Clontech® Laboratories, Inc.

Tet-One™ Inducible Expression System User Manual

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(010814)

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I. Introduction

A. Summary

The Tet-One Systems are inducible gene expression systems for mammalian cells that contain all the necessary components in a single plasmid, lentiviral, or retroviral vector. After transfecting target cells with plasmid (Tet-One System), or transducing them with lentivirus (Lenti-X™ Tet-One Systems) or retrovirus (Retro-X™ Tet-One systems), the cells will express the Tet-On® 3G transactivator protein and contain a gene of interest (GOI) under the tight control of a TRE3G promoter (P_{TRE3GS}). This manual describes the plasmid-based **Tet-One Inducible Expression System** (Cat. No. 634301). Using this system, your target cells will express high levels of your GOI, but only when cultured in the presence of doxycycline (Dox) (Figure 1).

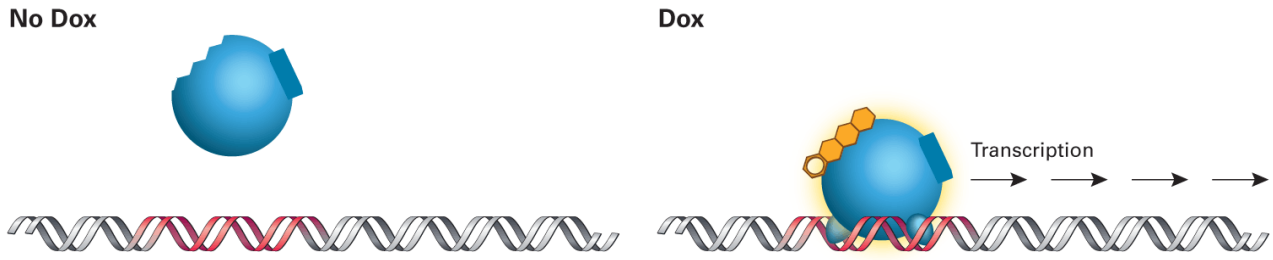

Figure 1. The Tet-On 3G and Tet-One Systems allow inducible gene expression in the presence of Dox.

B. Elements of Tet-One Systems

Tet-On 3G Transactivator Protein

Based on the transcriptional regulators described by Gossen & Bujard (1992), Gossen *et al.* (1995), and Urlinger *et al.* (2000), Tet-On 3G is a modified form of the Tet-On Advanced transactivator protein which has been evolved to display far higher sensitivity to doxycycline (Zhou *et. al,*. 2006).

P_{TRE3GS} Inducible Promoter

The inducible promoter P_{TRE3G} provides for very low basal expression and high maximal expression after induction (Loew *et. al.*, 2010). It consists of 7 repeats of a 19 bp *tet* operator sequence located upstream of a minimal CMV promoter. P_{TRE3GS} is a version of P_{TRE3G} that was modified for higher performance in a single vector context. In the presence of Dox, Tet-On 3G binds specifically to P_{TRE3GS} and activates transcription of the downstream GOI. P_{TRE3GS} lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction.

Tet-One Systems "All-in-One" Design

Before the Tet-One Systems were developed, Clontech's Tet-On and Tet-Off® products all required two separate vectors to introduce the transactivator protein and the inducible promoter controlling your gene of interest, respectively, into your target cells. The Tet-One Systems provide both of these components on a single vector. The Tet-On 3G transactivator is expressed in the forward direction from the human phosphoglycerate kinase 1 promoter, and the cloned gene of interest is expressed from the P_{TRE3GS} promoter in the reverse orientation. Compared to the two-vector Tet-On 3G Systems, all previously published all-in-one vectors have shown a low signal-to-noise ratio, typically providing only 50–100-fold induced expression, even in selected clones. Clontech's Tet-One Systems are based on an all-in-one design that has shown up to 25,000-fold induction (Heinz *et. al,*. 2011).

C. Doxycycline

Doxycycline is a synthetic tetracycline derivative that is the effector molecule for all Tet-On and Tet-Off Systems. When bound by Dox, the Tet-On 3G protein undergoes a conformational change that allows it to bind to *tet* operator sequences located in the P_{TRE3GS} promoter (Figure 1). The Dox concentrations required for induction are far below cytotoxic levels for either cell culture or transgenic studies, and Tet-On 3G responds to even lower concentrations than its predecessors (Zhou *et. al*,. 2006). Note that Tet-On Systems (including Tet-One) respond well only to doxycycline, and not to tetracycline (Gossen & Bujard, 1995). The half-life of Dox in cell culture medium is 24 hours. To maintain continuous inducible GOI expression in cell culture, the medium should be replenished with Dox every 48 hours.

II. List of Components

Store all components at -20°C.

