Takara Bio USA, Inc.

Lenti-X™ iDimerize™ Inducible Homodimer System (with Tet-On® 3G) User Manual User Manual

Cat. Nos. 635086, 635088, 635090, 635059, 635058, 635069, 632622
(011018)

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I. Introduction

The **Lenti-X iDimerize Inducible Homodimer System (with Tet-On 3G)** (Cat. No. 635086) is an optimized system which combines lentiviral gene delivery, Tet-On 3G inducible gene expression, and 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 Homodimer System with Tet-On 3G technology to reduce potential non-ligand induced dimerization events that occur due to the protein being dimerized, and lentiviral technology to deliver your GOI to almost any mammalian cell type, including dividing and nondividing cells, primary cell cultures, stem cells, and neurons. First, create your lentiviruses and transduce your target cells. Then use doxycycline (Dox) to optimize the DmrB-tagged protein's expression to a physiologically relevant level. Finally, induce dimerization using the B/B Homodimerizer ligand.

A. Summary: 4th Generation Lentiviral Packaging System

Lenti-X Packaging Single Shots (VSV-G), provided with the Lenti-X iDimerize Inducible Homodimer System (with Tet-On 3G) (Cat. No. 635086), can generate lentiviral titers that are superior to most other commercially available lentiviral packaging systems. The concerted effects of multiple components in an optimized five-vector plasmid mix, pre-aliquoted and lyophilized with Xfect™ Transfection Reagent, allow Lenti-X 293T Cells (sold separately; Cat. No. 632180) to produce the highest amounts of safe, replication-incompetent lentivirus (see **takarabio.com**).

B. 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} will express tightly controlled levels of your GOI when cultured in the presence of Dox (Figure 1).

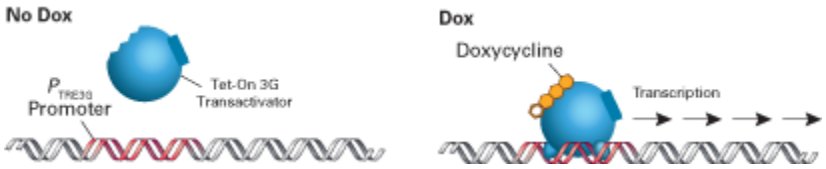


Figure 1. The Tet-On 3G Systems allow inducible gene expression in the presence of Dox.

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 they are 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.

C. 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 Homodimer Systems can be used to induce self-association of two or more copies of the same protein. The protein of interest is fused to the DmrB binding domain, and dimerization is induced by adding the B/B Homodimerizer (AP20187).This cell-permeable ligand induces two or more copies of the DmrB fusion protein to interact (Clackson *et al*. 1998). Many cellular processes are triggered by this type of interaction—for example, intracellular signaling (Crabtree *et al*. 1996; Figure 2).

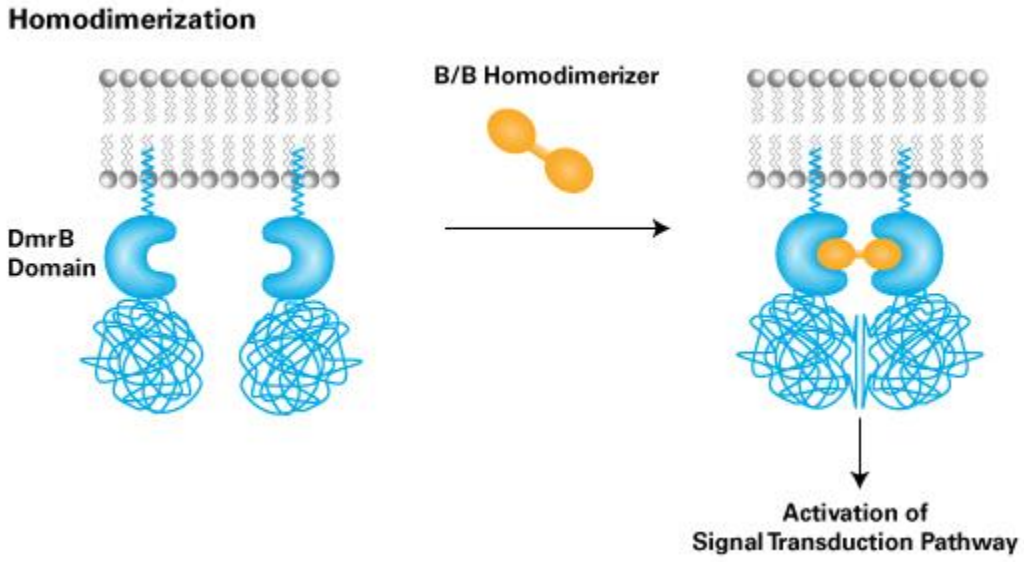


Figure 2. Controlling signal transduction using regulated homodimerization. This system can be used to induce any event that is controlled by self-association of two (or more) copies of the same protein, including proliferation, differentiation, adhesion, transformation, and apoptosis.

D. Lenti-X iDimerize Inducible Homodimer System (with Tet-On 3G)

The pLVX-TRE3G-Hom1 expression vector included in this system (Figure 3), in combination with the Lenti-X HTX Packaging System (which allows for packaging and delivery of high-titer lentivirus), enables your DmrB-tagged protein of interest to be expressed in a wide range of cell types, in a Dox-dependent manner.

