Takara Bio USA, Inc.

Lenti-X™ ProteoTuner™ Shield Systems User Manual

Cat. Nos. 631074, 631748, 631751, 631753, 632173 & 632175
(110416)

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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 **Lenti-X ProteoTuner Shield Systems**, which provide lentiviral delivery of DD-fusion proteins (via transduction) to your target cells. You can learn about all of our ProteoTuner Shield Systems at **takarabio.com**

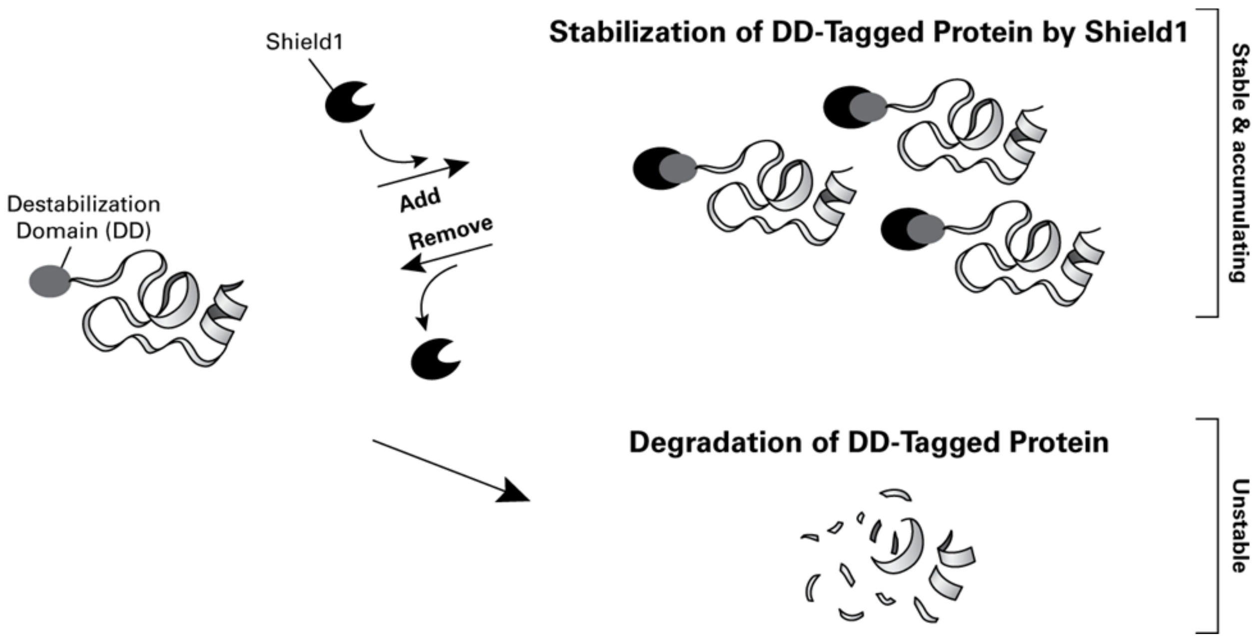

Figure 1. Ligand-dependent, targeted, and reversible protein stabilization. A small destabilization domain (DD; grey) is fused to a target protein of interest. The small membrane-permeable ligand Shield1 (black) binds to the DD and protects it from proteasomal degradation. Removal of Shield1, however, 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 Lentiviral ProteoTuner Expression System

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

1. Create and test Lenti-X ProteoTuner constructs containing your gene of interest (GOI).
   a. Clone your gene of interest into a Lenti-X ProteoTuner fusion vector (such as a pLVX-PTuner or pLVX-PTunerC) or a Lenti-X ProteoTuner reporter vector (such as pLVX-DD-ZsGreen1, pLVX-DD-Am Cyan1, or pLVX-DD-td Tomato) using fast, easy In-Fusion® HD cloning (Section V), or a standard ligation method.

      NOTE: All the Lenti-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 Lenti-X ProteoTuner-GOI constructs (Section VI).
2. Create Lenti-X ProteoTuner cell lines expressing your protein of interest.
   a. Produce supernatants containing lentiviral particles that express your protein of interest by transfecting your Lenti-X ProteoTuner-GOI constructs from Step 1 into the **Lenti-X 293T Cell Line** using the **Lenti-X Packaging Single Shots (VSV-G)** (Section VII).
   b. Determine the titer of your lentiviral supernatants (Section VIII).
   c. Infect (transduce) your target cells with your titered lentivirus (Section IX).
3. Perform protein stabilization and destabilization experiments using the Lenti-X ProteoTuner cell lines you created in Step 2 (Section X). See Section I.C for an overview of these experiments.

