Takara Bio USA, Inc.

Lenti-X™ Tet-One™ Inducible Expression Systems User Manual

Cat. Nos. 631844, 631847
(011118)

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: techUS@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

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I. Introduction

A. Summary

The **Tet-One Systems** are inducible gene expression systems for mammalian cells that contain all the necessary components in a single plasmid, lentiviral, or retroviral vector. After transfecting target cells with plasmid (Tet-One Systems), or transducing them with lentivirus (Lenti-X Tet-One Systems) or retrovirus (Retro-X™ Tet-One systems), the cells will express the Tet-On® 3G transactivator protein and contain a gene of interest (GOI) under the tight control of a TRE3G promoter (P_{TRE3GS}).). This manual describes the lentivirus-based **Lenti-X Tet-One Inducible Expression System** (Cat. No. 634844) and **Lenti -X Tet-One Inducible Expression System (Puro)** (Cat. No. 634847). Using these systems, your target cells will express high levels of your GOI, but only when cultured in the presence of doxycycline (Dox) (Figure 1).

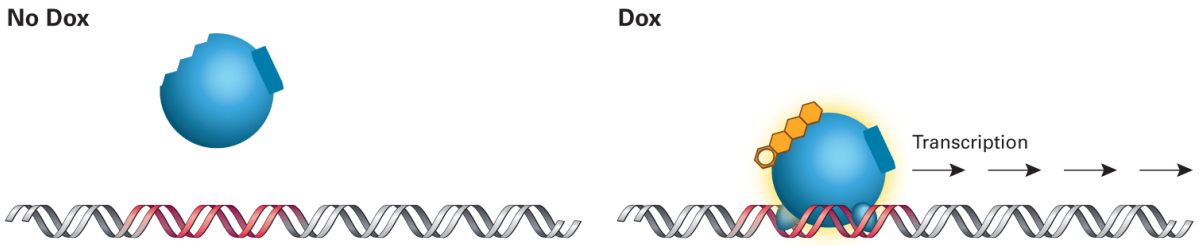

Figure 1. The Tet-On 3G and Tet-One Systems allow inducible gene expression in the presence of Dox.

B. Elements of Lenti-X Tet-One Systems

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_{TRE3GS} 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_{TRE3GS} is a version of P_{TRE3G} that was modified for higher performance in a single vector context. In the presence of Dox, Tet-On 3G binds specifically to P_{TRE3GS} and activates transcription of the downstream GOI. P_{TRE3GS} lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction.

Tet-One Systems "All-in-One" Design

Before the Tet-One Systems were developed, our Tet-On and Tet-Off® products all required two separate vectors to introduce the transactivator protein and the inducible promoter controlling your gene of interest, respectively, into your target cells. The Tet-One Systems provide both of these components on a single vector. The Tet-On 3G transactivator is expressed in the forward direction from the human phosphoglycerate kinase 1 promoter, and the cloned gene of interest is expressed from the P_{TRE3GS} promoter in the reverse orientation. Compared to the two-vector Tet-On 3G Systems, all previously published all-in-one vectors have shown a low signal-to-noise ratio, typically providing only 50–100-fold induced expression, even in selected clones. Our Tet-One Systems are based on an all-in-one design that has shown up to 25,000-fold induction (Heinz *et. al*,. 2011).

4th Generation Lentiviral Packaging System

Our **Lenti-X Packaging Single Shots (VSV-G)**, provided with the Lenti-X Tet-One Inducible Expression System and the Lenti-X Tet-One Inducible Expression System (Puro), can generate lentiviral titers that are superior to all 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 all 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_{TRE3GS} promoter (Figure 1). The Dox concentrations required for induction 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 and Tet-One 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.

Lenti-X Tet-One Inducible Expression System (Cat. No. 631844)

- 1 each pLVX-TetOne Vector Set (Cat. No. 631846; not sold separately)
  - 10 µg pLVX-TetOne Vector (500 ng/µl)
  - 10 µg pLVX-TetOne-Luc Control Vector (500 ng/µl)
- 16 rxns Lenti-X Packaging Single Shots (Cat. No. 631275)
- 3 tests Lenti-X GoStix Plus (Sample) (Cat. No. 631279; not sold separately)

Lenti-X Tet-One Inducible Expression System (Puro) (Cat. No. 631847)

