Tet-Off® Advanced Inducible Gene Expression System User Manual

PT3945-1 (PR6Y2133)
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I. Introduction

A. Summary

The Tet-Off Advanced Inducible Gene Expression System gives researchers ready access to the tightly regulated, high-level gene expression system described by Gossen, *et al.* (1992) with modifications made by Urlinger, *et al.* (2000; *Clontechniques,* January 2007). In the Tet-Off Advanced System, the basal state is maintained by the presence of doxycycline (Dox, a tetracycline derivative) in the culture medium, while induction is activated by removing Dox (Figure 1). The "On/Off" performance of the Tet-Off Advanced System permits gene expression to be tightly regulated in response to varying concentrations of Dox. Maximal expression levels in the Tet-Off Advanced System, in the absence of Dox, are very high and compare favorably to 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 results are not complicated by pleiotropic effects or nonspecific induction.

B. The Tet-Off Advanced System

In *E. coli*, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon on the Tn10 transposon. TetR blocks transcription of these genes by binding to the *tet* operator sequences (*tet*O) in the absence of tetracycline (Tc). In the presence of Tc, TetR dissociates from *tet*O and transcription of resistance-mediating genes begins. Together, TetR and *tet*O provide the basis of regulation and induction for both the Tet-Off Advanced and Tet-On Advanced Systems used in mammalian cells: the TetR protein has been converted into a transcriptional activator, and *tet*O sequences have been used to create an inducible promoter. The central concepts of each system are shown in Figure 1 and discussed below to provide a brief overview.

The Tet-Off Advanced Transactivator: tTA-Advanced. One key component of the improved Tet-Off Advanced System is a transcription-activating fusion protein, tTA-Advanced (Urlinger, *et al.,* 2000). This tetracycline-controlled transactivator consists of TetR, including its DNA binding domain (BD), fused to three minimal "F"-type activation domains (AD) derived from herpes simplex virus VP16 (Baron *et al.,* 1997, Triezenberg *et al.*, 1988). These 3 minimal ADs provide the same activation as the full-length VP16 AD, yet reduce the potential for cytotoxicity. The gene for tTA-Advanced is also fully synthetic and utilizes human codon preferences to increase the expression and stability of the protein in mammalian cells, and it lacks cryptic splice sites in its mRNA sequence. Expression of tTA-Advanced is typically high enough to be detected easilyon a Western blot using the **TetR Monoclonal Antibody**. Like TetR, tTA-Advanced is unable to bind *tet*O sequences in the presence of Tc or Dox.

The Regulator Plasmid: pTRE-Tight. The second key component of the Tet-Off Advanced System is the pTRE-Tight plasmid which is used to control expression of your gene of interest (GOI) (*Clontechniques,* April 2003). The TRE-Tight composite promoter sequence was originally developed as the $P_{tet\text{-}14}$ promoter in the laboratory of Dr. H. Bujard. It consists of a modified Tet-Responsive Element (TRE_{mod}), made up of seven direct repeats of an altered *tet*O sequence, joined to a modified minimal CMV promoter ($P_{minCMV\Delta}$). TRE-Tight also lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction, thus preventing basal expression of your downstream GOI. This is especially useful in cases where residual background expression is unacceptable, as in the study of toxic or apoptotic proteins.

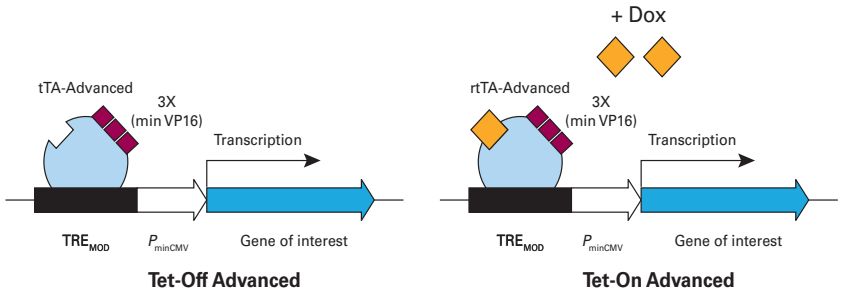

Figure 1. Gene induction in the Tet-Off Advanced and Tet-On Advanced Systems. The Tet-controlled transactivators for these systems (tTA-Advanced and rtTA-Advanced) are fusion proteins derived from a wild-type or mutant Tet repressor (TetR), respectively. Each DNA-binding TetR domain is joined to three minimal transcription activation domains of the herpes simplex virus VP16 protein, and each has been optimized for expression in mammalian cells. For Tet-Off Advanced, the uninduced basal state is maintained in the presence of doxycycline, which prevents the tTA-Advanced protein from binding to TRE_{mod} sequences in pTRE-Tight. Removal of doxycycline permits tight binding and induces high-level transcription. In exact contrast, the Tet-On Advanced System is activated by doxycycline

I. Introduction continued

C. Tet-Off Advanced Stable Cell Lines

The ultimate goal in setting up a complete Tet-Off Advanced Inducible Gene Expression System is to create a double-stable Tet-Off Advanced cell line which contains integrated copies of both the regulatory (pTet-Off Advanced) and response (pTRE-Tight-GOI) plasmids. In such a cell line, the GOI will be expressed only in the absence of Dox when the tTA-Advanced protein binds to the TRE_{mod} sequences in TRE-Tight (Figure 1).

D. Advantages of the Advanced Tet Expression Systems

The combined major improvements of the Tet-Off Advanced and Tet-On Advanced Systems yield robust induction, extremely low basal expression, and facilitate the process of developing stable host cell lines. These systems have numerous advantages over other inducible mammalian gene expression systems. See Appendix A, the Vector Information Packets provided, and our website **www.clontech.com** for maps and detailed information on these and other Tet System Vectors.

- **Extremely tight regulation.** In the absence of induction, the Tet-Off Advanced transactivator has no residual binding to the TRE in TRE-Tight, thus basal expression is virtually nonexistent and generally undetectable.
- **No pleiotropic effects.** When introduced into mammalian cells, the prokaryotic DNA BD of the Advanced acts very specifically on the *tet*O target sequences of TRE-Tight. The likely reason for this is that eukaryotic genomes lack these prokaryotic regulatory DNA sequences (Harkin, *et al.,* 1999).
- **High inducibility and fast response times.** Induction is often several thousand-fold and can be detected within 30 minutes after withdrawing Dox from the culture medium. In contrast, other mammalian systems often exhibit slow induction (up to several days), incomplete induction (compared to repressor-free controls), and low overall induction (often no more than 100-fold). Other systems may also require high, nearly cytotoxic levels of inducer (reviewed by Gossen, *et al.,* 1993; Yarronton, 1992).
- **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 inducers used in other systems, such as in the ecdysone system, Tc and Dox are inexpensive, well-characterized, and yield highly reproducible results. We recommend using Dox due to its longer half-life and increased potency, relative to Tc.
- **Promoter activation, rather than repression.** To completely shut off transcription, repression-based systems require very high levels of repressor to ensure 100% occupancy of the regulatory sites. The presence of high repressor levels also 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).
- **The Tet-On and Tet-Off expression systems are superlative control systems for transgenic mice.** The Tet System has become the *de facto* method of choice for generating inducible transgenic lines (Gossen & Bujard, 2002). No other inducible system has proved as successful. Indeed, more than 80 mouse lines have been described expressing the tTA/rtTA genes under the control of a variety of tissue-specific promoters, and approximately 100 mouse lines have been described expressing various target genes under control of Tet-inducible promoters. A list of these mouse lines can be found on the TET Systems website (http://www.tetsystems.com/main_transgenic.htm). With its greatly increased sensitivity to Dox, the Tet-On Advanced System (Urlinger, *et al.*, 2000) brings additional advantages to researchers wishing to develop inducible transgenic mice; particularly when control of gene expression in the brain is required, because the presence of the blood-brain barrier limits the concentration of Dox present in the brain.

