Clontech Laboratories, Inc.

Lenti-X™ iDimerize™ Inducible Homodimer System User Manual

Cat. Nos. 635072, 635088, 635090, 635059, 635058, 635069, 632622 (111115)

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Table of Contents

Table of Figures

Table of Tables

I. Introduction

A. Summary

ARGENT cell signaling regulation kits from ARIAD are now available exclusively from Clontech, as the iDimerize Inducible Dimerization Systems. This manual describes the **Lenti-X iDimerize Inducible Homodimer System** (Cat. No. 635072), which contains reagents for bringing together two molecules of an engineered fusion protein by adding a small molecule "dimerizer"—and introducing these dimerization components into a wide variety of cell types via Clontech's lentiviral expression technology—using the **Lenti-X Packaging Single Shots (VSV-G)** (available separately; Cat. Nos. 631275 & 631276). The kit can be used to create conditional alleles of receptors, signaling molecules, and any other protein normally regulated by protein-protein interactions, allowing complex cellular events to be brought under small molecule control. The Lenti-X iDimerize Inducible Homodimer System contains homodimerization components consisting of lentiviral expression vectors encoding the homodimerization domain, and the B/B homodimerization ligand. For a comparison of iDimerize vs. ARGENT nomenclature, see Section II of this manual.

B. Overview of Dimerization

Many cellular processes are triggered by the induced interaction, or "dimerization", of signaling proteins (Crabtree, *et al.*, 1996). Examples include the clustering of cell surface receptors by extracellular growth factors, and the subsequent stepwise recruitment and activation of intracellular signaling proteins. A chemical inducer of dimerization, or "dimerizer", is a cell-permeant organic molecule with two separate motifs that each bind with high affinity to a specific protein module tagged onto the protein of interest. Any cellular process activated by protein-protein interactions can in principle be brought under dimerizer control, by fusing the protein(s) of interest to the binding module. Addition of the dimerizer then brings the chimeric signaling protein subunits into very close proximity to each other, mimicking the activation of the cellular event that the protein of interest controls. Different types of dimerizer (Figures 1 & 2) are available:

- **Homodimerizers** incorporate two identical binding motifs, and can therefore be used to induce self-association of a single signaling domain or other protein of interest. The Lenti-X iDimerize Inducible Homodimer System provides the homodimerizer ligand—as well as DNA vectors for making fusion proteins consisting of the dimerization domain fused to a single signaling protein. This dimerization domain (DmrB) is able to bind to the B/B Homodimerizer ligand.
- **Heterodimerizers** contain two different binding motifs, allowing the dimerization of two different proteins of interest when each is fused to a different dimerization domain (DmrA and DmrC) recognized by the heterodimerizer. For applications requiring heterodimerization, we provide a separate kit, the **Lenti-X iDimerize Inducible Heterodimer System** (Cat. No. 635074), that includes the heterodimerizer ligand.
- **Reverse dimerizers** promote the dissociation of proteins that have been engineered to self-associate because they are tagged with "conditional aggregation domains" (DmrD), as in our **Lenti-X iDimerize Reverse Dimerization System** (Cat. No. 635076).

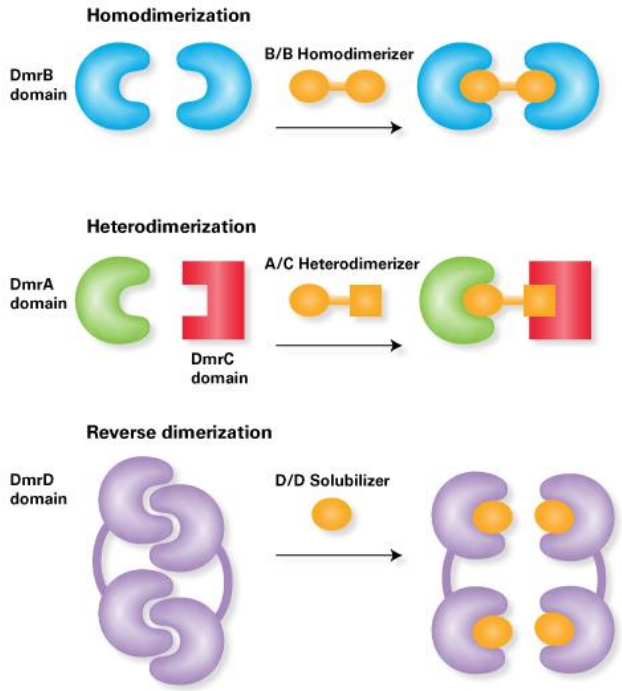

Figure 1. Different iDimerize kits use different dimerization strategies: homodimerization, heterodimerization, and reverse dimerization. Separate kits are available from Clontech.