- 1 each pLVX-TetOne-Puro Vector Set (Cat. No. 631849; not sold separately)
  - 10 µg pLVX-TetOne-Puro Vector (500 ng/µl)
  - 10 µg pLVX-TetOne-Puro-Luc Control Vector (500 ng/µl)
- 16 rxns Packaging Single Shots (Cat. No. 631275)
- 3 tests Lenti-X GoStix Plus (Sample) (Cat. No. 631279; not sold separately)

NOTE: The only difference between the two systems is the presence of a puromycin resistance cassette in the pLVX-TetOne-Puro Vector to allow for selection of stable clones using antibiotic selection (Section IX.C). pLVX-TetOne does not contain a selection marker gene and as a result allows for a larger transgene to be cloned (up to 4 kb, compared to ~3 kb for pLVX-TetOne-Puro). Transduced clones created using pLVX-TetOne can instead be isolated by limiting dilution (Section IX.D).

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)
631101	Tet System Approved FBS, US-Sourced (500 ml)
631105	Tet System Approved FBS, US-Sourced (50 ml)

B. Antibiotic for Selecting Stable Cell Lines

The pLVX-TetOne-Puro Vector contains a puromycin resistance marker for selection of stable clones or populations (Section IX.C). Use the following recommended puromycin concentrations:

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

		Recommended Concentration (µg/ml)	
Cat. No.	Antibiotic	Selecting Colonies[1]	Maintenance
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.

pLVX-TetOne does not contain a selection marker. However, clones can instead be isolated using limiting dilution (Section IX.D).

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 stable Tet-One and 293T cell lines.
- 6-well, 12-well, 24-well and 96-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 or reduce sample volume, 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.

J. 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)

K. 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)

L. 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)

M. 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)

N. Plasmid Purification (Transfection-Grade)

Cat. No.	Product	Size
740412.10	NucleoBond Xtra Midi Plus	10 preps
740416.10	NucleoBond Xtra Maxi Plus	10 preps
740422.10	NucleoBond Xtra Midi EF Plus	10 preps
740426.10	NucleoBond Xtra Maxi EF Plus	10 preps

O. Luciferase Assay and Luminometer

These items are required when using the pLVX-TRE3G-Luc Vector or the pLVX-TRE3G-Luc-Puro 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-TetOne Vector using In-Fusion HD (Section V).
2. Pilot test Tet-based induction of your construct using transient transfection (Section VI).
3. Produce lentiviral supernatants using Lenti-X Packaging Single Shots (VSV-G) (Section VII).
4. Transduce your target cells with TetOne virus (Section IX).