E. Tetracycline vs. Doxycycline

The Tet-Off Advanced System responds equally well to either Tc or Dox. The concentrations used are far below cytotoxic levels for either cell culture or transgenic studies, however the half-life of Dox (24 hr) is longer than Tc (12 hr). Of note, the Tet-On Advanced System responds only to Dox, and not to Tc (Gossen & Bujard, 1995).

I. Introduction continued

F. Additional Tet Response Vectors

The system is supplied with a luciferase reporter vector, pTRE-Tight-Luc, that can be used to screen the Tet-Off Advanced clones. Other reporter formats are also available. The pTRE-Tight-DsRed2 (Cat. No. 631061), pTRE-Tight-AcGFP1 (Cat. No. 631063), and pTRE-Tight-ZsGreen1 (Cat. No. 631062) Vectors offer regulated expression of our Living Colors® fluorescent proteins.

Additionally, several TRE vectors (not based on TRE-Tight) are available for expressing your GOI fused to a peptide tag to aid detection or purification of the induced protein. These vectors provide a way to screen colonies directly for protein expression by Western analysis using readily available antibodies, and are available with or without a mammalian selection marker. See Appendix A for additional information on these vectors.

- The pTRE-Myc (Cat. No. 631010), pTRE2hyg2-Myc (Cat. No. 631052), and pTRE2pur-Myc (Cat. No. 631055) Vectors encode a c-Myc tag, which is incorporated at the N-terminus of the expressed protein.
- The pTRE-HA (Cat. No. 631012), pTRE2hyg2-HA (Cat. No. 631051), and pTRE2pur-HA (Cat. No. 631054) Vectors encode an HA (hemagglutinin) epitope tag at the N-terminus of the expressed protein, allowing detection of the protein with anti-HA antibodies.
- The pTRE-6xHN (Cat. No. 631009), pTRE2hyg2-6xHN (Cat. No. 631053), and pTRE2pur-6xHN (Cat. No. 631056) Vectors express proteins that are fused with six His-Asn repeats (6xHN) and allow easy purification of your protein using TALON® Resin or any other immobilized metal affinity column.

G. Bidirectional Tet Expression Vectors

Bidirectional Tet Vectors are specially designed response vectors that allow coregulated expression of two genes from a single TRE (Baron, U., *et al.,* 1995). These are ideal TRE response vectors if a functional assay does not exist for the GOI since expression can be monitored indirectly through detection of the coregulated reporter gene.

- The pTRE-Tight-BI Vector (Cat. No. 631068) lacks a reporter, while the pTRE-Tight-BI-AcGFP1 (Cat. No. 631066), pTRE-Tight-BI-DsRed2 (Cat. No. 631064), pTRE-Tight-BI-DsRed-Express (Cat. No. 631065), and pTRE-Tight-BI-ZsGreen1 (Cat. No. 631067) Vectors express a Living Colors fluorescent protein.
- Bidirectional TRE vectors are also available with β–galactosidase (pBI-G Tet, Cat. No. 631004) or luciferase (pBI-L Tet, Cat. No. 631005).

Nuclear Localization Signals

Adding a nuclear localization sequence (NLS) to tTA-Advanced will alter the protein's function and is not recommended (M. Gossen & H. Bujard, personal communication). A NLS will increase maximum expression but will also elevate background expression due to altered binding affinity to the *tet*O sequences (unpublished observations).

II. List of System Components

Store frozen mammalian cell lines in liquid nitrogen (–196°C).

Store all plasmids and Fetal Bovine Serum at –20°C.

Tet-Off Advanced Inducible Gene Expression System (Cat. No. 630934)

- 20 µl pTet-Off Advanced Vector (0.5 µg/µl)
- 20 µl pTRE-Tight Vector (0.5 µg/µl)
- 20 µl pTRE-Tight-Luc Vector (0.5 µg/µl)
- 40 µl Linear hygromycin selection marker (0.05 µg/µl)
- 1 x 0.5 ml CHO-AA8-Luc Tet-Off Control Cell Line (1.0 x 10^6 cells/tube)
- 50 ml Tet System Approved FBS
- User Manual (PT3945-1)
- pTet-Off Advanced Vector Information Packet (PT3945-5)
- pTRE-Tight Vector Information Packet (PT3720-5)

Visit our Tet Systems product page **www.clontech.com** for a current list of cell lines and products available for the Tet Systems.

III. Additional Materials Required

A. Mammalian Cell Culture Supplies

- Alpha Minimal Essential Medium Eagle (Alpha-MEM) for the CHO-AA8-Luc Tet-Off Control Cell Line. The recommended culture medium for this cell line is: 90% Alpha-MEM, 10% Tet System Approved Fetal Bovine Serum, 4 mM L-glutamine, 100 µg/ml G418, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 100 µg/ml hygromycin B. The appropriate medium for growing Clontech's other premade Tet-Off, Tet-On, and Tet-On Advanced Cell Lines is described on the Certificate of Analysis provided with each cell line.

- Tc-Free fetal bovine serum (FBS). Many lots of bovine sera are contaminated with Tc or Tc-derivatives, and it is critical that the FBS used for cell culture not interfere with Tet-responsive expression. This problem can be eliminated by using a Tet System Approved FBS from Clontech (Cat. Nos. 631101 & 631106). These sera have been functionally tested in the Tet Systems and found to be free of contaminating Tc activity.
- L-glutamine, 200 mM (Sigma, Cat. No. G7513)
- Penicillin/Streptimycin solution of 10,000 units/ml penicillin G sodium and 10,000 µg/ml streptomycin sulfate (100X; Sigma, Cat. No. P0781)
- Trypsin-EDTA (Trypsin; Sigma, Cat. No. T3924)
- 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 many manufacturers
- Cloning cylinders or discs (PGC Scientific, Cat. No. 62-6150-40, -45 or Cat. No. 62-6151-12, -16)

B. Antibiotics for Clonal Selection

Prior to using these antibiotics, determine the optimal selection concentration for each one as described in Section VII.

- G418 is required for selection of your Tet-Off Advanced cell line and for maintenance of the CHO-AA8-Luc Control Cell Line. It is available in powdered form from Clontech (Cat. No. 631307). Make a 10 mg/ml stock solution by dissolving the appropriate amount in DMEM or alpha-MEM (without supplements). Filter sterilize and store at 4°C.
  - Recommended working concentration range: 50–800 µg/ml
  - Maintenance of stable cell lines: 100 µg/ml
  - Selection (e.g., HEK 293, HeLa or CHO cells): 400–500 µg/ml
- Hygromycin is required for selection of your double-stable Tet-Off Advanced/pTRE-Tight-GOI cell line and maintenance of the CHO-AA8-Luc Tet-Off Control Cell Line. Hygromycin B is available from Clontech (Cat. No. 631309).
  - Recommended working concentration range: 50–800 µg/ml
  - Maintenance of stable cell lines: 100 µg/ml
  - Selection (e.g., HEK 293, HeLa or CHO cells): 200 µg/ml

C. Transfection Reagents

- The CalPhos™ Mammalian Transfection Kit (Cat. No. 631312) or equivalent, for high-efficiency calcium-phosphate transfections.
- CLONfectin™ Transfection Reagent (Cat. No. 631301) or equivalent, for high-efficiency liposome-mediated transfections.

III. Additional Materials Required continued

D. Doxycycline

Doxycycline (Cat. No. 631311). Make a solution of 1–2 mg/ml in H_2O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.

