE-Tight-DsRed2*		Reporter vector containing a modified Tet Responsive Element (TRE_{mod}) for use in either Tet-Off or Tet-On
Tagged Vectors		
pTRE-Myc*		Response plasmids for use in either Tet-Off or Tet-On System
pTRE-HA*		Used for screening with antibodies or for purification
pTRE-6xHN*		
Bidirectional Tet Vectors		
pBI-G*		Response vectors for monitoring expression of a target gene via expression of a coregulated reporter
pBI-L*		
pBI-Tet*		
RevTet Basic Vectors		
pRevTet-Off*		Regulator vector for use in RevTet-Off System
pRevTet-On*		Regulator vector for use in RevTet-On System
pRevTRE*		Response vector for use in either RevTet-Off or RevTet-On Systems
RevTet Accessory Vectors		
pRevTet-Off-IN*		Can be used for quickly establishing a Tet-Off cell line

* Discontinued

TABLE II: TET SYSTEMS VECTOR INFORMATION

Name	Reference	Name in reference	Expressed protein	Mammalian selectable marker	Size (Kb)	Diagnostic restriction enzyme(s)	Fragment sizes (kb)
pTet-Off Vector	Resnitzky *et al.* (1994) Gossen & Bujard (1992)	pUHD15-1neo	tTA	neomycin	7.37	Xho I & Hind III	2.9, 2.2, 2.3
pTet-On Vector	Gossen *et al.* (1995)	pUHD17-1neo	rtTA	neomycin	7.37	Xho I & Hind III	2.9, 2.2 1.5, 0.9
pTet-tTS Vector	Freundlieb *et al.* (1999)	pUHS 6-1	tTS	none	4.3	EcoR I & Hind III	3.0, 1.3
pTRE2hyg Vector			protein X	hygromycin	5.3	Xho I BamH I	3.75, 1.55 5.3
pTRE2hyg-Luc (control provided with pTRE2hyg)			luciferase	hygromycin	6960	Xho I	5.4, 1.55
pTRE2pur Vector			protein X	puromycin	5.1	Xho I BamH I	5.4, 1.55 5.1
pTRE2pur-Luc (control provided with pTRE2pur)			luciferase	puromycin	6.73	Xho I BamH I	5.4, 1.55 4.5, 1.34
pTRE2 Vector			protein X	none	3.76	EcoR I	3.0, 0.7
pTRE2-Luc (control provided with pTRE2)			luciferase	none	5406	EcoR I	3.0, 1.7, 0.7
pTRE-Tight Vector			protein X	none	2.6	Xho I	2.0 & 0.6
pTRE-Tight-Luc (control provided with pTRE-Tight)				luciferase	none	4.2 Nhe I	BamH I & 2.6 & 1.6
pTRE-Myc Vector			Myc-protein X	none	3838	EcoR I	3.0, 0.8
pTRE-Myc-Luc (control provided with pTRE-Myc)			Myc-luciferase	none	5.5	BamH I Hind III	5.5 1.6, 3.8
pTRE2hyg2-Myc			Myc-protein X	hygromycin	5.4	Hind III EcoR V	3.6 & 1.8 5.4
pTRE2pur-Myc			Myc-protein X	puromycin	5.2	Hind III EcoR V	3.6 & 1.6 5.2

