Takara Bio USA

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

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(062819)

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I. Introduction

A. Summary

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

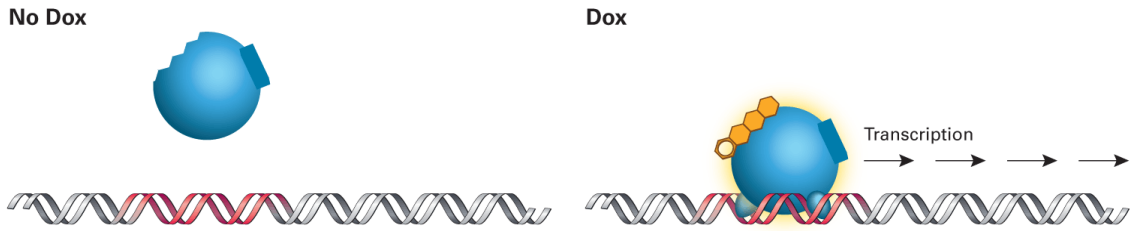

Figure 1. The Tet-On 3G Systems allow inducible gene expression in the presence of Dox.

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

Tet-On 3G Transactivator Protein

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

P_{TRE3GV} Inducible Promoter

The inducible promoter P_{TRE3G} provides for very low basal expression and high maximal expression after induction (Loew *et. al.*, 2010). It consists of 7 repeats of a 19 bp tet operator sequence located upstream of a minimal CMV promoter. P_{TRE3GV} is a version of P_{TRE3G} that was modified at Takara Bio for higher performance in retroviruses and lentiviruses. In the presence of Dox, Tet-On 3G binds specifically to P_{TRE3GV} and activates transcription of the downstream GOI. P_{TRE3GV} lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction.

Retro-X Universal Packaging System

The Retro-X Universal Packaging System 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.

C. Doxycycline

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

II. List of Components

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

Retro-X Tet-On 3G Inducible Expression System (Cat. No. 631188).

- **Retro-X Tet-On 3G Vector Set**
  - pRetroX-Tet3G Vector (10 µg)
  - pRetroX-TRE3G Vector (10 µg)
  - pRetroX-TRE3G-Luc Control Vector (10 µg)
- **Retro-X Universal Packaging Vector Set**
  - p10A1 Vector (20 µg)
  - pAmpho Vector (20 µg)
  - pEco Vector (20 µg)
  - pVSV-G Vector (20 µg)
  - pQCLIN Retroviral Vector (20 µg)
- **GP2-293 Packaging Cell Line** (1 ml)
- **Xfect™ Transfection Reagent** (100 rxns)
- **Tet System Approved FBS, US-Sourced** (50 ml)

III. Additional Materials Required

The following reagents are required but not supplied.

A. Tetracycline-Free Fetal Bovine Serum

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

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

*Product availability varies by region.

B. Antibiotics for Selecting Stable Cell Lines

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

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

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

C. Mammalian Cell Culture Supplies

- **Medium for 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 Tet-On 3G and GP2-293 cell lines.
- 6-well, 12-well, and 24-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 **www.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 without ultracentrifugation—visit **www.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 **www.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 **www.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 www.takarabio.com/in-fusion

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.

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

L. Luciferase Assay and Luminometer

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

IV. Protocol Overview

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

A. General Cell Culture

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

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

B. Safety Guidelines for Working with 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-TRE3G Vector using In-Fusion HD (Section V).
2. Pilot test Tet-based induction of your construct (Section VI).
3. Produce retroviral supernatants using Retro-X Universal Packaging System (Section VII).
4. Co-infect your target cells with Tet-On 3G virus and TRE3G virus (Section IX).

