Takara Bio USA, Inc.

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

Cat. No. 632162
(011518)

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I. Introduction

A. Summary

The **Lenti-X Tet-On Advanced Inducible Expression System** uses a highly efficient, lentiviral-based gene transfer technology to deliver our tightly regulated and highly responsive Tet-On Advanced Gene expression system into dividing and nondividing target cells, primary cells, and stem cells. While based on the original tetracycline (Tc)-regulated expression systems described by Gossen & Bujard (1992) and Gossen *et al.* (1995), the Tet-On Advanced System includes a novel, more highly developed and refined transactivator (Urlinger, *et al.* 2000), and incorporates an improved inducible promoter, P_{Tight}.

When transfected into 293T cells with **Lenti-X Packaging Single Shots (VSV-G)**, the Lenti-X Tet-On Advanced System Vectors in this kit produce very high titer supernatants containing infectious, replication-incompetent lentiviruses which are then used to transduce target cells. Cotransducing the regulator and response viruses into your target cells, creates a system that allows you to control the expression level of your gene of interest (GOI) by adjusting the concentration of the system's inducer, doxycycline (Dox; a tetracycline derivative) (Figure 1).

B. Elements of the Tet-On Advanced System

In *E. coli*, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon on the Tn10 transposon. TetR blocks transcription of these genes by binding to the tet operator sequences (*tet*O) in the absence of Tc. In the presence of Tc, TetR dissociates from *tet*O and transcription of resistance-mediating genes begins. Together, TetR and *tet*O provide the basis of regulation and induction for all of Takara Bio's Tet Systems.

- **The Regulator Vector: pLVX-Tet-On Advanced**. The pLVX-Tet-On Advanced vector constitutively expresses the tetracycline-controlled transactivator, rtTA-Advanced (Urlinger et al., 2000). This protein consists of a mutant TetR (rTetR) that possesses a "reverse" tetO binding phenotype (i.e., it binds to *tet*O sequences in the *presence of Dox*, rather than in its absence), which is fused to three minimal "F"-type activation domains derived from herpes simplex virus VP16 (Baron et al., 1997, Triezenberg et al., 1988). The gene for the rtTA-Advanced fusion protein is fully synthetic and utilizes human codon preferences to increase the protein's expression levels and its stability in mammalian cells. Cryptic splice sites were removed from the rtTAAdvanced cDNA sequence to increase production of mature mRNA. Expression of rtTA-Advanced protein is typically high enough to be readily detected by western analysis using the **TetR Monoclonal Antibody** (Cat. Nos. 631108 & 631109).
- **The Response Vector: pLVX-Tight-Puro**. The pLVX-Tight-Puro response vector contains P_{Tight}, the inducible promoter that controls expression of your GOI. The P_{Tight} composite promoter sequence was originally developed as the P_{tet-14} promoter in the laboratory of Dr. H. Bujard. It consists of a modified Tet-Responsive Element (TRE_{mod}), made up of seven direct repeats of an altered *tet*O sequence, joined to a modified minimal CMV promoter ($P_{minCMV\Delta}$). PTight also lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction. Upon induction in the presence of Dox, rtTA-Advanced binds to the P_{Tight} promoter on the response vector, activating transcription of the downstream gene (Figure 1).

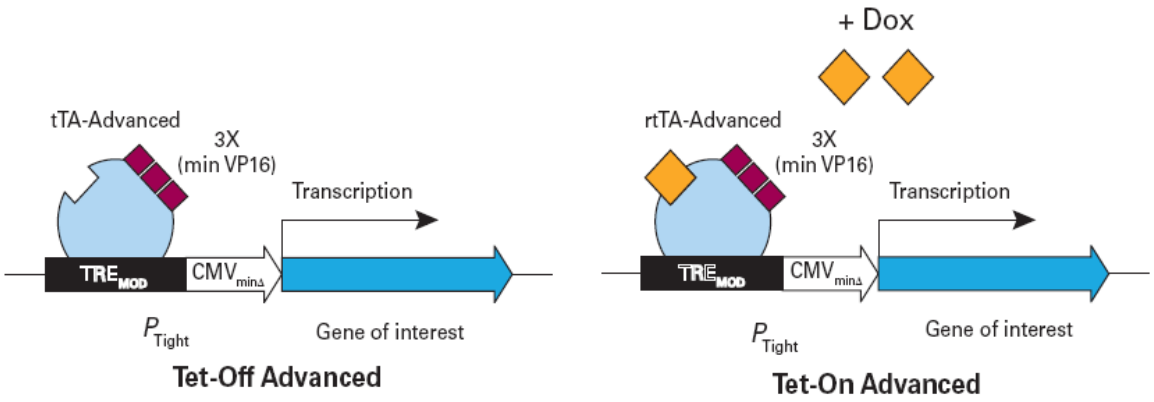

Figure 1. Gene induction in the Tet-Off Advanced and Tet-On Advanced Systems. The Tet-controlled transactivators for these systems (tTA-Advanced and rtTA-Advanced) are fusion proteins derived from either a wild-type or mutant version of the *E. coli* Tet repressor protein (TetR or rTetR, respectively). The modified TetR proteins are each joined to three minimal transcription activation domains which activate transcription when the transactivators are bound to the tetracycline response element (TRE) in P_{Tight}. In Tet-Off Advanced Systems, the inactive basal state is maintained in the presence of Dox, and the system is activated by Dox withdrawal. Tet-On Advanced Systems are active in the presence of Dox. Induction in either system produces high-level transcription of your gene from P_{Tight}.

