Takara Bio USA, Inc.

# iDimerize™ Inducible Heterodimer System (with Tet-On® 3G) User Manual

Cat. No. 635079
(013117)

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# I. Introduction

The **iDimerize Inducible Heterodimer System (with Tet-On 3G)** (Cat. No. 635079) is an optimized system which combines Tet-On 3G inducible gene expression with iDimerize inducible protein interactions in live cells. One challenge of ligand-dependent dimerization experiments is that non ligand-induced dimerization events may occur if the protein of interest is expressed at high levels. This is especially problematic if the target protein is a membrane protein, because the local concentration can increase quickly due to the limited space on the membrane. For example, overexpression of the Fas receptor can induce apoptosis due to ligand-independent receptor trimerization when there is a high abundance of the Fas receptor in the cell membrane.

We have combined the iDimerize Heterodimer System with Tet-On 3G technology to reduce potential non ligand-induced dimerization events that occur due to overexpression of the protein being dimerized. First, use doxycycline (Dox) to optimize the expression levels of the DmrA-tagged and DmrC-tagged proteins to physiologically relevant levels. Then induce dimerization using the A/C Heterodimerizer ligand.

## A. Summary: Tet-On 3G Inducible Gene Expression

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 the $P_{TRE3G}$ promoter will express tightly controlled levels of your GOI when cultured in the presence of Dox (Figure 1).

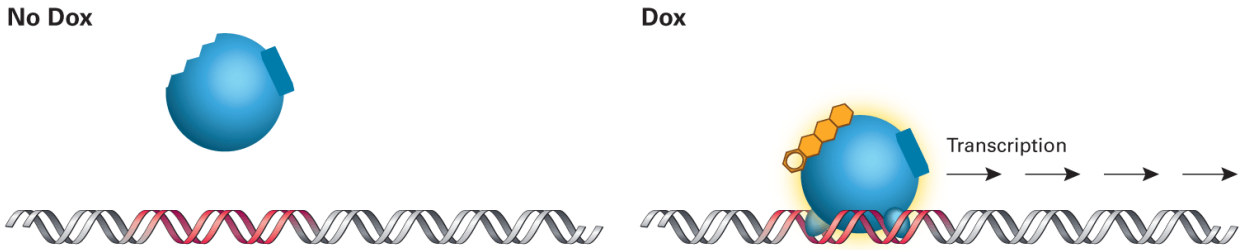



**Figure 1. The Tet-On 3G Systems allow inducible gene expression in the presence of Dox.**

### 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

$P_{TRE3G}$ provides for very low basal expression and high maximal expression after induction (Loew *et al*. 2010). It consists of 7 repeats of a 19 bp tet operator sequence located upstream of a minimal CMV promoter. In the presence of Dox, the Tet-On 3G transactivator 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.

### 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 *et al*. 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.

## B. Summary: iDimerize Inducible Protein Interactions

The iDimerize Systems are based on ARGENT dimerization control technology which was formerly available from ARIAD Pharmaceuticals, Inc. iDimerize systems bring protein-protein interactions under real-time, small molecule control. Any cellular process activated by protein-protein interactions can in principle be brought under dimerizer control, by fusing the protein(s) of interest to the binding module. Addition of the dimerizer then brings the chimeric signaling protein subunits into very close proximity to each other, mimicking the activation of the cellular event that the protein of interest controls.

The iDimerize Inducible Heterodimer Systems can be used to induce the association of two different proteins. The proteins of interest are fused to the DmrA and DmrC binding domains, respectively, and dimerization is induced by adding the A/C Heterodimerizer (AP21967). When A/C Heterodimerizer is added to live cells expressing DmrA and DmrC fusion proteins, it induces the heterodimerization of the fusion proteins (Graef *et al*., 1997, Castellano *et al*., 1999). Many cellular processes are triggered by this type of interaction—for example, protein localization at the plasma membrane (Figure 2).

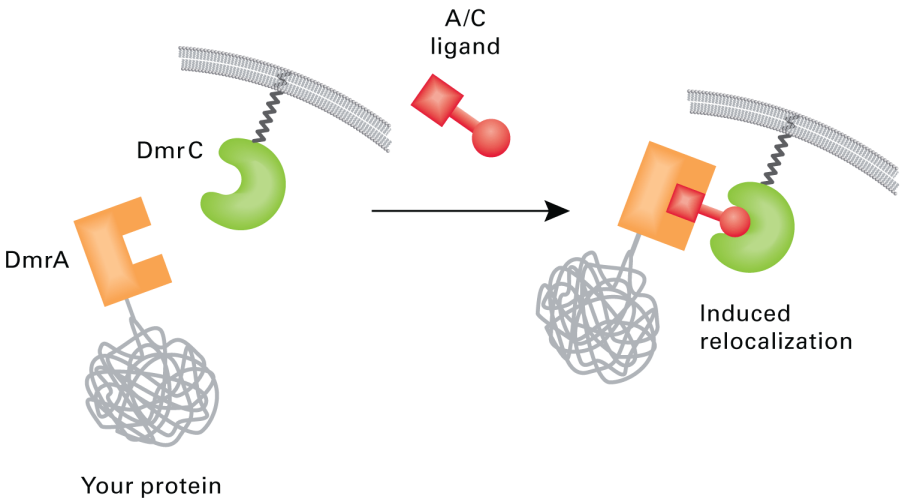



**Figure 2. Inducing protein accumulation at the plasma membrane using inducible heterodimerization.** This system can be used to induce any event that is controlled by the association of two different proteins, including cellular localization, conditional alleles of receptors, signaling molecules, and other proteins involved in a signaling pathway.