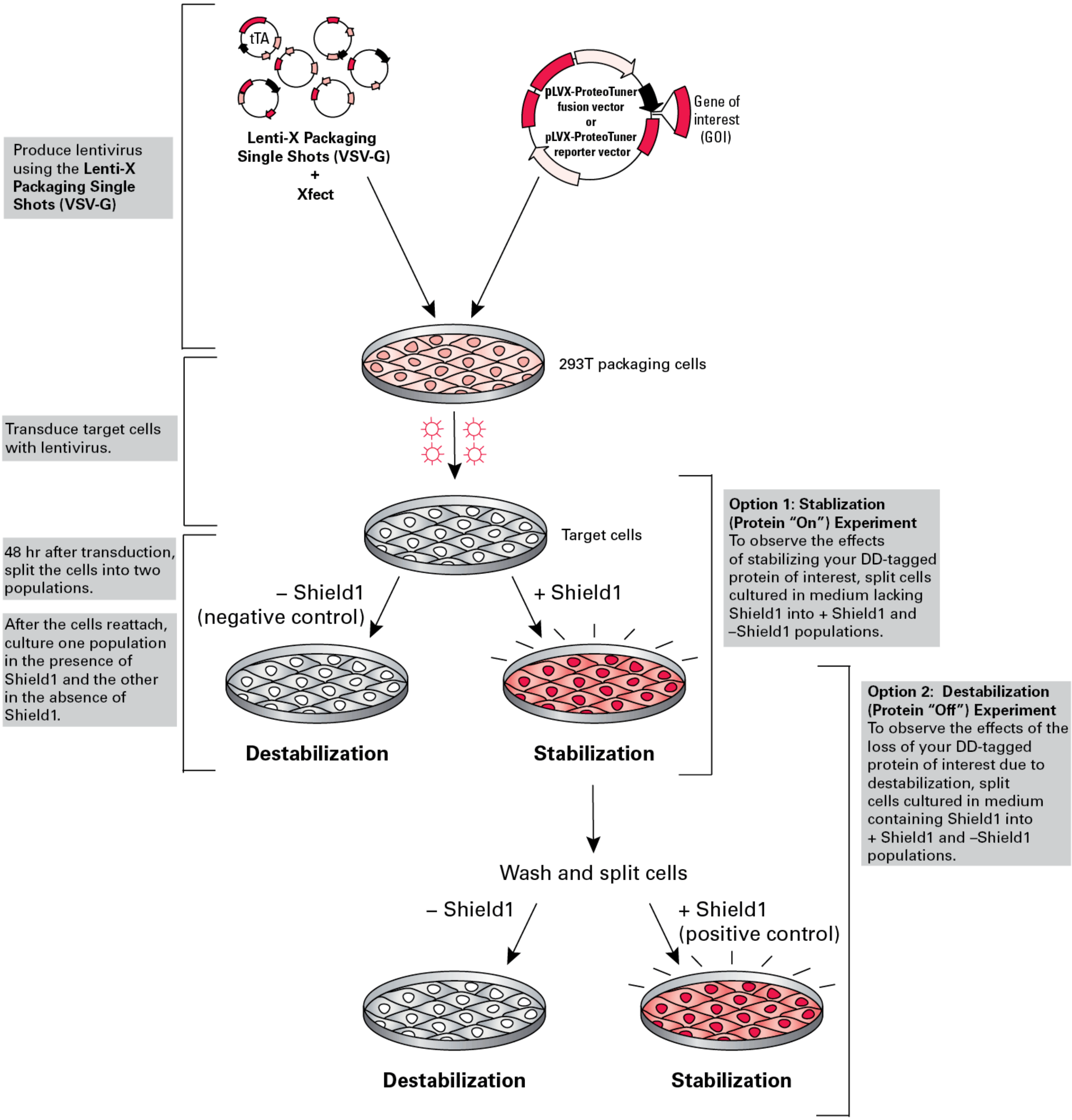

Figure 2. Establishing an expression system in target cells with Lenti-X ProteoTuner Shield Systems. The Lenti-X Packaging Single Shots (VSV-G) and 293T cells are used to generate a high-titer lentiviral supernatant from a Lenti-X ProteoTuner fusion vector or a Lenti-X ProteoTuner reporter vector which contains your gene or promoter of interest. Target cells are then transduced with these packaged lentiviral 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 Lenti-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 timecourse 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

Lenti-X ProteoTuner Shield System N (Cat. No. 632173)

- pLVX-PTuner Vector (20 µg) (Cat. No. 632174; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

Lenti-X ProteoTuner Shield System C (Cat. No. 631074)

- pLVX-PTunerC Vector (20 µg) (Cat. No. 631075; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

Lenti-X ProteoTuner Shield System N (w/ ZsGreen1) (Cat. No. 632175)

- pLVX-PTuner Green Vector (20 µg) (Cat. No. 632176; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

B. On Demand Reporter Systems

Lenti-X DD Green Reporter System (Cat. No. 631751)

- Lenti-X DD-ZsGreen1 Vector Set (Cat. No. 631752; not sold separately)
  - pLVX-DD-ZsGreen1 Reporter Vector (20 µg)
  - pLVX-DD-ZsGreen1 Control Vector (20 µg)
- Lenti-X Packaging Single Shots (VSV-G) (16 rxns) (Cat. No. 631275)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)
- Lenti-X GoStix™ (Sample) (3 tests) (Cat. No. 631242; not sold separately)

Lenti-X DD Cyan Reporter System (Cat. No. 631748)

