Clontech® Laboratories, Inc.

Lenti-X™ iDimerize™ Reverse Dimerization System User Manual

Cat. No. 635076
(031915)

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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.

The Lenti-X **iDimerize Reverse Dimerization System** (Cat. No. 635076) contains reagents for creating and expressing a fusion protein containing a tag that causes the fusion to automatically self-associate. If the fusion contains several of these self-association tags, the protein will form aggregates that can be dissociated by adding a cell-permeant ligand (the D/D Solubilizer) (Figure 2). These reverse dimerization components can be introduced 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 system can be used *in vitro* or *in vivo* to:

- induce protein secretion following accumulation of engineered proteins in the endoplasmic reticulum (Figure 3).
- achieve rapid, reversible changes in the subcellular location/biological activity of engineered proteins.

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 stepwise recruitment and activation of intracellular signaling molecules, and the subsequent activation of gene expression. Methods that allow such processes to be manipulated at will using small molecules are powerful tools for investigating and controlling cellular activities. The use of chemical inducers of dimerization, or "dimerizers", has proven to be a particularly versatile approach (Spencer *et al.*, 1993). Cells are engineered to express a protein of interest fused to a drug-binding domain; treatment with the bivalent dimerizer brings the chimeric signaling protein subunits into very close proximity to each other and initiates signaling. This approach has been used to control numerous cellular activities. Different types of dimerizer (Figures 1 & 2) are available:

- **Reverse dimerizers** promote the dissociation of proteins that have been engineered to self-associate because they are tagged with "conditional aggregation domains" (DmrD), The Lenti-X iDimerize Reverse Dimerization System provides the reverse dimerizer ligand (D/D Solubilizer) that binds to the DmrD domain in a manner that disrupts (reverses) the self-association—as well as vectors to express DmrD domains fused to a protein of interest.
- **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. For applications requiring homodimerization, we provide a separate kit, the Lenti-X **iDimerize Inducible Homodimer System** (Cat. No. 635072), that includes the 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 recognized by the heterodimerizer. For applications requiring heterodimerization, we provide a separate kit that includes the heterodimerizer ligand, the **Lenti-X iDimerize Inducible Heterodimer System** (Cat. No. 635074).

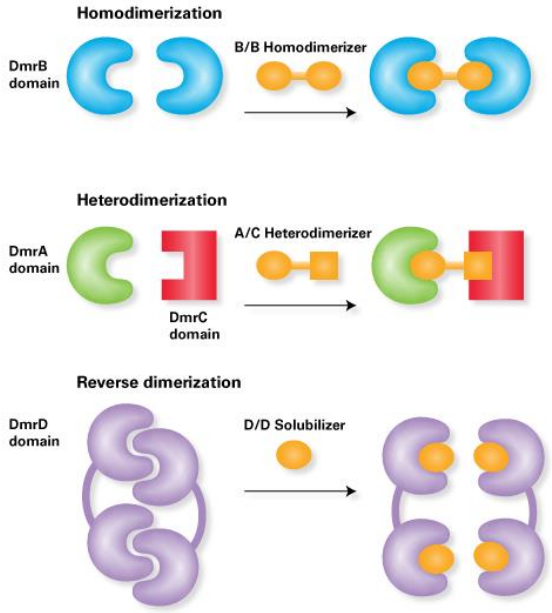

Figure 1. The different types of dimerization include homodimerization, heterodimerization, and reverse dimerization. Separate kits are available from Clontech.

C. Lenti-X iDimerize Reverse Dimerization System Overview

The Lenti-X iDimerize Reverse Dimerization System contains the reagents required to engineer a self-associating protein of interest. The system also contains "DD Solubilizer"—the small molecule used to induce protein dissociation—as well as three vectors that allow the creation of fusion proteins containing two (pLVX-rHom-1), three (pLVX-rHom-Nuc1), or four copies (pLVX-rHom-Sec1) of a DmrD self-dimerizing domain, each of which bind D/D Solubilizer. The resulting fusion proteins are localized to the cytoplasm (pLVX-rHom-1), nucleus (pLVX-rHom-Nuc1), or endoplasmic reticulum (pLVX-rHom-Sec1), where aggregation prevents transport through the secretory pathway unless D/D Solubilizer is added to dissolve the aggregates. The addition of D/D Solubilizer to live cells expressing self-associating fusion proteins induces the dissociation of the fusions to monomers by blocking these dimerization domains.