## C. iDimerize Inducible Heterodimer System (with Tet-On 3G)

The pTRE3G-BI-Het1 expression vector included in this system (Figure 3) allows you to express your DmrA- and DmrC-tagged proteins of interest in a Dox-dependent manner. After cloning your genes of interest into pTRE3G-BI-Het1, you have three options:

- Transient cotransfection of pTRE3G-BI-Het1 and pCMV-Tet3G into your cells of choice. *See Protocol VI.A.*
- Transient transfection of pTRE3G-BI-Het1 into a stable Tet-On 3G cell line. *See Protocol VI.B and Appendix A.*
- Creating a double-stable cell line expressing pTRE3G-BI-Het1 and the Tet-On 3G transactivator. *See Protocol VI.C and Appendices A and C.*

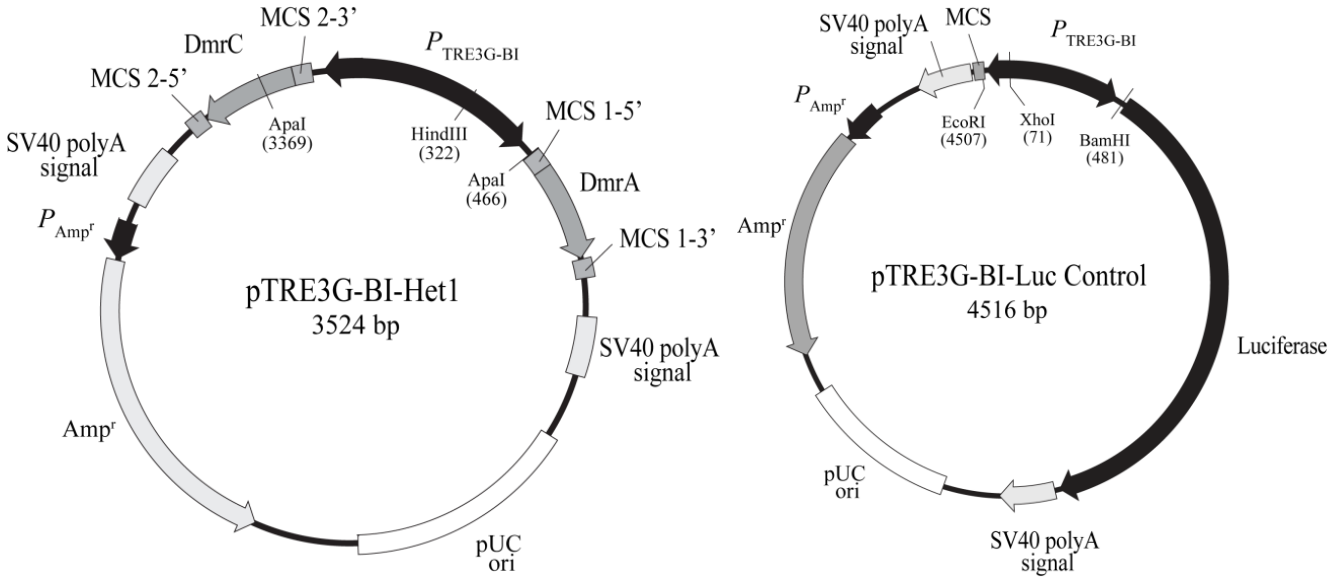



**Figure 3. pTRE3G-BI-Het1 and pTRE3G-BI-Luc Control vector maps.** pTRE3G-BI-Het1 allows simultaneous, dox-dependent expression of two proteins of interest, tagged with the DmrA and DmrC domains, respectively. pTRE3G-BI-Luc is a control vector that expresses firefly luciferase under the control of $P_{TRE3G}$. When used with standard luciferase detection reagents, this vector can be used as a reporter of induction efficiency.

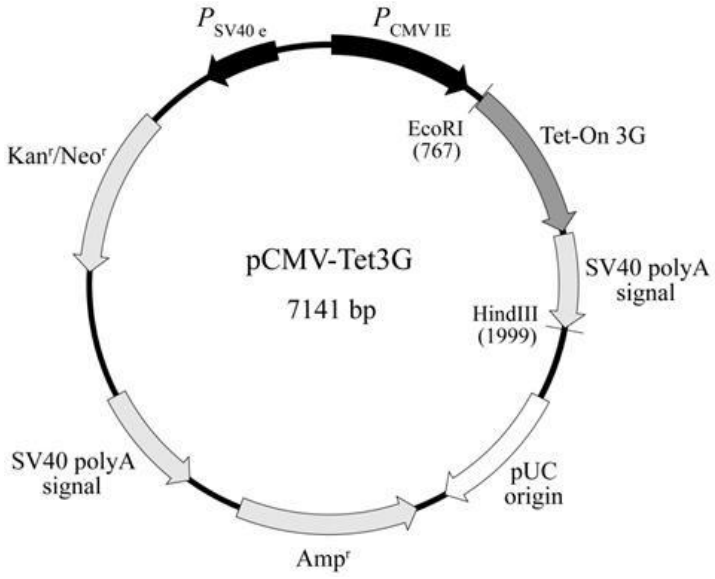



**Figure 4. pCMV-Tet3G vector map.** pCMV-Tet3G allows you to express the Tet-On 3G transactivator protein in your cells of interest.

# II. List of Components

Store all components at -20°C.