Tet-One Inducible Expression System (Cat. No. 634301)

- 1 each pTetOne Vector Set (Cat. No. 634303; not sold separately)
  - 20 µl pTetOne Vector (500 ng/µl)
  - 20 µl pTetOne-Luc Control Vector (500 ng /µl)
  - 40 µl Linear Hygromycin Marker (50 ng/µl) (also sold separately as Cat. No. 631625)
  - 40 µl Linear Puromycin Marker (50 ng/µl) (also sold separately as Cat. No. 631626)
- 100 rxns Xfect™ Transfection Reagent (Cat. No. 631317)
  - 2 tubes Xfect Polymer (75 µl each)
  - 2 tubes Xfect Reaction Buffer (12 ml each)
- 50 ml Tet System Approved FBS, US-Sourced (Cat. No. 631105)

III. Additional Materials Required

The following reagents are required but not supplied.

A. Tetracycline-Free Fetal Bovine Serum

Contaminating tetracyclines, often found in serum, will significantly elevate basal expression when using Tet-On 3G. The following functionally tested tetracycline-free sera are available from Clontech:

Cat. No.	Serum Name
631106	Tet System Approved FBS (500 ml)
631107	Tet System Approved FBS (50 ml)
631101	Tet System Approved FBS, US-Sourced (500 ml)
631105	Tet System Approved FBS, US-Sourced (50 ml)

B. Antibiotics for Selecting Stable Cell Lines

Table 1. Recommended Antibiotic Concentrations for Selecting & Maintaining Stable Cell Lines

		Recommended Concentration (µg/ml)	
Cat. No.	Antibiotic	Selecting Colonies[1]	Maintenance
631306	Puromycin (100 mg)	0.25–10	0.25
631305	Puromycin (25 mg)		
631309	Hygromycin B (1 g)	50–400	100

[1] When selecting for single colonies, the appropriate dose must be determined empirically for your specific cell line. Test a dosage range using dishes of untransfected cells and choose the dose that kills all of the cells in 3–5 days. If all the cells die in less than 24 hr, you should use a lower dose.

C. Mammalian Cell Culture Supplies

- Culture medium, supplies, and additives specific for your target cells
- Trypsin/EDTA (e.g., Sigma, Cat. No. T4049)
- Cloning cylinders or discs for isolating colonies of adherent cell lines (Sigma, Cat. No. C1059*)*
- Cell Freezing Medium, with or without DMSO (Sigma, Cat. Nos. C6164 or C6039), for freezing stable Tet-One cell lines.
- 6-well, 12-well & 24-well cell culture plates, 10 cm cell culture dishes

D. Doxycycline

- 5 g Doxycycline (Cat. No. 631311)

Dilute to 1 mg/ml in double distilled H_2O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.

E. Xfect Transfection Reagent

Xfect Transfection Reagent provides high transfection efficiency and low cytotoxicity for most commonly used cell types.

Cat. No.	Transfection Reagent
631317	Xfect Transfection Reagent (100 rxns)
631318	Xfect Transfection Reagent (300 rxns)

F. In-Fusion® HD Cloning System

In-Fusion is a revolutionary technology that greatly simplifies cloning.

For more information, visit www.clontech.com/infusion

Cat. No.	In-Fusion Cloning Kit
638909	In-Fusion HD Cloning Plus (10 rxns)
638910	In-Fusion HD Cloning Plus (50 rxns)
638911	In-Fusion HD Cloning Plus (100 rxns)

G. Stellar™ Competent Cells

We recommend using Stellar Competent Cells (see Section V), which are included in the In-Fusion HD Cloning Kits listed in Section III.F. You can also purchase these cells separately (Cat. No. 636763).

H. TetR Monoclonal Antibody

If you wish to confirm that Tet-On 3G is expressed in your cells, we recommend that you use the following antibody and detect the protein via Western Blot.

Cat. No.	Antibody
631131	TetR Monoclonal Antibody (Clone 9G9) (40 µg)
631132	TetR Monoclonal Antibody (Clone 9G9) (200 µg)

I. Plasmid Purification (Transfection-Grade)

Cat. No.	Product	Size
740412.10	NucleoBond Xtra Midi Plus	10 preps
740416.10	NucleoBond Xtra Maxi Plus	10 preps
740422.10	NucleoBond Xtra Midi EF Plus	10 preps
740426.10	NucleoBond Xtra Maxi EF Plus	10 preps

J. Luciferase Assay and Luminometer

These items are required when using the pTetOne-Luc Vector as a control to test for induction (Section VI.B). Use any standard luciferase assay system and luminometer.

IV. Protocol Overview

Please read each protocol completely before starting. Successful results depend on understanding and performing the following steps correctly.

A. General Cell Culture

This user manual provides only general guidelines for mammalian cell culture techniques. For users requiring more information on mammalian cell culture, transfection, and creating stable cell lines, we recommend the following general reference:

Freshney, R.I. (2005). *Culture of Animal Cells: A Manual of Basic Technique, 5th Edition* (Wiley-Liss, Hoboken, NJ).