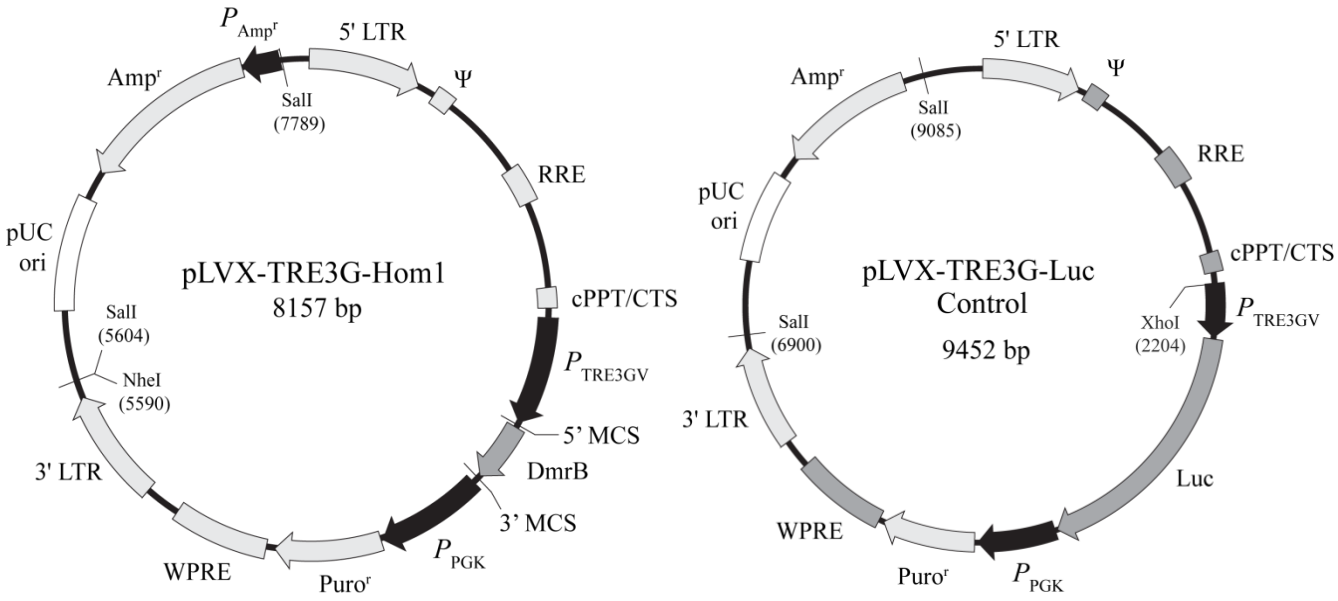


Figure 3. pLVX-TRE3G-Hom1 and pLVX-TRE3G-Luc Control vector maps. pLVX-TRE3G-Hom1 allows you to express your DmrB-tagged protein of interest in a Dox-dependent manner. pLVX-TRE3G-Luc is a control vector that expresses firefly luciferase under the control of P_{TRE3GV}. When used with standard luciferase detection reagents, this vector can be used as a reporter of induction efficiency.

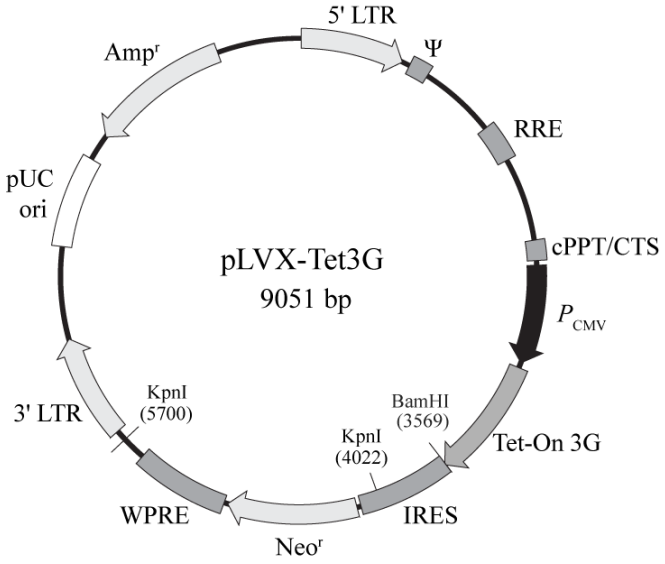


Figure 4. pLVX-Tet3G vector map. pLVX-Tet3G allows you to express the Tet-On 3G transactivator protein in your cells of interest.

II. List of Components

Store Lenti-X GoStix™ Plus at room temperature. Store all other components at -20°C.

- 1 each pLVX-TRE3G-Hom1 Vector Set (Cat. No. 635087; not sold separately)
  - 20 µl pLVX-TRE3G-Hom1 Vector (500 ng/µl)
  - 20 µl pLVX-TRE3G-Luc Control Vector (500 ng/µl)
- 20 µl pLVX-Tet3G Vector (500 ng/µl) (Cat. No. 631358; not sold separately)
- 16 rxns Lenti-X Packaging Single shots
- 1 each Lenti-X GoStix Plus (Sample) (Cat. No. 631279; not sold separately)
- 500 µl B/B Homodimerizer (0.5 mM; also sold separately as Cat. Nos. 635059, 635058, 635069 & 632622—see Section III.F)

NOTE: The B/B Homodimerizer is so named because it induces dimerization of two proteins that contain the DmrB dimerization 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/infusion. 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	Size
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

- **Medium for Lenti-X 293T Cells:**
  90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and sodium bicarbonate (Sigma-Aldrich, Cat. No. D5796); 10% Fetal Bovine Serum (FBS); 100 units/ml penicillin G sodium; and 100 µg/ml streptomycin sulfate.
- 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)
- 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:

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

F. B/B Homodimerizer

Each Lenti-X iDimerize Inducible Homodimer System (with Tet-On 3G) includes 500 µl B/B Homodimerizer. Additional B/B Homodimerizer can also be purchased separately in the following sizes:

Cat. No.	Product Name	Size
635059	B/B Homodimerizer (0.5 mM)	5 x 500 µl
635058	B/B Homodimerizer*	5 mg
635069		25 mg
632622		4 x 25 mg

*Supplied in a dry-down format.

G. B/B Washout Ligand

B/B Washout Ligand is a membrane-permeant ligand that dissociates protein interactions induced by the B/B Homodimerizer. It dissociates these interactions with a $T_{1/2}$ of ~10 minutes after adding it to target cells treated with B/B Homodimerizer.

Cat. No.	Product Name	Size
635088	B/B Washout Ligand (0.5 mM)	500 µl

H. DmrB Monoclonal Antibody

The DmrB Monoclonal Antibody recognizes the DmrB binding domain expressed using iDimerize Inducible Homodimer Systems, and is recommended for Western blot analysis.

