Tet-On® Advanced Inducible Gene Expression System User Manual

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I. Introduction

A. Summary

The Tet-On Advanced Inducible Gene Expression System gives researchers ready access to the tightly regulated, high-level gene expression system described by Gossen, *et al.* (1995) with modifications made by Urlinger, *et al.* (2000). In the Tet-On Advanced System, expression is activated by the addition of doxycycline (Dox), a tetracycline (Tc) deriviative, to the culture medium. This permits gene expression to be tightly regulated in response to varying concentrations of Dox. Maximal expression levels in the Tet-On Advanced System 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 results are not complicated by pleiotropic effects or nonspecific induction.

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

B. Tet Expression Systems

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 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 the mammalian Tet-Off and Tet-On Advanced Systems. The central concepts of each system are shown in Figure 1 and discussed below to provide a brief overview of these methodologies.

The Tet-Off System: One key component of the Tet-Off System is a 37 kDa transcription-activating fusion protein derived from TetR, including its DNA binding domain (BD), and the activation domain (AD) of the herpes simplex virus (HSV) VP16 protein (Triezenberg, *et al.,* 1988). The VP16 AD converts TetR from a repressor to a transcriptional activator, and the resulting hybrid protein is known as the tetracycline-controlled transactivator (tTA). Like TetR, tTA is unable to bind *tet*O sequences in the presence of Tc or Dox. The second key component of the Tet-Off System is the tetracycline-response element (TRE). This composite sequence consists of seven direct repeats of a 42 bp sequence containing *tet*O, and is located just upstream of a minimal CMV promoter (P_{minCMV}) that lacks the strong enhancer elements of the wild-type CMV immediate early promoter. Transcription of a "gene of interest" (GOI), located downstream of the TRE, is activated in the Tet-Off system by removing Tc from the culture medium (Figure 2A).

The Tet-On Advanced System: Induction of gene expression in the Tet-On and Tet-On Advanced Systems is similar to, but accomplished in a manner opposite to that of Tet-Off. The Tet-On and Tet-On Advanced regulatory proteins are based on two mutant "reverse" Tet repressors (rTetRs) that bind to *tet*O in the *presence* of Dox. These rTetRs, with their BD, have been fused to VP16 AD in the case of Tet-On or to minimal VP16 ADs in the case of Tet-On Advanced. The resulting transactivators (rtTA and rtTA-Advanced, respectively) activate transcription from a TRE as a consequence of Dox treatment, not from its withdrawal (Figure 2B; Hillen & Berens, 1994; Urlinger, *et al.*, 2000; Gossen *et al.,* 1995). Induction with Tet-On Advanced requires the use of Dox, not Tc, and utilizes the improved, low-background pTRE-Tight vector to control expression of the GOI.

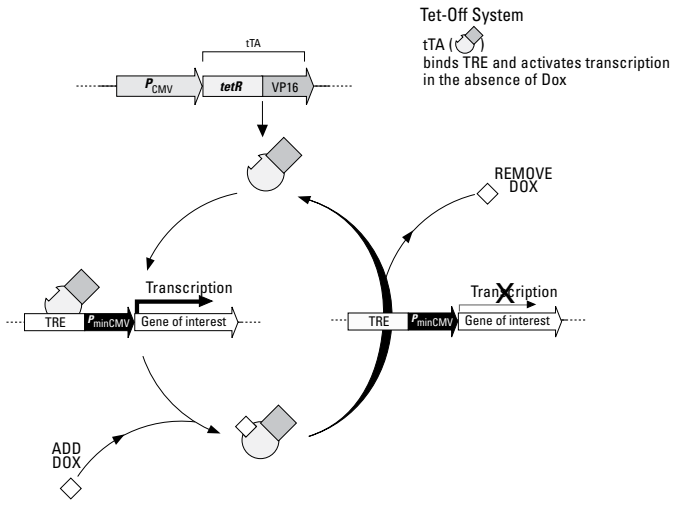
C. The Tet-On Advanced Transactivator

The rtTA-Advanced protein contains features that improve its performance over the original Tet-On rtTA (Urlinger, *et al.,* 2000):
Several specific amino acid substitutions enhance its sensitivity to Dox at least ten-fold and virtually eliminate its background binding to *tet*O in the absence of Dox. Thus, background TRE-dependent expression is dramatically reduced with rtTA-Advanced, when compared to Tet-On (Figure 3).

- The expression and stability of rtTA-Advanced in mammalian cells was optimized by replacing its original bacterial codons with mammalian ones and by removing cryptic splice sites from the rtTA-Advanced mRNA.

I. Introduction continued

A Tet-Off



B Tet-On Advanced

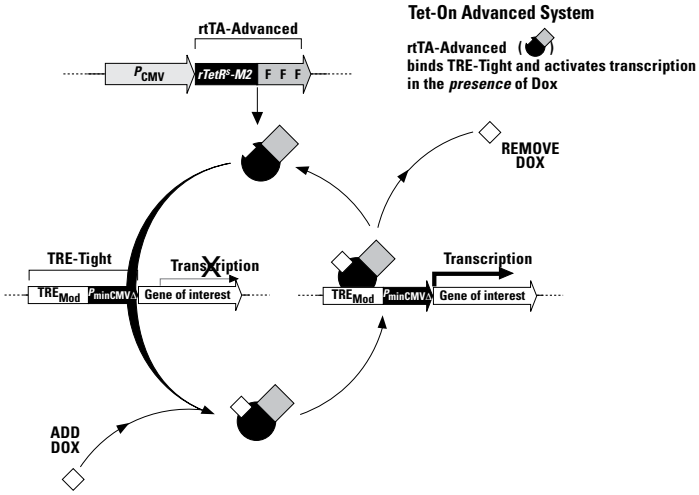


Figure 1. Schematic of gene regulation in the Tet-Off and Tet-On Advanced Systems. **A. Tet-Off:** The TRE is located upstream of the minimal immediate early promoter of cytomegalovirus (P_{minCMV}), which is silent in the absence of activation. The tTA protein binds the TRE—and thereby activates transcription of the gene of interest (GOI)—in the absence of Tc or Dox. **B. Tet-On Advanced:** The "reverse" Tet repressor domain (rTetR^S-M2) of rtTA-Advanced contains five amino acid changes that reverse the protein's original response to Dox. Further amino acid changes have made it more sensitive to Dox, reduced background binding to the TRE, and optimized its expression in mammalian cells. rtTA-Advanced binds TRE-Tight and fully activates transcription in the presence of low concentrations of Dox. Please see Appendix A for maps and detailed vector information.

