Takara Bio USA, Inc.

Lenti-X™ Tet-On® 3G Inducible Expression System User Manual

Cat. Nos. 631187, 631349, 631350, 631351, 631352, 631353, 631354, 631363 (030420)

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I. Introduction

A. Summary

The **Tet-On 3G Systems** are inducible gene expression systems for mammalian cells. Target cells that express the Tet-On 3G transactivator protein and contain a gene of interest (GOI) under the control of a TRE3G promoter (P_{TRE3G} or P_{TRE3GV}) will express high levels of your GOI, but only when cultured in the presence of doxycycline (Dox) (Figure 1).

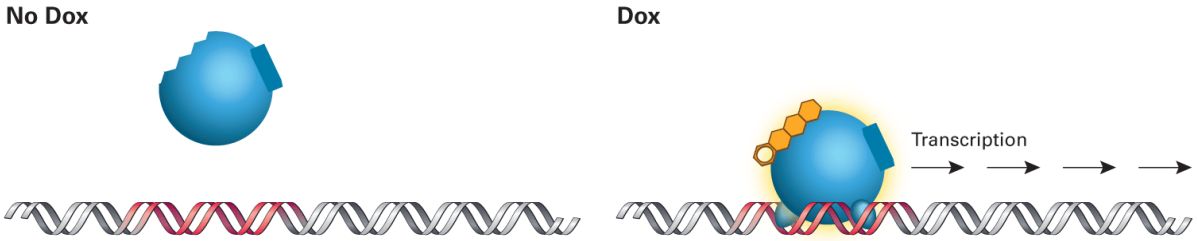

Figure 1. The Tet-On 3G Systems allow inducible gene expression in the presence of Dox.

B. Elements of Lenti-X Tet-On 3G

Tet-On 3G Transactivator Protein

Based on the transcriptional regulators described by Gossen & Bujard (1992), Gossen *et al.* (1995), and Urlinger *et al.* (2000), Tet-On 3G is a modified form of the Tet-On Advanced transactivator protein which has been evolved to display far higher sensitivity to doxycycline (Zhou *et al.*,. 2006).

P_{TRE3GV} Inducible Promoter

The 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. P_{TRE3GV} is a version of P_{TRE3G} that was modified at Takara Bio for higher performance in lentiviruses and retroviruses. In the presence of Dox, Tet-On 3G binds specifically to P_{TRE3GV} and activates transcription of the downstream GOI. P_{TRE3GV} lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction.

4th Generation Lentiviral Packaging System

Our **Lenti-X Packaging Single Shots (VSV-G)**, provided with the **Lenti-X Tet-On 3G Inducible Expression System** (Cat. No. 631187), 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)**.

C. Doxycycline

Doxycycline is a synthetic tetracycline derivative that is the effector molecule for the Tet-On and Tet-Off® Systems. When bound by Dox, the Tet-On 3G protein undergoes a conformational change that allows it to bind to *tet* operator sequences located in the P_{TRE3G} promoter (Figure 1). The Dox concentrations required for induction of Tet-On Systems are far below cytotoxic levels for either cell culture or transgenic studies, and Tet-On 3G responds to even lower concentrations than its predecessors (Zhou *et al.*, 2006). Note that Tet-On Systems respond well only to doxycycline, and not to tetracycline (Gossen & Bujard, 1995). The half-life of Dox in cell culture medium is 24 hr. To maintain continuous inducible GOI expression in cell culture, the medium should be replenished with Dox every 48 hr.

II. List of Components

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

A. Lenti-X Tet-On 3G Plasmid Systems

Cat. No.	System Name
631187	Lenti-X Tet-On 3G Inducible Expression System
631351	Lenti-X Tet-On 3G Inducible Expression System (Bicistronic Version)
631349	Lenti-X Tet-On 3G Inducible Expression System (with mCherry)
631350	Lenti-X Tet-On 3G Inducible Expression System (with ZsGreen1)
631363	Lenti-X Tet-On 3G Inducible Expression System (EF1alpha Version)
631354	Lenti-X Tet-On 3G Inducible Expression System (EF1alpha, Bicistronic)
631352	Lenti-X Tet-On 3G Inducible Expression System (EF1alpha, mCherry)
631353	Lenti-X Tet-On 3G Inducible Expression System (EF1alpha, ZsGreen1)