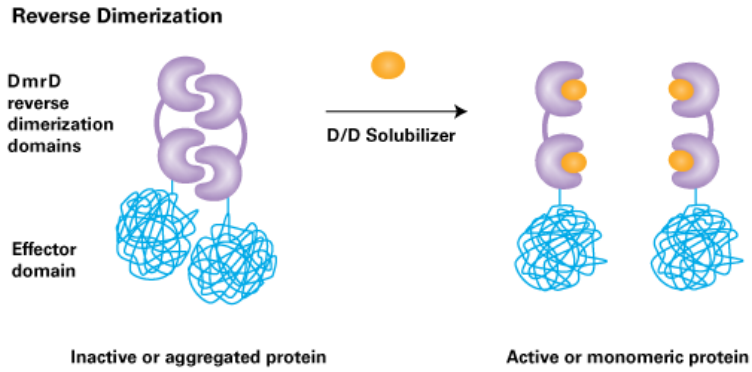

Figure 2. Controlling protein activity using regulated aggregation.

D. Controlling Secretion Using the Lenti-X iDimerize Reverse Dimerization System

The Lenti-X iDimerize Reverse Dimerization System can be used to turn *on* a process that is *inactivated* by oligomerization. A key example is the regulation of protein secretion through controlled aggregation in the endoplasmic reticulum (ER). The use of this system to control secretion (Figure 3) involves the addition of DmrD domains to a secreted protein of interest, i.e., between the signal sequence and the mature protein (Rivera *et al.*, 2000). The resulting fusion proteins localize and accumulate in the ER as aggregates. Addition of the D/D Solubilizer dissolves the aggregates and allows the protein to be exported through the secretory apparatus. To ensure secretion of the authentic protein, a cleavage site for the specific endopeptidase furin is interposed between the DmrD domains and the protein of interest. Since endogenous furin is exclusively expressed in the trans Golgi, the fusion protein will be processed as it traverses this compartment, resulting in the secretion of the correctly processed protein (as well as the separate DmrD moiety). Thus, this system allows ligand-dependent control of secretion.

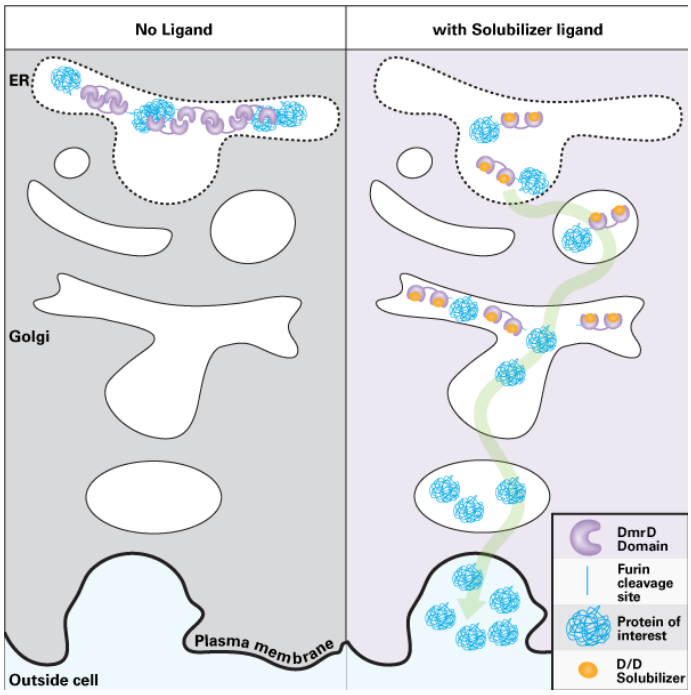

Figure 3. Induction of protein secretion by addition of D/D Solubilizer.

II. List of Components

Store all components at -20°C.

