Takara Bio USA, Inc.

Retro-X™ ProteoTuner™ Shield Systems User Manual

Cat. Nos. 632167 & 632171
(062719)

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

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

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I. Introduction

A. Summary

Analyzing protein function is a key focus in discovery-based cell biology research. ProteoTuner technology allows you to directly investigate the function of a specific protein of interest—by directly manipulating the level of the protein itself. This fast regulation occurs directly at the protein level, rather than at the mRNA or promoter induction level, and enables you to control the quantity of a specific protein in the cell, in as little as 15 to 30 minutes.

This revolutionary method takes advantage of ligand-dependent, tunable stabilization/destabilization of the protein of interest. It is based on a 12 kDa mutant of the FKBP protein (the destabilization domain, or DD) that can be expressed as a tag on your protein of interest. In the presence of the small (750 Da), membrane-permeant, stabilizing ligand Shield1, the DD-tagged protein of interest is stabilized (protected from proteasomal degradation) and accumulates inside the cell (Figure 1). Ligand-dependent stabilization occurs very quickly: DD fusion proteins have been shown to accumulate to detectable levels just 15–30 minutes after the addition of Shield1 (Banaszynski *et al.*, 2006).

The ProteoTuner method is not restricted to protein stabilization—it can also be used to **destabilize** the DD-tagged protein when you culture your cells in medium without Shield1, allowing proteasomal degradation of the DD-protein (Figure 1). This makes it possible to "tune" the amount of stabilized DD-tagged protein present in the cell by titrating the amount of Shield1 in the culture medium, and to repeatedly stabilize and destabilize the protein of interest using the same set of cells.

NOTE: To be degraded effectively, the DD fusion protein must have access to proteasomes within the cell. Cell regions that lack such access (e.g., the ER lumen) will not allow DD-tagged protein degradation.

A variety of ProteoTuner shield systems are available:

Your choices include N- or C-terminal DD fusions, conventional plasmid or viral delivery, and systems with or without a Living Colors® Fluorescent Protein marker for transfection. One system contains a tag for ProLabel quantitation. ProteoTuner technology also plays an important role in the On-Demand Fluorescent Reporter Systems. This manual describes the **Retro-X ProteoTuner Shield Systems**, which provide retroviral delivery of DD-fusion proteins (via transduction) to your target cells. You can learn about all of our ProteoTuner Shield Systems at **takarabio.com**

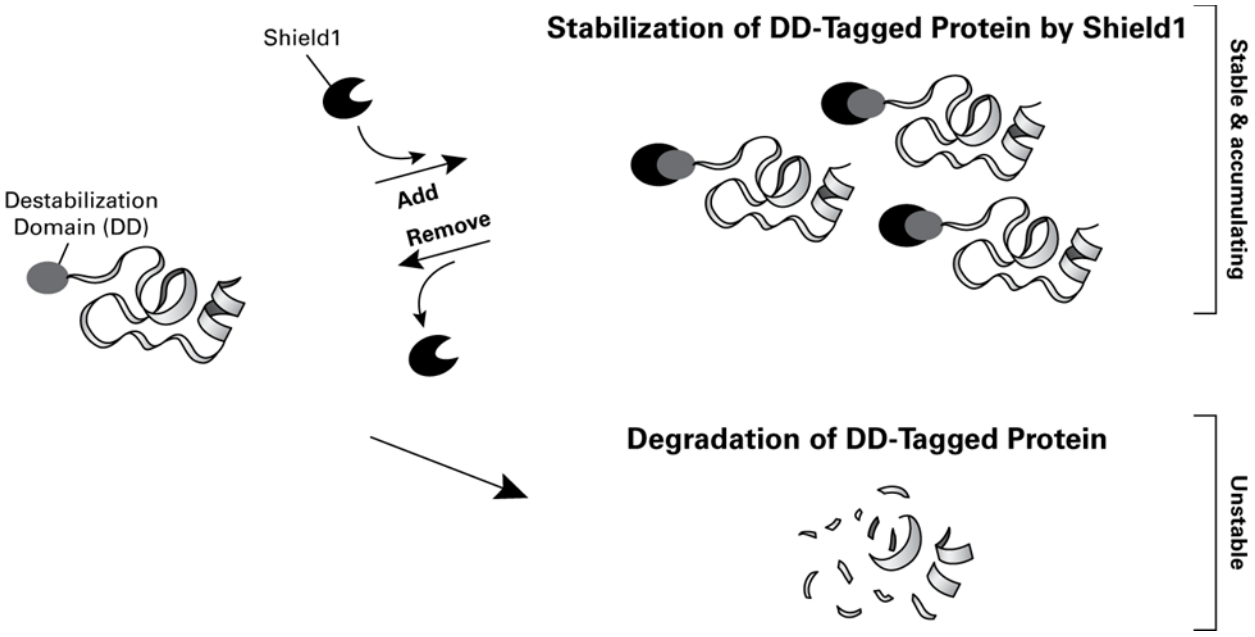

Figure 1. Ligand-dependent, targeted, and reversible protein stabilization. A small destabilization domain (DD; grey) is fused to a target protein of interest. The small membrane-permeable ligand Shield1 (black) binds to the DD and protects it from proteasomal degradation. Removal of Shield1, however, causes rapid degradation of the entire fusion protein. The default pathway for the ProteoTuner Shield Systems is the degradation of the DD-tagged protein, unless Shield1 is present to stabilize it.

B. Protocol Overview: Creating a Retroviral ProteoTuner Expression System

The following steps are required to create a ligand-dependent, tunable stabilization/destabilization system for your protein of interest using retrovirus. (Steps 2 and 3 are illustrated in Figure 2.)

1. Create and test Retro-X ProteoTuner constructs containing your gene of interest (GOI).
   a. Clone your gene of interest into a Retro-X ProteoTuner vector (such as pRetroX-PTuner or pRetroX-PTuner IRES) using fast, easy In-Fusion® HD cloning (Section V), or a standard ligation method.