TABLE II: TET SYSTEMS VECTOR INFORMATION *continued*

Name	Reference	Name in reference	Expressed protein	Mammalian selectable marker	Size (Kb)	Diagnostic restriction enzyme(s)	Fragment sizes (kb)
pTRE-HA Vector			HA-protein X	none	3.83	BamH I Mlu I	3.8 not cut
pTRE2hyg2-HA Vector			HA-protein X	hygromycin	5.4	Hind III EcoR V	3.6 & 1.8 5.4
pTRE2pur-HA Vector			HA-protein X	puromycin	5.2	Hind III EcoR V	3.6 & 1.6 5.2
pTRE-HA-Luc (control provided with pTRE-HA)			HA-luciferase	none	5489	BamH I Hind III	5.6 3.8, 1.8
pTRE-6xHN Vector			6xHN-protein X	none	3.8	BamH I EcoR I	3.8 3.0, 0.8
pTRE2hyg2-6xHN Vector			6xHN-protein X	hygromycin	5.4	Hind III EcoR V	3.6 & 1.8 5.4
pTRE2pur-6xHN Vector			6xHN-protein X	puromycin	5.2	Hind III EcoR V	3.6 & 1.6 5.2
pTRE-6xHN-Luc (control provided with pTRE-6xHN)			6xHN-luciferase	none	5.5	Hind III BamH I	3.8, 1.8 5.6
pTRE-Tight-DsRed2 Vector			DsRed2	none	3.3	Not I BamH I	2.6, 0.7
pBI Vector	Baron *et al.* (1995)	pBI-4	protein X, protein Y	none	4.36	Xba I	3.7, 0.7
pBI-G VEctor	Baron *et al.* (1995)	pBI-3	β-gal, protein X	none	7.73	Xba I	3.7, 3.5, 0.6
pBI-L Vector	Baron *et al.* (1995)	pBI-2	luciferase, protein X	none	6.08	Xba I	3.7, 2.4
pBI-GL (control provided with pBI, pBI-G, and pBI-L)	Baron *et al.* (1995)	pBI-1	β-gal, luciferase	none	9.5	Xba I	3.7, 3.5, 2.3
pTK-Hyg Vector			none	hygromycin	5.07	EcoR I	0.4, 1.0, 1.3, 2.3

Appendix A: Vector Information *continued*

You can obtain the sequences of the Tet-Off and Tet-On vectors at **www.clontech.com/manuals**.

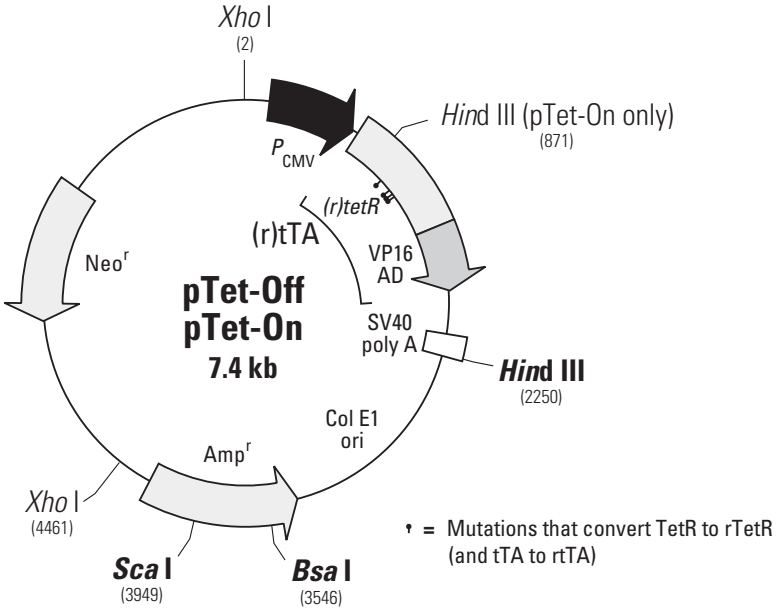


Figure 9. pTet-Off and pTet-On composite vector map. Unique sites are in bold. Only pTet-On contains the second *Hin*d III site at Position No. 871. This site can be used to distinguish pTet-Off from pTet-On. pTet-Off expresses the tTA (tet transactivator) regulator protein from the strong immediate early promoter of cytomegalovirus (P_{CMV}). pTet-On expresses the rtTA (reverse tTA), which contains four amino-acid mutations (as marked on the map). In addition, there are several silent mutations in pTet-On. In all other respects, the vectors are identical.

