Clontech Laboratories, Inc.

# Tet-On® 3G Inducible Expression Systems

PT5148-1 (PR053540)
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# I. Introduction

## A. Summary

The **Tet-On 3G Systems** are inducible gene expression systems for mammalian cells. Target cells that express the Tet-On 3G transactivator protein and contain a gene of interest (GOI) under the control of a TRE3G promoter ($P_{TRE3G)}$ will express high levels of your GOI, but only when cultured in the presence of doxycycline (Dox) (Figure 1).

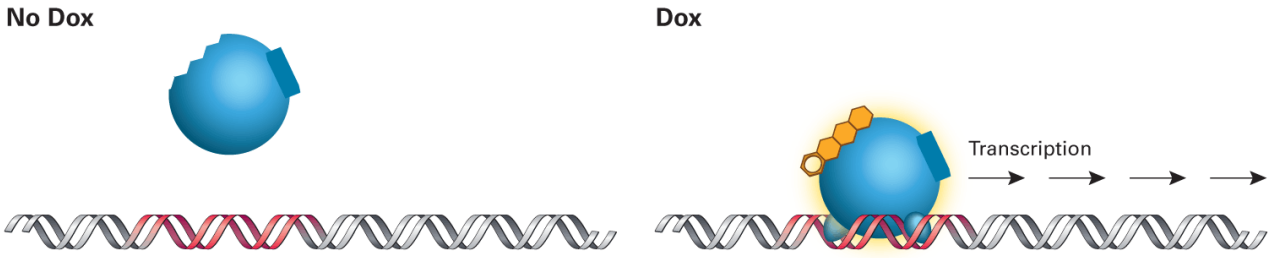


**Figure 1. The Tet-On 3G Systems allow inducible gene expression in the presence of Dox.**

## B. Two Elements of Tet-On 3G

### 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).

### $P_{TRE3G}$ Inducible Promoter

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

## C. Doxycycline

Doxycycline is a synthetic tetracycline derivative that is the effector molecule for the 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 $P_{TRE3G}$ promoter (Figure 1). The Dox concentrations required for induction of Tet-On Systems 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 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

## A. Available Tet-On 3G Plasmid Systems

| Cat. No. | System Name |
|---|---|
| 631168 | Tet-On 3G Inducible Expression System |
| 631167 | Tet-On 3G Inducible Expression System (EF1α Version) |
| 631166 | Tet-On 3G Inducible Expression System (Bicistronic Version) |
| 631165 | Tet-On 3G Inducible Expression System (with mCherry) |
| 631164 | Tet-On 3G Inducible Expression System (with ZsGreen1) |

## B. General System Components

All systems listed in Section II.A contain the following 7 components (store all components at -20°C):

- 10 µg regulator plasmid (see Section II.C)
- 10 µg response plasmid (see Section II.C)
- 10 µg pTRE3G-Luc (control response plasmid )
- 2 µg Linear Hygromycin Marker (also sold separately as Cat. No. 631625)
- 2 µg Linear Puromycin Marker (also sold separately as Cat. No. 631626)
- 100 rxns Xfect™ transfection reagent (also sold separately as Cat. No. 631320)
- 50 ml Tet System Approved FBS, US Sourced (also sold separately as Cat. No. 631105)

## C. System-Specific Regulator and Response Plasmids

| Cat. No. | Regulator Plasmid | Response Plasmid |
|---|---|---|
| 631168 | pCMV-Tet3G | pTRE3G |
| 631167 | pEF1α-Tet3G | pTRE3G |
| 631166 | pCMV-Tet3G | pTRE3G-IRES |
| 631165 | pCMV-Tet3G | pTRE3G-mCherry |
| 631164 | pCMV-Tet3G | pTRE3G-ZsGreen1 |

# III. Additional Materials Required

## 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) |
| 631040 | Tet System Approved FBS, Australia-Sourced (500 ml) |
| 631039 | Tet System Approved FBS, Australia-Sourced (50 ml) |
| 631158 | Tet System Approved FBS, ES Cell Qualified (500 ml) |
| 631157 | Tet System Approved FBS, ES Cell Qualified (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** |
| 631308 | G418 (5 g) | 100–800 | 200 |
| 631307 | G418 (1 g) | | |
| 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 Tet-On 3G cell lines.
- 6-well cell culture plates, 12-well cell culture plates, 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 Reagents