- 1 each Lenti-X iDimerize Reverse Dimerization Vector Set
  - pLVX-rHom-1 Vector (10 µg)
  - pLVX-rHom-Nuc1 Vector (10 µg)
  - pLVX-rHom-Sec1 Vector (10 µg)

500 µl D/D Solubilizer (0.5 mM)
(also sold separately as Cat. Nos. 635054 & 635053—see Section III.A)

Table 1. ARGENT and Clontech Nomenclature for Lenti-X iDimerize Reverse Dimerization System Components

Ligand Name in ARIAD Kit	Ligand Name in Clontech Kit
AP21998	D/D Solubilizer
Dimerization Domain Name in ARIAD Kit	**Dimerization Domain Name in Clontech Kit**
F_M	DmrD

NOTE: The D/D Solubilizer is so named because it induces the dissociation of a self-associating fusion protein possessing DmrD domains

III. Additional Materials Required

A. D/D Solubilizer

Each Lenti-X iDimerize Reverse Dimerization System includes 500 µl D/D Solubilizer (0.5 mM; see Section II). Additional D/D Solubilizer can also be purchased separately in the following sizes:

Cat. No.	Product Name	Size
635054	D/D Solubilizer (0.5 mM)	500 µl
635053	D/D Solubilizer (0.5 mM)	5 x 500 µl

B. 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

C. 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.D) to produce the highest amounts of safe, replication-incompetent lentivirus (see **www.clontech.com**).

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

D. 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).

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

E. 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.

F. 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)

G. 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)

H. Transduction Enhancers

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

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

I. 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
631317	Xfect Transfection Reagent (100 rxns)
631318	Xfect Transfection Reagent (300 rxns)

J. In-Fusion® HD Cloning System

In-Fusion is a revolutionary technology that 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)

K. 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 Reverse Dimerization Domains

A. General Considerations

1. Controlling secretion of a protein of interest

To control the secretion of your protein of interest, the gene of interest should be cloned in-frame into the NotI or BamHI site downstream of the fourth DmrD domain of pLVX-rHom-Sec1. If the gene of interest needs to be cloned upstream of the DmrD domains, it must be cloned in-frame into the Xba1 site between the secretory signal sequence and the DmrD domain. This is most easily accomplished by using Clontech's In-Fusion HD Cloning System (see Section III.J). If it is cloned upstream of the DmrD domains, the sequence encoding your protein must not contain a stop codon, and must be in-frame with the downstream DmrD domains. Since a fusion protein created using pLVX-rHom-Sec1 contains a secretory signal sequence, as well as four tandem DmrD domains, this protein will form aggregates in the endoplasmic reticulum that prevent it from being transported through the secretory pathway. Adding D/D Solubilizer dissolves the aggregates and allows the protein to be secreted by the cell.

2. Controlling localization of fusion proteins to the cytoplasm and nucleus

- Fusion proteins are created by cloning the gene encoding your protein of interest into the MCS of pLVX-rHom-1 or pLVX-rHom-Nuc1 on either the 5' or the 3' end of the DmrD domains shown in Figure 4.
- If the sequence encoding your protein of interest is cloned into the 5' MCS, it must be cloned in-frame with the downstream DmrD domains and must not contain a stop codon
- If the sequence encoding your protein of interest is cloned into the 3' MCS, it needs to be cloned in-frame with the encoding sequence of the upstream DmrD domains. 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.
- Fusion proteins localize to the cytoplasm when created using pLVX-rHom-1 (which contains no targeting signal) or to the nucleus when created using pLVX-rHom-Nuc1.

3. How many DmrD domains should I use?

The number of DmrD domains best suited for each application varies. For inducible secretion, we recommend constructing a fusion protein containing 4 tandem repeats of the DmrD domain. In some instances it may be desirable to have less efficient retention of the fusion protein in the ER to increase the rate of secretion in the presence of ligand. This can be achieved by constructing a fusion protein that contains fewer than 4 DmrD domains (see Section IV.B; Rivera *et al.*, 2000). Often the optimal configuration is best determined empirically.