Appendix A: Vector Information *continued*

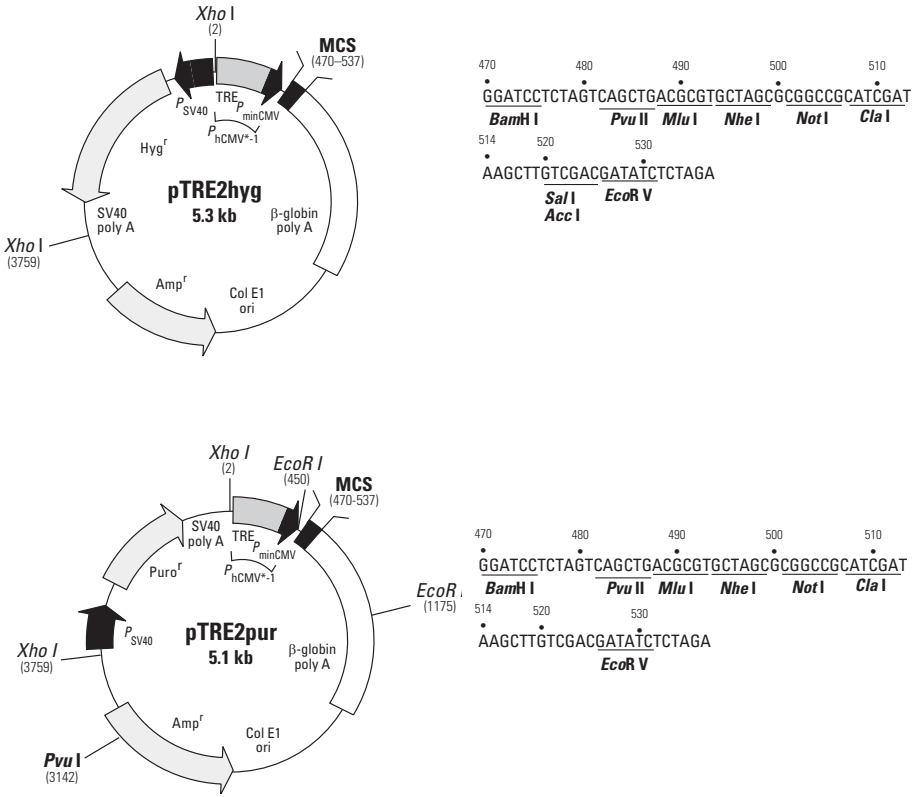


Figure 10. pTRE2hyg and pTRE2pur vector maps and MCSs. Both response vectors contain an MCS immediately downstream of the Tet-responsive $P_{hCMV*-1}$ promoter. $P_{hCMV*-1}$ contains the Tet response element (TRE), which consists of seven copies of a sequence containing the 19-bp tet operator sequence (*tetO*), and the minimal CMV promoter (P_{minCMV}), which lacks the enhancer that is part of the complete CMV promoter in the regulatory plasmids. Consequently, $P_{hCMV*-1}$ is silent in the absence of binding of TetR or rTetR to the *tetO* sequences. Genes inserted into one of the sites in the MCS will be responsive to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems, respectively. Note that the cloned insert must have an initiating ATG codon. The addition of a Kozak sequence is not required, but may improve expression levels. The addition of an internal selection element (Hyg^r or Puro^r) eliminates the need for cotransfection with pTK-Hyg. Complete sequence information is provided in the pTRE2hyg and pTRE2pur Vector Information Packets (pTRE2hyg: PT3521-5; pTRE2pur: PT3520-5).

pTRE2hyg-Luc and pTRE2pur-Luc contain the gene encoding firefly luciferase cloned into the *Bam*H I and *Nhe* I sites in the pTRE2hyg and pTRE2pur MCS. The *Nhe* I sites were destroyed during construction. The luciferase construct adds 1,649 bp to the vectors.