      NOTE: All the Retro-X ProteoTuner systems and their components are listed in Section II. Additional required materials are listed in Section III.

   b. Pilot test Shield1 protein stabilization of your protein of interest using your Retro-X ProteoTuner-GOI constructs (Section VI).
2. Create Retro-X ProteoTuner cell lines expressing your protein of interest.
   a. Produce supernatants containing retroviral particles that express your protein of interest by transfecting your Retro-X ProteoTuner-GOI constructs from Step 1 into the **GP2-293 Packaging Cell Line** using the **Retro-X Universal Packaging System** (Cat. No. 631530), which includes this cell line (Section VII).
   b. Determine the titer of retroviral supernatants (Section VIII).
   c. Infect (transduce) your target cells with your titered retrovirus (Section IX).
3. Perform protein stabilization and destabilization experiments using the Retro-X ProteoTuner cell lines you created in Step 2 (Section X). See Section I.C for an overview of these experiments.

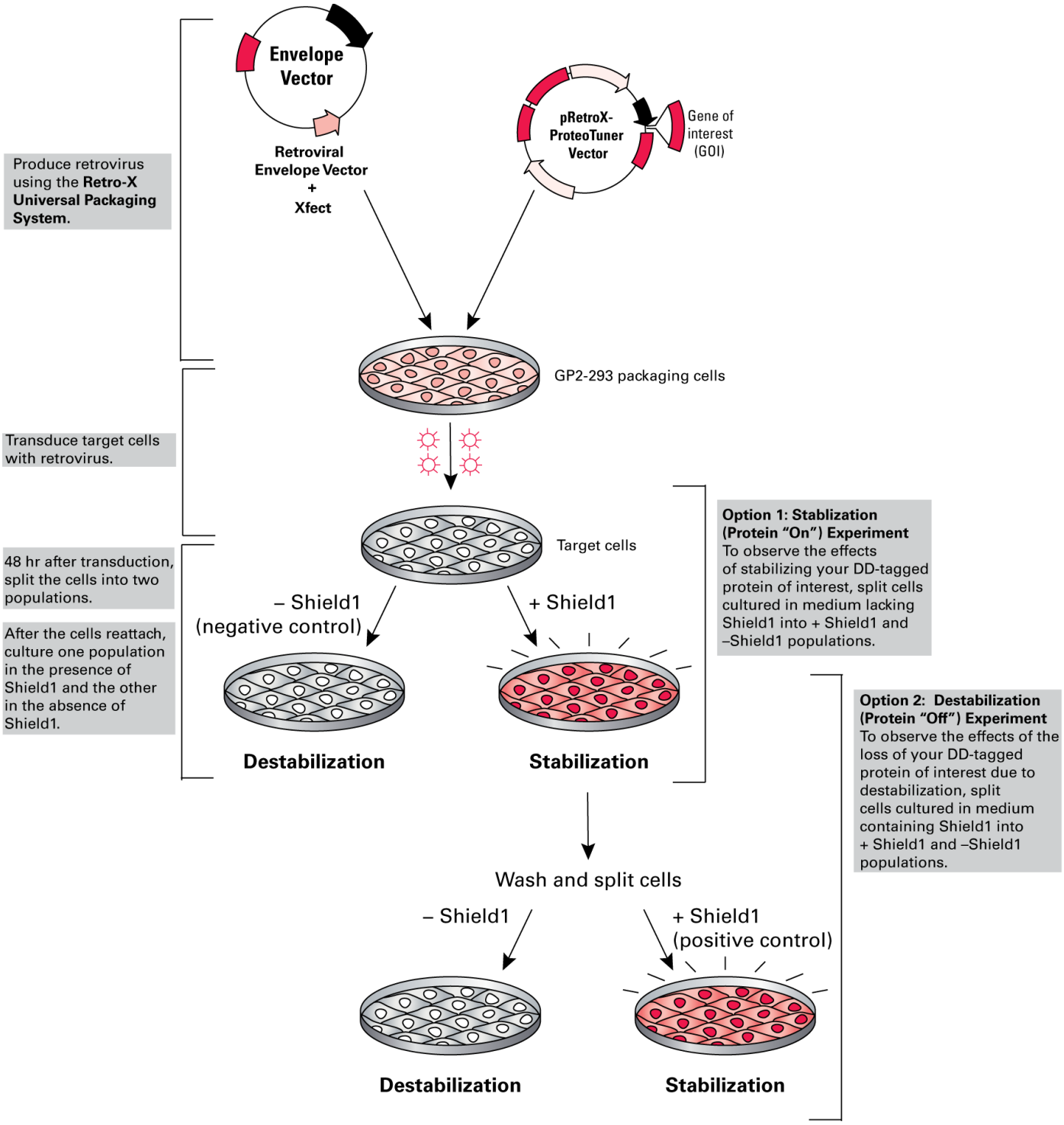

Figure 2. Establishing an expression system in target cells with Retro-X ProteoTuner Shield Systems. The Retro-X Universal Packaging System and GP-293 cells are used to generate a high-titer retroviral supernatant from a Retro-X ProteoTuner vector which contains your gene or promoter of interest. Target cells are then transduced with these packaged retroviral particles and your protein of interest can be stabilized or destabilized by adding or removing Shield1 from the culture medium.

C. Protocol Overview: The ProteoTuner Assay

After you have established a Retro-X ProteoTuner Shield System in your target cells (see Section I.B for an overview), you can perform a protein stabilization protocol to observe the effects of stabilizing your protein of interest, and a protein destabilization protocol to observe the effects of the loss of protein of interest (Figure 2). Both protocols are based on Shield1's ability to reversibly stabilize DD-tagged fusion proteins (Figure 1).

1. Protein Stabilization (Protein "On" Experiment)

In order to stabilize your protein of interest, you need to add the stabilizing ligand, Shield1, to one of two parallel cell cultures which were previously untreated with Shield1 (Figure 2). The other culture will be continuously cultured in the absence of Shield1 as a negative control.

- The added Shield1 will protect your DD-tagged protein of interest from proteasomal degradation, causing a dramatic increase in its level in the cell. Stabilization has been reported in as little as 15–30 minutes (Banaszynski *et al*., 2006) but we recommend performing a time course experiment in order to determine the Shield1-based stabilization rate for your protein of interest as well as testing different Shield1 concentrations (50–1,000 nM).
- At different time points, analyze the treated and control cells using your method of choice (e.g., Western blot or phenotypic analysis), depending on your experimental goals.