Cat. No.	Product Name	Size
635090	DmrB Monoclonal Antibody (0.5 µg/µl)	100 µg

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. Lenti-X 293T Cells

Getting the most from any lentiviral packaging system requires a host 293T cell line that transfects easily and supports high-level expression of viral proteins. Our Lenti-X 293T Cell Line was clonally selected to meet these requirements, allowing you to produce the highest possible lentiviral titers when combined with Lenti-X Packaging Single Shots (VSV-G), an optimized fourth-generation packaging system, pre-mixed and lyophilized with Xfect Transfection Reagent.

Cat. No.	Cell Line
632180	Lenti-X 293T Cell Line

K. High-Titer Packaging System

This 4th generation lentiviral packaging system can generate lentiviral titers that are superior to most other commercially available lentiviral packaging systems. The concerted effects of multiple components in an optimized five-vector plasmid mix, pre-aliquoted and lyophilized with Xfect Transfection Reagent, allow Lenti-X 293T Cells to produce the highest amounts of safe, replication-incompetent lentivirus (see takarabio.com).

Cat. No.	Packaging System
631275	Lenti-X Packaging Single Shots (VSV-G) (16 rxns)
631276	Lenti-X Packaging Single Shots (VSV-G) (96 rxns)

L. Lentiviral Titer Determination

For accurate and consistent transductions, we highly recommend titrating your lentiviral stocks. Various technologies are available; visit takarabio.com for details.

Cat. No.	Lentiviral Titration Technology
632200	Lenti-X p24 Rapid Titer Kit (96 rxns)
631235	Lenti-X qRT-PCR Titration Kit (200 rxns)
631280	Lenti-X GoStix Plus (20 tests)

M. Lentivirus Concentration

Use Lenti-X Concentrator to simply increase your available titer up to 100-fold without ultracentrifugation—visit takarabio.com for details.

Cat. No.	Concentrator
631231	Lenti-X Concentrator (100 ml)
631232	Lenti-X Concentrator (500 ml)

N. Transduction Enhancers

Use Polybrene (hexadimethrine bromide; Sigma-Aldrich, No. H9268), Lenti-X Accelerator, or RetroNectin®.

- Lenti-X Accelerator is a magnetic bead-based technology designed to accelerate lentiviral and retroviral transduction experiments; visit takarabio.comfor details.
- RetroNectin is a multivalent molecule that simultaneously binds virus particles and cell surface proteins, maximizing cell-virus contact. RetroNectin, in particular, is recommended for increasing the transduction efficiency of suspension cells and stem cells; visit takarabio.comfor details.

Cat. No.	Transduction Enhancer
631256	Lenti-X Accelerator (400 µl)
631257	Lenti-X Accelerator (1,000 µl)
631254	Lenti-X Accelerator Starter Kit (1 each)
T110A	RetroNectin Precoated Dish (10 dishes)
T100B	RetroNectin Recombinant Human Fibronectin Fragment (2.5 mg)
T100A	RetroNectin Recombinant Human Fibronectin Fragment (0.5 mg)

O. Antibiotics for Selecting Stable Cell Lines

Cat. No.	Antibiotic	Size
631306	Puromycin	100 mg
631305	Puromycin	25 mg
631308	G418	5 g
631307	G418	1 g

P. Luciferase Assay and Luminometer

These items are required when using the pLVX-TRE3G-Luc Vector to screen Tet-On 3G clones. Use any standard luciferase assay system and luminometer.

IV. Protocol Overview

A. General Cell Culture

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).

B. Safety Guidelines for Working with Lentiviruses

The protocols in this User Manual require the production, handling, and storage of infectious lentivirus. It is imperative to fully understand the potential hazards of, and necessary precautions for, the laboratory use of lentiviruses.

The National Institutes of Health and Centers for Disease Control have designated recombinant lentiviruses as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. The VSV-G pseudotyped lentiviruses packaged from the HIV-1-based vectors described here are capable of infecting human cells. The viral supernatants produced by these lentiviral systems could, depending on your insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

IMPORTANT: For these reasons, due caution must be exercised in the production and handling of any recombinant lentivirus. **The user is strongly advised not to create VSV-G pseudotyped lentiviruses capable of expressing known oncogenes.**

For more information on Biosafety Level 2 agents and practices, download the following reference:

Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (December 2009) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH.