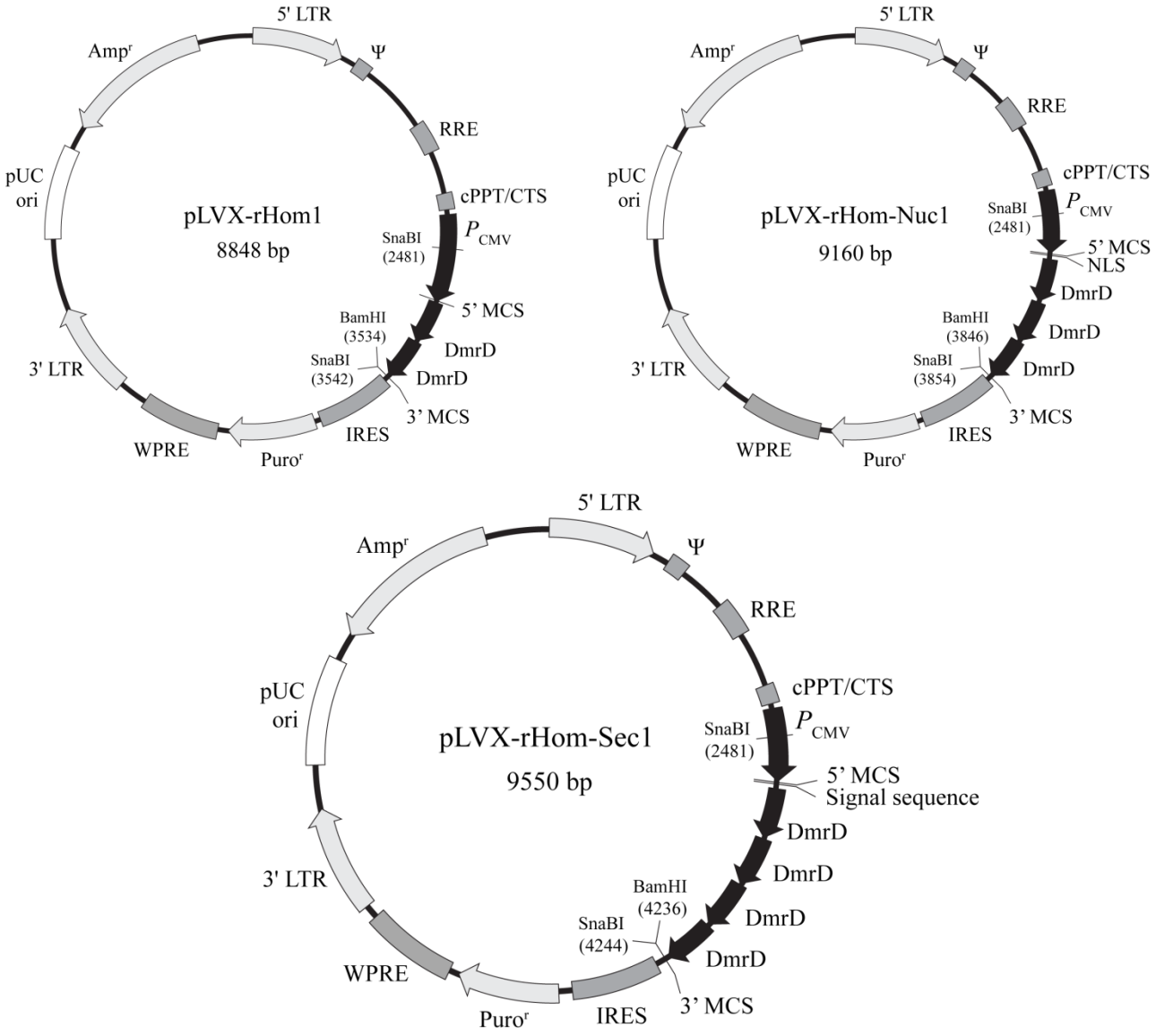

Figure 4. pLVX-rHom-1, pLVX-rHom-Nuc1 and pLVX-rHom-Sec1 Vector Maps.
For more detailed vector information, see **www.clontech.com**

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

We recommend using In-Fusion HD (Figure 5) for cloning your protein of interest into the pLVX-rHom-1, pLVX-rHom-Nuc1, or pLVX-rHom-Sec1 vectors. The technology is described at **www.clontech.com/infusion**

NOTE: Stellar Competent Cells (Section III.K) 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.

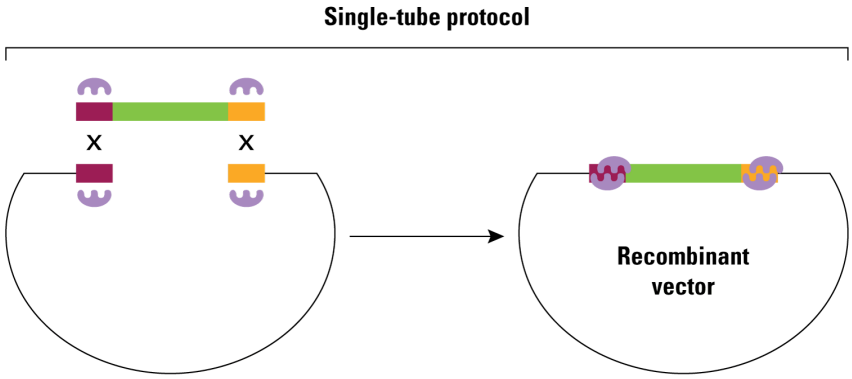

Figure 5. The In-Fusion HD Single-Tube Cloning Protocol.