C. Lenti-X iDimerize Inducible Homodimer System

The Lenti-X iDimerize Inducible Homodimer System contains reagents that induce the self-association of a single protein by the addition of a small molecule, the "B/B Homodimerizer". The system consists of the B/B Homodimerizer and three lentiviral vectors used to create fusion proteins that contain DmrB dimerization domains—and either lack a subcellular localization domain (pLVX-Hom-1), or include a plasma membrane targeting domain (pLVX-Hom-Mem1) or a nuclear targeting domain (pLVX-Hom-Nuc1). These optimized Lenti-X constructs, in combination with the Lenti-X HTX Packaging System (which allows for packaging and delivery of high-titer lentivirus), enable these chimeric proteins to be expressed in a wide range of cell types. The addition of B/B Homodimerizer to live cells expressing a DmrB-tagged fusion protein induces self-association of the fusion protein by promoting the interaction of the dimerization domains (Figure 2; Clackson *et al.*, 1998).

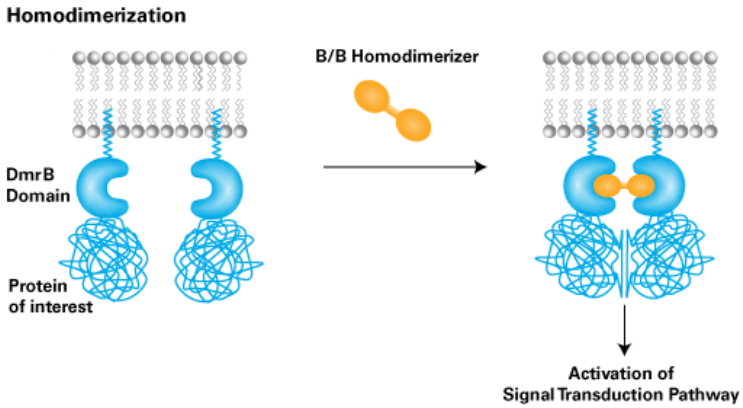

Figure 2. Controlling signal transduction using regulated homodimerization.

II. List of Components

Store all components at -20°C.

- 1 each Lenti-X iDimerize Inducible Homodimer Vector Set
  - pLVX-Hom-1 Vector (10 µg)
  - pLVX-Hom-Mem1 Vector (10 µg)
  - pLVX-Hom-Nuc1 Vector (10 µg)
- 500 µl B/B Homodimerizer (0.5 mM)
  (also sold separately as Cat. Nos. 635059, 635058, 635069 & 632622—see Section III.A)

Table 1. ARGENT and Clontech Nomenclature for Lenti-X iDimerize Inducible Homodimer System Components

Ligand Name in ARIAD Kit	Ligand Name in Clontech Kit
AP20187	B/B Homodimerizer
Dimerization Domain Name in ARIAD Kit	**Dimerization Domain Name in Clontech Kit**
F_V	DmrB

NOTES:

- The B/B Homodimerizer is so named because it induces dimerization of two proteins that contain the DmrB dimerization domain.

III. Additional Materials Required

A. B/B Homodimerizer

Each Lenti-X iDimerize Inducible Homodimer System includes 500 µl B/B Homodimerizer (0.5 mM; see Section II). Additional B/B Homodimerizer can also be purchased separately in the following sizes:

Cat. No.	Product Name	Size
635059	B/B Homodimerizer (0.5 mM)	5 x 500 µl
635058	B/B Homodimerizer*	5 mg
635069		25 mg
632622		4 x 25 mg

*Supplied in a dry-down format.

B. B/B Washout Ligand

B/B Washout Ligand is a membrane-permeant ligand that dissociates protein interactions induced by the B/B Homodimerizer. It dissociates these interactions with a $T_{1/2}$ of ~10 minutes after adding it to target cells treated with B/B Homodimerizer.

Cat. No.	Product Name	Size
635088	B/B Washout Ligand (0.5 mM)	500 µl

C. DmrB Monoclonal Antibody

The DmrB Monoclonal Antibody recognizes the DmrB binding domain expressed using iDimerize Inducible Homodimer Systems, and is recommended for Western blot analysis.