Available on the web at **http://www.cdc.gov/biosafety/publications/bmbl5/**

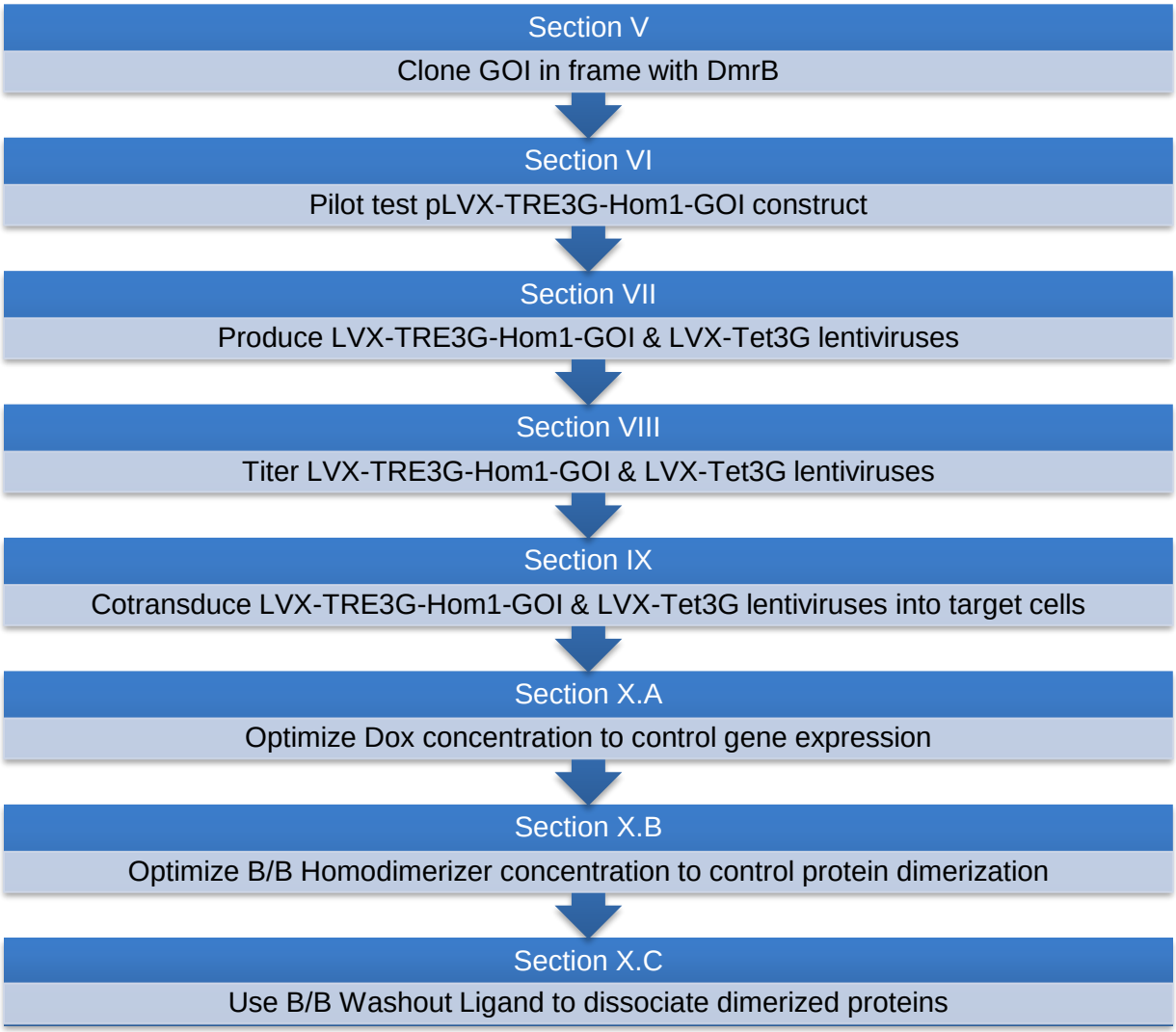
Biosafety Level 2: The following information is a brief description of Biosafety Level 2. *It is neither detailed nor complete.* Details of the practices, safety equipment, and facilities that combine to produce a Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with lentiviruses.

Summary of Biosafety Level 2:

- Practices:
  - Standard microbiological practices
  - Limited access to work area
  - Biohazard warning signs posted
  - Minimize production of aerosols
  - Decontaminate potentially infectious wastes before disposal
  - Use precautions with sharps (e.g., syringes, blades)
  - Biosafety manual defining any needed waste decontamination or medical surveillance policies
- Safety equipment:
  - Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is unrecirculated
  - PPE: protective laboratory coats, gloves, face protection as needed
- Facilities:
  - Autoclave available for waste decontamination
  - Chemical disinfectants available for spills

C. Protocol Summary

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



V. Creating your pLVX-TRE3G-Hom1-GOI Construct

We recommend using the In-Fusion HD Cloning System (Section III.A) to clone your gene of interest into pLVX-TRE3G-Hom1. 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.

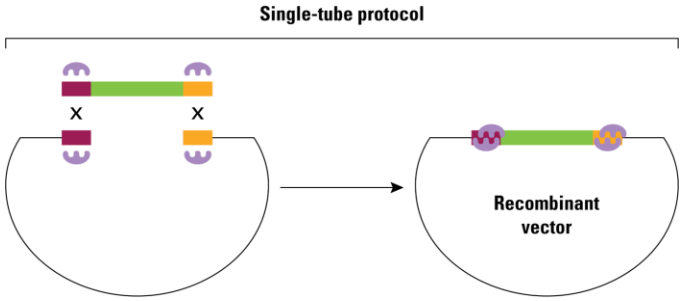


Figure 5. The In-Fusion HD single-tube cloning protocol.

VI. Pilot Testing Your pLVX-TRE3G-Hom1-GOI Construct for Tet-Based Induction & B/B Homodimerizer-Mediated Dimerization

Prior to lentivirus production, your pLVX-TRE3G-Hom1-GOI construct can be tested for functionality by plasmid transfection. Transiently cotransfect your pLVX-TRE3G-Hom1-GOI vector together with pLVX-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.

The optimal concentrations of doxycycline and B/B Homodimerizer that you determine in this pilot test will not apply directly to your final experiment (Section X), but they may serve as general guidelines for the concentrations to use in your final experiment.

Materials required

- pLVX-TRE3G-Hom1-GOI (Section V)
- pLVX-Tet3G
- Host cell line: *Choose an easy-to-transfect cell line such as HeLa or HEK 293, a premade Tet-On 3G cell line, or your target cell line*
- 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)
- B/B Homodimerizer (Section III.F)

A. Confirm Expression of Your pLVX-TRE3G-Hom1-GOI Construct

1. Seed your target cells in each well of a 6-well plate. When the cells reach 50–80% confluence, cotransfect pLVX-TRE3G-Hom1-GOI and pLVX-Tet3G into the target cells using Xfect Transfection Reagent. Use 1 µg of pLVX-Tet3G and 4 µg of pLVX-TRE3G-Hom1-GOI for each well and follow the Xfect Protocol.
   - To find the protocol, go to takarabio.com/manuals and type "Xfect" in the search box.
   - Use pLVX-TRE3G-Luc as a positive control
   - 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 pLVX-Tet3G and 4 µg pLVX-TRE3G-Hom1-GOI (no Dox)
Wells 3 & 4: 1 µg pLVX-Tet3G and 4 µg pLVX-TRE3G-Hom1-GOI (100–1,000 ng/ml Dox)
Well 5: 1 µg pLVX-Tet3G and 4 µg pLVX-TRE3G-Hom1-GOI empty (no Dox)
Well 6: 1 µg pLVX-Tet3G and 4 µg pLVX-TRE3G-Hom1-GOI empty (100–1,000 ng/ml Dox)

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 (Western blot, Northern blot, qRT-PCR, or a gene-specific functional assay).