- The AD of rtTA-Advanced consists of three minimal "F"-type activation domains from VP16 (Baron *et al.*, 1997) which provide full transcriptional activation of the full-length AD, yet reduce crossreactivity and cytotoxicity.
- The rtTA-Advanced protein's many functional improvements also elevate its nominal expression levels and facilitate the generation of host cell lines. Transfected mammalian cells tolerate the stabilized protein well.

I. Introduction continued

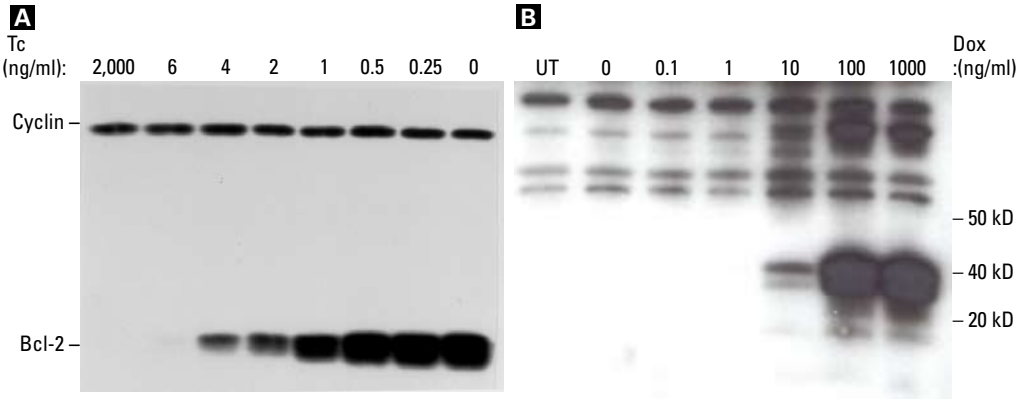


Figure 2. Inducible on/off control of gene expression in the Tet Systems. Panel A. HeLa S3 Tet-Off cells were stably transfected with a plasmid expressing Bcl-2 under control of the TRE and grown in the presence of the indicated amounts of Tc. A Western blot containing 100 µg of total protein from each 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). **Panel B.** The Tet-On Advanced System was used to generate a double-stable HEK 293 cell line capable of induced expression of procaspase-8 (~55/50 kD), which generates active forms of caspase-8 (40 kD and 23 kD) following proteolytic cleavage. Cells were treated for 12 hr with the indicated concentration of Dox, then harvested and subjected to Western blot analysis using an anti-caspase-8 antibody. UT: untransfected, untreated.

D. TRE-Tight Controls GOI Expression

The Tet-On Advanced System utilizes TRE-Tight to control gene expression. This element contains a modified TRE (TRE_{mod}) and an altered minimal CMV promoter ($P_{minCMV\Delta}$), which together further reduce basal expression of the GOI, i.e., that occurring from any given minimal promoter independent of the presence of a transcriptional transactivator. TRE-Tight abolishes background expression in certain cell lines, greatly reduces background activity in most others, and is especially useful in cases where residual background expression is unacceptable, such as the expression of proteins that are extremely potent or toxic to the host cell (*Clontechniques,* April 2003). The P_{tight} promoter was originally developed as the $P_{tet\text{-}14}$ promoter in the laboratory of Dr. H. Bujard.

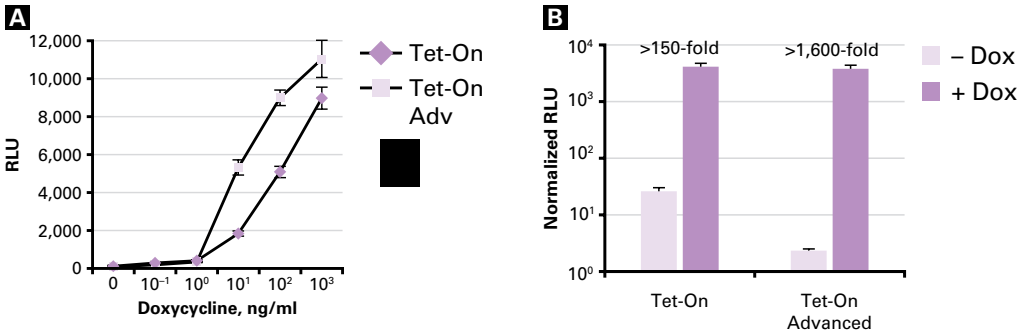


Figure 3. Tet-On Advanced is more sensitive to Dox and has lower background than Tet-On. Panel A. Two HEK 293 cell lines stably transformed with either pTet-On or pTet-On Advanced were transiently transfected with pTRE2-Luciferase. The cells were exposed to the indicated concentrations of Dox before being harvested and assayed for luciferase activity 48 hr after transfection. **Panel B.** MCF-7 cell lines capable of induced luciferase expression were generated with the Tet-On System or with the Tet-On Advanced System. Luciferase expression is controlled by the TRE2 promoter in Tet-On and by the TRE-Tight promoter in Tet-On Advanced. Cells were either treated with 1 µg/ml Dox or left untreated, prior to harvest and assay for luciferase activity. Maximum induction is similar for both cell lines, but reduced background results in higher fold-induction with Tet-On Advanced.

I. Introduction continued

E. Tet-On Advanced Stable Cell Lines

The ultimate goal in setting up a functional Tet-On Advanced System is to create a double-stable Tet-On Advanced cell line which contains both the regulatory (pTet-On Advanced) and response (pTRE-Tight-GOI) plasmids. In such a cell line, the GOI is only expressed when rtTA-Advanced binds to the TRE in the presence of Dox (Figure 1). Transcription is turned on in response to Dox in a precise and dose-dependent manner.

