User Manual

Mir-X™ Inducible miRNA Systems User Manual

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I. Introduction

A. Summary

The **Mir-X Inducible miRNA Systems (Red & Green)** employ elements of our tightly regulated and highly responsive Tet-On® Advanced Expression System to produce on-demand and high-level expression of miRNAs (*Clontechniques,* April 2006). Mir-X Inducible Systems are established by sequentially transfecting the provided vectors into your target cells and selecting stable cell lines. Cells that express the Tet-On Advanced transactivator, and that also bear an integrated **pmRi-mCherry** or **pmRi-ZsGreen1** miRNA expression vector, will express high levels of the miRNA along with a red or green fluorescent protein marker, when cultured in the presence of 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 inducible miRNA expression vectors: pmRi-mCherry and pmRi-ZsGreen1.** The central feature of these vectors is the inducible promoter, P_{Tight}, which controls transcription of the mCherry or ZsGreen1 fluorescent protein mRNAs that also contain your miRNA sequence embedded in their 3' UTRs. The P_{Tight} composite promoter sequence 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}) joined to a minimal CMV promoter ($P_{minCMV\Delta}$) (*Clontechniques,* April 2003). P_{Tight} also 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 the P_{Tight} promoter and activates transcription of the fluorescent protein/miRNA transcript (Figure 1).

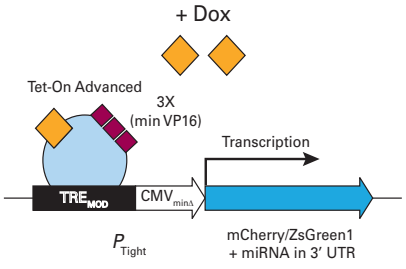

Figure 1. Induction in the Tet-On® Advanced System. The Tet-controlled transactivator, Tet-On Advanced, is a fusion protein derived from a mutant version of the *E. coli* Tet repressor protein, rTetR, which is joined to three minimal transcription activation domains. In the presence of doxycycline (Dox), Tet-On Advanced binds to the tetracycline response element (TRE_{Mod}) in P_{Tight}, and produces high-level transcription of the downstream gene. In the Mir-X miRNA Inducible Systems, the mRNA transcript encodes a fluorescent protein (mCherry or ZsGreen1) and an miRNA sequence in the 3' UTR.

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 expression 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 Tet-Advanced TetR portion of the transactivator binds 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 detectable effects 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 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 the Tet transactivator genes under the control of a variety of tissue-specific promoters or express various target genes under control of Tet-inducible promoters. A list of these mouse lines can be found on the TET Systems website (**http://www.tetsystems.com/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 all components at –20°C.

Mir-X Inducible miRNA System (Red), (Cat. No. 631118) and
Mir-X Inducible miRNA System (Green), (Cat. No. 631120)

Package Contents

- 20 µg pTet-On Advanced Vector (500 ng/µl; Cat. No. 631069)
- 20 µg pmRi-mCherry Vector (500 ng/µl; Cat. No. 631119) *Red system only,* **or**
  20 µg pmRi-ZsGreen1 Vector (500 ng/µl; Cat. No. 631121) *Green system only.*
  - 20 µg pTRE-Tight-Luc Vector (500 ng/µl)
  - 40 µl Linear Hygromycin Marker (50 ng/µl)
  - 40 µl Linear Puromycin Marker (50 ng/µl)
- 50 ml Tet System Approved FBS, US-Sourced (50 ml; Cat. No. 631105).
  (Also available in 500 ml size, Cat. No. 631101).

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

- Mir-X Inducible miRNA Systems User Manual (PT5050-1)
- pTet-On Advanced Vector Information (PT3899-5)
- pmRi-mCherry Vector Information (PT5048-5) *Red system only,* **or**
  pmRi-ZsGreen1 Vector Information (PT5049-5) *Green system only.*

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.
- 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 containing Tet Systems.
- 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 single-stable Tet-On Advanced cell lines and double-stable Mir-X 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.

- **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 Mir-X System 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 Mir-X System 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 activating Tet-On Advanced and inducing expression of the miRNA and fluorescent protein marker. 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 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

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.