B. Determine Optimal Doxycycline Concentration

To perform a study of this type, you must determine the maximum dose of doxycycline that *does not* result in dimerization of your protein of interest in the absence of B/B Homodimerizer to optimize the expression level of your protein of interest. You will use this concentration of doxycycline in subsequent studies.

1. Seed your target cells in each well of a 6-well plate. When the cells reach 50–80% confluence, cotransfect pLVX-TRE3G-Hom1-GOI and pLVX-Tet3G into the target cells using Xfect Transfection Reagent. Use 1 µg of pLVX-Tet3G and 4 µg of pLVX-TRE3G-Hom1-GOI for each well and follow the Xfect Protocol.
2. 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

3. Incubate your cells in media containing Dox for at least 24 hr.
4. At an appropriate time point (after at least 24 hr), test for the event of interest caused by ligand-independent dimerization of your protein of interest using an appropriate assay.
5. For your experiment using the B/B Homodimerizer 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.2.

C. Determine the Optimal B/B Homodimerizer Concentration

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

1. Seed your target cells in each well of a 6-well plate. When the cells reach 50–80% confluence, cotransfect pLVX-TRE3G-Hom1-GOI and pLVX-Tet3G into the target cells using Xfect Transfection Reagent. Use 1 µg of pCMV-Tet3G and 4 µg of pTRE3G-Hom1-GOI for each well and follow the Xfect Protocol.
2. 4 hr later, replace the media with fresh media containing the optimal concentration of doxycycline from Step B.5. Incubate your cells in media containing Dox for at least 12 hr.
3. Add a range of B/B Homodimerizer concentrations to the growth media containing Dox.

Each well should contain the optimal concentration of Dox determined in Step B.5 above, plus:

Well 1: 0 nM B/B Homodimerizer
Well 2: 10 nM B/B Homodimerizer
Well 3: 50 nM B/B Homodimerizer
Well 4: 100 nM B/B Homodimerizer
Well 5:250 nM B/B Homodimerizer
Well 6:1,000 nM B/B Homodimerizer

4. At an appropriate time point, test for the event caused by ligand-dependent dimerization of your protein 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 B/B Homodimerizer depends on the nature of the event caused by protein homodimerization. Rapid events such as phosphorylation may occur within 15–60 min. Slower events, such as differentiation, may require 1–5 days.

VII. Producing Lentivirus from Lenti-X Vectors

Follow the **Lenti-X Packaging Single Shots (VSV-G) Protocol-At-A-Glance**. (Locate this protocol by searching at **takarabio.com/manuals**).

VIII. Lentivirus Titration

A. Summary

1. Instant Qualitative Titer Test

You can detect and quantify your lentivirus stock in ten minutes with our Lenti-X GoStix Plus (Cat. Nos. 631280, 631281) and the related smartphone app. The GoStix detect lentiviral p24 in only 20 µl, and can be used to determine whether virus production is within a usable range or for selecting the best time to harvest your virus. A 3-prep sample of Lenti-X GoStix Plus is supplied for free with the Lenti-X iDimerize Inducible Homodimer System (with Tet-On 3G). Visit takarabio.com/gostixhelp for details.

2. Quantitative Titer Test

a. Determining the viral titer is necessary to obtain the following information:
   - Confirmation that viral stocks are viable
   - The proper transduction conditions for your particular cell type—obtained by adjusting the MOI for the desired transduction efficiency
     (MOI = No. of infectious virus particles per target cell)
   - The maximum number of target cells that can be transduced by a given virus volume

b. To transduce using a known multiplicity of infection (MOI), it is necessary to titrate your lentiviral stocks. We recommend the **Lenti-X qRT-PCR Titration Kit** (Cat. No. 631235) or **Lenti-X p24 Rapid Titer Kit** (Cat. No. 632200) for very rapid quantitative titrations of virus stocks (~4 hr), or a standard method that relies on infection.

c. The **standard viral titration protocol** consists of infecting cells with serial dilutions of the stock, selecting for stable transductants with antibiotic, and counting the resulting cell colonies (Section VIII.B).
   - Freshly harvested virus can be titered immediately, or frozen in aliquots at –80°C and then titrated. Note that each freeze-thaw cycle can reduce the functional titers of infectious virus by up to 2–4 fold.
   - Absolute titers will depend heavily on the cell type used for titration, and there may be significant differences between the titer values determined in cells typically used for lentiviral titration (i.e. HT-1080) and the number of target cells transduced by the titered virus. However, titrations serve to determine the relative virus content of different viral stocks prepared from different vectors.

B. Protocol: Determining Viral Titer by Colony Formation

NOTE: This protocol can be completed in 7–14 days.

1. Plate HT-1080 cells (or other) in 6-well plates the day before performing the titration infections. Plate 2 x 10^5 cells/well, in 2 ml of medium. Allow at least one well to be used as a "no infection" control.
2. Prepare 20 ml of complete medium and add 60 µl of 4 mg/ml Polybrene. This will be diluted 3-fold for a final Polybrene concentration of 4 µg/ml.

   NOTE: Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of Polybrene may be determined empirically but generally falls within a range of 2–12 µg/ml. Excessive exposure to Polybrene (>24 hr) can be toxic to cells.

3. Prepare filtered viral supernatant from packaging cells (Section VII). This is the virus stock.
4. Prepare six 10-fold serial dilutions of the virus stock as follows:
   a. Add 1.35 ml of medium containing Polybrene (Step 2) to each of six sterile and numbered 1.5 ml microfuge tubes.
   b. Add 150 µl of the virus stock (Step 3) to Tube 1. Mix.
   c. Transfer 150 µl from Tube 1 to Tube 2 and mix. Continue making serial dilutions by transferring 150 µl from each successive dilution into the next prepared tube.
5. Infect the HT-1080 cells by adding 1 ml of each viral dilution (Step 4) to each appropriate well. The final Polybrene concentration will be 4 µg/ml in ~3 ml. Centrifuge the cultures to improve infection efficiency*.