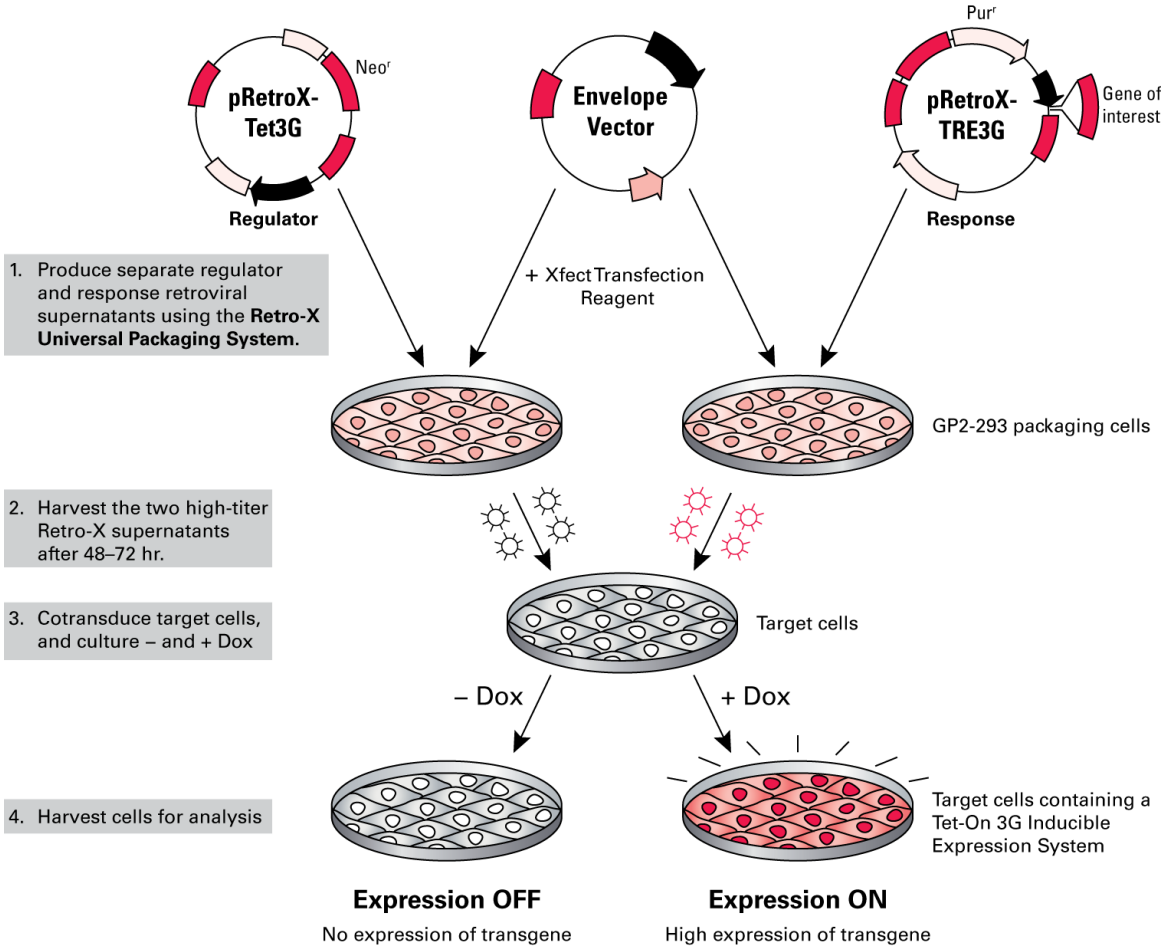

Figure 2. Establishing an inducible expression system in target cells with Retro-X Tet-On 3G. The Retro-X Universal Packaging System is used to generate high-titer retroviral supernatants from the pRetroX-Tet3G Vector and from the pRetroX-TRE3G Vector, which contains your gene of interest. Target cells are then simultaneously cotransduced with the two retroviruses (~8 hr). After culturing for an additional 48–72 hr (+ and – Dox), the cells are harvested for analysis.

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

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

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.

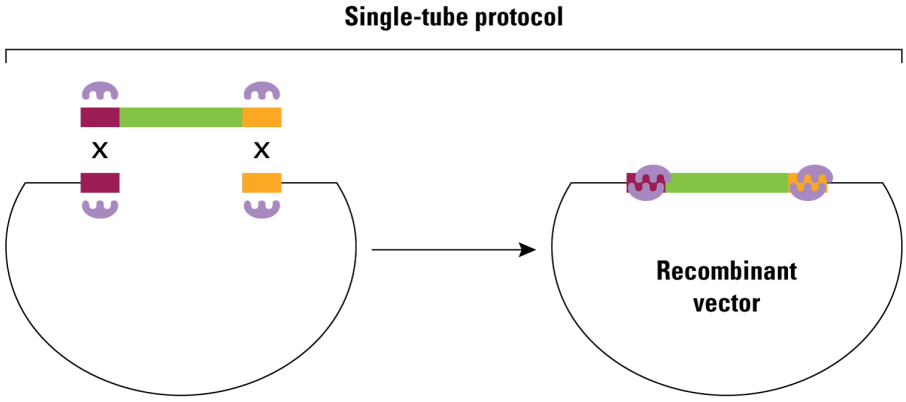

Figure 3. The In-Fusion HD Single-Tube Cloning Protocol.