E. Luciferase Assay

A method for assaying luciferase expression is required for use with the CHO-AA8-Luc Tet-Off Control Cell Line. Use any standard luciferase assay system for detecting firefly luciferase. For these assays, a luminometer is also required.

F. Primers for Sequencing the GOI Insert in pTRE-Tight

Following creation of your pTRE-Tight-GOI construct, the insertion junctions should be confirmed by sequencing. Specific primers for pTRE-Tight are:

- Forward primer: 5'–AGGCGTATCACGAGGCCCTTTCGT–3' (located at 2577–2600)
- Reverse primer: 5'–TATTACCGCCTTTGAGTGAGCTGA–3' (located at 683–660)

NOTE: The pTRE and pTRE2 Sequencing Primers sold by Clontech are not compatible with pTRE-Tight.

IV. Protocol Overview

An overview for creating a double-stable Tet-Off Advanced cell line that contains integrated copies of the regulatory vector and the TRE response vector is shown in Figure 2. For more detailed flow charts for each of the sequential transfection procedures, see Figure 4 (Section VIII) and Figure 5 (Section IX). When starting with a premade Tet-inducible Cell Line from Clontech, only the second transfection using a TRE- or TRE-Tight-GOI construct is required.

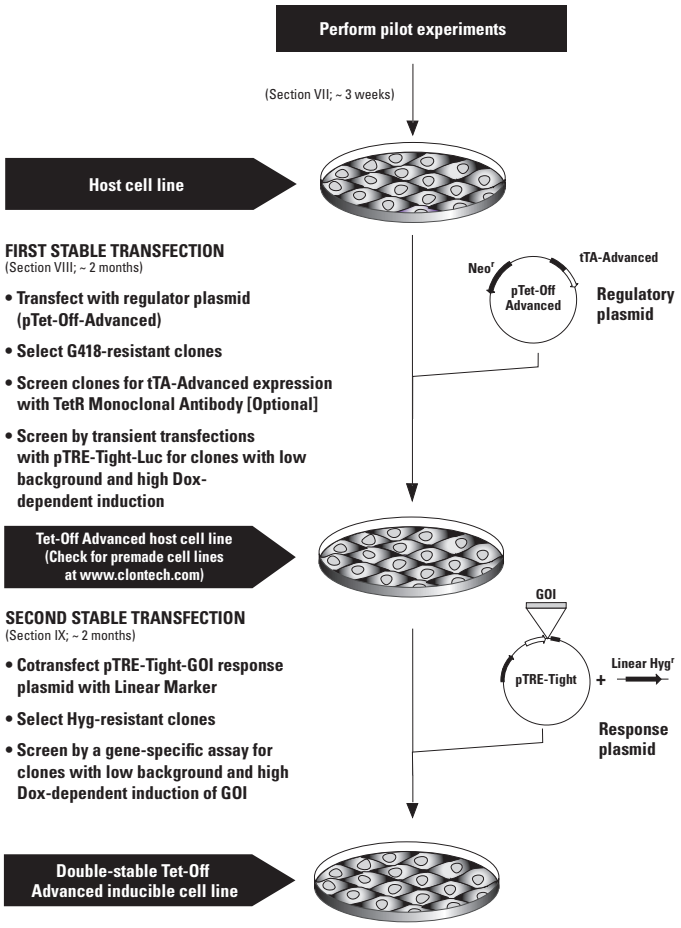

Figure 2. Overview of developing the stable cell lines of the Tet-Off Advanced System.

Cotransfection versus sequential transfections

For the following reasons, 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, so enhancer elements from the CMV promoter on the regulator plasmid (pTet-Off Advanced) can elevate uninduced basal expression of a nearby TRE-Tight-controlled GOI. To prevent plasmid cointegration, perform the transfections sequentially.
- Cotransfection prevents comparison of multiple clones. Differences in induction or absolute expression may arise due to clone-to-clone variation in tTA-Advanced expression, rather than from true differences in inducible TRE-Tight-dependent activity.
- A Tet-Off Advanced cell line is a versatile host that provides a proven genetic background in which to introduce other TRE-Tight-controlled constructs.

V. Plasmid Manipulations

A. Vector Propagation & Construction of the pTRE-Tight-GOI Plasmid

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. See the Vector Information Packet provided with each vector for further propagation details.
2. Using standard cloning techniques (Sambrook & Russell, 2001), insert your GOI into the pTRE-Tight Vector using the restriction sites contained in the multiple cloning site (MCS). You can also use an In-Fusion™ 2.0 CF Dry Down PCR Cloning Kit (Cat. No. 639607) which allows PCR products to be directly and directionally cloned into any linearized vector, without the need for restriction enzyme digestion, blunt-end polishing, or ligase.
3. Perform a midi- to large-scale plasmid DNA preparation for each plasmid that will be transfected into mammalian cells. To ensure that the purity of the DNA is transfection-grade, use a NucleoBond® or NucleoBond® Xtra Plasmid Midi or Maxi Kit. Visit **www.clontech.com** for complete Nucleobond product information.

VI. Cell Culture Guidelines & Protocols

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 users 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 Cell Lines

See the Certificate of Analysis (CofA) for information on each Tet Cell Line. Additional information for all the currently available Tet-Off, Tet-On, and Tet-On Advanced Cell Lines, including propagation information, is provided in the Tet Cell Lines Protocol-at-a-Glance (PT3001-2), available from our Tet Systems product page at **www.clontech.com.**

- **General cell culture conditions:** Premade Tet Cell Lines should be grown at 37°C in a humidified chamber with 5–10% CO_2. See the CofA for details particular to each cell line.
- **Relative growth rates:** The incubation times in this User Manual are provided for cells such as CHO or HeLa with relatively rapid doubling times. Other cell types will have different growth rates.
- **Selection in G418 and hygromycin:** Maintain stable and double-stable Tet Cell Lines in the appropriate selective medium; however, the concentration can be reduced from the higher levels normally used to select stably transfected clones to a lower maintenance concentration, typically 100 µg/ml for each drug.

VI. Cell Culture Guidelines & Protocols continued

Protocol
30 min
+ 2–3
days

C. Protocol: Starting Tet Cell Line Cultures from Frozen Stocks

The frozen aliquot of the CHO-AA8-Luc Tet-Off Control Cell Line should be thawed and cultured immediately upon receipt, or as soon as possible thereafter. Decreased cell viability may result if the culture is not initiated soon after receipt. To prevent osmotic shock and maximize cell survival, perform the following steps:

1. Thaw the vial of cells rapidly in a 37°C water bath with gentle agitation. Immediately upon thawing, wipe the outside of the vial with 70% ethanol. All of the operations from this point on should be carried out in a laminar flow tissue culture hood under strict aseptic conditions. Unscrew the top of the vial slowly and, using a pipet, transfer the contents of the vial to a 15 ml conical centrifuge tube containing 1 ml of prewarmed medium (without selective antibiotics, e.g. G418). Mix gently.
2. Slowly add an additional 4 ml of fresh, prewarmed medium to the tube and mix gently.
3. Add an additional 5 ml of prewarmed medium to the tube, mix gently. Centrifuge at 100 x g for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium lacking selective antibiotics and containing 100 ng/ml Dox to maintain luciferase expression in the "off" state. *(This method also removes the cryopreservative and can be beneficial when resuspending in small volumes. However, be sure to treat the cells gently to prevent damaging fragile cell membranes.)*
4. Mix the cell suspension thoroughly and add to a suitable culture vessel. 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% CO_2 as appropriate) for 24 hrs.

Note

Note: For HEK 293-based cell lines, we recommend using collagen-coated plates or flasks for efficient culturing of frozen stocks. Vessels coated with compounds other than collagen may also provide suitable growth substrates (e.g. poly-L-lysine), but only collagen has been tested at Clontech. Once recovered, the cells may be cultured directly on tissue culture plastic. However, if adherence is poor, we recommend using only collagen-coated vessels. Complete attachment of newly thawed HEK 293 cultures may require up to 48 hr.