   ***NOTE:** CULTURE CENTRIFUGATION INCREASES INFECTION EFFICIENCY. Centrifuging the plate at 1,200 x g for 60–90 min at 32°C can significantly increase infection efficiency. A room temperature centrifuge is acceptable if a 32°C unit is not available.

6. After infecting for 8–24 hours, remove supernatants and subject the cells to G418 or puromycin selection using the selection concentrations that are optimal for your cell line (Section III.O).
7. Allow colonies to form for 7–14 days. Stain the colonies with 1% crystal violet solution (in 10% ethanol) and count.
8. The titer of virus corresponds to the number of colonies generated by the highest dilution, multiplied by the dilution factor. For example, the presence of 4 colonies in the 10^6 dilution would represent a viral titer of 4 x 10^6 colony forming units.

IX. Transducing Target Cells with the Tet-On 3G Lentiviruses

A. Summary

- Simultaneous Cotransduction: To establish the complete Lenti-X iDimerize Tet-On 3G System, target cells must be cotransduced with both the LVX-Tet3G and LVX-TRE3G-Hom1-GOI lentiviruses. Using high titers of each virus ensures that the highest proportion of cells will contain both vectors. Depending on your application, transduced cells can either be treated immediately with Dox to induce expression of your GOI and then harvested for analysis, or the cells may be selected with G418 and puromycin to isolate doubly-transduced clones or to enrich the population for doubly-transduced cells (see Section III.O).
- Virus Ratio Optimization: It is possible to optimize the induction characteristics of your system by infecting target cells with different ratios of the regulator and response lentiviruses. The optimal ratio can be determined in pilot studies, but in general we find that a ratio of 1:1 works best.

B. Protocol: Cotransducing Target Cells with Lenti-X Tet-On 3G Lentiviruses

NOTE: This protocol can be completed in 2–3 days.

1. Plate target cells in complete growth medium 12–18 hr before transduction.
2. Thaw aliquots of your LVX-Tet3G and LVX-TRE3G-Hom1-GOI lentiviral stocks, or use filtered virus stocks freshly prepared from packaging cells (Section VII). Mix gently, but do not vortex.
3. Add Polybrene to the cell cultures to obtain the desired final concentration during the transduction step (e.g., 4 µg/ml).

   NOTE: Lenti-X Accelerator (Cat. Nos. 631256, 631257 & 631254) and RetroNectin (Cat. Nos. T110A, T100B & T100A) may be used as transduction enhancers instead of Polybrene (see Section III.N).

4. Combine the LVX-Tet3G and LVX-TRE3G-Hom1-GOI lentiviral stocks in the desired ratio and MOI. In general we find that an MOI ratio of 1:1 works best for most experiments. The optimal ratio should be determined empirically (for example, compare three different ratios, such as 1:1, 4:1, and 1:4). If titer values are unknown, use serial dilutions of the viruses mixed at a ratio of 1:1, such that the total volume of supernatant used makes up no more than 1/3 the final volume of culture medium used in the transduction. Centrifuge the cultures to improve transduction efficiency (see Section VIII.B).
5. Transduce the cells for 8–24 hr. If you are concerned that exposure to either the Polybrene or to the viral supernatant (which contains medium conditioned by the packaging cells) may adversely affect your target cells, limit the transduction to 6–8 hr.
6. Remove and discard the virus-containing medium and replace it with fresh growth medium. Proceed to Part X.

X. Combining iDimerize Homodimerization with the Tet-On 3G System

After cotransducing your target cells with your LVX-Tet3G and LVX-TRE3G-Hom1-GOI lentiviral stocks, you are ready to study the effects of dimerization in your cells of interest.

Materials Required

- Host cells cotransduced with LVX-Tet3G and LVX-TRE3G-Hom1-GOI lentiviruses(Section IX)
- Doxycycline (1 mg/ml) (Section III.C)
- Mammalian cell culture supplies (Section III.D)
- Tet Approved FBS (Section III.E)
- B/B Homodimerizer (Section III.F)

A. Determine the 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 B/B Homodimerizer to optimize the expression level of your protein of interest. You will use this concentration of doxycycline in subsequent studies.

1. Seed transduced cells expressing Tet-On 3G and TRE3G-Hom1-GOI in each well of a 6-well plate.
2. 6–12 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

3. Incubate your cells in media containing Dox for at least 12 hr.
4. At an appropriate time point, test for the event of interest caused by *ligand-independent* dimerization of your protein of interest using an appropriate assay.
5. For your experiment using the B/B Homodimerizer 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 X.B.2 below.

B. Determine the Optimal B/B Homodimerizer Concentration

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

1. Seed transduced cells expressing Tet-On 3G and TRE3G-Hom1-GOI in each well of a 6-well plate.
2. 6–12 hr later, add a range of B/B Homodimerizer concentrations to the growth media containing Dox:

Each well should contain the optimal concentration of Dox determined in Step A.5 above, plus:

Well 1:0 nM B/B Homodimerizer
Well 2:10 nM B/B Homodimerizer
Well 3: 50 nM B/B Homodimerizer
Well 4: 100 nM B/B Homodimerizer
Well 5: 250 nM B/B Homodimerizer
Well 6: 1,000 nM B/B Homodimerizer

3. At an appropriate time point, test for the event caused by ligand-dependent dimerization of your protein 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 B/B Homodimerizer depends on the nature of the event caused by protein homodimerization. Rapid events such as phosphorylation may occur within 15–60 min. Slower events, such as differentiation, may require 1–5 days.

C. Dissociate Dimerized Proteins with the B/B Washout Ligand

The B/B Washout Ligand (Cat. No. 635088) can be used to accomplish fast dissociation of protein interactions that were induced by the B/B Homodimerizer (Figure 7). When you remove the B/B Homodimerizer-containing medium from the cells and replace it with medium containing 1 µM B/B Washout Ligand, your dimerized proteins will dissociate from each other with a $T_{1/2}$ of ~10 to 12 minutes.