Cat. No.	Product Name	Size
635090	DmrB Monoclonal Antibody (0.5 µg/µl)	100 µg

D. 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 iDimerize cell lines.
- 6-well, 12-well, and 24-well cell culture plates; 10 cm cell culture dishes

E. High-Titer Packaging System

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, pre-aliquoted and lyophilized with Xfect™ Transfection Reagent, allow **Lenti-X 293T Cells** (Section III.F) to produce the highest amounts of safe, replication-incompetent lentivirus (see **www.clontech.com**).

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

F. Lenti-X 293T Cells

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

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

G. Antibiotics for Selecting Stable Cell Lines

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

[1] The appropriate dose must be determined empirically for your specific cell line.

H. Lentiviral Titer Determination

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

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

I. Lentivirus Concentration

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

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

J. Transduction Enhancers

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

- Lenti-X Accelerator is a magnetic bead-based technology designed to accelerate lentiviral and retroviral transduction experiments; visit **www.clontech.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 **www.clontech.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

K. Xfect Transfection Reagent

Xfect Transfection Reagent provides high transfection efficiency and low cytotoxicity for most commonly used cell types.

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

L. In-Fusion® HD Cloning System

In-Fusion 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
639645	In-Fusion HD Cloning System (10 rxns)
639646	In-Fusion HD Cloning System (50 rxns)
639647	In-Fusion HD Cloning System (100 rxns)

M. Stellar™ Competent Cells

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

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

IV. Creating Fusion Proteins Containing Dimerization Domains

A. General Considerations

1. Controlling localization of fusion proteins

NOTE: See vector information at **www.clontech.com** for additional details regarding each MCS.

a. Fusion proteins are created by cloning the gene encoding your protein of interest into the MCS on either the 5' or the 3' end of the DmrB domain(s) shown in Figure 3.

b. If the sequence encoding your protein of interest is cloned into the 5' MCS, it must be cloned in-frame with the downstream DmrB domain and must not contain a stop codon.

c. If the sequence encoding your protein of interest is cloned into the 3' MCS, it needs to be cloned in-frame with the upstream DmrB domain encoding gene. This will also enable the stop codon at the end of the 3'MCS to terminate translation should you choose not to include a stop codon at the end of your cloned sequence.

d. When cloning your gene of interest into the pLVX-Hom-Mem1 vector, make certain that the N-myr-signal is located on the N-terminus of the DmrB fusion protein. The N-myr-signal is NOT functional if localized in the middle or at the C-terminus of a protein of interest. Your gene must either be cloned into the 3'MCS, or if cloned into the 5' MCS, which is located upstream of the N-myr signal, you must include a separate N-myr signal sequence on the forward primer used to amplify your gene of interest.

   The N-myr sequence is as follows:
   5'- atg ggg agt agc aag agc aag cct aag gac ccc agc cag cgc-3'.

e. Fusion proteins localize to the cytoplasm when created using pLVX-Hom-1 (which contains no targeting signal), to the nucleus when created using pLVX-Hom-Nuc1, and to the inner leaflet of the plasma membrane when created using pLVX-Hom-Mem1 (See **1.d** regarding the cloning strategy necessary to achieve membrane localization of a protein of interest).

2. How many DmrB domains should I use?

The number of DmrB domains best suited for each application varies. Fusion to a single DmrB domain is generally preferred if dimer formation is sufficient to induce the desired signaling event. Fusion to multiple tandem DmrB domains may be required when the event studied requires the formation of higher order oligomers. Often the optimal configuration is best determined empirically.