Xfect provides high transfection efficiency and low cytotoxicity for most commonly used cell types. Xfect Stem is optimized for mouse embryonic stem cells.

| Cat. No. | Transfection Reagent |
|---|---|
| 631317 | Xfect (100 rxns) |
| 631318 | Xfect (300 rxns) |
| 631320 | Xfect Stem (100 rxns) |
| 631321 | Xfect Stem (300 rxns) |

### F. In-Fusion® Advantage Cloning Kits and Stellar™ Competent Cells

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

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

| Cat. No. | In-Fusion Cloning Kit |
|---|---|
| 639629 | In-Fusion Advantage PCR Cloning Kit w/Stellar Competent Cells (10 rxns) |
| 639630 | In-Fusion Advantage PCR Cloning Kit w/Cloning Enhancer and Stellar Competent Cells (10 rxns) |
| 639631 | In-Fusion Advantage PCR Cloning Kit w/NucleoSpin® and Stellar Competent Cells (10 rxns) |

### G. 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) |

### H. Luciferase Assay and Luminometer

These items are required when using the pTRE3G-Luc Vector to screen Tet-On 3G clones (Section VII.C). 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 a pTRE3G Vector using In-Fusion Advantage (Section V).
2. Pilot test Tet-based induction of your construct (Section VI).
3. Create a Tet3G-expressing stable cell line (Section VII).
4. Create and screen for a double-stable clone capable of high induction of your GOI (Section VIII).