5. The next day, examine the cells under a microscope. If the cells are well-attached, and confluent, they can be passaged for use. If the majority of cells are not well-attached, continue culturing for another 24 hr.
6. After 48–72 hr, add 100 µg/ml G418 and 100 µg/ml hygromycin. Expand the culture as needed.

Protocol
1 hr

D. Protocol: Preparing Frozen Stocks of Tet Cell Lines

Once you have started growing a Tet System cell line—either a premade one from Clontech or one of your own cell lines—prepare frozen aliquots to ensure a renewable source of cells.Use the freezing medium specified on the CofA provided with each Tet Cell Line.

1. Trypsinize the desired number of flasks or plates.
2. Pool cell suspensions together, count cells, and calculate total viable cell number.
3. Centrifuge cells at 100 x g for 5 min. Aspirate the supernatant.
4. Resuspend the pellet at a density of at least 1–2 x 10^6 cells/ml in freezing medium. Freezing medium can be purchased from Sigma (Cat. Nos. C6164 & C6039). Alternatively, freeze cells in 70–90% FBS/0–20% culture medium (without selective antibiotics), and 10% DMSO.
5. Dispense 1 ml aliquots into sterile cryovials.
6. Freeze slowly (1°C per min). For this purpose, you can place the vials in Nalgene cryo-containers (Nalgene Cat. No. 5100) and 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 and freeze overnight. Remove vials from the cryo-containers or styrofoam containers the following day, and place in liquid nitrogen storage or ultralow-temperature freezer (–150°C) for storage.
7. Two or more weeks later, plate a vial of frozen cells to confirm viability.

VII. Pilot Experiments

A. Luciferase Induction

Before you perform any other experiments, we strongly recommend that you perform an induction experiment using the CHO-AA8-Luc Tet-Off Cell Line provided with the Tet-Off Advanced Expression System. This double-stable Tet-Off Cell Line routinely exhibits a greater than 1,000-fold induction of luciferase when Dox is removed from the culture medium. This experiment provides a "hands-on" introduction to the Tet System and verifies that your culture system, induction conditions, and reagents are working properly.

B. Protocols: Luciferase induction in the CHO-AA8-Luc Tet-Off Control Cell Line

Protocol 2 days each

1. **Doxycycline Dose Response.** Full supression of gene expression in this Tet-Off cell line is generally obtained with 10–100 ng/ml Dox.

   a. After thawing and establishing the CHO-AA8-Luc Tet-Off Cell Line (Section VI), maintain it in the "off" state by including 100 ng/ml Dox in the culture medium. To ensure full induction, passage the cells using the following method:

      i. Wash the cells on the plate 2X with PBS before trypsinizing.

      ii. After trypsinizing and collecting the cells, wash them in suspension 1X with PBS. Plate 5 x 10^4 cells in a volume of 2–3 ml of complete culture medium without Dox into the wells of 6-well culture plates.

      iii. 3–6 hr after plating the cells, and after they have reattached to the substrate, wash them on the plate 1X with PBS and add fresh medium with or without Dox.

   b. Test Dox in duplicate or triplicate wells at final concentrations of 0, 1 x 10^{-3}, 1 x 10^{-2}, 0.1, 1.0, 10 and 100 ng/ml.

   c. After 48 hr of growth, assay each sample for luciferase activity using a standard luciferase assay. Plot your results and compare to Figure 3A.

2. **Serum (FBS) Compatability Testing.** *We recommend using Tet System Approved FBS (Cat. No. 631101 or Cat. No. 631106), which has been functionally tested and is guaranteed to support the full range of induction with all Tet System Cell Lines.* If alternative sources of FBS are used, they should be tested for Tc contamination. As shown in Figure 3B, different lots of FBS may prevent full induction in the CHO-AA8-Luc Cell Line, presumably due to residual tetracyclines present in the serum and used in the diet of cattle. This test should be repeated with each new or different lot of serum.

   – To perform the test, passage the cells and plate 5 x 10^4 cells in 6-well plates in a volume of 2–3 ml of complete culture medium containing either the Tet System Approved FBS or the sera to be tested, with and without 100 ng/ml Dox.

   – After 48 hr of growth, assay each sample for luciferase activity. Compare the maximum induction and fold induction values obtained from cells grown in media containing the Tet System Approved FBS to those obtained from cells grown in media containing the test sera. Compare your results to Figure 3B.

Attention

Ensuring Induction in the Tet-Off Advanced System

Residual Dox that remains bound to cells or the extracellular matrix can prevent full gene induction in Tet-Off Systems (Rennel & Gerwins, 2002). Cells that have been maintained in the "off" state with 10–100 ng/ml Dox should be passaged as follows:

- Wash the cells on the plate 2X with PBS before trypsinizing.
- After trypsinizing and collecting the cells, wash them in suspension 1X with PBS, and plate in fresh medium without Dox.
- 3-6 hr after plating the cells, and after they have reattached to the substrate, wash them on the plate 1X with PBS and add fresh medium with or without Dox.

VII. Pilot Experiments continued

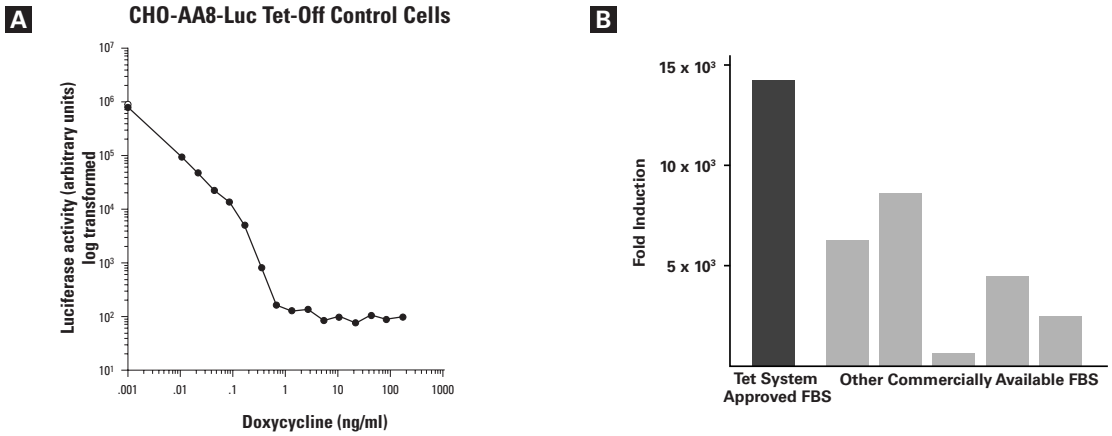

Figure 3. Luciferase induction in the CHO-AA8-Luc Control Cell Line under standard conditions and with different lots of FBS. Panel A. Dox dose-response curve for the CHO-AA8-Luc Tet-Off Control Cell Line. **Panel B.** The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different 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.

C. Titrating Antibiotics for Selection (Kill Curves)

Prior to using the antibiotics G418 and hygromycin (or puromycin) to establish your single-stable Tet-Off Advanced cell line and double-stable Tet-Off Advanced/TRE-Tight-GOI cell line, respectively, titrate these selection agents to determine the optimal concentration for each one in your target host cell line. Lot-to-lot variations in potency exist for these drugs, so each new lot of antibiotic should be titrated. Perform two experiments for each drug: (1) a titration to determine optimal drug concentration, and (2) a determination of the optimal plating density. These steps are recommended even for premade Tet Cell Lines.

Protocol 7–14 days

D. Protocol: Antibiotic Titrations at Fixed Cell Density.

For selecting stable transformants, use the lowest concentration that results in 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 about 1 µg/ml.