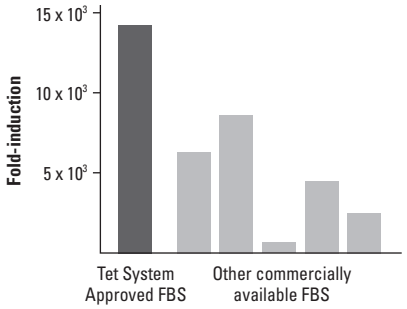

Figure 2. Tetracycline activity in bovine sera. The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different lots of FBS. Average uninduced expression level = 0.21 RLU (n=21, S.D.=0.07); maximum expression levels varied from 123 to 3,176 RLU.

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 Mir-X Inducible miRNA System in Target Cells

The general strategy for establishing the Mir-X Inducible miRNA 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, then this cell line is stably transfected with your customized pmRi-mCherry (or -ZsGreen1) vector.

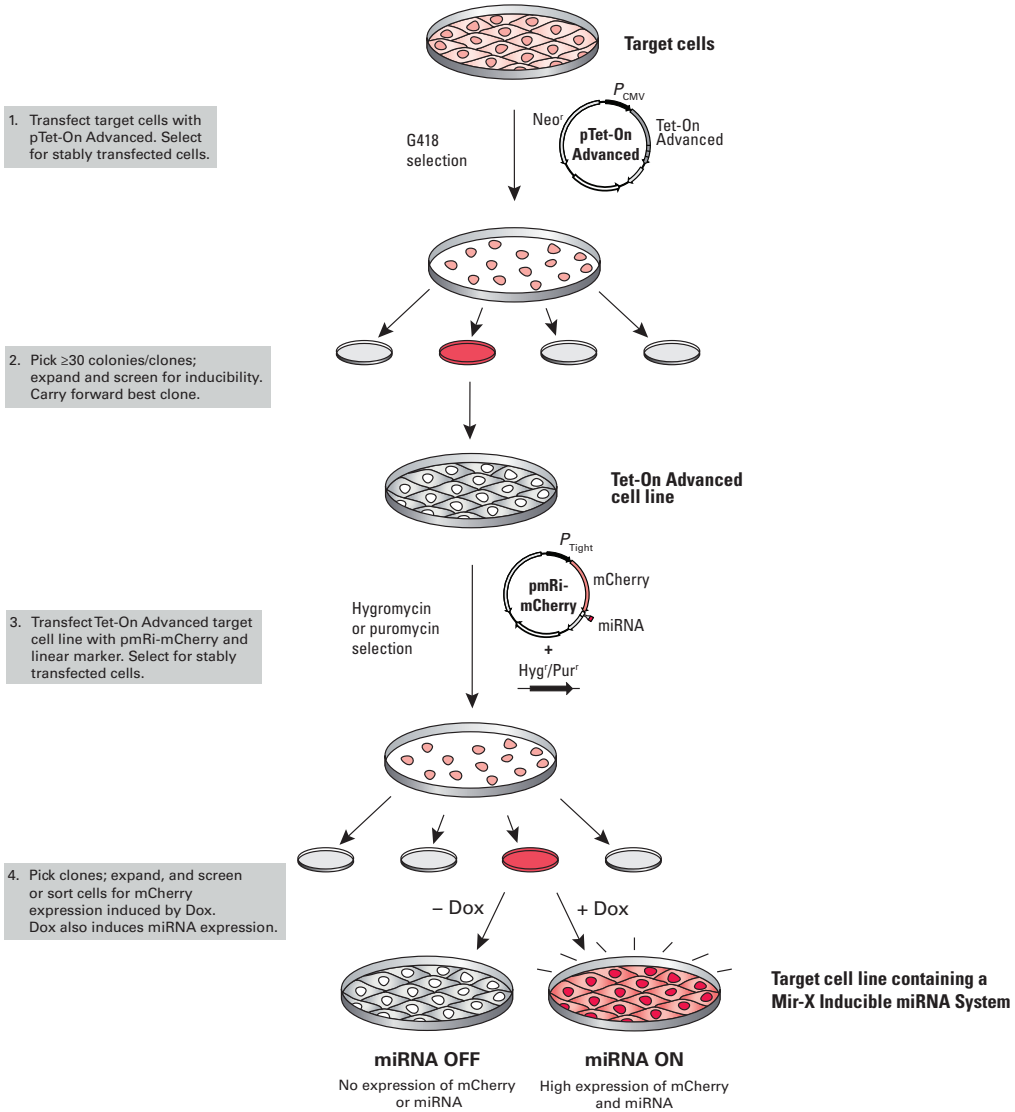

Figure 3. Establishing the Mir-X Inducible miRNA System (Red) in target cells. Target cells are transfected with the pTet-On Advanced plasmid and selected with G418 to generate a stable Tet-On Advanced cell line. This cell line serves as the host for the pmRi-mCherry miRNA expression vector, which is transfected into the Tet-On Advanced cell line along with one of the linear markers. After a second round of drug selection, a stable cell line is produced which will coexpress the miRNA and mCherry in response to doxycycline (Dox).

V. Plasmid and miRNA Vector Preparation

A. General Molecular Biology Techniques

Only general information for propagating 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. Isolating Your miRNA Sequence

We recommend that you use PCR and specific primers to amplify and isolate a small genomic fragment containing your miRNA precursor of interest. This fragment will be cloned in the pmRi-mCherry (or -ZsGeen1) vector and should be positioned in the same orientation as the fluorescent protein. In addition to the miRNA precursor sequence itself, we recommend that you include 100-300 bp of flanking genomic DNA sequence to ensure that the miRNA will be efficiently processed. The sequence of miRNA precursors and flanking genomic DNA can be obtained from a number of public databases including:

- GenBank (http://www.ncbi.nlm.nih.gov/)
- EMBL-Bank (http://www.ebi.ac.uk/embl/).
- The UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/); an easy-to-navigate genomic database with tracks for miRNAs.
- The Sanger Institute hosts miRBase, a compilation of known miRNA sequences (http://microrna.sanger.ac.uk/).

C. Plasmid Propagation & Construction of Your miRNA Expression 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 each plasmid's Vector Information Packet 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 (derived from your PCR primers or from the genomic DNA sequence), clone your miRNA precursor fragment (Section V.B) in the multiple cloning site (MCS) of the pmRi-mCherry or -ZsGreen1 vector. 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. You must synthesize PCR primers that are specifically designed for In-Fusion cloning. 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).