B. Protocol Summary

The following are the steps required to create a doxycycline-responsive cell line capable of inducible expression of your gene of interest (GOI) (see Figure 2).

1. Clone your gene of interest into the pTetOne Vector using In-Fusion HD (Section V).
2. Pilot test Tet-based induction of your construct (Section VI).
3. Create and screen for a stable TetOne clone capable of high induction of your GOI (Section VII).

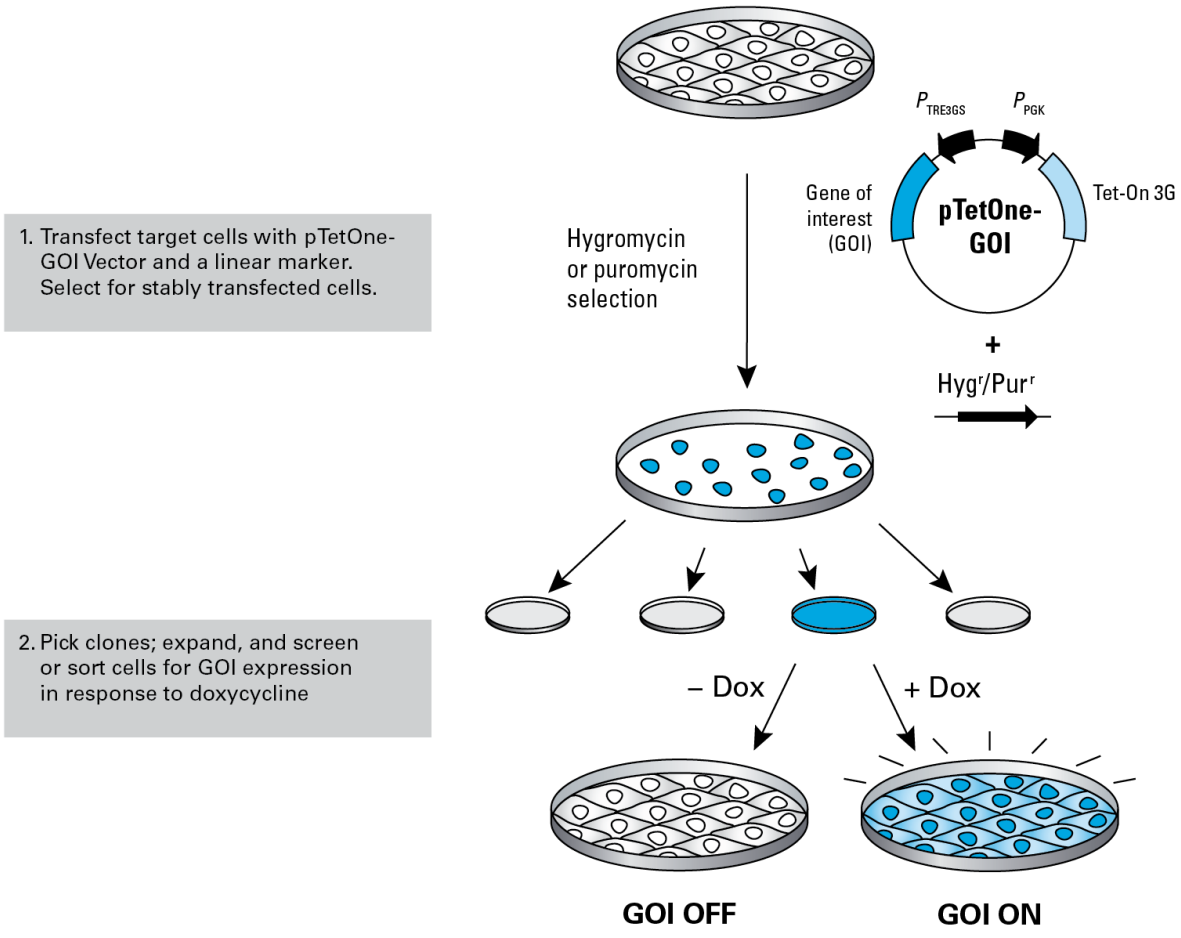

Figure 2. Establishing an inducible expression system in target cells with Tet-One. Target cells are cotransfected with the pTetOne plasmid containing your gene of interest and one of the linear selection markers (Hyg^R or $Puro^R$). Clones are then selected with antibiotic, expanded, and screened for doxycycline-inducible expression of your gene of interest.

V. Cloning Your Gene of Interest into a pTetOne Vector using In-Fusion HD

We recommend using In-Fusion HD for all cloning. Follow the protocol outlined in the In-Fusion HD user manual at **www.clontech.com/infusion**

NOTE: Stellar Competent Cells (Section III.G) are recommended by Clontech for In-Fusion cloning. Stellar Competent Cells are provided with all In-Fusion HD Cloning Systems.