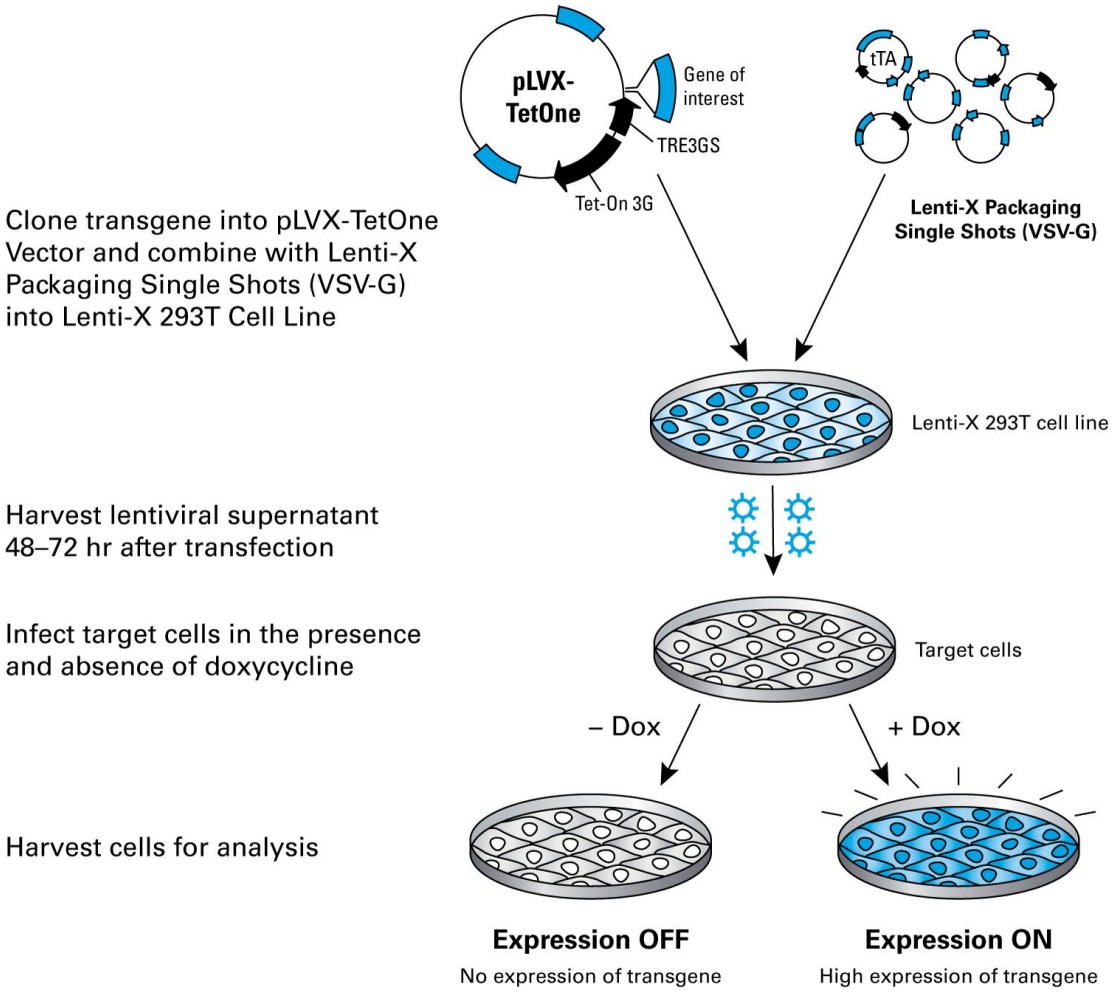

Figure 2. Establishing an inducible expression system in target cells with Lenti-X Tet-One. The pLVX-TetOne plasmid containing your gene of interest and Lenti-X Packaging Single Shots (VSV-G), an optimized packaging pre-mix lyophilized with Xfect Transfection Reagent, are contransfected into Lenti-X 293T target cell lines, and used to generate a high-titer lentiviral supernatant (Section VII). The lentiviral supernatant is used to transduce your target cells (Section IX). Clones are then selected, expanded, and screened for doxycycline-inducible expression of your gene of interest.

V. Cloning Your Gene of Interest into a pLVX-TetOne 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.

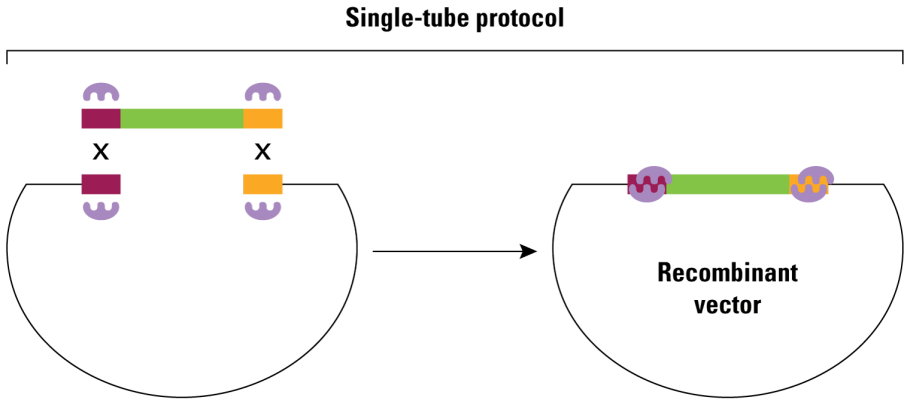

Figure 3. The In-Fusion HD Single-Tube Cloning Protocol.

The recommended linearization sites and forward/reverse primer designs are as follows:

Vector	Linearize with	Forward Primer*	Reverse Primer**
pLVX-TetOne	EcoRI & BamHI	CCCTCGTAAAGAATTC 111 222 333 444 555 666 777 888	GAGGTGGTCTGGATCCSSS NNN NNN NNN NNN NNN NNN NNN
pLVX-TetOne-Puro	EcoRI & BamHI	CCCTCGTAAAGAATTC 111 222 333 444 555 666 777 888	GAGGTGGTCTGGATCC 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-TetOne or pLVX-TetOne-Puro construct should be tested for functionality by plasmid transfection. Transiently transfect your vector 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

pLVX-TetOne-Luc or pLVX-TetOne-Puro-Luc can be used as a positive control.