C. Lenti-X Vectors

The Lenti-X Expression Vectors used in this kit possess a lentiviral packaging signal (Ψ) and the LTRs necessary for vector replication and packaging, as well as elements to improve transgene expression, titer, and overall vector function (see Appendix A). A woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) believed to promote the processing and nuclear export of RNAs, imparts a dual benefit (Zufferey *et al.* 1999). First, it acts within the context of viral genomic RNAs to enhance packaging, which helps to increase the viral titers of supernatants produced from 293T packaging cells. Second, it boosts expression of your cDNA transgene in target cells by facilitating the production of mature mRNA from transcripts initiated by the vector's internal promoter (e.g. P_{CMV} or P_{Tight}). Lenti-X vectors also contain a central polypurine tract (cPPT; also called a "DNA flap") that increases nuclear importation of the viral genome during target cell infection, resulting in improved vector integration and more efficient transduction (Zennou *et al*., 2000). The Rev-responsive element (RRE) is essential for the nuclear export of unspliced viral genomic RNA (Cochrane *et al.*, 1990).

D. 4th-Generation Lentiviral Packaging System

Our **Lenti-X Packaging Single Shots (VSV-G)**, provided with the **Lenti-X Tet-On Advanced Inducible Expression System** (Cat. No. 632162), can generate lentiviral titers that are superior to most other commercially available lentiviral packaging systems. The concerted effects of multiple components in an optimized five-vector plasmid mix, pre-aliquoted and lyophilized with Xfect Transfection Reagent, allow **Lenti-X 293T Cells** (sold separately; Cat. No. 632180) to produce the highest amounts of safe, replication-incompetent lentivirus (see takarabio.com).

E. Benefits of the Tet-Advanced Expression Systems

The Tet-Off Advanced and Tet-On Advanced Systems yield very high maximal expression levels and extremely low basal expression, coupled with high sensitivity and control. Advantages over other inducible mammalian gene expression systems are listed below.

- **Extremely tight regulation**. In the absence of induction, the Tet-On Advanced transactivator has no residual binding to the TRE in P_{Tight}; thus basal expression is often undetectable.

- **Highly specific**. The binding of the Tet-Advanced TetR-derived domains to the *tet*O target sequences of P_{Tight} is highly specific and does not activate off-target cellular genes. This high degree of specificity may be due in part to the prokaryotic nature of these components acting within the context of a large eukaryotic genome lacking similar elements (Harkin *et al.*, 1999).
- **No pleiotropic effects**. Tc and Dox are prokaryotic antibiotics and when used at the concentrations required by the Tet-Advanced Systems, they have no known effects on eukaryotic cells.
- **High inducibility and fast response times**. In properly screened clones, maximal induction of the Tet-On Advanced System is often several thousand-fold and can be detected within 30 minutes after addition of Dox to the culture medium. In contrast, other mammalian systems often exhibit slow induction (up to several days), incomplete induction (compared to repressor-free controls), and low overall induction (often no more than 100-fold). Other systems may also require high, nearly cytotoxic levels of inducer (reviewed by Gossen *et al*., 1993; Yarronton, 1992).
- **Highest absolute expression levels.** Maximal expression levels in the Tet Systems can be higher than expression levels obtained from the CMV promoter or other constitutive promoters. For example, Yin *et al.* (1996) reported that the maximal level of luciferase expression in HeLa Tet-Off cells transiently transfected with pTRE-Luc is 35-fold higher than that obtained with HeLa cells transiently transfected with a plasmid expressing luciferase from the wild-type CMV promoter.
- **Well-characterized effector**. In contrast to effectors used in other systems, such as ecdysone, Tc and Dox are inexpensive, well-characterized, and yield highly reproducible results. Dox binds with high affinity to either transactivator with and is essentially nontoxic at the effective concentrations. It is therefore the preferred effector substance and we recommend using it for all Tet-Advanced Systems. Note that Tet-On Advanced Systems will respond only to Dox, and not to Tc (Gossen & Bujard, 1995).
- **Promoter activation, rather than repression**. To completely shut off transcription, repression-based systems require very high levels of repressor to ensure 100% occupancy of the regulatory sites. The presence of high repressor levels also makes it difficult to achieve rapid, high-level induction (Yao *et al.*, 1998). For a more complete discussion of the advantages of transcription activation versus repression, see Gossen *et al.* (1993).
- **The Tet-On Advanced and Tet-Off Advanced Expression Systems are superlative control systems for transgenic mice.** The Tet System has become the *de facto* method of choice for generating inducible transgenic lines (Gossen & Bujard, 2002). No other inducible system has proved as successful. Indeed, more than 180 mouse lines have been described that express the tTA/rtTA genes under the control of a variety of tissue-specific promoters or express various target genes under control of Tet-inducible promoters. A list of these mouse lines can be found on the TET Systems website (http://www.tetsystems.com/main_transgenic.htm). With its greatly increased sensitivity to Dox, the Tet-On Advanced System brings additional advantages to the development of inducible transgenic mice. This may be particularly helpful when control of gene expression in the brain is required, as the presence of the blood-brain barrier limits the concentration of Dox that can be attained in the brain.

F. Doxycycline

The doxycycline concentrations used with our Tet-On Advanced and Tet-Off Advanced Expression Systems are far below cytotoxic levels for either cell culture or transgenic studies. Of note, Tet-On Systems respond only to Dox, and not to Tc (Gossen & Bujard, 1995).

G. Inducible Luciferase Control Vector

The system is supplied with a luciferase reporter vector, pLVX-Tight-Puro-Luc, that can be used to test the inducibility of the Tet-On Advanced System in target cells, and optimize transduction conditions.

NOTE: Nuclear Localization Signals. Adding a nuclear localization sequence (NLS) to either tTA-Advanced or rtTA-Advanced will alter the protein's function and is not recommended (M. Gossen & H. Bujard, personal communication). An NLS will increase maximum expression but will also elevate background expression due to altered binding affinity to the *tet*O sequences (unpublished observations).

II. List of Components

Store all components at –20°C.