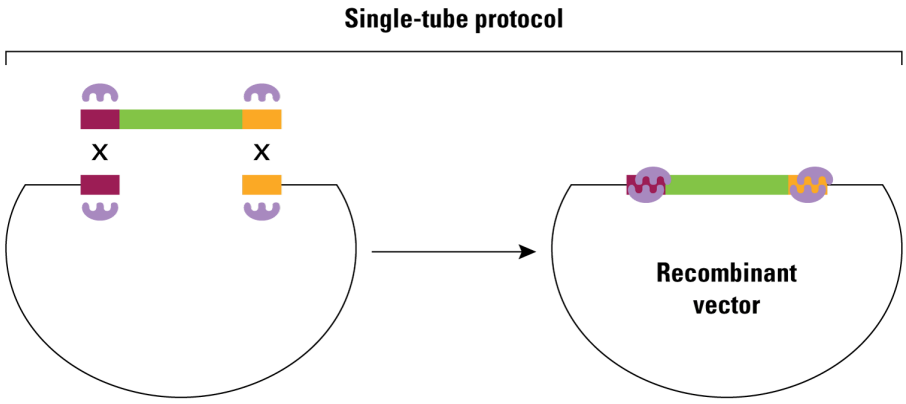

Figure 3. The In-Fusion HD Single-Tube Cloning Protocol.

The recommended linearization sites and forward/reverse primer designs are as follows:

Vector	Linearize w/	Forward Primer*	Reverse Primer**
pTetOne	EcoRI & BamHI	CCCTCGTAAAGAATTC 111 222 333 444 555 666 777 888	GCAGAGATCTGGATCC sss nnn nnn nnn nnn nnn nnn nnn

*111 = Start codon of your gene; 222 = 2nd codon of your gene; etc.

**SSS = reverse compliment of the stop codon of your gene; NNN = reverse compliment of the end of your gene.

VI. Pilot Testing Tet-Based Induction of Your Construct

Prior to establishing the stable Tet-One cell line for your GOI, your pTetOne construct should be tested for functionality. Transiently transfect your pTetOne vector into an easy-to-transfect cell line such as HeLa or HEK 293, or your target cell line, and test for GOI induction with Dox. You will need an appropriate gene-specific assay to test for induction, such as:

- Western blot
- Northern blot
- qRT-PCR
- Gene-specific functional assay

pTetOne-Luc can be used as a positive control.

A. Materials Required

1. pTetOne Vector containing your gene of interest (Section V)
2. Host cell line
3. Xfect Transfection Reagent (Section III.E)
4. Doxycycline (1 mg/ml) (Section III.D)
5. Mammalian cell culture supplies (Section III.C)
6. Tet Approved FBS (Section III.A)

B. Protocol

1. Transfect the pTetOne Vector into your target cells (in a 6-well plate) using Xfect Transfection Reagent. Follow the **Xfect Transfection Reagent Protocol-At-A-Glance**. (Locate this protocol by searching at **www.clontech.com/manuals**).
   - Use 5 µg of pTetOne-GOI for each well (GOI = gene of interest).
   - We recommend performing the test in duplicate with negative controls: 3 wells containing 100 ng/ml of Dox, and 3 wells without Dox.



Wells 1 & 2: 5 µg pTetOne-GOI (no Dox)

Wells 3 & 4: 5 µg pTetOne-GOI (100 ng/ml Dox)

Well 5: 5 µg pTetOne empty (no Dox)

Well 6: 5 µg pTetOne empty (100 ng/ml Dox)

Figure 4. Transfection of the pTetOne plasmids into target cells in a 6-well plate.

2. After 24 hr, harvest the cell pellets from each well and compare induced expression levels to uninduced expression levels using a method appropriate for your GOI.

NOTE: Because transiently transfected cells contain more copies of the pTetOne plasmid than do stable cell lines, fold induction (ratio of maximal to basal GOI expression) levels are almost always lower in transient assays (e.g., by 10–100 fold) than in properly selected stable clonal cell lines.

VII. Creating & Screening for a Tet-One Stable Cell Line Capable of High Induction of your GOI

A. Materials Required

1. pTetOne-GOI Vector (Section V)
2. Linear Hygromycin/Puromycin Marker (Section II)
3. Target Cell Line
4. Xfect Transfection Reagent (Section III.E)
5. Hygromycin or Puromycin (Section III.B)
6. Doxycycline (1 mg/ml) (Section III.D)
7. Mammalian cell culture supplies (Section III.C)
8. Tet Approved FBS (Section III.A)

B. Protocol: Creating a Stable Tet-One Inducible Cell Line

To generate a highly inducible stable Tet-On 3G inducible cell line, transfect your customized pTetOne vector into your target cell line along with a linear selection marker (Hyg^r or Pur^r). Select stable transfectants by screening for hygromycin or puromycin resistance, and inducibility.

