Takara Bio USA

Retro-X™ Tet-One™ Inducible Expression System User Manual

Cat. Nos. 634304, 634307
(030920)

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Table of Contents

Table of Figures

Table of Tables

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 retrovirus (Retro-X Tet-One systems) or lentivirus (Lenti-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 retrovirus-based **Retro-X Tet-One Inducible Expression System** (Cat. No. 634304) and **Retro-X Tet-One Inducible Expression System (Puro)** (Cat. No. 634307). 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).

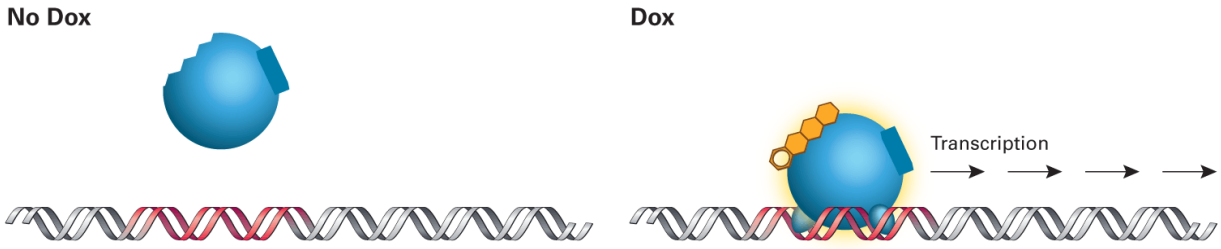

Figure 1. The Tet-On 3G and Tet-One Systems allow inducible gene expression in the presence of Dox.

B. Elements of Retro-X Tet-One Systems

Tet-On 3G Transactivator Protein

Based on the transcriptional regulators described by Gossen and 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).

Retro-X Universal Packaging System

The **Retro-X Universal Packaging System**, provided with the Retro-X Tet-One Inducible Expression System and the Retro-X Tet-One Inducible Expression System (Puro), is our premium retroviral packaging system, featuring the high titer GP2-293 packaging cell line. All four commonly used envelopes are supplied on separate vectors, including VSV-G, eco, ampho and 10A1, to allow you to choose the tropism that is most appropriate for your target cells. The envelope vector is cotransfected with your retroviral expression vector, and high titers of pantropic, ecotropic, amphotropic, or dualtropic virions can be obtained in less than 48 hr (see Table 2).

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 et al. 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 the GP2-293 Packaging Cell Line at –196°C and all other components at –20°C.

Retro-X Tet-One Inducible Expression System (Cat. No. 634304)

- 1 each pRetroX-TetOne Vector Set (Cat. No. 634306; not sold separately)
  - 20 µl pRetroX-TetOne Vector (500 ng/µl)
  - 20 µl pRetroX-TetOne-Luc Control Vector (500 ng /µl)
- 1 each Retro-X Universal Packaging Vector Set (Cat. No. 631457; not sold separately)
  - 20 µl p10A1 Vector (500 ng/µl)
  - 20 µl pAmpho Vector (500 ng /µl)
  - 20 µl pEco Vector (500 ng /µl)
  - 20 µl pVSV-G Vector (500 ng /µl)
  - 20 µl pQCLIN Retroviral Vector (500 ng /µl))
- 1 ml GP2-293 Packaging Cell Line (2 x 10^6 cells/ml) (Cat. No. 631458; not sold separately)
- 100 rxns Xfect™ Transfection Reagent (Cat. No. 631317)
  - 2 tubes Xfect Polymer (75 µl each)
  - 2 tubes Xfect Reaction Buffer (12 ml each)
- 50 ml Tet System Approved FBS, US-Sourced (Cat. No. 631105)

Retro-X Tet-One Inducible Expression System (Puro) (Cat. No. 634307)