2. Protein Destabilization (Protein "Off" Experiment)

The default pathway of the ProteoTuner shield systems in the absence of the ligand Shield1 is rapid destabilization and degradation of the DD-tagged protein. In order to destabilize/degrade a protein of interest that has been stabilized with Shield 1, split the cells expressing the stabilized protein into two parallel cell cultures (Figure 2). One culture will continue to be maintained in the presence of Shield1 as a positive control, and the second (experimental) culture will be maintained without the stabilizing ligand, Shield1.

- In the absence of Shield1, the DD-tagged protein of interest will be rapidly degraded. Degradation half-lives of one to two hours have been reported (Banaszynski *et al*., 2006), but we recommend performing a time-course assay in order to assess the rate of degradation of your protein of interest.
- At different time points, analyze the treated and control cells using your method of choice (e.g., Western blot or phenotypic analysis), depending on your experimental goals.

II. List of Components

Store all components at -20°C.

A. Expression Systems

Retro-X ProteoTuner Shield System N (Cat. No. 632171)

- pRetroX-PTuner Vector (20 µg) (Cat. No. 632169; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

Retro-X ProteoTuner Shield System N (w/ ZsGreen1) (Cat. No. 632167)

- pRetroX-PTuner IRES Vector (20 µg) (Cat. No. 631035; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

III. Additional Materials Required

A. Shield1

Each Retro-X ProteoTuner Shield System includes 500 µl of Shield1 (0.5 mM; see Section II). Additional Shield1 can also be purchased separately in the following sizes:

Cat. No.	Product Name	Size
632189	Shield1 (0.5 mM)	500 µl
632188	Shield1*	5 mg

* Designed for *in vivo* use; supplied in a dry-down format.

B. ProteoTuner Accessory Products

Cat. No.	Product Name	Size
631073	DD Monoclonal Antibody	50 µl

C. Mammalian Cell Culture Supplies

- **Medium for GP2-293 Cells:**
  90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and sodium bicarbonate (Sigma-Aldrich, D5796); 10% Fetal Bovine Serum (FBS); 100 units/ml penicillin G sodium & 100 µg/ml streptomycin sulfate.
- Culture medium, supplies, and additives specific for your target cells
- Trypsin/EDTA (e.g., Sigma, Cat. No. T4049)
- Cloning cylinders or discs for isolating colonies of adherent cell lines (Sigma, Cat. No. C1059)
- Cell Freezing Medium, with or without DMSO (Sigma, Cat. Nos. C6164 or C6039), for freezing Tet-On 3G and GP2-293 cell lines.
- 6-well, 12-well, and 24-well cell culture plates; 10-cm cell-culture dishes

D. Retro-X Universal Packaging System

We highly recommend using the Retro-X Universal Packaging System, which includes the GP2-293 packaging cell line, to package your retroviruses. This system supplies all four commonly used envelopes on separate vectors, including VSV-G, eco, ampho and 10A1, to allow you to choose the tropism that is most appropriate for your target cells.

Cat. No.	Packaging System
631530	Retro-X Universal Packaging System

E. Antibiotics for Selecting Stable Cell Lines

Table 1. Recommended antibiotic concentrations

		Recommended Concentration (µg/ml)	
Cat. No.	**Antibiotic**	**Selecting Colonies[1]**	**Maintenance**
631306	Puromycin (100 mg)	0.25–10	0.25
631305	Puromycin (25 mg)		
631306	Puromycin (100 mg)	0.25–10	0.25
631305	Puromycin (25 mg)		

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

F. Retroviral Titer Determination

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

Cat. No.	Retroviral Titration Technology	Size
631453	Retro-X qRT-PCR Titration Kit	200 rxns

G. Retrovirus Concentration

Use Retro-X Concentrator to easily increase your available titer up to 100-fold without ultracentrifugation—see **takarabio.com** for details.

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

H. Transduction Enhancers

Use Polybrene (hexadimethrine bromide; Sigma-Aldrich, No. H9268), Lenti-X Accelerator (see below), or RetroNectin® (see below).

- Lenti-X Accelerator is a magnetic bead-based technology designed to accelerate lentiviral and retroviral transduction experiments; visit **takarabio.com** for details.
- RetroNectin is a multivalent molecule that simultaneously binds virus particles and cell surface proteins, maximizing cell-virus contact. RetroNectin, in particular, is recommended for increasing the transduction efficiency of suspension cells and stem cells; see **takarabio.com** for details.

Cat. No.	Transduction Enhancer	Size
631256	Lenti-X Accelerator	400 µl
631257	Lenti-X Accelerator	1,000 µl
631254	Lenti-X Accelerator Starter Kit	Each
T110A	RetroNectin Precoated Dish	10 dishes
T100B	RetroNectin Recombinant Human Fibronectin Fragment	2.5 mg
T100A	RetroNectin Recombinant Human Fibronectin Fragment	0.5 mg

I. Xfect™ Transfection Reagent

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

Cat. No.	Transfection Reagent	Size
631317	Xfect Transfection Reagent	100 rxns
631318	Xfect Transfection Reagent	300 rxns

J. In-Fusion HD Cloning System

In-Fusion is a revolutionary technology that greatly simplifies cloning.
For more information, visit takarabio.com/in-fusion

Cat. No.	In-Fusion Cloning Kit	Size
639645	In-Fusion HD Cloning System	10 rxns
639646	In-Fusion HD Cloning System	50 rxns
639647	In-Fusion HD Cloning System	100 rxns

K. Stellar™ Competent Cells

Stellar Competent Cells are recommended by Takara Bio for cloning of lentiviral and retroviral vectors. Propagation of vectors containing repeat sequences such as viral LTRs using other strains of *E.coli* may result in plasmid rearrangements. Stellar Competent Cells are sold separately and provided with all In-Fusion HD Cloning Systems.