It is possible to reduce the time needed to establish a Tet-On Advanced cell line by purchasing one of our premade lines. A list of available Tet-On Advanced, Tet-Off, and Tet-On Cell Lines may be found on our website: **www.clontech.com.**

NOTE: The addition of a nuclear localization sequence (NLS) to rtTA-Advanced alters the protein's regulatory function and is not recommended (M. Gossen & H. Bujard, pers. comm.). An NLS modification will increase maximum expression but will also elevate background expression due to altered binding affinity to tetO (unpublished observations).

F. Advantages of Tet-On Advanced Over Other Expression Systems

The combined major improvements implemented in the Tet-On Advanced System yield robust induction capabilities, extremely low basal expression, and facilitate the process of developing a stable Tet-On Advanced host cell line. The Tet-On Advanced System has several advantages over other regulated mammalian gene expression systems:

- Extremely tight regulation. Background expression of the GOI in the absence of induction is extremely low due to the combined effects of the improved Tet-On Advanced rtTA and TRE-Tight.
- No pleiotropic effects. When introduced into mammalian cells, the prokaryotic DNA BD of rtTA-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 over 1000-fold and can be detected within 30 minutes using nontoxic levels of Dox (Figure 3B; results not shown). 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). These 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. *Use only Dox with Tet-On Advanced.*
- 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).

I. Introduction continued

- The Tet On and Tet Off expression systems have proven to be superlative control systems for the generation of transgenic mice; to the extent 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 TET Systems home page (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 advantage 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.
- The Tet-On Advanced System is a premier gene expression system that possesses all of these essential characteristics, making it vastly preferable over existing systems (Gossen *et al.,* 1993; 1994).

G. Tetracycline vs. Doxycycline

While the original Tet-Off system responds equally well to either Tc or Dox, the Tet-On Advanced System responds only to Dox, and not to Tc (Gossen & Bujard, 1995). In both systems, the concentrations used are far below cytotoxic levels for either cell culture or transgenic studies and Dox has a longer half-life (24 hours) than Tc (12 hours).

H. Additional Tet Response Vectors

The system is supplied with a luciferase reporter vector, pTRE-Tight-Luc, that is used to screen the Tet-On 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 express our Living Colors® fluorescent proteins.

Additionally, several TRE vectors (not based on TRE-tight) are available for expressing your GOI with a tag to aid detection or purification of the induced protein. These vectors, which provide a way to screen colonies directly for protein expression by Western analysis using readily available antibodies, 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.

I. 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 because its expression can be monitored indirectly through expression of the coregulated reporter gene.

- The pTRE-Tight-BI (Cat. No. 631068) Vector 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 without a reporter (pBI-Tet, Cat. No. 631006), or with either β–galactosidase (pBI-G Tet, Cat. No. 631004) or luciferase (pBI-L Tet, Cat. No. 631005).

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-On Advanced Inducible Gene Expression System (Cat. No. 630930)

- 20 µl pTet-On-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 U2-OS-Luc Tet-On Control Cell Line (~1.0 x 10^6 cells/tube)
- 50 ml Tet System Approved FBS
- User Manual (PT3898-1)
- pTet-On-Advanced Vector Information Packet (PT3899-5)
- pTRE-Tight Vector Information Packet (PT3720-5)

NOTE: 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

- Dulbecco's Modified Eagle's Medium (DMEM) for the U2-OS-Luc Tet-On Control Cell Line. The appropriate medium for growing other Clontech premade Tet-Off and Tet-On cell lines is described on the Certificate of Analysis provided with each cell line.
- Fetal bovine serum (FBS), Tc-Free. It is critical that the FBS used not interfere with Tet-responsive expression. Many bovine sera are contaminated with Tc. This problem can be eliminated by using a Tet System Approved FBS from Clontech (see Section XI). These sera have been functionally tested in the Tet Systems and found to be free of contaminating Tc activity. Alternatively, use the U2-OS-Luc Tet-On Control Cell Line to test for Tc contamination in other sera, as described in Section VII.A.
- 200 mM L-Glutamine (Sigma, Cat. No. G7513)
- Pen/Strep solution of 10,000 units/ml penicillin G sodium and 10,000 µg/ml streptomycin sulfate (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)

III. Additional Materials Required continued

B. Antibiotics for Clonal Selection

Prior to antibiotic use, determine the optimal selection concentration for each antibiotic as described in Section VII.

- G418 (for selection of the Tet-On Advanced cell line), 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 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 (for selection of double-stable Tet-On Advanced cell lines and maintenance of the U2-OS-Luc Tet-On 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.

D. Doxycycline

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.

E. Luciferase Assay

A method for assaying luciferase expression is required for use with the U2-OS-Luc Tet-On Control Cell Line. Use any standard luciferase assay system. For these assays, a luminometer is also required.

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

Following cloning of pTRE-Tight-GOI plasmid, 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: Do not use the pTRE or pTRE2 Sequencing Primers. They are incompatible with pTRE-Tight.

IV. Protocol Overview

An overview for creating a double-stable Tet-On Advanced cell line that contains integrated copies of the regulatory vector and the response vector is shown in Figure 4.

For more detailed flow charts of each of the transfection procedures see Figure 7 (Section VIII) for generation of transactivator stable lines and Figure 8 (Section IX) for generation of GOI cell lines. When using a premade Tet-On Advanced Cell Line from Clontech, only the second transfection using the pTRE-Tight-GOI construct is required (see Figure 4 below and Figure 8, p. 20).

Attention

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-On Advanced) can elevate basal expression of the colocalized TRE-controlled GOI.
- Cotransfection prevents comparison of multiple clones. Differences in induction or absolute expression may arise due to clone-to-clone variation in rtTA-Advanced expression, rather than from true differences in TRE activity.
- Sequential transfection yields lower background expression, since the response plasmid generally will not cointegrate with the CMV-driven regulator.
- A Tet-On Advanced cell line is a versatile host that provides a proven genetic background in which to introduce other TRE-controlled constructs.