B. General System Components

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

- 10 µg regulator vector (see Section II.C)
- 10 µg response vector (see Section II.C)
- 10 µg pLVX-TRE3G-Luc Control Vector
- 16 rxns Lenti-X Packaging Single Shots (VSV-G) (Cat. No. 631275)
- Lenti-X GoStix Plus (Sample) (Cat. No. 631279; not sold separately)

C. System-Specific Regulator and Response Vectors

Cat. No.	Regulator Vector	Response Vector
631187	pLVX-Tet3G	pLVX-TRE3G
631351	pLVX-Tet3G	pLVX-TRE3G-IRES
631349	pLVX-Tet3G	pLVX-TRE3G-mCherry
631350	pLVX-Tet3G	pLVX-TRE3G-ZsGreen1
631363	pLVX-pEF1a-Tet3G	pLVX-TRE3G
631354	pLVX-pEF1a-Tet3G	pLVX-TRE3G-IRES
631352	pLVX-pEF1a-Tet3G	pLVX-TRE3G-mCherry
631353	pLVX-pEF1a-Tet3G	pLVX-TRE3G-ZsGreen1

III. Additional Materials Required

The following reagents are required but not supplied.

A. Tetracycline-Free Fetal Bovine Serum

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

Cat. No.	Serum Name
631106	Tet System Approved FBS (500 ml)
631107	Tet System Approved FBS (50 ml)
631367	Tet System Approved FBS (3 x 500 ml)
631101	Tet System Approved FBS, US-Sourced (500 ml)
631105	Tet System Approved FBS, US-Sourced (50 ml)
631368	Tet System Approved FBS, US-Sourced (3 x 500 ml)

B. Antibiotics for Selecting Stable Cell Lines

Table 1. Recommended Antibiotic Concentrations for Selecting & Maintaining Stable Cell Lines

		Recommended Concentration (µg/ml)	
Cat. No.	Antibiotic	Selecting Colonies[1]	Maintenance
631308	G418 (5 g)	100–800	200
631307	G418 (1 g)		
631306	Puromycin (100 mg)	0.25–10	0.25
631305	Puromycin (25 mg)		

[1] When selecting for single colonies, the appropriate dose must be determined empirically for your specific cell line. Test a dosage range using dishes of untransfected cells and choose the dose that kills all of the cells in 3–5 days. If all the cells die in less than 24 hr, you should use a lower dose.

C. Mammalian Cell Culture Supplies

- **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, D5796); 10% Fetal Bovine Serum (FBS); 100 units/ml penicillin G sodium & 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), for freezing Tet-On 3G and 293T cell lines.
- 6-well, 12-well, and 24-well cell culture plates; 10 cm cell culture dishes

D. Lenti-X 293T Cells

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

  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 meets 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.

E. High-Titer Packaging System

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

F. Lentiviral Titer Determination

For accurate and consistent transductions, we highly recommend titrating your lentiviral stocks. Various technologies are available from Takara Bio; 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)

G. 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)

H. Transduction Enhancers

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

- Lenti-X Accelerator is a magnetic bead-based technology designed to accelerate lentiviral and retroviral transduction experiments; visit **takarabio.com** for 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.com** for details.

Cat. No.	Transduction Enhancer	Size
631256	Lenti-X Accelerator	400 µl
631257	Lenti-X Accelerator	1,000 µl
631254	Lenti-X Accelerator Starter Kit	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

I. Doxycycline

- 5 g Doxycycline (Cat. No. 631311)

Dilute to 1 mg/ml in double distilled H_2O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.

I. Xfect Transfection Reagent

Xfect provides high transfection efficiency for most commonly used cell types.

Cat. No.	Transfection Reagent
631317	Xfect Transfection Reagent (100 rxns)
631318	Xfect Transfection Reagent (300 rxns)

J. In-Fusion® HD Cloning System

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

For more information, visit takarabio.com/infusion

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)

K. Stellar™ Competent Cells

We recommend Stellar Competent Cells for cloning of lentiviral and retroviral vectors. Propagation of vectors containing repeat sequences such as viral LTRs using other strains of *E.coli* may result in plasmid rearrangements. Stellar Competent Cells are sold separately and provided with all In-Fusion HD Cloning Systems.

Cat. No.	Competent Cells
636763	Stellar Competent Cells (10 x 100 µl)
636766	Stellar Competent Cells (50 x 100 µl)

L. TetR Monoclonal Antibody

If you wish to confirm that Tet-On 3G is expressed in your cells, we recommend that you use the following antibody and detect the protein via Western Blot.

