User Manual

Tet-On® Advanced Inducible Gene Expression Systems User Manual

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I. Introduction

A. Summary

The **Tet-On Advanced Inducible Gene Expression System** is a tightly regulated and highly responsive system that produces on-demand, robust expression of your gene of interest (GOI) in target cells (*Clontechniques,* April 2006). The system is established in target cells by sequentially transfecting them with the provided vectors and selecting stable cell lines. Target cells that express the Tet-On Advanced transactivator, and that also contain an integrated TRE-based expression vector (e.g., pTRE-Tight) will express high levels of your GOI when cultured in the presence of the system's inducer, doxycycline (Dox) (Figure 1).

B. Elements of Tet-On Advanced Induction

Based on the original tetracycline (Tc)-regulated transcriptional transactivators described by Gossen & Bujard (1992) and Gossen *et al.* (1995), Tet-On Advanced is a modified transactivator protein that is optimized for expression in mammalian cells, and which demonstrates higher sensitivity and fidelity than previous versions (Urlinger, *et al.* 2000; *Clontechniques,* January 2007). The inducible promoter, P_{Tight}, provides for very low basal expression and tightly controlled induction.

- **The Tet-On Advanced transactivator.** The pTet-On Advanced vector constitutively expresses the tetracycline-controlled transcriptional transactivator, Tet-On Advanced (Urlinger *et al.*, 2000). This engineered protein consists of a mutant *E. coli* TetR protein (rTetR) fused to three minimal "F"-type activation domains derived from the herpes simplex virus VP16 protein (Baron et al., 1997, Triezenberg et al., 1988). In the presence of Dox, Tet-On Advanced binds to the *tet*O sequences in P_{Tight}, and activates high level transcription from this inducible promoter. The Tet-On Advanced coding sequence is fully synthetic and utilizes human codon preferences to increase its expression level and stability in mammalian cells.
- **The P_{Tight} inducible promoter.** This is an inducible promoter that controls transcription of your GOI. The P_{Tight} composite promoter was originally developed as the P_{tet-14} promoter in the laboratory of Dr. H. Bujard and consists of a modified Tet-Responsive Element (TRE_{mod}) containing 7 direct repeats of the *tet* operator sequence, *tet*O, which is joined to a minimal CMV promoter ($P_{minCMV\Delta}$) (*Clontechniques,* April 2003). P_{Tight} lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction. In the presence of Dox, Tet-On Advanced binds tightly and specifically to P_{Tight} and activates transcription of the downstream GOI (Figure 1).

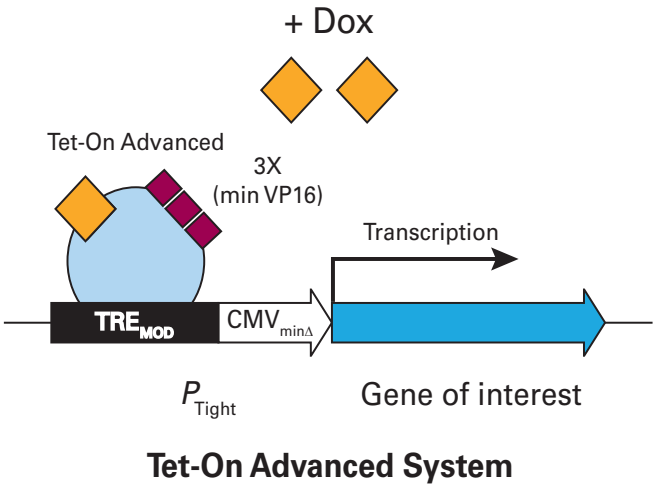

Figure 1. Induction in the Tet-On Advanced System. The Tet-controlled transactivator, Tet-On Advanced, is a fusion protein derived from a mutant version of the *E. coli* Tet repressor protein, rTetR, which is joined to three minimal transcription activation domains from the HSV VP16 protein. In the presence of doxycycline (Dox), Tet-On Advanced binds to the tetracycline response element (TRE_{Mod}) in P_{Tight}, and produces high-level transcription of the downstream gene of interest.

I. Introduction continued

C. Benefits of the Tet-Advanced Expression Systems

The Tet-On Advanced System produces very high maximal expression coupled with extremely low basal promoter activity to yield very high induction levels that are both highly sensitive and concentration dependent. Advantages over other inducible mammalian gene expression systems are listed below.

- **Extremely tight regulation.** In the absence of induction, the Tet-On Advanced transactivator shows virtually no residual binding to the TRE in P_{Tight}. Thus, basal expression is extremely low and often undetectable.
- **Highly specific.** The rTetR portion of the Tet-On Advanced transactivator bind very specifically to the *tet*O target sequences of P_{Tight} and does not activate off-target cellular genes. This high degree of specificity may be due in part to the prokaryotic nature of these components acting within the context of a large eukaryotic genome lacking similar elements (Harkin *et al.,* 1999).
- **No pleiotropic effects.** Tc and Dox are prokaryotic antibiotics that have no known effects on eukaryotic cells when used at the concentrations required by the Tet-Advanced Systems,
- **High inducibility and fast response times.** In properly screened clones, maximal induction of the Tet-On Advanced System is often several thousand-fold and can be detected within 30 minutes after addition of Dox to the culture medium. In contrast, other mammalian systems often exhibit slow induction (up to several days), incomplete induction (compared to repressor-free controls), and low overall induction (often no more than 100-fold). Other systems may also require high, nearly cytotoxic levels of inducer (reviewed by Gossen *et al.,* 1993; Yarronton, 1992).
- **Highest 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 effector.** In contrast to effectors used in other systems, such as ecdysone, Dox is inexpensive, well-characterized, and yields highly reproducible results. Dox binds with high affinity to Tet-On Advanced and is essentially nontoxic at the effective concentrations. Note that Tet-On Advanced Systems respond only to Dox, and not to Tc (Gossen & Bujard, 1995).
- **Promoter activation is superior to repression.** Repression-based systems require very high levels of repressor to ensure 100% occupancy of the regulatory sites and fully shut-off transcription. The presence of high repressor levels also prevents rapid, high-level induction (Yao *et al.*, 1998). For a more complete discussion of the advantages of transcription activation versus repression, see Gossen *et al.* (1993).
- **The Tet-On Advanced and Tet-Off Advanced Expression Systems offer versatile expression control strategies for transgenic mice.** The Tet System has become the *de facto* method of choice for generating reversibly inducible transgenic lines (Gossen & Bujard, 2002). More than 280 mouse lines have been described that express Tet transactivator genes under the control of a variety of tissue-specific promoters or that express target genes under control of Tet-inducible promoters. A list of these mouse lines can be found on the TET Systems website (**http://www.tetsystems.com/support/transgenic-mouse-lines/**). With its greatly increased sensitivity to Dox, the Tet-On Advanced System brings additional advantages to the development of inducible transgenic mice. This may be particularly helpful when control of gene expression in the brain is required, as the presence of the blood-brain barrier limits the concentration of Dox that can be attained in the brain.