- 1 each pRetroX-TetOne-Puro Vector Set (Cat. No. 634309; not sold separately)
  - 20 µl pRetroX-TetOne-Puro Vector (500 ng/µl)
  - 20 µl pRetroX-TetOne-Puro-Luc Control Vector (500 ng /µl)
- 1 each Retro-X Universal Packaging Vector Set (Cat. No. 631457; not sold separately)
  - 20 µl p10A1 Vector (500 ng/µl)
  - 20 µl pAmpho Vector (500 ng /µl)
  - 20 µl pEco Vector (500 ng /µl)
  - 20 µl pVSV-G Vector (500 ng /µl)
  - 20 µl pQCLIN Retroviral Vector (500 ng /µl)
- 1 ml GP2-293 Packaging Cell Line (2 x 10^6 cells/ml) (Cat. No. 631458; not sold separately)
- 100 rxns Xfect Transfection Reagent (Cat. No. 631317)
  - 2 tubes Xfect Polymer (75 µl each)
  - 2 tubes Xfect Reaction Buffer (12 ml each)
- 50 ml Tet System Approved FBS, US-Sourced (Cat. No. 631105)

NOTE: The only difference between the two systems is the presence of a puromycin resistance cassette in the pRetroX-TetOne-Puro Vector to allow for selection of stable clones using antibiotic selection (Section IX.C). pRetroX-TetOne does not contain a selection marker gene and as a result allows for a larger transgene to be cloned (up to 3.1 kb, compared to ~2.1 kb for pRetroX -TetOne-Puro). Transduced clones created using pRetroX -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 RetroX-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.

pRetroX-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 GP2-293 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 GP2-293 cell lines.
- 6-well, 12-well, 24-well, and 96-well cell culture plates; 10 cm cell culture dishes

D. Retroviral Titer Determination

For accurate and consistent transductions, we highly recommend titrating your retroviral stocks. Visit **takarabio.com** for details.

Cat. No.	**Retroviral Titration Technology**
631453	Retro-X qRT-PCR Titration Kit (200 rxns)

E. Retrovirus Concentration

Cat. No.	Concentrator
631455	Retro-X Concentrator (100 ml)
631456	Retro-X Concentrator (500 ml)

Use Retro-X Concentrator to simply increase your available titer up to 100-fold or reduce sample volume, without ultracentrifugation—visit **takarabio.com** for details.

F. 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

G. 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.

H. Xfect Transfection Reagent

Xfect provides high transfection efficiency for most commonly used cell types, including GP2-293 cells.

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

I. 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)

J. Stellar™ Competent Cells

Stellar Competent Cells are recommended by Takara Bio 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)

K. 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. We do not recommend using the TetR Monoclonal Antibody to screen clones.

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

L. 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

M. Luciferase Assay and Luminometer

These items are required when using the pRetroX-TRE3G-Luc Vector or the pRetroX-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 Retroviruses

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

The National Institute of Health and Center for Disease Control have designated retroviruses such as Moloney murine leukemia virus (MMLV) as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. MMLV does not naturally infect human cells; however, virus packaged from the MMLV-based vectors described here is capable of infecting human cells. The viral supernatants produced by these retroviral systems could, depending on your retroviral 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 retrovirus. **The user is strongly advised not to create retroviruses capable of expressing known oncogenes in amphotropic or polytropic host range viruses.**

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

> Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (Revised December 2009) HHS Pub. No. (CDC) 21-1112. U.S. Department of Health and Human Services Public Health Service, Centers for Disease Control and Prevention, and NIH.

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

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 retrovirus (see Figure 2).

1. Clone your gene of interest into the pRetroX-TetOne Vector using In-Fusion HD (Section V).
2. Pilot test Tet-based induction of your construct using transient transfection (Section VI).
3. Produce retroviral supernatants using the Retro-X Universal Packaging System (Section VII).
4. Transduce your target cells with TetOne virus (Section IX).