Cat. No.	Antibody
631131	TetR Monoclonal Antibody (Clone 9G9) (40 µg)
631132	TetR Monoclonal Antibody (Clone 9G9) (200 µg)

M. Luciferase Assay and Luminometer

These items are required when using the pLVX-TRE3G-Luc Vector as a control to test for induction (Section VI.B). Use any standard firefly luciferase assay system and luminometer.

IV. Protocol Overview

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

A. General Cell Culture

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

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

B. 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 Institute of Health and Center 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 (February 2007) 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/od/ohs/biosfty/bmbl5/bmbl5toc.htm**

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

The following are the steps required to create a doxycycline-inducible expression system using lentivirus (see Figure 2).

1. Clone your gene of interest into the pLVX-TRE3G Vector using In-Fusion HD (Section V).
2. Pilot test Tet-based induction of your construct (Section VI).
3. Produce lentiviral supernatants using Lenti-X Packaging Single Shots (VSV-G) (Section VII).
4. Co-infect your target cells with Tet-On 3G virus and TRE3G virus (Section IX).

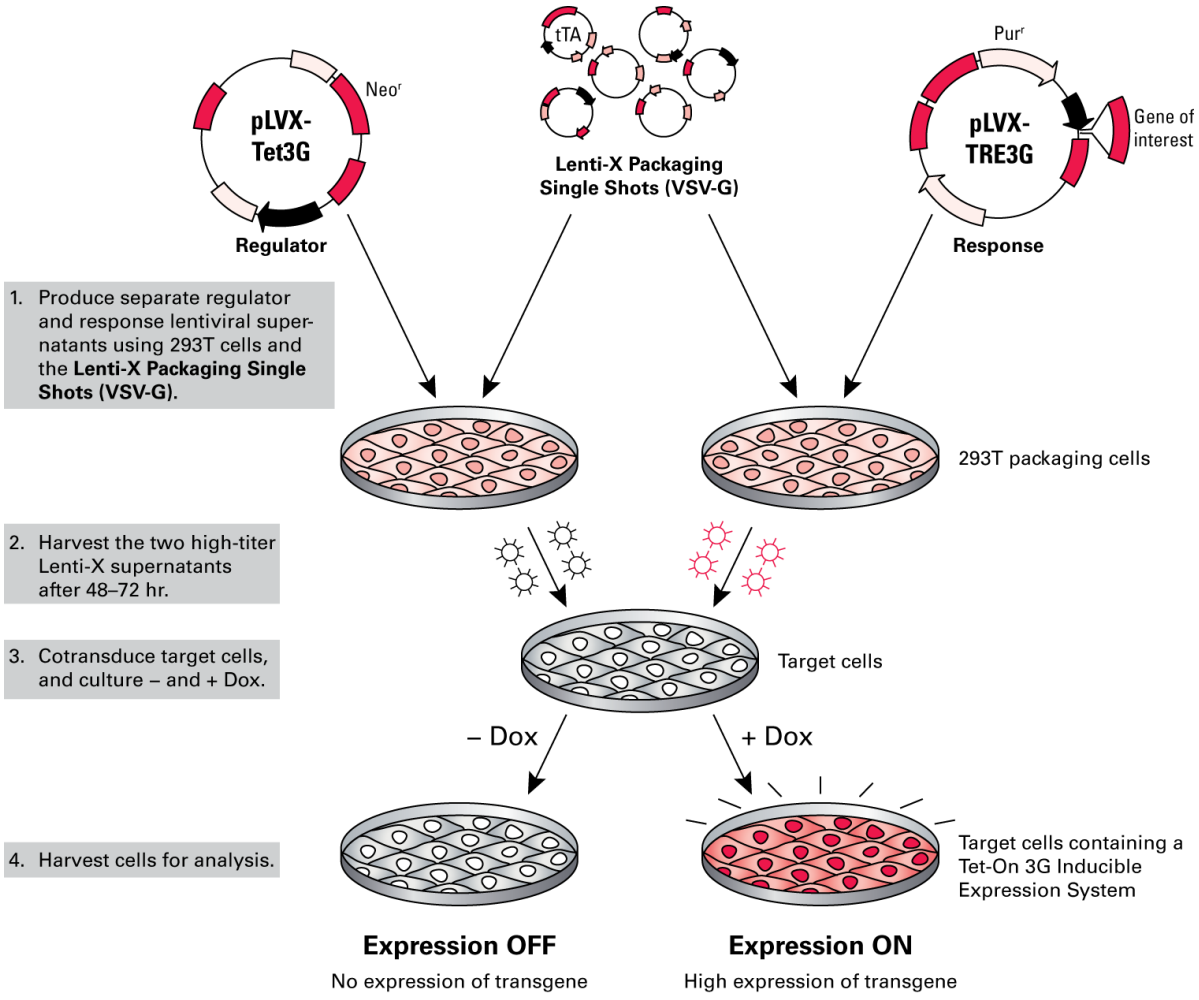

Figure 2. Establishing an inducible expression system in target cells with Lenti-X Tet-On 3G. Lenti-X Packaging Single Shots (VSV-G), an optimized packaging pre-mix lyophilized with Xfect Transfection Reagent, and 293T cells are used to generate high-titer lentiviral supernatants from the pLVX-Tet3G Vector and from the pLVX-TRE3G Vector, which contains your gene of interest. Target cells are then simultaneously cotransduced with the two lentiviruses (~8 hr). After culturing for an additional 48–72 hr (+ and – Dox), the cells are harvested for analysis.