Cat. No.	Competent Cells	Size
636763	Stellar Competent Cells	10 x 100 µl
636766	Stellar Competent Cells	50 x 100 µl

IV. General Considerations

A. General Cell Culture

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

Freshney, R. I. in *Cult. Anim. Cells* (John Wiley & Sons, Inc., 2005). doi:10.1002/0471747599.cac034

B. Safety Guidelines for Working with Retroviruses

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

The National Institute of Health and Center for Disease Control have designated retroviruses such as Moloney murine leukemia virus (MMLV) as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. MMLV does not naturally infect human cells; however, virus packaged from the MMLV-based vectors described here is capable of infecting human cells. The viral supernatants produced by these retroviral systems could, depending on your retroviral insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

IMPORTANT: For these reasons, due caution must be exercised in the production and handling of any recombinant retrovirus. **The user is strongly advised not to create retroviruses capable of expressing known oncogenes in amphotropic or polytropic host range viruses.**

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

Biosafety in Microbiological and Biomedical Laboratories. *Public Heal. Serv. Centers Dis. Control Prev. Natl. Institutes Heal. HHS Publ. No.* 21–1112

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 retroviruses.

Summary of Biosafety Level 2:

1. **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
2. **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
3. **Facilities:**
   - Autoclave available for waste decontamination
   - Chemical disinfectants available for spills

V. Creating Vector Constructs Encoding DD-Tagged Proteins of Interest

A. Protocol: Creating ProteoTuner Vector Constructs using In-Fusion HD

You need to clone your gene of interest into the corresponding pRetroX-PTuner vectors so it is in-frame with the destabilization domain (DD) encoding sequence. We recommend using the In-Fusion HD Cloning System (Section III.K; Figure 3), a revolutionary technology that permits highly efficient, seamless, and directional cloning. The technology is described at **takarabio.com/in-fusion**

NOTE: Stellar Competent Cells (Section III.L) are recommended by Takara Bio for cloning of retroviral vectors. Propagation of vectors containing repeat sequences such as viral LTRs using other strains of *E. coli* may result in plasmid rearrangements. Stellar Competent Cells are provided with all In-Fusion HD Cloning Systems.

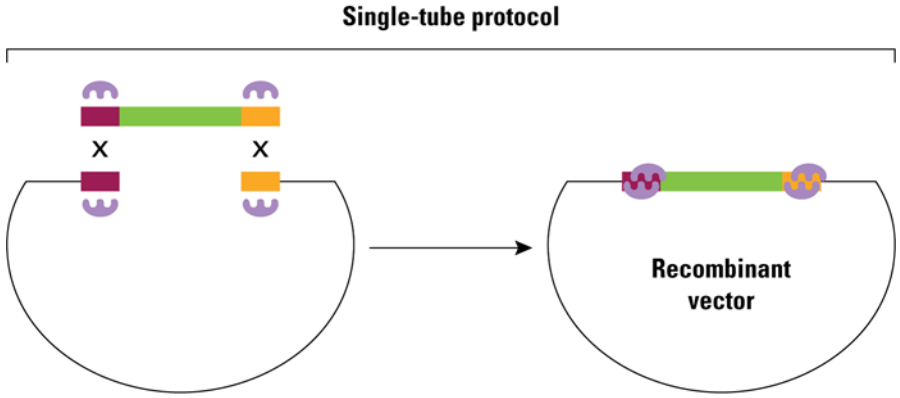

Figure 3. The In-Fusion HD Single-Tube Cloning Protocol.

VI. Pilot Expression Testing of Your Construct

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

A. Protocol: Transient Transfection of Retro-X ProteoTuner Constructs

Prior to retroviral production, your retroviral vector construct, containing the gene or promoter of interest, should be tested for functionality by standard plasmid transient transfection. If it is transfected into target cells, the plasmid will express your DD-tagged protein of interest in a transient fashion. For your initial *in vitro* experiments, we recommend testing medium containing different concentrations of Shield1 with your transfected cells in order to determine the sensitivity of the system containing your protein(s) of interest.

1. In a well of a 6-well plate, use Xfect Transfection Reagent (Section III.I) to transfect your target cells with 5 µg of the Retro-X ProteoTuner vector construct of interest. Follow the **Xfect Transfection Reagent Protocol-At-A-Glance**. (Locate this protocol by searching at **takarabio.com/manuals**).
2. After transfecting overnight, **split transfected cells** into different plates or separate wells of a 6-well plate, or your preferred plate format.

3. Incubate the transfected cells with Shield1 at specific time intervals and concentrations. **Replace the medium** in the plates holding the transfected cells with medium containing the appropriate amount of Shield1, diluted as described below. Maintain at least one culture in medium containing no Shield1 as a negative control.

   NOTE: In the case of adherent cells, **let the cells reattach after the split before removing medium**.

   a. Recommended Shield1 Concentrations and Time Points

   - Try Shield1 concentrations between 50 nM and 1,000 nM for different lengths of time (30 minutes to 12+ hours) to determine the best experimental conditions.

   b. General Guidelines for Preparing Medium Containing Shield1

   - Dilute the supplied Shield1 stock solution (0.5 mM, supplied in ethanol) in tissue culture media to the final concentration(s) needed in your experiment.

     EXAMPLE: Preparation of 10 ml of medium containing 500 nM of Shield1: Dilute 10 µl of Shield1 stock solution (500 µM) in 10 ml of medium.
   - Working concentrations of Shield1 can be obtained by adding it directly from ethanol stocks, or by diluting it serially in culture medium just before use.
   - Dilute the Shield1 stock solution using one of the two following types of culture medium:
     1) **Culture medium that has already been used to culture the cells:** Collect the media supernatant from your cell culture into a clean and sterile container and add the appropriate amount of Shield1 to reach the appropriate final concentration. After mixing, add the medium containing Shield1 back into the plate.
     2) **Fresh culture medium:** Warm up the appropriate volume of fresh culture media needed for your experiment to ~37°C. Then add the appropriate volume of Shield1 stock solution, to obtain the final concentration of Shield1 to be used in the experiment.
   - If you are making serial dilutions of Shield1 into culture medium, we recommend that the highest concentration not exceed 5 µM, to ensure complete solubility in the (aqueous) culture medium.
   - In either case, the final concentration of ethanol in the medium added to mammalian cells should be kept below 0.5% (a 200-fold dilution of a 100% ethanol solution) to prevent this solvent from having a detrimental effect on the cells.

4. After adding the medium containing Shield1 at the appropriate concentration and for the appropriate length of time, the effect and extent of protein stabilization can be analyzed with an assay that is appropriate for your experiment, e.g., Western blot.