Lenti-X Tet-On Advanced Inducible Expression system (Cat. No. 632162)

Package Contents

- Lenti-X Tet-On Advanced Vector Set
  - 10 µg pLVX-Tet-On Advanced Vector (0.5 µg/µl)
  - 10 µg pLVX-Tight-Puro Vector (0.5 µg/µl)
  - 10 µg pLVX-Tight-Puro-Luc Control Vector (0.5 µg/µl)
- 16 rxns Lenti-X Packaging Single Shots (VSV-G) (Cat. No. 631275)
- Lenti-X GoStix™ Plus (Sample)

Product User Manuals

- Lenti-X Tet-On Advanced Inducible Expression System User Manual (PT5144-1)
- Lenti-X Lentiviral Expression Systems User Manual (PT5135-1)
- pLVX-Tet-On Advanced Vector Information (PT3990-5)
- pLVX-Tight-Puro Vector Information (PT3996-5)
- pLVX-Tight-Puro-Luc Vector Information (PT3997-5)
- Lenti-X Packaging Single Shots (VSV-G) Protocol-at-a-Glance
- Lenti-X GoStix Plus Protocol-at-a-Glance

Visit takarabio.com for a current list of products and cell lines available for the Tet Systems.

III. Additional Materials Required

The following reagents are required but not supplied.

A. Cell Lines for Lentivirus Packaging and Titration

- **Lenti-X 293T Cell Line** (Cat. No. 632180): Getting the most from any lentiviral packaging system requires a host 293T cell line that transfects easily and supports high-level expression of viral proteins. Our Lenti-X 293T Cell Line was clonally selected to meets these requirements, allowing you to produce the highest possible lentiviral titers when combined with Lenti-X Packaging Single Shots (VSV-G), an optimized fourth-generation packaging system, pre-mixed and lyophilized with Xfect Transfection Reagent.
- **HT-1080 cell line:** American Type Culture Collection HT-1080 (ATCC No. CCL-121) [Recommended]. This cell line is easily transduced by recombinant lentiviruses and is frequently used for lentiviral titration. Alternatively, virus stocks can be titrated with the **Lenti-X qRT-PCR Titration Kit** (Cat. No. 632165) or the **Lenti-X p24 Rapid Titer Kit** (Cat. No. 632200). Or, you can save time and quantify your lentiviral supernatant in 10 minutes using **Lenti-X Go Stix Plus** (Cat. Nos. 631280 & 631281) and its smartphone app. Visit takarabio.com/gostixhelp for details.

B. Mammalian Cell Culture Supplies

- **Lenti-X 293T Cell Line growth medium:** 90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and 3.7 g/L sodium bicarbonate (Sigma-Aldrich Co., No. D5796); and 10% tetracycline-free fetal bovine serum. *Add 1 mM sodium pyruvate*.
- **HT-1080 growth medium:** 90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and 3.7 g/L sodium bicarbonate (Sigma-Aldrich Co., No. D5796); and 10% fetal bovine serum. *Add 1 mM sodium pyruvate.*
- **Tetracycline-free fetal bovine serum** (FBS; see important information below). We strongly recommend using **Tet System Approved FBS** (Cat. Nos. 631101 & 631106) for all packaging cell transfections and for culturing target cells when using a Lenti-X Tet-Advanced Inducible Expression System.

NOTE: Tetracycline-Free Fetal Bovine Serum (FBS) for Packaging Cell and Target Cell Culture. Many lots of bovine sera are contaminated with Tc or Tc-derivatives which can affect basal expression or inducibilty in Tet Expression Systems (Figure 2). It is critical that the FBS used for cell culture not interfere with Tet-responsive expression.

- Lenti-X Packaging Single Shots (VSV-G) utilize Tet-Off transactivation to drive highlevel expression of specific viral packaging proteins. The presence of Tc contaminants will reduce expression of these components and will negatively affect viral titers. *Therefore, lentiviral packaging in 293T cells must be performed using Tc-free FBS.*
- Tc-contaminants will diminish the performance of the Tet-On Advanced System in target cells by elevating basal expression and reducing fold-induction.
- These problems can be eliminated by using a **Tet System Approved FBS** (Cat. Nos. 631101, 631105, 631106 & 631107) from Takara Bio. These sera have been functionally tested in our Tet Systems and found to be free of contaminating Tc activity.

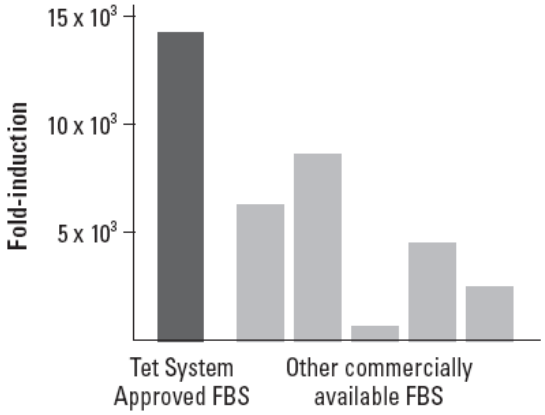

Figure 2. Tetracycline activity in bovine sera. The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different lots of FBS. Average uninduced expression level = 0.21 RLU (n=21, S.D.=0.07); maximum expression levels varied from 123 to 3,176 RLU.