A. Materials Required

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

B. Protocol

1. Transfect the TetOne vector 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 5 µg of pLVX-TetOne-GOI (or pLVX-TetOne-Puro-GOI) for each well (GOI = gene of interest).
   - We recommend performing the test in duplicate with negative controls: 3 wells containing 100 ng/ml of Dox, and 3 wells without Dox.
   - Use pLVX-TetOne-Luc (or pLVX-TetOne-Puro-Luc) as a positive control (Section III.N)

Wells 1 & 2: 5 µg pLVX-TetOne-GOI (no Dox)

Wells 3 & 4: 5 µg pLVX- TetOne-GOI (100 ng/ml Dox)

Well 5: 5 µg pLVX-TetOne empty (no Dox)

Well 6: 5 µg pLVX-TetOne empty (100 ng/ml Dox)

Figure 4. Transfection of the pLVX-TetOne 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 **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 included 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, concentrated, 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 Using Antibiotic Selection

This protocol can only be performed with LVX-TetOne-Puro lentiviral supernatants.

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 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-One Lentiviruses

A. Summary

- **Creating a mixed population of transduced cells without selection:** To very rapidly establish an inducible system in your cell line or primary cells, you can transduce your entire cell population with an MOI (multiplicity of infection) of 1–10 and analyze your results 3 days post-transduction (Section IX.B). This method relies on producing sufficient lentivirus to transduce your entire cell population. For long-term studies in a dividing cell line, we recommend screening a single inducible clone for high inducibility (Section IX.E).
- **Screening for single clones by antibiotic selection:** After transduction of LVX-TetOne-Puro virus, it is possible to select and screen single clones with puromycin (see Section IX.C).
- **Screening for single clones by limiting dilution:** pLVX-TetOne does not contain a cassette for antibiotic selection, but it is possible to screen single clones using a limiting dilution technique (see Section IX.D).

B. Protocol: Transducing a Mixed Population without Clonal Selection

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

1. Plate target cells in complete growth medium 12–18 hr before transduction. The cells should be 70–80% confluent at the time of transduction.
2. Thaw aliquots of your LVX-TetOne or LVX-TetOne-Puro lentiviral stock, or use filtered virus stocks freshly prepared from packaging cells (Section VII).
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.G).
4. Transduce your target cells at an MOI of 1–10 so that every cell is transduced at least once. Make sure that the total volume of viral 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.

   *__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.
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 (100 ng/ml), as appropriate. Alternatively, expand and freeze to create a cell line stock (Appendix C).

   CAUTION: Discarded medium contains infectious lentivirus.
7. Continue to incubate the cells for 24–48 hr to allow the expressed protein to accumulate.
8. Harvest the cells for analysis.

C. Protocol: Screening Single Clones Using Puromycin Selection

NOTE: This protocol can be completed in ~2 weeks.

1. Plate target cells in a well of a 6-well plate in complete growth medium 12–18 hr before transduction. The cells should be 70–80% confluent at the time of transduction.
2. Thaw aliquots of your LVX-TetOne-Puro lentiviral stock, or use filtered virus stocks freshly prepared from packaging cells (Section VII).

   NOTE: pLVX-TetOne does not contain an antibiotic selection cassette; single clones must be screened using limiting dilution (Section IX.D)
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.G).
4. Transduce your target cells at an MOI of 1–10. Make sure that the total volume of viral supernatant used makes up no more than 1/3 the final volume of culture medium used in the transduction.
5. Transduce the cells for 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, change the medium at 6–8 hr.
6. At 24 hr post-transduction, remove medium, trypsinize and split the cells. Cells from a single well of a 6-well plate should be split into 4 x 10 cm dishes containing complete growth medium supplemented with 0.1–1 µg/ml puromycin (Section III.B).

   CAUTION: Discarded medium contains infectious lentivirus.
7. After ~2 weeks, puromycin-resistant colonies should begin to appear.
8. When the colonies are large enough to transfer, use cloning cylinders or disks to harvest (i.e., “pick”) large, healthy colonies, and transfer each into a separate well of a 24-well plate.
9. Isolate 10–20 colonies and culture in a maintenance concentration of puromycin (Section III.B).
10. When confluent, split the cells from each well into three wells of a 6-well plate for testing and maintenance (Section IX.E).

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

D. Protocol: Screening Single Clones Using Limiting Dilution

NOTE: This protocol can be completed in 2 weeks.