NOTE: Working with mixed (polyclonal) populations of transfected cells rather than selecting for single clones can affect the consistency of induction, due to the possible outgrowth of poorly inducing clones as the cells are passaged.

Why use linear selection markers? See Appendix B.

1. Plate (seed) your target cell line in a single well of a 6-well plate at a density sufficient to reach near confluence at 48 hr after transfection.
2. Using Xfect transfection reagent (PT5003-2 from www.clontech.com/manuals), cotransfect the following:
   - 2 µg pTRE3G-GOI
   - 100 ng Linear Selection Marker (puromycin or hygromycin)

   NOTE: Always combine your customized pTetOne vector and either the Linear Hygromycin Marker or the Linear Puromycin Marker at a ratio of 20:1 (i.e., use 20-fold less of the linear marker).
3. After 48 hr, split the confluent cells into 4 x 10 cm dishes (do not add the selective antibiotic yet).
4. After an additional 48 hr, add hygromycin or puromycin at the selection concentration that is optimal for your cell line (Section III.B).
5. Replace medium with fresh complete medium plus hygromycin (or puromycin) every four days, or more often if necessary.

6. Cells that have not integrated the plasmid should begin to die after ~3–5 days.

   NOTE: Avoid passaging the cells a second time, since replating cells under selection may result in plates containing too many colonies for effective colony isolation (because individual colonies are not well-separated).

7. After ~2 weeks, drug-resistant colonies should begin to appear.

8. When the colonies are large enough to transfer, use cloning cylinders or disks to harvest (i.e. "pick") large, healthy colonies, and transfer each into a separate well of a 24-well plate.

   - Isolate as many clones as feasible, so that 10– 20 clones are available for testing.
   - Suspension cultures must be cloned using a limiting dilution technique (see Appendix C).

9. Culture the clones in maintenance concentrations of hygromycin (or puromycin) (Section III.B). When confluent, split the cells from each well into three wells of a 6-well plate for testing and maintenance (Section VII.C).

C. Protocol: Screening Your Tet-One Inducible Clones

Test individual stable clones for expression of your GOI in the presence and absence of Dox. Choose clones that generate the highest maximal and lowest basal expression levels, i.e., the highest fold induction.

1. For each clone to be tested, seed 1/3 of the total amount of cells (see Section VII.B, Step 9) into a single well of a 6-well plate. The cells in this "stock plate" may be propagated, depending upon the results of the inducibility assay.

2. Divide the remaining 2/3 of the cells between duplicate wells of a second 6-well plate. Add Dox (100 ng/ml) to one of the wells and incubate the cells for 48 hr.

3. Harvest the cells and use an assay specific for your GOI to compare induced to uninduced expression of your GOI.

4. Select clones with the highest fold induction for propagation and further testing.

5. Expand and freeze stocks of each promising clone as soon as possible (Appendix D).

   NOTE: Once you have chosen the best clone(s), you may choose to determine the minimal concentration of Dox that is required for high inducible expression and use that minimal concentration for all subsequent experiments. Remove the cells from one nearly confluent well (of a 6-well plate) and divide them among six wells of a 24-well plate. Titrate doxycycline concentrations across these 6 wells (e.g., 0, 1, 10, 50, 100, and 1,000 ng/ml), and assay for induced expression after 24 hr. Typically, there is no need to use more than 100 ng/ml Dox, since maximal expression is often obtained with just 10 ng/ml Dox.

VIII. References

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 bibliography 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 website.)

Freshney, R.I. (2005). *Culture of Animal Cells: A Manual of Basic Technique, 5th Edition* (Wiley-Liss, Hoboken, NJ).

Sambrook, J., Fritsch, E. F. & Maniatis, T., eds. (2001) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY).

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Loew, R., Heinz, N., Hampf, M., Bujard, H. & Gossen, M. (2010) Improved Tet-responsive promoters with minimized background expression. *BMC Biotechnol.* **10**:81 (24 November 2010).

Sambrook, J., Fritsch, E. F. & Maniatis, T., eds. (2001). *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY).

Urlinger, S., Baron, U., Thellmann, M., Hasan, M.T., Bujard, H. & Hillen, W. (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: Novel mutations yield expanded range and sensitivity. *Proc. Natl. Acad. Sci. USA* **97**(14):7963–7968.

Zhou, X., Vink, M., Klave, B., Berkhout, B. & Das, A. T. (2006) Optimization of the Tet-On system for regulated gene expression through viral evolution. *Gene Ther.* **13**(19):1382–1390.

