

Takara Bio USA, Inc.

Retro-X™ Tet-Off® Advanced Inducible Expression System User Manual

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I. Introduction

A. Summary

The Retro-X Tet-Off Advanced Inducible Expression System provides the tightly regulated, high-level gene expression system originally described by Gossen & Bujard (1992), including the system improvements described by Urlinger, *et al.* (2000; *Clontechniques*, January 2007), in a highly efficient retroviral vector format. The vectors provided produce infectious, replication-incompetent retroviruses when transfected into an appropriate MMLV packaging cell line, such as GP2-293 (supplied with Retro-X Universal Packaging System, Cat. No. 631530). The retroviruses are then used to deliver the system into your target cells. Once established in your cell line, either through sequential infection or by coinfection, the system 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).

In the Tet-Off Advanced System, the basal "off" state is maintained by the presence of low concentrations of Dox in the culture medium, while induction is achieved by the removal of Dox (Figure 1). Expression levels can be fine-tuned by titrating the Dox concentration. The maximum expression levels obtained with the Tet-Off Advanced System are very high, and often exceed those obtained by constitutive mammalian promoters, such as CMV (Yin, *et al.*, 1996). Basal (uninduced) expression levels are extremely low and often undetectable. Unlike in other inducible mammalian expression systems, gene regulation in all of our Tet Systems is highly specific, so results are not complicated by pleiotropic effects or nonspecific induction.

B. Elements of Tet-Off Advanced Systems

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 tetracycline (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 our Tet Systems.

1. The Retro-X Tet-Off Advanced Transactivator: tTA-Advanced

The pRetroX-Tet-Off Advanced vector constitutively expresses the tetracycline-controlled transactivator tTA-Advanced (Urlinger, *et al.*, 2000). This protein consists of TetR fused to three minimal "F"-type transcription activation domains derived from herpes simplex virus VP16 (Baron *et al.*, 1997, Triezenberg *et al.*, 1988). The gene for tTA-Advanced is fully synthetic and utilizes human codon preferences to increase the protein's expression levels and stability in mammalian cells. Cryptic splice sites were removed from its cDNA sequence to increase production of mature mRNAs. Expression of tTA-Advanced is typically high enough to be easily detectable by Western analysis using the **TetR Monoclonal Antibody** (Cat. No. 631108). Like TetR, tTA-Advanced is unable to bind *tet*O sequences in the presence of Dox.

2. The Response Vector: pRetroX-Tight-Pur

The pRetroX-Tight-Pur vector contains P_{Tight} , the inducible response promoter that controls expression of your GOI (*Clontechniques*, April 2003). 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 ($P_{minCMV\Delta}$). P_{Tight} also lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction. Upon induction (i.e., in the absence of Dox), tTA-Advanced binds to the P_{Tight} promoter on the response vector, activating transcription of the downstream gene (Figure 1).

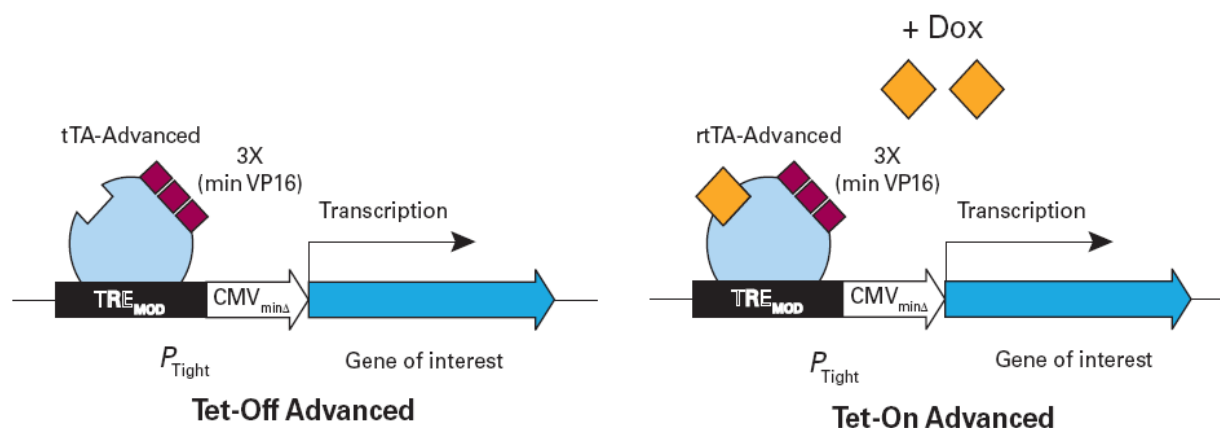


Figure 1. Gene induction in the Tet-Off Advanced and Tet-On Advanced Systems. The Tet-controlled transactivators for these systems (tTA-Advanced and rtTA-Advanced) are fusion proteins derived from a wild-type or mutant Tet repressor (TetR), respectively. Each TetR DNA-binding domain is joined to three minimal transcription activation domains which activate transcription when bound to the tetracycline response element (TRE) in P_{Tight} . For Tet-Off Advanced, removal of doxycycline permits tight binding and induces high-level transcription. In exact contrast, the Tet-On Advanced System is activated by doxycycline.

C. Retro-X Q Vectors

The Retro-X Q retroviral vectors used to establish this system have been engineered to:

- Produce the highest titers from packaging cells
- Produce the highest transgene expression in target cells (*Clontechniques*, April 2007; Julius, M.A. *et al.*, 2000).

Retro-X Q vectors have self-inactivating LTRs that eliminate promoter interference. In LTR self-inactivation, the mechanism of viral integration is used to introduce a deletion into the CMV/MSV promoter in the 5' LTR. As a result of this deletion, the strong CMV/MSV promoter that drives high expression of the replicative viral genome in packaging cells, is inactivated in your target cells after infection. Thus, the only vector-derived promoters that remain active in your target cells are those driving expression of either the Tet-On/Off Advanced regulator (P_{CMV}), or your gene of interest (P_{Tight}). Retro-X Q bicistronic expression vectors (i.e., pRetroX-Tet-On/Off Advanced) use an internal ribosome expression sequence (IRES) to coexpress tTA-Advanced and the Neo^r marker as a single transcription unit, ensuring that a high frequency of G418 resistant clones express the Tet-Off Advanced transactivator.

D. Benefits of the Tet-Advanced Expression Systems

The improved Tet-Off Advanced and Tet-On Advanced Systems yield robust induction, extremely low basal expression, and facilitate host cell line development. These systems have numerous advantages over other inducible mammalian gene expression systems: (See Appendix A, the Vector Information Packets provided, and takarabio.com for maps and detailed information on these and other Tet System Vectors.)