Appendix A: Vector Information *continued*

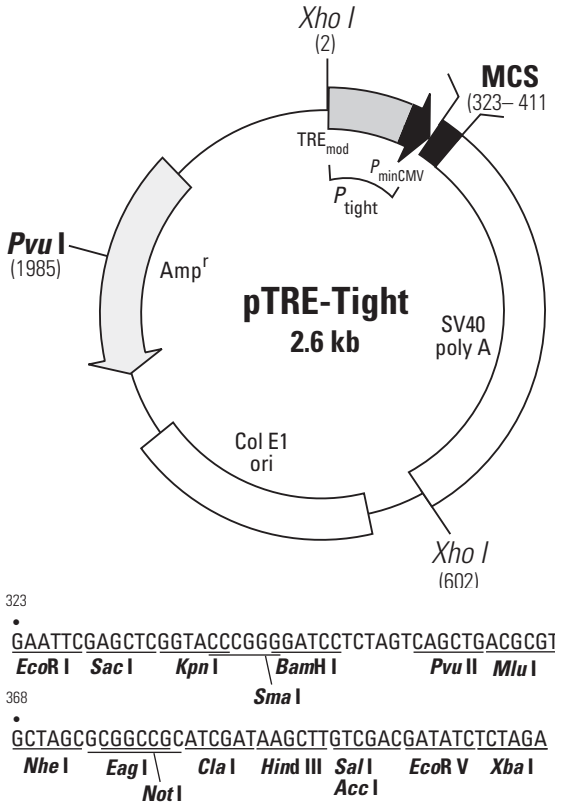
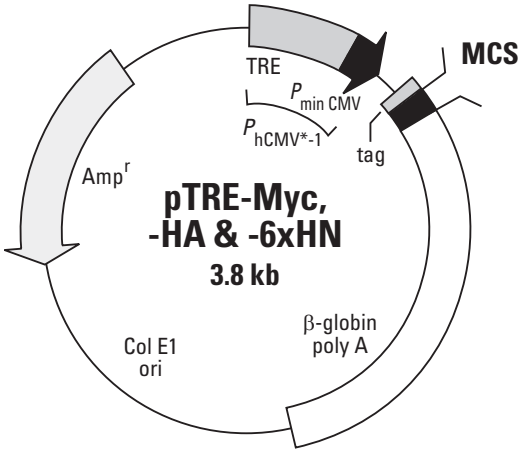


Figure 11. pTRE-Tight vector map and MCS. This response plasmid contains an MCS immediately downstream of the Tet-responsive P_{tight} promoter. P_{tight} contains a modified Tet response element (TRE_{mod}), which consists of seven direct repeats of a 36-bp sequence that contains the 19-bp tet operator sequence (*tetO*) and the minimal CMV promoter ($P_{minCMV\Delta}$), which lacks the enhancer that is part of the complete CMV promoter. Consequently, P_{tight} is silent in the absence of binding of TetR or rTetR to the *tetO* sequences. Genes inserted into the MCS will be responsive to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems, respectively. Note that the cloned insert must have an initiating ATG codon. The addition of a Kozak sequence is not required, but may improve expression levels. pTRE-Tight-Gene X plasmids should be cotransfected with the Linear Hygromycin Marker (Cat. No. 631625, not included) or Linear Puromycin Marker (Cat. No. 631626, not included) to permit selection of stable transfectants. Complete sequence information is provided in the pTRE-Tight Vector Information Packet (PT3720-5).

The **pTRE-Tight-Luc** Control Vector, packaged with the pTRE-Tight Vector, contains an additional 1,649 bp encoding firefly luciferase inserted into the MCS. This vector can be used as a reporter of induction efficiency. It is not intended as a cloning vector.

pTRE-Tight-DsRed2 (discontinued) contains the gene encoding DsRed2 cloned into the *Bam*H I and *Not* I sites in the pTRE-Tight MCS. DsRed2 is a variant of the red fluorescent protein isolated from the IndoPacific sea anemone relative *Discosoma sp.*

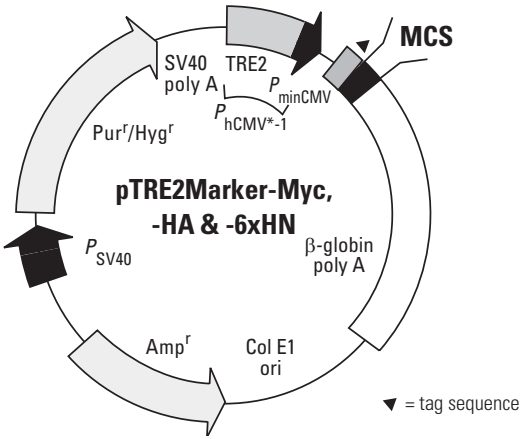
Appendix A: Vector Information *continued*