- 1 each iDimerize Inducible Heterodimer Vector Set 2 (Cat. No. 635080; not sold separately)
  - 20 µl pTRE3G-BI-Het1 Vector (500 ng/µl)
  - 20 µl pTRE3G-BI-Luc Control Vector (500 ng/µl)
  - 40 µl Linear Hygromycin Marker (50 ng/µl) (also sold separately as Cat. No. 631625)
  - 40 µl Linear Puromycin Marker (50 ng/µl) (also sold separately as Cat. No. 631626)
- 20 µl pCMV-Tet3G Vector (500 ng/µl) (Cat. No. 631335; not sold separately)
- 100 rxns Xfect™ Transfection Reagent (Cat. No. 631317)
- 50 ml Tet System Approved FBS, US-Sourced (also sold separately as Cat. No. 631105)
- 500 µl A/C Heterodimerizer (0.5 mM)
  (also sold separately as Cat. Nos. 635056, 635055 & 635095—see Section III.F)

**NOTE:** The A/C Heterodimerizer is so named because it induces dimerization of a protein possessing the DmrA domain and a second protein containing the DmrC domain.

# III. Additional Materials Required

## A. In-Fusion® HD Cloning System & Stellar™ Competent Cells

In-Fusion is a revolutionary technology that greatly simplifies cloning. For more information, visit takarabio.com/in-fusion. We recommend using Stellar Competent Cells, which are included in the In-Fusion HD Cloning Kits listed below. You can also purchase Stellar Competent Cells separately as Cat. No. 636763.

| Cat. No. | In-Fusion Cloning Kit |
|---|---|
| 638909 | In-Fusion HD Cloning Plus (10 rxns) |
| 638910 | In-Fusion HD Cloning Plus (50 rxns) |
| 638911 | In-Fusion HD Cloning Plus (100 rxns) |
| 638920 | In-Fusion HD Cloning Plus (96 rxns) |
| 638916 | In-Fusion HD Cloning Plus CE (10 rxns) |
| 638917 | In-Fusion HD Cloning Plus CE (50 rxns) |
| 638918 | In-Fusion HD Cloning Plus CE (100 rxns) |
| 638919 | In-Fusion HD Cloning Plus CE (96 rxns) |

## B. Xfect Transfection Reagents

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

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

## C. Doxycycline

| Cat. No. | Transfection Reagent |
|---|---|
| 631311 | Doxycycline (5 g) |

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.

## D. 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)
- Dulbecco's phosphate buffered saline (DPBS; VWR, Cat. No. 82020-066 or Sigma, Cat. No. D8662)
- 6-well, 12-well, and 24-well cell culture plates, 10 cm cell culture dishes

## E. Tetracycline-Free Fetal Bovine Serum

Contaminating tetracyclines, which are often found in serum, significantly elevate basal expression when using Tet-On 3G. The following functionally tested tetracycline-free sera are available from Takara Bio USA, Inc. (TBUSA):

| Cat. No. | Serum Name | Size |
|---|---|---|
| 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 |

## F. A/C Heterodimerizer

Each iDimerize Inducible Heterodimer System (with Tet-On 3G) includes 500 µl A/C Heterodimerizer (0.5 mM, see Section II). Additional A/C Heterodimerizer can also be purchased separately in the following sizes:

| Cat. No. | Product Name | Size |
|---|---|---|
| 635056 | A/C Heterodimerizer (0.5 mM) | 5 x 500 µl |
| 635055 | A/C Heterodimerizer* | 5 mg |
| 635095 | A/C Heterodimerizer* | 5 x 5 mg |

* Designed for *in vivo* use; supplied in a dry-down format.

## G. DmrA & DmrC Antibodies

The DmrA and DmrC Antibodies recognize the respective DmrA and Dmr C dimerization domains expressed using any iDimerize Inducible Heterodimer System, and are recommended for Western blot analysis.

| Cat. No. | Product Name | Size |
|---|---|---|
| 635089 | DmrA Monoclonal Antibody (0.5 µg/µl) | 100 µg |
| 635091 | DmrC Polyclonal Antibody (0.5 µg/µl) | 100 µg |

## H. Antibiotics for Selecting Stable Cell Lines

| Cat. No. | Antibiotic | Size |
|---|---|---|
| 631308 | G418 | 5 g |
| 631307 | | 1 g |
| 631306 | Puromycin | 100 mg |
| 631305 | | 25 mg |
| 631309 | Hygromycin B | 1 g |

## I. Tet-On 3G Cell Lines

| Cat. No. | Cell Line |
|---|---|
| 631181 | Jurkat Tet-On 3G Cell Line |
| 631182 | HEK 293 Tet-On 3G Cell Line |
| 631183 | HeLa Tet-On 3G Cell Line |
| 631195 | CHO Tet-On 3G Cell Line |
| 631197 | NIH/3T3 Tet-On 3G Cell Line |

## J. 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 | Size |
|---|---|---|
| 631131 | TetR Monoclonal Antibody (Clone 9G9) | 40 µg |
| 631132 | | 200 µg |

## K. Luciferase Assay and Luminometer

These items are required when using the pTRE3G-BI-Luc Vector to screen Tet-On 3G clones. Use any standard luciferase assay system and luminometer.