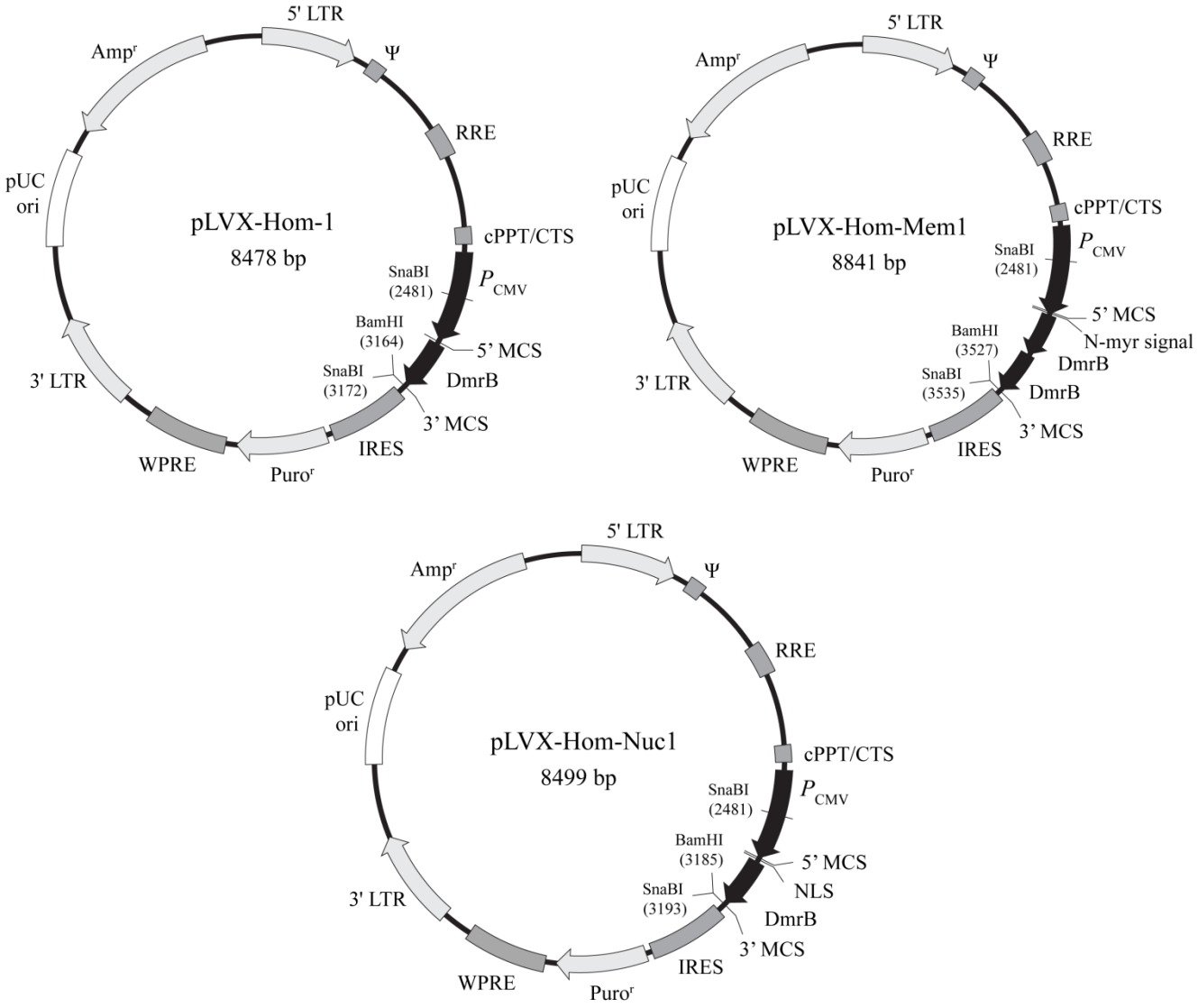

Figure 3. pLVX-Hom-1, pLVX-Hom-Mem1, and pLVX-Hom-Nuc1 Vector Maps.
For more detailed vector information, see **www.clontech.com**

B. Protocol: Creating Fusion Proteins using In-Fusion HD

We recommend using In-Fusion HD (Figure 4) for cloning your protein of interest into the pLVX-Hom-1, pLVX-Hom-Mem1, and pLVX-Hom-Nuc1 vectors. The technology is described at **www.clontech.com/infusion**

NOTE: Stellar Competent Cells (Section III.M) are recommended by Clontech 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.

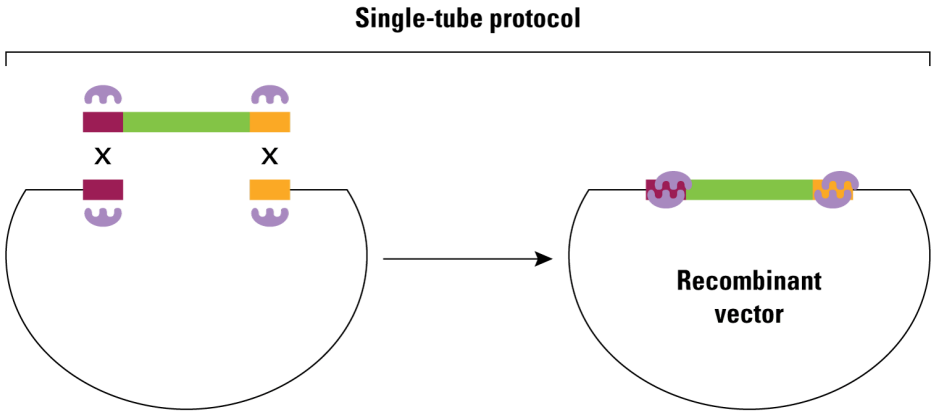

Figure 4. The In-Fusion HD Single-Tube Cloning Protocol.