- Cell growth medium and supplies specific for your target cells
- Sodium pyruvate solution, 100 mM, sterile filtered (Sigma-Aldrich Co., No. S8636), for supplementing cell culture media
- Penicillin/streptomycin solution of 10,000 units/ml penicillin G sodium and 10,000 µg/ml streptomycin sulfate (100X; Sigma-Aldrich Co., No. P0781)
- Trypsin-EDTA (Trypsin; Sigma-Aldrich Co., No. T3924)
- Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich Co., No. D8662)
- L-glutamine solution, 200 mM, sterile filtered (Sigma-Aldrich Co., No. G7513) [Optional]
- Cell Freezing Medium, with or without DMSO (Sigma-Aldrich Co., No. C6164 or C6039)
- Tissue culture plates (100 mm) for packaging cell transfections; other plates and flasks as required
- Polystyrene culture tubes, 12 x 75 mm (e.g., BD Falcon No. 352054), for packaging cell transfections
- Sterile microfuge tubes (1.5 ml) for use in titrating virus stocks; and cryovials for freezing virus stocks
- Crystal violet (Sigma-Aldrich Co., No. C3886), 1% solution prepared in ethanol, for staining colonies of transduced cells in the virus titration protocol (Section VIII.B)
- Cloning cylinders (PGC Scientific, No. CORN31666, -31668, or -316610), for isolating clones of stable transductants

C. Antibiotics for Selecting Transduced Cells

Prior to using these antibiotics for selecting transduced cells, determine the optimal selection concentration for each cell type as shown in Appendix B.

- G418 (Cat. No. 631307) is used for selecting cells transduced with the LVX-Tet-On Advanced virus and for titrating the corresponding lentivirus stock.
    - Recommended working concentration range: 50–800 µg/ml
    - Maintenance of stable cell lines: 100 µg/ml
    - Selection (e.g., HEK 293, HeLa or CHO cells): 400–500 µg/ml

- Puromycin (Cat. Nos. 631305 & 631306) is used for selecting cells transduced with the LVX-Tight-Puro-GOI or LVX-Tight-Puro-Luc viruses, and for titrating the corresponding lentivirus stocks.
    - Recommended working concentration range: 0.25–2 µg/ml
    - Maintenance of stable cell lines: 0.25 µg/ml
    - Selection (e.g., HEK 293, HeLa or CHO cells): 0.5–10 µg/ml

D. High-Titer Packaging System

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

E. Lentivirus Titration Kits

For accurate and consistent transductions, we highly recommend titrating your lentiviral stocks. The **Lenti-X qRT-PCR Titration Kit** (Cat. No. 631235) provides a fast and simple qRT-PCR-based titration method. The kit determines viral RNA genome content using qRT-PCR and SYBR® technologies, and titrates virus stocks in ~4 hr. The **Lenti-X p24 Rapid Titer Kit** (Cat. No. 632200) uses ELISA to specifically measure the amount of p24 capsid protein present in your viral supernatant, and then correlates the level of p24 directly to virus titer. Alternatively, you can use **Lenti-X GoStix Plus** (Cat. Nos. 631280 & 631281) with its related smartphone app to quantify your viral supernatant in just 10 minutes. The GoStix detect lentiviral p24 in only 20 µl, and can be used to determine whether virus production is within a usable range or for selecting the best time to harvest your virus. Visit takarabio.com/gostixhelp for details.

F. Lentivirus Purification

Virus purification enables you to remove cellular contaminants that could otherwise adversely affect your transduction experiments. **Lenti-X Maxi Purification Kits** (Cat. Nos. 631233 & 631234) produce outstanding yields of highly purified virus from crude supernatants. The gravity column-based protocol is fast, simple, and effective, and produces virus that is fully intact and fully functional.

G. Lentivirus Concentration

Use **Lenti-X Concentrator** (Cat. Nos. 631231 & 631232) to increase your available titer up to 100-fold without ultracentrifugation. Concentrated virus allows you to infect target cells at higher MOIs without making more virus or transfecting additional packaging cells.

H. Polybrene for Viral Transductions

Polybrene (hexadimethrine bromide; Sigma-Aldrich, No. H9268) is needed for the standard infection/transduction protocol to facilitate lentiviral gene transfer. Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimal polybrene concentration for your target cells (maximal infectivity with minimal toxicity) should be determined empirically by testing concentrations in the range of 2–12 µg/ml. For cells that are especially sensitive to polybrene, consider using **RetroNectin® Reagent**.

I. Retronectin Reagent for Enhanced Viral Transductions

RetroNectin Reagent (Cat. Nos. TAK 100A, TAK 100B) is a recombinant fragment of fibronectin (CH-296) that can be used to greatly improve retroviral and lentiviral transduction efficiencies. RetroNectin is coated onto tissue culture plates to provide a substratum that binds both viruses and cells. The colocalization of virus and cells on this novel substratum improves cell-virus contact and enhances transduction. This is especially useful for cells grown in suspension (e.g. lymphocytes and lymphocyte cell lines) and other cells that are difficult to transduce, such as hematopoietic stem cells; or for cells that may be especially sensitive to polybrene. Visit takarabio.com for more information.

J. Doxycycline

Doxycycline (Cat. No. 631311) is required for inducing gene expression in cells transduced with the complete Lenti-X Tet-On Advanced System. Make a solution of 1–2 mg/ml in H_2O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.

K. Xfect Transfection Reagent

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

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

L. Luciferase Assay

A method for assaying luciferase expression is required for use with the LVX-Tight-Puro-Luc Control Vector. Use any standard luciferase assay system for detecting firefly luciferase. A luminometer is also required.

IV. Safety Guidelines for Working with Lentiviruses

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

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

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

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

- *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, Fifth Edition (February 2007) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH. Available on the web at http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm.

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

Summary of Biosafety Level 2:

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

V. Protocol Overview

PLEASE READ THESE PROTOCOLS IN THEIR ENTIRETY BEFORE STARTING. Successful results depend on understanding and performing the following steps correctly.