The recommended linearization sites and forward/reverse primer designs for cloning into **pRetroX-TRE3G Vector** are as follows:

Response Plasmid	Linearize with	Forward Primer*	Reverse Primer**
pRetroX-TRE3G	BamHI & MluI	tcttatacttggatcc 111 222 333 444 555 666 777 888	gcgcggccgcacgcgt 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 pRetro-TRE3G construct should be tested for functionality by plasmid transfection. Transiently cotransfect your pRetroX-TRE3G-GOI vector together with pRetroX-Tet3G (in a 1:4 ratio for best inducibility) into an easy-to-transfect cell line such as HeLa or HEK 293, or your target cell line, and test for transgene induction with Dox. You will need an appropriate gene-specific assay to test for induction, such as:

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

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

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

A. Materials Required

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

B. Protocol

1. Cotransfect both the regulator and response plasmids 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 **www.takarabio.com/manuals**).
   - Use 1 µg of pRetroX-Tet3G and 4 µg of pRetroX-TRE3G-GOI for each well (GOI = gene of interest).
   - We recommend performing the test in duplicate with negative controls: 3 wells containing 100–1,000 ng/ml of Dox, and 3 wells without Dox.
   - Use pRetroX-TRE3G-Luc instead of pRetroX-TRE3G as a positive control (Section III.L)

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

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

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

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

Figure 4. Transfection of the regulator and response plasmids 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 Takara Bio's **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, which is available at **www.takarabio.com/manuals.** The system includes a selection of 4 *env* expression vectors; consult Table I 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. Retro-X Universal Packaging System

env Expression Vector	Tropism	Envelope	Receptors	Host Cell
pEco	Ecotropic	gp70	mCAT1	Rat and mouse
pAmpho	Amphotropic	4070A	Ram-1 (rPit-2)	Many mammalian cell types
p10A1	Dualtropic	10A1	GALV, Ram-1	Many mammalian cell types
pVSV-G	Pantropic	VSV-G	n/a*	All cell types

* The VSV-G envelope protein mediates retroviral entry through lipid binding and plasma membrane fusion, and does not depend on a cell surface receptor.

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
- 60 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-On 3G 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: Transfection of Retroviral Vectors

NOTE: This protocol can be completed in 2–4 days.

1. Approximately 24 hr before transfection, seed 1.5 x 10^6 GP2-293 cells in a 60 mm plate, in 3 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 60-80% confluent at the time of transfection.
2. Thoroughly vortex Xfect Polymer.
3. For each transfection sample, prepare two microcentrifuge tubes by adding reagents in the following order:

Tube 1 (Plasmid DNA)		Tube 2 (Polymer)	
200 µl	Xfect Reaction Buffer		
10 µl	Envelope vector (0.5 µg/µl)	217 µl	Xfect Reaction Buffer
10 µl	Retro-X Vector DNA (0.5 µg/µl)	3 µl	Xfect Polymer
220 µl	Total Volume	220 µl	Total Volume

NOTE: It is crucial that the Xfect Polymer does not remain in aqueous solution for longer than 30 min at room temperature.

4. Vortex each tube well to mix.
5. Add the Xfect Polymer solution (Tube 2) to the Plasmid DNA solution (Tube 1) and vortex well at a medium speed for 10 sec.
6. Incubate each DNA-Xfect mixture for 10 min at room temperature to allow nanoparticle complexes to form.
7. Add the entire 440 µl of DNA-Xfect solution (Step 5) 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.
9. After 4 hr to overnight, replace the transfection medium with 3 ml fresh complete growth medium (containing Tet System Approved FBS) 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 x 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 polysulfonic (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 store at –80°C. If smaller volumes are required for transduction, Retro-X Concentrator (Section III.E) 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-On 3G System.