- **Extremely tight regulation.** In the absence of induction, the Tet-Off Advanced transactivator has no residual binding to the TRE in P_{Tight} ; thus basal expression is virtually nonexistent and generally undetectable.
- **No pleiotropic effects.** When the Advanced transactivators are introduced into mammalian cells, their prokaryotic DNA-binding domains bind very specifically to the tetO target sequences in P_{Tight} . The likely reason for this is that eukaryotic genomes lack these prokaryotic regulatory DNA sequences (Harkin, *et al.*, 1999).
- **High inducibility and fast response times.** Maximal induction is often several thousand-fold and can be detected within 30 minutes after complete withdrawal of Dox from 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).
- **High 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 inducer.** In contrast to inducers used in other systems, such as ecdysone, Tc and Dox are inexpensive, well-characterized, and yield highly reproducible results. We recommend using Dox due to its longer half-life and increased potency, relative to Tc.
- **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 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 80 mouse lines have been described expressing the tTA/rtTA genes under the control of a variety of tissue-specific promoters, and approximately 100 mouse lines have been described expressing 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 researchers wishing to develop inducible transgenic mice; particularly when control of gene expression in the brain is required, because the presence of the blood-brain barrier limits the concentration of Dox present in the brain.

E. 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).

F. Inducible Luciferase Control Vector

The system is supplied with a luciferase reporter vector, pRetroX-Tight-Pur-Luc, that can be used to screen the Tet-Off Advanced clones.

G. Bidirectional Tet Expression Vectors

Bidirectional Tet Vectors are specially designed response vectors that allow coregulated expression of two genes from a single TRE (Baron, U., *et al.*, 1995). These are ideal TRE response vectors if a functional assay does not exist for your GOI since expression can be monitored indirectly through detection of the coregulated reporter gene. Note that the following are not retroviral vectors and must be transfected into a single-stable Tet-Off or Tet-On Advanced cell line to be effective. Visit takarabio.com for a complete selection of inducible vectors and expression systems.

- The pTRE-Tight-BI Vector (Cat. No. 631068) lacks a reporter, while the pTRE-Tight-BI-AcGFP1 (Cat. No. 631066), pTRE-Tight-BI-DsRed2 (Cat. No. 631064), pTRE-Tight-BI-DsRed-Express (Cat. No. 631065), and pTRE-Tight-BI-ZsGreen1 (Cat. No. 631067) Vectors express a Living Colors® fluorescent protein.
- Bidirectional TRE vectors are also available with β -galactosidase (pBI-G Tet, Cat. No. 631004) or luciferase (pBI-L Tet, Cat. No. 631005).

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 *tetO* sequences (unpublished observations).

II. List of Components

Retro-X Tet-Off Advanced Inducible Expression System (Cat. No. 632105)

Package 1 (Store all plasmids and Fetal Bovine Serum at –20°C.)

pRetroX-Tet-Off Advanced Vector (0.5 µg/µl)	10 µg
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pRetroX-Tight-Pur Vector (0.5 µg/µl)	10 µg
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pRetroX-Tight-Pur-Luc Control Vector (0.5 µg/µl)	10 µg
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Tet System Approved FBS	50 ml
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Package 2 (Store HBS at –20°C, store all other components at 4°C or –20°C.)

CalPhos™ Mammalian Transfection Kit (also available as Cat. No. 631312)	1 ea
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Product Documents

Documents for our products are available for download at takarabio.com/manuals

The following additional documents apply to this product:

- pRetroX-Tet-Off Advanced Vector Information (PT3969-5)
- pRetroX-Tight-Pur Vector Information (PT3960-5)
- CalPhos Mammalian Transfection Kit User Manual (PT3025-1)
- CalPhos Mammalian Transfection Kit Protocol-at-a-Glance (PT3025-2)

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

III. Additional Materials Required

The following reagents and materials are required but not supplied. The named products have been validated to work with this protocol.

A. Retroviral Packaging System

We highly recommend using our Retro-X Universal Packaging System (Cat. No. 631530). This system provides consistently high titers, and the ability to tailor the host range of the packaged virus to suit your needs.

B. Mammalian Cell Culture Supplies

- Culture medium and additives specific for your cell line(s)
- Cell culture plates and dishes (e.g. 6-well plates, 100 mm dishes, etc.)
- Cloning cylinders or discs for isolating colonies of adherent cell lines (PGC Scientific, Cat. No. 62-6150-40, -45 or Cat. No. 62-6151-12, -16)
- Cell Freezing Medium, with or without DMSO (Sigma, Cat. No. C6164 or Cat. No. C6039) for freezing your single-stable Retro-X Tet-Off Advanced clone(s) and your double-stable Retro-X Tet-Off Advanced/GOI clone(s).
- Tetracycline-Free Fetal Bovine Serum (FBS)

NOTE: Tetracycline-Free Fetal Bovine Serum (FBS). Many lots of bovine sera are contaminated with Tc or Tc-derivatives which can affect basal expression or inducibility in Tet Expression Systems (Figure 2). It is critical that the FBS used for cell culture not interfere with Tet-responsive expression. This problem can be eliminated by using a **Tet System Approved FBS** (Cat. Nos. 631101 & 631106) from Takara Bio. These sera have been functionally tested in our Tet Systems and found to be free of contaminating Tc activity.

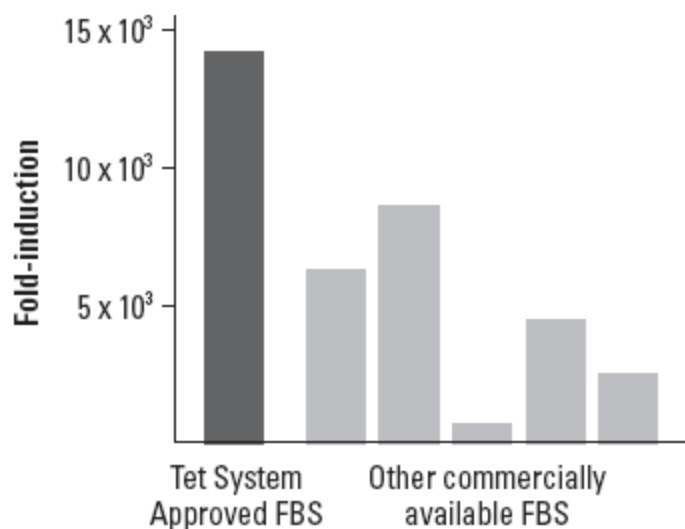


Figure 2. Tetracycline activity in bovine sera. The CHO-AA8-Luc Tet-Off Control Cell Line was grown in media prepared with different lots of FBS. Average uninduced expression level = 0.21 RLU (n=21, S.D.=0.07); maximum expression levels varied from 123 to 3,176 RLU.

C. Antibiotics for Clonal Selection

Prior to using these antibiotics, determine the optimal selection concentration for each one as described in Section IX.

- G418 (Cat. No. 631307) is required for selection of your Retro-X Tet-Off Advanced cell line, and is available in powdered form from Takara Bio.
 - 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 required for selection of your double-stable cell line expressing the tTA Advanced and your gene of interest.
 - 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. Polybrene

Polybrene (hexadimethrine bromide; Sigma-Aldrich, Cat. No. H9268) is needed to facilitate the infection of target cells with retrovirus. The concentration of polybrene that is optimal for your target cells may be determined empirically by testing a concentration range of 2–12 µg/ml.

E. Doxycycline

Doxycycline (Cat. No. 631311) is required to maintain your Retro-X Tet-Off Advanced System in the “off” state, thus preventing your GOI from being expressed. Make a solution of 1–2 mg/ml in H₂O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.