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-rHom-1, pLVX-rHom-Nuc1 or pLVX-rHom-Sec1 vector constructs 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 DmrD fusion proteins of interest in a transient fashion and can be tested for reverse dimerization (dissociation) in response to D/D Solubilizer. For your initial *in vitro* experiments, we recommend testing medium containing different concentrations of D/D Solubilizer with your transfected cells in order to determine the sensitivity of the system containing your protein(s) of interest.

1. In a well of a 6-well plate, use Xfect Transfection Reagent (Section III.I) to transfect your target cells with 5 µg of the pLVX-rHom-1, pLVX-rHom-Nuc1, or pLVX-rHom-Sec1 vector containing your genes 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 D/D Solubilizer at specific time intervals and concentrations, replace the medium in the plates containing the transfected cells with medium containing the appropriate amount of D/D Solubilizer diluted as described below. Maintain at least one culture in medium containing no D/D Solubilizer as a negative control.

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

a. Recommended D/D Solubilizer **Concentrations and Time Points**

- Try D/D Solubilizer 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 D/D Solubilizer

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

- Working concentrations of D/D Solubilizer 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 D/D Solubilizer 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 D/D Solubilizer at the appropriate concentration and for the appropriate length of time, the effect of dissociation can be analyzed with an assay that is appropriate for your experiment.

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 reverse dimerization expression system using lentivirus (Figure 6):

1. Clone your gene of interest into the pLVX-rHom-1, pLVX-rHom-Nuc1 or pLVX-rHom-Sec1 vector using fast, easy In-Fusion HD cloning (Section IV) or a standard ligation method.
2. Produce Lenti-X Reverse iDimerize lentiviral supernatant (lentivirus encoding your DmrD-tagged protein of interest) by transfecting the lentiviral vectors encoding for the DmrD fusion protein 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 Reverse iDimerize lentiviral supernatant from Step 2 (Section IX).