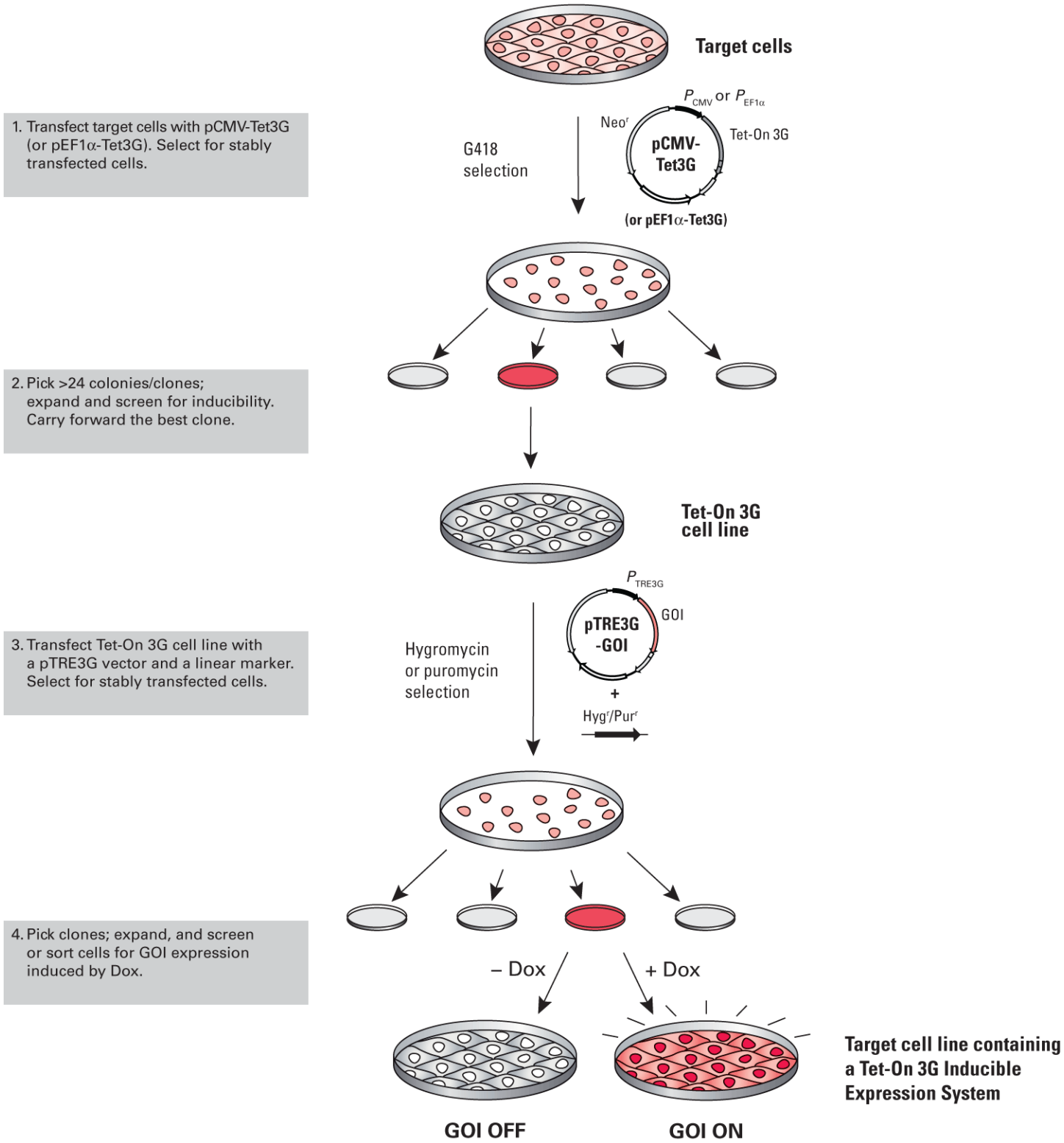


**Figure 2. Establishing the Tet-On 3G System in target cells.** Target cells are transfected with the pCMV-Tet3G (or pEF1α-Tet3G) plasmid and selected with G418 to generate a stable Tet-On 3G cell line constitutively expressing Tet-On 3G transactivator. This cell line serves as the host for a $P_{TRE3G}$-based expression vector, which is transfected into the Tet-On 3G cell line along with a linear selection marker ($Hyg^r$ or $Pur^r$). After a second round of drug selection, a double-stable cell line is established which expresses high levels of the GOI in response to doxycycline (Dox).

# V. Cloning Your Gene of Interest into a pTRE3G Vector using In-Fusion® Advantage

We recommend using In-Fusion Advantage for all cloning. Follow the protocol outlined in the In-Fusion Advantage user manual (Type PT4065-1 in the keyword field at www.clontech.com/manuals).

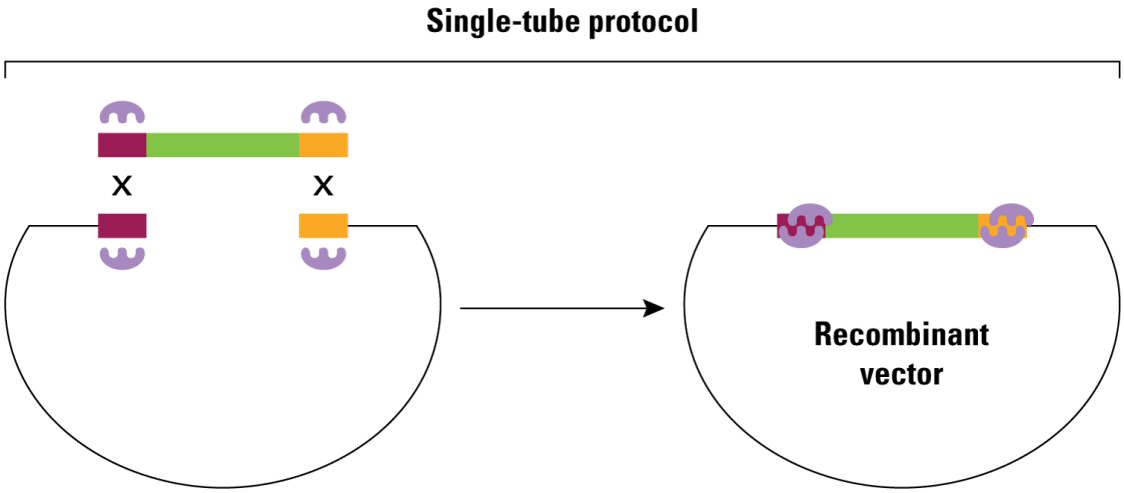


**Figure 3. The In-Fusion Advantage Single-Tube Cloning Protocol.**

Depending on which pTRE3G vector you are using, the recommended linearization sites and forward/reverse primer designs are as follows:

| Response Plasmid | Linearize with | Forward Primer* | Reverse Primer** |
|---|---|---|---|
| pTRE3G | SalI & BamHI | ccctcgtaaagtcga 111 222 333 444 555 666 777 888 | cagttacattggatc SSS NNN NNN NNN NNN NNN NNN NNN |
| pTRE3G-IRES (MCSI) | SalI & EagI | ccctcgtaaagtcga 111 222 333 444 555 666 777 888 | ggagaggggccggcc SSS NNN NNN NNN NNN NNN NNN NNN |
| pTRE3G-IRES (MCSII) | MluI & BamHI | gccggatatcacgcgt 111 222 333 444 555 666 777 888 | cagttacattggatc SSS NNN NNN NNN NNN NNN NNN NNN |
| pTRE3G-mCherry | MluI & BamHI | gccggatatcacgcgt 111 222 333 444 555 666 777 888 | cagttacattggatc SSS NNN NNN NNN NNN NNN NNN NNN |
| pTRE3G-ZsGreen1 | MluI & BamHI | gccggatatcacgcgt 111 222 333 444 555 666 777 888 | cagttacattggatc 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 double-stable Tet-On 3G cell line for your GOI, your pTRE3G construct should be tested for functionality. Transiently cotransfect your pTRE3G-GOI vector together with pCMV-Tet3G (in a 1:4 ratio for best inducibility) 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

Alternatively you can perform a single vector transfection of pTRE3G-GOI into a newly created Tet-On 3G cell line (Section VII).

## A. Materials Required

1. pTRE3G Vector containing your gene of interest (Section V)
2. pCMV-Tet3G (or pEF1α-Tet3G) (Section II.C)
3. Host cell line
4. Xfect transfection reagent (Section III.E)
5. Doxycycline (1 mg/ml) (Section III.D)
6. Mammalian cell culture supplies (Section III.C)
7. Tet Approved FBS (Section III.A)

## B. Protocol

1. Cotransfect both the regulator and response plasmids into your target cells (in a 6-well plate) using Xfect transfection reagent. Follow the Xfect Protocol (Type PT5003-2 in the keyword field at www.clontech.com/manuals).
   - Use 1 µg of pCMV-Tet3G and 4 µg of pTRE3G-GOI for each well (GOI = gene of interest).
   - We recommend performing the test in duplicate with negative controls: 3 wells containing 100–1,000 ng/ml of Dox, and 3 wells without Dox.


**Wells 1 & 2:** 1 µg pCMV-Tet3G and 4 µg pTRE3G-GOI (no Dox)

**Wells 3 & 4:** 1 µg pCMV-Tet3G and 4 µg pTRE3G-GOI (100–1,000 ng/ml Dox)

**Well 5:** 1 µg pCMV-Tet3G and 4 µg pTRE3G empty (no Dox)

**Well 6:** 1 µg pCMV-Tet3G and 4 µg pTRE3G empty (100–1,000 ng/ml Dox)

**Figure 4. Transfection of the regulator and response 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 TRE-containing 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 and double-stable clonal cell lines.

# VII. Creating a Tet-On 3G-Expressing Stable Cell Line

The first step in establishing the Tet-On 3G System in your cells is creating a stable cell line that: (1) expresses the Tet-On 3G transactivator; (2) demonstrates high levels of induction from $P_{TRE3G}$; and (3) exhibits low basal expression from $P_{TRE3G}$. This Tet-On 3G cell line will be frozen in aliquots and can be used to create individual inducible cell lines for all your genes of interest.

Transfect using Xfect transfection reagent and select for colonies with G418 selection. In general, isolate enough colonies to be able to test at least 24 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 24 clones, our experience has shown that testing this many clones yields a high rate of success and will prevent significant delays.

Your panel of 24 clones should then be screened by transient transfection with pTRE3G-Luc Control Vector to test for high induction and low basal expression using luciferase activity as a reporter. When you have identified a clone that demonstrates ideal induction characteristics, proceed to Section VIII to develop the double-stable Tet-On 3G inducible cell line. Be sure to freeze aliquots of your Tet-On 3G cell line(s) (Appendix D, Section A).

**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.

## A. Materials Required

1. pCMV-Tet3G (or pEF1α-Tet3G) (Section II.C)
2. pTRE3G-Luc Control Vector (Section II.B)
3. Host cell line
4. Xfect transfection reagent (Section III.E)
5. G418 (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: Transfect and Select for 24 Independent Clones

1. Seed your target cells in a single well of a 6-well plate at a density sufficient to reach near confluence at 48 hr after transfection. Then transfect pCMV-Tet3G (or pEF1α-Tet3G) into your target cells using Xfect transfection reagent.
2. Follow the Xfect Protocol (PT5003-2 from www.clontech.com/manuals), except use 2 µg of plasmid per well.