VII. Producing Retrovirus from the Retro-X Vectors

We highly recommend using Takara Bio's Retro-X Universal Packaging System (Section III.D) to package your retroviruses. The protocol for packaging retrovirus is outlined below, but more detailed procedures may be found in the Retroviral Gene Transfer and Expression User Manual, which is available at **takarabio.com/manuals**. The system includes the GP2-293 Packaging Cell Line and a selection of four envelope expression vectors. Consult Table 1 to determine which envelope protein is best suited for your target cell line, and transfect using Xfect Transfection Reagent (Section III.I). You may wish to perform separate tests of different envelope proteins to optimize the infectivity of your viruses.

Table 2. Retro-X Universal Packaging System

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

A. General Considerations

1. Optimizing Retroviral Titer

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

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

2. Required Materials & Precautions

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

- 60-mm culture plates
- Transfection-grade DNA

IMPORTANT: Perform all steps in a sterile tissue culture hood. Retrovirus requires the use of a Biosafety Level 2 facility. Depending on which viral envelope is selected (see Table 1), recombinant pseudotyped retroviruses packaged from this system are capable of infecting human cells. Know and use appropriate safety precautions (see Section IV.B).

B. Protocol: Transfecting Retroviral Vectors into GP2-293 Packaging Cells

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

1. Approximately 24 hr before transfection, seed 4–5 x 10^6 GP2-293 cells in a 100-mm plate, in 10 ml of growth medium. Make sure that the cells are plated evenly. Incubate at 37°C, 5% CO_2 overnight. Continue to incubate the cells until you are ready to add the transfection mixture in Step 7. The cells should be 80–90% confluent at the time of transfection.
2. Thoroughly vortex Xfect Polymer.
3. In a microcentrifuge tube, dilute your retroviral plasmid DNA with Xfect Reaction Buffer to a final volume of 600 µl, using the following amounts of DNA:

   15 µg of retroviral plasmid + 15 µg of envelope plasmid (e.g., pVSV-G; see Table 1)

   NOTES:

   - Always add your plasmid(s) to the Xfect Reaction Buffer before adding Xfect Polymer.
   - At least 50% of the solution must consist of Xfect Reaction Buffer.
4. Mix well by vortexing for 5 seconds at high speed.
5. Add 9 µl of Xfect Polymer to the diluted retroviral plasmid DNA and mix well by vortexing for 10 seconds at high speed.

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

   NOTE: It is not necessary to remove serum from your cell culture medium. It is normal for the medium to change color slightly upon addition of the DNA-Xfect solution.
8. Incubate the plate at 37°C, 5% CO_2.
9. After 4 hr to overnight, replace the transfection medium with 10 ml fresh complete growth medium and incubate at 37°C for an additional 24–48 hr. Virus titers will generally be highest 48 hr after the start of transfection. *Caution: discarded medium contains infectious retrovirus*.
10. Harvest the retroviral supernatants and pool similar stocks, if desired. *Caution: supernatants contain infectious retrovirus*. Centrifuge briefly (500 *g* for 10 min) or filter through a 0.45 µm filter to remove cellular debris.

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

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

VIII. Retrovirus Titration

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

A. Titrating Your Retroviral Supernatants by qRT-PCR

The **Retro-X qRT-PCR Titration Kit** (Section III.F) provides a fast and simple method for titrating retroviral supernatants. The kit employs a quick RNA purification step and determines viral RNA genome content using qRT-PCR and TB Green® technologies. Titration can be completed in only 4 hr, which reduces time delays between virus harvest and target cell infection, allowing you to do both on the same day. It is designed for use with all MMLV-based vectors, including those in the Retro-X Tet-On 3G System.

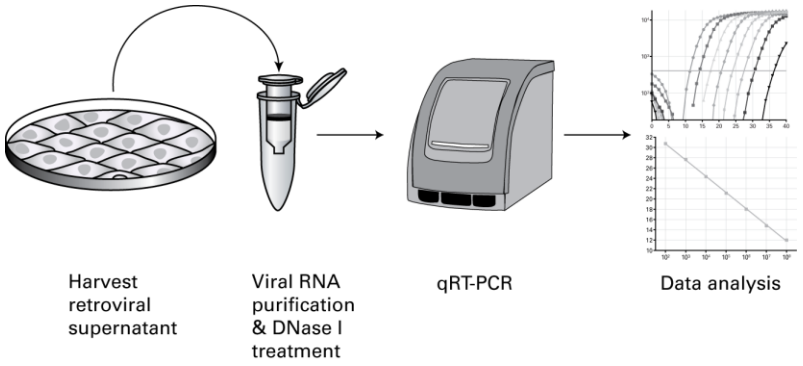

Figure 4. Schematic for titering retrovirus supernatants with the Retro-X qRT-PCR Titration Kit.

B. Protocol: Determining Viral Titer by Colony Formation

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

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

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

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

   NOTE: Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of Polybrene may be determined empirically but generally falls within a range of 2–12 µg/ml. Excessive exposure to Polybrene (>24 hr) can be toxic to cells.

3. Prepare filtered viral supernatant from the transfected Retro-X packaging cells (Section VII). This is your virus stock.

4. Prepare six 10-fold serial dilutions of the virus stock as follows:

   a. Add 1.35 ml of medium containing Polybrene (from Step 2) to each of six sterile and numbered 1.5 ml microfuge tubes.

   b. Add 150 µl of the virus stock (from Step 3) to Tube 1. Mix gently.

   c. Transfer 150 µl from Tube 1 to Tube 2 and mix. Continue making serial dilutions by transferring 150 µl from each successive dilution into the next prepared tube.

5. Infect the HT-1080 cells by adding 1 ml from each of the five least concentrated viral dilutions (Step 4) to the appropriately labeled wells. The final Polybrene concentration will be 4 µg/ml in ~3 ml. Centrifuge the cultures to improve transduction efficiency*.

   *** Culture Centrifugation During Infection Increases Transduction Efficiency**

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

6. After infecting for 8–24 hr, remove the supernatants and begin antibiotic selection using the concentration of antibiotic that is optimal for your cell line (Section III. E). ***Caution: discarded medium contains infectious retrovirus.***

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

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

IX. Transducing Target Cells with a Retro-X ProteoTuner Retrovirus

A. Protocol: Transducing Target Cells with Retro-X ProteoTuner Retroviruses

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

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

NOTE: Lenti-X Accelerator and **RetroNectin** (Section III.H) may be used as transduction enhancers instead of Polybrene.