# IV. Protocol Overview

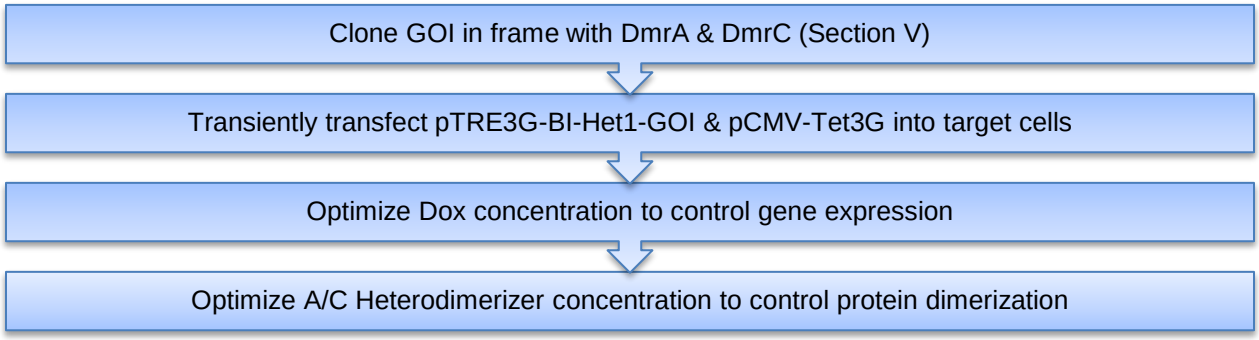
Please read each protocol completely before starting. Successful results depend on understanding and performing all the steps correctly.

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

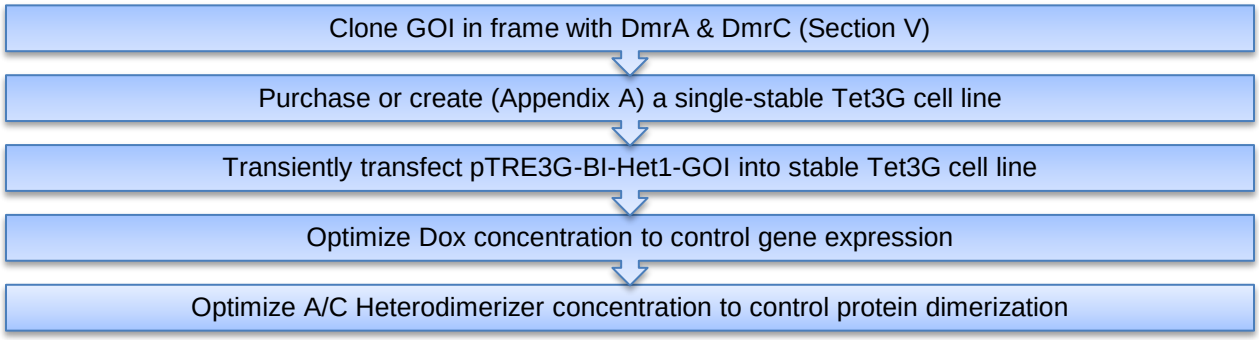
Freshney, R.I. (2005). *Culture of Animal Cells: A Manual of Basic Technique, 5th Edition* (Wiley-Liss, Hoboken, NJ).

Below you will find protocols for three different experimental approaches:

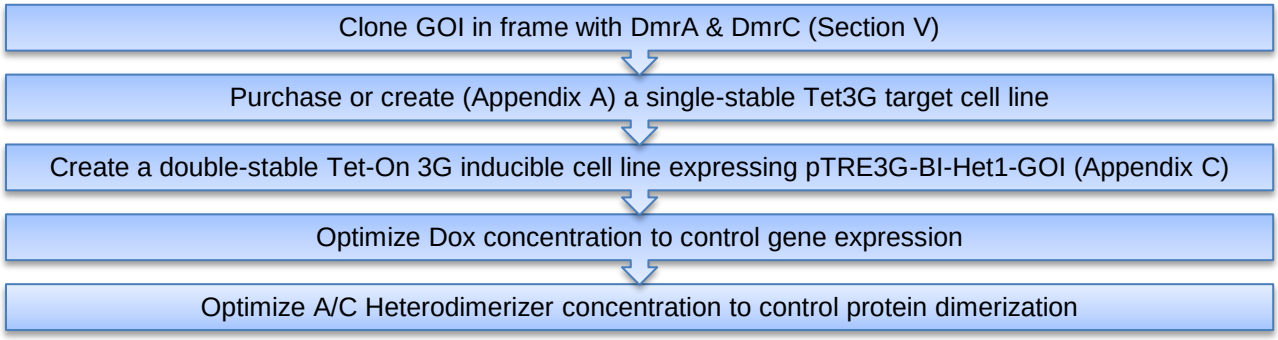
- **Transient cotransfection of pTRE3G-BI-Het1-GOI and pCMV-Tet3G into your cells of choice (see Protocol VI.A).**




- **Transient transfection of pTRE3G-BI-Het1-GOI into a stable Tet-On 3G cell line (see Protocol VI.B and Appendix A).**




- **Creating a double-stable cell line expressing pTRE3G-BI-Het1-GOI and the Tet-On 3G transactivator (see Protocol VI.C and Appendices A and C).**



# V. Creating a pTRE3G-BI-Het1 Construct Encoding your GOIs

We recommend using the In-Fusion HD Cloning System (Section III.A) to clone your genes of interest into pTRE3G-BI-Het1. In-Fusion HD cloning is generally recommended over ligation-based cloning because it is directional, unaffected by internal cut sites, and highly efficient (most clones contain the correct insert).

Follow the protocol outlined in the In-Fusion HD user manual. To find the manual, go to takarabio.com/manuals and type "In-Fusion HD" in the search box.