1. For each antibiotic to be tested, plate 2 x 10^5 cells in each of six 10 cm tissue culture dishes containing 10 ml of the appropriate complete medium plus increasing concentrations (0, 50, 100, 200, 400, and 800 µg/ml) of G418 or hygromycin. For puromycin, add the drug at 0, 1, 2.5, 5, 7.5, and 10 µg/ml.
2. Incubate the cells for 5–14 days, and examine the dishes for viable cells every two days. Replace the selective medium every four days (or more often if necessary), until the optimal concentration is determined.

Note

Note: Our HEK 293 Tet-On, Tet-Off, or Tet-On Advanced Cell Lines (Cat. Nos. 630903, 630908 & 630931, respectively), are especially sensitive to hygromycin. When creating a double-stable cell line from these cells, test a concentration range with a midpoint of 25 µg/ml. Saos-2 Tet-Off cells (Cat. No. 630911) are resistant to hygromycin; test a concentration range with a midpoint of 800 µg/ml.

VII. Pilot Experiments continued

Protocol 7–14 days

E. Protocol: Determining Optimal Plating Density.

Once you have determined the optimal concentrations for G418 and hygromycin selection, determine the best cell densities for each drug by plating cells at several different densities in the presence of the ideal drug concentration. When selecting stable transfectants, use a plating density that allows the cells to reach ~80% confluence before massive cell death begins (~5 days). If cells are plated too densely, they will become confluent before the selection takes effect. Optimal plating density will depend 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. For HeLa cells, we have found 2×10^5 cells/10 cm dish to be a good plating density.

1. For each antibiotic (G418 and hygromycin), 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): 5×10^6, 1×10^6, 5×10^5, 2×10^5, 1×10^5, and 5×10^4.
2. Incubate the cells for 5–14 days, and examine the dishes for viable cells every two days. Replace the selective medium every four days (or more often if necessary), until the optimal plating density is determined.
3. The density determined in this experiment will provide an estimate of the target density you will use for antibiotic selection after the transfections. In this case, selection should only begin 48–72 hr after replating the transfected cells (Section VIII.A, steps 2 & 3; and Section IX.B, steps 2 & 3).

F. Testing Host Cells by Transient Cotransfection with pTet-Off-Advanced and pTRE-Tight-Luc

Performing a transient expression assay with pTet-Off-Advanced and pTRE-Tight-Luc provides a quick functional test of the Tet-Off Advanced System in your particular cell line. This test is not necessary if you have purchased a premade Tet Cell Line. Transfect cells using different ratios of pTet-Off-Advanced and pTRE-Tight-Luc. For example, try the following ratios:

pTet-Off-Advanced : pTRE-Tight-Luc

1. 1 : 1
2. 1 : 5
3. 5 : 1

Note

NOTE: Fold-induction levels are generally 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 indistinguishable from untransfected controls. Therefore, an apparent low level of induction response in the transient assay should not be the sole reason for not using a particular cell line.

VIII. Developing a Stable Tet-Off Advanced Cell Line

The protocols in this section describe how to develop a Tet-Off Advanced cell line. The protocols should be optimized for each cell type by adjusting parameters such as plating densities, transfection method, G418 concentrations used for selection, and incubation and growing times. For an overview of this protocol, see the flow chart in Figure 4.

- Regardless of the cell type and transfection method, the goal is to generate a cell line that produces only very low basal expression and high induction of luciferase activity when tested by transient transfection with pTRE-Tight-Luc (Section C). The site of plasmid integration may profoundly affect the ultimate level of expression.
- For these reasons, we recommend that as many clones as possible be isolated in Section A, Step 7. Some clones may not survive isolation and expansion. **In general, isolate enough colonies in order to test at least 30 clones**. Though it is possible that an optimal clone may be found by screening fewer colonies, an unsuccessful screen will cause significant delays.

ATTENTION: Do not work with a mixed population.

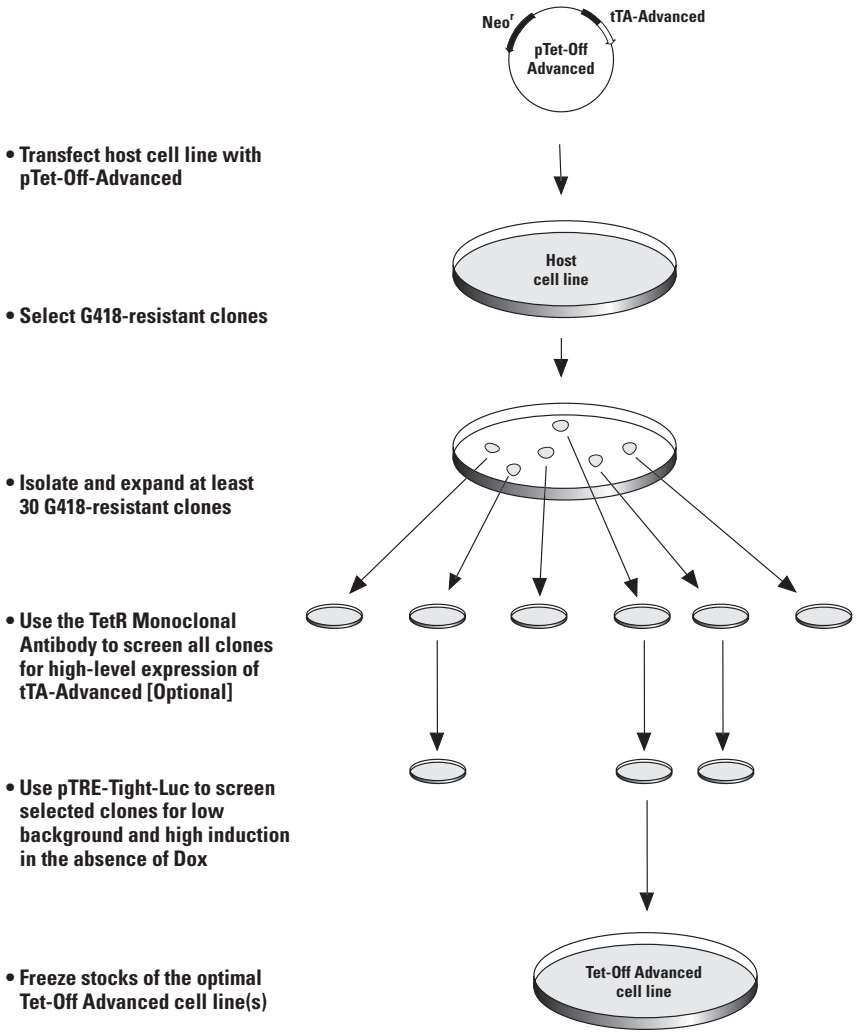

Figure 4. Flow chart for developing a Tet-Off Advanced cell line

VIII. Developing a Stable Tet-Off Advanced Cell Line continued

Protocol 2–4 weeks

A. Protocol: Transfection and Selection of Tet-Off Advanced Stable Cell Lines

1. Grow cells to ~80% confluency in complete medium or to a density appropriate for your transfection method, and transfect the pTet-Off-Advanced Vector by the desired method.

   NOTE: If desired, the regulator plasmid can be linearized by digestion with a restriction enzyme to increase integration efficiency (Sca I for pTet-Off-Advanced).