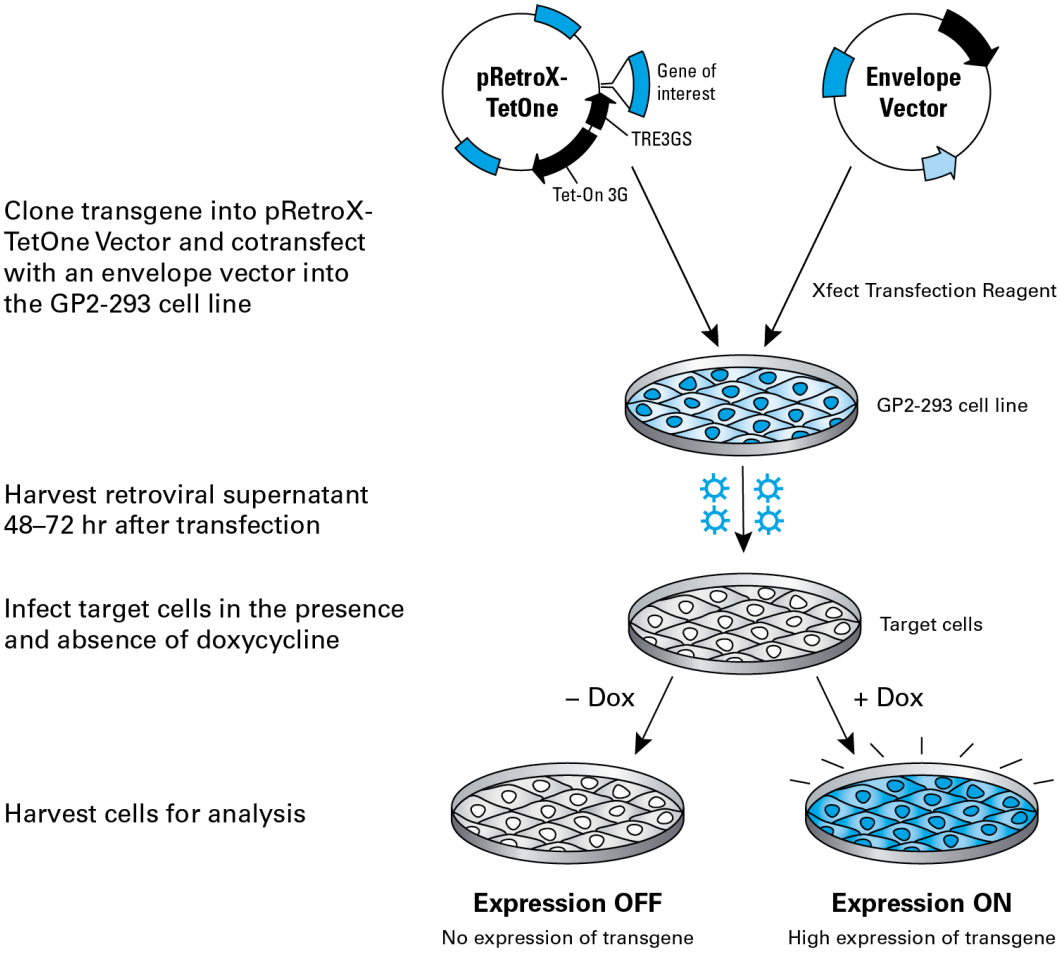

Figure 2. Establishing an inducible expression system in target cells with Retro-X Tet-One. The pRetroX-TetOne plasmid containing your gene of interest and an envelope vector are cotransfected into GP2-293 target cell lines, and used to generate a high-titer retroviral supernatant (Section VII). The retroviral 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 pRetroX-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: Stellar Competent Cells (Section III.J) are recommended by Takara Bio 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.

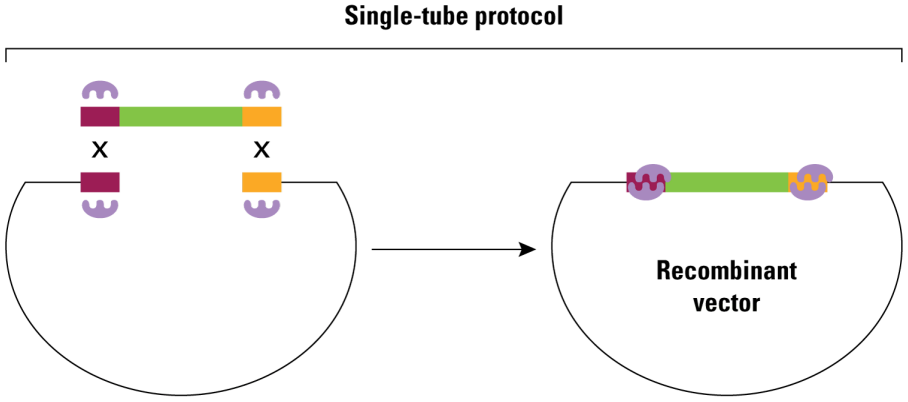

Figure 3. The In-Fusion HD Single-Tube Cloning Protocol.