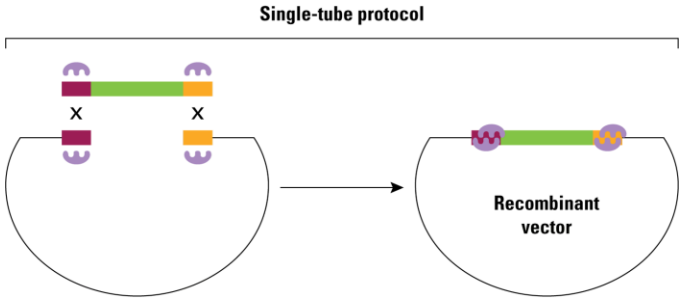



**Figure 5. The In-Fusion HD single-tube cloning protocol.**

# VI. Combining iDimerize Heterodimerization with the Tet-On 3G System

After cloning your gene of interest into pTRE3G-BI-Het1, you have three options:

A. **Transiently cotransfect pTRE3G-BI-Het1 and pCMV-Tet3G into your cells of choice.**
*Use these cells directly to perform your actual ligand-induced dimerization experiment, or for pilot testing before creating a stable cell line.*

B. **Transiently transfect pTRE3G-BI-Het1 into a stable Tet-On 3G cell line.**
*Make your own Tet-On 3G-expressing stable cell line using pCMV-Tet3G, or purchase a premade stable cell line from TBUSA. See section III.I for information on our premade Tet-On 3G cell lines.*

C. **Create a double-stable cell line by transfecting pTRE3G-BI-Het1 into a cell line expressing the Tet-On 3G transactivator, using two different selection markers.**
*First, make a single-stable Tet-On 3G cell line. Then transfect pTRE3G-BI-Het1 into the single-stable Tet-On 3G cell line along with a linear selection marker ($Hyg^r$ or $Pur^r$) and select double-stable transfectants by screening for hygromycin or puromycin resistance, and inducibility.*
*Alternatively, you can begin with a single-stable premade cell line from TBUSA (see section III.I) and transfect pTRE3G-BI-Het1 and the appropriate resistance cassette into the premade Tet-On 3G cell line.*

# A. Protocol: Study the Effects of Dimerization in Cells Transiently Cotransfected with pTRE3G-BI-Het1 and pCMV-Tet3G

### Materials required

- pTRE3G-BI-Het1 construct encoding your GOIs (Section V)
- pCMV-Tet3G
- Host cell line: *If this is a pilot test prior to creating a double-stable cell line, choose an easy-to-transfect cell line such as HeLa or HEK 293.*
- Xfect Transfection Reagent (Section III.B)
- Doxycycline (1 mg/ml) (Section III.C)
- Mammalian cell culture supplies (Section III.D)
- Tet Approved FBS (Section III.E)
- A/C Heterodimerizer (Section III.F)

### 1. Determine optimal doxycycline concentration

The first step in a study of this type is to determine the maximum dose of doxycycline that *does not* result in dimerization of your proteins of interest in the absence of A/C Heterodimerizer (that is, to optimize the expression level of your protein of interest). You will use this concentration of doxycycline in all subsequent cotransfection studies.

a. Seed your target cells in each well of a 6-well plate. When the cells reach 50–80% confluence, cotransfect your pTRE3G-BI-Het1 construct and pCMV-Tet3G into the target cells using Xfect Transfection Reagent. Use 1 µg of pCMV-Tet3G and 4 µg of your pTRE3G-BI-Het1 construct for each well and follow the Xfect Protocol.
To find the protocol, go to takarabio.com/manuals and type "Xfect" in the search box.

b. 4 hr later, replace the media with fresh media with or without doxycycline, as shown:


**Well 1:** No Dox
**Well 2:** 1 ng/ml Dox
**Well 3:** 10 ng/ml Dox
**Well 4:** 25 ng/ml Dox
**Well 5:** 100 ng/ml Dox
**Well 6:** 1,000 ng/ml Dox

c. Incubate your cells in media containing Dox for at least 24 hr.

d. At an appropriate time point (after at least 24 hr), test for the event of interest caused by *ligand-independent* dimerization of your proteins of interest using an appropriate assay.

e. For your experiment using the A/C Heterodimerizer ligand, choose the maximum concentration of Dox at which you do **not** observe the ligand-independent dimerization event. Use this concentration of Dox in Step A.2.

### 2. Determine optimal A/C Heterodimerizer concentration

Now that you have determined the optimal level of Dox that induces expression of your proteins of interest *without* causing ligand-independent dimerization, you can determine the optimal amount of ligand to add in order to induce dimerization.

a. Seed your target cells in each well of a 6-well plate. When the cells reach 50–80% confluence, cotransfect your pTRE3G-BI-Het1construct and pCMV-Tet3G into the target cells using Xfect Transfection Reagent. Use 1 µg of pCMV-Tet3G and 4 µg of pTRE3G-BI-Het1construct for each well and follow the Xfect Protocol.

b. 4 hr later, replace the media with fresh media containing the optimal concentration of doxycycline from Step A.1.e. Incubate your cells in media containing Dox for at least 12 hr.

c. Add a range of A/C Heterodimerizer concentrations to the growth media containing Dox.


Each well should contain the optimal concentration of Dox determined in Step A.1.e above, plus:

**Well 1:** 0 nM A/C Heterodimerizer
**Well 2:** 10 nM A/C Heterodimerizer
**Well 3:** 50 nM A/C Heterodimerizer
**Well 4:** 100 nM A/C Heterodimerizer
**Well 5:** 250 nM A/C Heterodimerizer
**Well 6:** 1,000 nM A/C Heterodimerizer

d. At an appropriate time point, test for the event caused by ligand-dependent dimerization of your proteins of interest.

**Notes:**

- Test for the effects of dimerization using any assay that is appropriate for your experiment.
- The amount of time you should wait to perform your analysis after adding A/C Heterodimerizer depends on the nature of the event caused by protein heterodimerization. Rapid events such as phosphorylation may occur within 15–60 min. Slower events, such as differentiation, may require 1–5 days.