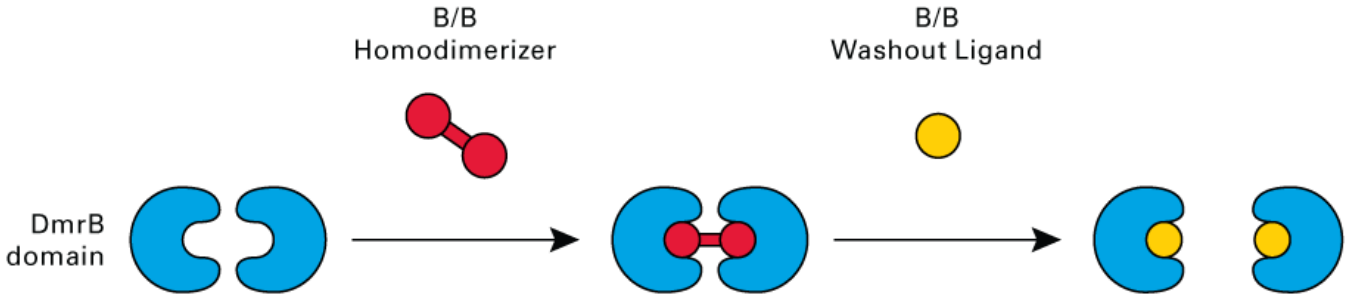


Figure 6. B/B Washout Ligand can be used to disrupt protein interactions that were induced by the B/B Homodimerizer.

1. Adherent cells

a. Remove the medium from your transfected and B/B Homodimerizer-treated cells.
b. Rinse cells with warm TC-grade PBS with Ca^{2+} and Mg^{2+}, or culture medium.
c. Add medium containing 1 µM B/B Washout Ligand to the cells. Total dissociation of dimerized proteins can be observed about 30 minutes after the medium change.
d. Analyze the effect of B/B Washout Ligand-induced protein dissociation with your method/assay of choice.

2. Suspension cells

a. Collect the cells via centrifugation.
b. Resuspend the cells in medium containing 1 µM B/B Washout Ligand. Total dissociation of dimerized proteins can be observed about 30 minutes after the medium change.
c. Analyze the effect of B/B Washout Ligand-induced protein dissociation with your method/assay of choice.

Results Obtained Using the B/B Washout Ligand to Dissociate Protein Dimers

Figure 8 shows the release of a B/B Homodimerizer-induced DmrB-DmrB interaction. Lentiviral delivery was used to create a stable HeLa cell line (mixed population) expressing the two parts of a "split" version of a red fluorescent protein. The two monomeric proteins are non-fluorescent until they are forced to undergo dimerization via the B/B Homodimerizer. When the B/B Washout Ligand was added, red fluorescence diminished quickly due to the dissociation of the split fluorescent protein (solid line). Simply replacing medium containing B/B Homodimerizer with medium without B/B Homodimerizer had only a minor dissociative effect (dashed line).

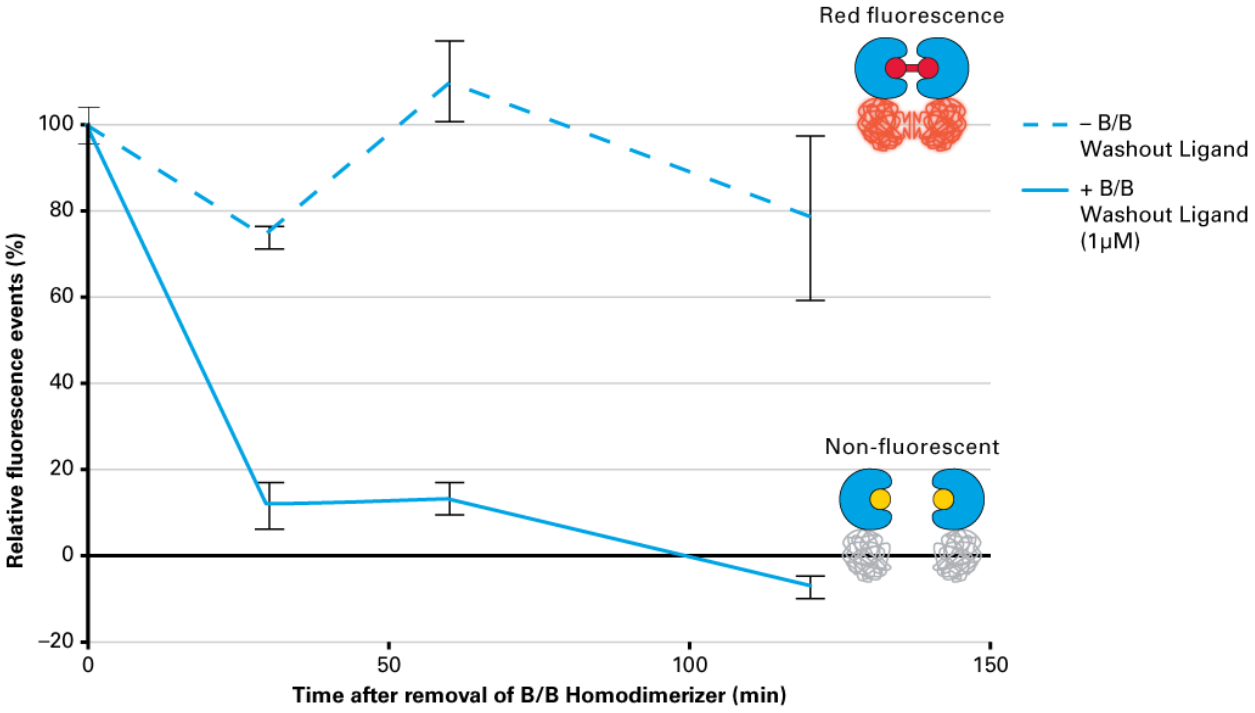


Figure 7. B/B Washout Ligand has a much more dissociative effect than simple removal of the B/B Homodimerizer. A stable HeLa cell line expressing DmrB-tagged versions of the two parts of a split red fluorescent protein was treated with 0.1 µM B/B Homodimerizer for 3 hr. The medium was then removed and replaced with medium ± 1 µM B/B Washout Ligand. After 30 min, the fluorescence level in the "+ B/B Washout Ligand" sample had dropped virtually to the background level, indicating that the two parts of the red fluorescent protein were completely dissociated from each other.