   **NOTE:** We use less DNA for stable transfections than required by the general Xfect protocol, to ensure that individual colonies are well-separated after G418 selection.

3. After 48 hr, split the confluent well into 4 x 10 cm dishes (do not add G418 yet).

4. After an additional 48 hr, add G418 at the selection concentration that is optimal for your cell line. For most cell lines, this is usually 400–500 µg/ml (Section III.B).
5. Replace medium with fresh complete medium plus G418 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, G418-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 at least 24 clones are available for testing. Suspension cultures must be cloned using a limiting dilution technique (see Appendix C).

9. Culture the clones in a maintenance concentration of G418 (100–200 µg/ml). When confluent, split the cells from each well into three wells of a 6-well plate for testing and maintenance (Section VII.C).

   **NOTE:** You may wish to use TetR monoclonal antibody (Section III.G) to determine, via Western blot, which clones express the Tet-On 3G protein. However, Western analysis should not be used to substitute for a functional test for inducibility (Section VII.C), since the highest expressing Tet-On 3G clones often do not provide the highest fold inducibility.

## C. Protocol: Testing Your Tet-On 3G Clones for Induction

1. For each clone to be tested, seed 1/3 of the total amount of cells (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 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 5 µg of pTRE3G-Luc using Xfect transfection reagent.
3. After 4 hr, replace the culture medium with fresh medium and add Dox (100–1,000 ng/ml) to one of the duplicate wells, while leaving the second well Dox-free.
4. After 24 hr, assay for luciferase activity and calculate fold induction (e.g., +Dox RLU/–Dox RLU).
5. Select clones with the highest fold induction (ratio of maximal to basal gene expression) for propagation and further testing.

   **NOTE:** When testing clones via transient transfection, you can expect lower fold induction levels than in double-stable clones. This is because transiently transfected cells contain more copies of the TRE-containing plasmid than do stable cell lines.

6. Freeze stocks of each promising clone as soon as possible after expanding the culture (Appendix D).

# VIII. Creating & Screening for a Double-Stable Cell Line Capable of High Induction of your GOI

## A. Materials Required

1. pTRE3G-GOI Vector (Section V)
2. Linear Hygromycin/Puromycin Marker (Section II.B)
3. Tet-On 3G Cell Line (Section VII)
4. Xfect transfection reagent (Section III.E)
5. G418 (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 Double-Stable Tet-On 3G Inducible Cell Line

To generate a double-stable Tet-On 3G inducible cell line, cotransfect your customized pTRE-3G vector into your Tet-On 3G cell line along with a linear selection marker ($Hyg^r$ or $Pur^r$). Select double-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 Tet3G-expressing 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 pTRE3G 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 at least 24 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 both G418 and 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 VIII.C).

## C. Protocol: Screening Your Double-Stable Tet-On 3G Inducible Cell Lines

Test individual double-stable clones for expression of your GOI in the presence and absence of Dox (100–1,000 ng/ml). 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 VIII.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 (100–1,000 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 & 1,000 ng/ml) and assay for induced expression after 24 hr).

# IX. 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.)

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# X. Troubleshooting

## A. Low Fold Induction of Transient Expression

| Description of Problem | Possible Explanation | Solution |
|---|---|---|
| Low fold induction (ratio of maximal to basal expression of the GOI) | A suboptimal ratio of cotransfected vectors was used. | We generally recommend a co-transient transfection vector ratio of 1:4 for pCMV-Tet3G:pTRE3G-GOI (Section VI.B). Different vector ratios may result in different maximal/basal gene expression ratios. |
| | Cells were harvested and analyzed too soon or too late. | Harvest and analyze cells between 18–48 hr. |
| | Poor transfection efficiency | Optimize transfection protocol.<br>Optimize density of cell plating; use at 60–90% confluency. |
| | Poor target cell viability | Optimize passage number of target cells.<br>Optimize culture conditions of target cells.<br>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 double-stable CHO-AA8-Luc Tet-Off Control Cell Line. |
| | Transiently transfected cells contain more copies of the TRE-containing plasmid than do stable cell lines. | When testing clones via transient transfection, expect lower fold induction levels than in double-stable clones (sometimes only ~100-fold). |