## B. Protocol: Study the Effects of Dimerization in a Stable Tet3G Cell Line Transiently Transfected with pTRE3G-BI-Het1

### Materials required

- pTRE3G-BI-Het1 construct encoding your GOIs (Section V)
- Stable Tet-On 3G Cell Line made with pCMV-Tet3G (Appendix A) or a premade Tet-On 3G Cell Line (Section III.I)
- Xfect Transfection Reagent (Section III.B)
- Doxycycline (1 mg/ml) (Section III.C)
- Mammalian cell culture supplies (Section III.D)
- Tet Approved FBS (Section III.E)
- A/C Heterodimerizer (Section III.F)

### 1. Create a single-stable cell line

If you are not using a premade Tet-On 3G Cell Line from TBUSA, please see Appendix A for instructions about creating a Tet-On 3G-Expressing Stable Cell Line.

### 2. Determine optimal doxycycline concentration

The first step in a study of this type is to determine the maximum dose of doxycycline that *does not* result in dimerization of your proteins of interest in the absence of A/C Heterodimerizer (that is, to optimize the expression levels of your proteins of interest). You will use this concentration of doxycycline in all subsequent studies.

a. Seed your stable Tet-On 3G-expressing cells in each well of a 6-well plate. When they reach 50–80% confluence, transfect your pTRE3G-BI-Het1 construct into the Tet-On 3G-expressing cells using Xfect Transfection Reagent. Use 5 µg of pTRE3G-BI-Het1 for each well, and follow the Xfect Protocol.
To find the protocol, go to takarabio.com/manuals and type "Xfect" in the search box.

b. 4 hr later, replace the media with fresh media with or without doxycycline, as shown:


**Well 1:** No Dox
**Well 2:** 1 ng/ml Dox
**Well 3:** 10 ng/ml Dox
**Well 4:** 25 ng/ml Dox
**Well 5:** 100 ng/ml Dox
**Well 6:** 1,000 ng/ml Dox

c. Incubate your cells in media containing Dox for at least 12 hr.

d. At an appropriate time point, test for the event of interest caused by *ligand-independent* dimerization of your proteins of interest using an appropriate assay.

e. For your experiment using the A/C Heterodimerizer ligand, choose the maximum concentration of Dox at which you do **not** observe the *ligand-independent* dimerization event. Use this concentration of Dox in Step B.3.

### 3. Determine optimal A/C Heterodimerizer concentration

Now that you have determined the optimal level of Dox that induces expression of your proteins of interest *without* causing ligand-independent dimerization, you can determine the optimal amount of ligand to add in order to induce dimerization.

a. Seed your cells that are stably expressing Tet-On 3G in each well of a 6-well plate. When the cells reach 50–80% confluence, transfect your pTRE3G-BI-Het1 construct into your Tet-On 3G Cell Line using Xfect Transfection Reagent. Use 5 µg of pTRE3G-BI-Het1 for each well, and follow the Xfect Protocol.
   To find the protocol, go to takarabio.com/manuals and type "Xfect" in the search box.
b. 4 hr later, replace the media with fresh media containing the optimal concentration of doxycycline from Step B.2.e. Incubate your cells in media containing Dox for at least 12 hr.
c. Add a range of A/C Heterodimerizer concentrations to the growth media containing Dox:


Each well should contain the optimal concentration of Dox determined in Step B.2.5 above, plus:

**Well 1:** 0 nM A/C Heterodimerizer
**Well 2:** 10 nM A/C Heterodimerizer
**Well 3:** 50 nM A/C Heterodimerizer
**Well 4:** 100 nM A/C Heterodimerizer
**Well 5:** 250 nM A/C Heterodimerizer
**Well 6:** 1,000 nM A/C Heterodimerizer

d. At an appropriate time point, test for the event caused by ligand-dependent dimerization of your proteins of interest.

**Notes:**

- Test for the effects of dimerization using any assay that is appropriate for your experiment.
- The amount of time you should wait to perform your analysis after adding A/C Heterodimerizer depends on the nature of the event caused by protein heterodimerization. Rapid events such as phosphorylation may occur within 15–60 min. Slower events, such as differentiation, may require 1–5 days.

## C. Protocol: Study the Effects of Dimerization in a Double-Stable Cell line expressing your pTRE3G-BI-Het1 construct

### Materials required

- pTRE3G-BI-Het1 construct encoding your GOIs (Section V)
- Linear Hygromycin/Puromycin Markers (Section II and Appendix D)
- Stable Tet-On 3G Cell Line made with pCMV-Tet3G or a premade Tet-On 3G Cell Line (Appendix A or Section III.I, respectively)
- Xfect Transfection Reagent (Section III.B)
- G418 (Section III.H)
- Doxycycline (1 mg/ml) (Section III.C)
- Mammalian cell culture supplies (Section III.D)
- Tet Approved FBS (Section III.E)
- A/C Heterodimerizer (Section III.F)

### 1. Create a single-stable cell line

If you are not using a premade Tet-On 3G Cell Line from TBUSA, please see Appendix A for instructions about creating a Tet-On 3G-Expressing Stable Cell Line.

### 2. Create a double-stable Tet-On 3G inducible cell line

Please see Appendix C for instructions about creating a double-stable Tet-On 3G inducible cell line expressing pTRE3G-BI-Het1.