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XII. Troubleshooting

A. Lenti-X Troubleshooting

1. Vector Cloning

Problem	Possible Explanation	Solution
Plasmid is difficult to grow or clone	Some viral vectors may undergo rearrangement between the 5' and 3' LTRs when propagated in less-than-optimal *E. coli* host strains	Use Stellar Competent Cells (Cat. No. 636763) to produce high DNA yields and to minimize the potential for DNA rearrangements.

2. Lenti-X 293T Packaging Cells

Problem	Possible Explanation	Solution
Poor viability upon thawing	Improper thawing techniques	Use thawing procedure in Appendix A, and/or consult the Lenti-X 293T Cell Line Protocol-at-a-Glance.
	Incorrect culture medium	Use DMEM with additives listed in Section III.D. Use 10% Tet System Approved FBS (Tc-free).
	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Slow growth	Incorrect culture medium	Use DMEM with additives listed in Section III.D. Use 10% Tet System Approved FBS (Tc-free).
Cells do not attach to plate	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Cells appear morphologically different	Passage of cell culture is too high (old cells)	Thaw/purchase new aliquot of Lenti-X 293T cells.

3. Virus Production

Problem	Possible Explanation	Solution
Poor transfection efficiency (as determined by GOI or marker expression in the Lenti-X 293T cell line)	Cells plated too densely	Plate 4–5 x 10^6 cells/100 mm plate, or fewer if the cells divide rapidly. Use at 50–80% confluency. See Section VII.
	Transfection is toxic to cells	Use the optimized conditions provided in Section VII.
	Cells harvested or analyzed too soon after transfection	Wait 48 hr after transfection for maximal expression of GOI or marker to determine efficiency.
Low titers (<10^5cfu/ml)	Serum in medium contains tetracycline contaminants, which can interfere with the expression of viral proteins, resulting in lower titers	Use Tet System Approved FBS (Cat. Nos. 631101 & 631106) in the Lenti-X 293T cell culture medium.
	Virus was harvested too early	Harvest virus 48–72 hr after the start of transfection.
	Vector is too large	The limit for efficient packaging function is 9.7 kb from the end of the 5'-LTR to the end of the 3'-LTR.
	Polybrene is missing or at suboptimal concentration	Add Polybrene (4 µg/ml) during transduction or optimize the concentration (2–12 µg/ml).
Low titers (<10^5cfu/ml)	Virus was exposed to multiple freeze-thaw cycles	Each cycle reduces titer by approximately 2–4 fold. Limit the number of freeze-thaws.
	Suboptimal selection procedure during titration	Perform an antibiotic kill curve on the cell line prior to using it for titration.

4. Transduction of Target Cells

Problem	Possible Explanation	Solution
Poor transduction efficiency	Low titer	See Section C or use the Lenti-X Concentrator (Section III.M) to increase your available titer up to 100-fold without ultracentrifugation.
	Poor transfection efficiency	Follow the protocol in Section VII.B. Be sure to use 5 µg of transfection-grade plasmid.
	Low viability of target cells during transduction	Optimize culture conditions for target cells prior to infection.
		Packaging cell line-conditioned media may affect cell growth; dilute viral supernatant or shorten exposure time to viral supernatant. Consider using RetroNectin Reagent and the RetroNectin-Bound Virus transduction protocol or purify your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. Nos. 631233 & 631234).
		Excessive exposure to Polybrene: optimize amount (titrate) or shorten exposure time to viral supernatant.
	Viral supernatant contains transduction inhibitors	Use RetroNectin Reagent or RetroNectin-coated plates in the RetroNectin-Bound Virus transduction protocol, which allows virions to bind the RetroNectin substratum and be washed free of inhibitors prior to target cell infection; or, purify your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. Nos. 631233 & 631234).

B. Tet-On 3G Troubleshooting

1. Low Fold Induction of Transient Expression

Description of Problem	Possible Explanation	Solution
Decrease in fold induction after several passages	The appropriate antibiotics are missing from the cell culture medium.	Maintain optimal antibiotic concentrations (Section III.O).
or Loss of inducibility after passaging of a (previously frozen) double-stable cell line.	Mixed cell population	Reselect the current cell line through single colony selection using selective concentrations of puromycin and G418, (Section IX).

1. Low Fold Induction of Transient Expression (continued)

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-GOI (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 • Optimize density of cell plating; use at 60–90% confluency
	Poor target cell viability	• Optimize target cell passage number • Optimize target cell culture conditions • Optimize tissue culture plasticware
	The FBS used in the cell culture medium contains tetracycline derivatives	Use our Tet System Approved FBS (Section III.E). Only Takara Bio 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. 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. 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. • 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.

D. iDimerize Troubleshooting

Description of Problem	Possible Explanation	Solution
Dimerization is observed in the absence of the B/B Homodimerizer	The expression level of the protein of interest fused to the DmrB domain is too high, especially in the case of a DmrB-tagged protein of interest localized to the plasma membrane.	Use a lower concentration of Dox to limit the gene expression level: • Transient cotransfections of pTRE3G-Hom1-GOI and pCMV-Tet3G: See Part VI, Protocol A.1 • Stable Tet3G cell lines transiently transfected with pTRE3G-Hom1-GOI: See Part VI, Protocol B.2 • Double-stable cell lines expressing Tet3G and pTRE3G-Hom1-GOI: See Part VI, Protocol C.3
Addition of B/B Homodimerizer does not result in the expected effect(s)	The B/B Homodimerizer concentration is too low.	Increase the amount of B/B Homodimerizer added.
	The monitoring assay is not sensitive enough.	Include a positive control when performing your assay.
	The volume of B/B Homodimerizer used causes cells to die, due to high solvent concentration. Low expression level	Prepare a more concentrated stock solution. Monitor protein expression in your cells by Western blot using the anti-DmrB antibody (Section III.H).

Appendix A: 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 2x10^5 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: 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 puromycin, as appropriate (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)		

[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.

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