F. Luciferase Assay

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

G. Retroviral Titration [Recommended]

To achieve consistent infections, we highly recommend titrating your retroviral stocks. The **Retro-X qRT-PCR Titration Kit** (Cat. No. 631451) provides a fast and simple method for titrating retroviral stocks (*Clontechiques*, April 2007). The kit employs a quick RNA purification step and determines viral RNA genome content using qRT-PCR and TB Green® technologies, and titers virus stock in ~4 hr.

H. Primers for Sequencing your GOI Insert in pRetroX-Tight-Pur

Following the creation of your pRetroX-Tight-Pur-GOI construct, the insertion junctions should be confirmed by sequencing. Specific primers for pRetroX-Tight-Pur should have the following sequences:

- Forward primer: 5' –ATCTGAGGCCCTTTTCGTCTTCACT–3' (located at 1579–1602)
- Reverse primer: 5' –TGTGTGCGAGGCCAGAGGCCACTT–3' (located at 2073–2050)

IV. Protocol Overview

An overview for creating a double-stable Retro-X Tet-Off Advanced cell line that contains integrated copies of the regulatory vector and the pRetroX-Tight-Pur-GOI response vector containing the gene of interest is shown in Figure 3. For more detailed flow charts for each of the sequential infection procedures, see Figure 5 (Section XI) and Figure 6 (Section XII). When starting with a premade Tet-On- or Tet-Off Advanced Inducible Cell Line from Takara Bio, only the second infection using a RetroX-Tight-Pur-GOI retrovirus is required.

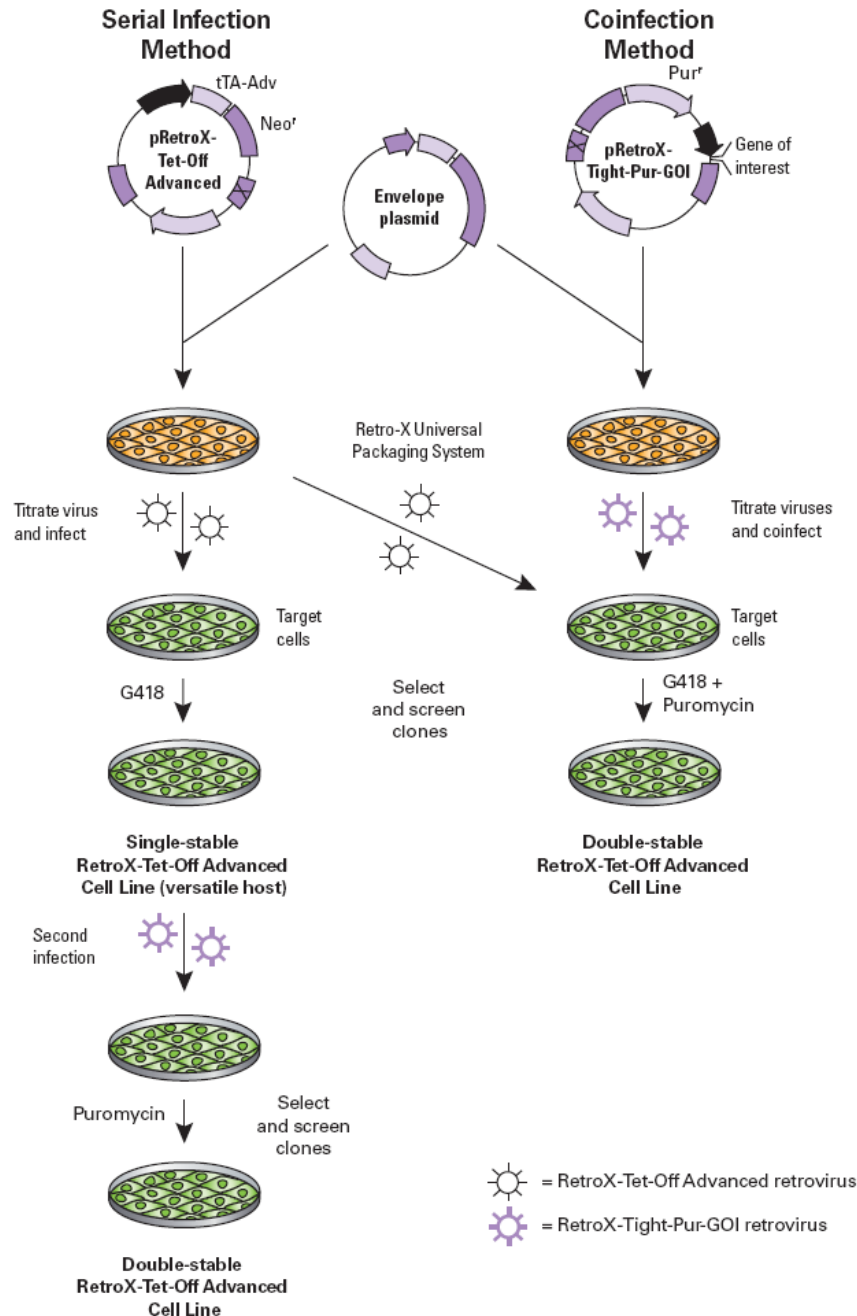


Figure 3. Establishing an inducible gene expression system with Retro-X Tet-Off Advanced. Each of your Retro-X System vectors along with a viral envelope expression vector, are first transiently cotransfected into packaging cells from the Retro-X Universal Packaging System (Cat. No. 631530) to generate retrovirus stocks which are then titrated to determine virus content. For the serial infection method (**left**), the RetroX-Tet-Off Advanced virus (regulator) is used to develop a single-stable RetroX-Tet-Off Advanced cell line from the target cell line, which is then superinfected with a RetroX-Tight-Pur-GOI virus (response) containing your gene of interest. This produces a double-stable cell line that expresses your gene in the absence of Dox. Target cells may also be coinfecting with the two retroviruses and infected cells selected simultaneously with G418 and puromycin to produce the desired double-stable cell line (**right**).

V. Vector Manipulations

A. Vector Propagation & Construction of pRetroX-Tight-Pur-GOI

1. Transform each of the plasmid vectors provided in this kit into a suitable E. coli host strain (e.g., DH5 α) to ensure that you have a renewable source of plasmid DNA. See the enclosed Vector Information Packets for further DNA propagation details.
2. Using standard cloning techniques (Sambrook & Russell, 2001), insert your cDNA into the multiple cloning site (MCS) of pRetroX-Tight-Pur. You can also use one of our In-Fusion® cloning kits which allow 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).

3. Perform a maxi-scale plasmid DNA preparation for each plasmid that will be transfected into the packaging cell line. To ensure that the purity of the DNA is transfection-grade, we recommend using NucleoBond Xtra Maxi Plus (Cat. Nos. 637109 and 637110).