D. Doxycycline

The doxycycline concentrations required for induction with Tet-On Advanced Systems are far below cytotoxic levels for either cell culture or transgenic studies. Of note, Tet-On Systems respond only to Dox, and not to Tc (Gossen & Bujard, 1995).

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)

Package Contents

- 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 Marker (50 ng/µl)
- 0.5 ml U2-OS-Luc Tet-On Control Cell Line (~1.0 x 10^6 cells/tube)
- 50 ml Tet System Approved FBS

Product Documents and Manuals (available at www.clontech.com/manuals)

- Tet-On Advanced Inducible Gene Expression System User Manual (PT3898-1)
- pTet-On-Advanced Vector Information Packet (PT3899-5)
- pTRE-Tight Vector Information Packet (PT3720-5)

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

III. Additional Materials Required

A. Mammalian Cell Culture Supplies

- Culture medium, supplies, and additives specific for your target cells.
- Culture medium for the U2-OS-Luc Tet-On Control Cell Line: 90% Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4.5 g/L), 10% Tet System Approved Fetal Bovine Serum (FBS), 4 mM L-glutamine, 100 units/ml penicillin (optional), 100 µg/ml streptomycin (optional), 200 µg/ml G418, and 100 µg/ml hygromycin.
- Tetracycline-free fetal bovine serum (FBS; see important information below). We *strongly recommend* using **Tet System Approved FBS** (Cat. Nos. 631101 & 631106) for culturing target cells.
- Cloning cylinders or discs for isolating colonies of adherent cell lines (PGC Scientific, Cat. No. 62-6150-40, -45 or Cat. No. 62-6151-12, -16)
- Cell Freezing Medium, with or without DMSO (Sigma, Cat. No. C6164 or Cat. No. C6039) for freezing Tet-On Advanced cell lines and double-stable Tet-On Advanced cell lines [Optional].

B. Antibiotics for Selecting Stable Cell Lines

Prior to using antibiotics to select stable cell lines from your transfected cells, determine the optimal selection concentration for each antibiotic as described in Appendix A. For example, the G418 concentration range for selecting stable HeLa cell lines is 400–500 µg/ml. However, each new lot of any selection antibiotic should be titrated.

- **G418,** for selecting single-stable Tet-On Advanced cell lines. G418 is available from Clontech (Cat. No. 631307). Note that the effective weight is approximately 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 culture medium (without supplements). Filter sterilize and store at 4°C.
  - Concentration range for selecting stable cell lines: 50–800 µg/ml
  - Maintenance of stable cell lines: 100 µg/ml
  - Selection concentration (e.g., HEK 293, HeLa cells): 400–500 µg/ml

III. Additional Materials Required continued

B. Antibiotics for Selecting Stable Cell Lines (cont'd)

- **Hygromycin**, for selecting double-stable Tet-On Advanced cell lines transfected with the Linear Hygromycin Marker. Hygromycin B is available from Clontech (Cat. No. 631309).
  - Concentration range for selecting stable cell lines: 50–800 µg/ml
  - Maintenance of stable cell lines: 100 µg/ml
- **Puromycin**, for selecting double-stable Tet-On Advanced cell lines transfected with the Linear Puromycin Marker. Puromycin is available from Clontech (Cat. Nos. 631305 & 631306)
  - Concentration range for selecting stable cell lines: 0.25–10 µg/ml
  - Maintenance of stable cell lines: 0.25 µg/ml

C. Transfection Reagents

- **Xfect™** is a novel, highly efficient, and versatile transfection reagent that forms biodegradable nanoparticles and produces superior transfection results for a wide variety of mammalian cell types (Cat. Nos. 631317 & 631318).
- The **CalPhos™ Mammalian Transfection Kit** is a highly efficient calcium-phosphate-based transfection system (Cat. No. 631312).

D. Doxycycline

Doxycycline (Cat. No. 631311) is needed for inducing expression of your GOI from the transfected pTRE-Tight vector. 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 when testing induction in the U2-OS-Luc Tet-On Control Cell Line, or when using the pTRE-Tight-Luc vector to screen Tet-On Advanced clones. Use any standard luciferase assay system and luminometer.

F. Tetracycline-Free Fetal Bovine Serum (FBS) for Target Cell Culture

Attention

Many lots of bovine sera are contaminated with tetracycline (Tc) or Tc-derivatives which can affect basal expression or inducibility in Tet Systems (Figure 2). *It is critical that the FBS used for cell culture not interfere with Tet-responsive expression.*

- Tc-contaminants will diminish the performance of Tet-On Advanced-based systems by elevating basal expression and reducing fold-induction.
- These problems can be eliminated by using a Tet System Approved FBS (Cat. Nos. 631101 & 631106) from Clontech. These sera have been functionally tested in our Tet Systems and found to be free of contaminating Tc activity.