V. 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 Homodimerizer Constructs

Prior to lentiviral production, your pLVX-Hom-1, pLVX-Hom-Mem1, or pLVX-Hom-Nuc1 vector construct, containing the gene of interest, should be tested for functionality by standard plasmid transient transfection. If transfected into the cell line of interest, the plasmid will express your DmrB fusion protein of interest in a transient fashion and can be tested for dimerization in response to B/B Homodimerizer. For your initial *in vitro* experiments, we recommend testing medium containing different concentrations of B/B Homodimerizer 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.K) to transfect your target cells with 5 µg of the pLVX-Hom-1, pLVX-Hom-Mem1, or pLVX-Hom-Nuc1 vector containing your gene of interest. Follow the **Xfect Transfection Reagent Protocol-At-A-Glance**. (Locate this protocol by searching at **www.clontech.com/manuals**).

2. At 12 hours after transfection, split transfected cells into different plates or separate wells of a 6-well plate, or your preferred plate format.

   To begin incubation of the transfected cells with B/B Homodimerizer at specific time intervals and concentrations, replace the medium in the plates containing the transfected cells with medium

containing the appropriate amount of B/B Homodimerizer, diluted as described below. Maintain at least one culture in medium containing no B/B Homodimerizer as a negative control.

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

a. Recommended B/B Homodimerizer Concentrations and Time Points

- Try B/B Homodimerizer concentrations between 0.1 nM and 500 nM for different lengths of time (30 minutes to 12+ hours) to determine the best experimental conditions.

b. General Guidelines for Preparing Medium Containing B/B Homodimerizer

- Dilute the supplied B/B Homodimerizer 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 B/B Homodimerizer: Dilute 10 µl of B/B Homodimerizer stock solution (500 µM) in 10 ml of medium to yield a final concentration of 500 nM.

- Working concentrations of B/B Homodimerizer can be obtained by adding it directly from ethanol stocks, or by diluting it serially in culture medium just before use.
- If you are making serial dilutions of B/B Homodimerizer 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.

3. After adding the medium containing B/B Homodimerizer at the appropriate concentration and for the appropriate length of time, the effect of dimerization can be analyzed with an assay that is appropriate for your experiment.

B. Protocol: Dissociating Dimerized Proteins with the B/B Washout Ligand

The B/B Washout Ligand (Cat. No. 635088) can be used to accomplish fast dissociation of protein interactions that were induced by the B/B Homodimerizer (Figure 5). When you remove the B/B Homodimerizer-containing medium from the cells and replace it with medium containing 1 µM B/B Washout Ligand, your dimerized proteins will dissociate from each other with a $T_{1/2}$ of ~10 to 12 minutes.

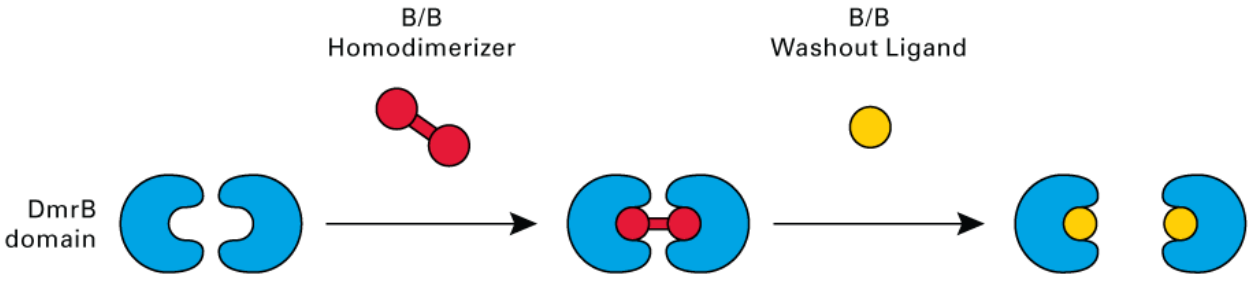

Figure 5. B/B Washout Ligand can be used to disrupt protein interactions that were induced by the B/B Homodimerizer.

1. Adherent cells

a. Remove the medium from your transfected and B/B Homodimerizer-treated cells.
b. Rinse cells with warm TC-grade PBS with Ca^{2+} and Mg^{2+}, or culture medium.
c. Add medium containing 1 µM B/B Washout Ligand to the cells. Total dissociation of dimerized proteins can be observed about 30 minutes after the medium change.
d. Analyze the effect of B/B Washout Ligand-induced protein dissociation with your method/assay of choice.