VI. 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*.

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, see the following reference:

Biosafety in Microbiological and Biomedical Laboratories, Third Edition (May 1993) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services, PHS, CDC, NIH. (available on the web at <http://bmb1.od.nih.gov>)

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:
 - perform work in a limited access area
 - post biohazard warning signs
 - minimize production of aerosols
 - decontaminate potentially infectious wastes before disposal
 - take precautions with sharps (e.g., syringes, blades)
- Safety equipment:
 - biological safety cabinet, preferably Class II (i.e., a laminar flow hood with a microfilter [HEPA filter])
 - that prevents release of aerosols; not a standard tissue culture hood)
 - protective laboratory coats, face protection, double gloves
- Facilities:
 - autoclave for decontamination of wastes
 - unrecirculated exhaust air
 - chemical disinfectants available for spills

VII. Retrovirus Production Recommendations

A. Producing Retrovirus with the Retro-X Universal Packaging System

We highly recommend using our Retro-X Universal Packaging System (Cat. No. 631530) to package your retroviruses. Detailed procedures for using this packaging system and other Takara Bio retroviral packaging cell lines may be found in the Retroviral Gene Transfer and Expression User Manual (PT3132-1), which is available at 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. You may wish to perform separate tests of different Env proteins to optimize the infectivity of your viruses. For high titers, use the CalPhos Mammalian Transfection Kit (provided) to transfect your retroviral vectors into your HEK 293-based packaging cell line.

Table I. Retro-X Universal Packaging System.

<i>env</i> 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	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.

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. 631451) provides a fast and simple method for titrating retroviral supernatants (*Clontech*, April 2007). 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-Advanced Systems.

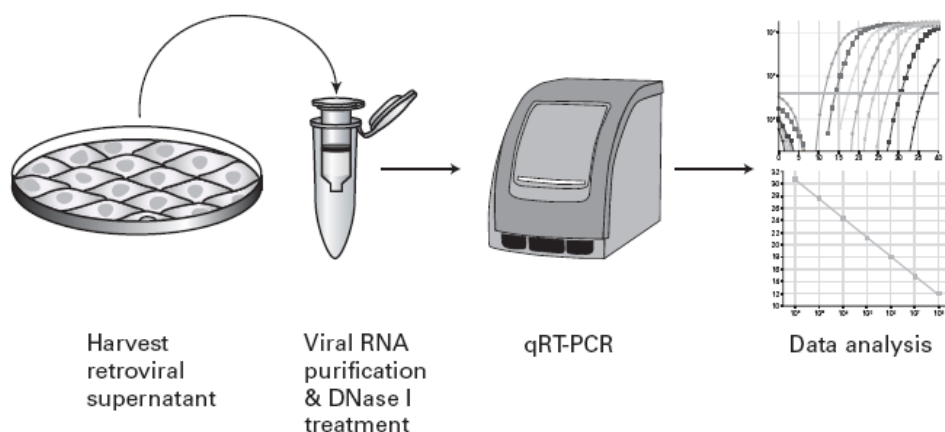


Figure 4. Flowchart of the procedures used for titrating retrovirus supernatants with the Retro-X qRT-PCR Titration Kit.

B. Protocol: Determining Viral Titer by Colony Formation

1. Plate cells in 6-well plates the day before performing the titration infections. For NIH-3T3 cells, plate 5×10^4 – 1×10^5 cells per well, in 3 ml of medium. Allow at least one well to be used as a no infection control.
2. Collect virus-containing medium (supernatant) from packaging cells. Add polybrene to a final concentration of 4 $\mu\text{g}/\text{ml}$ and filter medium through a 0.45- μm filter.

NOTE: The filter used should be cellulose acetate or polysulfonic (low protein binding) but not nitrocellulose. Nitrocellulose binds proteins present in the membrane of retrovirus and destroys the virus.

3. Prepare serial dilutions of the supernatant in 3 ml aliquots of fresh medium containing 4 $\mu\text{g}/\text{ml}$ of polybrene (e.g. six tenfold serial dilutions).
4. Infect target cells by replacing culture medium with the supernatant dilutions.

NOTE: Centrifuging the plate at 1,200g 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. After infecting for 8–24 hours, remove supernatants and subject cells to G418 or puromycin selection using the optimal selection concentrations for your cell line (Section IX).
6. Allow colonies to form for 7–14 days
7. 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 10^5 dilution would represent a viral titer of 4×10^5 colony forming units.

IX. Optimizing Antibiotic Selection of Target Cells

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 Retrovirus Information

The protocols in this User Manual provide only general guidelines for retrovirus use and mammalian cell culture techniques. Perform all steps involving cell culture using sterile technique in a tissue culture hood approved for use with retroviruses. For users requiring more information on 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*, Fourth Edition, ed. by R. I. Freshney (2000, Wiley-Liss, NY)
- *Current Protocols in Molecular Biology*, ed. by F. M. Ausubel, *et al.* (1995, Wiley & Sons)

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

Prior to using the antibiotics G418 and puromycin to establish your single-stable Retro-X-Tet-Off Advanced cell line and double-stable Retro-X-Tet-Off Advanced/GOI cell line, respectively, it is necessary to titrate each selection agent to determine its optimal concentration for your target cell line. With HeLa cells, for example, we have found 400 $\mu\text{g/ml}$ G418 and 1.0 $\mu\text{g/ml}$ 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 2×10^5 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 $\mu\text{g/ml}$). For puromycin, add the drug at 0, 1.0, 2.5, 5.0, 7.5, and 10.0 $\mu\text{g/ml}$.
 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.

X. Pilot Test of Inducibility in Your Target Cell Line

A. Protocol: Using Brief Coinfections to Test Induction in Your Target Cells

Performing a pilot assay with your RetroX-Tet-Off Advanced and RetroX-Tight-Pur-Luc retroviruses provides a quick functional test of the Retro-X Tet-Off Advanced System in your target cell line. This experiment also provides a "hands-on" introduction to the Tet System and verifies that your culture system, induction conditions, and reagents are working properly. After performing the experiment below, you may wish to perform a dose-response with Dox, using an optimal coinfection ratio of the two retroviruses.

1. Plate $\sim 5 \times 10^4$ cells in 1 ml medium. Use a sufficient number of 6-well plates to infect cells at the following virus MOI ratios, in the presence and absence of 100 ng/ml Dox, testing all conditions in duplicate (i.e., a minimum of 4 wells per infection ratio).

pRetroX-Tet-Off Advanced : pRetroX-Tight-Pur-Luc

- | | |
|----|-------|
| 1. | 1 : 1 |
| 2. | 1 : 5 |
| 3. | 5 : 1 |
2. Using the appropriate infection protocol in Section VIII, infect the cells with titered RetroX-Tet-Off Advanced and RetroX-Tight-Pur-Luc retroviral supernatants at the indicated ratios. Use the maximum possible total MOI in each case to ensure that many cells are coinfecting.
 3. Immediately add Dox to the appropriate wells and incubate the infected cells for 48–72 hr.
 4. Harvest the cells and assay for luciferase activity. Calculate fold-induction (e.g., $-\text{Dox RLU}/+\text{Dox RLU}$).