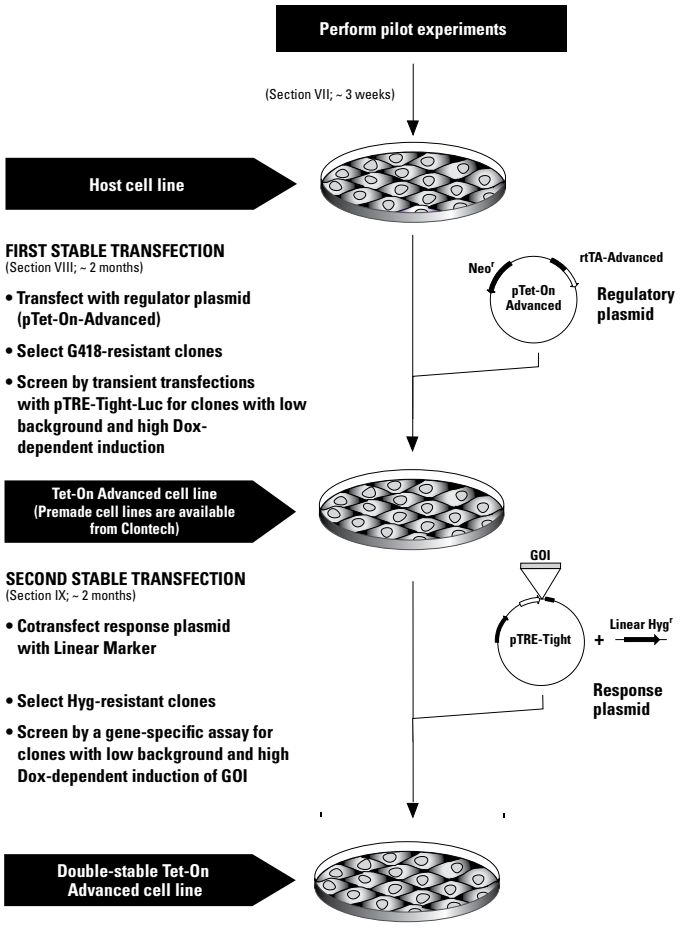


Figure 4. Overview of developing the Tet-On Advanced and double-stable Tet-On Advanced cell lines

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.
2. Perform large-scale plasmid preparations of any plasmid that will be transfected into mammalian cells. To ensure the purity of the DNA, prepare transfection-grade plasmid by purification on a NucleoBond® or NucleoBond® Xtra column. Visit **www.clontech.com** for complete product information.

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 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 documents PT3001-1and 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 PAC 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 differ in their 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 levels used to select stably transfected clones to a maintenance concentration, typically 100 µg/ml for each drug.

VI. Cell Culture Guidelines continued

Protocol

Protocol: Starting Tet Cell Cultures from Frozen Stocks

Frozen Tet Cell Lines, e.g., the U2-OS-Luc Tet-On Cell Line, should be cultured as soon as possible after receipt. Cell viability may decrease rapidly over time after the cells have been shipped.

1. Thaw vial of cells rapidly in a 37°C water bath. Immediately upon thawing, wipe the outside of the vial with 70% EtOH. Transfer the contents of the vial to a 10-cm dish containing 9 ml of medium **(without selective antibiotics).** Mix gently.

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

2. Mix the cell suspension thoroughly, but gently. 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).

[Alternative method] The cells can also be rinsed prior to incubation to remove the cryopreservative (DMSO). 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 can be beneficial when resuspending in small volumes; however, viability may decrease due to cell membrane damage, so handle the cells with care.

3. The next day, examine the cells under a microscope. If the cells were not rinsed before plating, remove the medium, and replace with fresh, prewarmed, complete medium (without selective antibiotics)
4. Expand the culture as needed. The appropriate selective antibiotic(s) should be added to the medium after 48–72 hr in culture.

Once you have started growing a Tet Cell Line from Clontech, be certain to prepare frozen aliquots to ensure a renewable source of cells. Similarly, prepare frozen aliquots of any single or double-stable Tet cell line that you make. Use the freezing medium specified on the certificate of analysis.

Protocol

Protocol: Preparing Frozen Stocks of Tet Cell Lines

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 an ultra low-temperature freezer (–150°C).
7. (Two or more weeks later) Plate a vial of frozen cells, as described in Section 6, to confirm viability.

VII. Pilot Experiments

A. Luciferase Induction

Before you perform any other experiments, we strongly recommend that you perform a Dox dose-response curve using the U2-OS-Luc Tet-On Cell Line provided with the Tet-On Advanced kit. This premade double-stable Tet-On Cell Line can exhibit over 500-fold induction of luciferase upon addition of Dox to the culture medium. In addition to providing a "hands-on" introduction to the Tet System, this experiment serves two critical functions:

- Determine effective concentrations of Dox stocks: The concentrations of Dox listed throughout this protocol are approximate. The optimal concentration may vary with different cell lines and with different lots of this antibiotic. In general, full activation of gene expression in Tet-On cell lines can be obtained with 100–1000 ng/ml Dox.
- Test serum for Tc contamination: As shown in Figure 5 using a Tet-Off cell line, different lots of FBS vary significantly in how they affect Tet System expression. This is presumably due to the widespread use of tetracyclines in the diet of cattle. The 500-fold induction of luciferase in U2-OS-Luc Tet-On Control Cells in response to Dox is highly reproducible. If a significantly lower level of induction is observed, it is possible that the serum is contaminated with 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.

Protocol: Induction of Luciferase in U2-OS-Luc Tet-On Cells

Protocol

1. After thawing and establishing the cell line (Section VI), plate 0.5 x 10^5 U2-OS-Luc Tet-On cells in a volume of 5 ml of complete culture medium into 8–12 wells of two 6-well culture dishes.
2. Add Dox to a series of wells at final concentrations of 0, 1 x 10^{-3}, 1 x 10^{-2}, 0.1, 1.0, 10, 100, and 1000 ng/ml, respectively.
3. Allow the cells to grow for 48 hr.
4. Assay each sample for luciferase activity using any standard luciferase assay. Plot your results and compare to Figure 6.