A. General Cell Culture and Lentivirus Information

The protocols in this User Manual provide only general guidelines for lentivirus use and mammalian cell culture techniques. Perform all steps involving cell culture using sterile technique in a Biosafety Level 2 tissue culture hood that has been approved for use with lentiviruses. For users requiring more information on lentiviruses, retroviruses, and mammalian cell culture, we recommend the following general references:

- *Retroviruses*, ed. by J. M. Coffin, S. H. Hughes & H. E. Varmus (1997, Cold Spring Harbor Laboratory Press, NY)
- *Culture of Animal Cells*, 5th Edition, by R. I. Freshney (2005, Wiley-Liss, NY)

B. Establishing Your Inducible System

The general strategy for establishing an inducible Lenti-X Tet-On Advanced System for expressing a GOI is shown in Figure 3, in which target cells are cotransduced with lentiviruses derived from the pLVX-Tet-On Advanced regulator vector and the pLVX-Tight-Pur-GOI response vector.

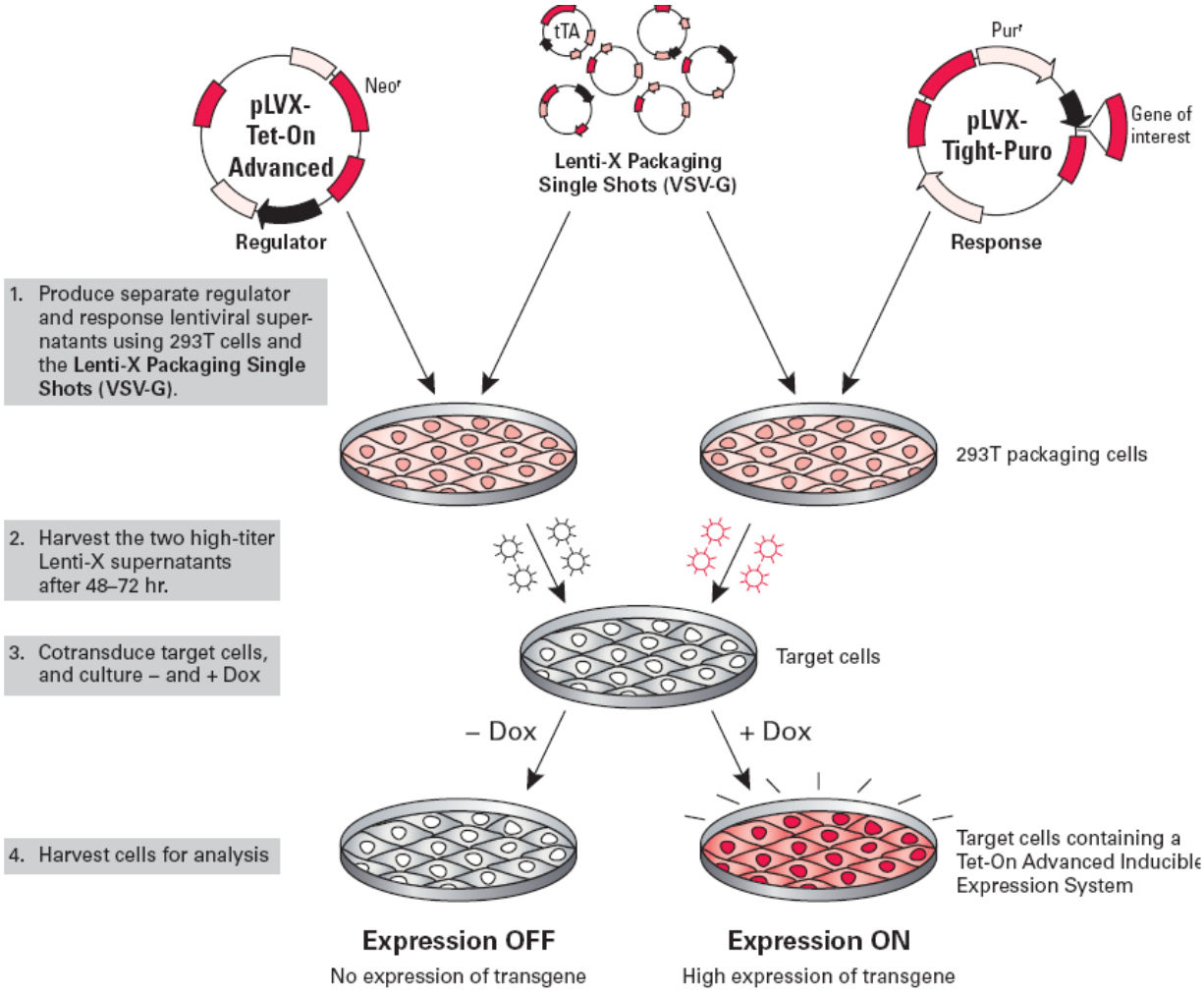

Figure 3. Establishing an inducible expression system in target cells with Lenti-X Tet-On Advanced. The Lenti-X Packaging Single Shots (VSV-G), an optimized packaging premix lyophilized with Xfect Transfection Reagent, and 293T cells are used to generate high-titer lentiviral supernatants from the pLVX-Tet-On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

VI. Plasmid Vector Manipulations

A. General Molecular Biology Techniques

These protocols contain only general information for propagating plasmid vectors and for preparing your customized pLVX-Tight-Puro expression construct. For users requiring more information on standard molecular biology practices and cloning techniques, we recommend the following laboratory references:

- *Current Protocols in Molecular Biology* ed. by F. M. Ausubel et al. (1995, John Wiley & Sons, NY).
- *Molecular Cloning: A Laboratory Manual* ed. by J. Sambrook et al. (2001, Cold Spring Harbor Laboratory Press, NY).

B. Plasmid Vector Propagation & Construction of pLVX-Tight-Puro-GOI

1. To ensure that you have a renewable source of plasmid DNA, transform each of the plasmid vectors provided in this kit into a suitable E. coli host strain [e.g., **Stellar™ Electrocompetent Cells** (Cat. No. 636765)]. See the enclosed Vector Information Packets for further DNA propagation details.