1. Plate target cells in complete growth medium 12–18 hr before transduction. The cells should be 70–80% confluent at the time of transduction.
2. Thaw aliquots of your LVX-TetOne lentiviral stock, or use filtered virus stocks freshly prepared from packaging cells (Section VII).
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.G).
4. Transduce your target cells at an MOI of 1–10 so that every cell is transduced at least once. Make sure that the total volume of viral supernatant used makes up no more than 1/3 the final volume of culture medium used in the transduction.
5. Transduce the cells for 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, change the medium at 6–8 hr.
6. At 24 hr post-transduction, remove medium, trypsinize and count the cells using a hemocytometer.

   CAUTION: Discarded medium contains infectious lentivirus.
7. Dilute your cells using 10 ml serial dilutions in complete growth media, until you obtain a 10 ml aliquot containing only 100–150 cells.
8. Transfer 100 µl of this dilution (containing 1–2 cells on average) to each well of a 96-well culture dish.
9. After ~2 weeks, approximately half of the wells should contain a colony expanded from a single cell.
10. Transfer 10–20 healthy colonies into separate wells of a 24-well plate.
11. When confluent, split the cells from each well into three wells of a 6-well plate for testing and maintenance (Section IX.E).

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

E. Protocol: Testing Your Tet-One Clones for Induction

NOTE: This protocol can be completed in 2 days.

1. For each clone to be tested, seed 1/3 of the total amount of cells (Section IX.C, Step 10, or Section IX.D, Step 11) into a single well of a 6-well plate. The cells in this “stock plate” may be propagated, depending upon the results of the screening assay.

2. Divide the remaining 2/3 of the cells between duplicate wells of a second 6-well plate. Allow the cells to adhere overnight and replace the culture medium with fresh medium and add Dox (100 ng/ml) to one of the duplicate wells, while leaving the second well Dox-free.
3. After 24 hr, assay induced expression of your gene of interest.
4. Select clones with the highest fold induction (ratio of maximal to basal gene expression) for propagation and further testing.
5. Freeze stocks of each promising clone as soon as possible after expanding the culture (Appendix C).

NOTE: Once you have chosen the best clone(s), you may choose to determine the minimal concentration of Dox that is required for high inducible expression and use that minimal concentration for all subsequent experiments. Remove the cells from one nearly confluent well (of a 6-well plate) and divide them among six wells of a 24-well plate. Titrate doxycycline concentrations across these 6 wells (e.g., 0, 1, 10, 50, 100, and 1,000 ng/ml), and assay for induced expression after 24 hr. Typically, there is no need to use more than 100 ng/ml Dox, since maximal expression is often obtained with just 10 ng/ml Dox.

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-One 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 or not densely enough	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.
	Cloned transgene 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 7 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)	Cellular sequences adjacent to integration site of some clones may affect the expression profile.	Screen additional clones (Section IX).
	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) stable cell line.	Mixed cell population	Reselect the current cell line through single colony selection (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 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.	Add a ProteoTuner™ destabilization domain to your protein of interest and control its stability through the addition/removal of Shield1 ligand.
	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-One System Vector Information

For complete descriptions of the vectors provided with each system, refer to the enclosed Certificate of Analysis, which is also available at takarabio.com