V. Cloning Your Gene of Interest into the pLVX-TRE3G Vector using In-Fusion HD

We recommend using In-Fusion HD for all cloning. Follow the protocol outlined in the In-Fusion HD user manual at **takarabio.com/infusion**

NOTE: We recommend Stellar Competent Cells (Section III.K) for cloning of lentiviral and retroviral vectors. Propagation of vectors containing repeat sequences such as viral LTRs using other strains of *E. coli* may result in plasmid rearrangements. Stellar Competent Cells are provided with all In-Fusion HD Cloning Systems.

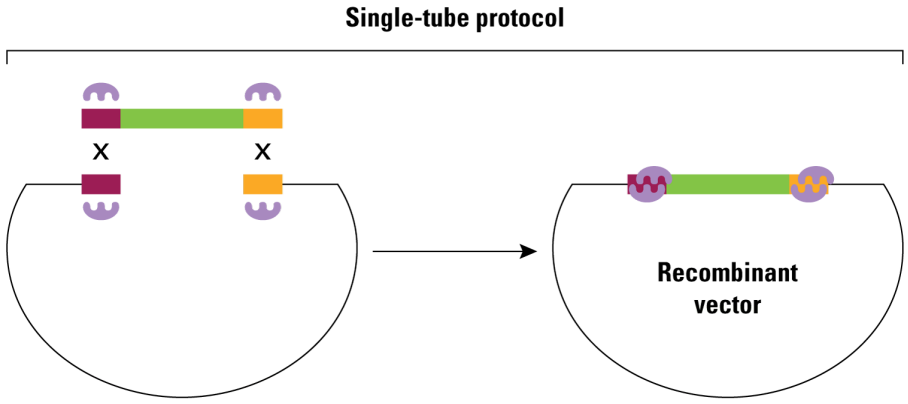

Figure 3. The In-Fusion HD Single-Tube Cloning Protocol.

Depending on which pLVX-TRE3G vector you are using, the recommended linearization sites and forward/reverse primer designs are as follows:

Response Vector	Linearize with	Forward Primer*	Reverse Primer**
pLVX-TRE3G	BamHI & MluI	tcttatacttggatcc 111 222 333 444 555 666 777 888	attccatatgacgcgt SSS NNN NNN NNN NNN NNN NNN NNN
pLVX-TRE3G-IRES (MCS-1)	BamHI & NotI	tcttatacttggatcc 111 222 333 444 555 666 777 888	cggccggggcggccgc SSS NNN NNN NNN NNN NNN NNN NNN
pLVX-TRE3G-IRES (MCS-2)	MluI & EcoRI	gcccccgggacgcgt 111 222 333 444 555 666 777 888	ctacccggtagaattc SSS NNN NNN NNN NNN NNN NNN NNN
pLVX-TRE3G-mCherry	MluI & EcoRI	gcccccgggacgcgt 111 222 333 444 555 666 777 888	ctacccggtagaattc SSS NNN NNN NNN NNN NNN NNN NNN
pLVX-TRE3G-ZsGreen1	MluI & EcoRI	gcccccgggacgcgt 111 222 333 444 555 666 777 888	ctacccggtagaattc SSS NNN NNN NNN NNN NNN NNN NNN

*111 = Start codon of your gene; 222 = 2nd codon of your gene; etc.