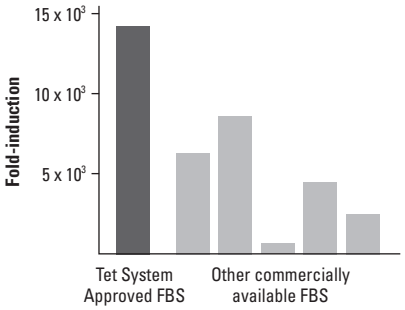

Figure 2. Tetracycline activity in bovine sera. The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different lots of FBS. Average uninduced expression level = 0.21 RLU (n=21, S.D.=0.07); maximum expression levels varied from 123 to 3,176 RLU.

IV. Protocol Overview

PLEASE READ THESE PROTOCOLS IN THEIR ENTIRETY 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. Perform all steps involving cell culture using sterile technique in a tissue culture hood. For users requiring more information on mammalian cell culture, transfection, and creating stable cell lines, we recommend the following general reference:

- *Culture of Animal Cells,* 5th Edition, by R. I. Freshney (2005, Wiley-Liss, NY)

B. Establishing the Tet-On Advanced System in Target Cells

The general strategy for establishing the Tet-On Advanced System is shown in Figure 3, in which target cells are first transfected with pTet-On Advanced to create a stable cell line. Once a suitable Tet-On Advanced cell line (clone) is identified, the cell line is then stably transfected with your customized TRE-based vector containing your GOI.

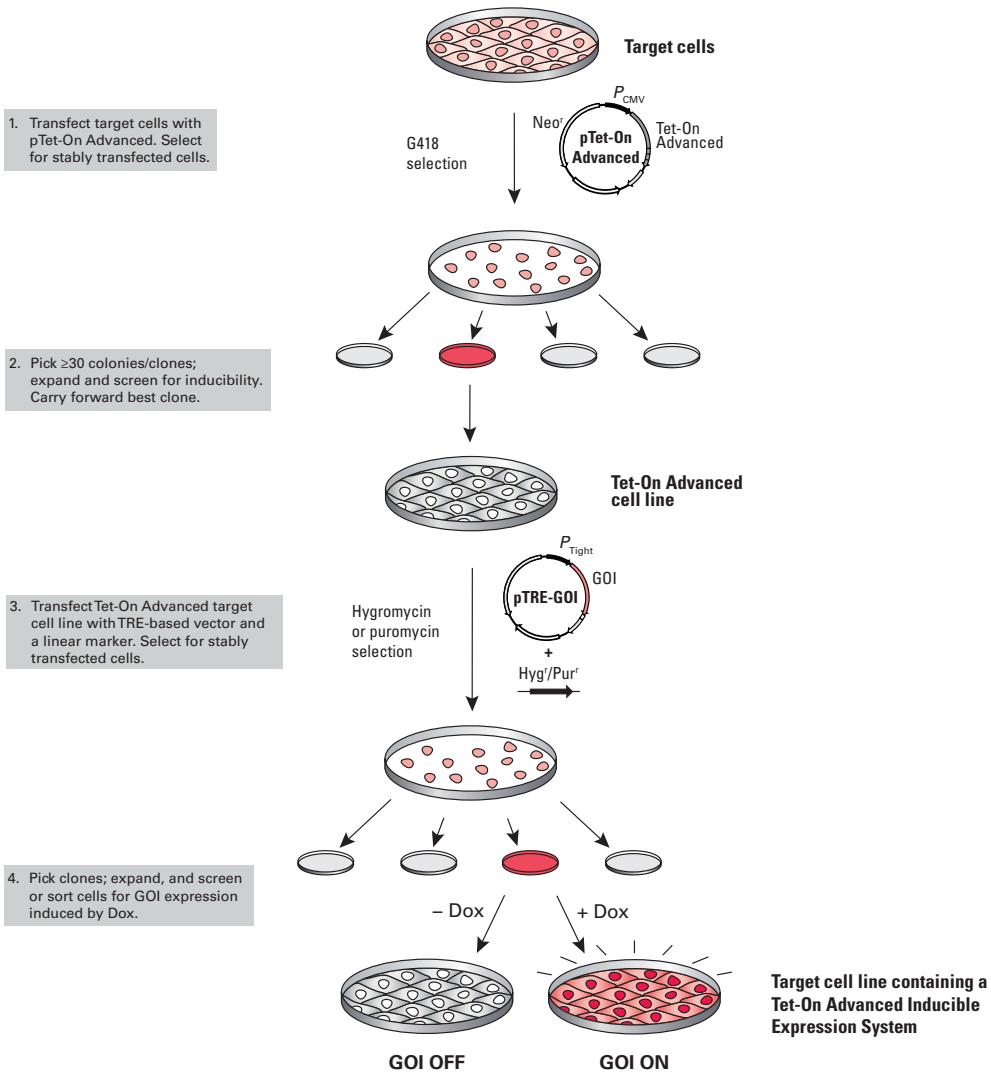

Figure 3. Establishing the Tet-On Advanced System in target cells. Target cells are transfected with the pTet-On Advanced plasmid and selected with G418 to generate a stable Tet-On Advanced cell line. This cell line serves as the host for a TRE-based expression vector, which is transfected into the Tet-On Advanced cell line along with a linear selection marker (Hyg or Pur). After a second round of drug selection, a stable cell line is produced which expresses high levels of the GOI in response to doxycycline (Dox).