- Lenti-X DD- AmCyan1 Vector Set (Cat. No. 631749; not sold separately)
  - pLVX-DD-AmCyan1 Reporter Vector (20 µg)
  - pLVX-DD-AmCyan1 Control Vector (20 µg)
- Lenti-X Packaging Single Shots (VSV-G) (16 rxns) (Cat. No. 631275)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)
- Lenti-X GoStix (Sample) (3 tests) (Cat. No. 631242; not sold separately)

Lenti-X DD Red Reporter System (Cat. No. 631753)

- Lenti-X DD-tdTomato Vector Set (Cat. No. 631754; not sold separately)
  - pLVX-DD-tdTomato1 Reporter Vector (20 µg)
  - pLVX-DD-tdTomato1 Control Vector (20 µg)
- Lenti-X Packaging Single Shots (VSV-G) (16 rxns) (Cat. No. 631275)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)
- Lenti-X GoStix (Sample) (3 tests) (Cat. No. 631242; not sold separately)

III. Additional Materials Required

A. Shield1

Each Lenti-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 Lenti-X 293T 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 ProteoTuner cell lines.
- 6-well, 12-well, and 24-well cell culture plates; 10 cm cell culture dishes

D. Lenti-X Packaging Single Shots (VSV-G)

This 4th generation lentiviral packaging system can generate lentiviral titers that are superior to most other commercially available lentiviral packaging systems. The concerted effects of multiple components in an optimized five-vector plasmid mix allow **Lenti-X 293T Cells** (Section III.E) to produce the highest amounts of safe, replication-incompetent lentivirus (see **takarabio.com**).

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

E. Lenti-X 293T Cell Line

Getting the most from any lentiviral packaging system requires a host 293T cell line that transfects easily and supports high-level expression of viral proteins. Our Lenti-X 293T Cell Line was clonally selected to meet these requirements, allowing you to produce the highest possible lentiviral titers when combined with the Lenti-X Packaging Single Shots (VSV-G).

Cat. No.	Cell Line	Size
632180	Lenti-X 293T Cell Line	1 ml

F. Antibiotics for Selecting Stable Cell Lines

Table 1. Recommended antibiotic concentrations

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

* The appropriate dose must be determined empirically for your specific cell line.

G. Lentiviral Titer Determination

For accurate and consistent transductions, we highly recommend titrating your lentiviral stocks. Various technologies are available from Takara Bio; visit **takarabio.com** for details.

Cat. No.	Lentiviral Titration Technology	Size
632200	Lenti-X p24 Rapid Titer Kit	96 rxns
631235	Lenti-X qRT-PCR Titration Kit	200 rxns
631243	Lenti-X GoStix	20 tests

H. Lentivirus Concentration

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

Cat. No.	Concentrator	Size
631231	Lenti-X Concentrator	100 ml
631232	Lenti-X Concentrator	500 ml

I. 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

J. Xfect™ Transfection Reagent

Xfect Transfection Reagent provides high transfection efficiency and low cytotoxicity for most commonly used cell types, including 293T cells.

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

K. In-Fusion® HD Cloning System

In-Fusion Cloning technology is a revolutionary technology that permits highly efficient, seamless, and directional cloning.
For more information, visit **www.clontech.com/infusion**

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

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

B. Safety Guidelines for Working with Lentiviruses

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

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

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

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

Biosafety in Microbiological and Biomedical Laboratories. *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 lentiviruses.

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 pLVX-DD 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 **www.clontech.com/infusion**

NOTE: Stellar Competent Cells (Section III.L) are recommended by Takara Bio for cloning of lentiviral 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.

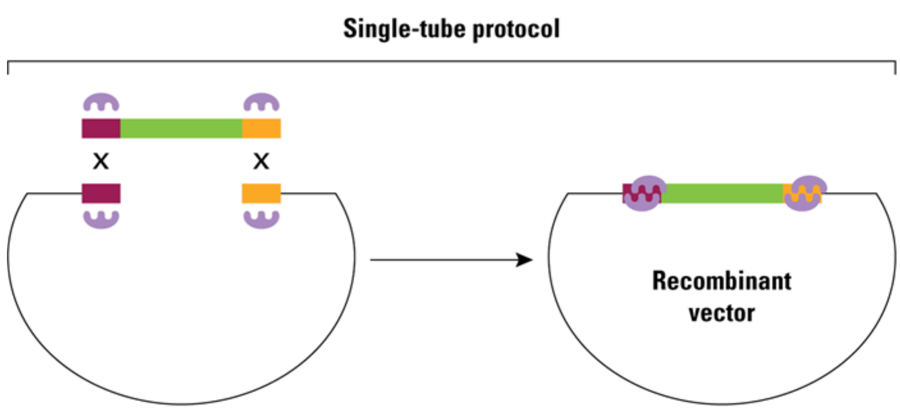

Figure 3. The In-Fusion HD Single-Tube Cloning Protocol.