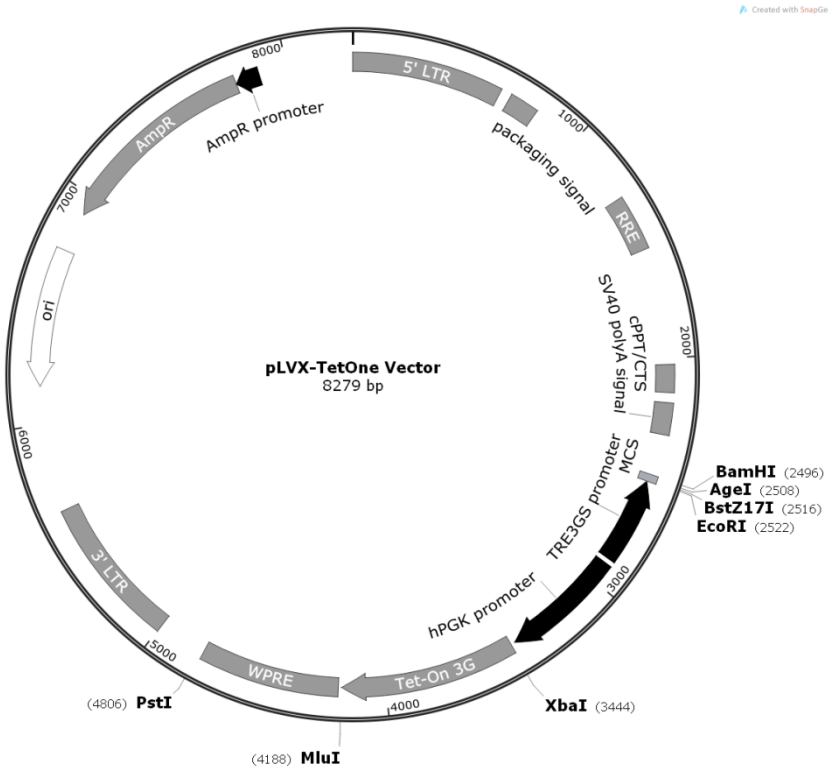

Figure 5. pLVX-TetOne Vector Map

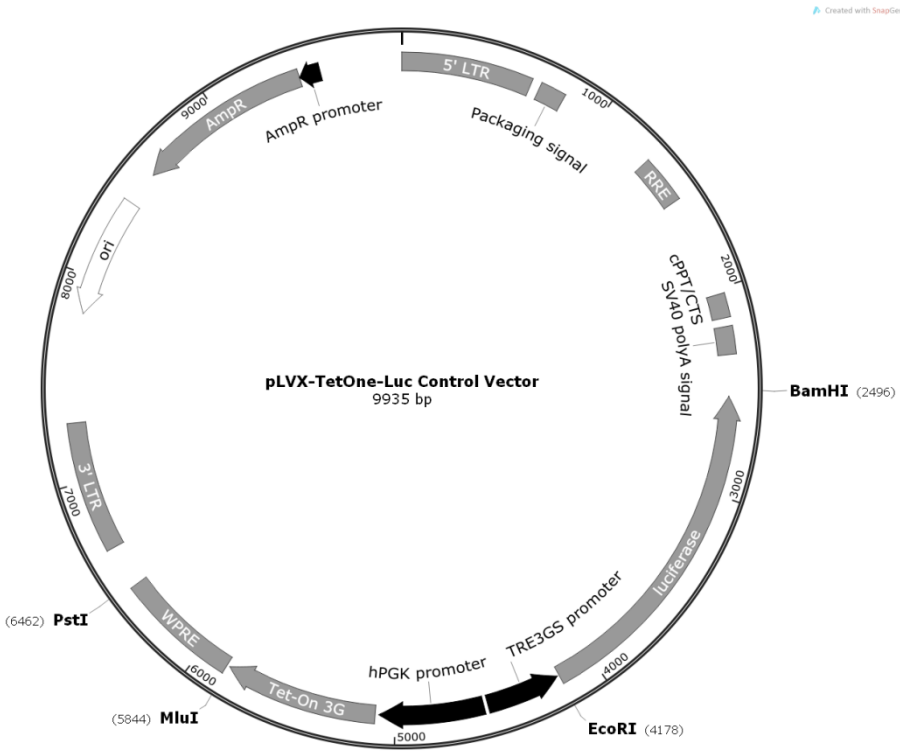

Figure 6. pLVX-TetOne-Luc Control Vector Map

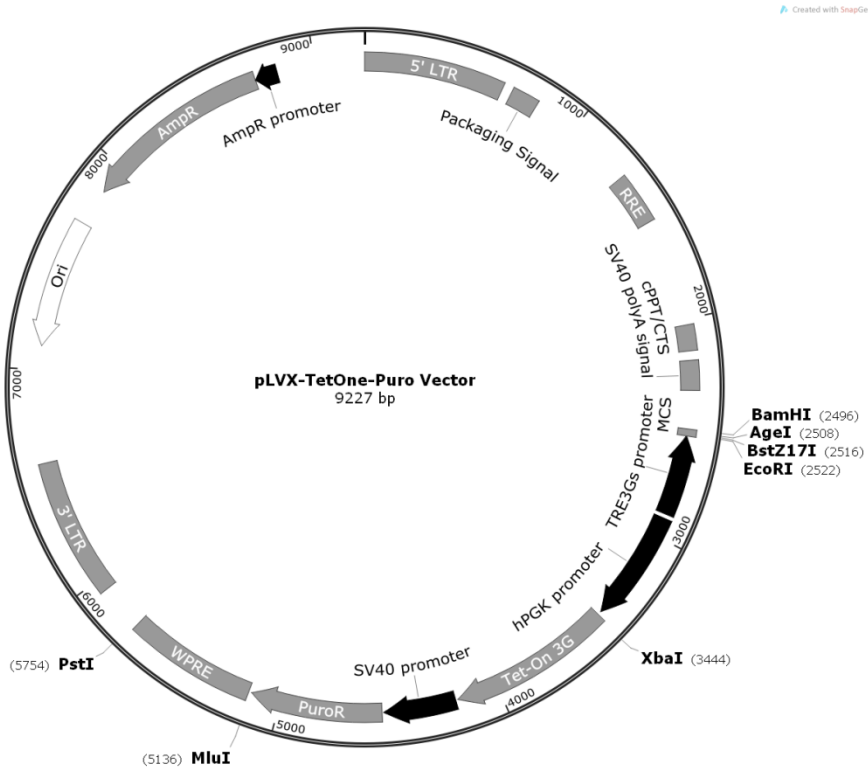

Figure 7. pLVX-TetOne-Puro Vector Map.

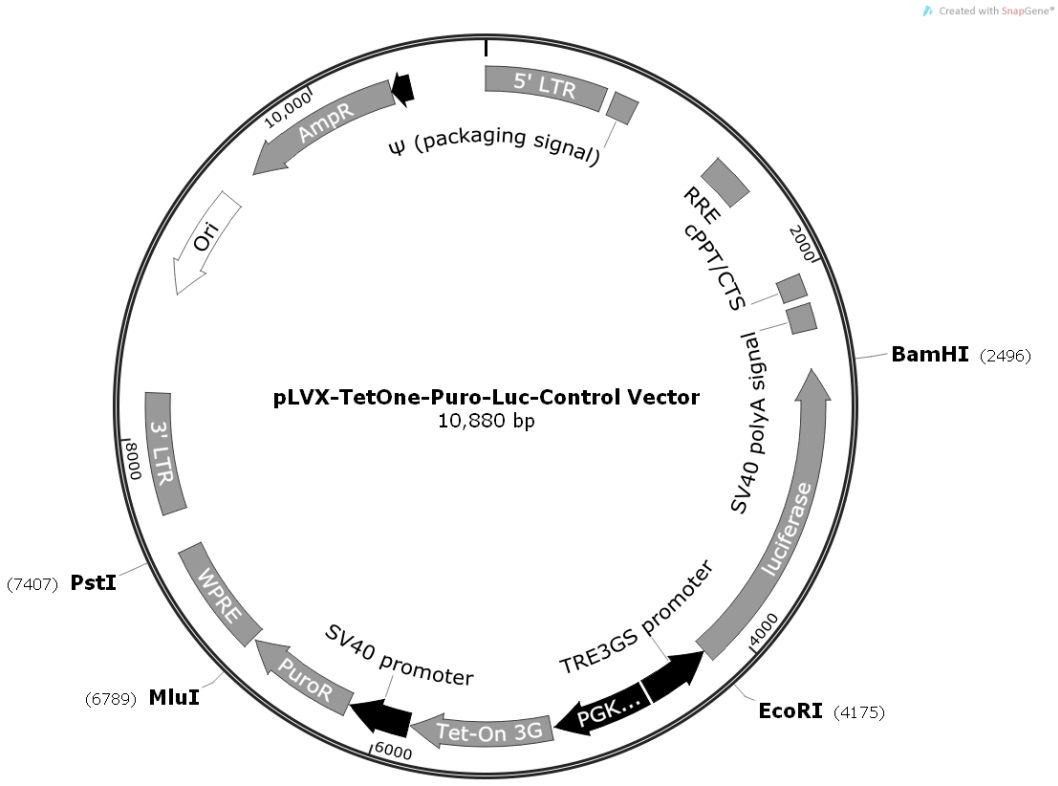

Figure 8. pLVX-TetOne-Puro-Luc Control Vector Map.

Appendix C: Preparing and Handling Cell Line Stocks

A. Protocol: Freezing Cell Line Stocks

Once you have created and tested your Tet-One 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). 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. For cell lines created using pLVX-TetOne-Puro, puromycin should be added to the medium after 48–72 hr in culture. Maintain in complete culture medium containing an appropriate maintenance concentration of puromycin (see Section III.B).

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