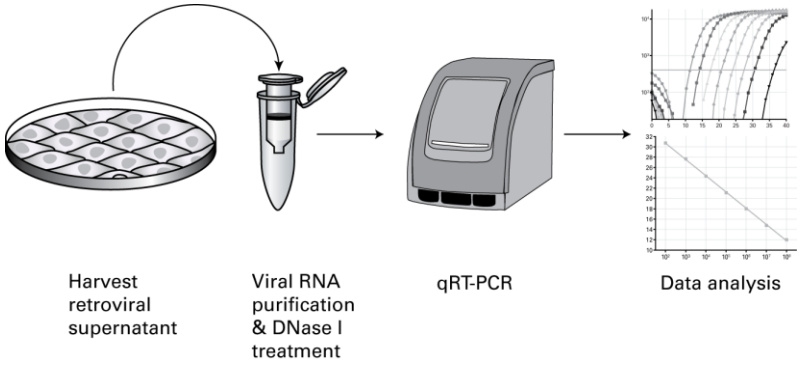

Figure 5. Flowchart of the procedures used for titering retrovirus supernatants with the Retro-X qRT-PCR Titration Kit.

B. Protocol: Determining Viral Titer by Colony Formation

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

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

   NOTE: Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of Polybrene may be determined empirically but generally falls within a range of 2–12 µg/ml. Excessive exposure to Polybrene (>24 hr) can be toxic to cells.
3. Prepare filtered viral supernatant from packaging cells (Section VII). This is the virus stock.
4. Prepare six 10-fold serial dilutions of the virus stock as follows:
   a. Add 1.35 ml of medium containing Polybrene (Step 2) to each of six sterile and numbered 1.5 ml microfuge tubes.
   b. Add 150 µl of the virus stock (Step 3) to the tube 1. Mix.
   c. Transfer 150 µl tube 1 to tube 2 and mix. Continue making serial dilutions by transferring 150 µl from each successive dilution into the next prepared tube.

5. Infect the HT-1080 cells by adding 1 ml of each viral dilution (Step 4) to each appropriate well. The final Polybrene concentration will be 4 µg/ml in ~3 ml. Centrifuge the cultures to improve infection efficiency*.

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

6. After infecting for 8–24 hours, remove supernatants and subject the cells to G418 or puromycin selection using the selection concentrations that are optimal for your cell line (Section III.B).

7. Allow colonies to form for 7–14 days. Stain the colonies with 1% crystal violet solution (in 10% ethanol) and count.

8. The titer of virus corresponds to the number of colonies generated by the highest dilution, multiplied by the dilution factor. For example, the presence of 4 colonies in the 10^6 dilution would represent a viral titer of 4 x 10^6 colony forming units.

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

A. Summary

- **Simultaneous Cotransduction:** To establish the complete Tet-On 3G System, target cells must be cotransduced with both the RetroX-Tet3G and RetroX-TRE3G-GOI retroviruses. Using high titers of each virus ensures that the highest proportion of cells will contain both vectors. Depending on your application, transduced cells can be either treated immediately with Dox to induce expression of your GOI and then harvested for analysis, or the cells may be selected with G418 and puromycin to isolate doubly-transduced clones or to enrich the population for doubly-transduced cells (see Section III.B).

- **Virus Ratio Optimization:** It is possible to optimize the induction characteristics of your system by infecting target cells with different ratios of the regulator and response retroviruses. Using a ratio that favors the response vector (RetroX-TRE3G-GOI) over the regulator vector (RetroX-Tet3G) can produce maximum overall expression of the GOI. Alternatively, using a ratio that favors the regulator vector will generally produce the absolute lowest basal expression levels and can achieve maximum fold-induction. The optimal ratio can be determined in pilot studies, but in general we find that a ratio of 1:1 works best.