2. Suspension cells

a. Collect the cells via centrifugation.
b. Resuspend the cells in medium containing 1 µM B/B Washout Ligand. Total dissociation of dimerized proteins can be observed about 30 minutes after the medium change.
c. Analyze the effect of B/B Washout Ligand-induced protein dissociation with your method/assay of choice.

Results Obtained Using the B/B Washout Ligand to Dissociate Protein Dimers

Figure 6 shows the release of a B/B Homodimerizer-induced DmrB-DmrB interaction. Lentiviral delivery was used to create a stable HeLa cell line (mixed population) expressing the two parts of a "split" version of a red fluorescent protein. The two monomeric proteins are non-fluorescent until they are forced to undergo dimerization via the B/B Homodimerizer. When the B/B Washout Ligand was added, red fluorescence diminished quickly due to the dissociation of the split fluorescent protein (solid line). Simply replacing medium containing B/B Homodimerizer with medium without B/B Homodimerizer had only a minor dissociative effect (dashed line).

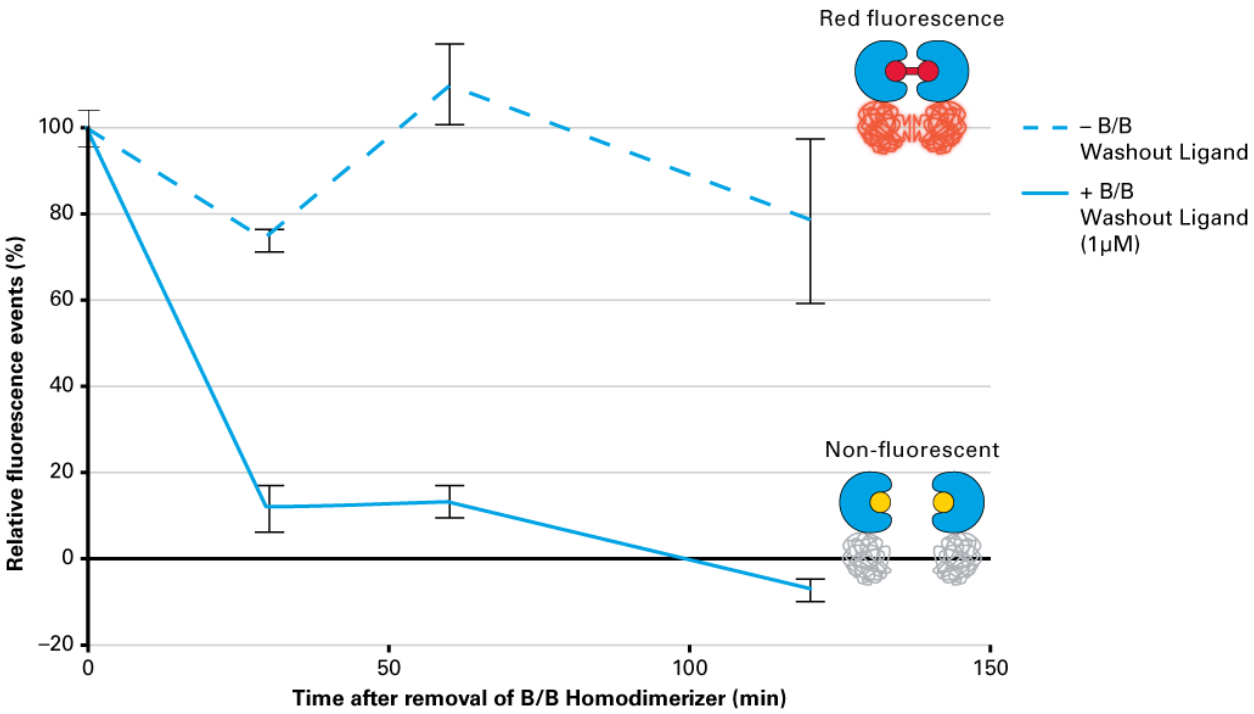

Figure 6. B/B Washout Ligand has a much more dissociative effect than simple removal of the B/B Homodimerizer. A stable HeLa cell line expressing DmrB-tagged versions of the two parts of a split red fluorescent protein was treated with 0.1 µM B/B Homodimerizer for 3 hr. The medium was then removed and replaced with medium ± 1 µM B/B Washout Ligand. After 30 min, the fluorescence level in the "+ B/B Washout Ligand" sample had dropped virtually to the background level, indicating that the two parts of the red fluorescent protein were completely dissociated from each other.