4. In general, we find that an MOI of 5–20 works best. If titer values are unknown, use serial dilutions of the virus supernatant, 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.
   - Use the transduced cells to optimize Shield 1 concentration and incubation time (Section X.A) in preparation for protein stabilization and destabilization experiments using Shield1 (Sections X.B & X.C).
   - Alternatively, passage the cultures and subject the cells to selection using puromycin to establish a stable cell population or cell line. (Instructions for expansion and freezing of cell line stocks are provided in Appendix B.)

X. Protein Stabilization & Destabilization Using Retro-X ProteoTuner Cell Lines

A. Protocol: Optimizing Shield1 Concentration and Incubation Time of Transduced Cells

1. Split the transduced cells from Section IX.A, Step 6 into different plates or separate wells of a 6-well plate, or your preferred plate format.

   To begin incubation of the transduced cells with Shield1 at predetermined time intervals and concentrations (these can be determined using transient transfection—see Section VI), replace the medium in the plates containing the transduced cells with medium containing the appropriate amount of Shield1, diluted as described below. Maintain at least one culture in medium containing no Shield1 as a negative control.

   NOTE: In the case of adherent cells, let the cells reattach after the split before removing the medium.

   a. Recommended Shield1 Concentrations and Time Points

   - Try Shield1 concentrations between 0.1 nM and 1,000 nM for different lengths of time (30 minutes to 12+ hr) to determine the best experimental conditions.

   b. General Guidelines for Preparing Medium Containing Shield1

   - Dilute the supplied Shield1 stock solution (0.5 mM, supplied in ethanol) in tissue culture media to the final concentration(s) needed in your experiment.

     EXAMPLE: Preparation of 10 ml of medium containing 500 nM of Shield1: Dilute 10 µl of Shield1 stock solution (500 µM) in 10 ml of medium to yield a final concentration of 500 nM.
   - Working concentrations of Shield1 can be obtained by adding it directly from ethanol stocks, or by diluting it serially in culture medium just before use.

- Dilute the Shield1 stock solution using one of the two following types of culture medium:
    1) **Culture medium that has already been used to culture the cells:** Collect the media supernatant from your cell culture into a clean and sterile container and add the appropriate amount of Shield1 to reach the appropriate final concentration. After mixing, add the medium containing Shield1 back into the plate.
    2) **Fresh culture medium:** Warm up the appropriate volume of fresh culture media needed for your experiment to ~37°C. Then add the appropriate volume of Shield1 stock solution, to obtain the final concentration of Shield1 to be used in the experiment.
- If you are making serial dilutions of Shield1 into culture medium, the highest concentration should not exceed 5 µM, to ensure complete solubility in the (aqueous) culture medium.
- In either case, the final concentration of ethanol in the medium added to mammalian cells should be kept below 0.5% (a 200-fold dilution of a 100% ethanol solution) to prevent this solvent from having a detrimental effect on the cells.

2. After adding the medium containing Shield1 at the appropriate concentration and for the appropriate length of time, the effect of stabilizing your DD-tagged protein of interest can be analyzed with an assay that is appropriate for your experiment, e.g., Western blot.

B. Protocol: DD-Protein Stabilization of Transduced Cells

Before you begin, transduce your DD construct of interest into your cells of interest (Section IX.A) and determine the optimal Shield1 concentration and incubation time (see Section X.A).

Stabilizing a protein of interest in attached cells

1. 12–24 hr posttransduction, split the cells into at least two parallel cultures (the number of plates depends on the number of samples you would like to collect).
2. Culture the cells (all plates) in medium without Shield1 until the cells are attached to each plate.

   NOTE: Shield1 does not interfere with the attachment process. Therefore, Shield1 can be added immediately after splitting if required for your experimental needs.
3. Dilute the Shield1 to the optimal concentration determined in Section X.A. We recommend final concentrations of ~50–1,000 nM Shield1 in the cell culture medium.
4. Remove the culture medium and replace it with warm medium with or without Shield1. Shield1 added to the experimental plate(s) will protect the DD-tagged protein of interest from proteasomal degradation, causing a rapid increase in its level in the cell.
5. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD fusion protein of interest using the assay appropriate for your experiment, e.g., Western blot.

Stabilizing a protein of interest in cells grown in suspension

1. 12–24 hr posttransduction, divide the cell suspension evenly into at least two tubes. (The number of tubes depends on the number of samples you would like to collect.)
2. Dilute Shield1 to the optimal concentration determined in Section X.A. We recommend final concentrations of ~50–1,000 nM Shield1 in the cell culture medium.
3. Centrifuge the tubes (from Step 1) for 5 minutes at ≤1,000 rpm.
4. Remove the culture medium and replace with warm media with or without Shield1 (prepared in Step 2) as determined by your needs.

   NOTE: The added Shield1 will protect your DD-tagged protein of interest from proteasomal degradation, causing a rapid increase in its level in the cell.
5. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD fusion protein of interest using the assay appropriate for your experiment, e.g., Western blot.

C. Protocol: DD-Protein Destabilization

Before you begin, transduce your DD construct of interest into your cells of interest (Section IX.A). Culture your cells in medium containing Shield1 at the optimal concentration determined in Section X.A to stabilize your protein of interest.

Destabilizing a protein of interest in attached cells

Method A

Requires splitting cells (for quickest destabilization)

1. After stabilizing the protein of interest for the desired length of time via Shield1, remove the medium containing Shield1.
2. Rinse the cells with warm Dulbecco's Phosphate Buffered Saline (TC grade).
3. Detach the cells by your method of choice (trypsin, cell dissociation buffer, etc.) and split them into at least two new cell culture plates (the number of plates depends on the number of samples you would like to collect).
4. Culture the cells in one plate in medium containing Shield1 (positive control) and culture the cells in the other plate(s) in medium without Shield1.

   NOTE: Growing the cells in the absence of Shield1 causes the fast degradation of the previously stabilized protein of interest.
5. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD fusion protein of interest using the assay appropriate for your experiment, e.g., Western blot.