323
•
GAATTCGAGCTCGGTACCCGGGGATCCTCTAGTCAGCTGACGCGT
*Eco*R I *Sac* I *Kpn* I *Bam*H I *Pvu* II *Mlu* I
368 *Sma* I
•
GCTAGCGCGGCCGCATCGATAAGCTTGTCGACGATATCTCTAGA
Nhe I *Eag* I *Cla* I *Hin*d III *Sal* I *Eco*R V *Xba* I
Not I *Acc* I

Figure 12. pTRE-Myc, -HA and -6xHN composite vector map and multiple clone site (MCS). These tagged pTRE vectors contain an MCS immediately downstream of the Tet-responsive $P_{hCMV*-1}$ promoter. $P_{hCMV*-1}$ contains the Tet response element (TRE), which consists of seven copies of a sequence containing the 19-bp tet operator sequence (*tetO*), and the minimal CMV promoter (P_{minCMV}), which lacks the enhancer that is part of the complete CMV promoter in the regulatory plasmids. Consequently, $P_{hCMV*-1}$ is silent in the absence of binding of TetR or rTetR to the *tetO* sequences. Genes inserted into one of the sites in the MCS will be responsive to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems, respectively. Note that the cloned insert must be in frame with the tag and need not have an ATG or Kozak sequence, as these are provided at the start of the tag. The tagged fusion protein can be efficiently detected and purified using antibodies and resins optimized against the different markers. Complete sequence information is provided in the pTRE-Myc, -HA, and -6xHN Vector Information Packets (pTRE-Myc: PT3398-5; pTRE-HA: PT3462-5; pTRE-6xHN: PT3463-5). Please note that these vectors have been discontinued.

Appendix A: Vector Information *continued*



504

c-Myc, HA or 6xHN epitope tag

ATG XXX XXX XXX XXX XXX XXX XXX XXX XXX XXX XXX XXX XXX CTT ATG GCC ACT

GAC GCG TTG CTA GCG CAG CTG GAA GCT TAT CGA TTG CGG CCG CGT CGA CGA TAT C

***Mlu* I** ***Nhe* I** ***Cla* I** ***Not* I** ***Eco*R V**

Figure 13. pTRE2hyg2-Myc, -HA & -6xHN and pTRE2pur-Myc, -HA & -6xHN composite vector map and multiple clone site (MCS). These tagged pTRE vectors contain a protein tag sequence followed by an MCS immediately downstream of the Tet-responsive $P_{hCMV*-1}$ promoter. $P_{hCMV*-1}$ contains the Tet response element (TRE), which consists of seven copies of a sequence containing the 19-bp tet operator sequence (*tetO*), and the minimal CMV promoter (P_{minCMV}), which lacks the enhancer that is part of the complete CMV promoter in the regulatory plasmids. Consequently, $P_{hCMV*-1}$ is silent in the absence of binding of tTA or rtTA to the *tetO* sequences. Genes inserted into one of the sites in the MCS will be responsive to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems, respectively. The tagged fusion protein can be efficiently detected and purified using antibodies and resins optimized against the different markers. Complete sequence information is provided in the Vector Information Packets (pTRE2hyg2-Myc: PT3685-5; pTRE2hyg2-HA: PT3684-5; pTRE2hyg2-6xHN: PT3686-5; pTRE2pur-Myc: PT3688-5; pTRE2pur-HA: PT3687-5; pTRE2pur-6xHN: PT3689-5). Please note that these vectors have been discontinued.