### 3. Determine optimal doxycycline concentration

The first step in a study of this type is to determine the maximum dose of doxycycline that *does not* result in dimerization of your protein of interest in the absence of A/C Heterodimerizer (that is, to optimize the expression level of your protein of interest). You will use this concentration of doxycycline in all subsequent co-transfection studies.

a. Seed your double-stable cell line in each well of a 6-well plate at a density of 50% and grow in fresh media with or without doxycycline, as shown:


**Well 1:** No Dox
**Well 2:** 1 ng/ml Dox
**Well 3:** 10 ng/ml Dox
**Well 4:** 25 ng/ml Dox
**Well 5:** 100 ng/ml Dox
**Well 6:** 1,000 ng/ml Dox

b. Incubate your cells in media containing Dox for at least 12 hr.
c. At an appropriate time point, test for the event of interest caused by *ligand-independent* dimerization of your proteins of interest using an appropriate assay.
d. For your experiment using the A/C Heterodimerizer ligand, choose the maximum concentration of Dox at which you do **not** observe the *ligand-independent* dimerization event. Use this concentration of Dox in Step C.4.

### 4. Determine optimal A/C Heterodimerizer concentration

Now that you have determined the optimal level of Dox that induces expression of your proteins of interest *without* causing ligand-independent dimerization, you can determine the optimal amount of ligand to add in order to induce dimerization.

a. Seed your double-stable cell line in each well of a 6-well plate at a density of 50%.

b. After the cells have attached, replace the media with fresh media containing the optimal concentration of doxycycline from Step C.3.d. Incubate your cells in media containing Dox for at least 12 hr.

c. Add a range of A/C Heterodimerizer concentrations to the growth media containing Dox:


Each well should contain the optimal concentration of Dox determined in Step C.3.d above, plus:

**Well 1:** 0 nM A/C Heterodimerizer
**Well 2:** 10 nM A/C Heterodimerizer
**Well 3:** 50 nM A/C Heterodimerizer
**Well 4:** 100 nM A/C Heterodimerizer
**Well 5:** 250 nM A/C Heterodimerizer
**Well 6:** 1,000 nM A/C Heterodimerizer

d. At an appropriate time point, test for the event caused by ligand-dependent dimerization of your proteins of interest.

**Notes:**

- Test for the effects of dimerization using any assay that is appropriate for your experiment.
- The amount of time you should wait to perform your analysis after adding A/C Heterodimerizer depends on the nature of the event caused by protein heterodimerization. Rapid events such as phosphorylation may occur within 15–60 min. Slower events, such as differentiation, may require 1–5 days.

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# VIII. Troubleshooting

## A. iDimerize Troubleshooting

| Description of Problem | Possible Explanation | Solution |
|---|---|---|
| Dimerization is observed in the absence of the A/C Heterodimerizer | The expression level of the proteins of interest fused to the DmrA and C domains is too high, especially in the case of a tagged protein of interest that localizes to the plasma membrane. | Use a lower concentration of Dox to limit the gene expression level:<br>• Transient cotransfections of pTRE3G-BI-Het1 and pCMV-Tet3G: See Part VI, Protocol A.1<br>• Stable Tet3G cell lines transiently transfected with pTRE3G-BI-Het1: See Part VI, Protocol B.2<br>• Double-stable cell lines expressing Tet3G and pTRE3G-BI-Het1: See Part VI, Protocol C.3 |
| Addition of A/C Heterodimerizer does not result in the expected effect(s) | The A/C Heterodimerizer concentration is too low. | Increase the amount of A/C Heterodimerizer added. |
| | The monitoring assay is not sensitive enough. | • Include a positive control when performing your assay.<br>• Monitor protein expression in your cells by Western blot using the anti-DmrA and anti-DmrC antibodies (section III.G). |
| | The volume of A/C Heterodimerizer used causes cells to die, due to high solvent concentration. | Prepare a more concentrated stock solution. |

## B. Tet-On 3G Troubleshooting

### 1. 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 recommend a cotransient transfection vector ratio of 1:4 for pCMV-Tet3G: pTRE3G-BI-Het1 (Section VI.A). Different vector ratios may result in different maximal to basal gene expression ratios. |
| | Cells were harvested and analyzed too early or too late. | Harvest and analyze cells between 18–48 hr post transfection. |
| | Poor transfection efficiency | • Optimize transfection protocol<br>• Optimize density of cell plating; use at 60–90% confluency |
| | Poor target cell viability | • Optimize target cell passage number<br>• Optimize target cell culture conditions<br>• Optimize tissue culture plasticware |
| | The FBS used in the cell culture medium contains tetracycline derivatives. | Use Tet System Approved FBS (Section III.E). Only TBUSA performs actual inducibility tests on a sensitive Tet-inducible cell line in order to provide an absolute guarantee that your serum is tetracycline-free. |
| | 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). |

### 2. 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.<br>• Mixed cell population in the selected clone [1]. | Screen additional individual drug-resistant cell clones to ensure optimal fold induction. |
| 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-BI-Luc. |

[1] 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.

### 2. Low fold induction of stable expression, continued

| Description of Problem | Possible Explanation | Solution |
|---|---|---|
| Decrease in fold induction after several passages<br>or<br>Loss of inducibility after passaging of a (previously frozen) double-stable cell line | Appropriate antibiotics are missing from the cell culture medium. | Maintain optimal antibiotic concentrations (see Table I in Appendix A). |
| | Mixed cell population in the selected clone [1]. | Reselect the current cell line through single colony selection using selective concentrations of both antibiotics, and screen again with pTRE3G-BI-Luc. |

[1] 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.