- **Sequential Transduction:** If you are working with a clonable cell line, we recommended that you perform sequential transductions with the retroviruses so that you can obtain clones that have optimal inducibility characteristics. This method produces clones that have the highest expression levels, lowest backgrounds, and highest fold-induction. Briefly, cells are first transduced with only the RetroX-Tet3G retrovirus, followed by selection with G418. Resistant clones are then screened for expression of Tet-On 3G and tested for inducibility (e.g., using the supplied control vector and measuring induced luciferase expression). A favorable, Tet-On 3G clone is then transduced with the RetroX-TRE3G-GOI retrovirus. Doubly-transduced cells are selected using puromycin and the resulting puromycin-resistant clones are then screened for GOI inducibility.

B. Protocol: Cotransducing Target Cells with the Retro-X Tet-On 3G Retroviruses

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

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

   NOTE: Lenti-X Accelerator (Cat. Nos. 631256, 631257 & 631254) and **RetroNectin** (Cat. Nos. T110A, T100B & T100A) may be used as transduction enhancers instead of Polybrene (see Section III.F).
4. Combine the RetroX-Tet-On 3G and RetroX-TRE3G-GOI retroviral stocks in the desired ratio and MOI. In general, we find that an MOI ratio of 1:1 works best. For your cells and transgenes, the optimal ratio can be determined empirically (for example, compare three different ratios, such as 1:1, 4:1, and 1:4)**.** If titer values are unknown, use serial dilutions of the viruses mixed at a ratio of 1:1, such that the total volume of supernatant used makes up no more than 1/3 the final volume of culture medium used in the transduction. Centrifuge the cultures to improve transduction efficiency (see Section VIII.B).
5. Transduce the cells for 8–24 hr. If you are concerned that exposure to either the Polybrene or to the viral supernatant (which contains medium conditioned by the packaging cells) may adversely affect your target cells, limit the transduction to 6–8 hr.
6. Remove and discard the virus-containing medium and replace it with fresh growth medium, with or without Dox, as appropriate. Alternatively, passage the cultures and subject the cells to selection using G418 and puromycin, followed by expansion and freezing of cell line stocks (Appendix C).
7. Continue to incubate the cells for 24–48 hr to allow the expressed protein to accumulate.
8. Harvest the cells for analysis.

X. References

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

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Appendix A. Troubleshooting Guide

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

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

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 5 µg of transfection-grade plasmid.
	Low viability of target cells during transduction	Optimize culture conditions for target cells prior to infection
		Packaging cell line-conditioned media may affect cell growth; dilute viral supernatant or shorten exposure time to viral supernatant. Consider using RetroNectin Reagent and the RetroNectin-Bound Virus transduction protocol.
		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)	A suboptimal co-infection ratio was used.	We generally recommend a co-infection ratio of 1:1 for RetroX-Tet3G: RetroX-TRE3G-GOI (Section IX.B). Different vector ratios may result in different maximal/basal gene expression ratios.
	Cells were harvested and analyzed too soon or too late.	Harvest and analyze cells between 18–48 hr after addition of doxycycline
	Poor infection efficiency	• Confirm virus titers using a titration kit (Section III.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 Takara Bio's Tet System Approved FBS (Section III.A), which was functionally tested with Takara Bio's double-stable CHO-AA8-Luc Tet-Off Control Cell Line.
Decrease in fold induction after several passages **or** Loss of inducibility after passaging of a (previously frozen) double-stable cell line.	The appropriate antibiotics are missing from the cell culture medium.	Maintain optimal antibiotic concentrations (Section III.B).
	Mixed cell population	Reselect the current cell line through single colony selection using selective concentrations of puromycin and G418, (Section IX).