Method B

No splitting required (for slower destabilization)

1. After stabilizing the protein of interest for the desired length of time via Shield1, remove the medium containing Shield1.

2. In order to destabilize the protein of interest, wash the cells in the plates by rinsing them three times with warm culture medium without Shield1.
3. Culture the cells in culture medium without Shield1.
4. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD fusion protein of interest using the assay appropriate for your experiment, e.g., Western blot.

Destabilizing a protein of interest in cells grown in suspension

1. After stabilizing the protein of interest for the desired length of time via Shield1, distribute the cell suspension evenly into at least two tubes (the number of tubes depends on the number of samples you would like to collect).
2. Centrifuge the tubes for 5 min at ≤1,000 rpm and remove the culture medium.
3. Resuspend one pellet in culture medium with Shield1 at the appropriate concentration (positive control) and resuspend the remaining pellet(s) in culture medium without Shield1.
4. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD fusion protein of interest using the assay appropriate for your experiment, e.g., Western blot.

D. Protocol: Working with Stable Cell Lines Expressing a DD-Tagged Protein of Interest

1. After establishing a stable cell line, you can culture your cells either in the absence or the presence of Shield1, depending on your experimental needs.
2. If you grow your cells in the absence of Shield1, your protein of interest will be destabilized and expressed only at a very low level in your stable cell line. Then Shield1 can be added to rapidly increase the amount of your protein of interest (Section X.B).
3. Maintenance in, or addition of Shield1 to a stable cell line will stabilize your protein of interest and quickly increase its level in the cell (Section X.C).

E. Protocol: *In Vivo* Use of Shield1

General Methods

Because Shield1 is suitable for use *in vivo*, studies can also be performed in a whole animal context via one of the following commonly used methods:

- Generate transgenic mice in which expression of the DD-tagged fusion protein is restricted to a tissue of interest by a tissue-specific promoter.
- Subcutaneously xenograft cells into nude mice.

Preparation and Injection of Shield1

1. Preparing solutions for injection
    - Shield1 can be reconstituted into DMA or ethanol at various concentrations up to 10 mg/ml. This stock solution may be kept for several months at −20 °C.
    - Make up a fresh solution of 9:1 PEG 400: Tween 80 before each injection.

2. Injecting Shield1 into mice

    - Inject Shield1 at a concentration of 3–10 mg/kg body weight using a 1:1 mixture of the appropriate amount of Shield1 stock solution and the fresh 9:1 PEG 400:Tween 80 solution (Step 1). Control mice should be injected with a 1:1 mixture of DMA (without any Shield1 added) and the PEG 400:Tween 80 solution.
    - Shield1 may be injected intravenously; however, intraperitoneal injections often produce more reliable results.
    - The injection regiment can be repeated every 48 hr in order to maintain strong stabilization of the DD-tagged protein of interest.

Effects of Shield1 *In Vivo*

Nude mice were injected with Shield1 every 48 hr for 2 months and showed no signs of toxicity (e.g., changes in feeding behavior, grooming, or activity levels) (Sellmyer *et al.*, 2009).

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Appendix A. Troubleshooting Guide

Table 3. Troubleshooting Guide

Problem	Possible Explanation	Solution
A. Vector Cloning		
Plasmid is difficult to grow or clone	Some viral vectors may undergo rearrangements between the 5' and 3' LTRs when propagated in less-than-optimal E. coli host strains	Use Stellar Competent Cells (Cat. No. 636763) to produce high DNA yields and to minimize the potential for DNA rearrangements.
B. GP2-293 Packaging Cells		
Poor viability upon thawing	Improper thawing techniques	Use thawing procedure in Appendix C.
	Incorrect culture medium	Use DMEM with additives listed in Section III.C.
	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Slow growth	Incorrect culture medium	Use DMEM with additives listed in Section III.C.
Cells do not attach to plate	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Cells appear morphologically different	Passage of cell culture is too high (old cells)	Thaw/purchase new aliquot of GP2-293 cells.
C. Virus Production		
Poor transfection efficiency (as determined by GOI or marker expression in the GP2-293 cell line)	Cells plated too densely	Plate 4–5 x 10^6 cells/100-mm plate, or fewer if the cells divide rapidly. Use at 80–90% confluency. See Section VII.
	Transfection is toxic to cells	Use the optimized conditions provided in Section VII.
	Cells harvested or analyzed too soon after transfection.	Wait 48 hr after transfection for maximal expression of GOI or marker to determine efficiency.
Low titers (<10^5 cfu/ml)	Poor transfection efficiency	See above section. Concentrate the virus using the **Retro-X Concentrator** (Section III.G) to increase your available titer up to 100-fold without ultracentrifugation.
	Virus was harvested too early	Harvest virus 48–72 hr after the start of transfection.
	Vector is too large	The limit for efficient packaging function is 8.3 kb from the end of the 5'-LTR to the end of the 3'-LTR.
	Polybrene is missing or at suboptimal concentration	Add Polybrene (4 µg/ml) during transduction or optimize the concentration (2–12 µg/ml)
	Virus was exposed to multiple freeze-thaw cycles	Each cycle reduces titer by approximately 2–4 fold. Limit the number of freeze-thaws.
	Suboptimal selection procedure during titration	Perform an antibiotic kill curve on the cell line prior to using it for titration.

Problem	Possible Explanation	Solution
D. Transduction of Target Cells		
Poor transduction efficiency	Low titer	See Section C or use the Retro-X Concentrator (Section III.H) to increase your available titer up to 100-fold without ultracentrifugation.
	Low viability of target cells during transduction	Optimize culture conditions for target cells prior to infection
		Packaging cell line-conditioned media may affect cell growth; dilute viral supernatant or shorten exposure time to viral supernatant. Consider using RetroNectin reagent and the RetroNectin-Bound Virus transduction protocol.
		Excessive exposure to Polybrene: optimize amount (titrate) or shorten exposure time to viral supernatant.
	Viral supernatant contains transduction inhibitors	Use RetroNectin reagent or RetroNectin-coated plates in the RetroNectin-Bound Virus transduction protocol, which allows virions to bind the RetroNectin substratum and be washed free of inhibitors prior to target cell infection.
Low expression of GOI	Low transduction efficiency	See Section D above.
	Promoter may be weak or possibly inactivated in target cells	Insert a tissue-specific promoter for GOI expression.
	Poor target cell viability	Check growth parameters.
Infection is toxic to target cells	MOI too high (i.e. too much virus used)	Dilute virus stock; titrate the virus.
	Polybrene toxicity	Reduce or optimize polybrene concentration; reduce infection time.
	Packaging cell supernatant or medium is toxic to cells	Dilute virus stock using target cell culture medium; harvest virus from packaging cells using target cell medium. Consider using RetroNectin reagent and the RetroNectin-Bound Virus transduction protocol