VI. Developing a Stable Tet-On Advanced Cell Line

The first step in establishing the Mir-X Inducible miRNA 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 pmRi vector, which will ultimately enable your target cells to coexpress the mCherry or ZsGreen1 fluorescent protein and your miRNA 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 VI.B, 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 either pTRE-Tight-Luc to test for high induction and low basal expression of luciferase activity, or with your pmRi vector to test for high induction and low basal expression of fluorescent protein expression. When you have identified a clone that demonstrates ideal induction characteristics, proceed to Section VII to develop the double-stable, Mir-X inducible cell line. Be sure to freeze aliquots of your Tet-On Advanced cell line(s).

Protocol 2–3 days

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

While many cell backgrounds have been shown to support Tet-based expression control, these systems have not been tested in all types of cell lines. Performing a transient expression assay with pTet-On Advanced and pTRE-Tight-Luc, or your pmRi-mCherry or -ZsGreen1 vector, is the best way to provide a quick indication of how well the Mir-X Inducible miRNA System will work in your target cell line. Transfected cells are treated with Dox to induce luciferase or fluorescent protein expression.

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* either pTRE-Tight-Luc, pmRi-Cherry, or pmRi-ZsGreen1. Use several different Tet/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. Leave 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 or measure the fluorescent protein expression using flow cytometry. 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.

VI. Developing a Stable Tet-On Advanced Cell Line continued

Protocol 2–4 weeks

B. 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.
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 C.

Protocol 2–3 days

C. 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 either pTRE-Tight-Luc, pmRi-mCherry, or pmRi-ZsGreen1, using the amount of DNA appropriate for your 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 reporter activity (e.g. luciferase or fluorescent protein) and calculate fold-induction (e.g., luciferase assay: +Dox RLU/–Dox RLU)
6. Select clones with the highest fold-induction for propagation and further testing. In general, select 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.