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

Vector	Linearize with	Forward Primer*	Reverse Primer**
pRetroX-TetOne	EcoRI & BamHI	CCCTCGTAAAGAATTC 111 222 333 444 555 666 777 888	GAGGTGGTCTGGATCCSSS NNN NNN NNN NNN NNN NNN NNN
pRetroX -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 retrovirus production, your pRetroX-TetOne or pRetroX-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

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

A. Materials Required

1. pRetroX-TetOne (or pRetroX-TetOne-Puro) Vector containing your gene of interest, and pRetroX-TetOne-Luc (or pRetroX-TetOne-Puro-Luc) Vector as a positive control.
2. Host cell line
3. Xfect Transfection Reagent (Section III.H)
4. Doxycycline (1 mg/ml) (Section III.G)
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 pRetroX-TetOne-GOI (or pRetroX-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 pRetroX-TetOne-Luc (or pRetroX-TetOne-Puro-Luc) as a positive control (Section III.M)

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

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

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

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

Figure 4. Transfection of the pRetroX-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 Retrovirus from the Retro-X Vectors

We highly recommend using the supplied **Retro-X Universal Packaging System** (Cat. No. 631530) to package your retroviruses. The protocol for packaging retrovirus is outlined below, but more detailed procedures may be found in the Retroviral Gene Transfer and Expression User Manual, available at **takarabio.com/manuals.** The system includes a selection of 4 *env* expression vectors; consult Table 2 to determine which envelope protein is best suited for your target cell line and transfect using Xfect Transfection Reagent. You may wish to perform separate tests of different Env proteins to optimize the infectivity of your viruses.

Table 2. Tropisms associated with commonly used retroviral envelopes

Envelope		VSV-G	4070A (Ampho)	gap70 (Eco)	10A1 (Dual)
Tropism		Pantropic	Amphotropic	Ecotropic	Dualtropic
Receptor (target cell)		Unknown[b]	RAM1 (Pit2)	mCAT-1	GALV (Pit1), RAM1 (Pit2)
Possible target cell types[a]	Human	+	+	–	+
	Mouse	+	+	+	+
	Rat	+	+	+	+
	Hamster	+	+/–	–	+
	Cat	+	+	–	+
	Dog	+	+	–	+
	Monkey	+	+	–	+
	Avian	+	–	–	–
	Fish	+	–	–	–
	Insect	+	–	–	–

[a]This listing of the most commonly transduced target cell types is not intended to be exclusive.
[b]It appears likely that a gp96 chaperone client is responsible for binding (Bloor et al. 2010).

A. General Considerations

1. Optimizing Retroviral Titer

To obtain the highest titers from the Retro-X Universal Packaging System, adhere strictly to the following protocol, especially with respect to:

- Culture size and volume
- DNA amounts and transfection-grade quality
- Tetracycline-free serum in GP2-293 growth media
- Incubation times

2. Required Materials & Precautions

All Xfect transfection reagents, volumes, and conditions are optimized for use with Retro-X Vectors, the envelope vector of choice, and GP2-293 cells. For optimal results, it is also necessary to use:

- Tet System Approved FBS
- 100 mm culture plates
- Transfection-grade DNA

Be sure to use Tet System Approved FBS*, both in the transfection medium (**Step 1**) and in the medium used to collect the virus (**Step 9**).

*Tet-One System performance will be negatively affected by serum containing tetracycline contaminants (see Section III.A).

IMPORTANT: Perform all steps in a sterile tissue culture hood. Retrovirus requires the use of a Biosafety Level 2 facility. Depending on which viral envelope is selected (see Table 2), recombinant pseudotyped retroviruses packaged from this system are capable of infecting human cells. Know and use appropriate safety precautions (see Section IV).

B. Protocol: Packaging Retroviral Vectors Using Xfect Transfection Reagent

NOTE: This protocol is applicable to all packaging cell types and can be completed in 2–4 days.