Appendix A: Vector Information *continued*

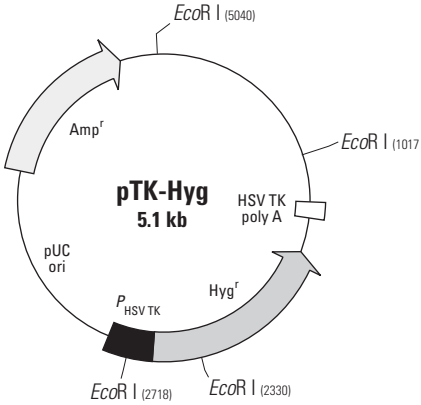


Figure 14. pTK-Hyg plasmid map. pTK-Hyg is cotransfected with pTRE-derived plasmids (but **not** with **pTRE2hyg** and **pTRE2pur** vectors) to allow selection of stably transformed cell lines in the presence of hygromycin. The absence of an enhancer element on pTK-Hyg prevents the unwanted activation of pTRE-derived plasmids upon cointegration into the genome. The sequence of pTK-Hyg has been deposited in GenBank (Accession No. U40398).

Appendix A: Vector Information *continued*

Bidirectional (pBI) Tet Vectors

The Bidirectional Tet Vectors are used to simultaneously express two genes under control of a single TRE (Baron *et al.*, 1995; for more information, see *Clontechniques*, October 1996, p. 8). After a Tet-Off or Tet-On cell line is established, a pBI vector is cotransfected with pTK-Hyg to permit selection of a double-stable tet-responsive cell line that coexpresses two genes.

pBI-G, and pBI-L can be used to indirectly monitor expression of a gene of interest for which there is no direct or convenient assay. These vectors express β-galactosidase, or luciferase as the reporter gene located on one side of the TRE. Gene X can be expressed at the same time as the reporter when cloned into the MCS flanking the other side of the TRE. When screening double-stable cell lines (Section IX.D), you can monitor expression of the reporter from the vector that also simultaneously expresses the gene of interest. Expression levels of the gene of interest can be inferred from reporter gene expression in response to Tc or Dox.

The pBI Vector lacks reporter sequences and instead contains two separate MCSs in opposite orientation driven by two identical, inducible promoters. pBI allows for coexpression of two genes of interest in the same cell. For instance, the interaction of two proteins or two subunits of a complex protein can be investigated by simultaneous expression in pBI.

Visit **www.clontech.com** for complete vector information. Please note that while these specific vectors have been discontinued, we do offer other Bidirectional Tet Vectors.

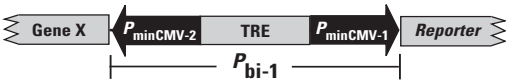


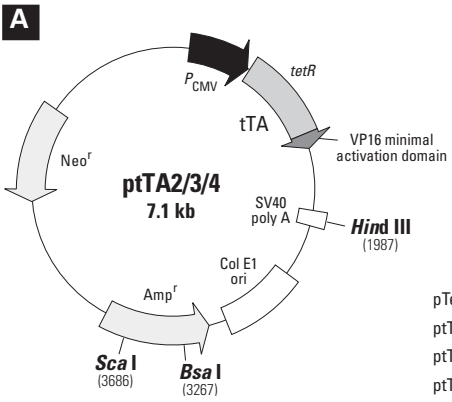
Figure 15. The pBI expression cassette. Two genes—either two genes of interest, a gene of interest and a reporter, or two reporters—can be expressed simultaneously from the P_{bi} promoter. Reporters are firefly luciferase, or β-galactosidase.

Appendix A: Vector Information *continued*

VP16 Minimal Domain Vectors

The VP16 Minimal Domain Vector Set (Cat. No. 631019, now discontinued)—ptTA2, ptTA3, and ptTA4—expresses tetracycline-controlled transactivators containing modified VP16 activation domains (Figure 17; Baron *et al.*, 1997). Overexpression of unmodified VP16 can have negative pleiotropic effects due to interactions with essential components of the transcriptional machinery. This generally does not interfere with *in vitro* expression, but can pose problems *in vivo* when tTA transcription is driven by a strong tissue-specific promoter.

The modified VP16 moieties contained in these transactivators allows their expression at higher intracellular levels, potentially allowing increased stability for cell culture and transgenic applications (Baron *et al.,* 1997). Furthermore, each vector allows protein expression over a different induction range (Panel B). Applications such as knock-in/knock-out experiments rely on site-specific integration and thus are dependent on the transcriptional activity of the particular locus. In these situations, the VP16 Minimal Domain Vectors may enable you to obtain optimal expression levels by adapting the activation potential of the transactivator to the expression level of the locus.