E. Establishment of Stable Cell Lines		
Untransduced cells do not die at the high antibiotic concentration established via titration in Section III.E	The cells have not been recently passaged, so they remain well-attached to the plate surface even when they are dead.	To determine the appropriate antibiotic concentration, use cells that have been split within the last 2–3 days.
	You have achieved 100% transduction efficiency.	
There are no surviving cells after transduction followed by selection	The antibiotic concentration which caused massive cell death when determining the appropriate dose via titration could be too high.	Use a lower antibiotic concentration for selection of stably transfected cell clones.
Poor cell viability	Cells were not properly frozen.	See Appendix C, Section A.
	Cells were not properly thawed.	See Appendix C, Section B.
F. Inducing Protein Stabilization		
The DD-tagged protein is already detectable in the absence of Shield1.	The expression level of the protein of interest fused to the DD domain is too high, especially in the case of a DD-tagged protein of interest localized to the plasma membrane.	Transfect cells with a lower amount of plasmid (in the case of transient transfection—Section VI.A) or establish stable clones after transduction and screen for the lowest background (Section X.A).
Addition of Shield1 does not result in any of the expected effect(s).	The Shield1 concentration is too low.	Increase the amount of Shield1 added.
	The monitoring assay is not sensitive enough.	Make sure to include a positive control when performing your assay.
	The volume of Shield1 used causes cells to die due to high solvent concentration.	Prepare a more concentrated stock solution.
	Poor infection efficiency	Confirm virus titers using a titration kit (Section VIII.A.)
		Increase amount of virus applied to target cells
		Optimize density of cells when transducing
	Poor target cell viability	Optimize passage number of target cells.
		Optimize culture conditions of target cells.
		Optimize tissue culture plasticware.

Appendix B. Retro-X ProteoTuner Vector Maps

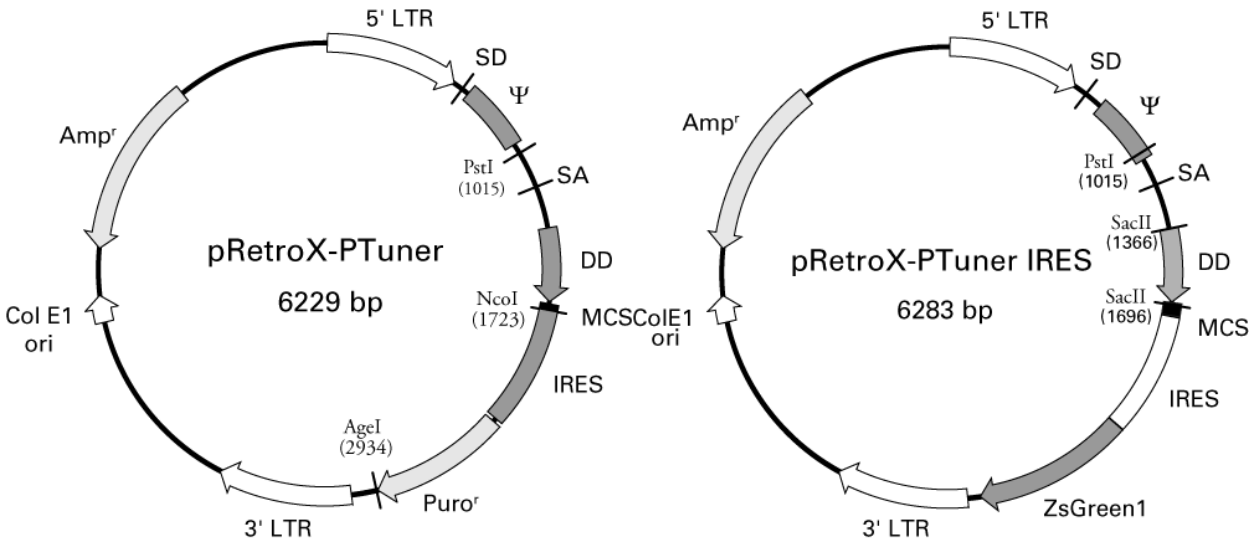

Figure 5. pRetroX-PTuner and pRetroX-PTuner IRES Vector maps.
For more detailed vector information, see **takarabio.com**

Appendix C: Preparing and Handling Cell Line Stocks

A. Protocol: Freezing Cell Line Stocks

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

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

B. Protocol: Thawing Cell Line Frozen Stocks

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

1. Thaw the vial of cells rapidly in a 37°C water bath with gentle agitation. Immediately upon thawing, wipe the outside of the vial with 70% ethanol. All of the operations from this point on should be carried out in a laminar flow tissue culture hood under strict aseptic conditions.
2. Unscrew the top of the vial slowly and, using a pipet, transfer the contents of the vial to a 15-ml conical centrifuge tube containing 1 ml of prewarmed medium (without selective antibiotics such as puromycin). Mix gently.
3. Slowly add an additional 4 ml of fresh, prewarmed medium to the tube and mix gently.
4. Add an additional 5 ml of prewarmed medium to the tube and mix gently.
5. Centrifuge at 100*g* for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium without selective antibiotics. (This method removes the cryopreservative and can be beneficial when resuspending in small volumes. However, be sure to treat the cells gently to prevent damaging fragile cell membranes.)
6. Mix the cell suspension thoroughly and add to a suitable culture vessel. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place in a 37°C humidified incubator (5–10% CO_2 as appropriate) for 24 hr.

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

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

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

8. Expand the culture as needed. The appropriate selective antibiotic(s) should be added to the medium after 48–72 hr in culture. Maintain cell lines in complete culture medium containing a maintenance concentration of puromycin, as appropriate (Section III.F).

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