1. Approximately 24 hr before transfection, seed 4–5 x 10^6 cells/100 mm plate, in 10 ml of growth medium. Make sure that the cells are plated evenly. Incubate at 37°C, 5% CO_2 overnight. Continue to incubate the cells until you are ready to add the transfection mixture in Step 7. The cells should be 80–90% confluent at the time of transfection.
2. Thoroughly vortex Xfect Polymer.
3. In a microcentrifuge tube, dilute your retroviral plasmid DNA with Xfect Reaction Buffer to a final volume of 600 µl. Use the following amounts of DNA for the indicated cell lines:
    - **GP2-293:** 15 µg retroviral plasmid + 15 µg envelope plasmid (e.g., pVSV-G)
    - **AmphoPack-293, EcoPack2-293, and RetroPack PT67:** 15 µg retroviral plasmid

    NOTES:
    - Always add your plasmid(s) to the Xfect Reaction Buffer before adding Xfect Polymer.
    - At least 50% of the solution must consist of Xfect Reaction Buffer.
4. Mix well by vortexing for 5 sec at high speed.
5. Add the following amounts of Xfect Polymer (for the indicated cell lines) to the diluted retroviral plasmid DNA and mix well by vortexing for 10 sec at high speed.
    - **GP2-293:** 9 µl Xfect Polymer
    - **AmphoPack-293, EcoPack 2-293, and RetroPack PT67:** 4.5 µl Xfect Polymer

    NOTE: Always keep the ratio of Xfect Polymer:DNA the same. Use 0.3 µl of Xfect Polymer per 1 µg of plasmid DNA.
6. Incubate DNA-Xfect mixture for 10 min at room temperature to allow nanoparticle complexes to form.
7. Add the entire 600 µl of DNA-Xfect solution (Step 6) dropwise to the cell culture medium from Step 1. Rock the plate gently back and forth to mix.

    NOTE: It is not necessary to remove serum from your cell culture medium. It is normal for the medium to change color slightly upon addition of the DNA-Xfect solution.

8. Incubate the plate at 37°C, 5% CO_2.
9. After 4 hr to overnight, replace the transfection medium with 10 ml fresh complete growth medium and incubate at 37°C for an additional 24–48 hr. Virus titers will generally be highest 48 hr after the start of transfection. *Caution: discarded medium contains infectious retrovirus*.
10. Harvest the retroviral supernatants and pool similar stocks, if desired. *Caution: supernatants contain infectious retrovirus*. Centrifuge briefly (500*g* for 10 min) or filter through a 0.45 µm filter to remove cellular debris.

    NOTE: The filter used should be made of cellulose acetate, or polysulfonate (low protein binding), instead of nitrocellulose. Nitrocellulose binds proteins present in the membrane of retrovirus and destroys the virus.
11. Verify virus production by titrating the virus stock (see Section VIII), then use the virus to transduce target cells, or aliquot and store at –80°C. If smaller volumes are required for transduction, Retro-X Concentrator (Section III.D) can be used.

    NOTE: Titers can drop as much as 2–4 fold with each freeze-thaw cycle.

VIII. Retrovirus Titration

To produce consistent infection results at a known multiplicity of infection (MOI), it is necessary to titrate each of your retroviral supernatants. Freshly harvested virus can be titered immediately, or frozen in aliquots and then titrated. Note that each freeze-thaw cycle will reduce the functional titers of infectious virus by approximately 2–4 fold. Functional titers will depend largely on the cell type used for titration and may vary significantly between cells commonly used for functional titration (i.e. NIH-3T3) and your target cell line.

A. Titrating Your Retroviral Supernatants by qRT-PCR

The **Retro-X qRT-PCR Titration Kit** (Cat. No. 631453) provides a fast and simple method for titrating retroviral supernatants. The kit employs a quick RNA purification step and determines viral RNA genome content using qRT-PCR and TB Green® technologies. Titration can be completed in only 4 hours, which reduces time delays between virus harvest and target cell infection, allowing you to do both on the same day. It is designed for use with all MMLV-based vectors, including those in the Retro-X Tet-One System.