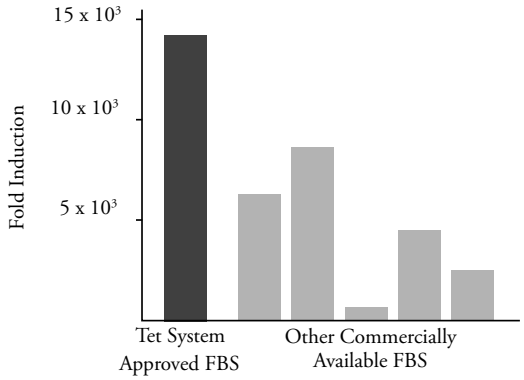


Figure 5. Fold induction of luciferase activity in different lots of FBS. The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different lots of FBS. Average uninduced expression level = 0.21 RLU (n=21, S.D.=0.07); maximum expression levels varied from 123 to 3,176 RLU.

VII. Pilot Experiments continued

B. Titrating Antibiotics for Selection (Kill Curves)

Prior to using an antibiotic (G418, hygromycin, or puromycin) to establish stable and double-stable cell lines, titrate the selection agent to determine the optimal concentration for selection in the 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

Protocol: Antibiotic Titration at Fixed Cell Density.

For selecting stable transformants, use the lowest concentration that begins to result 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 around 1 µg/ml.

1. 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, and 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, respectively) 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.

2. Incubate the cells for 5–14 days, replacing the selective medium every four days (or more often if necessary).
3. Examine the dishes for viable cells every two days.

Important information about Tet System transfections

Transient and stable transfections can be performed using various methods, and transfection efficiencies vary greatly among different cell lines. Therefore, when working with a cell line for the first time, it is useful to compare the efficiencies of several transfection methods in transient assays.

- CalPhos and CLONfectin produce reliable results for either calcium-phosphate or liposome-mediated transfections, respectively. Other similar methods or electroporation may also work well for the cell line in question.
- Optimize transfection efficiency with a noninducible 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, in transient transfections, and assay for reporter gene activity.
- Once a preferred method of transfection is identified, 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.
- Cotransfection with a selectable marker is required to create a stable cell line using the pTRE-Tight vector supplied with the Tet-On Advanced Kit, and may also be required for other TRE-based vectors. We recommend cotransfecting with our 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 the pTK-Hyg Vector (Cat. No. 631750) or pPUR Vector (Cat. No. 631601).
- **Note:** If a selection vector other than a Linear Selection Marker, pTK-Hyg, or pPUR, is used, the marker's promoter should not contain an enhancer element. Cointegration of the response plasmid and selection plasmids containing an enhancer may lead to high background expression of the GOI in the uninduced state.

VII. Pilot Experiments continued

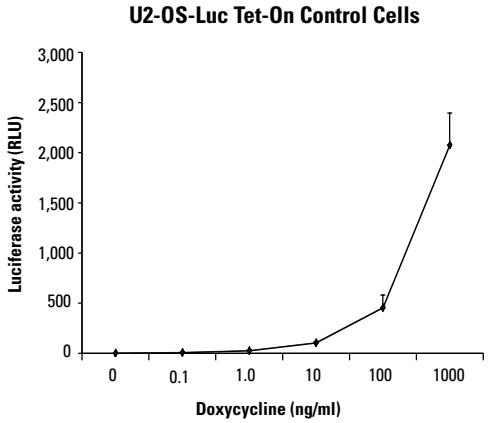


Figure 6. Dose-response curve for the U2-OS-Luc Tet-On Control Cell Line

Protocol

Protocol: Determine Optimal Plating Density.

When selecting stable transfectants, use a plating density that allows the cells to reach ~80% confluence 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.

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 confluence 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.

1. 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.
2. Incubate the cells for 5–14 days, replacing the selective medium every four days.
3. Examine the dishes for viable cells every two days.

VIII. Development of a Stable Tet-On Advanced Cell Line

For an overview of this protocol, see the flow chart in Figure 7.

A. Test Potential Host Cells by Transient Cotransfection with pTet-On-Advanced and pTRE-Tight-Luc

Tet expression systems have been established in numerous cell lines including HeLa, CHO, MCF7, HEK 293, and HepG2. However, quantitative differences in the performance of the Tet system have been attributed to specific cell types. Performing a transient expression assay with pTet-On-Advanced and pTRE-Tight-Luc may provide a quick indication of whether or not the Tet-On Advancecd Systems will work in a particular cell line. This test is not necessary if you have purchased a premade Tet-On Advanced Cell Line.

You should transfect cells using varying ratios of pTet-On-Advanced and pTRE-Tight-Luc. For example, try the following ratios:

pTet-On-Advanced : pTRE-Tight-Luc

1 : 1

1 : 5

5 : 1

NOTE: Due to higher background expression, 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 low level of induction response in the transient assay should not be the sole reason for not using a particular cell line.

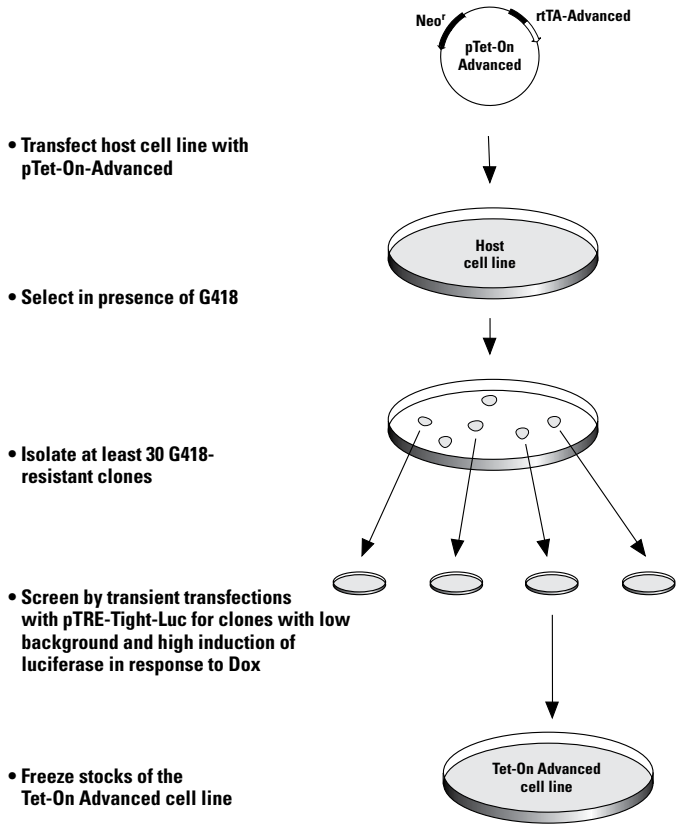


Figure 7. Flow chart for developing a Tet-On Advanced cell line

VIII. Development of a Stable Tet-On Advanced Cell Line continued

- The following protocols describe the development of a Tet-On Advanced cell line. The protocol must be optimized for each cell type. Some of the parameters that may 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 pTRE-Tight-Luc in the next section. While the Tet-On Advanced rtTA has been optimized for expression in mammalian cells, its expression has not been tested in all cell types. Further, the site of plasmid integration may profoundly affect its ultimate level of expression.
- For these reasons, we recommend that as many clones as possible be isolated at Step 8. 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.