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 Lenti-X ProteoTuner Constructs

Prior to lentiviral production, your lentiviral 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 or the DD-tagged fluorescent protein reporter 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.J) to transfect your target cells with 5 µg of the Lenti-X ProteoTuner vector construct of interest. Follow the **Xfect Transfection Reagent Protocol-At-A-Glance**. (Locate this protocol by searching at **www.clontech.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 Lentivirus from the Lenti-X Vectors

A. General Considerations

1. Optimizing Lentiviral Titer

To obtain the highest titers from the Lenti-X Packaging Single Shots (VSV-G), use the Lenti-X 293T Cell Line (Section III.E) and adhere strictly to the following protocol, especially with respect to:

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

2. Required Materials & Precautions

All Xfect transfection reagents, volumes, and conditions are optimized for use with Lenti-X Vectors, the Lenti-X Packaging Single Shots (VSV-G), and Lenti-X 293T cells. For optimal results, it is also necessary to use:

- Tetracycline-free FBS
- 10-cm culture plates
- Transfection-grade DNA
- Be sure to use Tetracycline-free FBS*, both in the transfection medium (**Step 1**) and in the medium used to collect the virus (**Step 9**).

* Tetracycline-contaminated serum is detrimental to the expression of essential packaging components in the Lenti-X Packaging System.

IMPORTANT: Perform all steps in a sterile tissue culture hood. Lentivirus requires the use of a Biosafety Level 2 facility. Recombinant pseudotyped lentiviruses packaged from HIV-1-based vectors are capable of infecting human cells. Know and use appropriate safety precautions (see Section IV.B).

3. Optimal Cell Densities at Transfection & Harvest

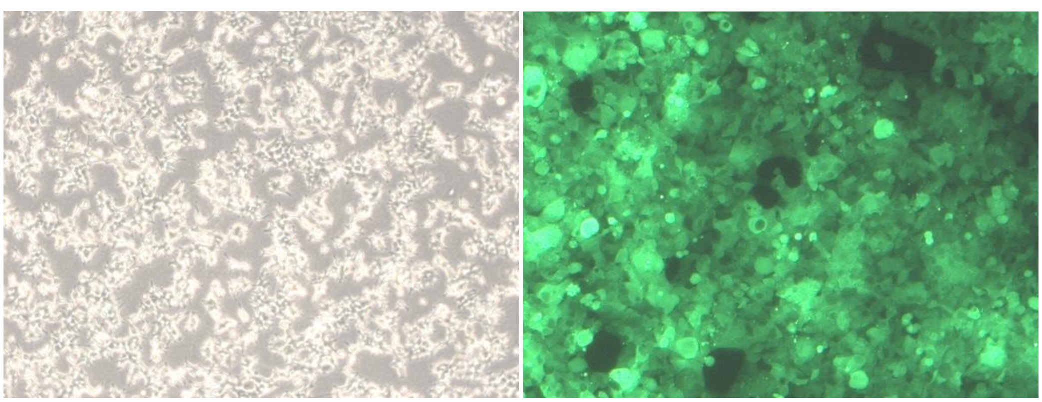

Figure 4. Optimal density of Lenti-X 293T cells at point of transfection (left panel) and harvest time (right panel), shown here using a transfer vector containing ZsGreen1.

B. Protocol: Transfecting Lentiviral Vectors into Lenti-X 293T Packaging Cells

NOTE: To achieve the highest titers, it is critical to pay close attention to the transfection. You may want to perform a cotransfection with a lentiviral vector that contains a fluorescent protein. You should be able to achieve transfection efficiencies of greater than 90%.

1. Approximately 24 hr before transfection, seed 4–5 x 10^6 Lenti-X 293T cells/10-cm plate, in 8 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 5. The cells should be 80–90% confluent at the time of transfection.
2. In a sterile microfuge tube, dilute 7.0 µg of your lentiviral vector plasmid DNA with sterile water to a final volume of 600 µl. Mix thoroughly by vortexing.

   NOTE: Always dilute your DNA in water prior to adding it to a Lenti-X Packaging Single Shot. Do not add water and DNA separately (since undiluted DNA should not be mixed with Xfect Transfection Reagent).
3. Add the 600 µl of diluted DNA to a tube of Lenti-X Packaging Single Shots, replace the cap, and vortex well at a high speed for 20 seconds. The pellet should dissolve completely.

   NOTE: In some cases some insoluble material may be visible after vortexing. This material does not have a negative effect on transfection efficiency or virus yields.
4. Incubate the samples for 10 min at room temperature to allow nanoparticle complexes to form. After the 10 min incubation, centrifuge the tube for 2 seconds to bring the sample to the bottom of the tube.

   NOTE: Sample tubes can be inserted into 1.5-ml microfuge tubes for a brief centrifugation.
5. Add the entire 600 µl of nanoparticle complex solution dropwise to the 8 ml of cell culture prepared in Step 1. Rock the plate gently back and forth to mix.

   NOTE: It is normal for the medium to change color slightly upon addition of nanoparticle complex solution.
6. Incubate the cells at 37°C supplied with 5% CO_2.