NOTE: Serial infection versus coinfection. While coinfection with the two retroviruses followed by simultaneous selection with G418 and puromycin may produce a double-stable Retro-X Tet-Off Advanced/GOI cell line more quickly than serial infection, there are some important considerations:

- Coinfection generally requires the screening of significantly more clones in order to find those with optimal inducibility.
- The intermediate single-stable Retro-X-Tet-Off Advanced cell line, created during the serial infection protocol, is a highly useful and versatile host cell line for all TRE-based vectors. Consider this option if you plan to test or study more than one GOI in your cell line.

XI. Developing a Single-Stable RetroX-Tet-Off Advanced Cell Line

This section describes how to infect your target cells with your RetroX-Tet-Off Advanced virus to create a single-stable RetroX-Tet-Off Advanced cell line as shown in Figure 6. This is the most reliable method of establishing a complete inducible system. Stably transduced target cells are selected with G418 and individual clones are isolated and expanded for screening with the TetR Monoclonal Antibody and/or pRetroX-Tight-Pur-Luc. The RetroX-Tet-Off Advanced clone with the best inducibility profile will then be superinfected with your RetroX-Tight-Pur-GOI virus and selected with puromycin to create the final double-stable RetroX-Tet-Off Advanced cell line that expresses your GOI under the control of Dox.

- If desired, you may use a mixed population of clones. However, stable cell lines with the lowest background and highest inducibility are obtained after isolating and screening individual clones. It is also possible that a poorly inducing clone may eventually overgrow the culture.
- An alternative strategy is to simultaneously infect with both viruses (RetroX-Tet-Off Advanced and RetroX-Tight-Pur-GOI) and perform a double selection with G418 and puromycin to create the double-stable cell line in one step. This method is generally less reliable however, and may require screening more than the usual number of clones (>30) to identify one with optimum properties.
- Use the infection protocol in Section A for adherent cells, and the protocol in Section B for non-adherent cells. For more information about retroviral infections, refer to our Retroviral Gene Transfer and Expression User Manual (PT3132-1).

NOTE: Growth of some target cells can be affected by media conditioned by the packaging cells due to nutrient depletion. If this appears to be the case in your system, the following precautions can be taken to avoid an adverse effect induced by the packaging cell-derived supernatants:

- Dilute virus-containing media at least twofold with fresh medium
- 4–6 hr after exposure of target cells to virus, replace with fresh medium

A. Protocol: Infecting Adherent Target Cells and Selecting Stable Clones

1. Plate target cells 12–18 hr before infection in several wells of a 6-well plate. Plate a sufficient number of cells in each well to attain a confluency of 40–60% at the time of infection ($\sim 2 \times 10^5$).
2. Collect retroviral supernatant from your transfected packaging cells, filter through a 0.45- μm filter. Add polybrene to the culture to a final concentration of 4 $\mu\text{g}/\text{ml}$.

NOTE: Use a cellulose acetate or polysulfonic (low protein binding) filter but not nitrocellulose. Nitrocellulose binds proteins present in the membrane of retrovirus and destroys the virus. The concentration of polybrene may be titrated from 2–12 $\mu\text{g}/\text{ml}$ to optimize the infection efficiency.

3. To obtain approximately one insert per cell, use a multiplicity of infection (MOI) of 0.5–3 colony forming units per cell. If you have not determined the viral titer, use as much virus-containing medium as possible.

NOTE: Centrifuging the plate at 1,200g 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.

4. Allow infection to proceed for 24 hr, then passage the cells onto several 100 mm plates. Use a range of cell dilutions that will allow you to isolate ~ 30 individual colonies on one or more plates once G418 selection is complete. The number of plates used will depend on the MOI of your virus. Limiting dilutions in 96-well plates can also be used to isolate clones.
5. Allow cells to grow for 24 hrs, then add G418 to the concentration previously determined to be optimal for your cell line. Treat the cells with G418 for 7–14 days, changing medium as necessary (every 2–3 days).
6. Once colonies are visible and contain ~ 100 cells, use cloning cylinders or cloning discs to isolate large, healthy colonies and transfer them to individual plates or wells. Continue with the clone screening protocols in Sections C & D.

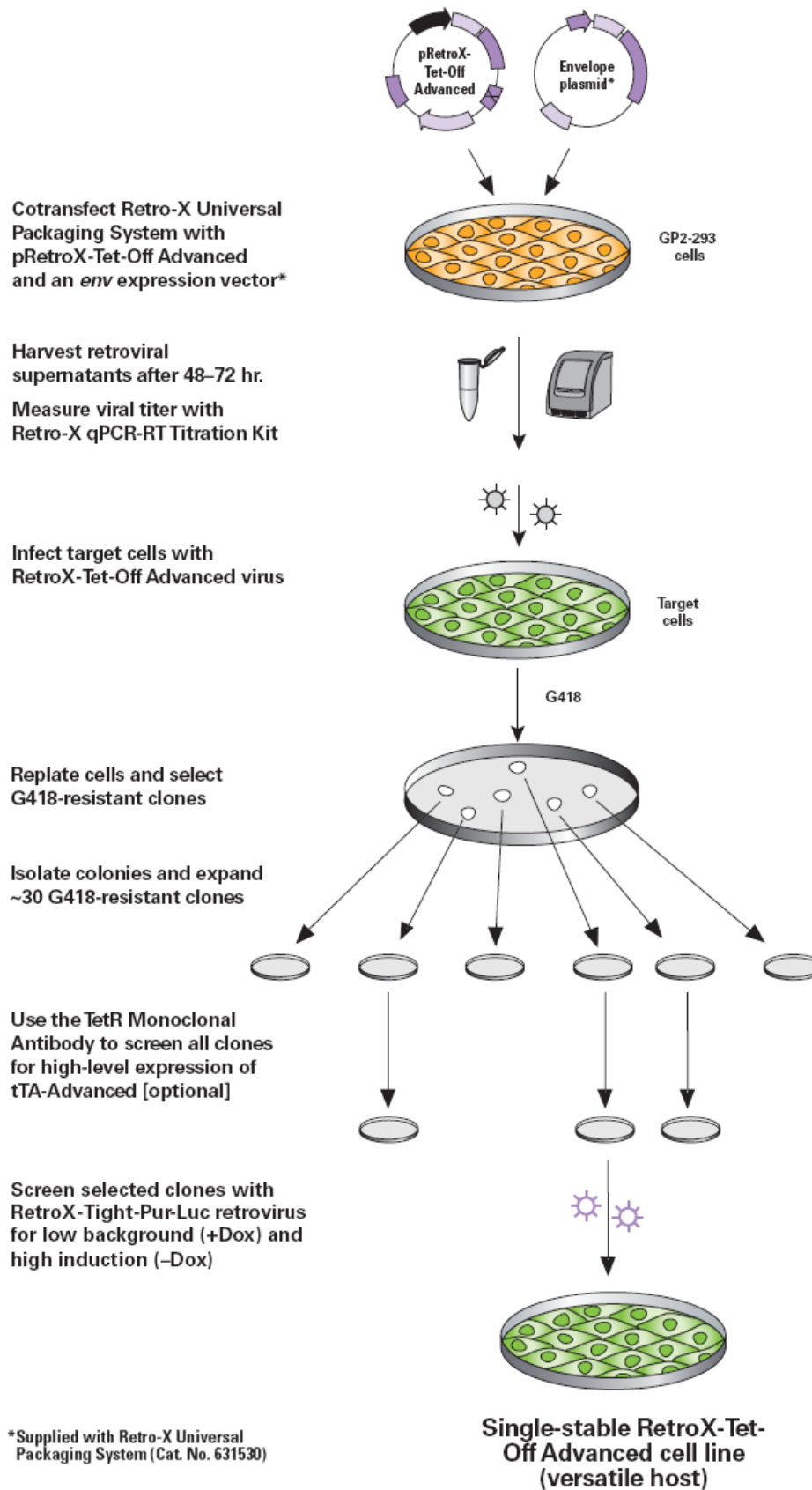


Figure 5. Flowchart for developing a single-stable RetroX-Tet-Off Advanced cell line.