Protocol

Protocol: Transfection and Selection of Tet-On Advanced Stable Cell Lines

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

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 the concentration determined in Section VII for selection. This is usually 400–500 µg/ml.
5. Replace medium with fresh complete medium plus G418 every four days, or more often if necessary.
6. After about five days, cells that have not taken up the plasmid should start to die. If 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.
7. After 2–4 weeks, isolated G418-resistant colonies should begin to appear.
8. 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. Isolate as many clones as possible, typically at least 30.

NOTE: Once the candidate stable cell lines produced in Section VIII have been isolated and sufficiently expanded, the next step is to perform transient transfection assays with pTRE-Tight-Luc (or another reporter vector, see Appendix A) to identify G418-resistant clones that meet the criteria for a stable Tet-On Advanced cell line. Tet-On Advanced clones are ready to be tested once they reach 50–80% confluence in a 6-well plate. In addition, the response plasmid, pTRE-Tight-GOI, should be tested for functionality.

VIII. Development of a Stable Tet-On Advanced Cell Line continued

Protocol: Early Screening of Tet-On 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 (see Testing Tet-On Advanced Clones for Induction Protocol below).

Protocol: Testing Tet-On Advanced Clones for Induction

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 DNA appropriate for the desired transfection method.
3. To one of the two wells from Step 2, add Dox at the concentration found to give maximal activity in the pilot experiment described in Section VII. For example, suitable concentrations are: for HEK293 cells, 1,000 ng/ml; for HeLa cells, 300 ng/ml.
4. Incubate the transfected cells for 48 hr.
5. Assay for reporter activity and calculate fold-induction (e.g., luciferase assay: +Dox RLU/–Dox RLU)
6. 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.

NOTE: You can expect when testing clones via transient infection of a TRE reporter that you will see higher background than in the double-stable clones which you will make subsequently in Section IX.

7. Freeze stocks of each clone as soon as possible after expanding the culture.

IX. Development of a Double Stable Tet-On Advanced Cell Line

For an overview of this protocol, see the flow chart in Figure 8.