   NOTE: A 4-hr incubation with Xfect-DNA nanoparticles is sufficient for optimal transfection. Incubation may be continued overnight for convenience but does not generally increase transfection efficiency or titer.
7. After 4 hr to overnight, add an additional 6 ml of fresh complete growth medium and incubate at 37°C, 5% CO_2 for an additional 24–48 hr. Virus titers will generally be highest 48 hr after the start of transfection.
8. Harvest the lentiviral supernatants and pool similar stocks, if desired (a 48-hr sample may be stored at 4°C until a 72-hr sample is harvested).

   CAUTION: Supernatants contain infectious lentivirus.

   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 polysulfone (low protein binding), instead of nitrocellulose. Nitrocellulose binds proteins present in the membrane of lentivirus and destroys the virus.
9. Verify virus production using Lenti-X GoStix (Section III.G; see **Lenti-X GoStix (50 & 200) Protocol-At-A-Glance** at **www.clontech.com/manuals** for details). Alternatively, titrate the virus stock, then use the virus to transduce target cells, or store at –80°C.

   NOTE: Titers can drop as much as 2–4 fold with each freeze-thaw cycle.
10. For protocols describing how to transduce your target cells or create frozen stocks, see the Lenti-X Lentiviral Expression Systems User Manual at **www.clontech.com/manuals**

VIII. Lentivirus Titration

A. Summary

1. Instant Qualitative Titer Test

You can assess the quality of your lentivirus stock in 30 seconds with Takara Bio's **Lenti-X GoStix** (Cat. Nos. 631241, 631243 & 631244). The GoStix detect lentiviral p24 in only 20 µl, and can be used to determine whether virus production is within a usable range or for selecting the best time to harvest your virus. A 3-prep sample is supplied for free with the **Lenti-X Packaging Single Shots (VSV-G)** (Section III.D).

2. Quantitative Titer Test

a. **Determining the viral titer** is necessary to obtain the following information:

- Confirmation that viral stocks are viable
- The proper transduction conditions for your particular cell type by adjusting the MOI for the desired transduction efficiency. MOI = No. of infectious virus particles per target cell at the time of infection.
- The maximum number of target cells that can be transduced by a given virus volume.

b. To transduce using a known multiplicity of infection (MOI), it is necessary to titrate your lentiviral stocks. We recommend the **Lenti-X qRT-PCR Titration Kit** (Cat. No. 631235) or **Lenti-X p24 Rapid Titer Kit** (Cat. No. 632200) for very rapid quantitative titrations of virus stocks (~4 hr), or a standard method that relies on infection.

c. The **standard viral titration protocol** consists of infecting cells with serial dilutions of the stock, selecting for stable transductants with antibiotic, and counting the resulting cell colonies (Section VIII.B).

- Freshly harvested virus can be titrated immediately, or frozen in aliquots at –80°C and then titrated. Note that each freeze-thaw cycle can reduce the functional titers of infectious virus by up to 2–4 fold.
- Absolute titers will depend heavily on the cell type used for titration, and there may be significant differences between the titer values determined in cells typically used for lentiviral titration (i.e., HT-1080) and the number of target cells transduced by the titrated virus. However, titrations serve to determine the relative virus content of different viral stocks prepared from different vectors.

B. Protocol: Determining Viral Titer by Colony Formation

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

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

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

3. Prepare filtered viral supernatant from packaging cells (Section VII). This is the virus stock.
4. Prepare six 10-fold serial dilutions of the virus stock as follows:
   a. Add 1.35 ml of medium containing Polybrene (Step 2) to each of six sterile and numbered 1.5 ml microfuge tubes.
   b. Add 150 µl of the virus stock (Step 3) to the tube 1. Mix.
   c. Transfer 150 µl tube 1 to tube 2 and mix. Continue making serial dilutions by transferring 150 µl from each successive dilution into the next prepared tube.
5. Infect the HT-1080 cells by adding 1 ml of each viral dilution (Step 4) to each appropriate well. The final Polybrene concentration will be 4 µg/ml in ~3 ml. Centrifuge the cultures to improve infection efficiency*.

   * **NOTE:** CULTURE CENTRIFUGATION INCREASES INFECTION EFFICIENCY. Centrifuging the plate at 1,200*g* for 60–90 min at 32°C can significantly increase infection efficiency. A room temperature centrifuge is acceptable if a 32°C unit is not available.
6. After infecting for 8–24 hours, remove supernatants and subject the cells to puromycin selection using the selection concentrations that are optimal for your cell line (Section III.F).
7. Allow colonies to form for 7–14 days. Stain the colonies with 1% crystal violet solution (in 10% ethanol) and count.
8. The titer of virus corresponds to the number of colonies generated by the highest dilution, multiplied by the dilution factor. For example, the presence of 4 colonies in the 10^6 dilution would represent a viral titer of 4 x 10^6 colony forming units.

IX. Transducing Target Cells with a Lenti-X ProteoTuner Lentivirus

A. Protocol: Transducing Target Cells with Lenti-X ProteoTuner Lentiviruses

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 Lenti-X ProteoTuner lentiviral stocks, or use filtered virus stocks freshly prepared from packaging cells (Section VII). Mix gently, but do not vortex.
3. Add Polybrene to the cell cultures to obtain the desired final concentration during the transduction step (e.g., 4 µg/ml).