**SSS = reverse complement of the stop codon of your gene; NNN = reverse complement of the end of your gene.

VI. Pilot Testing Tet-Based Induction of Your Construct

Prior to lentivirus production, your pLVX-TRE3G construct should be tested for functionality by plasmid transfection. Transiently cotransfect your pLVX-TRE3G-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, and test for transgene induction with Dox. You will need an appropriate gene-specific assay to test for induction, such as:

- Western blot
- Northern blot
- qRT-PCR
- Gene-specific functional assay

Alternatively you can perform a single vector transfection of pLVX-TRE3G-GOI into a newly created Tet-On 3G cell line (Section VII).

pLVX-TRE3G-Luc can be used as a positive control.

A. Materials Required

1. pLVX-TRE3G Vector containing your gene of interest (or pLVX-TRE3G-Luc as a positive control)
2. pLVX-Tet3G
3. Host cell line
4. Xfect Transfection Reagent (Section III.I)
5. Doxycycline (1 mg/ml) (Section III.H)
6. Mammalian cell culture supplies (Section III.C)
7. Tet Approved FBS (Section III.A)

B. Protocol

1. Cotransfect both the regulator and response vectors into your target cells (in a 6-well plate) using Xfect Transfection Reagent. Follow the **Xfect Transfection Reagent Protocol-At-A-Glance**. (Locate this protocol by searching at **takarabio.com/manuals**).
   - Use 1 µg of pLVX-Tet3G and 4 µg of pLVX-TRE3G-GOI for each well (GOI = gene of interest).
   - We recommend performing the test in duplicate with negative controls: 3 wells containing 100–1,000 ng/ml of Dox, and 3 wells without Dox.
   - Use pLVX-TRE3G-Luc instead of pLVX-TRE3G as a positive control (Section III.M)

Wells 1 & 2: 1 µg pLVX-Tet3G and 4 µg pLVX-TRE3G-GOI (no Dox)

Wells 3 & 4: 1 µg pLVX-Tet3G and 4 µg pLVX- TRE3G-GOI (100–1,000 ng/ml Dox)

Well 5: 1 µg pLVX-Tet3G and 4 µg pLVX-TRE3G empty (no Dox)

Well 6: 1 µg pLVX-Tet3G and 4 µg pLVX-TRE3G empty (100–1,000 ng/ml Dox)

Figure 4. Transfection of the regulator and response vectors into target cells in a 6-well plate.

2. After 24 hr, harvest the cell pellets from each well and compare induced expression levels to uninduced expression levels using a method appropriate for your GOI.

VII. Producing Lentivirus from the 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 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 is supplied for free with many of our Lenti-X systems. 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 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 the tube 1. Mix.
   c. Transfer 150 µl 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.B).
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 Tet-On 3G System, target cells must be cotransduced with both the LVX-Tet3G and LVX-TRE3G-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 be either 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.B).

- **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.

- **Sequential Transduction:** If you are working with a clonable cell line, we recommended that you perform sequential transductions with the lentiviruses so that you can obtain clones that have optimal inducibility characteristics. This method produces clones that have the highest expression levels, lowest backgrounds, and highest fold-induction. Briefly, cells are first transduced with only the LVX-Tet3G lentivirus, followed by selection with G418. Resistant clones are then screened for expression of Tet-On 3G and tested for inducibility. For example, as a control experiment, transfect clones with pLVX-TRE3G-Luc and choose the best clone that exhibits inducible luciferase expression. A favorable Tet-On 3G clone is then transduced with the LVX-TRE3G-GOI lentivirus. Doubly-transduced cells are selected using puromycin and the resulting puromycin-resistant clones are then screened for GOI inducibility.

B. Protocol: Cotransducing Target Cells with the 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-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). Add the predetermined optimal concentration of Dox to the appropriate cultures.

   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.G).

4. Combine the LVX-Tet3G and LVX-TRE3G-GOI lentiviral stocks in the desired ratio and MOI. In general we find that an MOI ratio of 1:1 works best for your cells and transgenes. 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, with or without Dox, as appropriate. Alternatively, passage the cultures and subject the cells to selection using G418 and puromycin, followed by expansion and freezing of cell line stocks (Appendix C).
7. Continue to incubate the cells for 24–48 hr to allow the expressed protein to accumulate.
8. Harvest the cells for analysis.