2. For pLVX-Tight-Puro cloning, prepare purified plasmid DNA using a suitable **NucleoBond or NucleoSpin Kit** (see takarabio.com)

3. Using standard cloning techniques, insert your cDNA into the multiple cloning site (MCS) of pLVX-Tight-Puro. You can also use an **In-Fusion® PCR Cloning Kit** (Cat. Nos. 639616-639624) which allows PCR products to be easily cloned into any vector.

   NOTE: The cDNA or gene fragment must contain an ATG initiation codon. In some cases, addition of a Kozak consensus ribosome binding site (Kozak, 1987) may improve expression levels, but is often unnecessary. **The fragment or cDNA should not contain a polyadenylation signal.** The inclusion of such sequences between retroviral LTRs can cause premature polyadenylation during virus transcription, which interferes with the production of vector-containing virions (Coffin & Varmus, 1996).

4. Perform a midi- or maxi-scale plasmid DNA preparation for each plasmid that will be transfected into the packaging cells. For guaranteed transfection-grade plasmid DNA, we recommend using NucleoBond Xtra Midi Plus or Maxi Plus Kits (Figure 4; Cat. Nos. 740412.10 and 740416.10). For rapid production of endotoxin-free, transfection-grade plasmid DNA, use NucleoBond Xtra Midi EF Plus or Maxi EF Plus Kits (Cat. Nos. 740422.10 and 740426.10).

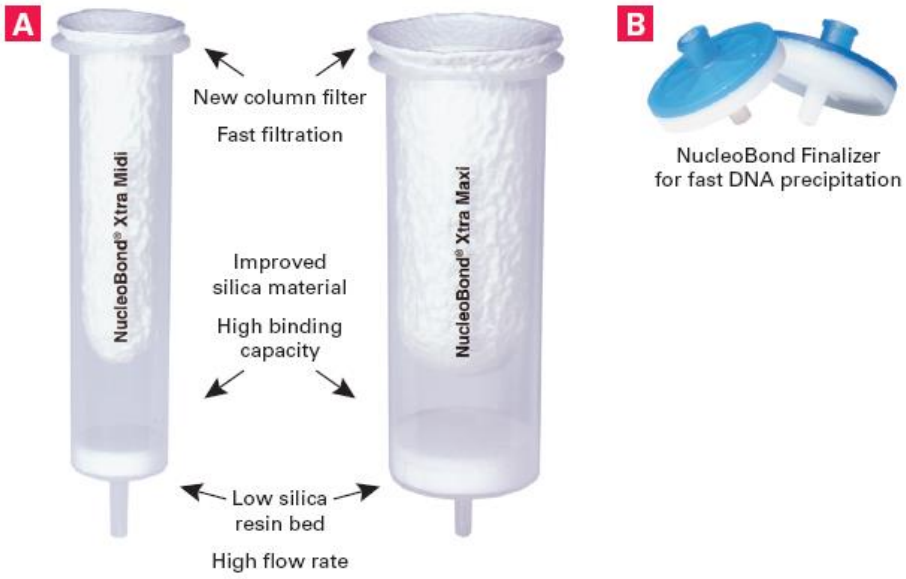

Figure 4. Advanced features of NucleoBond Xtra Maxi and Midi Columns and NucleoBond Finalizer. NucleoBond Xtra columns contain a high-flow column filter that minimizes clogging and clears debris from cell lysates during column loading. An improved silica resin provides high DNA-binding capacity, and a wide column diameter keeps the resin bed low for maximum flow rates **(Panel A)**. The NucleoBond Finalizer system speeds preparation and increases purity by capturing precipitated DNA on a syringe filter where it can be easily washed and eluted (**Panel B**).

VII. Producing Lentivirus from the Lenti-X Vectors

A. Protocol: Using Lenti-X Packaging Single Shots (VSV-G) to Produce Lentiviral Supernatants

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

VIII. Lentivirus Titration

A. Summary

Instant quantitative titer test: You can quantify your lentivirus stock in 10 minutes with our **Lenti-X GoStix Plus** (Cat. Nos. 631280 & 631281) and the related smartphone app. The GoStix detect lentiviral p24 in only 20 µl, and can be used to determine whether virus production is within a usable range or for selecting the best time to harvest your virus. A 3-prep sample is supplied for free with many of our Lenti-X systems. Visit takarabio.com/gostixhelp for details.

Standard quantitative titer test: To transduce using a known multiplicity of infection (MOI), it is necessary to titrate your lentiviral stocks. We recommend the **Lenti-X qRT-PCR Titration Kit** (Cat. No. 632165) or **Lenti-X p24 Rapid Titer Kit** (Cat. No. 632220) for very rapid quantitative titrations of virus stocks (~4 hr), or a traditional method that relies on infection. The latter consists of infecting cells with serial dilutions of the stock, selecting for stable transductants with antibiotic and counting the resulting cell colonies (Section B). Freshly harvested virus can be titered immediately, or frozen in aliquots at –80°C and then titrated. Note that each freeze-thaw cycle can reduce the functional titers of infectious virus by up to 2–4 fold. Absolute titers will depend heavily on the cell type used for titration, and there may be significant differences between the titer values determined in cells typically used for lentiviral titration (i.e. HT-1080) and the number of target cells transduced by the

titered virus. However, titrations serve to determine the relative virus content of different viral stocks prepared from different vectors. Determining the viral titer is necessary for the following reasons:

- Confirming that viral stocks are viable.
- Determining the proper transduction conditions for your particular cell type by adjusting the MOI for the desired transduction efficiency. MOI = No. of infectious virus particles per target cell
- Determining the precise regulator-to-response ratio that produces the optimum induction profile.
- Determining the maximum number of target cells that can be transduced by a given virus volume.