V. Plasmid Propagation and Vector Construction

A. General Molecular Biology Techniques

Only general information for propagating, cloning, and purifying plasmid vectors is provided below. For users requiring detailed information on plasmid propagation and cloning, we recommend the following laboratory references:

- *Current Protocols in Molecular Biology* ed. by F. M. Ausubel *et al.* (1995, John Wiley & Sons, NY).
- *Molecular Cloning: A Laboratory Manual* ed. by J. Sambrook *et al.* (2001, Cold Spring Harbor Laboratory Press, NY).

B. Plasmid Propagation & Construction of Your pTRE-Tight Vector

1. To ensure that you have a renewable source of plasmid DNA, transform each of the plasmids provided into a suitable *E. coli* host strain (e.g., **Supercharge EZ10 Electrocompetent Cells**, Cat. No. 636756 or DH5α). See the specific Vector Information Packet supplied with each vector for further DNA propagation details.
2. For plasmids to be used in cloning, grow a sufficient culture volume of transformed bacteria, and purify the plasmid DNA using an appropriate **NucleoBond® Xtra** or **NucleoSpin®** kit (see **www.clontech.com**), or an equivalent purification method.
3. Using standard cloning techniques and appropriate directional restriction sites, clone your GOI (cDNA) fragment in the multiple cloning site (MCS) of pTRE-Tight, or your TRE-based vector of choice (see Appendix A). You may also use Clontech's In-Fusion technology, such as the **In-Fusion™ Advantage PCR Cloning Kit** (Cat. No. 639619). In-Fusion allows PCR products to be easily cloned, without restriction enzyme digestion or ligation, into any linearized vector. To use In-Fusion, you must synthesize PCR primers that are specifically designed for this purpose. For more information, see the In-Fusion Advantage PCR Cloning Kit User Manual (PT4065-1).
4. Perform a midi- or maxi-scale plasmid DNA preparation for each plasmid that will be transfected into target cells. For guaranteed transfection-grade plasmid DNA, we recommend using **NucleoBond® Xtra Midi Plus** or **Maxi Plus Kits** (Cat. Nos. 740412.10 and 740416.10). For rapid production of endotoxin-free, transfection-grade plasmid DNA, use **NucleoBond® Xtra Midi EF Plus** or **Maxi EF Plus Kits** (Cat. Nos. 740422.10 and 740426.10).

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. These primer sets are incompatible with pTRE-Tight.

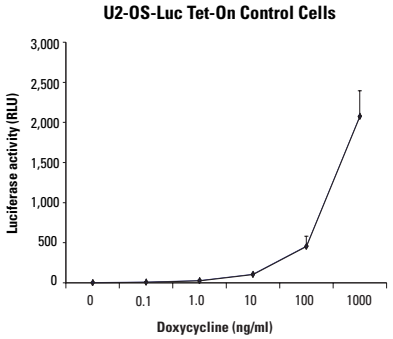

Figure 4. Doxycycline dose-response curve for the U2-OS-Luc Tet-On Control Cell Line

VI. Culturing Premade Tet Cell Lines

A. Characteristics of Tet Cell Lines

Clontech premade Tet System Cell Lines, such as the U2-OS-Luc Tet-On Control Cell Line, have been developed and functionally tested for use with Tet expression systems and a wide variety of Tet and TRE-based vectors. See the Certificate of Analysis of each individually purchased cell line for specific information regarding its culture, maintenance, and drug resistance. Detailed instructions for the use of these cell lines are available in the Tet Systems User Manual (PT3001-1) and the Tet Cell Lines Protocol-at-a-Glance (PT3001-2). A complete listing of all available cell lines can be found at **www.clontech.com.**

Protocol 2–3 days

B. Protocol: Starting Tet Cell Cultures from Frozen Stocks

Important: Frozen cells should be cultured immediately upon receipt, or as soon as possible thereafter. If culturing after shipping is significantly delayed, decreased cell viability may result. To prevent osmotic shock and maximize cell survival, follow the steps below to begin 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. 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 pre-warmed medium (without selective antibiotics, e.g. G418). Mix gently.
2. Slowly add an additional 4 ml of fresh, pre-warmed medium to the tube and mix gently.
3. Add an additional 5 ml of pre-warmed medium to the tube, mix gently. Centrifuge at 100 x g for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium 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.)
4. Mix the cell suspension thoroughly and add to a suitable culture vessel. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place it in a 37°C humidified incubator (5–10% CO2 as appropriate) for 24 hrs.

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

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

5. The next day, examine the cells under a microscope. If the cells are well-attached, and confluent, they can be passaged for use. If the majority of cells are not well-attached, continue culturing for another 24 hrs.

   Note: For HEK 293-based cell lines, complete attachment of newly thawed cultures may require up to 48 hrs.

6. Expand the culture as needed.

   Note: The appropriate selective antibiotic(s) should be added to the medium after 48–72 hr in culture. Maintain stable and double-stable Tet Cell Lines in complete culture medium containing a maintenance concentration G418 and/or hygromycin, as appropriate. Typically, this is 100 µg/ml for each drug.

VI. Culturing Premade Tet Cell Lines continued

C. Protocol: Freezing Tet Cell Line Cultures

Once you have started growing a Tet System cell line—either a premade one from Clontech or one of your own cell lines—prepare frozen aliquots to ensure a renewable source of cells.