A. Functional Testing of pTRE-Tight-GOI in Tet-On Advanced Cell Lines

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

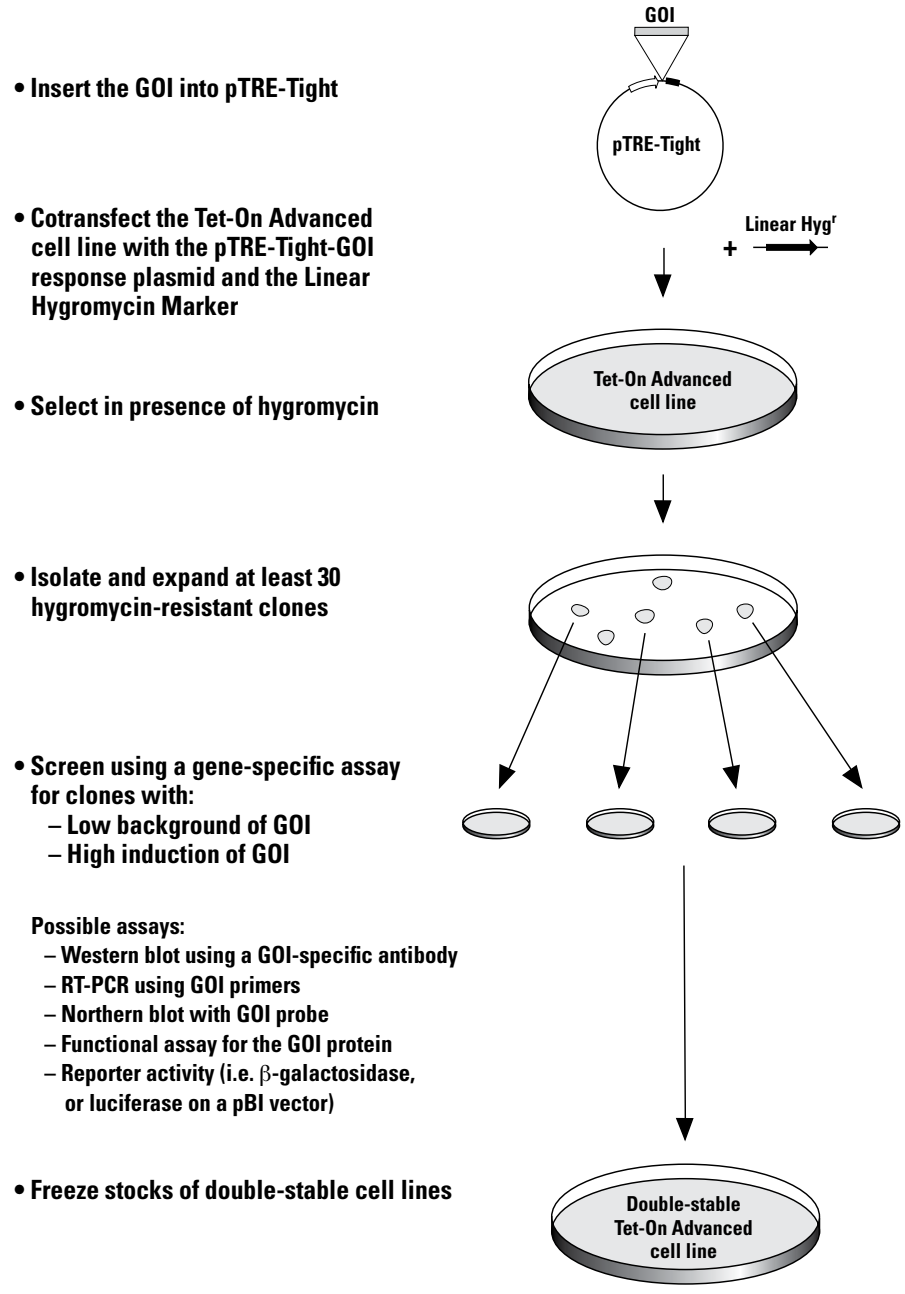


Figure 8. Flow chart for developing a double-stable Tet-On Advanced cell line

IX. Development of a Double Stable Tet-On Advanced Cell Line continued

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

Protocol

Protocol: Stably Transfect and Select a Double-Stable Cell Line by Cotransfection

To generate a double-stable Tet-On Advanced cell line containing pTRE-Tight-GOI, the response plasmid must be cotransfected either with the Linear Hygromycin Marker or another selectable marker using the following protocol.

1. Grow cells to ~80% confluence in complete medium or to a density appropriate for your transfection method.
2. Transfect pTRE-Tight-GOI and the Linear Hygromycin Marker at a ratio of 20:1 (i.e., 20x less Linear Hygromycin Marker) by the desired method. 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).

3. 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.
4. Allow cells to divide twice (24–48 hr; time may vary with cell line) before adding hygromycin to 200–400 µg/ml (or the optimal concentration determined in Section VII).
5. Replace medium with fresh complete/G418 medium containing hygromycin every four days. After about five days, cells should start to die. Split cells if they reach confluence 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, Hyg-resistant 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. Isolate as many clones as possible, typically at least 30.

ATTENTION: Do not work with a mixed population.

B. 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 presence and absence of 100–1000 ng/ml Dox. As with the development of the Tet-On Advanced cell line, choose a clone that generates the highest overall induction and lowest background (i.e., uninduced expression level) of the GOI.
- Allow the cells to grow for at least 48 hr, then assay each sample for expression of the GOI using a gene-specific assay.
- Once a suitable double-stable Tet-On Advanced cell line has been established, prepare frozen aliquots to ensure a renewable source of the cells (Section VI).

IX. Development of a Double Stable Tet-On Advanced Cell Line continued

C. 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, failure to obtain a cell line exhibiting low background expression of the GOI is a result of a poor integration site in the tested lines, and can be overcome simply by screening more clones.
- Perform a dose-response curve (see Figure 6) similar to the experiments described in Section VII. 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.

D. Other Concerns

- **Loss of regulation:** Occasionally, well-characterized double-stable Tet cell lines may lose their responsiveness to Tc or Dox. This often occurs after changing to a lot of calf or fetal bovine serum that is contaminated with Tc. If sudden loss of responsiveness is observed, check the serum by performing a dose-response curve as described in Section VII (and Figure 6). Methylation of the viral promoter in the TRE may occur to "switch off" the promoter, resulting in loss of regulation. It is recommended that the cell lines be periodically subcloned and frozen at various stages.
- **Toxicity of the VP16 activation domain:** Some researchers may be concerned about the possible toxic effects of expressing the VP16 AD in mammalian cells. However, the Tet-On Advanced rtTA already includes three minimal VP16 "F"-type activation domains in place of the full-length VP16 AD, and thus, its potential for toxicity is reduced. Tet-On Advanced is well-suited for *in vivo* applications and is tolerated at higher intracellular concentrations to allow activation over different ranges. Furthermore, Tet transactivators do not require high levels of expression in order to elicit very high-level expression of the genes they regulate. Gossen and Bujard have characterized HeLa Tet-Off cell lines that contain 6,000–10,000 molecules of tTA per cell and produce 10^5-fold induction of the Tet-regulated genes (pers. comm.).

X. References

You can access an extensive Tet System bibliography from the Tet Systems product page at **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 is available at the site maintained by TET Systems:

http://www.tetsystems.com

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

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XI. Related Products

For a complete listing of all Clontech products, please visit **www.clontech.com**. Additional vector information can be found in Appendix A.

Product	Cat. No.
Tet Cell Lines:	
CHO AA8 Tet-Off Cell Line	630904
CHO-K1 Tet-On Cell Line	630917
HEK 293 tTS Cell Line	630927
HeLa S3 Tet-Off Cell Line	630902
HeLa Tet-Off Cell Line	630905
HeLa Tet-On Cell Line	630901
HeLa tTS Cell Line	630928
HT-1080 Tet-Off Cell Line	630916
Jurkat Tet-Off Cell Line	630909
Jurkat Tet-On Cell Line	630915
MCF7 Tet-Off Cell Line	630907
MCF7 Tet-On Cell Line	630918
MCF7 tTS Cell Line	630929
MDCK Tet-Off Cell Line	630913
MEF/3T3 Tet-Off Cell Line	630914
PC12 Tet-Off Cell Line	630906
PC12 Tet-On Cell Line	630912
Saos-2 Tet-Off Cell Line	630911
U2-OS Tet-On Cell Line	630919
293 Tet-Off Cell Line	630908
293 Tet-On Cell Line	630903
T-47D Tet-On Cell Line	630923
T-47D Tet-Off Cell Line	630924
Serum:	
Tet System Approved FBS, US-Sourced (500 ml)	631101
Tet System Approved FBS, US-Sourced (50 ml)	631105
Tet System Approved FBS, USDA-Approved (500 ml)	631106
Tet System Approved FBS, USDA-Approved (50 ml)	631107