X. References

Takara Bio's Tet Systems were developed in cooperation with Dr. Bujard and his colleagues at the Center for Molecular Biology in Heidelberg (ZMBH) and in Dr. Wolfgang Hillen's laboratory at the University of Erlangen, Germany. Additional background information on Tet-regulated gene expression systems and an extensive bibliography are available at the website maintained by TET Systems: http://www.tetsystems.com (Please note that Takara Bio is not responsible for the information contained on this website.)

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Appendix A. Troubleshooting Guide

Table 2. Troubleshooting Guide for the Lenti-X Tet-On 3G Inducible Expression System

Problem	Possible Explanation	Solution
A. Vector Cloning		
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.
B. Lenti-X 293T Packaging Cells		
Poor viability upon thawing	Improper thawing techniques	Use thawing procedure in Appendix C, and/or consult the Lenti-X 293T Cell Line Protocol-at-a-Glance
	Incorrect culture medium	Use DMEM with additives listed in Section III.C. 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.C. 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.
C. Virus Production		
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 (<10^5 cfu/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 293T 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)
	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.

Problem	Possible Explanation	Solution
D. Transduction of Target Cells		
Poor transduction efficiency	Low titer	See Section C or use the Lenti-X Concentrator (Section III.F) 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).

Problem	Possible Explanation	Solution
E. Inducing Expression		
Low fold induction (ratio of maximal to basal expression of the GOI)	A suboptimal co-infection ratio was used.	We generally recommend a co-infection ratio of 1:1 for LVX-Tet3G: LVX-TRE3G-GOI (Section IX.B). Different vector ratios may result in different maximal/basal gene expression ratios.
	Cells were harvested and analyzed too soon or too late.	Harvest and analyze cells between 18–48 hr after addition of doxycycline
	Poor infection efficiency	• Confirm virus titers using a titration kit (Section III.E) • Increase amount of virus applied to target cells • Optimize density of cells when transducing
	Poor target cell viability	• Optimize passage number of target cells. • Optimize culture conditions of target cells. • Optimize tissue culture plasticware
	The FBS used in the cell culture medium contains tetracycline derivatives.	Use our Tet System Approved FBS (Section III.A), which was functionally tested with our double-stable CHO-AA8-Luc Tet-Off Control Cell Line.
Decrease in fold induction after several passages **or** Loss of inducibility after passaging of a (previously frozen) double-stable cell line.	The appropriate antibiotics are missing from the cell culture medium.	Maintain optimal antibiotic concentrations (Section III.B).
	Mixed cell population	Reselect the current cell line through single colony selection using selective concentrations of puromycin and G418, (Section IX).

Description of Problem	Possible Explanation	Solution
F. Establishment of Stable Cell Lines		
Untransduced cells do not die at the high antibiotic concentration established via titration in Section III.B	• The cells have not been recently passaged, so they remain well-attached to the plate surface even when they are dead. • You have achieved 100% transduction efficiency.	To determine the appropriate antibiotic concentration, use cells that have been split within the last 2–3 days.
There are no surviving cells after transduction followed by selection	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.
Poor cell viability	Cells were not properly frozen.	See Appendix C, Section A.
	Cells were not properly thawed.	See Appendix C, Section B.
G. Detection and Inhibition of Expression		
No detectable GOI expression by Western Blot.	Low sensitivity of detection method.	Check sensitivity of primary and secondary antibodies. Analyze GOI expression by qRT-PCR, using different sets of primers to ensure optimal detection of GOI expression.
Continuous GOI/Fluorescent Protein expression after the removal of doxycycline	Depending on the stability of the protein, it may persist in the cell in the absence of gene induction and de novo synthesis of GOI mRNA. Fluorescent proteins tend to have long half-lives.	Upon degradation, GOI/Fluorescent Protein expression will not be detectable in cells in the absence of induction. For faster degradation of an inducible GOI, use pTRE-Cycle Vectors (see 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.

Appendix B: Lenti-X Tet-On 3G System Vector Information

The Lenti-X Tet-On 3G Inducible Expression System (Section II) contains three vectors (Figures 6 & 7), a regulator vector (pLVX-Tet3G), a response vector (pLVX-TRE3G), and a control response vector (pLVX-TRE3G-Luc). For complete descriptions of the vectors provided with each system, refer to the enclosed Certificate of Analysis, which is also available at takarabio.com