IX. Troubleshooting

Table 2.Troubleshooting Guide for the Tet-One Inducible Expression System

Description of Problem	Possible Explanation	Solution
A. Inducing Expression		
Low fold induction (ratio of maximal to basal expression of the GOI)	Cellular sequences adjacent to the integration site of some clones may affect their expression profiles.	Screen additional clones (Section VII)
	Cells were harvested and analyzed too soon or too late.	Harvest and analyze cells between 18–48 hr.
	Poor transfection efficiency	• Optimize transfection protocol. • Optimize density of cell plating; use at 60–90% confluency.
	Poor target cell viability	• Optimize passage number of target cells. • Optimize culture conditions of target cells. • Optimize tissue culture plasticware
	The FBS used in the cell culture medium contains tetracycline derivatives.	Use Clontech's Tet System Approved FBS (Section III.A), which was functionally tested with Clontech's stable CHO-AA8-Luc Tet-Off Control Cell Line.
Low fold induction of GOI expression in selected drug-resistant stable cell clones.	Cellular sequences flanking the integrated pTetOne expression construct may affect GOI expression.	Screen additional individual drug-resistant cell clones to ensure optimal fold induction.
	Mixed cell population in the selected clone (see Section VII.B Note).	
Decrease in fold induction after several passages or Loss of inducibility after passaging of a (previously frozen) stable cell line.	The appropriate antibiotics are missing from the cell culture medium.	Maintain optimal antibiotic concentrations (Section III.B).
	Mixed cell population in the selected clone (see Section VII.B Note).	Reselect the current cell line through single colony selection (Section VII.C).

Description of Problem	Possible Explanation	Solution
B. Establishment of Stable Cell Lines		
Untransduced cells do not die at the high antibiotic concentration established via titration in Section II.B	The cells have not been recently passaged, so they remain well-attached to the plate surface even when they are dead.	To determine the appropriate antibiotic concentration, use cells that have been split within the last 2–3 days.
There are no surviving cells after transfection/cotransfection with a drug-resistant expression cassette at the antibiotic concentration determined to be optimal in Section II.B	The antibiotic concentration which caused massive cell death when determining the appropriate dose via titration could be too high.	Use a lower antibiotic concentration for selection of stably transfected cell clones.
Low number of drug resistant clones	Transfection was inefficient because cells used for transfection were of unsatisfactory quality, resulting in inefficient uptake of DNA during transfection.	Use cells for transfection at passages no higher than 15–17 since defrosting, and no older than 2–3 days since the last split. Passage cells 3–4 times after defrosting to allow a complete cell recovery prior to transfection experiments.
	Inefficient transfection due to using the wrong ratio of Vector/Linear Selection Marker.	Check the ratio of Vector/Linear Selection Marker. Retransfect Vector/Linear Selection Marker at a ratio of 20:1 (Section VII.B).
	Antibiotic was added too soon.	See protocol in Sections VII.B.
	Used wrong antibiotic concentration.	See Section III.B.
Too many colonies for effective colony isolation (individual colonies are not well-separated)	• Cells were not split and/or diluted correctly. • Antibiotic was added too late. • Transfected cells were passaged a second time after addition of antibiotic.	See protocols in Section VII.B.
	Used wrong antibiotic concentration.	See Section III.B
Poor cell viability	Cells were not properly frozen.	See Appendix D, Section A.
	Cells were not properly thawed.	See Appendix D, Section B.
C. Detection and Inhibition of Expression		
No detectable GOI expression by Western Blot.	Low sensitivity of detection method.	Check sensitivity of primary and secondary antibodies. Analyze GOI expression by qRT-PCR, using different sets of primers to ensure optimal detection of GOI expression.
Continuous protein expression after the removal of doxycycline	Depending on the stability of the protein, it may persist in the cell in the absence of gene induction and de novo synthesis of GOI mRNA. Fluorescent proteins tend to have long half-lives.	Add a ProteoTuner™ destabilization domain to your protein of interest and control its stability through the addition/removal of Shield1 ligand
	Doxycyline was not completely removed from the cell culture medium.	Wash cells three times with PBS, followed by trypsinization and replating in fresh medium supplemented with Clontech's Tet System Approved FBS. If trypsinization is undesirable, wash cells three times with medium and three times with PBS, then replace with fresh medium supplemented with Tet System Approved FBS.