VI. Lentiviral Vector Guidelines & Protocol Overview

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 (BMBL), Fifth Edition (February 2007) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH.

Available on the web at **http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**

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

Summary of Biosafety Level 2:

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

- **Safety equipment:**
  - Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is unrecirculated
  - PPE: protective laboratory coats, gloves, face protection as needed
- **Facilities:**
  - Autoclave available for waste decontamination
  - Chemical disinfectants available for spills

C. Protocol Overview

The following steps are required to create an inducible homodimer expression system using lentivirus (Figure 5):

1. Clone your gene of interest into the pLVX-Hom-1, pLVX-Hom-Mem1, or pLVX-Hom-Nuc1 vector using fast, easy In-Fusion HD cloning (Section IV) or a standard ligation method.
2. Produce Lenti-X iDimerize lentiviral supernatants (lentivirus encoding your DmrB-tagged protein of interest) by transfecting the lentiviral vectors from Step 1 into Lenti-X 293T Packaging Cells using the Lenti-X Packaging Single Shots (VSV-G) (Section VII).
3. Infect (transduce) your target cells with the Lenti-X iDimerize lentivirus from Step 2 (Section IX).

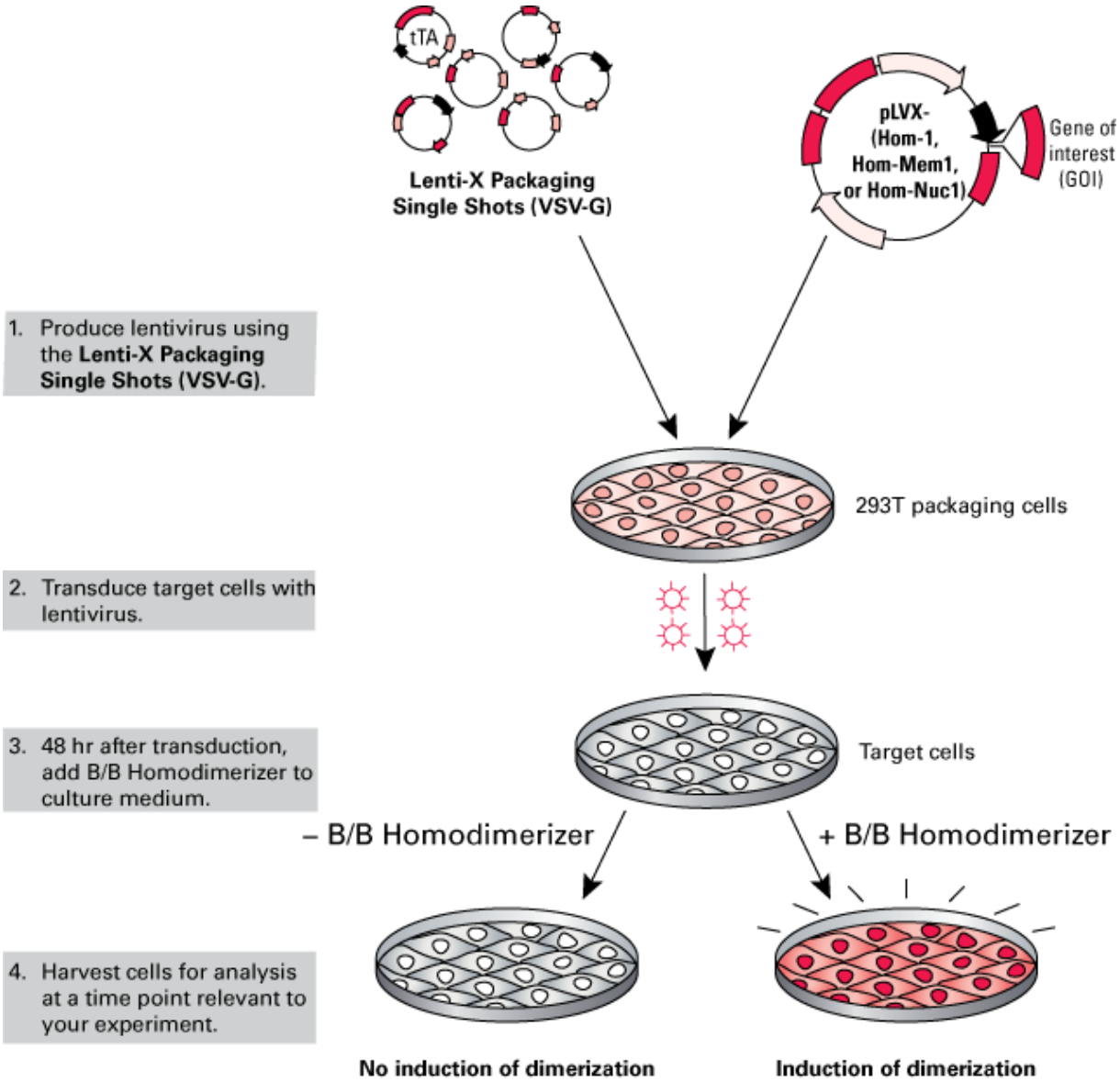

Figure 7. Establishing an expression system in target cells with the Lenti-X iDimerize Inducible Homodimer System. Lenti-X Packaging Single Shots (VSV-G), an optimized packaging pre-mix lyophilized with Xfect Transfection Reagent, and 293T cells are used to generate a high-titer lentiviral supernatant from the pLVX-Hom-1, pLVX-Hom-Mem1, or pLVX-Hom-Nuc1 vector, which contains your gene of interest. Target cells are then transduced with this lentivirus and your protein of interest is induced to dimerize using B/B Homodimerizer.

VII. Producing Lentivirus from the Lenti-X Vectors

Follow the **Lenti-X Packaging Single Shots (VSV-G) Protocol-At-A-Glance**. (Locate this protocol by searching 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 ten minutes with Clontech'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 many of Clontech's Lenti-X systems.

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 x 10^5 cells/well, in 2 ml of medium. Allow at least one well to be used as a "no infection" control.
2. Prepare 20 ml of complete medium and add 60 µl of 4 mg/ml Polybrene. This will be diluted 3-fold for a final Polybrene concentration of 4 µg/ml.

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

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

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

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

A. Protocol: Transducing Target Cells with LVX-Hom1, LVX-Hom-Mem1, or LVX-Hom-Nuc1 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 iDimerize 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.J) 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. 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 A.)
7. Split transduced cells into different plates or separate wells of a 6-well plate, or your preferred plate format.

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