XI. Related Products, continued

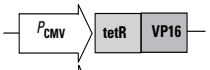
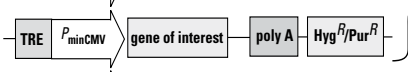
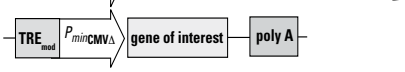
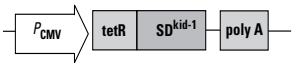
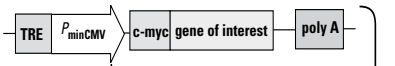
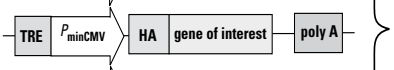
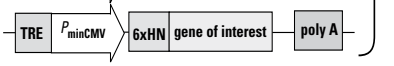
Product	Cat. No.
Tet Vectors:	
Response Vectors/Bidirectional	
pTRE-Tight-BI Vector	631068
pTRE-Tight-BI-AcGFP1 Vector	631066
pTRE-Tight-BI-DsRed2 Vector	631064
pTRE-Tight-BI-DsRed-Express Vector	631065
pTRE-Tight-BI-ZsGreen1 Vector	631067
pBI Tet Vector	631006
pBI-G Tet Vector	631004
pBI-L Tet Vector	631005
Response Vectors/Control	
pTRE-Tight-DsRed2 Vector	631061
pTRE-Tight-ZsGreen1 Vector	631062
pTRE-Tight-AcGFP Vector	631063
Response Vectors/Tag	
pTRE2hyg2-Myc Vector	631052
pTRE2hyg2-HA Vector	631051
pTRE2hyg2-6xHN Vector	631053
pTRE2pur-Myc Vector	631055
pTRE2pur-HA Vector	631054
pTRE2pur-6xHN Vector	631056
pTRE-HA Vector	631012
pTRE-Myc Vector	631010
pTRE-6xHN Vector	631009
Response Vectors/Others	
pTRE2hyg Vector	631014
pTRE2pur Vector	631013
pTRE2 Vector	631008
pLP-TRE2 Acceptor Vector	631016
Regulator Vectors	
pTet-Off Vector	631017
VP16 Minimal Domain Vector Set	631019
pTet-tTS Vector	631011

XI. Related Products, continued

Product	Cat. No.
Selection Markers:	
Linear Hygromycin Marker	631625
Linear Puromycin Marker	631626
pTK-Hyg Vector	631750
pPUR Vector	631601
Other Systems:	
Knockout Inducible RNAi Systems	
Knockout Tet RNAi System H	630925
Knockout Tet RNAi System P	630926
Adenoviral Expression Systems	
Adeno-X™ Tet-Off Expression System 1	631022
Adeno-X™ Tet-On Expression System 1	631050
Adeno-X™ Tet-Off Expression System 2	631058
Retroviral Expression Systems	
RevTet-Off™ System	631020
RevTet-On™ System	631021
Creator-Compatible RevTet-Off™ Gene Expression System	631023
Creator-Compatible RevTet-On™ Gene Expression System	631024
pRevTet-Off Vector	631003
pRevTet-On Vector	631007
pRevTet-Off-IN Vector	631001
pRevTRE Vector	631002
pLP-RevTRE Acceptor Vector	631015
Packaging Cell Lines	many
Additional Products:	
Cell Culture	
CalPhos™ Mammalian Transfection Kit	631312
CLONfectin™ Transfection Reagent	631301
G418	631307
Hygromycin B	631309
Doxycycline	631311
Puromycin	631305
Other Related Products	
NucleoBond® and NucleoSpin® Columns	many
NucleoSpin Extract II Kit	636972
Luciferase Reporter Assay Kit	631714
Creator™ pDNR Cloning Kit	631615
pCMVβ Vector	631719
Living Colors® pAcGFP-N1 Vector	632469
AcGFP Vector Set	632426
pSEAP2-Control Vector	631717

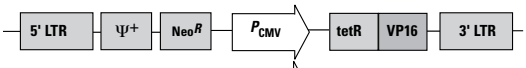
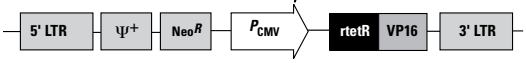
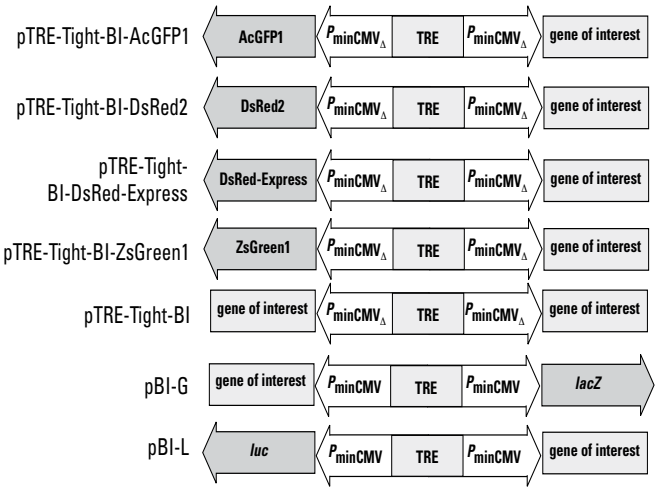
Appendix A: Vector Information

Table I: Tet Systems Vector Alignments

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		

Appendix A: Vector Information continued

Table I: Tet Systems Vector Alignments continued

Name	Applications
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
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	
pBI-G	
pBI-L	

Appendix A: Vector Information continued

Table II: Tet Systems Vector Information

Vector Name	Reference	Name in Reference	Expressed Protein	Selectable Marker
pTet-On-Advanced	Urlinger, *et al.* (2000)	pUHrT62-1	rtTAs-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
pBI	Baron, *et al.* (1995)	pBI-4	GOI1, GOI2	none
pBI-G	Baron, *et al.* (1995)	pBI-3	β-gal, GOI	none
pBI-L	Baron, *et al.* (1995)	pBI-2	luciferase, GOI	none
pBI-GL (control)	Baron, *et al.* (1995)	pBI-1	β-gal, luciferase	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

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 X construct. Gene X 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. A pTRE-derived plasmid can be used in both Tet-Off and Tet-On systems.
rTetR	The reverse Tet repressor protein. In *E. coli,* rTetR binds specifically to tetO and blocks transcription of the tet operon in the presence of Tc. It is the tetO-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 tetO and blocks transcription of the tet operon in the absence of Tc. It is the tetO-binding component of rtTA in Tet-Off Systems.
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.

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