   NOTE: Lenti-X Accelerator and **RetroNectin** (Section III.I) 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 Lenti-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 min 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 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 min 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) to ensure complete removal of Shield1.
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.

- Shield-1 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).

XI. References for ProteoTuner

Armstrong, C. M. & Goldberg, D. E. An FKBP destabilization domain modulates protein levels in Plasmodium falciparum. *Nat. Methods* **4,** 1007–1009 (2007).

Banaszynski, L. A., Chen, L. chun, Maynard-Smith, L. A., Ooi, A. G. L. & Wandless, T. J. A Rapid, Reversible, and Tunable Method to Regulate Protein Function in Living Cells Using Synthetic Small Molecules. *Cell* **126,** 995–1004 (2006).

Banaszynski, L. a, Sellmyer, M. a, Contag, C. H., Wandless, T. J. & Thorne, S. H. Chemical control of protein stability and function in living mice. *Nat. Med.* **14,** 1123–1127 (2008).

Banaszynski, L. a & Wandless, T. J. Conditional control of protein function. *Chem. Biol.* **13,** 11–21 (2006).

Berdeaux, R. *et al.* SIK1 is a class IIHDAC kinase that promotes survival of skeletal myocytes. *Nat. Med.* **13,** 597–603 (2007).

Grimley, J. S., Chen, D. A., Banaszynski, L. A. & Wandless, T. J. Synthesis and analysis of stabilizing ligands for FKBP-derived destabilizing domains. *Bioorganic Med. Chem. Lett.* **18,** 759–761 (2008).

Herm-Götz, A. *et al.* Rapid control of protein level in the apicomplexan Toxoplasma gondii. *Nat. Methods* **4,** 1003–1005 (2007).

Maynard-Smith, L. A., Chen, L. C., Banaszynski, L. A., Ooi, A. G. L. & Wandless, T. J. A directed approach for engineering conditional protein stability using biologically silent small molecules. *J. Biol. Chem.* **282,** 24866–24872 (2007).

Schoeber, J. P. H. *et al.* Conditional fast expression and function of multimeric TRPV5 channels using Shield-1. *Am. J. Physiol. Renal Physiol.* **296,** F204–F211 (2009).

Sellmyer, M. A., Thorne, S. H., Banaszynski, L. A., Contag, C. H. & Wandless, T. J. A general method for conditional regulation of protein stability in living animals. *Cold Spring Harb. Protoc.* **2009,** pdb.prot5173 (2009).

Appendix A. Troubleshooting Guide

Table 2. 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. Lenti-X 293T Packaging Cells		
Poor viability upon thawing	Improper thawing techniques	Use thawing procedure in Appendix B, and/or consult the Lenti-X 293T Cell Line Protocol-at-a-Glance
	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 Lenti-X 293T cells.
C. Virus Production		
Poor transfection efficiency (as determined by GOI or marker expression in the Lenti-X 293T 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 50–80% confluency. See Section VII.
	Transfection is toxic to cells	Use the optimized conditions provided in Section VII.
	Cells harvested or analyzed too soon after transfection	Wait 48 hr after transfection for maximal expression of GOI or marker to determine efficiency.
Low titers (<10^5 cfu/ml)	Virus was harvested too early	Harvest virus 48–72 hr after the start of transfection.
	Vector is too large	The limit for efficient packaging function is 9.7 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 Lenti-X Concentrator (Section III.H) to increase your available titer up to 100-fold without ultracentrifugation.
	Poor transfection efficiency	Follow the protocol in Section VII.B. Be sure to use 5 µg of transfection-grade plasmid.
	Low viability of target cells during transduction	Optimize culture conditions for target cells prior to infection
		Packaging cell line-conditioned media may affect cell growth; dilute viral supernatant or shorten exposure time to viral supernatant. Consider using RetroNectin reagent and the RetroNectin-Bound Virus transduction protocol or purify your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. Nos. 631233 & 631234).
		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; or, purify your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. Nos. 631233 & 631234).
E. Establishment of Stable Cell Lines		
Untransduced cells do not die at the high antibiotic concentration established via titration in Section III.F	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.