B. Protocol: Infecting Non-Adherent Target Cells and Selecting Stable Clones

1. Plate target cells in a six well plate in 1 ml medium. Plate a sufficient number of cells to attain a density of $0.5\text{--}1 \times 10^6$ cells per well at the time of infection.
2. For infection, collect medium from the packaging cells, and filter through a 0.45- μm filter. Add polybrene to the culture to a final concentration of 4 $\mu\text{g}/\text{ml}$.

NOTE: Use a cellulose acetate or polysulfonic (low protein binding) filter instead of nitrocellulose. Nitrocellulose binds proteins present in the membrane of retrovirus and destroys the virus. The concentration of polybrene may be titrated from 2–12 $\mu\text{g}/\text{ml}$ to optimize the infection efficiency.

3. To obtain approximately one insert per cell, use a multiplicity of infection (MOI) of 0.5–3 colony forming units per cell. If you have not determined the viral titer, use as much virus-containing medium as possible (~2 ml).
4. Centrifuge the plate at 1,200g for 60–90 min at 32°C. A room temperature centrifuge is acceptable if a 32°C unit is not available.
5. Continue to incubate cells for 6–8 hr at 37°C.
6. To remove virus-containing supernatant, transfer cell culture to a 15 ml conical tube, and centrifuge at 500g for 10 min. Carefully collect the supernatant.
7. Resuspend cells in a 50/50 mixture of conditioned and fresh media. Incubate cells overnight.
8. Allow cells to grow for 24 hrs, then add G418 to the concentration previously determined to be optimal for your cell line. Treat the cells with G418 for 7–14 days, changing medium as necessary.
9. Isolate individual clones using the limiting dilution technique. Continue with the clone screening protocols in Sections C & D.

C. Protocol: Early Screening of Retro-X Tet-Off Advanced Clones using the TetR Antibody (Optional)

It is advantageous in terms of time and effort, to screen your clones as soon as possible and identify a few clones that are likely to produce optimal results. This strategy will reduce the numbers of individual clones that need to be maintained for subsequent functional screening. It is possible to perform early screening of many clones on a single Western blot by using the TetR Monoclonal Antibody (Cat. No. 631108). This antibody detects the Tet-Off Advanced transactivator protein (tTA-Advanced), the Tet-On Advanced transactivator, and the tTS transcriptional silencer (*Clontechniques*, January 2007.)

1. Harvest a sufficient number of cells from each clonal culture to generate 25–50 μg of total protein for SDS-PAGE. This amount of protein can typically be produced from a single confluent well of cells (i.e., HeLa) from a 12- or 24-well plate.
2. Prepare the cell extracts for SDS-PAGE, and perform a Western analysis using standard techniques. The tTA-Advanced protein is easily detectable in 25 μg of total cell protein when using the TetR antibody diluted 1:1,000 and a chemiluminescent detection method for the secondary antibody.
3. Expression of the tTA-Advanced protein should be evident in many clones. Select the best-expressing clones and test their capacity for induced expression of luciferase using the RetroX-Tight-Pur-Luc virus (Section D).

D. Protocol: Testing the Inducibility of RetroX-Tet-Off Advanced Clones

To identify clones that have the best inducibility (low basal expression and high maximum expression) among your pool of G418-resistant RetroX-Tet-Off Advanced cell clones, you can prescreen a few of them first with the TetR Antibody (as in Section C), or test 30 clones by infecting with the RetroX-Tight-Pur-Luc luciferase expression virus and assaying for luciferase activity in the presence and absence of 100 ng/ml Dox. Alternatively, you may also use the original plasmid (pRetroX-Tight-Pur-Luc) in transient transfections in 6-well plates using a standard transfection protocol.

Be sure to read the information highlighted in “Ensuring Induction with the Tet-Off Advanced System”. Clones are ready to be functionally tested once they reach 50–80% confluence in a 6-well plate. Your customized response vector, pRetroX-Tight-Pur-GOI, should also be functionally tested in a RetroX-Tet-Off Advanced clone (see Section XII.A).

1. Passage the clones, reserving ~1/3 of the total in a single well of a 6-well plate. The cells in this "stock plate" will be propagated depending upon the results of the screening assay.
2. Divide the remaining 2/3 of the cells between two wells of a 6-well plate. To one well, add 100 ng/ml Dox to determine basal, uninduced luciferase expression (“off”). Luciferase gene expression will be induced in the second well, without Dox.
3. Infect each well with equivalent amounts of RetroX-Tight-Pur-Luc virus, adapting the protocols in Sections A & B as appropriate.
4. Incubate the infected cells for 48–72 hr.
5. Harvest the cells and assay for luciferase activity. Calculate fold-induction (e.g., –Dox RLU/+Dox RLU).
6. Select clone(s) with the highest fold-induction (highest expression with lowest background) for propagation and further testing with your RetroX-Tight-Pur-GOI virus or vector (Section XII.A). In general, select only those clones that exhibit 20- to 50-fold induction in this rapid screening assay.
7. Freeze stocks of each promising clone as soon as possible after expanding the culture.

NOTE: When testing clones in transient assays, expect to see higher basal expression than in the double-stable clones which you will make in Section XII.

NOTE: Ensuring Induction in a Tet-Off Advanced System. Residual Dox that remains bound to cells or the extracellular matrix can prevent full gene induction in Tet-Off Systems (Rennel & Gerwins, 2002). Cells that have been maintained in the “off” state with 10–100 ng/ml Dox should be passaged as follows:

- Wash the cells on the plate 2X with PBS before trypsinizing
- After trypsinizing and collecting the cells, wash them in suspension 1X with PBS, and plate in fresh medium without Dox
- After the cells have reattached, 3–6 hr after plating, wash them 1X with PBS and add fresh medium without Dox

XII. Developing a Double-Stable RetroX-Tet-Off Advanced Cell Line

A. Functional Testing of pRetroX-Tight-Pur-GOI in a RetroX-Tet-Off Advanced Cell Line

Prior to establishing a double-stable RetroX-Tet-Off Advanced cell line for your GOI, the pRetroX-Tight-Pur-GOI construct should be functionally tested for inducible expression. Infect one or more of the single-stable RetroX-Tet-Off Advanced cell lines created in Section XI with your RetroX-Tight-Pur-GOI virus and assay for GOI expression in the presence and absence of 100 ng/ml Dox (see Section XI.D).