1. Trypsinize the desired number of flasks or plates.
2. Pool cell suspensions together, count cells, and calculate total viable cell number.
3. Centrifuge cells at 100 x g for 5 min. Aspirate the supernatant.
4. Resuspend the pellet at a density of at least 1–2x10^6 cells/ml in freezing medium. Freezing medium can be purchased from Sigma (Cat. Nos. C6164 & C6039), or freeze cells in 70–90% FBS, 0–20% medium (without selective antibiotics), and 10% DMSO.
5. Dispense 1 ml aliquots into sterile cryovials.
6. Freeze slowly (1°C per min). For this purpose, you can place the vials in Nalgene cryo-containers (Nalgene Cat. No. 5100) and freeze at –80°C overnight. Alternatively, place vials in a thick-walled styrofoam container at –20°C for 1–2 hr. Transfer to –80°C and freeze overnight. Remove vials from the cryo-containers or styrofoam containers the following day, and place in liquid nitrogen storage or ultralow-temperature freezer (–150°C) for storage.
7. Two or more weeks later, plate a vial of frozen cells to confirm viability.

VII. Luciferase Induction in the U2-OS-Luc Tet-On Cell Line

A. Testing Luciferase Induction

Before you develop your own Tet-On Advanced System, we strongly recommend that you perform a Dox dose-response curve using the U2-OS-Luc Tet-On Cell Line. This premade double-stable Tet-On/pTRE-Luc cell line exhibits >500-fold induction of luciferase when cultured in the presence of Dox. In addition to providing a "hands-on" experience with a Tet-On system, this experiment (1) calibrates the effective concencentration of your Dox, i.e., full activation should be achieved with 100–1000 ng/ml Dox; and (2) verifies that your tissue culture medium and serum are free of tetracycline contamination. Luciferase induction in the U2-OS-Luc Tet-On cell line is highly reproducible. If induction is significantly lower than 500-fold, or if a high level of basal expression is observed, it is possible that your serum is contaminated with Tc.

Protocol 2–3 days

B. Protocol: Inducing Luciferase Expression in U2-OS-Luc Tet-On Cells

1. After thawing and establishing the cell line, plate 5 x 10^4 U2-OS-Luc Tet-On cells in a volume of 2–3 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 4, page 8.

VIII. Developing a Tet-On Advanced Cell Line

A. Summary

The first step in establishing the Tet-On Advanced Inducible Expression System is creating a Tet-On Advanced stable cell line that: (1) expresses the Tet-On Advanced transactivator; (2) demonstrates high levels of P_{Tight} induction; and (3) exhibits low basal expression from P_{Tight}. This Tet-On Advanced cell line will be subsequently transfected with your customized pTRE-Tight-GOI vector, which will ultimately enable your target cells to express your GOI when the cells are treated with Dox.

For best results, we suggest that you use a high-efficiency transfection method such as Clontech's **Xfect™** (Cat. No. 631317) or **CalPhos™ Mammalian Transfection Kit** (Cat. No. 631312) and optimize the transfection conditions for your target cell type. Parameters to be optimized include: initial plating density, transfection time, plating density for drug selection, G418 concentration for selection, etc.

Once G418 selection has been completed, we recommend that you isolate as many clones/colonies as possible in Section VIII.C, Step 7. In general, isolate and expand enough colonies to be able to *test* at least 30 clones. Note that not all picked colonies will survive isolation and expansion. While it is possible to identify an optimal clone by screening fewer than 30 clones, our experience has shown that testing this many clones yields a high rate of success and will prevent significant delays.

Your panel of Tet-On Advanced cell line clones should be screened by transiently transfecting them with pTRE-Tight-Luc to test for high induction and low basal expression of luciferase activity. When you have identified a clone that demonstrates ideal induction characteristics, proceed to Section IX to develop the *double-stable* Tet-On Advanced inducible cell line. Be sure to freeze aliquots of your Tet-On Advanced cell line(s).

Protocol 2–3 days

B. Protocol: Pilot Testing Tet-Based Induction in Target Cells

While many cell backgrounds have been shown to support Tet-based expression control, Tet systems have not been tested in all cell lines. Performing a transient expression assay with pTet-On Advanced and pTRE-Tight-Luc provides a quick indication of how well the Tet-On Advanced System will work in your target cell line. Transfected cells are treated with Dox to induce expression of luciferase from pTRE-Tight-Luc.

1. Using conditions and transfection methods appropriate for your cell line, cotransfect duplicate wells of cells in 6-well plates with pTet-On Advanced *and* pTRE-Tight-Luc. Use several different Tet-On/TRE vector ratios, e.g. at 1:1, 1:5, and 5:1, to ensure that a functional induction system is attained in the transfected cells.
2. When transfection has been completed, replace the transfection medium with fresh culture medium. Add Dox (0.01–1.0 µg/ml) to **one** of the duplicate wells for each vector ratio being tested. Use the second well in each duplicate as an untreated control. If multiple wells are available for each ratio, test a range of Dox concentrations.
3. After 12–24 hr of treatment with Dox, harvest the cells and assay for luciferase activity. Compare "+Dox" cells to "–Dox" cells to determine fold induction.

NOTE: Due to the very high plasmid copy numbers contained in transiently transfected cells, fold-induction levels are almost always lower in transient assays than in properly screened stable and double-stable clonal 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 have basal expression levels that are indistinguishable from control background expression. Therefore, an apparent low level of induction is not necessarily a true indication of the inducibility that can be ultimately attained in a particular cell line.

VIII. Developing a Tet-On Advanced Cell Line continued

Protocol 2–4 weeks

C. Protocol: Creating a Stable Tet-On Advanced Cell Line from Your Target Cell Line

1. Plate target cells at a density appropriate for your transfection method. After 12–24 hr, transfect them with the pTet-On Advanced Vector by your preferred method.

   NOTE: Using an alternative Tet-On Advanced expression vector, such as pTet-DualON (Cat. No. 631112), requires that a linear selection marker (hygromycin or puromycin) be cotransfected along with the vector plasmid. Use a vector-to-marker molar ratio of 20:1, i.e. 20-fold less marker than plasmid. A different marker must be used when transfecting the TRE-based expression vector in Section IX.