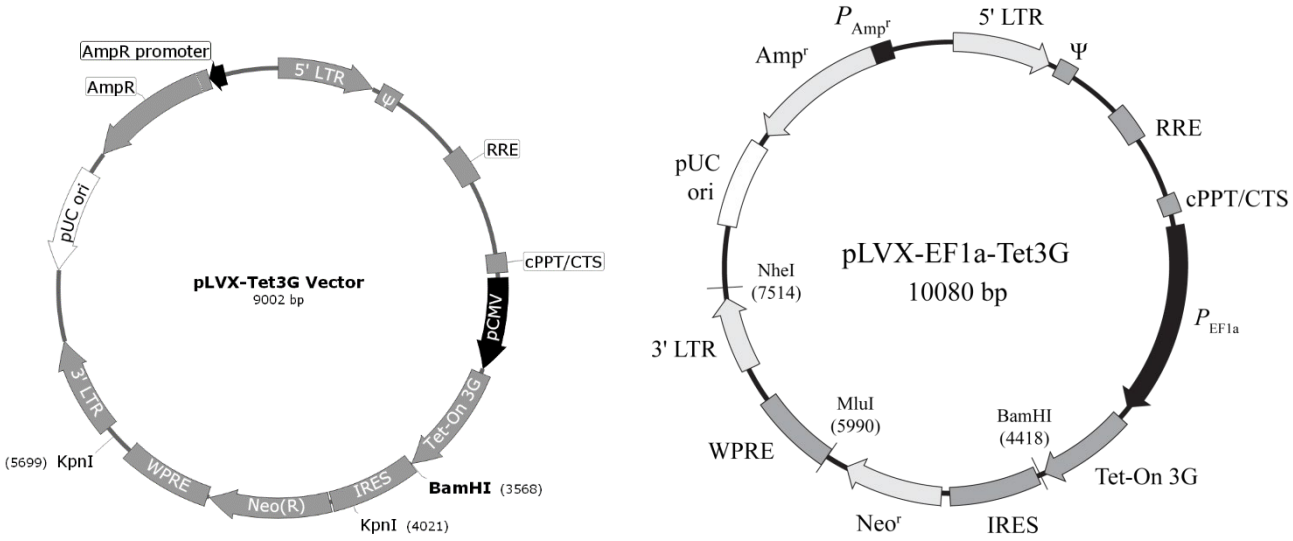

Figure 5. pLVX-Tet3G Vector and pLVX-EF1a-Tet3G Vector Maps.

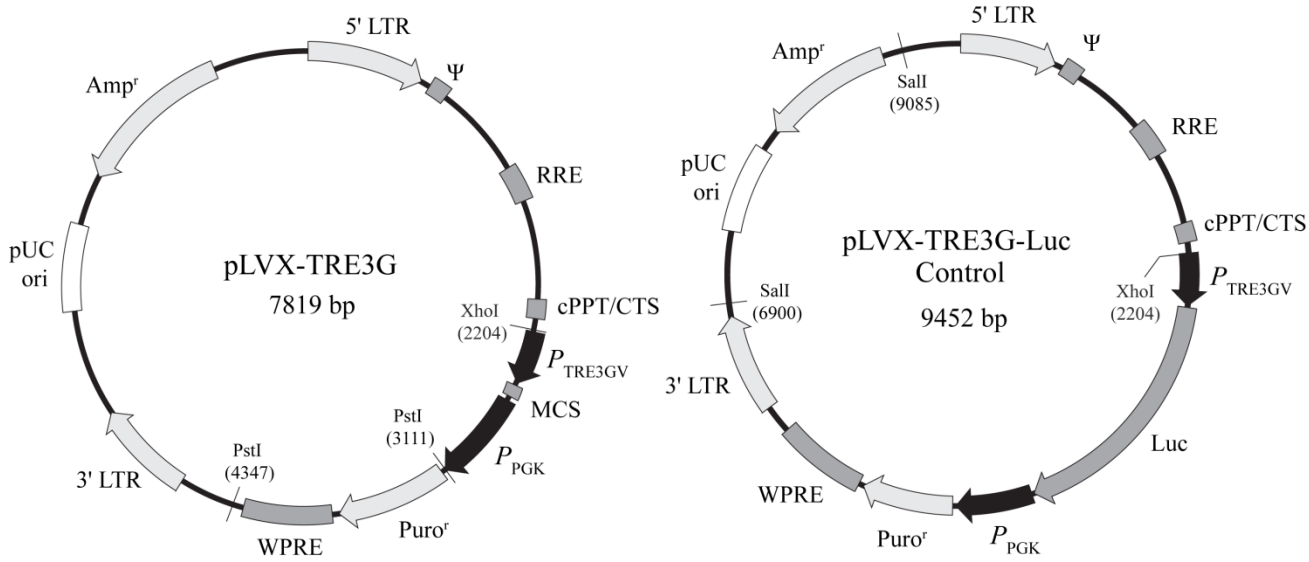

Figure 6. pLVX-TRE3G Vector & pLVX-TRE3G-Luc Control Vector Maps.