Alternatively, the corresponding plasmid construct may be transfected. Use an appropriate GOI-specific assay, for example:

- Western analysis with an antibody that recognizes the GOI protein
- RT-PCR using GOI-specific primers.

NOTE: Be sure you can discriminate among the various possible GOI-related RT-PCR and PCR products that may be generated from the following: (1) genomic DNA, (2) integrated cDNA, (3) retroviral RNA, (4) endogenous mRNA, and (5) integrated cDNA-derived mRNA.

- Northern analysis with a GOI-specific probe
- Functional assay for the GOI protein

B. Protocol: Infecting and Selecting a Double-Stable RetroX-Tet-Off Advanced/GOI Cell Line

To generate a double-stable Retro-X Tet-Off Advanced cell line, infect your single-stable Retro-X Tet-Off Advanced cell line with your functionally tested RetroX-Tight-Pur-GOI retrovirus. Then, select for stably transduced cells with puromycin. To maintain the expression system in the “off” state and prevent untimely expression of your GOI, include 100 ng/ml Dox in all culture media through infection, selection, and clone expansion. Use the following protocol for adherent cells. For non-adherent cells, adapt Section XI.B for RetroX-Tight-Pur-GOI infection and puromycin selection.

1. Plate Retro-X Tet-Off Advanced cells 12–18 hr before infection in several wells of a 6-well plate in medium containing 100 ng/ml Dox. Plate a sufficient number of cells in each well to attain a confluency of 40–60% at the time of infection ($\sim 2 \times 10^5$).
2. Collect retroviral supernatant from your transfected packaging cells, filter through a 0.45 μm filter. Add polybrene to the culture to a final concentration of 4 $\mu\text{g/ml}$ and Dox to 100 ng/ml.

NOTE: Use a cellulose acetate or polysulfonic (low protein binding) filter instead of nitrocellulose. Nitrocellulose binds proteins present in the membrane of retrovirus and destroys the virus. The concentration of polybrene may be titrated from 2–12 $\mu\text{g/ml}$ to optimize the infection efficiency.

3. To obtain approximately one insert per cell, use a multiplicity of infection (MOI) of 0.5–3 colony forming units per cell. If you have not determined the viral titer, use as much virus-containing medium as possible.
4. Add sufficient filtered and titered RetroX-Tight-Pur-GOI supernatant to the cells and allow infection to proceed for 24 hr.

NOTE: Centrifuging the plate at 1,200 x g for 60–90 min at 32°C can significantly increase infection efficiency. A room temperature centrifuge is acceptable if a 32°C unit is not available.

5. Remove supernatant and passage the cells onto several 100 mm plates using fresh medium containing 100 ng/ml Dox. Plate a range of cell dilutions that will allow you to isolate ~30 individual colonies on one or more plates once puromycin selection is complete. The number of plates used will depend on the MOI of your virus. Limiting dilutions in 96-well plates can also be used to isolate clones.
6. Allow cells to grow for 24 hrs, then add puromycin to the concentration previously determined to be optimal for your cell line. Treat the cells with puromycin for 4–5 days, changing medium as necessary.
7. Once colonies are visible and contain ~100 cells (in ~2 weeks), use cloning cylinders or cloning discs to isolate large, healthy colonies and transfer them to individual plates or wells for growth. For non-adherent cells, use a limiting dilution technique to isolate individual clones.
8. When clones have expanded sufficiently, continue with the screening protocol in Section C.

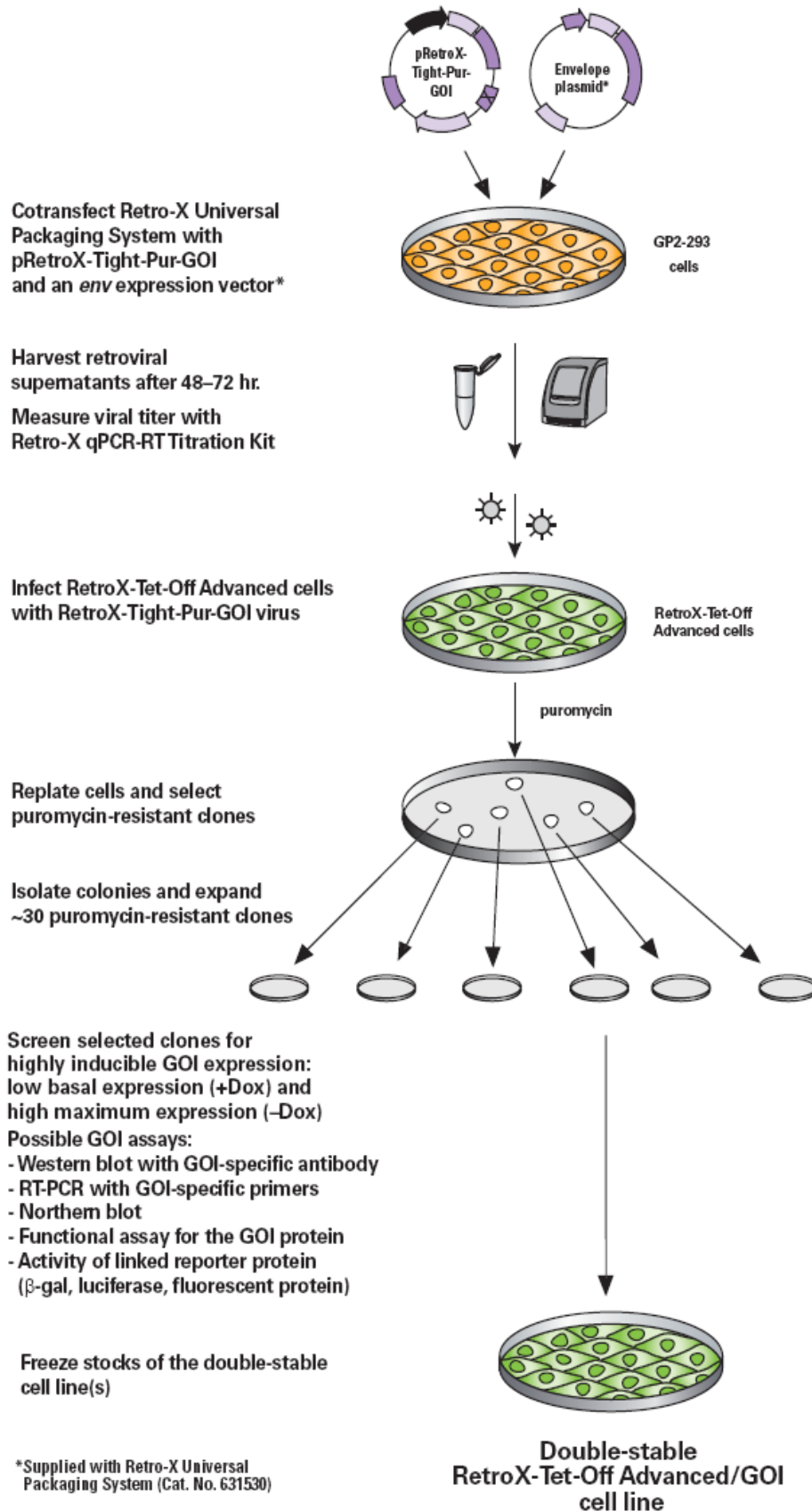


Figure 6. Flowchart for developing a double-stable RetroX-Tet-Off Advanced cell line.