2. When transfection is complete, reseed the transfected cells in 10 cm plates in complete culture medium. Use the plating density for your cell line that is optimal for G418 selection (Appendix B).
3. Allow cells to divide twice (24–48 hr), then add G418 at the selection concentration that is optimal for your cell line. For most cell lines, this is usually 400–500 µg/ml.
4. Replace medium with fresh complete medium plus G418 every four days, or more often if necessary.
5. Cells that have not taken up the plasmid should begin to die after ~5 days. Avoid passaging the cells a second time since replating cells under selection may result in plates containing too many colonies for effective colony isolation.
6. After ~2 weeks, G418-resistant colonies should begin to appear.
7. When the colonies are large enough to transfer, use cloning cylinders or disks to harvest (i.e. "pick") large, healthy colonies and transfer them to individual plates or tissue culture wells. Isolate as many clones as feasible, so that at least 30 clones are available for testing. Suspension cultures must be cloned using a limiting dilution technique.
8. Culture the clones in a maintenance concentration of G418 (100–200 µg/ml). When they have grown sufficiently, proceed with testing the clones for induction as described in Section VIII.D.

Protocol 2–3 days

D. Protocol: Testing Your Tet-On Advanced Clones for Induction

1. For each clone to be tested, seed 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 duplicate wells of a second 6-well plate. Allow the cells to adhere overnight, and transfect each well with pTRE-Tight-Luc using the amount of DNA appropriate for your preferred transfection method.
3. When transfection is complete, replace the transfection medium with fresh culture medium and add Dox (0.01–1.0 µg/ml) to **one** of the duplicate wells, while leaving the second well Dox-free.
4. Incubate the cells with Dox for 48 hr.
5. Assay for luciferase activity and calculate fold-induction (e.g., +Dox RLU/–Dox RLU)
6. Select clones with the highest fold-induction for propagation and further testing, i.e., clones that exhibit >20-fold induction.

   NOTE: When testing clones via transient transfection, you can expect lower fold-induction levels than in double-stable clones. This is due to the far higher copies of the TRE-containing plasmid present in transiently transfected cells, compared to the copy numbers in stable cell lines.

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

IX. Developing the Double-Stable Tet-On Advanced Inducible Cell Line

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

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

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

B. Protocol: Creating the Double-Stable Tet-On Advanced Inducible Cell Line

Protocol 2–4 weeks

To generate the double-stable Tet-On Advanced inducible cell line, your customized pTRE-Tight vector (or any other TRE-based vector) is cotransfected along with the selection marker into your Tet-On Advanced cell line. Stable transfectants are selected using hygromycin or puromycin.

1. Plate your Tet-On Advanced cell line at a density appropriate for your preferred transfection method.
2. Combine your customized pTRE-Tight vector and either the Linear Hygromycin or Puromycin Marker at a ratio of 20:1 (i.e., 20-fold less linear marker), and transfect the Tet-On Advanced cells using your preferred method.

   NOTE: If the Linear Hygromycin Marker was used to create the Tet-On Advanced cell line, you must cotransfect the Linear Puromycin Marker with the TRE-based vector to create the double-stable cell line.

3. When transfection is complete, seed the transfected cells in 10 cm plates. Use complete medium containing an appropriate maintenance concentration of G418 (100–200 µg/ml). Use the plating density for your cell line that is optimal for hygromycin or puromycin selection (Appendix B).
4. Allow cells to divide twice (24–48 hr; time will vary with cell line) before adding hygromycin (200–400 µg/ml) or puromycin (1–10 µg/ml) to the culture medium. Use the drug concentration optimal for your cell line (Appendix B).
5. Continue drug selection until colonies are visible and all untransfected cells have died. Avoid passaging the cells a second time since replating cells under selection may result in plates containing too many colonies for effective colony isolation. Colonies should be visible in 2–4 weeks.
6. When colonies are large enough to transfer, use cloning cylinders or disks to isolate large, healthy colonies and transfer them to individual plates or tissue culture wells. Harvest as many clones as feasible, so that at least 30 clones are available for testing. Suspension cultures must be cloned using a limiting dilution technique.
7. Culture the clones in medium containing maintenance concentrations of G418 and hygromycin or puromycin. When they have grown sufficiently, test the clones for induction as described in Section C.

Attention

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.

IX. Developing the Double-Stable Tet-On Advanced Inducible Cell Line

Protocol 2–3 days

C. Protocol: Screening Your Panel of Double-Stable Tet-On Advanced Inducible Cell Lines

Test individual double-stable clones for expression of your GOI in the presence and absence of several concentrations of Dox (10–1000 ng/ml). Choose clones that generate the highest overall induction and lowest basal expression, i.e., highest fold-induction.

1. For each clone to be tested, seed an aliquot of cells in 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. Distribute the remaining cells among the wells of a tissue culture plate (24–96 wells) so that a range of Dox concentrations (10–1000 ng/ml) can be tested in duplicate versus an uninduced (No Dox) control.
3. Add Dox to the appropriate wells and incubate the cells for 48 hr.
4. Harvest the cells and use an assay specific for your GOI to quantify the expression of your GOI.
5. Select clones with the highest fold-induction for propagation and further testing.
6. Freeze stocks of each promising clone as soon as possible.

X. References

You can access further information on Tet Systems products on our website: **www.clontech.com.** Clontech's Tet Systems were developed in cooperation with Dr. Bujard and his colleagues at the Center for Molecular Biology in Heidelberg (ZMBH) and in Dr. Wolfgang Hillen's laboratory at the University of Erlangen, Germany. Additional background information on Tet-regulated gene expression systems and an extensive 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.)

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Appendix A: Tet Vector Information

Clontech offers a wide variety of inducible expression vectors designed for use with Tet Expresssion Systems (Figure 8). Visit **www.clontech.com** for a complete list of currently available vectors. The vectors below are supplied with the Tet-On Advanced Gene Expression System (Cat. No. 630930).