Appendix A: Tet-One Vector Information

For complete descriptions of the vectors provided with each system, refer to the enclosed Certificate of Analysis, which is also available at www.clontech.com

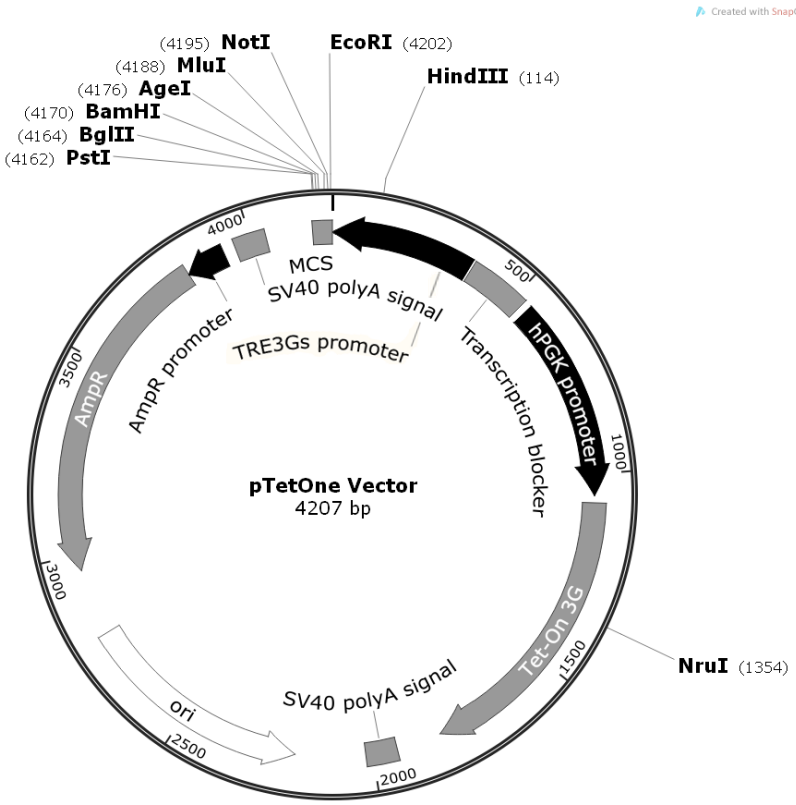

Figure 5. pTetOne Vector Map.

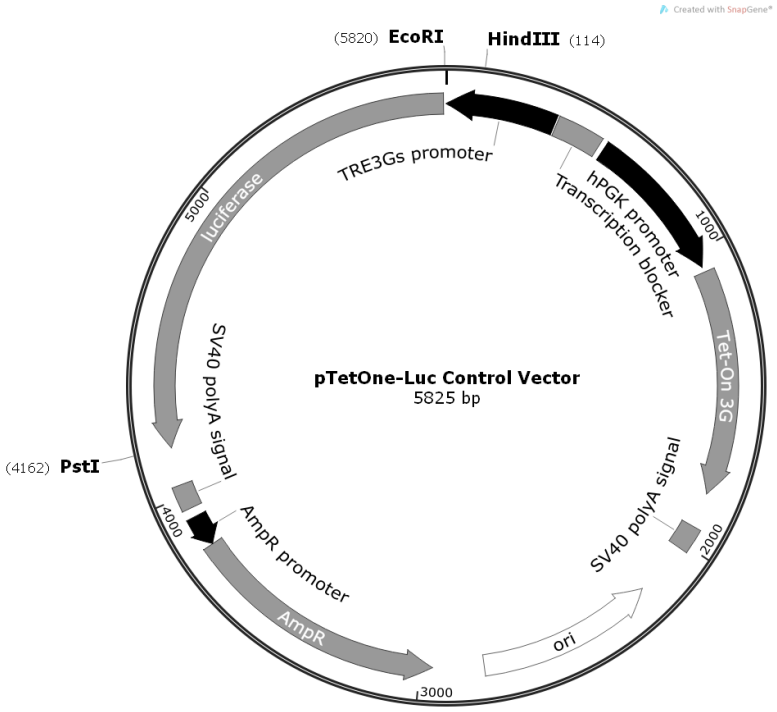

Figure 6. pTetOne-Luc Control Vector Map

Appendix B: Why Use a Linear Selection Marker?

Linear selection markers are short, purified linear DNA fragments that consist of the marker gene (Hyg^r or Pur^r), an SV40 promoter, and the SV40 polyadenylation signal. Use of a linear selection marker allows you to screen fewer clones to obtain your desired clone; plus, you'll observe a higher fold induction in the clones that you select.

Why is this? Because there is **less interference with basal expression of the gene of interest** from the promoter of a cotransfected linear selection marker than would result from the promoter of a selection marker present on the pTRE3G-GOI plasmid itself.

This is due to the fact that stable integration of plasmids usually results in co-integration of multiple copies of that plasmid at a single locus. If pTetOne were supplied with a constitutive selectable marker included on the plasmid backbone (i.e., a constitutive promoter at an automatic 1:1 ratio to the TRE promoter), the constitutive promoter used for the selection marker could affect basal expression in many of the clones by a combination of:

- its juxtaposition with the TRE in one or more of the tandem integrations **or**
- the recruitment of a high concentration of endogenous transcription factors to the region

However, since the **linear selection markers are cotransfected at a decreased ratio of 1:20** relative to the pTREG-GOI plasmid (i.e., 20-fold less of the linear marker), these types of interference are less likely to occur.