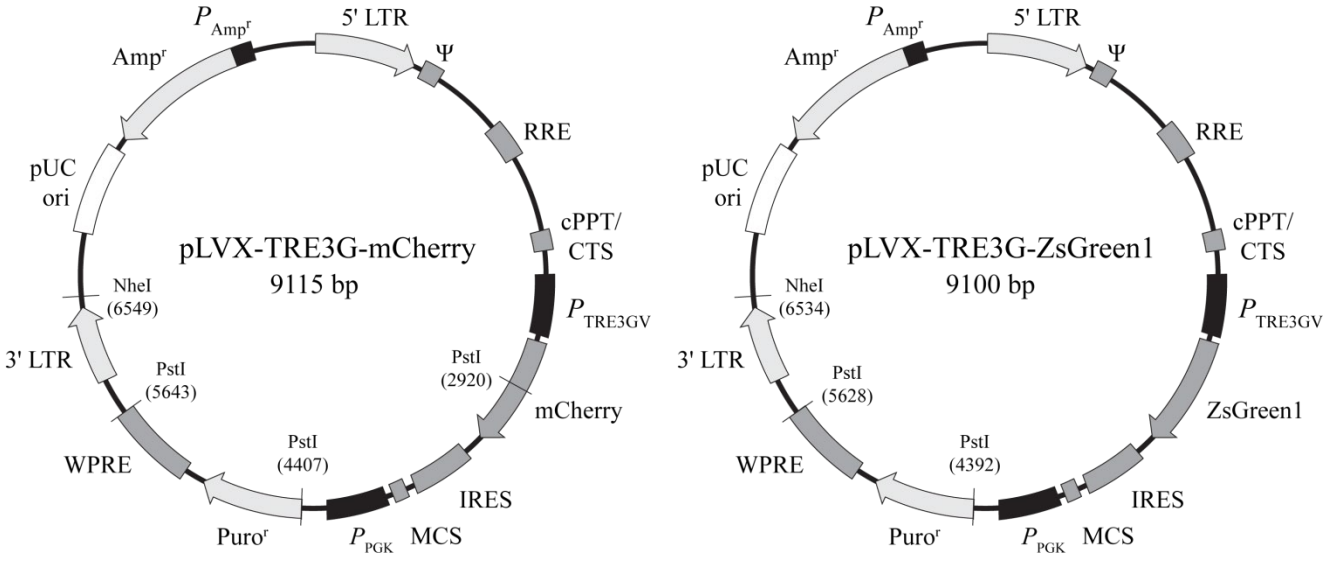

Figure 7. pLVX-TRE3G-mCherry Vector and pLVX-TRE3G-ZsGreen1 Vector Maps.

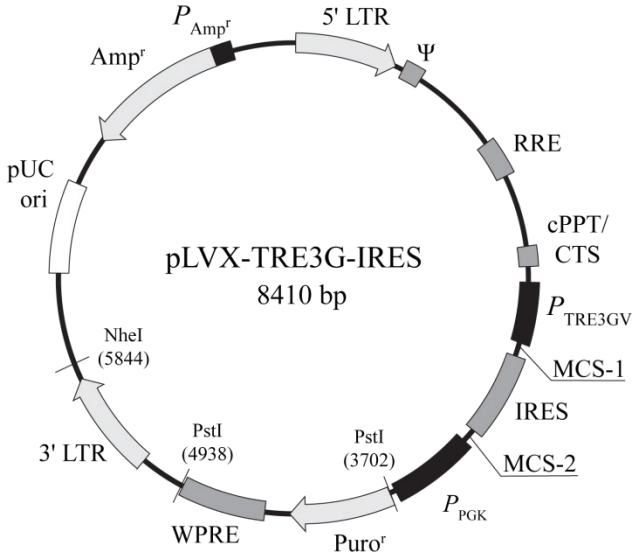

Figure 8. pLVX-TRE3G-IRES Vector Map.

Appendix C: Preparing and Handling Cell Line Stocks

A. Protocol: Freezing 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. 5100-001) 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 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 of G418 and/or puromycin), as appropriate (Section III.B).

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