C. Protocol: Screening Double-Stable Cell Lines

- Test your puromycin-resistant clones for inducible GOI expression by testing equivalent numbers of cells in 6-well plates for expression of the GOI in the absence and presence of 100 ng/ml Dox. Be sure to read the information highlighted in “Ensuring Induction with the Tet-Off Advanced System” prior to plating the cells. Choose a clone that generates the highest overall induction and lowest background expression of the GOI.
- Allow the cells to grow for at least 48 hr in the presence and absence of Dox, then assay each sample for expression of the GOI using your gene-specific assay.
- Once a suitable double-stable RetroX-Tet-Off Advanced/GOI clone has been identified, store several frozen aliquots of the cells to ensure a renewable source.

D. Working with Double-Stable Tet Cell Lines

- Tet Systems have been established successfully in many cell types, as well as in transgenic mice, rats, plants, and yeast. In general, difficulties in obtaining a cell line that exhibits low background expression arise from suboptimal construct integration events, and can be overcome by screening more clones.
- Perform a time course of induction using the techniques described in Section VII, being sure to wash the cells free of residual Dox before beginning the assay. Be sure to read the information highlighted in “Ensuring Induction with the Tet-Off Advanced System”. Because the kinetics of induction depend on the stability of the mRNA and the GOI protein, stably expressed proteins will achieve equilibrium at different times after induction.

NOTE: Ensuring Induction in a Tet-Off Advanced System. Residual Dox that remains bound to cells or the extracellular matrix can prevent full gene induction in Tet-Off Systems (Rennel & Gerwins, 2002). Cells that have been maintained in the “off” state with 10–100 ng/ml Dox should be passaged as follows:

- Wash the cells on the plate 2X with PBS before trypsinizing
- After trypsinizing and collecting the cells, wash them in suspension 1X with PBS, and plate in fresh medium without Dox
- After the cells have reattached (3–6 hr after plating) wash them 1X with PBS and add fresh medium with or without Dox

XIII. 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 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 site.)

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Appendix A. Vector Information

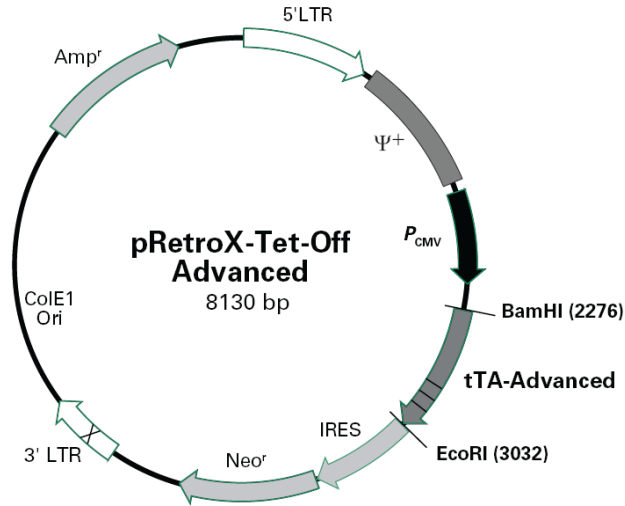


Figure 7. Map of the pRetroX-Tet-Off Advanced Vector. Unique restriction sites are in bold. For a complete vector description, refer to the enclosed pRetroX-Tet-Off Advanced Vector Information Packet (PT3969-5).

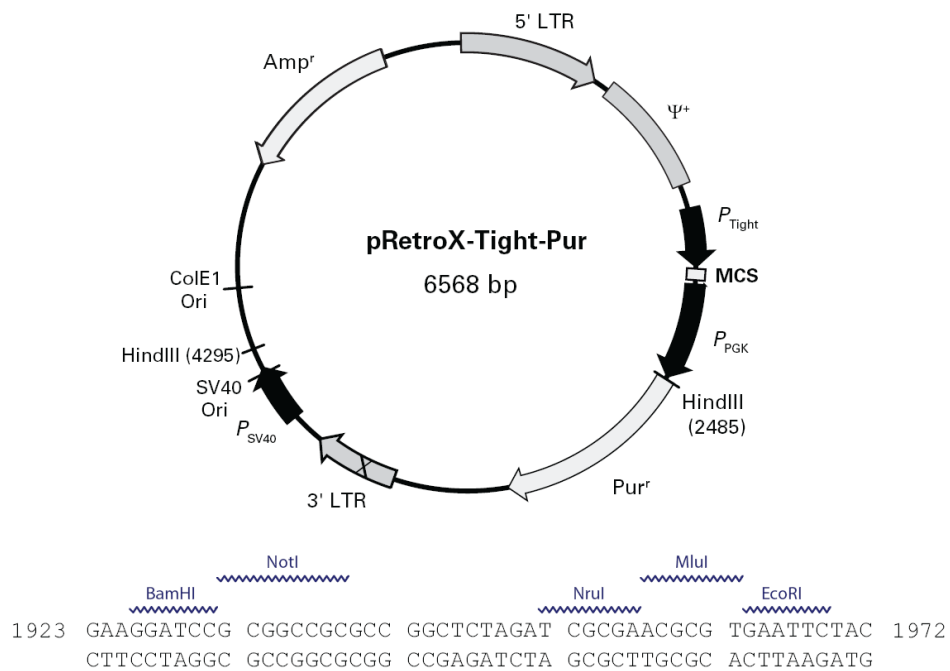


Figure 8. Map and multiple cloning site of the pRetroX-Tight-Pur Vector. Unique restriction sites are in bold. For a complete vector description, refer to the enclosed Vector Information Packet (PT3960-5).

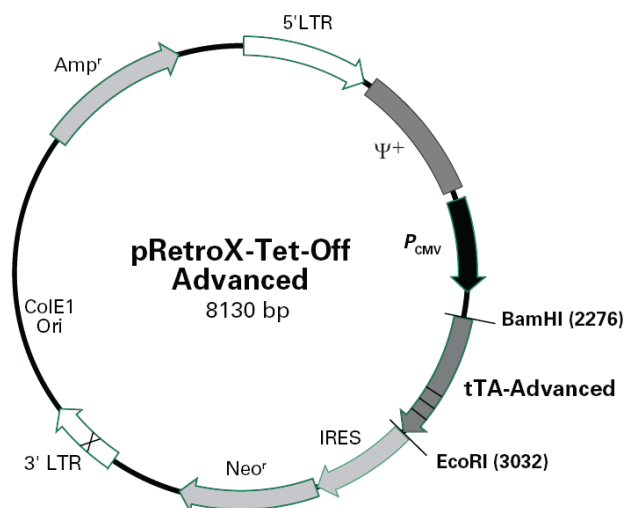


Figure 9. Map of the pRetroX-Tight-Pur-Luc Vector. Unique restriction sites are in bold. For a complete vector description, refer to the Vector Information Packet (PT3961-5) available at takarabio.com

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