B. Protocol: Determining Viral Titer by Colony Formation

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*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 (Appendix A).
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. Testing the Inducibility of Tet-On Advanced in Your Target Cells

A. Summary

Performing pilot experiments using the LVX-Tet-On Advanced lentivirus, the LVX-Tight-Puro-Luc control lentivirus, and your target cells provides a means of rapidly testing the infectivity of your lentiviral stocks and the induction capabilities of the Lenti-X Tet-On Advanced System in these cells.

Other points to consider:

- The experiment outlined below also provides a "hands-on" introduction to the Tet System and verifies that your culture system, induction conditions, and reagents are working properly.
- In order to characterize your system more rapidly, you can perform the pilot experiment by using virus ratios based on volumes of virus stock rather than on titer values.
- If your target cells are especially rare or difficult to prepare, you may prefer to test the system using a readily available cell line that is compatible with lentiviral transduction.
- Once the stocks are titered, we suggest that you perform a Dox dose-response experiment, using the best coinfection ratio of the two lentiviruses, to determine the maximum fold-induction conditions.
- You should also test the induction properties of your LVX-Tight-Puro-GOI lentivirus, either in a readily available cell line or in the target cell of your choice.

B. Protocol: Testing Luciferase Induction in Target Cells

1. Using 6-well plates, seed 0.5–2 x 10^5 target cells/well in 2 ml medium. Use a sufficient number of plates to infect cells at the following virus ratios, each with and without Dox (500 ng/ml), and testing all conditions in duplicate (i.e. a minimum of 4 wells per virus ratio).

   pLVX-Tet-On Advanced : pLVX-Tight-Puro-Luc

   a. 0 : 0 (no virus)

   b. 1 : 1

   c. 1 : 4

   d. 4 : 1

2. Prepare two volumes of culture medium (allow 2 ml/well), each containing one of the following sets of additives:

   - 6 µg/ml polybrene and 750 ng/ml Dox (for +Dox treatments)
   - 6 µg/ml polybrene only (for –Dox treatments)

3. Exchange the cell culture medium in the plates with 2 ml of the appropriate medium prepared in Step 2. Return the plates to the incubator.

   NOTE: In this case, polybrene will be diluted to a final concentration 4 µg/ml after adding 1 ml of virus stock (Step 6). Adjust the polybrene concentration accordingly if a different concentration is known to produce optimal results.

4. Thaw aliquots of virus stocks or use filtered virus stocks prepared from 293T packaging cells transfected with pLVX-Tet-On Advanced and pLVX-Tight-Puro-Luc (Section VII). If the stocks have been titered, adjust them to contain equal amounts of infectious virus. If the stocks are untitered you may also need to dilute the stocks for optimum results (see the Information Box on page 15). *Be sure to use culture medium containing Tet System Approved FBS (see Section III.B).*
5. In 15-ml conical tubes, prepare 5 ml of combined virus stock for each virus ratio below. Mix gently, do not vortex:
    a. 1:1 = combine 2.5 ml of each virus stock
    b. 1:4 = combine 1.0 ml of LVX-Tet-On Advanced with 4.0 ml LVX-Tight-Puro-Luc
    c. 4:1 = combine 4.0 ml of LVX-Tet-On Advanced with 1.0 ml LVX-Tight-Puro-Luc
6. Add 1 ml of the combined virus stocks to the appropriate wells and infect the cells for 8–24 hr.

    NOTE: Centrifuging the plates 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.
7. After the infection, remove and discard the virus-containing culture medium. Feed the cells using fresh medium, with or without Dox as appropriate, and incubate the infected cells for 48–72 hr.
8. Harvest the cells and assay for luciferase activity. Calculate the fold-induction obtained for each condition (i.e., +Dox RLU/–Dox RLU).

NOTE: Using Untitered Lenti-X Packaging Single Shots (VSV-G) Stocks and Supernatants
Lenti-X Packaging Single Shots (VSV-G) are capable of producing very high titers of virus. Using large excesses of virus can be detrimental to target cell performance. If you have not determined the titer of your virus stock, perform transduction experiments using several different fold-dilutions to test a range of MOIs. At Takara Bio, our scientists often transduce Attention an entire 100 mm dish of target cells using only 10–100 µl of unconcentrated supernatant.

X. Transducing Target Cells with the Tet-On Advanced Lentiviruses

A. Summary

- **Simultaneous Cotransduction:** To establish the complete Tet-On Advanced System, target cells must be cotranduced with the LVX-Tet-On Advanced and LVX-Tight-Puro-GOI lentiviruses. Using high titers of each virus ensures that the highest proportion of cells will contain both vectors. Depending on your application, transduced cells can be either treated immediately with Dox to induce expression of your GOI and then harvested for analysis, or the cells may be selected with G418 and puromycin to isolate doubly transduced clones or to enrich the population for doubly transduced cells (see Appendix B).
- **Virus Ratio Optimization:** It is possible to optimize the induction characteristics of your system by infecting target cells with different ratios of the regulator and response lentiviruses. Using a ratio that favors the response vector (LVX-Tight-Puro-GOI) over the regulator vector (LVX-Tet-On Advanced) 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 (Section IX).

- **Sequential Transduction:** If you are working with a clonable cell line, we recommended that you perform sequential transductions with the lentiviruses so that you can obtain clones that have optimal inducibility characteristics. This method produces clones that have the highest expression levels, lowest backgrounds, and highest fold-induction. Briefly, cells are first transduced with only the LVX-Tet-On Advanced lentivirus, followed by selection with G418. Resistant clones are then screened for expression of Tet-On Advanced and tested for inducibility. A favorable, Tet-On Advanced clone is then transduced with the LVX-Tight-Puro-GOI lentivirus. Doubly-transduced cells are selected using puromycin and the resulting puromycin-resistant clones are then screened for GOI inducibility. *For more information on sequential transduction, clone selection, and clone screening, consult our Retro-X™ Tet-On Advanced Inducible Expression System User Manual (PT3958-1) available at takarabio.com.*