Description of Problem	Possible Explanation	Solution
F. Establishment of Stable Cell Lines		
Untransduced cells do not die at the high antibiotic concentration established via titration in Section III.B	• The cells have not been recently passaged, so they remain well-attached to the plate surface even when they are dead. • You have achieved 100% transduction efficiency.	To determine the appropriate antibiotic concentration, use cells that have been split within the last 2–3 days.
There are no surviving cells after transduction followed by selection	The antibiotic concentration which caused massive cell death when determining the appropriate dose via titration could be too high.	Use a lower antibiotic concentration for selection of stably transfected cell clones.
Poor cell viability	Cells were not properly frozen.	See Appendix C, Section A.
	Cells were not properly thawed.	See Appendix C, Section B.
G. Detection and Inhibition of Expression		
No detectable GOI expression by Western Blot.	Low sensitivity of detection method.	Check sensitivity of primary and secondary antibodies. Analyze GOI expression by qRT-PCR, using different sets of primers to ensure optimal detection of GOI expression.
Continuous GOI/Fluorescent Protein expression after the removal of doxycycline	Depending on the stability of the protein, it may persist in the cell in the absence of gene induction and de novo synthesis of GOI mRNA. Fluorescent proteins tend to have long half-lives.	Upon degradation, GOI/Fluorescent Protein expression will not be detectable in cells in the absence of induction. For faster degradation of an inducible GOI, use pTRE-Cycle Vectors (see www.takarabio.com).
	Doxycycline was not completely removed from the cell culture medium.	Wash cells three times with PBS, followed by trypsinization and replating in fresh medium supplemented with Takara Bio's 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-On 3G System Vector Information

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

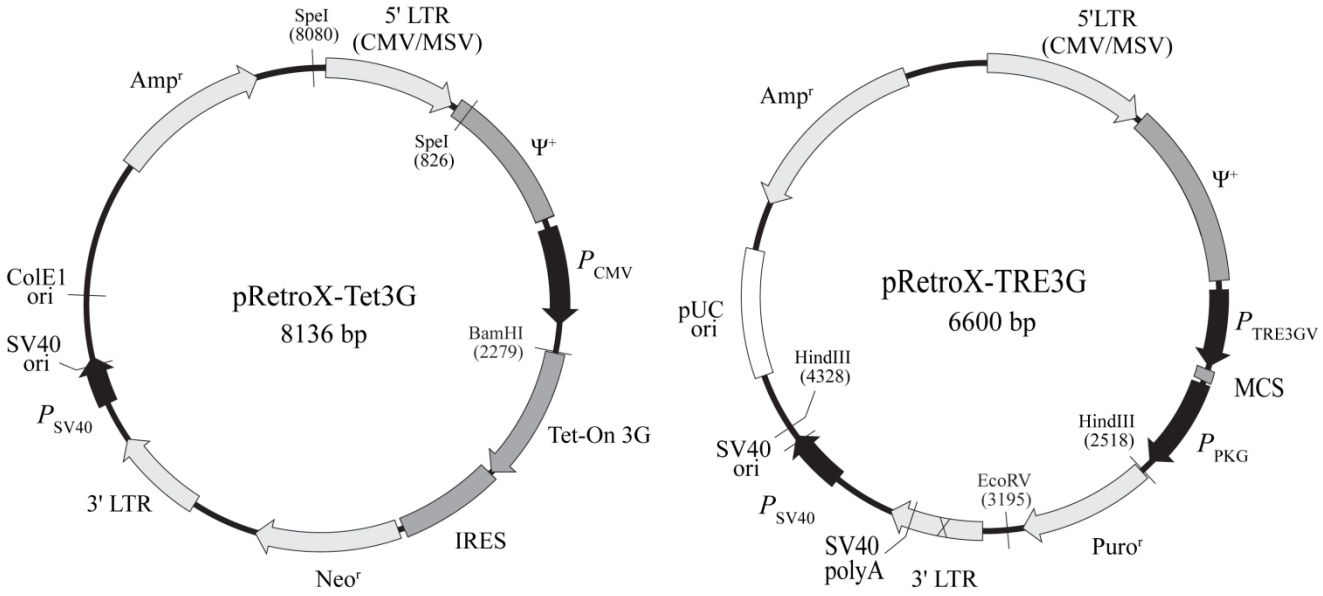

Figure 6. pRetroX-Tet3G and pRetroX-TRE3G Vector Maps.