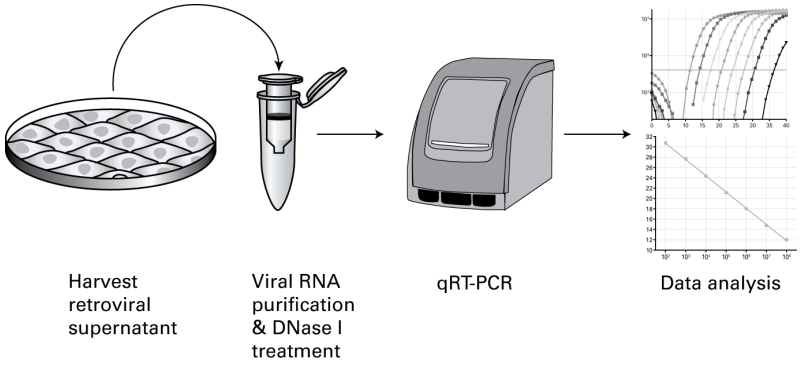

Figure 5. Flowchart of procedures for titering retrovirus supernatants with the Retro-X qRT-PCR Titration Kit.

B. Protocol: Determining Viral Titer Using Antibiotic Selection

This protocol can only be performed with pRetroX-TetOne-Puro retroviral supernatants.

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

1. Plate HT-1080 cells (or NIH/3T3 if you are using ecotropic virus) in one 6-well plate the day before performing the titration infections. Plate 2 x 10^5 cells/well, in 2 ml of medium. Reserve at least one well for a "no infection" control.

   NOTE: You can use other cell lines to determine viral titer, but HT-1080 cells are widely accepted as the standard target cell for titrating retrovirus (NIH/3T3 cells for ecotropic virus) because these cells are transduced very efficiently. Note that the same virus preparation can yield different "apparent" titers in different cells lines due to host cell factors that can produce very different transduction efficiencies and hence different titer measurements. Thus, it is important to use the same cell line when comparing titers across experiments.

2. Prepare 20 ml of complete medium and add 60 µl of 4 mg/ml Polybrene. This concentration of Polybrene (12 µg/ml) will be eventually diluted 3-fold for a final concentration of 4 µg/ml during transduction.

   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 the transfected Retro-X packaging cells (Section VII). This is your virus stock.
4. Prepare six 10-fold serial dilutions of the virus stock as follows:
   a. Add 1.35 ml of medium containing Polybrene (from Step 2) to each of six sterile and numbered 1.5 ml microfuge tubes.
   b. Add 150 µl of the virus stock (from Step 3) to Tube 1. Mix gently.
   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 from each of the five least concentrated viral dilutions (Step 4) to the appropriately labeled wells. The final Polybrene concentration will be 4 µg/ml in ~3 ml. Centrifuge the cultures to improve transduction efficiency*.

   ***Culture centrifugation during infection increases transduction efficiency**

   Centrifuging the plate at 1,200*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 the supernatants and begin antibiotic selection using the concentration of antibiotic that is optimal for your cell line (Section III. F). ***Caution: discarded medium contains infectious retrovirus.***

7. Allow drug-resistant colonies to form for 7–14 days. Change media every 2 days, or add fresh antibiotic every fourth day to maintain selection pressure. Stain the colonies with 1% crystal violet solution (in 10% ethanol), and count.
8. The titer of the virus stock corresponds to the number of colonies generated by the least concentrated dilution, multiplied by the dilution factor. For example, the presence of 4 colonies in the 10^6 dilution would represent a titer of 4 x 10^6 colony forming units.

IX. Transducing Target Cells with the Tet-One Retroviruses

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 retrovirus 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 RetroX-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:** RetroX-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 RetroX-TetOne or RetroX-TetOne-Puro retroviral 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.F).

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*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 retrovirus.
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 RetroX-TetOne-Puro retroviral stock, or use filtered virus stocks freshly prepared from packaging cells (Section VII).

   NOTE: RetroX-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.F).
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.

   NOTE: Use **Retro-X Concentrator** (Section III.E) if you think that your viral titer may be too low.
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 retrovirus.
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.M) 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 RetroX-TetOne retroviral 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.F).

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.

NOTE: Use **Retro-X Concentrator** (Section III.E) if you think that your viral titer may be too low.

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 retrovirus.