VII. Developing the Double-Stable Mir-X System Cell Line

Protocol 2–4 weeks

A. Protocol: Creating the Double-Stable Mir-X System Cell Line

To generate the double-stable Mir-X System cell line, your customized pmRi-mCherry or -ZsGreen1 vector is cotransfected along with a linear 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 pmRi 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.
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 B.

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.

Protocol 2–3 days

B. Screening Your Panel of Double-Stable Mir-X Cell Lines

Test individual double-stable clones for expression of mCherry or ZsGreen1 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 of the fluorescent protein.

1. For each clone to be tested, seed an aliquot 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 assay for fluorescent protein expression using flow cytometry or visual inspection using fluorescence microscopy.
5. Select clones with the highest fold-induction for propagation and further testing.
6. Freeze stocks of each promising clone as soon as possible.

VIII. 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: Vector Information

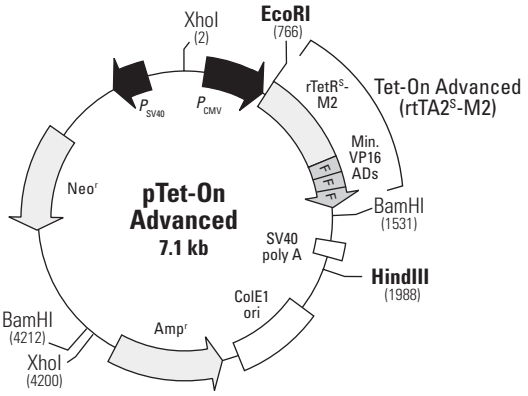

Figure 4. Map of pTet-On Advanced. For a complete vector description, refer to the enclosed pTet-On Advanced Vector Information Packet (PT3899-5).

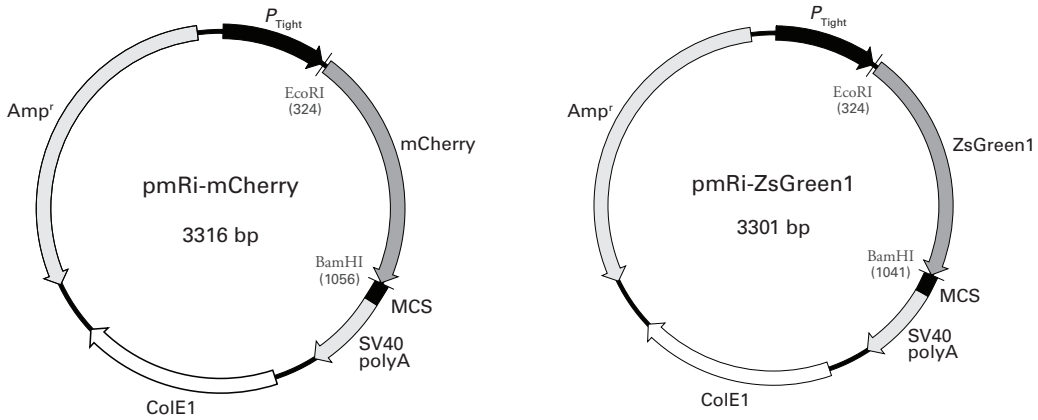

Figure 5. Map of the pmRi-mCherry and pmRi-ZsGreen1 Vectors. For a complete vector description and MCS diagram, refer to the enclosed Vector Information Packet (PT5048-5 or PT5049-5, respectively).

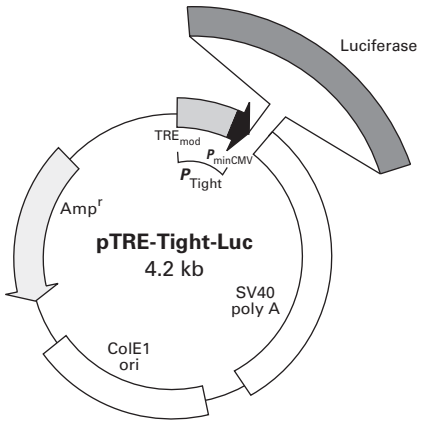

Figure 6. Map of the pTRE-Tight-Luc Vector.

Appendix B: Additional Protocols

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.

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