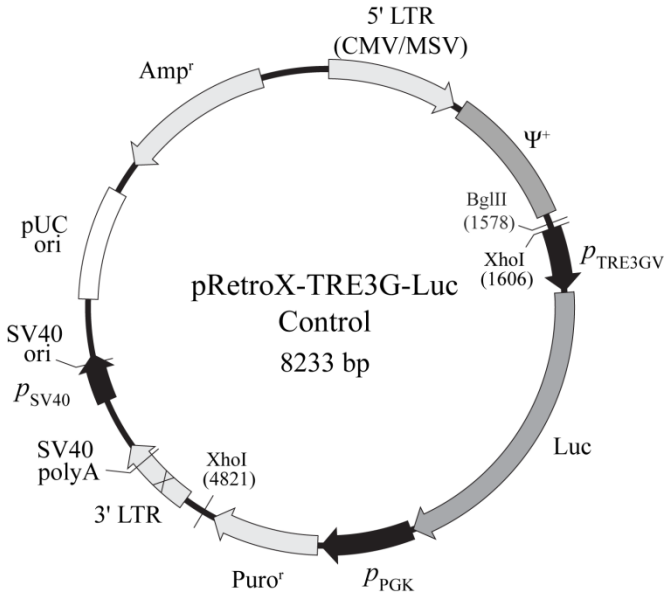

Figure 7. pRetroX-TRE3G-Luc Control Vector Map.

Appendix C: Preparing and Handling Cell Line Stocks

A. Protocol: Freezing Cell Line Stocks

Once you have created and tested your Tet-On 3G cell line, you must prepare multiple frozen aliquots to ensure a renewable source of cells, according to the following protocol:

1. Expand your cells to multiple 10 cm dishes or T75 flasks.
2. Trypsinize and pool all of the cells, then count the cells using a hemocytometer.
3. Centrifuge the cells at 100 x g for 5 min. Aspirate the supernatant.
4. Resuspend the pellet at a density of at least 1–2 x 10^6 cells/ml in freezing medium. Freezing medium can be purchased from Sigma (Cat. Nos. C6164 & C6039), or use 70–90% FBS, 0–20% medium (without selective antibiotics), and 10% DMSO.
5. Dispense 1 ml aliquots into sterile cryovials and freeze slowly (1°C per min). For this purpose, you can place the vials in Nalgene cryo-containers (Nalgene, Cat. No. 5100-001) and freeze at –80°C overnight. Alternatively, place vials in a thick-walled styrofoam container at –20°C for 1–2 hr. Transfer to –80°C and freeze overnight.
6. The next day, remove the vials from the cryo-containers or styrofoam containers, and place in liquid nitrogen storage or an ultra-low temperature freezer (–150°C) for storage.
7. Two or more weeks later, plate a vial of frozen cells to confirm viability.

B. Protocol: Thawing Cell Line Frozen Stocks

To prevent osmotic shock and maximize cell survival, use the following procedure to start a new culture from frozen cells:

1. Thaw the vial of cells rapidly in a 37°C water bath with gentle agitation. Immediately upon thawing, wipe the outside of the vial with 70% ethanol. All of the operations from this point on should be carried out in a laminar flow tissue culture hood under strict aseptic conditions.
2. Unscrew the top of the vial slowly and, using a pipet, transfer the contents of the vial to a 15 ml conical centrifuge tube containing 1 ml of prewarmed medium (without selective antibiotics such as G418). Mix gently.
3. Slowly add an additional 4 ml of fresh, prewarmed medium to the tube and mix gently.
4. Add an additional 5 ml of prewarmed medium to the tube and mix gently.
5. Centrifuge at 100 x g for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium without selective antibiotics. (This method removes the cryopreservative and can be beneficial when resuspending in small volumes. However, be sure to treat the cells gently to prevent damaging fragile cell membranes.)

6. Mix the cell suspension thoroughly and add to a suitable culture vessel. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place in a 37°C humidified incubator (5–10% CO_2 as appropriate) for 24 hr.

   NOTE: For some loosely adherent cells (e.g. HEK 293-based cell lines), we recommend using collagen-coated plates to aid attachment after thawing. For suspension cultures, suspend cells at a density of no less than 2 x 10^5 cells/ml.

7. The next day, examine the cells under a microscope. If the cells are well-attached and confluent, they can be passaged for use. If the majority of cells are not well-attached, continue culturing for another 24 hr.

   NOTE: Note: For some loosely adherent cell lines (e.g., HEK 293-based cell lines), complete attachment of newly thawed cultures may require up to 48 hr.

8. Expand the culture as needed. The appropriate selective antibiotic(s) should be added to the medium after 48–72 hr in culture. Maintain stable and double-stable Tet Cell Lines in complete culture medium containing a maintenance concentration of G418 and/or puromycin), as appropriate (Section III.B).

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