### 3. Establishment of stable cell lines

| Description of Problem | Possible Explanation | Solution |
|---|---|---|
| Cells do not die at the high antibiotic concentration established via titration in Appendix E. | 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 optimal antibiotic concentration. | 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 complete recovery prior to transfection experiments. |
| | Transfection was inefficient because the wrong ratio of Vector/Linear Selection Marker was used. | Retransfect Vector/Linear Selection Marker at a ratio of 20:1. |
| | Antibiotic was added too soon. | See Appendix A, Protocol B & Appendix C, Protocol B. |
| | Used wrong antibiotic concentration. | See Appendix C, Protocol 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 of the Tet-On 3G User Manual. |
| | Used wrong antibiotic concentration | See Table I in Appendix A |
| Poor cell viability | • Cells were not properly frozen<br>• Cells were not properly thawed | See Appendix B |

### 4. 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.<br>• Monitor protein expression in your cells by Western blot using the anti-DmrA and anti-DmrC antibodies (section III.G). |
| 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. For example, 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 takarabio.com). |
| | Doxycycline 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 our Tet System Approved FBS.<br>• 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: Creating a Single-Stable Cell Line Expressing Tet-On 3G

This appendix explains how to create 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-BI-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 VI.B to transiently transfect pTRE3G-BI-Het1 into your stable Tet-On 3G cell line or Section VI.C to develop a double-stable Tet-On 3G inducible cell line with pTRE3G-BI-Het1. Be sure to freeze aliquots of your Tet-On 3G cell line(s) (Appendix B).

**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

- pCMV-Tet3G
- pTRE3G-BI-Luc Control Vector
- Host cell line
- Xfect Transfection Reagent (Section III.B)
- G418 (Section III.H)
- Doxycycline (1 mg/ml; Section III.C)
- Mammalian cell culture supplies (Section III.D)
- Tet Approved FBS (Section III.E)

## 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 48 hr after transfection. Then transfect pCMV-Tet3G into your target cells using Xfect Transfection Reagent. Follow the Xfect Protocol, except use 2 µg of plasmid per well.
   To find the protocol, go to takarabio.com/manuals and type "Xfect" in the search box

   **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.

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

3. After an additional 48 hr, add G418 at the selection concentration that is optimal for your cell line. For most cell lines, this is between 400–500 µg/ml (Table I).

   **Table 1. Recommended antibiotic concentrations for selecting and maintaining stable clones.**

| Cat. No. | Antibiotic | Recommended Concentration (µg/ml) | |
|---|---|---|---|
| | | Selection[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, you must determine the appropriate dose for your specific cell line empirically. 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.

4. Replace medium with fresh complete medium plus G418 every four days, or more often if necessary.

5. 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).

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 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 (Appendix E).

8. 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 (Appendix A, Protocol C).

   **NOTE:** You may wish to use TetR monoclonal antibody (Section III.J) 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, 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 (from Step B. 8 above) 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-BI-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 B).

## Appendix B: 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:

1. Expand your cells to multiple 10 cm dishes or T75 flasks.
2. Trypsinize and pool all of the cells, then count the cells using a hemocytometer.
3. Centrifuge the cells at 100 x g for 5 min. Aspirate the supernatant.
4. Resuspend the pellet at a density of at least 1–2 x $10^6$ 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.
5. Dispense 1 ml aliquots into sterile cryovials and freeze slowly (1°C per min). For this purpose, you can place the vials in Nalgene cryo-containers (Nalgene Cat. No. 5100) and freeze at –80°C overnight. Alternatively, place vials in a thick-walled styrofoam container at –20°C for 1–2 hr. Transfer to –80°C and freeze overnight.
6. The next day, remove the vials from the cryo-containers or styrofoam containers, and place in liquid nitrogen storage or an ultra-low temperature freezer (–150°C) for storage.
7. Two or more weeks later, plate a vial of frozen cells to confirm viability.

## B. Protocol: Thawing 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 (Table I in Appendix A).

# Appendix C: Creating & Screening for a Double-Stable Cell Line Capable of High Induction of TRE3G-BI-Het1

## A. Materials Required

- pTRE3G-BI-Het1 construct (Section V)
- Linear Hygromycin/Puromycin Marker (Section II)
- Tet-On 3G Cell Line (Appendix A or Section III.I)
- Xfect Transfection Reagent (Section III.B)
- G418 (Section III.H)
- Doxycycline (1 mg/ml) (Section III.C)
- Mammalian cell culture supplies (Section III.D)
- Tet Approved FBS (Section III.E)

## 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 pTRE3G-BI-Het1 construct into your Tet-On 3G cell line (generated according to Appendix A above, or purchased separately from TBUSA) along with a linear selection marker (Hyg$^r$ or Pur$^r$). Select double-stable transfectants by screening for hygromycin or puromycin resistance, and inducibility.

**NOTES:**

- 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 D.

1. 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, cotransfect 2 µg pTRE3G-BI-Het1 with 100 ng linear selection marker (puromycin or hygromycin).
   To find the Xfect protocol, go to takarabio.com/manuals and type "Xfect" in the search box.

   **NOTE:** Always combine your customized pTRE3G-BI-Het1 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 (see Table I in Appendix A).
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 E).

9. Culture the clones in maintenance concentrations of both G418 and either hygromycin or puromycin (see Table I in Appendix A). When confluent, split the cells from each well into three wells of a 6-well plate for testing and maintenance (Part C, below).

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

Test individual double-stable clones for expression of your genes of interest 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 (Appendix C.B, Step 9 above) 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 B).

   **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).

## Appendix D: 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 genes 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-BI-Het1 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-BI-Het1 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-BI-Het1 plasmid (i.e., 20-fold less of the linear marker), these types of interference are less likely to occur.

## Appendix E: 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.

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.
   To find the protocol, go to takarabio.com/manuals and type "Xfect" in the search box.
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).
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 to determine the optimal concentrations of Dox (Part VI.B. for single-stable cell lines or Part VI.C for double-stable cell lines).


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