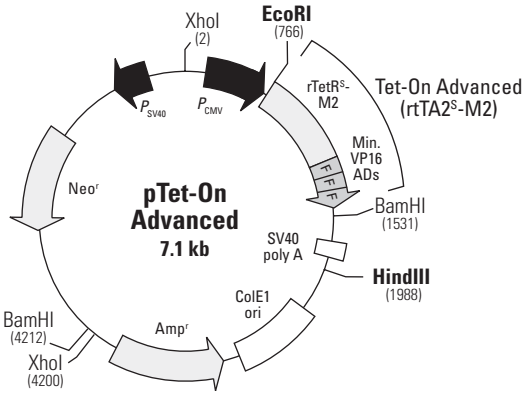

Figure 5. Map of pTet-On Advanced. For a complete vector description, refer to the enclosed pTet-On Advanced Vector Information Packet (PT3899-5).

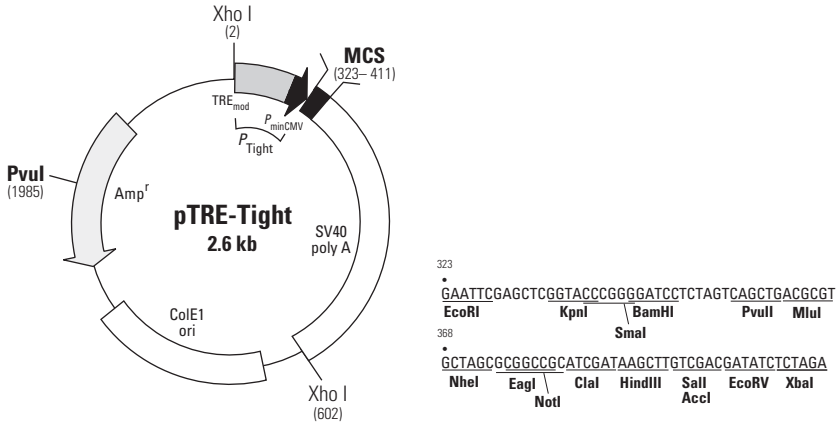

Figure 6. Map and MCS of pTRE-Tight. For a complete vector description, refer to the enclosed Vector Information Packet (PT3720-5).

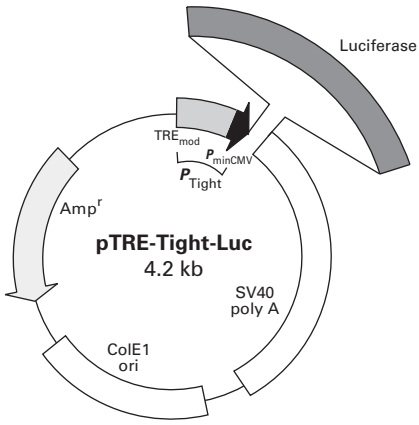

Figure 7. Map of pTRE-Tight-Luc.