## B. Low Fold Induction of Stable Expression

| Description of Problem | Possible Explanation | Solution |
|---|---|---|
| Low fold induction of GOI expression in selected drug-resistant double-stable cell clones. | Cellular sequences flanking the integrated TRE3G 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 VIII.B Note). | |
| Low fold induction of GOI expression in selected drug-resistant cell clones expressing Tet-On 3G transactivator, as detected by TetR Monoclonal Antibody | There is no direct correlation between the amount of expressed Tet-On 3G transactivator and induction efficiency. | Perform functional screening of selected drug-resistant clones using pTRE3G-Luc (Section VII.C). |
| Decrease in fold induction after several passages<br><br>or<br><br>Loss of inducibility after passaging of a (previously frozen) double-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 VIII.B Note). | Reselect the current cell line through single colony selection using selective concentrations of both antibiotics, and screen again with pTRE3G-Luc (Section VII.C). |

## C. Establishment of Stable Cell Lines

| Description of Problem | Possible Explanation | Solution |
|---|---|---|
| Cells do not die at the high antibiotic concentration established via titration in Section III.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 III.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 VIII.B). |
| | Antibiotic was added too soon. | See protocols in Sections VII.B & VIII.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.<br>• Antibiotic was added too late.<br>• Transfected cells were passaged a second time after addition of antibiotic. | See protocols in Sections VII.B & VIII.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. |

## D. Detection and Inhibition of Expression

| Description of Problem | Possible Explanation | Solution |
|---|---|---|
| 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 GOI/Fluorescent 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. | Upon degradation, GOI/Fluorescent Protein expression will not be detectable in cells in the absence of induction. For faster degradation of an inducible GOI, use pTRE-Cycle Vectors (see www.clontech.com). |
| | 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-On 3G Systems Vector Information

The Tet-On 3G Inducible Expression Systems (Section II) each contain one of two possible regulator plasmids (Figure 5) and one of four possible response plasmids (Figures 6 & 7), as well as a pTRE3G-Luc control response plasmid (Figure 8). 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