Appendix B. Lenti-X ProteoTuner Vector Maps

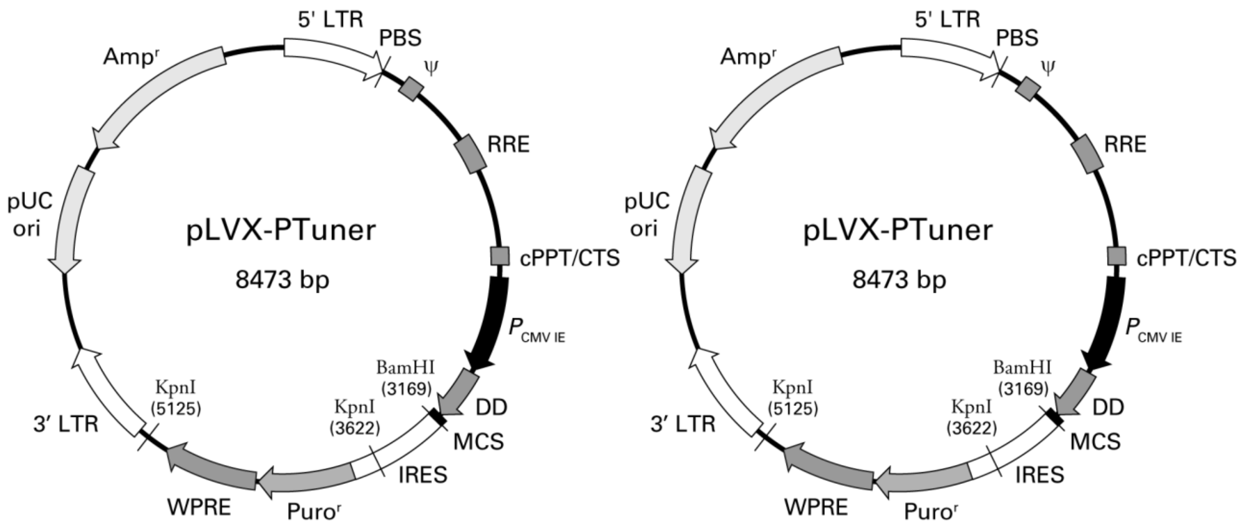

Figure 5. pLVX-PTuner and pLVX-PTunerC Vector maps.
For more detailed vector information, see **takarabio.com**

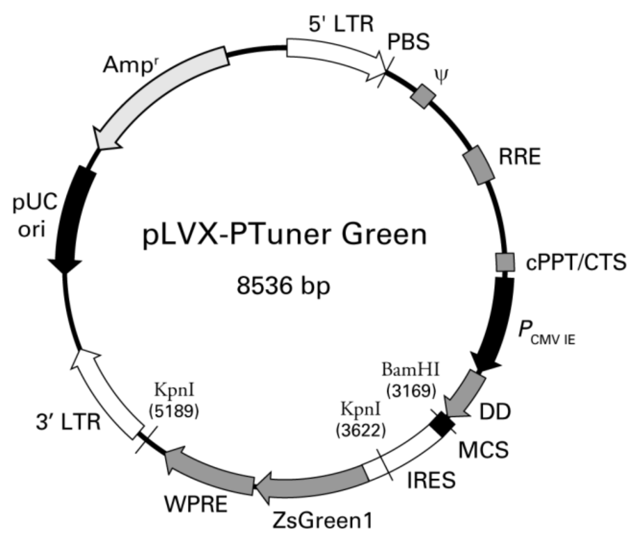

Figure 6. pLVX-PTuner Green Vector map.
For more detailed vector information, see **takarabio.com**

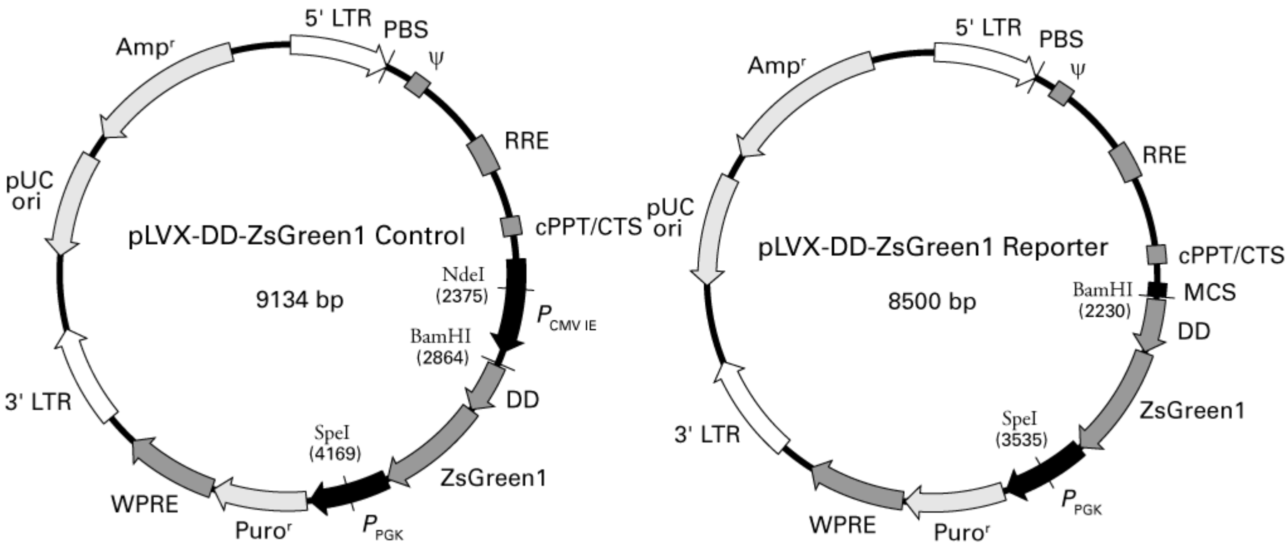

Figure 7. pLVX-DD-ZsGreen1 Control and pLVX-DD-ZsGreen1 Reporter Vector maps.
For more detailed vector information, see **takarabio.com**