Appendix A: Tet Vector Information

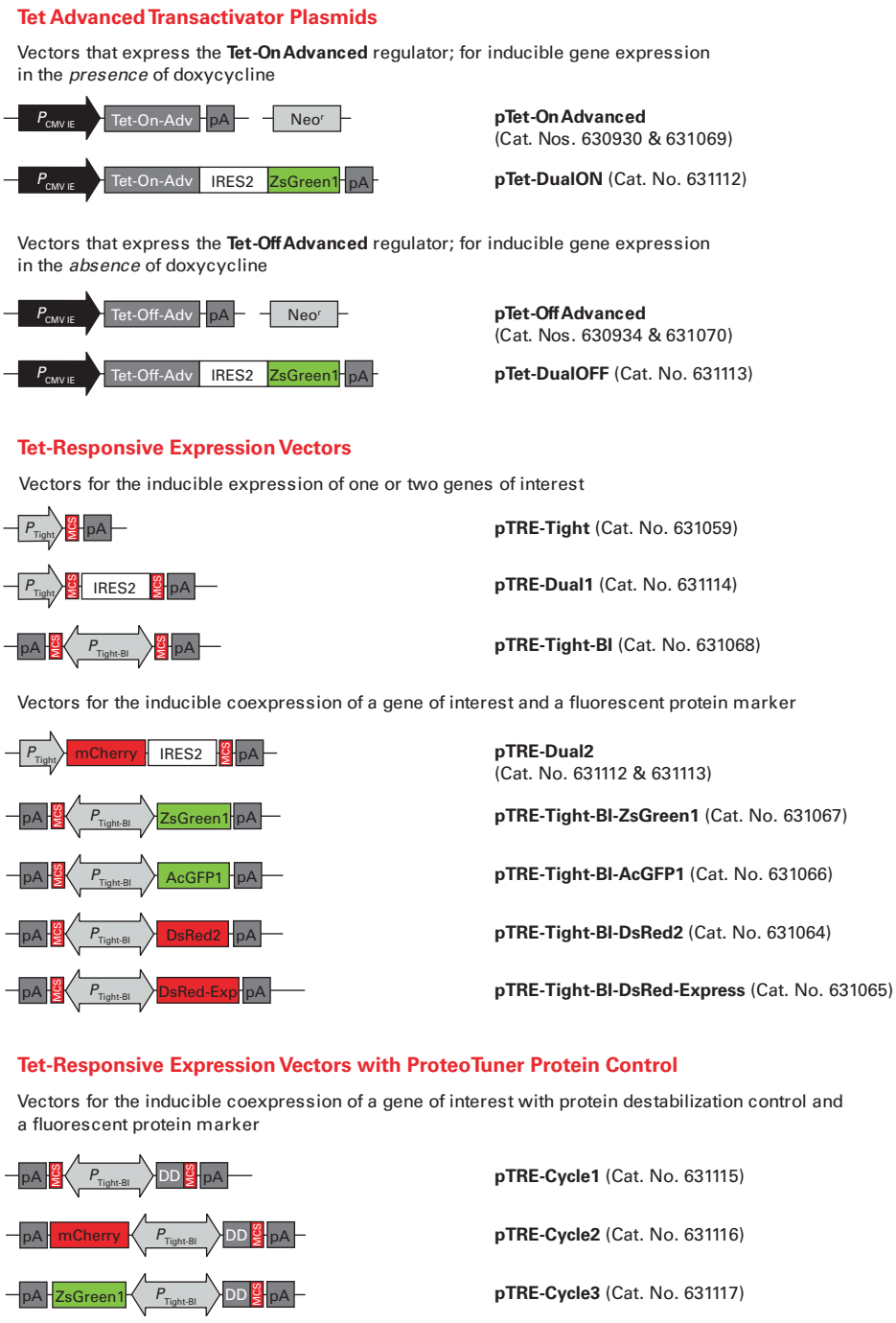

Figure 8. Tet System vectors. For a complete list of vectors and their descriptions, visit www.clontech.com.

Appendix B: Titrating Antibiotics for Selecting Stable Cell Lines

Protocol 3–5 days

A. Protocol: Titrating Antibiotics for Selecting Stable Cell Lines.

Prior to using G418, hygromycin, or puromycin to select stably transfected cell lines, it is necessary to titrate each selection agent to determine the optimal concentration for your target cell line. Also, the absolute activity of the antibiotic can vary from lot to lot. With HeLa cells, for example, we have found 400 µg/ml G418 and 1.0 µg/ml puromycin to be optimal.

- For selecting stable cell lines with G418 or hygromycin, use the lowest concentration that results in widespread cell death in ~5 days and kills all the cells within two weeks.
- Puromycin selection occurs more rapidly; use a concentration that will kill all cells within 3–4 days.
- If possible, test several plating densities versus each antibiotic concentration. If cells become heavily confluent before they begin to die, viable clones may be lost if they detach from the plate. Also, passaging cells while they are under selection is not recommended.
- IMPORTANT: Lot-to-lot variations in potency exist for all selection drugs, so each new lot of antibiotic should be titrated.

1. For each antibiotic to be tested, plate 2 x 10^5 cells in each well of a 6-well plate containing 3 ml of the appropriate complete medium plus increasing concentrations of G418 (0, 50, 100, 200, 400, and 800 µg/ml). For puromycin, add the drug at 0, 1.0, 2.5, 5.0, 7.5, and 10.0 µg/ml.
2. For G418, incubate the cells for 5–10 days or until all cells are dead. Examine the dishes for viable cells every two days. Replace the selective medium every four days (or more often if necessary), until the optimal concentration is determined.
3. For puromycin, incubate the cells 4–7 days. Replace medium after 2 days to remove dead cells.

Appendix C: Using pTRE-Cycle Vectors to Rapidly Control Protein Levels

A. Summary

Clontech's pTRE-Cycle vectors allow precise rapid control of inducible expression by using a multi-tiered approach that combines the powerful transcriptional control of the Tet-Advanced System with the controlled protein destabilization of the ProteoTuner™ System. Transcriptional control of your GOI is achieved with Dox, while the stability of your protein is controlled with the ProteoTuner ligand, **Shield1** (Cat. No. 631037). With this combination, it is possible to rapidly and completely eliminate your expressed protein from cells by removing Dox and Shield 1, and have it reappear by adding Dox and Shield1. The pTRE-Cycle vectors possess the following two key features:

- The $P_{\text{Tight-BI}}$ promoter provides tight, bidirectional control of transcription mediated by Tet-Advanced transactivators;
- The ProteoTuner destabilizing domain (DD) allows the stability of a protein of interest to be precisely controlled by Shield1.

Below is a brief outline of the steps needed to perform protein cycling studies. We strongly recommend that you consult this Tet-On Advanced Systems user manual and the ProteoTuner Systems User Manual (PT4039-1) for detailed protocols describing the independent aspects of these technologies.

B. Screening for Highly Inducible Clones

1. Create and select double-stable pTet-On Advanced/ pTRE-Cycle-GOI clonal cell lines (see Section IX). When testing these clones for induction, include both Dox (0.1–1 µg/ml) and Shield1 (50–1000 nM), and compare to cells treated with Shield1 only.
2. Select a clone that has high GOI expression in the presence of Dox + Shield1, and very low basal expression with Shield1 alone. This ensures that you have the tightest control of transcription.

Appendix C: Using pTRE-Cycle Vectors to Rapidly Control Protein Levels

C. Performing a protein cycling experiment

1. Expression ON: Using the double-stable pTet-On Advanced/ pTRE-Cycle-GOI clone (as selected in Step A), induce expression in the presence of both Dox and Shield1.
2. Expression OFF: Remove the medium containing both Dox and Shield1. Protein levels should decline very rapidly*. Dox and Shield1 are effectively removed by the following treatment:
   a. Wash the cells on the plate 2X with PBS. Trypsinize and collect the cells, wash them in suspension 1X with PBS, and plate in fresh medium without Dox.
   b. After the cells have reattached to the substrate, gently wash them on the plate 1X with PBS and add fresh medium without Dox.
3. Expression ON: Add Dox and Shield1 again

*In fact, there are two options for control in Step C.2:

- *Rapid control* = Turn OFF by removing *Shield1 only*, and retain Dox in the medium. Depending on mRNA stability, this may result in a more rapid subsequent ON rate, since high-level transcriptional activity is maintained for Step 3.
- *Tightest control* = Turn OFF by removing *both Dox and Shield1*. This will result in the lowest possible level of your protein of interest.