Appendix C: Selecting Stable Clones via Limited Dilution of Suspension Cells

To avoid creating a cell line containing a mixture of clones, suspension cells must be selected using a limited dilution technique. The following protocol allows you to dilute stably transfected cells in a manner ensuring that only one stable cell clone is seeded per well in a 96-well plate—and then use that clone to test for inducible expression.

A. Protocol

1. Seed one well of a 6-well plate with 1–1.5 x 10^6 cells in 3 ml of complete growth medium.
2. Using Xfect transfection reagent, transfect these cells with 2 µg of pTetOne-GOI + 100 ng of the puromycin or hygromycin linear selection marker according to the **Xfect Transfection Reagent Protocol-At-A-Glance**. (Locate this protocol by searching at **www.clontech.com/manuals**).
3. 48 hr after transfection, centrifuge at 1,100 rpm to harvest the cells, and resuspend them in 6 ml of medium in a T25 flask containing the appropriate antibiotic to select for stable integrants.
4. Allow the cells to grow for 1 week.
5. Dilute the cells from Step 4 to 1 cell per well in a 96-well plate as follows:
    a. Dilute a 100 µl aliquot of the cells in 2 ml of complete medium (1/20 stock dilution).
    b. Set up four vials containing 5 ml of complete growth medium. From the 1/20 stock dilution created in Step 5.a, add:
        i. 10 µl to Vial 1
        ii. 20 µl to Vial 2
        iii. 30 µl to Vial 3
        iv. 40 µl to Vial 4
    c. Mix well.
    d. From Vial 1, add one 50 µl aliquot per well to each well of a 96-well plate. Repeat this process for Vials 2–4 on separate 96-well plates (four plates total—one for each vial).
6. Allow the cells on each of the four 96-well plates to grow until growth is visible in half of the wells on one of the plates.
7. Choose 24 clones only from the plate that shows growth in approximately half of its wells. Expand each of these clones to fill one well of a 24-well plate and then one well of a 6-well plate.

    NOTE: If one of the 96-well plates shows growth in only half of its wells, this means that on average there was less than one cell per well on that plate when they were seeded (Step 5.d), so the cells in the wells that show growth are likely to have been derived from a single cell clone.

8. When each of the 24 clones in Step 7 has grown sufficiently to fill 3 wells of a 6-well plate, maintain the cells from one well as the reference stock, and test the cells in the other two wells for inducible expression with and without Dox (see SectionVII.C).

Appendix D: Preparing and Handling Cell Line Stocks

A. Protocol: Freezing Cell Line Stocks

Once you have created and tested your Tet-One cell line, you must prepare multiple frozen aliquots to ensure a renewable source of cells, according to the following protocol:

1. Expand your cells to multiple 10 cm dishes or T75 flasks.
2. Trypsinize and pool all of the cells, then count the cells using a hemocytometer.
3. Centrifuge the cells at 100 x g for 5 min. Aspirate the supernatant.
4. Resuspend the pellet at a density of at least 1–2 x 106 cells/ml in freezing medium. Freezing medium can be purchased from Sigma (Cat. Nos. C6164 & C6039), or use 70–90% FBS, 0–20% medium (without selective antibiotics), and 10% DMSO.
5. Dispense 1 ml aliquots into sterile cryovials and freeze slowly (1°C per min). For this purpose, you can place the vials in Nalgene cryo-containers (Nalgene 6. 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.
6. The next day, remove the vials from the cryo-containers or styrofoam containers, and place in liquid nitrogen storage or an ultra-low temperature freezer (–150°C) for storage.
7. Two or more weeks later, plate a vial of frozen cells to confirm viability.

B. Protocol: Thawing Cell Line Frozen Stocks

To prevent osmotic shock and maximize cell survival, use the following procedure to start a new culture from frozen cells:

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.
2. 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 such as G418). Mix gently.
3. Slowly add an additional 4 ml of fresh, prewarmed medium to the tube and mix gently.
4. Add an additional 5 ml of prewarmed medium to the tube and mix gently.
5. Centrifuge at 100 x g for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium without selective antibiotics. (This method 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.)

6. 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 in a 37°C humidified incubator (5–10% CO_2 as appropriate) for 24 hr.

   NOTE: For some loosely adherent cells (e.g. HEK 293-based cell lines), we recommend using collagen-coated plates to aid attachment after thawing. For suspension cultures, suspend cells at a density of no less than 2 x 10^5 cells/ml.

7. 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.

   NOTE: Note: For some loosely adherent cell lines (e.g., HEK 293-based cell lines), complete attachment of newly thawed cultures may require up to 48 hr.

8. Expand the culture as needed. The appropriate selective antibiotic(s) should be added to the medium after 48–72 hr in culture. Maintain in complete culture medium containing a maintenance concentration of hygromycin (or puromycin), as appropriate (Section III.B).

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