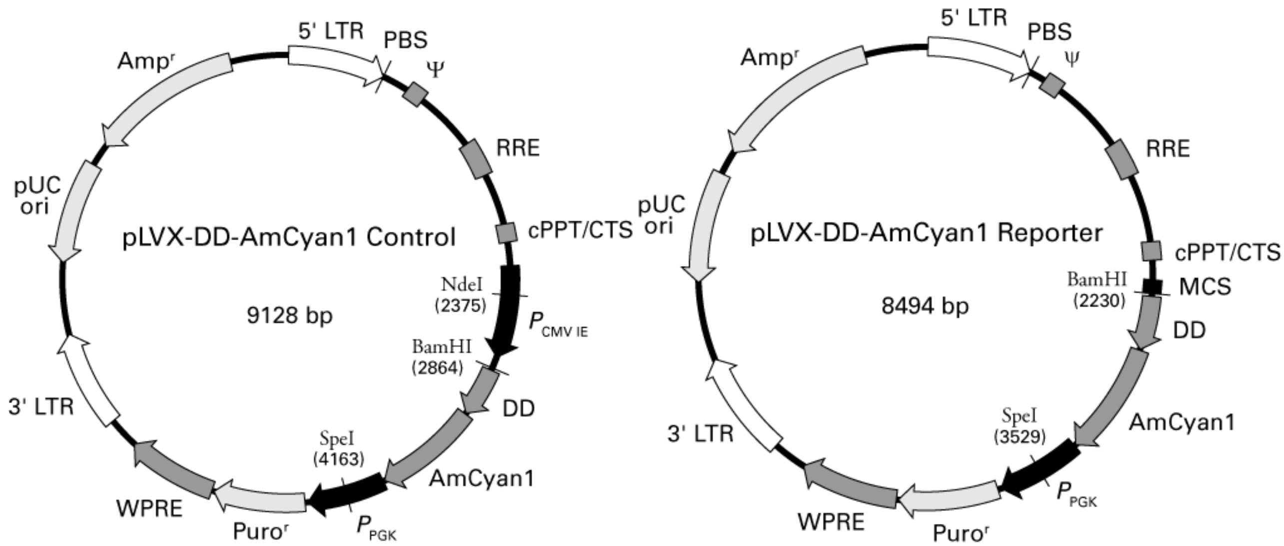

Figure 8. pLVX-DD-AmCyan1 Control and pLVX-DD-AmCyan1 Reporter Vector maps.
For more detailed vector information, see **takarabio.com**

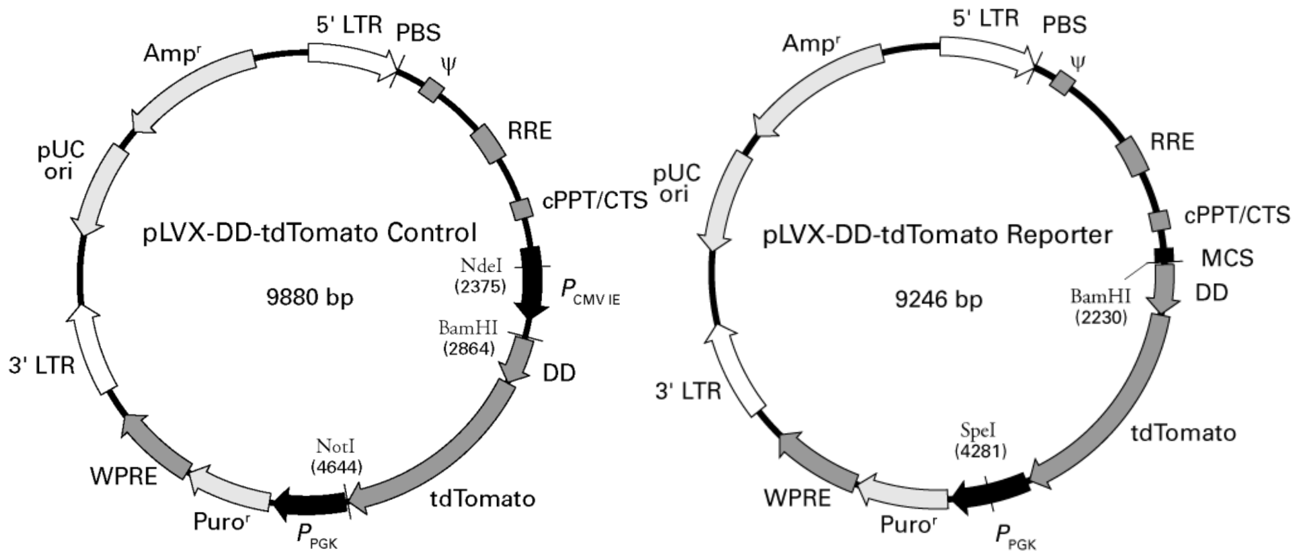

Figure 9. pLVX-DD-tdTomato Control and pLVX-DD-tdTomato Reporter Vector maps.
For more detailed vector information, see **takarabio.com**

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: 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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