2. Plate transfected cells in ten 10 cm culture dishes, each containing 10 ml of the appropriate complete medium. Use an initial plating density of 25–50% of the optimal density determined in Section VII, so that the cells will expand to the ideal density after 2 rounds of cell division.
3. To permit expression of antibiotic resistance, allow cells to divide twice (24–48 hr) after transfection before adding G418 to the optimal selection concentration determined in Section VII. This is usually 400–500 µg/ml.
4. Replace medium with fresh complete medium plus G418 every four days, or more often if necessary.
5. After about five days, cells that have not taken up or integrated the plasmid should start to die. If absolutely necessary, you can split the cells if they reach confluency before massive cell death begins. However, this should be avoided, since replating cells at this point may result in plates containing too many colonies for effective colony isolation.
6. After 2–4 weeks, isolated G418-resistant colonies should begin to appear.
7. Using cloning rings or discs, isolate large, healthy colonies and transfer them to small individual plates or wells. Suspension cultures must be cloned using the limiting dilution technique. Isolate as many clones as possible, typically at least 30.

Important information about Tet System transfections

The efficiencies of available transfection methods will vary greatly among different cell lines. When transfecting a cell line for the first time, it is useful to compare the efficiencies of several methods in transient assays.

- CalPhos and CLONfectin produce reliable results for calcium-phosphate or liposome-mediated transfections, respectively. Other reagent-based methods or electroporation may also work well for the cell line in question.
- Optimize transfection parameters (i. e. cell density, amount of DNA, media conditions, transfection time) in transient assays using a constitutive reporter/expression vector, such as pCMVβ (Cat. No. 631719) for β-galactosidase expression, pSEAP2-Control (Cat. No. 631717) for secreted alkaline phosphatase expression, or pAcGFP1-N1 (Cat. Nos. 632469 & 632426) for fluorescent protein expression.
- Cotransfecting pTRE-Tight with a selectable marker is required to create a stable cell line with the Tet-Off Advanced System, and may be required for other TRE-based vectors. We recommend using the Linear Hygromycin Marker supplied with the kit or our Linear Puromycin Marker (Cat. No. 631626). Both are short, purified DNA fragments comprised of the antibiotic resistance gene, an SV40 promoter, and the SV40 polyadenylation signal. These small markers are highly effective at generating stable transfectants and will not interfere with the inducibility of pTRE-Tight. Alternatively, you can use the pTK-Hyg Vector (Cat. No. 631750) or pPUR Vector (Cat. No. 631601).
- **NOTE:** The selection marker(s) used must not contain an enhancer element in its regulatory sequences. Localized cointegration of the TRE response plasmid and a marker gene containing an enhancer may lead to elevated background expression of the GOI in the uninduced state.

VIII. Developing a Stable Tet-Off Advanced Cell Line continued

Protocol 1–2 days

B. Protocol: Early Screening of Tet-Off Advanced Clones using the TetR Antibody [Optional]

It is advantageous in terms of time and effort, to screen your clones as soon as possible and identify a few clones that are likely to produce optimal results. This will reduce the size of the clone pool that you will retain for functional analysis. Such screening is possible with the TetR Monoclonal Antibody (Cat. No. 631108) that detects the Tet-Off Advanced transactivator protein, tTA-Advanced, as well as the Tet-On Advanced transactivator, and the tTS transcriptional silencer.

1. Harvest sufficient cells from each clonal culture to generate 25–50 µg of total protein for SDS-PAGE. This amount of protein can typically be produced from a single confluent well of cells (i.e. HeLa) from a 12- or 24-well plate.
2. Prepare the cell extract for SDS-PAGE, and prepare a Western blot using standard techniques. The tTA-Advanced protein is easily detectable in 25 µg of total cell protein when using the TetR antibody diluted 1:1,000 and a chemilumenescent detection method for the secondary antibody.
3. Expression of the tTA-Advanced protein should be evident in many clones. Select the best-expressing clones and test their capacity for induced expression of luciferase using pTRE-Tight-Luc in a transient transfection assay (Section C).

Protocol 2–3 days

C. Protocol: Testing Tet-Off Advanced Clones for Induction

Once you have a pool of candidate Tet-Off Advanced cell clones, you can prescreen a few of them first with the TetR Antibody (as in Section B), or test 30 clones in transient transfection assays using the pTRE-Tight-Luc plasmid to identify clones that best meet the criteria for a stable Tet-Off Advanced cell line. Be sure to read the information highlighted in "Ensuring Induction with the Tet-Off Advanced System". Advanced clones are ready to be functionally tested once they reach 50–80% confluence in a 6-well plate. Your customized response plasmid, pTRE-Tight-GOI, should also be tested for functionality in a Tet-Off Advanced clone (see Section IX.A).

1. Trypsinize the cells and split about 1/3 of the total 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.
2. Divide the remaining 2/3 of the cells between two wells of a 6-well plate and transfect each well with pTRE-Tight-Luc (or another TRE reporter vector), using the amount of plasmid DNA appropriate for the desired transfection method.
3. To one of the two transfected wells, add 100 ng/ml Dox to reduce expression to basal levels ("off").
4. Incubate the transfected cells for 48–72 hr.
5. Harvest the cells and assay for luciferase activity. Calculate fold-induction (e.g., –Dox RLU/+Dox RLU).
6. Select clones with the highest fold-induction (highest expression with lowest background) for propagation and further testing. In general, select only those clones that exhibit 20- to 50-fold induction
7. Freeze stocks of each clone as soon as possible after expanding the culture.

Note

NOTE: When testing clones via transient transfection of a TRE reporter, expect to see higher basal expression than in the double-stable clones which you will make in Section IX.

VIII. Developing a Stable Tet-Off Advanced Cell Line continued

Ensuring Induction in the Tet-Off Advanced System

Residual Dox that remains bound to cells or the extracellular matrix can prevent full gene induction in Tet-Off Systems (Rennel & Gerwins, 2002). Cells that have been maintained in the "off" state with 10–100 ng/ml Dox should be passaged as follows:

- Wash the cells on the plate 2X with PBS before trypsinizing.
- After trypsinizing and collecting the cells, wash them in suspension 1X with PBS, and plate in fresh medium without Dox.
- 3–6 hr after plating the cells, and after they have reattached to the substrate, wash them on the plate 1X with PBS and add fresh medium with or without Dox.

IX. Developing a Double-Stable Tet-Off Advanced Cell Line

For an overview of this protocol, see the flow chart in Figure 5.

A. Functional Testing of pTRE-Tight-GOI in the Tet-Off Advanced Cell Line

Prior to establishing the double-stable Tet-Off Advanced cell line for your GOI, the pTRE-Tight-GOI construct should be tested for functionality. Transiently transfect your pTRE-Tight-GOI into one or more stable cell lines created in Section VIII and tested for induction in the absence of Dox. An appropriate gene-specific assay to test for the GOI will be needed. For example:

- Western blotting with an antibody to the GOI protein
- RT-PCR using GOI-specific primers. Be sure you can discriminate between PCR products generated from genomic DNA and true RT-PCR products.
- Northern blotting with a GOI-specific probe
- Functional assay for the GOI protein

Protocol 2–4 weeks

B. Protocol: Stably Transfect and Select a Double-Stable Tet-Off Advanced Cell Line

To generate a double-stable Tet-Off Advanced cell line, your pTRE-Tight-GOI response plasmid must be cotransfected either with the Linear Hygromycin Marker provided in the kit or with another selectable marker. Use the following protocol.

1. Using the optimized conditions and method for your cell line, transfect your pTRE-Tight-GOI plasmid and the Linear Hygromycin Marker at a ratio of 20:1 (i.e., 20-fold less Linear Hygromycin Marker). This ratio may be optimized for the cell type being used. Optimal ratios typically lie between 10:1 and 20:1.

   

   NOTE: If desired, linearize pTRE-Tight-GOI by digestion with a unique restriction enzyme to improve integration efficiency (check your GOI sequence and the pTRE-Tight Vector Information Packet to find an appropriate restriction site).