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.M) 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 3. Troubleshooting guide for the Retro-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. GP2-293 Packaging Cells		
Poor viability upon thawing	Improper thawing techniques	Use thawing procedure in Appendix C.
	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 GP2-293 cells.
C. Virus Production		
Poor transfection efficiency (as determined by GOI or marker expression in the GP2-293 cell line)	Cells plated too densely or not densely enough	Plate 1.5 x 10^6 cells/60 mm plate, or fewer if the cells divide rapidly. Use at 60–80% 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)	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 8.3 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.
	Target cells are not actively dividing.	Make sure that the cells are in log phase during transduction.

Problem	Possible Explanation	Solution
D. Transduction of Target Cells		
Poor transduction efficiency	Low titer	See Section C or use the Retro-X Concentrator (Section III.E) 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 15 µg of transfection-grade plasmid.
	Low viability of target cells during transduction	Optimize culture conditions for target cells prior to infection; cells must be actively dividing to be infected with retrovirus.
		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.
		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.
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.D) • 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).

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: Retro-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**

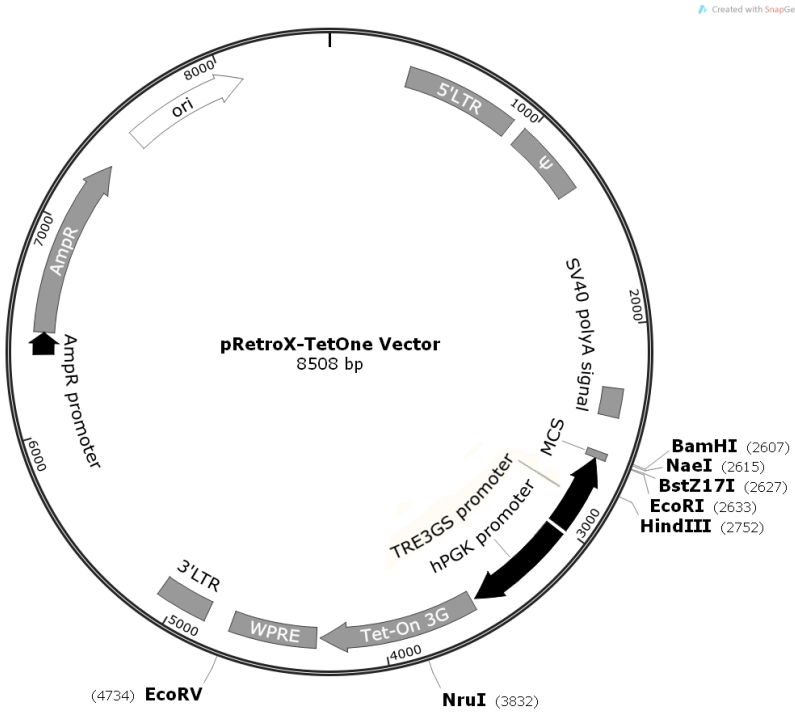

Figure 6. pRetroX-TetOne Vector Map

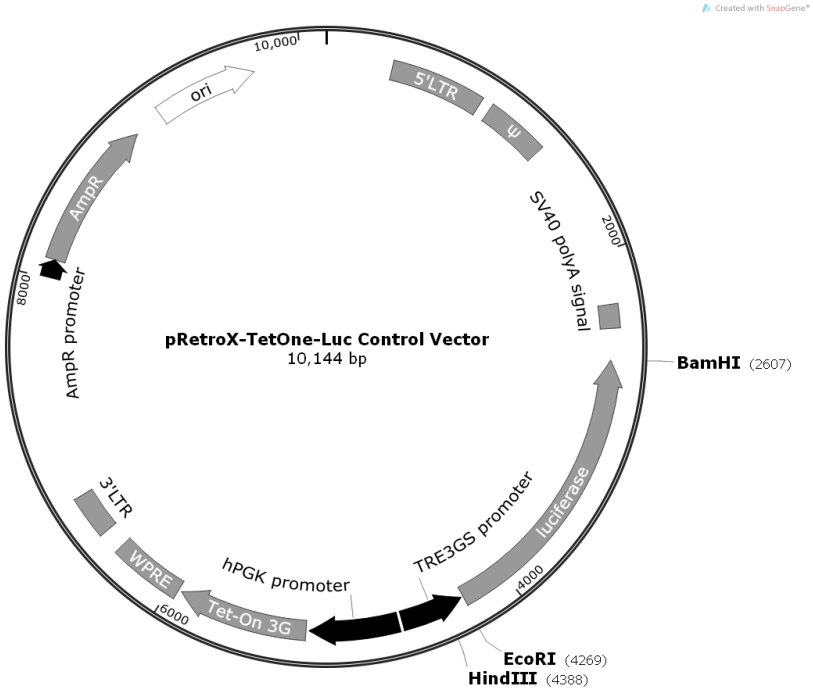

Figure 7. pRetroX-TetOne-Luc Control Vector Map

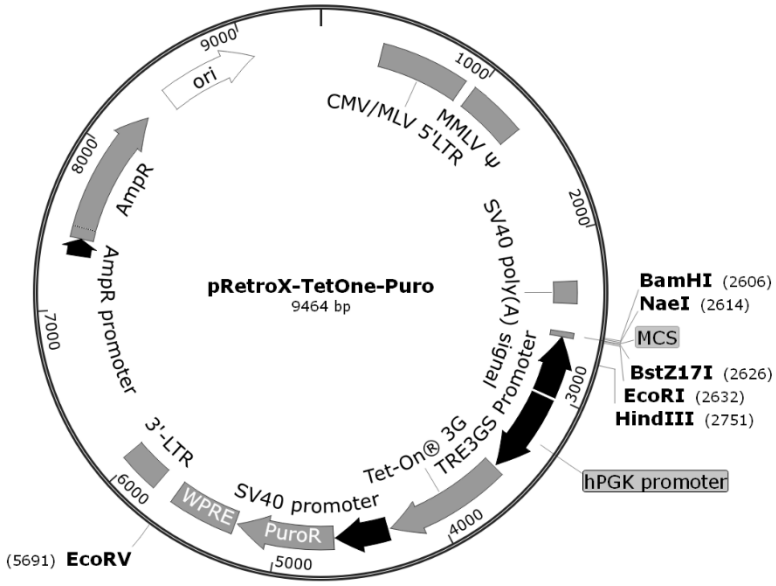

Figure 8. pRetroX-TetOne-Puro Vector Map

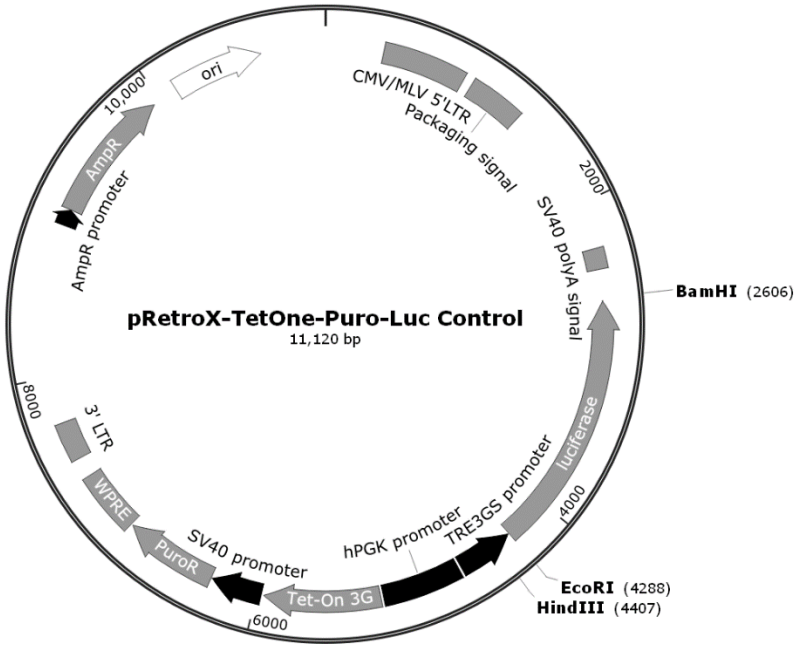

Figure 9. pRetroX-TetOne-Puro-Luc Control Vector Map

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*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*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 pRetroX-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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