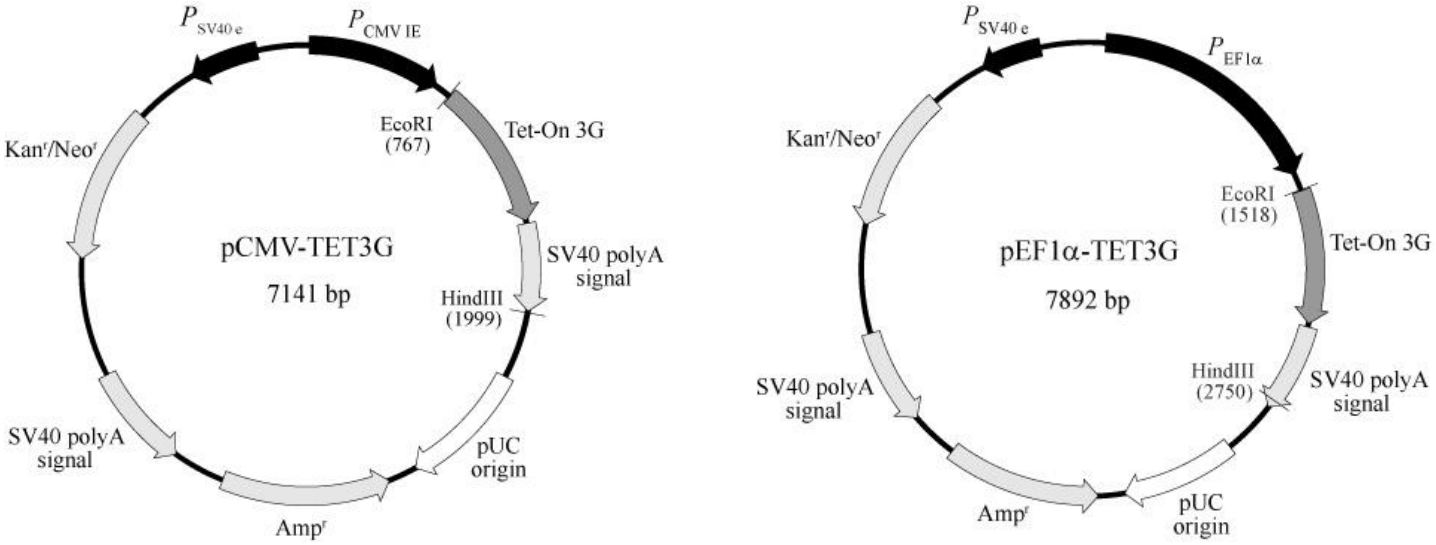


**Figure 5. pCMV-Tet3G Vector and pEF1α-Tet3G Vector Maps.**

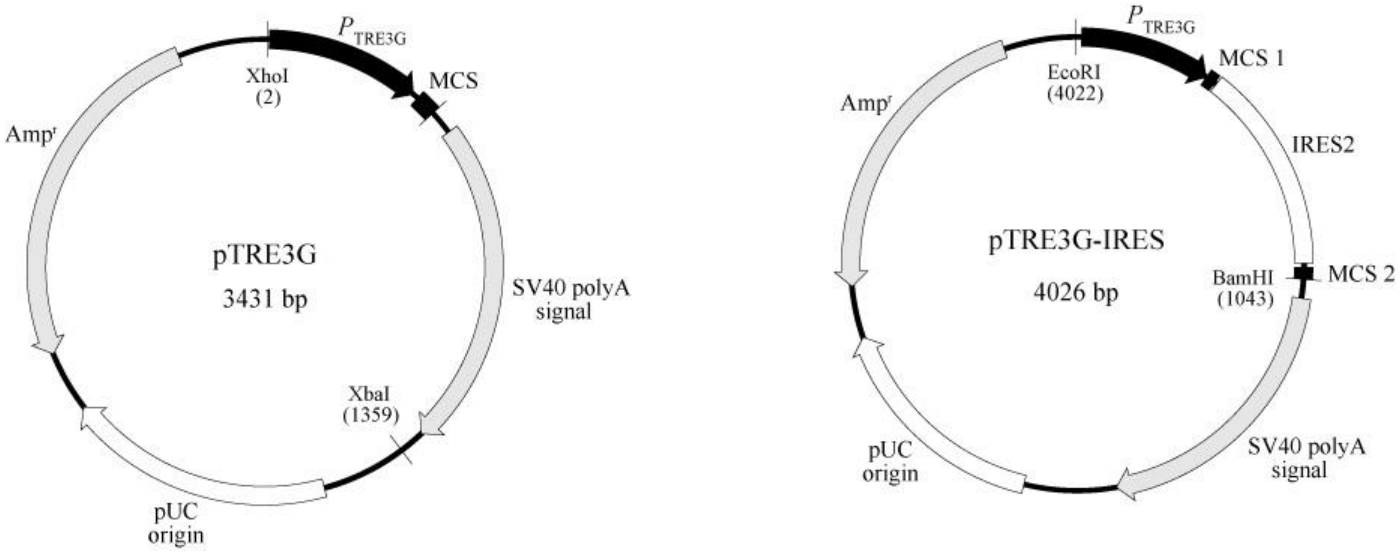


**Figure 6. pTRE3G Vector and pTRE3G-IRES Vector Maps.**

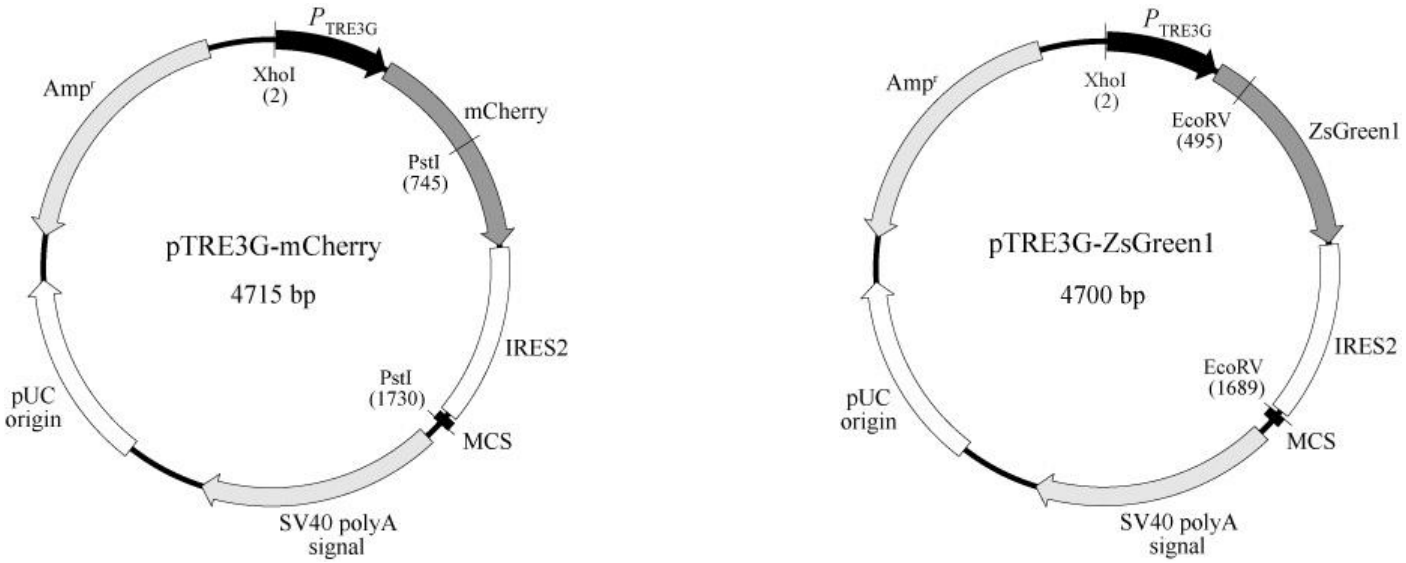


**Figure 7. pTRE3G-mCherry Vector and pTRE3G-ZsGreen1 Vector Maps.**

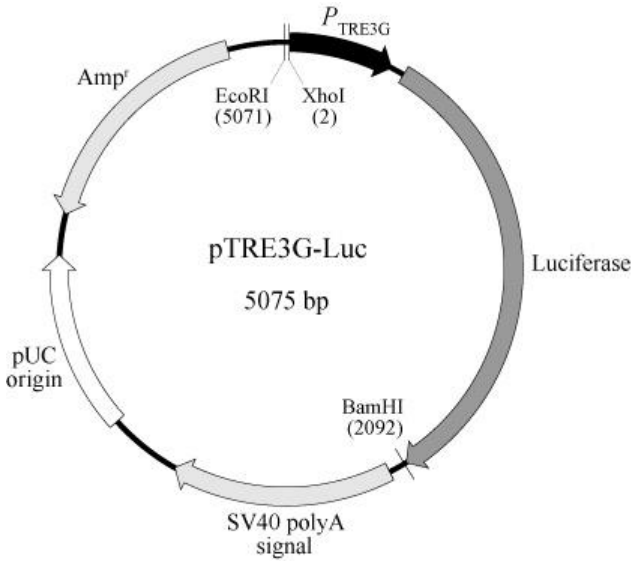


**Figure 8. pTRE3G-Luc Control Vector Map.**

## 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 pTRE3G 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 5 µg of your plasmid according to the Xfect protocol (type PT5003-2 in the keyword field 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 (e.g., use G418 to select for pCMV-Tet3G or pEF1α-Tet3G).
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 Sections VII.C and VIII.C).

# Appendix D: Preparing and Handling Tet-On 3G Cell Line Stocks

## A. Protocol: Freezing Tet-On 3G Cell Line Stocks

Once you have created and tested your Tet-On 3G cell line, you must prepare multiple frozen aliquots to ensure a renewable source of cells, according to the following protocol:

9. Expand your cells to multiple 10 cm dishes or T75 flasks.
10. Trypsinize and pool all of the cells, then count the cells using a hemocytometer.
11. Centrifuge the cells at 100 x g for 5 min. Aspirate the supernatant.
12. Resuspend the pellet at a density of at least 1–2 x $10^6$ cells/ml in freezing medium. Freezing medium can be purchased from Sigma (Cat. Nos. C6164 & C6039), or use 70–90% FBS, 0–20% medium (without selective antibiotics), and 10% DMSO.
13. 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.
14. 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.
15. Two or more weeks later, plate a vial of frozen cells to confirm viability.

## B. Protocol: Thawing Tet-On 3G 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 stable and double-stable Tet Cell Lines in complete culture medium containing a maintenance concentration G418 and/or hygromycin (or puromycin), as appropriate (Section III.B).

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