2. Plate transfected cells in ten 10 cm culture dishes at the optimal density determined in Section VII. Use complete medium containing an appropriate maintenance concentration of G418. Include 100 ng/ml Dox to prevent expression of the GOI. Use an initial plating density of 25–50% of the optimal density determined for hygromycin selection in Section VII, so that the cells will expand to the ideal density after 2 rounds of cell division.
3. To permit expression of antibiotic resistance, allow cells to divide twice after transfection(24–48 hr; time may vary with cell line) before adding the optimal hygromycin concentration determined in Section VII (200–400 µg/ml).
4. Replace medium with fresh complete/G418 medium containing hygromycin and 100 ng/ml Dox every four days. After about five days, cells should start to die. If absolutely necessary, you can split the cells if they reach confluency before massive cell death begins. However, this should be avoided, since replating cells at this point may result in plates containing too many colonies for effective colony isolation. After 2–4 weeks, hygromycin-resistant colonies should begin to appear.
5. Using cloning rings or discs, isolate large, healthy colonies and transfer them to small individual plates or wells. Suspension cultures must be cloned using the limiting dilution technique. Isolate as many clones as possible, typically at least 30.

ATTENTION: Do not work with a mixed population.

IX. Developing a Double Stable Tet-Off Advanced Cell Line continued

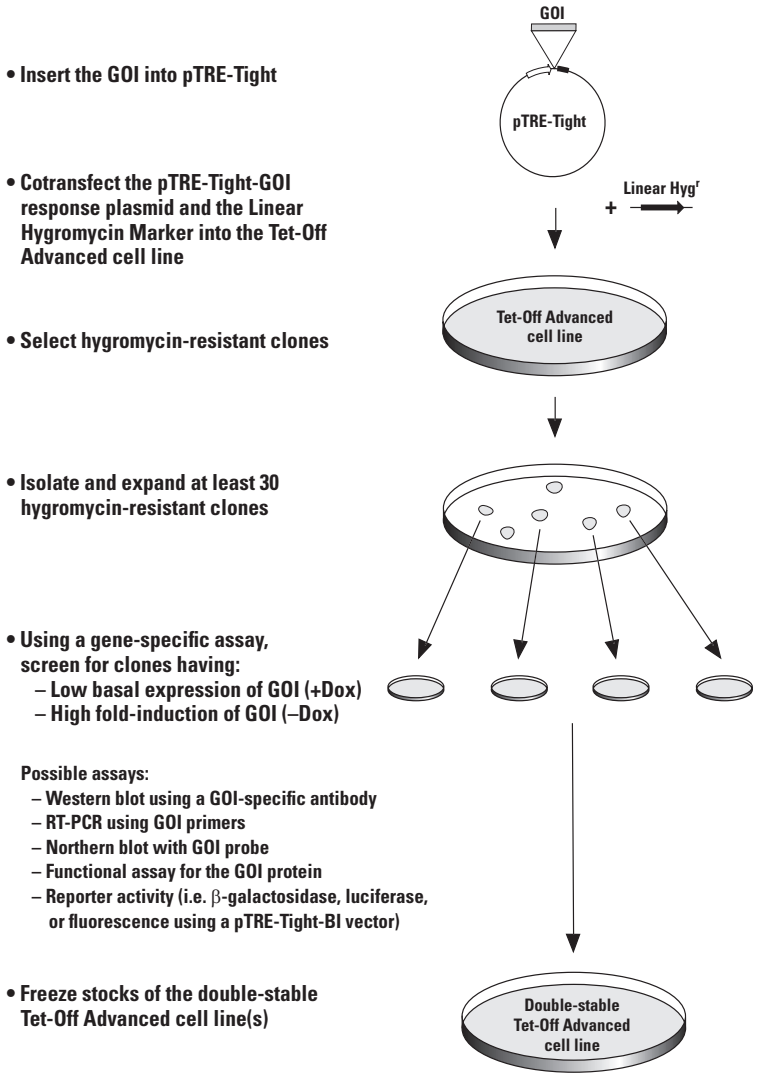

Figure 5. Flow chart for developing a double-stable Tet-Off Advanced cell line

IX. Developing a Double Stable Tet-Off Advanced Cell Line continued

Protocol
2 days

C. Protocol: Screening Double-Stable Cell Lines

- Test isolated resistant clones for Dox-regulated gene expression by testing equivalent numbers of cells for expression of the GOI in the absence and presence of 100 ng/ml Dox. As with the development of the single-stable Tet-Off Advanced cell line, choose a clone that generates the highest overall induction and lowest background expression of the GOI.
- Allow the cells to grow for at least 48 hr, with and without Dox, then assay each sample for expression of the GOI using a gene-specific assay.
- Once a suitable double-stable Tet-Off Advanced cell line has been identified, prepare frozen aliquots to ensure a renewable source of the cells (Section VI).

D. Working with Double-Stable Tet Cell Lines

- Tet Systems have been established successfully in many cell types, as well as in transgenic mice, rats, plants, and yeast. In general, difficulties in obtaining a cell line that exhibits low background expression, arise from suboptimal plasmid integration events, and can be overcome simply by screening more clones.
- Perform a time course of induction using the techniques described in Section VII, being sure to wash the cells free of residual Dox before beginning the assay. Be sure to read the information highlighted in "Ensuring Induction with the Tet-Off Advanced System". Because the kinetics of induction depend on the stability of the mRNA and the GOI protein, stably expressed proteins will achieve equilibrium at different times after induction.

X. References

You can access further information on Tet Systems products on our website: **www.clontech.com.** Clontech's Tet Systems were developed in cooperation with Dr. Bujard and his colleagues at the Center for Molecular Biology in Heidelberg (ZMBH) and in Dr. Wolfgang Hillen's laboratory at the University of Erlangen, Germany. Additional background information on Tet-regulated gene expression systems and an extensive bibliograpy are available at the website maintained by TET Systems: **http://www.tetsystems.com**. (Please note that Clontech is not responsible for the information contained on this site.)

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Appendix A: Vector Information

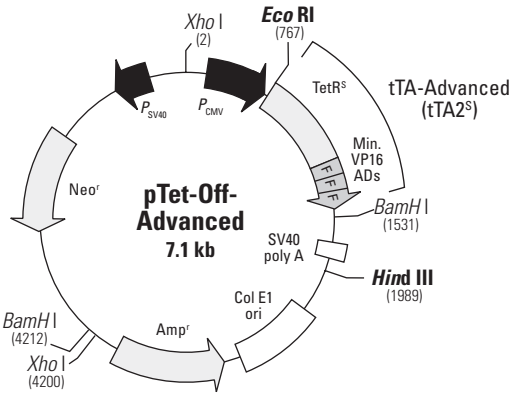


Figure 6. Map of the pTet-Off-Advanced Vector. Unique restriction sites are in bold. For a complete vector description, refer to the enclosed pTet-Off-Advanced Vector Information Packet (PT3945-5).