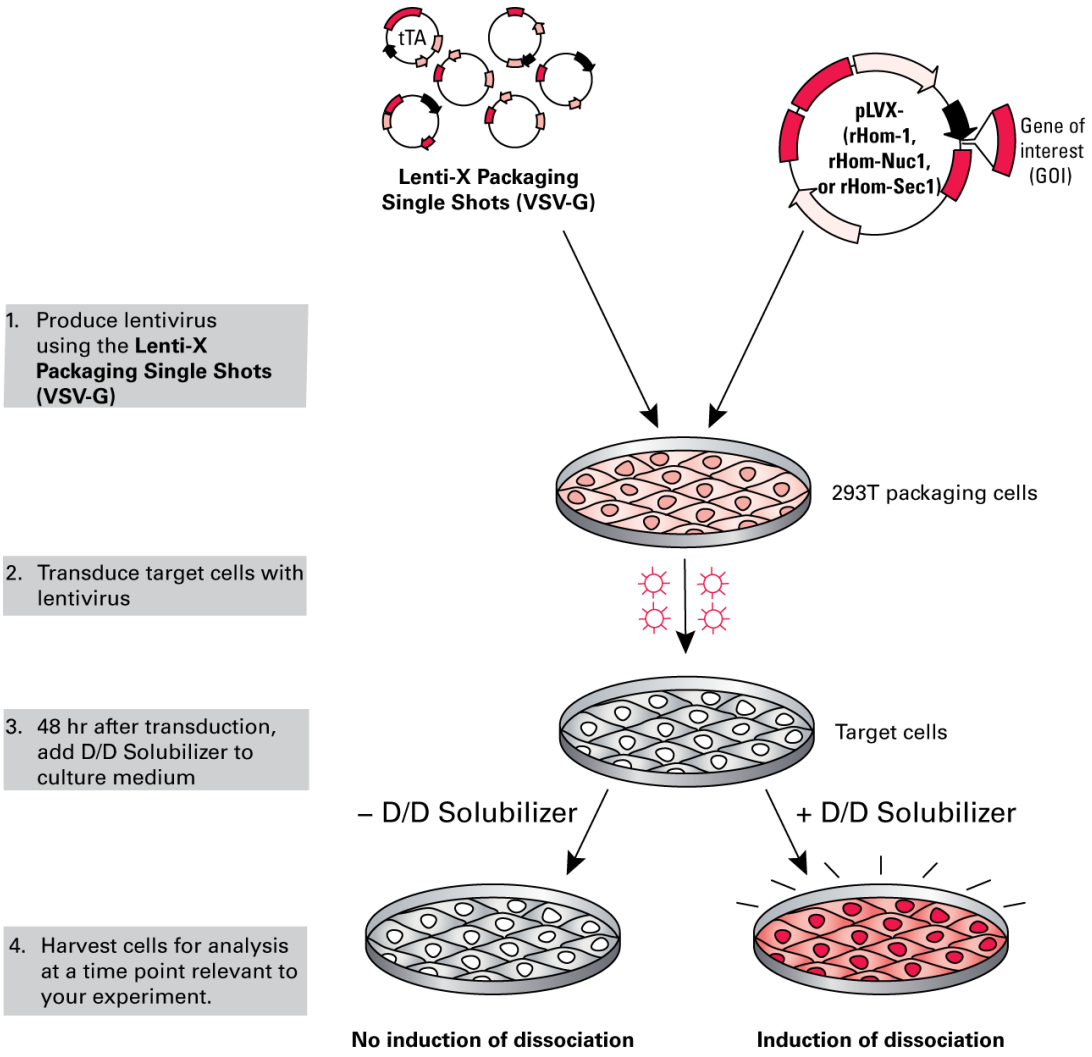

Figure 6. Establishing an expression system in target cells with the Lenti-X iDimerize Reverse Dimerization System. The Lenti-X Packaging Single Shots (VSV-G), an optimized packaging premix lyophilized with Xfect Transfection Reagent, and 293T cells are used to generate a high-titer lentiviral supernatant from the pLVX-rHom-1, pLVX-rHom-Nuc1 or pLVX-rHom-Sec1 vector, which contains your gene of interest. Target cells are then transduced with this lentivirus and your protein of interest is induced to dissociate using D/D Solubilizer.

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 10 minutes with Clontech's **Lenti-X GoStix** (Section III.F; 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.E).
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 pLVX-rHom-1, pLVX-rHom-Nuc1 or pLVX-rHom-Sec1 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 Reverse 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.H) may be used as transduction enhancers instead of Polybrene.

4. In general, we find that an MOI of 5–20 works best. If titer values are unknown, use serial dilutions of the virus supernatant, such that the total volume of supernatant used makes up no more than 1/3 the final volume of culture medium used in the transduction. Centrifuge the cultures to improve transduction efficiency (see Section VIII.B).

5. Transduce the cells for 8–24 hr. If you are concerned that exposure to either the Polybrene or to the viral supernatant (which contains medium conditioned by the packaging cells) may adversely affect your target cells, limit the transduction to 6–8 hr.
6. Remove and discard the virus-containing medium and replace it with fresh growth medium. 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 D/D Solubilizer 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 D/D Solubilizer, diluted as described below. Maintain at least one culture in medium containing no D/D Solubilizer as a negative control.

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

   a. Recommended D/D Solubilizer Concentrations and Time Points

   - Try D/D Solubilizer 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 D/D Solubilizer

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

   - Working concentrations of D/D Solubilizer 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 D/D Solubilizer 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 D/D Solubilizer at the appropriate concentration and for the appropriate length of time, the effect of dissociation can be analyzed with an assay that is appropriate for your experiment.

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.B.
	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.B.
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.G) 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 dissociation is observed in the absence of D/D Solubilizer	The expression level of the proteins of interest fused to the DmrD domain, is too high.	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 D/D Solubilizer does not result in any of the expected effect(s).	The D/D Solubilizer concentration is too low.	Increase the amount of D/D Solubilizer added.
	The monitoring assay is not sensitive enough.	Make sure to include a positive control when performing your assay.
	The volume of D/D Solubilizer 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.F) • 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.E).

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