B

	VP16 activation domain	Tolerated level of activator	Relative transient activation (%)	Stable regulatior factor
pTet-Off	F	1X	100	2.2×10^5
ptTA2	F F F	3X	98	nd
ptTA3	F F	5X	39	1.5×10^5
ptTA4	G F Y	9X	14	4.4×10^4

Figure 16. VP16 Minimal Domain Vectors. The three vectors differ in the sequence of their VP16 activation domains. The letters in the first column of Panel B indicate the amino acid at the key functional position of a 13 amino acid repeat that composes the minimal domains. The rest of the vector is identical to pTet-Off. The activation domain from each vector is tolerated at different levels and causes activation at different levels relative to pTet-Off (Panel B). nd = not determined.

Appendix A: Vector Information *continued*

The pTet-tTS Vector

The pTet-tTS Vector (Cat. No. 631011) is designed for use with the Tet-On System. It is not suitable for use with the Tet-Off System. pTet-tTS prevents unregulated gene expression in the absence of Dox. It expresses the tetracycline-controlled transcriptional silencer (tTS), which is a fusion of TetR and the KRAB-AB domain of the Kid-1 protein (Freundlieb *et al.*, 1999; Witzgall *et al.* , 1994). In the absence of Dox, tTS binds the *tetO* sequence in the TRE and actively silences transcription of Gene X (Figure 18). As Dox is added to the culture medium, the tTS dissociates from the TRE, relieving transcriptional suppression. At sufficient concentrations of Dox, the rtTA transactivator binds the TRE and activates transcription of Gene X.

For additional information on pTet-tTS, including a vector map, please refer to the pTet-tTS Vector Information Packet (PT3334-5), available at **www.clontech.com/manuals.**

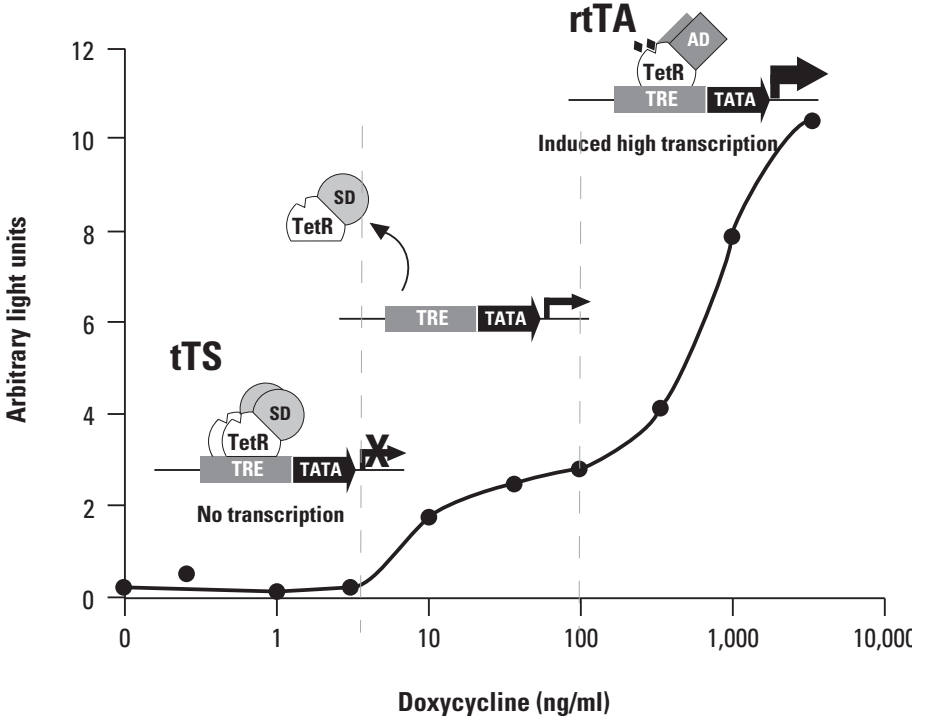


Figure 17. Dose response curve demonstrating controlled expression in a cell line coexpressing tTS and rtTA. HR5 cells, which constitutively express rtTA, were transiently transfected with a plasmid expressing tTS and a control vector expressing luciferase downstream of the TRE. Cells were cultured in the indicated levels of Dox. After 24 hr, cells were harvested and assayed for luciferase activity. SD = silencing domain. AD = activation domain. Data provided courtesy of S. Freundlieb, Zentrum für Molekulare Biologie (ZMBH), Universität Heidelberg.