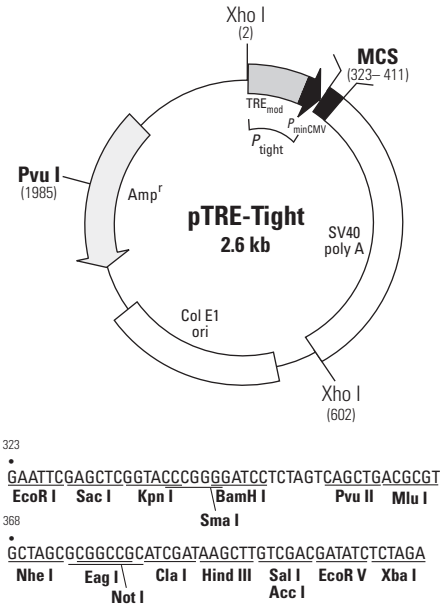
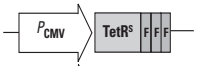
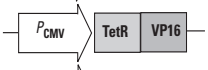
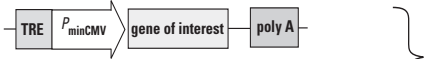
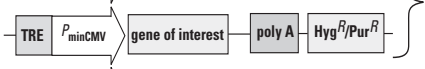
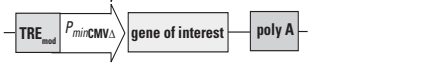
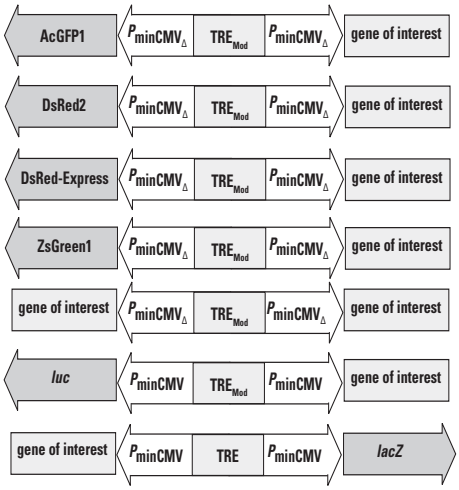
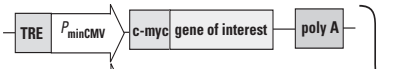


Figure 7. Map and Multiple Cloning Site of pTRE-Tight Vector. Unique restriction sites are in bold. For a complete vector description, refer to the enclosed pTRE-Tight Vector Information Packet (PT3720-5).

Appendix A: Vector Information continued

Table I: Tet Systems Vector Alignments

Name		Applications
Basic Vectors		
pTet-Off-Advanced		Regulator vector used in the Tet-Off Advanced System
pTet-On-Advanced		Regulator vector used in the Tet-On Advanced System
pTet-Off		Regulator vector used in the Tet-Off System
pTet-On		Regulator vector used in the 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}). Use with Tet-Off Advanced and Tet-On Advanced
Bidirectional Tet Vectors		
pTRE-Tight-BI-AcGFP1		Response vectors for monitoring expression of a target gene via expression of a coregulated reporter
pTRE-Tight-BI-DsRed2		
pTRE-Tight-BI-DsRed-Express		
pTRE-Tight-BI-ZsGreen1		
pTRE-Tight-BI		
pTRE-Tight-BI-Luc		
pBI-G		
Accessory Vectors		
pTet-tTS		Encodes the tTS transcriptional silencer for tighter control of gene expression in Tet-On Systems
ptTA-2, 3, 4		Minimal domain vectors used with the Tet-Off System; minimizes VP16 toxicity
pTRE-Tight-DsRed2		Fluorescent reporter vector containing a modified Tet Responsive Element (TRE_{mod}) for use in either Tet-Off or Tet-On Systems
Tagged Vectors		
pTRE-Myc		Response plasmids for use in either Tet-Off or Tet-On Systems
pTRE-HA		Used for screening for GOI expression with antibodies or for GOI purification
pTRE-6xHN		

Appendix A: Vector Information continued

Table II: Tet Systems Vector Information

Vector Name	Reference	Name in Reference	Expressed Protein	Selectable Marker
pTet-Off-Advanced	Urlinger, *et al.* (2000)	pUHT61-1	tTA2	neomycin
pTet-On-Advanced	Urlinger, *et al.* (2000)	pUHrT62-1	rtTA2-M2	neomycin
pTet-Off	Resnitzky, *et al.* (1994), Gossen & Bujard (1992)	pUHD15-1neo	tTA	neomycin
pTet-On	Gossen, *et al.* (1995)	pUHD17-1neo	rtTA	neomycin
pTet-tTS	Freundlieb, *et al.* (1999)	pUHS 6-1	tTS	none
pTRE2hyg			GOI	hygromycin
pTRE2hyg-Luc (control)			luciferase	hygromycin
pTRE2pur			GOI	puromycin
pTRE2pur-Luc (control)			luciferase	puromycin
pTRE2			GOI	none
pTRE2-Luc			luciferase	none
pTRE-Tight			GOI	none
pTRE-Tight-Luc (control)			luciferase	none
pTRE-Myc			Myc-GOI	none
pTRE-Myc-Luc (control)			Myc-luciferase	none
pTRE2hyg2-Myc			Myc-GOI	hygromycin
pTRE2pur-Myc			Myc-GOI	puromycin
pTRE-HA			HA-GOI	none
pTRE2hyg2-HA			HA-GOI	hygromycin
pTRE2pur-HA			HA-GOI	puromycin
pTRE-HA-Luc (control)			HA-luciferase	none
pTRE-6xHN			6xHN-GOI	none
pTRE2hyg2-6xHN			6xHN-GOI	hygromycin
pTRE2pur-6xHN			6xHN-GOI	puromycin
pTRE-6xHN-Luc (control)			6xHN-luciferase	none
pTRE-Tight-DsRed2			DsRed2	none
pTRE-Tight-AcGFP1			*AcGFP1*	none
pTRE-Tight-ZsGreen1			*ZsGreen1*	none
pTRE-Tight-BI			GOI1, GOI2	none
pTRE-Tight-BI-AcGFP1			GOI, AcGFP1	none
pTRE-Tight-BI-DsRed2			GOI, DsRed2	none
pTRE-Tight-BI-DsRed-Express			GOI, DsRed-Express	none
pTRE-Tight-BI-ZsGreen1			GOI, ZsGreen1	none
pTRE-Tight-BI-Luc (control)			GOI, luciferase	none

Appendix B: Glossary

Dox	Doxycycline, a derivative of Tc that is the preferred effector substance for Tet experiments and essential for use with Tet-On and Tet-On Advanced Systems.
Double-stable Tet Cell Line	A Tet-Off or Tet-On cell line that has been stably transfected with the pTRE2-Gene -GOI construct. The GOI is induced by the removal (for Tet-Off) or addition (for Tet-On) of Dox from the media.
"F-type" AD	A minimal activation domain derived from the HSV VP16 protein.
GOI	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 and used to drive the expression of rTA, rtTA, and rtTA-Advanced.
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-On-Advanced
Response Plasmid	A pTRE-derived plasmid that expresses a gene of interest from the $P_{hCMV*-1}$ promoter. and can be used in both Tet-Off and Tet-On systems.
rTetR	The reverse Tet repressor protein. In *E. coli,* rTetR binds specifically to *tet*O and blocks transcription of the tet operon in the presence of Tc. It is the *tet*O-binding component of rtTA in Tet-On Systems.
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.
rtTA-Advanced	Mutated and optimized reverse tetracycline-controlled transactivator of Tet-On Advanced: A 28 kDa fusion protein consisting of the $rTetR^{S}$-M2 and three minimal "F-type" activation domains from VP16. Binds very 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.)
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.

Appendix B: Glossary continued

*tet*O	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 protein. In *E. coli,* TetR binds specifically to *tet*O and blocks transcription of the tet operon in the absence of Tc. It is the *tet*O-binding component of tTA-Advanced in Tet-Off Advanced Systems.
TRE	Tet-Response Element. A regulatory sequence consisting of seven direct repeats of a 42 bp sequence that contains the *tet*O.
TRE_{mod}	Modified Tet-Response Element. A regulatory sequence consisting of seven direct repeats of a 36 bp sequence that contains the *tet*O.
tTA	Tetracycline-controlled transactivator of Tet-Off Systems: 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.
tTA-Advanced	The expression-optimized, tetracycline-controlled transactivator of Tet-Off Advanced. A 28 kDa fusion protein consisting of $TetR^S$ and three minimal "F-type" activation domains from VP16. 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.