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

   a. Recommended B/B Homodimerizer Concentrations and Time Points

   - Try B/B Homodimerizer concentrations between 0.1 nM and 500 nM for different lengths of time (30 minutes to 12+ hours) to determine the best experimental conditions.

   b. General Guidelines for Preparing Medium Containing B/B Homodimerizer

   - Dilute the supplied B/B Homodimerizer 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 B/B Homodimerizer: Dilute 10 µl of B/B Homodimerizer stock solution (500 µM) in 10 ml of medium to yield a final concentration of 500 nM.

   - Working concentrations of B/B Homodimerizer can be obtained by adding it directly from ethanol stocks, or by diluting it serially in culture medium just before use.
   - If you are making serial dilutions of B/B Homodimerizer 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.
8. After adding the medium containing B/B Homodimerizer at the appropriate concentration and for the appropriate length of time, the effect of dimerization can be analyzed with an assay that is appropriate for your experiment.
9. To dissociate dimerized proteins with the B/B Washout Ligand, please refer to section V.B.

X. References

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XI. Troubleshooting

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 A, and/or consult the Lenti-X 293T Cell Line Protocol-at-a-Glance
	Incorrect culture medium	Use DMEM with additives listed in Section III.D.
	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.D.
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.I) 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).

Problem	Possible Explanation	Solution
E. Inducing Dimerization		
The effect of dimerization is observed in the absence of B/B Homodimerizer.	The expression level of the protein of interest fused to the DmrB domain(s) is too high, especially in the case of a DmrB-tagged protein of interest localized to the plasma membrane.	Transfect cells with a lower amount of plasmid (in the case of transient transfection—Section V.A) or establish stable clones after transduction and screen for the lowest background (Section IX.A).
Addition of B/B Homodimerizer does not result in any of the expected effect(s).	The B/B Homodimerizer concentration is too low.	Increase the amount of B/B Homodimerizer added.
	The monitoring assay is not sensitive enough.	Make sure to include a positive control when performing your assay.
	The volume of B/B Homodimerizer 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 III.H) • 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 A: Preparing and Handling Cell Line Stocks

A. Protocol: Freezing Cell Line Stocks

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

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

B. Protocol: Thawing Cell Line Frozen Stocks

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

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

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

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

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

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

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

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