B. Protocol: Cotransducing Target Cells with the Lenti-X Tet-On Advanced Lentiviruses

1. Plate target cells in complete growth medium 12–18 hr before transduction.
2. Thaw aliquots of your LVX-Tet-On Advanced and LVX-Tight-Puro-GOI lentiviral stocks, or use filtered virus stocks freshly prepared from packaging cells (Section VII). Mix gently, but do not vortex.
3. Add polybrene to the cell cultures to obtain the desired final concentration during the transduction step (e.g., 4 µg/ml). Add the predetermined optimal concentration of Dox to the appropriate cultures.
4. Combine the LVX-Tet-On Advanced and LVX-Tight-Puro-GOI lentiviral stocks in the desired ratio and MOI, as determined from pilot studies. 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.
7. Continue to incubate the cells for 24–48 hr to allow the expressed protein to accumulate.
8. Harvest the cells for analysis.

XI. References

You can access further information on Tet Systems products on our website: takarabio.com. Our 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 bibliograpy 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. Vector Information

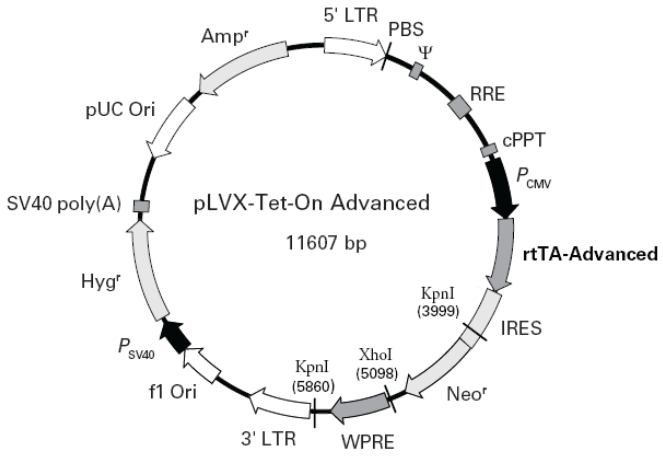

Figure 5. Map of the pLVX-Tet-On Advanced Vector. For a complete vector description, refer to the enclosed pLVX-Tet-On Advanced Vector Information Packet (PT3990-5).

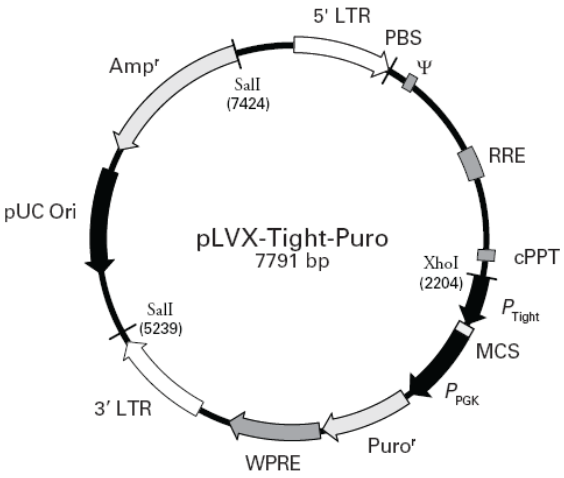

Figure 6. Map of the pLVX-Tight-Puro Vector. For a complete vector description and MCS diagram, refer to the enclosed Vector Information Packet (PT3996-5).

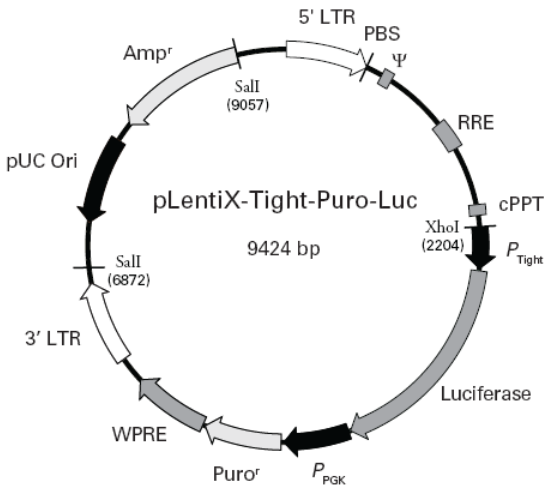

Figure 7. Map of the pLVX-Tight-Puro-Luc Vector. For a complete vector description, refer to the Vector Information Packet (PT3997-5) available at takarabio.com

Appendix B. Additional Protocols

A. Protocol: Titrating Antibiotics for Selecting Single- and Double-Stable Cell Lines

Prior to using the antibiotics G418 and/or puromycin to select either singly- or doubly-transduced cells, it is necessary to titrate each selection agent to determine the optimal concentration for your target cell line. With HeLa cells, for example, we have found 400 µg/ml G418 and 1.0 µg/ml puromycin to be optimal.

- For selecting stable transformants with G418, use the lowest concentration that results in massive cell death in ~5 days and kills all the cells within two weeks.
- Puromycin selection occurs more rapidly; use a concentration that will kill all cells within 3–4 days.
- IMPORTANT: Lot-to-lot variations in potency exist for all selection drugs, so each new lot of antibiotic should be titrated.

1. For each antibiotic to be tested, plate 2 x 10^5 cells in each well of a 6-well plate containing 3 ml of the appropriate complete medium plus increasing concentrations of G418 (0, 50, 100, 200, 400, and 800 µg/ml). For puromycin, add the drug at 0, 1.0, 2.5, 5.0, 7.5, and 10.0 µg/ml.

2. For G418, incubate the cells for 5–10 days or until all cells are dead. Examine the dishes for viable cells every two days. Replace the selective medium every four days (or more often if necessary), until the optimal concentration is determined.

3. For puromycin, incubate the cells 4–7 days. Replace medium after 2 days to remove dead cells.

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