Appendix B: Glossary

Dox	Doxycycline, a derivative of Tc that is the preferred effector substance for Tet experiments.
Double-stable Tet Cell Line	A Tet-Off or Tet-On cell line that has been stably transfected with pTRE2-Gene X construct. Gene X is induced by the removal (for Tet-Off) or addition (for Tet-On) of Dox from the media.
Gene X	The gene of interest, cloned into the Response Plasmid.
P_{CMV}	The complete immediate early promoter of cytomegalovirus. This is a proven strong promoter in many mammalian cell types.
P_{minCMV}	The minimal immediate early CMV promoter. This promoter lacks the strong CMV enhancer, and is therefore silent in the absence of binding of tTA or rtTA to the TRE.
$P_{minCMV\Delta}$	An altered minimal immediate early CMV promoter. This promoter is used in the pTRE-Tight vector series.
$P_{hCMV*-1}$	The compound promoter in pTRE and related vectors that consists of the TRE element located just upstream of $P_{minCMV.}$
P_{tight}	The compound promoter in the pTRE-Tight vectors that consists of the TRE_{mod} element located just upstream of $P_{minCMV\Delta.}$
Regulator Plasmid	The plasmid that encodes the hybrid regulatory protein (tTA or rtTA) in a Tet-Off or Tet-On System – i.e., pTet-Off or pTet.
Response Plasmid	A pTRE-derived plasmid that expresses a gene of interest from the $P_{hCMV*-1}$ promoter. A pTRE-derived plasmid can be used in both Tet-Off and Tet-On systems.
rTetR	The reverse Tet repressor. In *E. coli*, rTetR binds specifically to *tetO* and blocks transcription of the *tet* operon in the presence of Tc.
rtTA	Reverse tetracycline-controlled transactivator: A 37 kDa fusion protein consisting of the rTetR and the VP16 activation domain (AD). Binds specifically to TRE and activates transcription in the presence of Dox.
Tc	The chemical compound tetracycline
Tet	Tetracycline, as in the tet operon or the Tet repressor. (The compound tetracycline is abbreviated Tc.)

Appendix B: Glossary *continued*

Tet-Off Cell Lines	Any cell line that stably expresses tTA from integrated copies of pTet-Off. Tet-Off cell lines can either be made by the researcher or purchased from Clontech.
Tet-On Cell Lines	Any cell line that stably expresses rtTA from integrated copies of pTet-On. Tet-On cell lines can either be made by the researcher or purchased from Clontech.
tetO	The tet operator, a 19-bp, cis-acting regulatory DNA sequence from the bacterial *tet* operon, where it is the natural binding site for TetR. See TRE.
TetR	The Tet repressor component of tTA and rtTA. In *E. coli*, TetR binds specifically to *tetO* and blocks transcription of the tet operon in the absence of Tc.
TRE	Tet-Response Element. A regulatory sequence consisting of seven direct repeats of a 42-bp sequence that contains the *tetO*.
TRE_{mod}	Modified Tet-Response Element. A regulatory sequence consisting of seven direct repeats of a 36-bp sequence that contains the *tetO*.
tTA	Tetracycline-controlled transactivator: A 37 kDa fusion protein consisting of the TetR and the VP16 activation domain (AD). Binds specifically to the TRE and activates transcription in the absence of Tc or Dox.
tTS	Tetracycline-controlled transcriptional silencer, a fusion protein consisting of the TetR and the KRAB-AB domain of Kid-1. Binds specifically to the TRE and suppresses transcription in the absence of Dox.
VP16 AD	The activation domain of the VP16 protein from herpes simplex virus.